Management of Insect Pests: Nuclear and Related Molecular and Genetic Techniques

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OF A SYMPOSIUM
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FOREWORD

The balance of the relationship between people and insects is quite unacceptable in many regions of the world. The incidence of malaria, which is transmitted by mosquitoes, is far greater today than thirty years ago, when the *Anopheles* vectors were being suppressed strongly with DDT and other organochlorines in a co-ordinated global campaign. At that time, many hoped that the mosquitoes that transmit malaria would be reduced to such low numbers that this deadly disease would be eradicated from large sections of the globe. Subsequently, the programmes against these vectors lost their effectiveness and there has been a widespread resurgence of the disease. Similarly in Africa, ground has been lost against tsetse flies and trypanosomiasis in livestock and man. Tropical fruit flies destroy fruit and vegetables and they are a serious barrier for exports from developing countries to markets in several industrialized countries. Some major fruit fly pests have spread to other continents and threaten to spread even more widely as international travel continues to increase. Intermittently, locusts and other acridids devastate crops in Africa, the Middle East and Asia. The dreaded cotton boll weevil expanded its range into the cotton growing areas of Brazil, where it has resulted in widespread economic losses. The populations of more than six hundred species of insects have developed resistance to insecticides, still the major weapon used to combat them. The severity of certain insect pests, such as the whitefly, has increased as older insecticides have been replaced with synthetic pyrethroids, which decimate some of the natural enemies that have hitherto effectively kept some of these pests under a modicum of control.

Yet there are substantial grounds for optimism, based on some major and lasting advances against certain dangerous pests, and on very promising developments in science and technology. Thus, the New World screwworm has been eradicated by means of the sterile insect technique (SIT) from all of Mexico and the United States of America, and this campaign is rapidly advancing toward Panama. This deadly parasite was also eradicated from the Libyan Arab Jamahiriya, where it posed a tremendous threat to the livestock, wildlife and people of Africa and the Mediterranean region. The SIT was also used to eradicate both the melon fly and the Oriental fruit fly from Japan. The greatest single achievement in classical biological control occurred recently in Africa, where the ravages of the cassava mealybug were brought under lasting control throughout the cassava growing zone extending over 34 sub-Saharan countries. This was accomplished by mass rearing its natural enemy, an encyrtid wasp from South America, and distributing it over this vast region. In Indonesia, the brown plant hopper and other insects on rice were controlled by fostering the resurgence of their natural enemies, brought about by strongly limiting the excessive use of insecticides.

Progress has been made during the past decade in overcoming many of the impediments of biologically based methods of pest management. The reliability and economy of mass rearing many insects have been improved significantly. Significant advances have been made in formulating naturally occurring compounds, such as pheromones and biological control agents. It has become possible to develop robust genetic sexing strains of insects, so that only sexually sterile males can be released. This will definitely increase the effectiveness and economy of SIT.

A topic of particular importance, dealt with at length in these Proceedings, concerns the advances made in the field of molecular technology and biotechnology. DNA probes and other molecular techniques are being used to identify cryptic species and individual insects with genes for resistance to insecticides. Bacteria and several crops have been genetically engineered to express the δ endotoxin of *Bacillus thuringiensis* for effective insect control on a commercial basis. The excessively narrow host range of some nuclear polyhedrosis viruses and other biological control agents is being overcome through genetic engineering. However, many of the major benefits from such approaches will not be realized until practical technology for the genetic transformation of economically important arthropods has been developed.

The presentations in this Symposium focused on advances and trends in insect control and eradication, genetic engineering and molecular biology, insect genetics, operational SIT programmes, F_1 sterility and behaviour, biocontrol, tsetse fly R&D and quarantine. The Symposium was attended by 83 participants from 36 countries and 3 international organizations. Sixty papers and four posters were presented and are included in these Proceedings.

The Symposium participants expressed their desire to preserve in memory the life and work of the late André Van Der Vloedt. Dr. Van Der Vloedt pioneered in the development and use of the sterile insect technique in the combat of tsetse fly vectors of trypanosomiasis. He became ill while on a mission in the United Republic of Tanzania and Zambia and died in Vienna, after a brief illness, on 31 December 1991. Dr. Van Der Vloedt was an outstanding leader, teacher and researcher. His infectious enthusiasm and boundless good will enabled him to reach across the chasms of race and culture and to stimulate scientific advancement on several continents.

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ADVANCES AND TRENDS IN MANAGING INSECT PESTS

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Abstract

ADVANCES AND TRENDS IN MANAGING INSECT PESTS.

Providing the needed food, clothing and shelter for the world's peoples, while maintaining a long term sustainable natural resource base and a healthful environment, will be a major challenge. One very important aspect of this challenge is the protection of plants and animals from insect pests. Although many different insect control methods are currently in use, there has been a heavy reliance on conventional insecticides for more than forty years. Because of the development of insecticide resistance, the public concern about environmental and health risks, and the high cost of development, approvals for specific insecticide uses are now being removed at a faster rate than they are being replaced. Additional alternative insect management technologies will be required, and a strategy for their use should be selected based on the characteristics of the technology to be used and the pest involved. However, the basic methods available for insect control in the future are not likely to change drastically. Included will be natural enemies, semiochemicals and other natural products, synthetic insect toxicants, host plant resistance, autocidal methods and cultural controls. Since a number of alternative pest controls, such as importation of natural enemies, autocidal methods and cultural controls, usually do not have a significant market value, the research and development responsibilities necessary to bring such components into practice will rest primarily with the public sector. Also, desirable area-wide management and eradication programmes often will entail major involvement of public institutions. Most insect problems can best be dealt with by a pest management programme that includes integration of two or more suppression methods with the aid of a strong population dynamics knowledge base, decision support systems, economic analyses and environmental assessments. Although trends cannot be predicted with certainty, the market for conventional insecticides probably will decline and the market for biologically based products probably will increase. Thus, an increased number of biological suppression methods will probably be available in the future. However, advances in production, storage and delivery to reduce costs and increase efficacy are needed in order for these methods to produce the desired result. Therefore, increased efforts by the public sector, closely co-ordinated with the private sector, will likely be necessary to meet future needs for economically feasible and socially acceptable pest control products. Additional emphasis should also be placed on the development of non-product oriented, biologically based control methods.

4. SUPPRESSION METHODS AND STRATEGIES

Suppression methods can be placed into three broad categories: biological, cultural and chemical [11]. However, many methods cannot be exclusively classed in a single category. For instance, *Bt* is a biological organism, but much of its insect suppression activity is associated with a protein toxin that is a naturally occurring chemical. Therefore, an in depth understanding of the characteristics and properties of various suppression methods, particularly those that are biologically based, is usually necessary in order to develop and successfully utilize a suppression method. The various suppression methods to be considered include microbial agents, nematodes, arthropods (augmentation), pheromones, botanicals, insect growth regulators, synthetic toxicants, host plant resistance, arthropods (importation of exotics), autocidal (genetic) methods and various cultural controls.

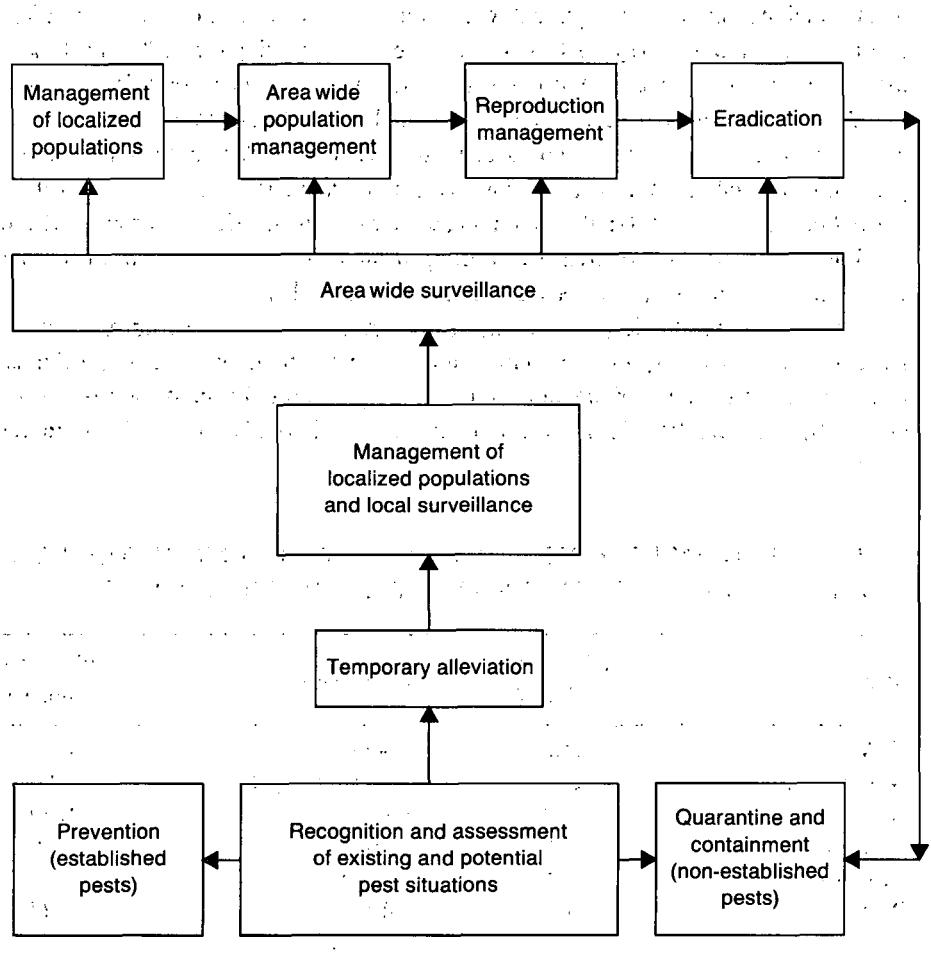

FIG. 1. Insect management strategies.

Suppression methods should be utilized within a deliberate strategy. The articulation of the various strategies has evolved during recent years; these now include prevention, quarantine and containment, temporary alleviation, management of localized populations and total population management, including area wide population management, reproduction management and eradication (Fig. 1) [12–14]. The successful implementation of a particular suppression method will be facilitated by the selection of the most appropriate strategy after careful consideration of the characteristics of the method to be employed and of the population dynamics of the insect to be managed.

5. PRODUCT ORIENTED TECHNOLOGIES

A wide range of biologically based product oriented technologies are in limited use and include products based on *Bt* [15], insect viruses [16], fungi [17], parasitic nematodes [18] and mass reared arthropods [19]. Other technologies of natural origin, but more closely associated with defined chemistry, include microbially produced toxins (other than *Bt* endotoxin) [20], pheromones [21] and botanical insecticides [22]. Other products include synthetic insect growth regulators [23] and synthetic organic toxicants [10], with the latter comprising the vast majority of products currently being sold.

6. NON-PRODUCT-ORIENTED TECHNOLOGIES

A number of insect suppression technologies that have made a major contribution are not particularly product related. For instance, biological controls with imported natural enemies may provide considerable public good [24], particularly through the control of exotic pests on perennial crops, but these controls do not usually have significant market value. Therefore, the research and development responsibilities necessary to bring these components into practice rest primarily with the public sector. Also, area wide management and eradication programmes [25] will continue to play a significant role in insect control and often will involve institutions that are not market oriented. For example, autocidal methods, such as the sterile insect technique, have had major impacts, but application of these methods usually requires the involvement of governmental or grower organizations.

Host plant resistance, although not usually considered to involve an insect control product, often comprises a commercial product if the seed or other plant material is sold. On the other hand, some seeds and other plant materials can be propagated by individual growers or by grower organizations. However, regardless of the source of the resistant plant material, there are many opportunities for reducing pests through the use of resistant plants [26].

Finally, cultural controls [27] such as tillage, crop rotation, irrigation and fertilization management, manure management, trap cropping and companion cropping provide a wide range of options for reducing insect populations, but they do not have a direct market value.

7. INTEGRATED PEST MANAGEMENT

7.1. Integrated pest management defined

During the debate in the 1970s and 1980s, when IPM was frequently touted as the preferred approach to pest control, many different interpretations were placed on IPM. These ranged from 'eliminate all pesticides' to 'make some changes, but don't reduce pesticide market size', and often did not include total population management. However, now, in the early 1990s, two predominant interpretations of IPM have emerged: (1) improved pesticide use management, which emphasizes application only when needed, with a wide range of precautions to reduce exposure of both workers and the environment, and (2) reduction in pest losses and/or pesticide use through expanded use of alternative pest control methods.

Thus, a common goal, which can encompass various insect management strategies designed to suppress insect populations, appears to be evolving and pest management, as earlier defined by the Council on Environmental Quality [28], can still provide the framework for moving toward that common goal:

> "Integrated pest management is the selection, integration, and implementation of pest control methods based on predicted economic, ecological, and sociological consequences."

Within this context, regardless of the strategy and methods to be employed, the basic components of an IPM programme are similar. They include: (a) suppression methods; (b) decision support technologies (sampling, treatment thresholds, decision guides and population models); and (c) integration into production systems, including environmental assessments and economic analyses. In addition, a thorough knowledge of population dynamics (host/pest interactions, pest density/damage/yield relationships and management of pests and their natural enemies) is often needed or useful in designing and implementing IPM programmes. Perhaps the single most important element common to all strategies is monitoring or sampling. The development of pheromones and other attractants for use in traps is a major advancement in that area [29]. Two examples of insect pest management are presented here to illustrate local management and reproduction management/eradication programmes.

7.2. Local management

Advanced local management programmes often include the use of computerized decision support, expert or knowledge based systems [30]. For instance, an IPM programme for apples has been developed that includes a computerized Expert Advisory System for Managing Apple Cropping Systems (EASY-MACS) [31]. This system provides a programme for the management of aphids, leafminers, leafrollers, mites and fruit flies. The system provides for inputs of phenological data, pest density information and weather data and for outputs on additional sampling recommendations if needed, damage forecasting, treatment recommendations and record keeping. A companion guide for sampling has also been developed which includes sampling data forms that enable the grower to make decisions without access to the computerized expert system [32].

7.3. Reproduction management/eradication

A number of very important programmes involving autocidal methods for eradication of both dipteran and lepidopteran pests are discussed elsewhere in these Proceedings, so a programme for reproduction management and eradication of a coleopteran pest, the boll weevil, *Anthonomus grandis*, the Southeastern Boll Weevil Eradication Program in the USA, has been selected here for illustration. The components of the programme include: (a) pheromone trap sampling for treatment decisions; (b) fall diapause control with insecticides; (c) selected presquaring (flower bud) control with insecticides; (d) fall diapause control as needed; and (e) pheromone trap monitoring to detect reinfestation. The programme, with these components, eliminated boll weevil reproduction in North Carolina and essentially eliminated it in eastern South Carolina between 1978 and 1986 [33]. In 1987, the programme was expanded throughout Florida, most of Georgia and a major portion of Alabama. Over 400 000 ha (1 million acres) were involved throughout the southeastern USA in 1991 [34].

8. TRENDS

Worldwide numerical data available for analysing insect management trends are limited primarily to information on sales of insecticides and other pest control methods that have a market value. However, these data do provide some insight into overall trends. For instance, the rapid growth in pesticide sales that occurred in the 1960s (11%) slowed in the 1970s (7%) and 1980s (3%) [7]. Also, a recent market study on insect control products indicates that the conventional insecticide market in the USA and the world will likely decline over the next ten years and that the market for biologically based products will increase significantly (Table II) [35, 36].

TABLE II. ESTIMATED AND PROJECTED SALES OF INSECT CONTROL PRODUCTS AT THE USER LEVEL (FROM REFS [35, 36])

	US market (millions of constant US dollars)		
Product group	1991	1996	2001
Microbial agents[a,b]	79	118	232
Nematodes[c]	3	15	35
Arthropods[c,d]	8	12	18
Behaviour modifying chemicals[b]	30	45	111
Botanicals[b]	45	51	55
Subtotal	165	241	451
Speciality chemicals[b,e]	22	30	57
Conventional insecticides[b]	2 119	2 021	1 600
Total	2 306	2 292	2 108
	World market (millions of constant US dollars)		
Product group	1991	1996	2001
Microbial agents[a,b]	157	219	381
Nematodes[c]	4	30	70
Arthropods[c,d]	35	47	60
Behaviour modifying chemicals[b]	60	80	158
Botanicals[b]	70	81	90
Subtotal	326	457	759
Speciality chemicals[b,e]	100	116	181
Conventional insecticides[b]	9 358	9 597	9 212
Total	9 784	10 170	10 152

[a] Bacteria, viruses and fungi, and toxins produced by these organisms.

[b] Estimates from Ref. [35].

[c] Estimates from Ref. [36].

[d] Includes primarily predators and parasitoids; some pollinators for use in glasshouses are also included.

[e] Includes primarily insect growth regulators.

Although the use of biological insect control products is likely to increase, their cost and the complexity of their use will likely limit the extent to which they will replace conventional insecticides. In addition, the extent to which new insecticidal chemistries become available will significantly influence the rate of increase in the use of biologically based insect suppression methods. Several new organic synthetic insecticides and miticides from several chemical classes, such as pyrroles, nitroguanidines, phenylpyrazoles, dibenzoylhydrazines, substituted triazones and quinazolines, are in various stages of development.

The differences in the total values for insect control product sales in Tables I and II should be noted. Even though both values are at the end user level, the values in Table I are calculated on the basis that all materials are sold at the farm level and the values in Table II include some sales at the consumer and professional pest control levels. Also, estimates in Table II on projected sales for arthropods and nematodes are from two different sources that used different criteria for the projections. Therefore, the estimates for arthropods are more conservative than the estimates for nematodes.

In addition to changing trends in the use of insect control products, there are numerous examples of the application of alternative insect control methods that are not product oriented which have resulted in reductions in insecticide use [37], even though quantitative worldwide data on the extent of use of these methods are not available.

9. OPPORTUNITIES FOR BIOLOGICALLY BASED METHODS

Conventional insecticides have received wide acceptance because they provide a means of rapidly reducing pest populations in highly manipulated agroecosystems in which natural regulation of pest populations does not maintain populations at acceptable levels. If biologically based pest controls are to be substituted for conventional insecticides, they need to be modified and/or managed in such a manner as to produce a similar result either by rapidly reducing a pest population or by preventing it from reaching damaging levels.

Therefore, the perception that biological pest controls are readily available, easy to use and effective, and that their use in lieu of conventional insecticides should therefore be simple and rather straightforward, is not consistent with actual circumstances. The characteristics generally associated with biologically based products, which often require different approaches to development and delivery compared with conventional insecticides, include: (a) narrow spectrum of activity; (b) short shelf life (c) slow action; (d) lower efficacy; (e) relatively small market size and fewer opportunities for proprietary protection. Although it is important to be cognizant of these characteristics, there are opportunities for overcoming some of these limitations. For instance, in vitro virus production [38] and improved artificial diets [39]

offer considerable potential for reducing the costs of virus and insect production. Further, chemical enhancers are showing considerable promise for increasing the effectiveness of insect viruses [40]. Also, nutrient based phagostimulants can be used to improve the efficacy of microbial agents [41], while some semiochemical baits hold promise for controlling insects with very small quantities of insecticide [42]. Also, the development of total population management programmes would favour the use of biologically based suppression methods [25].

Transgenic plants may also be a significant component in agricultural product sales in the future, but their role in insect control continues to be uncertain because of the time required to incorporate new traits and the concern about their potential to increase the rate of resistance development. However, the great increase in our ability to manipulate genes and, therefore, to transfer various traits from one organism to another, likely will greatly increase our ability to utilize various genetic traits. Therefore, the biologically based methods available in the future probably will include increased numbers of genetically modified organisms or products of such organisms [14, 43]. See related papers by Oakeshott[1] and others in these Proceedings.

10. VALUE COMPONENTS

Economic analyses and environmental assessments should be an integral part of programme development and these analyses and assessments, coupled with social and political considerations, should have a major influence on insect control decisions, since almost all pest control decisions affect people other than those who make the decisions. The value judgements made will vary greatly from country to country, so no attempt will be made here to review the total process. However, an example can be used to illustrate the value of obtaining applicable quantitative data on the impacts of pest control actions. An economic study of the US Southeastern Boll Weevil Eradication Program, discussed in Section 7.3, which included an analysis of the numbers of insecticide applications, cotton acreage and cotton yields, indicated that the programme in North Carolina, Virginia and South Carolina increased annual returns by about US $187/ha [33]. Also, the 50–70% reduction in insecticides associated with this programme represents a substantial and continuing environmental benefit. These results were particularly useful in guiding programme expansion.

[1] Paper IAEA-SM-327/2.

11. CONCLUSIONS

If current trends continue, a substantial increase in biologically based methods of insect control will be needed to replace conventional insecticides that are being lost. However, because of the characteristics of most alternative technologies, increased efforts by the public sector will likely be necessary to meet future needs for economically feasible and socially acceptable pest control products. Additional emphasis should also be placed on the development of non-product oriented biologically based pest control methods.

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BIOTECHNOLOGICAL PROSPECTS FOR MANAGING INSECT PESTS

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Abstract

BIOTECHNOLOGICAL PROSPECTS FOR MANAGING INSECT PESTS.

Mounting problems with resistance and residues threaten the long term utility of many chemical insecticides and drive the search for biotechnological alternatives. The potential impact that molecular and other biotechnologies may have on three pest control strategies, involving biological insecticides, insect resistant hosts and genetically engineered insects, is discussed. Regarding biological insecticides, it is argued that recent improvements in production technologies and relatively low development costs should allow exploitation of a number of non-engineered microbials, generally for situations involving relatively high damage thresholds where speed of action is not the major priority. The much greater development costs associated with engineered microbials may restrict their use in the short term to situations involving large markets in the developed world. Nevertheless, there are now excellent prospects for genetically engineering insect viruses to achieve kill times approaching those of many chemicals. Likewise, genetic engineering now permits the transfer of various insecticidal delta endotoxin genes from *Bacillus thuringiensis* (*Bt*) to other bacteria with different ecological niches. Concerning insect resistant hosts, the prospects of developing vaccines against several haematophagus animal ectoparasites such as ticks are now good, but genetic engineering offers the possibility of heritable protection against a wider range of pests in both animals and plants. The basic engineering technology is now available for many production animals and non-cereal crops, but major difficulties remain in developing an engineering technology for cereals; achieving adequate control of transgene expression in both plants and animals; the shortage of orally active alternatives to *Bt* genes to engineer into plants; and the lack of any efficacious insecticidal genes to engineer into animals. With regard to genetically engineered insects, two of three basic components needed to generalize the existing capability for *Drosophila* transformation to other insects are now available. These are marker genes to identify transformants and promoters to drive the expression of foreign genes in these insects. The third component is a transposable element system to act as a vector for transferring foreign DNA into the recipient's genome. This component has proved more problematic, but recent developments indicate that the *hobo* or *mariner* elements could function as such vectors for non-drosophilid transformation. The resulting ability to analyse specific genes within pest species will greatly enhance the ability to use these genes, or mutant alleles of them, as the basis for future control strategies.

1. INTRODUCTION

Several problems are now emerging with chemical insecticides which jeopardize their continued use and efficacy. Perhaps the most important of these has been the evolution of target pest resistance; over eight hundred cases are now reported and no major class of chemicals has proved immune to the problem. A second issue arises from their broad spectrum activity; while of value in many multipest situations, there are also many untoward effects on beneficial species. With such chemicals, implementation of insect pest management (IPM) programmes often becomes impractical and problems with secondary pests can also arise. Moreover, the persistence of chemical residues both in commodities and in the environment can present real or perceived downstream problems for human health. On top of all these problems, new chemical insecticides are becoming more expensive to develop and the probability of finding new classes may be diminishing.

As the problems with chemical insecticides have mounted, so has the pressure to develop biotechnological alternatives. This pressure intensified with the advent of recombinant DNA technologies in the early 1970s, but two decades later very few biotechnological alternatives have yet become available, although several may do so within the next decade. It is therefore timely to assess the prospects for these alternatives. In this paper, we argue that the alternatives under development do have the potential to deliver effective control, but only if their development and management incorporate the lessons learned from the misuse of chemical insecticides.

In the following sections, we deal with three major biotechnological approaches that should bring tangible benefits over the next 20 years. The first of these involves the development of biological insecticides, which could be used like conventional chemicals and may or may not be genetically engineered. The second involves the development of hosts carrying in built protection against insect attack, which could be achieved in non-heritable form by vaccination or in heritable form by the engineering of either plants or animals with protective genes. The third approach involves genetic manipulation of the insects themselves, which could lead to biotechnological versions of genetic control programmes, but could have other applications as well.

2. BIOLOGICAL INSECTICIDES

2.1. Non-engineered biological insecticides

Most insect species are susceptible to infection by a range of viruses, bacteria, protozoans, fungi and nematodes. However, few of these agents will meet all the criteria for use as a microbial insecticide. Some of the salient criteria concern pathogenicity and speed of action, host range and safety issues, ability of the

microorganism to be mass produced and stability under realistic storage and application conditions.

The majority of microbials have only limited pathogenicity and/or speed of action, presumably because they have evolved together with their host as a balanced host–parasite system. In some cases, these problems may be remedied by genetic engineering technologies and we discuss these in Section 2.2. In other pest control situations, factors such as slow speed of action are less of a problem because the host has a relatively high damage threshold and the losses incurred between application and control can be tolerated. In the latter situations at least, there are significant prospects of finding a pathogen that is sufficiently active in its wild type form to provide effective control.

The next set of criteria that determine the suitability of a pathogen as a biological insecticide concerns host range and safety issues. Before any field trial of a candidate pathogen can be contemplated it is necessary to have some information on these two issues. Most entomopathogens have fairly restricted host ranges and for some groups, such as baculoviruses, there are now extensive data to show that this range is limited at the broadest to the ordinal level [1]. At least for many non-engineered forms of such entomopathogens, their vertebrate safety can generally be demonstrated with only the minimum of acute toxicity testing. However, this will not be true for other groups of entomopathogens. For instance, the type virus of the Nodaviridae, Nodamura virus, is capable of replicating after inoculation into the brains of suckling mice [2]; obviously, any attempt to use a nodavirus as an insecticide would require more extensive safety testing before release. Such safety testing can form a significant part of total development costs.

The next criterion that a new insecticide must meet concerns the means of its mass production, which could entail the use of either living insects or artificial culture systems (in vivo or in vitro systems, respectively). In vivo systems have predominated historically, but they are only possible when a suitable host can be easily reared and even then problems with quality control can occur [1]. One example of a successful in vivo method concerns the baculovirus of the soybean looper, *Anticarsia gemmatalis*, which was produced under field conditions by a government agency in Brazil. A different in vivo production system was used by Sandoz in the United States of America to produce a viral insecticide marketed under the trade name of ELCAR™ for use against *Heliothis* and *Helicoverpa* species. Instead of infecting insects that had naturally infested field crops like the Brazilian system, a dedicated large scale 'caterpillar factory' was constructed to provide larvae for infection with the virus.

Nevertheless, there remain few cases where in vivo production systems have proved adequate for large scale and/or commercial production. Indeed, the successful commercial development of a biological insecticide has often been predicated on the development of a reliable, cost effective in vitro production system. The lack of such a system has been a major reason why virtually no protozoan insecticides have

been commercialized. Conversely, recent improvements in in vitro production systems have been important to the commercialization of the bacterium *Bacillus thuringiensis* (*Bt*), various nematodes directed against horticultural pests and fungi such as *Metarhizium* [3]. As yet, no viral insecticides have been produced commercially in vitro, although recent interest in the large scale production of pharmaceutical peptides using baculovirus expression systems in insect cell culture has led to qualitative improvements in the large scale production of baculoviruses and now makes the cost effective production of baculovirus insecticides in vitro a feasible proposition [4].

Another crucial criterion which a candidate biological insecticide must meet is its stability under realistic conditions of storage and use. Formulation technology for biologicals has generally lagged behind that for chemical insecticides and remains limiting for some fungi and nematodes with critical ecological requirements. On the other hand, the intense recent interest in *Bt* and engineered baculoviruses has led to improvements in their formulation, which now enable their use under broadly comparable application technologies to those used for conventional chemicals.

The future for some non-engineered biological insecticides therefore looks extremely promising. Many have economic levels of pathogenicity and kill times for particular situations, safety testing for some can be considerably cheaper than for broad spectrum chemicals, and production technologies are rapidly improving for some bacteria, fungi and viruses, as are the formulations for bacteria and viruses. In some instances, like *Bt*, the markets will be large enough that they will be developed and produced by large multinational companies. However, since development costs can be relatively small compared with chemicals and some engineered biologicals, some will also be suitable for smaller 'niche' markets where development and production could be undertaken by government agencies and/or small companies.

As more biological insecticides become available it will become critical to learn the lessons from the misuse of chemicals. Problems of *Bt* resistance have already emerged in the field for the Indian mealmoth, almondmoth [5] and diamondback moth [6] and laboratory studies indicate a high probability that it will quickly emerge in other major pests such as the Colorado potato beetle and *Heliothis* [7]. Unless the use of microbial *Bt* is integrated into a soundly based resistance management strategy, the exciting prospects for crop plants engineered with *Bt* toxin genes may be stymied before they even become commercially available.

2.2. Engineered biological insecticides

For those instances in which wild type microbials are incapable of delivering acceptable levels of control (e.g. in high value crops with low damage thresholds such as cotton), genetic engineering may be required to accelerate their pathogenic effects. The technology has two parts: the ability to engineer the agent and a gene encoding an insecticidal protein with which to engineer it.

At present, the most advanced genetic engineering technologies for entomopathogens have been developed for insect viruses and, in particular, the baculoviruses. Over five hundred isolates of baculoviruses have been recovered from a wide range of insects, albeit mostly lepidopterans. These viruses have large, double stranded DNA genomes and some are readily grown in cell culture, enabling the development of an engineering technology analogous to those previously developed for several mammalian viruses [8]. This technology was originally based on the promoter that drives the expression of polyhedrin, a structural protein produced late in the viral replicative cycle in very large amounts. The high level of expression from this promoter makes it attractive for producing insecticidal proteins, but the late timing of its expression limits the improvement in kill time over wild type virus. While 40% improvements have been achieved with one toxin [9], there is also intense interest in developing alternative promoters that are active earlier in the viral replicative cycle.

There are also other groups of insect viruses that should be amenable to genetic engineering [10]. For instance, the entomopoxviruses and iridoviruses are large double stranded DNA viruses and for some of them there are good cell culture systems. Neither of these virus groups are particularly pathogenic in their wild type forms, so engineering will be important for their exploitation. Moreover, these viruses infect many orthopteran and dipteran species for which there are no baculoviruses known. It is now also possible to engineer several genera of bacteria and much effort is currently being invested in the transfer of toxin genes from *Bt* to other bacteria with different environmental stabilities (e.g. pseudomonads) or ecological niches (e.g. cyanobacteria) [11]. The prospects for engineering other non-viral pathogens are not as clear; fungi, nematodes and protozoans have much larger and more complex genomes and their life histories and modes of action would make the manipulation of their kill times problematic. As discussed above, many have other problems associated with their production and formulation which may limit their potential anyway.

The second part of the technology for developing an engineered biological insecticide is the gene to be engineered into the pathogen to increase its control potential. Broadly speaking, two classes of genes are considered to have potential for insertion into viruses. The first of these are neurotoxins and the second are genes whose products disrupt hormonal functions. Two neourotoxin genes have been engineered into the baculovirus from *Autographa californica*, one encoding a toxin from the straw itch mite and the other encoding a venom protein from a scorpion [9, 12]; improvements in kill time of 40% and 25%, respectively, were obtained. Two hormone related genes, encoding the juvenile hormone esterase [13] and diuretic hormone [14], have also shown some promise in baculoviruses, but further work is required on these systems before their efficacy compares with the toxins above.

None of the neurotoxin or hormone related genes mentioned above are suitable for engineering into bacteria which, unlike viruses, cannot deliver gene products beyond the gut barrier. For bacteria, the only toxins currently available are from the *Bt* delta endotoxin group. These act directly on the midgut cells of a number of insects, causing cessation of feeding, lysis of the midgut cells and eventual death. Delta endotoxin genes specific for many lepidopterans, some coleopterans and dipterans are now available for engineering into various other bacteria [15].

Obviously, an engineered biological insecticide must meet similar downstream criteria regarding the safety, production and formulation as a wild type biological insecticide. In many respects, the technologies for production and formulation are the same as those used for the wild type forms. However, the safety issues are much more complex for a genetically modified organism (GMO). Many countries now have a legislative framework to govern the release of GMOs and the requirements relating to safety are more stringent than for non-engineered biologicals. For instance, host range studies need to be more comprehensive to ensure that the engineering has not significantly altered the host range. Environmental fate studies must also assess not only the way in which the organism disperses through the environment, but also whether the introduced gene can be transferred to other organisms. For some agents, it is likely that additional technology will require development to restrict their persistence and recombination potential. These clear needs for extra safety data and possible requirements for additional technology will add substantially to the development costs of such engineered biological insecticides. Consequently, they are less likely to be produced for small markets or by small companies than non-engineered alternatives, and are less likely to be developed for pest problems in developing nations in the short term.

The prospects of pests evolving resistance to engineered microbials have so far received much less attention than they have for the engineered hosts discussed below. This is a problem that needs urgent study, since several engineered viruses are well down the route to commercialization. Basic issues such as whether resistance would arise to viral functions or to the insecticidal proteins they express have important long term consequences but have so far not been explored in any depth.

3. BIOTECHNOLOGICAL APPROACHES TO HOST RESISTANCE

3.1. Vaccines

Vaccination technology is a major component of disease control strategies in both humans and production animals. There is now great interest in adapting this technology to provide at least short term protection against some ectoparasitic pests such as ticks and miasis flies. This approach will generally be more attractive for ectoparasites that cause production losses themselves than for insects which are

vectors for microbial pathogens. For the latter, it will generally be more appropriate to vaccinate directly against the microbe rather than its vector.

Successful adaptation of vaccine technology to control ectoparasitic insects clearly requires isolation of antigens which are capable of stimulating a vigorous immunological response to some aspect of the insect's physiology that is accessible via the digestive tract. For some haematophagus parasites such as ticks which have highly porous guts, ingestion of the antibody may give access to many internal organs; for other species such as miasis flies, gut porosity will generally be limited and the only accessible organ will be the digestive tract itself. Against this background, it is not surprising that the first ectoparasitic vaccines developed have been against ticks [16], although some progress has now also been made using essential gut antigens as the basis for vaccines against blowflies [17].

Modern biotechnologies could play several roles in the development of vaccines against ectoparasites. One lies in the identification of protective antigens and the cloning of cognate genes. A second lies in the expression of these genes in large scale in vitro expression systems to produce economic quantities of the antigens. Both of these functions have enabled the development of the tick vaccines discussed above. Eventually, a third role for molecular biology in this area could also arise as a result of emerging antibody library technologies [18]. These could allow genes for the host's antibodies to be be cloned and then engineered into the same, or another, host to confer long term and heritable protection. Particularly noteworthy here is the prospect of engineering such genes into other hosts; coupled with the recent demonstration that engineered plants can express functional antibodies [19], it now becomes possible to contemplate the transfer of protective antibody genes from laboratory animals into crop plants.

3.2. Engineered host resistance

Classical genetic procedures have been deployed with variable success for decades in efforts to build protection against insect pest attack into commercial varieties of plants and animals. The aim has been to produce varieties with sufficient in built protection that no further intervention is required to produce economic levels of control. In theory, the further attraction of genetic engineering solutions in this area is that protective genes which do not occur naturally within the species can be introduced into a commercial variety in a single generation, without the need for extensive wide crossing and back crossing programmes. As was the case with the engineered microbials above, progress towards this aim requires both the technology to engineer foreign genes into the organism and the availability of cloned insecticidal genes.

A basic engineering technology has now been developed for several production animals and broad acre crops. For production animals, the technology relies on

injection of the foreign gene located on a vector into the developing embryo and subsequent transplantation of the embryo [20]. For plants, the technology has generally used the Ti plasmid from the bacterium *Agrobacterium tumefaciens* as the transfer vector [21], while the host tissues used more protoplasts. *Agrobacterium tumefaciens* has a limited host range which does not include many important crops such as cereals, but recent advances in 'ballistic' technology, in which DNA is 'shot' into the plant cell, have led to the transformation of some cereals not tractable to engineering with the Ti element [22]. On the other hand, problems still remain in regenerating many species or varieties from transformed protoplasts.

Even when the basic engineering technology is available for commercial varieties, there are additional problems in adapting them for the expression of currently available insecticidal insert genes. One of these problems concerns the level and specificity of expression of the transgene product, which depend in large part on promoter technology. For plants, some high level promoters are now available which, together with enhancements in the post-transcriptional aspects of expression such as RNA processing and codon usage, achieve concentrations of transgene products that exceed 0.1% of the total cellular protein [23]. Unfortunately, these high level promoters are all systemic in their expression profiles and there are concerns about the public acceptability of commodities expressing high amounts of insecticidal proteins. However, currently available tissue specific promoters generally do not express at sufficient levels to be useful in this context. Some parallel problems have been met in the search for appropriate animal promoters, although some wound response and sweat gland promoters that express at relatively high levels are now under development [24].

Another problem in applying engineering technologies to the insertion of insecticidal genes relates to the specificity of the integration event. For no commercial crop or production animal is it yet possible to target the insertion of the foreign gene to a particular location in the host genome. This unpredictability means that a certain proportion of insertion events will either inhibit adequate expression of the inserted foreign gene or disrupt the functioning of an important host gene. This latter problem has already proved a significant impediment to the introduction of many desirable traits into both plants and animals.

While not dismissing some of the above problems, the major limitation in attempts to engineer pest protection into plants and animals is the shortage of cloned insecticidal genes. These genes must encode insecticidal proteins that are orally active and, as we discussed above, for most insects this means that they must disrupt the function of the digestive tract. Currently, the only group of genes that satisfies these criteria are those encoding *Bt* delta endotoxins. *Bt* toxin genes have now been expressed in many different plant species, providing effective protection from certain lepidopteran and coleopteran pests in the glasshouse, and in some cases in field trials as well. On the other hand, no currently identified *Bt* toxins are suitable for engineering into animals, for which many of the insect pests are members of the Diptera.

Some currently available *Bt* endotoxins display activity against particular dipteran pests such as mosquitoes and blackflies but, even then, their toxicity is too low for use in engineering pest reisistant animals.

Clearly, alternatives to *Bt* toxin genes are urgently needed, both in the event of resistance arising to *Bt* and to broaden the control prospects of the strategy against pests not susceptible to *Bt*. Some plant protease inhibitor genes show promise for use in plants [25], as do certain chitinases for animals [24], and we discussed the possibilities of antibody genes earlier. Another exciting prospect suggested by Sivasubramanian et al. [26] is to modify the mode of action of *Bt* delta endotoxins themselves. These toxins bind to a gut membrane receptor in a specific manner, the binding moeity of the toxin being located at its carboxy terminal. Sivasubramanian et al. [26] suggest that changing the binding moeity could change the specificity of the toxin. They constructed a chimeric *Bt* toxin composed of the amino terminal portion (the toxin moiety) of a *Bt* toxin and a carboxy terminus derived from the *gp67* gene of a baculovirus. They reported that the chimeric protein was then active in a normal host of the baculovirus, even though the toxin moiety in itself was not. This approach could be useful, both for extending the effective host range of *Bt* toxins to more pests and (because resistance generally seems to involve mutant receptors) for overcoming resistance problems with current targets.

Given that alternatives to *Bt* toxins for engineering into plants may not be available within the next ten years, good management of *Bt* in the interim is critical to minimize resistance problems. Two crucial 'design' issues impinge upon this problem, relating to the level and specificity of transgene expression. First, if sufficient expression levels are not achieved, then the situation is ripe for rapid selection of tolerance traits in the pest insect, in much the same way as continual use of sublethal doses of chemical insecticides selects effectively for tolerance. Indeed, such a situation has already been observed in insects selected on sublethal doses of the *Bt* delta endotoxin (e.g. Ref. [27]). Second, strong arguments have been made that the expression of the toxin should be restricted to those tissues of the plant requiring protection; other tissues would then be available as refuges for the pest, which theory suggests should impede the selection for resistance. Quite apart from these 'design' issues, many management issues are also critical to the likelihood of resistance evolving. There is fierce debate but little agreement over the benefits of rotations versus mixtures of protected and unprotected plants in this context [28, 29].

The development of genetically engineered host resistance is obviously an exciting prospect, but there are several technical problems that need to be overcome before this approach will realize its potential. Most of the problems with the engineering technologies should be soluble in the short term, but there are virtually no insecticidal genes suitable for engineering into production animals and there is a great need for alternatives to *Bt* toxins for engineering into plants. Until such alternatives are in commercial use, there is a great danger that misuse of *Bt* toxins, either delivered in transgenic plants or as microbial insecticides, could lead to resistance problems and the loss of *Bt* as a control option.

4. GENETIC ENGINEERING OF INSECTS

The availability of general insect genetic engineering technologies could have three potential benefits in pest management. First would be the engineering of pests to create genetic control systems such as have been developed with chromosomal translocations for the Australian sheep blowfly, *Lucilia cuprina* [30]. The second would be the engineering of beneficials to enhance their efficacy as natural enemies. An example would be the engineering of an insecticide resistance gene into a parasite/predator. Finally, a genetic engineering technology for insects would have value throughout applied entomology as a tool for understanding the function of strategic genes in insects of economic significance. For instance, characterization of genes involved in insecticide resistance would not only assist the management of current insecticides by providing tools for monitoring resistance, but could also lead to the design of new insecticides or synergists that break the resistance cycle. As another example, characterization of the genes involved in sexual differentiation could permit the apparent sex ratio to be manipulated, thereby providing an ability to rear only phenotypic males for use in the sterile insect technique (SIT).

So far, the only insects for which a reliable and repeatable engineering technology is available are certain *Drosophila* species. This technology has generally used the transposable element *P* as a vector. Briefly, the procedures involve the cloning of the gene of interest into a modified copy of the *P* element, injection of the gene–*P* element chimera into a preblastoderm embryo and then use of the transposition ability of the *P* element as a vector to insert the foreign gene into the host genome. However, the *P* element proves to have a highly restrictive functional range and efforts to use the element as a transfer vector to introduce marker genes into non-drosophilid insects have so far met with little success. Some transgenic mosquitoes have been recovered, but only at very low frequencies and subsequent analyses have shown that integration occurred independently of *P* element transposition (e.g. Ref. [31]).

Three other more mechanical approaches not based upon transposable elements have involved attempts to incorporate foreign DNA into sperm [32] and use of electroporation or ballistic technology to bombard embryos with foreign DNA in much the same way as has successfully been carried out with some plants [33]. To our knowledge, no insect species has been stably transformed by sperm absorption or electroporation as yet, although transient expression of foreign genes has recently been achieved in *Drosophila* embryos using electroporators [34]. However, ballistic technology has recently yielded putative germ line transformants in both *Drosophila* and a mosquito, *Anopheles gambiae* [35]. Transformation frequencies were very low and the technique would only be sufficient by itself for species where large numbers of synchronized eggs are relatively easily obtained. Nevertheless, the technique could still have value as a component of a transformation technology for other species; it could be a very useful alternative to microinjection as a means of introducing

the foreign DNA into the host nucleus, providing that an efficient biological vector system was then able to integrate the DNA into the host chromosome.

There have in fact been several recent advances in the biological aspects of the technology as well. These three critical aspects are: a suitable marker gene that allows transformants to be identified by a reliable and unambiguous phenotype; a promoter(s) to drive the expression of the marker and other foreign genes once integrated into the host genome; and the vector system to direct the incorporation of the foreign DNA into the host genome. Most marker systems in *Drosophila* have been based around eye pigmentation and antibiotic resistance; however, recent studies have shown that non-homologous markers such as the *E. coli β-glucuronidase* gene (GUS) may have generic application [36]. Likewise, the promoter system of first choice was based on the *Drosophila* heat shock protein 70 promoter, but this system is inadequate, at least in *L. cuprina* embryos, where the *Drosophila* actinC promoter gives better expression [37].

The vector system has remained the most elusive biological component of a non-drosophilid transformation technology. Nevertheless, the recent development of an in vitro transposable element excision assay has greatly accelerated work on this component. These assays, which detect and measure the excision of transposable elements in preblastoderm embryos, are both easy to perform and rapid — results are obtained within several days of microinjection. In these assays, a reporter plasmid containing a transposable element into which a genetic marker has been inserted is co-injected into preblastoderm embryos with a plasmid containing the functional transposase gene (the transposase enzyme is encoded by the transposable element and mediates the transposition event). The following day, the reporter plasmids are rescued from developed embryos and transformed into a strain of *E. coli* which permits the loss of the genetic marker to be assayed by a simple colour change. Candidate plasmids are then sequenced to determine whether the loss of the marker is associated with the excision of the transposable element. Results gained from these assays enable any particular transposable element's suitability as a transformation vector for a given species to be determined.

Excision assays have been used to demonstrate that the *P* element is capable of mobility in members of the Drosophilidae [38], but is effectively non-functional, or at least highly inefficient, in the various non-drosophilids examined. Major modifications to this transposable element would be needed if it is to be used to achieve transformation in these species.

Conversely, excision assays based on the *hobo* transposable element of *Drosophila* demonstrate that this element can be cross-mobilized in *L. cuprina* and *M. domestica* [39]. Furthermore, the ability of *hobo* to be cross-mobilized in non-drosophilids in the absence of *hobo* transposase indicates that these species may possess a similar transposable element system [39]. Another significant feature of *hobo* elements with respect to non-drosophilid transformation is that *hobo* transposase shows a high degree of amino acid similarity to both the *Ac* element of maize

and the *Tam3* element from snapdragons, suggesting a common phylogenetic origin [40]. These two plant transposable elements display the widest host range of all eukaryotic transposable elements so far examined. Our current understanding of *hobo* elements thus gives cause for optimism about their utility in the genetic transformation of *L. cuprina* and *M. domestica*, and perhaps other non-drosophilids as well.

There may also be cause for optimism about the use of *mariner*, another small transposable element originally isolated from *D. mauritiana* [41]. Like *P* and *hobo*, it transposes through a 'DNA only mechanism' but, unlike *P* and *hobo*, is capable of transposition in somatic as well as germ line tissue. *Mariner* is widely distributed in the *D. melanogaster* species group and has also been found in a distantly related genus, *Zaprionus*. *Mariner* can be used to achieve transformation of *D. melanogaster* using the same strategy as for *P* and *hobo* [42] and, given its wide distribution, may also be useful in achieving non-drosophilid transformation.

Once a transposable element based transformation system is available, two other recent developments can then be exploited to enhance both the efficiency and specificity of this technology. The first of these addresses the efficiency issue by overcoming the inability of injected plasmid DNA to replicate efficiently within insect nuclei. If the plasmids containing transposable elements could be made to replicate autonomously, there should be an increase in transformation frequency, simply because more plasmids survive through to the full development of germ line tissue. This prediction has been fulfilled in other organisms such as yeast, where use is made of autonomously replicating sequences (ARSs) which enable plasmids to replicate within yeast cells. Attempts to isolate analogous sequences from *Drosophila* have not been successful as yet; however, small palinodromic sequences have been isolated from the genome of a baculovirus from *A. californica* that, once cloned into plasmids, permit the autonomous replication of these plasmids in *Spodoptera frugiperda* cells [43]. This offers some hope that ARS like sequences from insects or their viruses may soon be available to increase the efficiency of insect transformation.

The second development which would improve insect transformation technology once established involves gene targeting technology. A major limitation of current transposable element based transformation technology in *Drosophila* has been that the transposable element, together with the foreign DNA contained in it, inserts randomly into the recipient genome. This makes it practically impossible to replace an endogenous gene with a custom mutagenized homologue; it means, for example, that the foreign gene will only affect the phenotype of the transformants if its expression is dominant over the existing endogenous gene. Recently, however, both the *P* element and a system from yeast called FLP recombinase have been manipulated so as to permit targeted gene replacement in *D. melanogaster* [44, 45]. The conditions under which gene replacements occur are still highly specific in that they require either the presence of a pre-existing *P* element or FRT sequence, respec-

tively, in the genome adjacent to the target sequence. Nevertheless, the FLP recombinase has been demonstrated to facilitate site directed plasmid to plasmid recombination in mosquitoes [46]. Clearly, neither the *hobo* equivalent of the *P* system or the FLP system are currently sufficient to provide a gene replacement technology for non-drosophilids. However, with further development of these systems, either could become the basis for highly specific gene replacement procedures in pest insects once the basic transformation technology has been established.

Finally, we consider some of the downstream research priorities that will arise once an efficient transformation technology has been developed for pest insects. Perhaps the most often discussed application is in the design of genetic control strategies. We would concur that genetic control strategies would be greatly aided by transformation technology. However, significant difficulties in the extension of this technology into the field would still remain. A system or gene must be identified which will lead to a significant and rapid decrease in the relevant insect population and this gene must first be efficiently spread throughout the population. In practice, this means that a conditional field lethal gene is needed. One such system which shows some potential in this respect is the process of somatic sexual differentiation. Investigations into this process in *Drosophila* have revealed that the apparent sex ratio of this species could be manipulated if so desired. Thus, a population of phenotypic males could be obtained and, if applied to insects such as the Mediterranean fruit fly which are currently the subject of SIT, would ensure the mass release of only phenotypically male flies.

While not devaluing such applications of transformation technology in genetic control, we believe that more powerful benefits may accrue in other areas. These benefits follow from the fact that the technology enables the molecular analysis of gene function in vivo. To give just one example, the study of *Plasmodium*–mosquito interactions will benefit greatly from the development of mosquito transformation. Genes expressed within the mosquito gut and salivary glands which are involved in *Plasmodium* transmission could be identified, isolated and analysed. Thus, the molecular basis for the *Plasmodium* refractory phenotype could be elucidated which, in turn, could provide new approaches to malaria control. For example, specific biorational insecticides could be designed which inhibit the function of those molecules and processes in the mosquito required for the passage of *Plasmodium*, thereby preventing the parasite from infecting livestock and man.

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GENETIC ENGINEERING OF INSECTS AND APPLICATIONS IN BASIC AND APPLIED ENTOMOLOGY

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Abstract

GENETIC ENGINEERING OF INSECTS AND APPLICATIONS IN BASIC AND APPLIED ENTOMOLOGY.

Insects are responsible for transmitting a wide variety of organisms to man and agricultural animals and are major agricultural pests. Control of such pest and vector populations has until now relied on the elimination of breeding sites and the widespread application of chemical insecticides. The appearance of insecticide resistance, coupled with rapidly escalating costs for developing new insecticidal compounds, and the increasing awareness of the detrimental effect insecticides have on the environment, has stimulated interest in alternative methods for controlling insect populations or their ability to transmit disease causing organisms to man and animals. What is required is the development and evaluation of a new generation of methodologies which will have a profound and long lasting effect on insect pest populations or their efficiency as vectors of disease. Biotechnology, genetic engineering and transgenic technology can play a central role in developing such methodologies. Transgenic technology in relation to insects may have potential application for population suppression. Perhaps, more interestingly, it may also provide a means for altering the vectorial capacity of insect populations. The various aspects of applying transgenic technology to insects are discussed in the paper, highlighting the requirements for being able to genetically manipulate insect genomes. The potential of the technology is then illustrated by outlining recent research by the author aimed at creating an 'incompetent' mosquito, i.e. one which blocks the transmission of malaria. There is clearly some way to go before any release of transgenic insects can be considered. The power of the technology is, however, so enormous that it must be explored not only for its potential for manipulating wild populations, but also as an analytical tool to help us understand the biology of insects and how they interact with man and his environment.

1. INTRODUCTION

Insects are responsible for transmitting a wide variety of organisms to man. This is particularly true in tropical countries where the diseases which these organisms cause, including malaria, filariasis, leishmaniasis, river blindness and

TABLE I. THE IMPORTANCE OF MOSQUITO VECTORS OF DISEASE

Disease	Causative organism	Persons infected annually	Principal mosquito vector
Malaria	Protozoa		
	Plasmodium	>500 million	*Anopheles*
Filariasis	Filarial worms		
	Wuchereria	>250 million	*Culex*
	Brugia	>5 million	
Yellow fever	Arbovirus	1 million	*Aedes*
Dengue	Arbovirus		
DHF	Arbovirus		
Japanese encephalitis	Neurotropic virus		
La Crosse	Neurotropic virus		

various viral diseases, represent the most important health care problems in the world today. Mosquitoes are particularly important as vectors of disease and Table I lists just some of the different organisms which they transmit to man and the estimated number of individuals affected by each disease annually.

The most important of the diseases transmitted by insects is malaria which, despite enormous efforts over many years, is again an increasingly important health problem. Recent estimates indicate that malaria is endemic in 102 countries with 2000 million people at risk from the disease, representing over half the world's population. There are perhaps 200 million malaria infections and 1–2 million deaths annually and clearly this one disease has an enormous impact on the health and economies of many tropical countries [1]. The incidence of malaria is increasing, due largely to the development of insecticide resistance by the mosquito vectors and by the appearance of drug resistance in the malaria parasite. These factors are exacerbated by climatic changes and migration of increasing numbers of people from non-endemic areas to regions where malaria is prevalent. Until now, control of malaria on a global scale has relied on the application of chemical insecticides to limit Anopheline vector populations. The appearance of insecticide resistance, coupled with rapidly escalating costs for developing new insecticidal compounds, and the increasing awareness of the detrimental effect insecticides have on the environment, has stimulated interest in alternative methods for malaria control. What is required is the development and evaluation of a new generation of methodologies which will

have a profound and long lasting effect on malaria transmission. Biotechnology and molecular biology can play a central role in the search for, and production of, such new tools. The development of potential anti-malarial vaccines and larvicidal compounds produced by biotechnology are obvious examples of the power of the approach. Perhaps, surprisingly, this technology has not been applied to the Anopheline vectors of malaria or other insect vectors of disease until very recently. Advances in the molecular analysis of vector–parasite relationships and vector molecular biology now make such an approach very attractive, especially as past experience has shown that vector control is an effective way of disrupting disease transmission.

2. GENETIC MANIPULATION OF INSECT VECTORS OF DISEASE

Control of insect populations by genetic means is not new. A number of vector control programmes have utilized the mass release of sterile males, the generation of cytogenetically induced sterility through translocation and the use of mating behaviour to transfer lethal or sterilizing agents between members of the populations. The strategy of sterile male release has proved to be particularly effective for the eradication of the screwworm fly from the southern part of the United States of America [2]. However, such strategies of autocidal population control have proved prohibitively expensive, since they involve the repeated mass release of treated males [3].

The advent of recombinant DNA and transgenic techniques now provides the means for the controlled genetic manipulation of insect vector genomes by the direct introduction of DNA into the germ line of these insects. Two particular advantages of using transgenic technology over classical genetics for future manipulation are evident and should be emphasized. One is the potential to exploit genes and gene constructs across species barriers and the other is the ability to introduce particular, defined sequences without the genome disruption of a conventional cross.

Transgenic technology in relation to insect vectors may have potential application for population suppression. Perhaps, more interestingly, it may also provide a means for altering the vectorial capacity of insect populations to transmit disease. For example, it may be possible to produce strains which are refractory for the pathogen, strains of the vector which have reduced vector competence or reproduction potential, or to increase the susceptibility of the vector to existing control strategies. These are clearly applications for the future. More immediately, this type of technology, and molecular biology in general, may be used as an exquisite analytical tool to begin to dissect the complex relationships between the insect vector and the disease causing organism which it transmits.

In any consideration of transgenic technology and how it may be applied to medically important insects, the following factors need to be considered:

(a) The practical requirements for creating transgenic insects,
(b) How best to apply the technology,
(c) What gene systems from insects or other organisms need to be defined in order to undertake the desired manipulations,
(d) Once transgenic insects incorporating the desired characteristics have been created, what further research needs to be carried out prior to their release into natural populations?

Each of these factors is considered in turn.

3. REQUIREMENTS FOR GENETIC MANIPULATION

Initially, we have concentrated our studies on the mosquito *Aedes aegypti*, the major urban vector of arboviral diseases such as yellow fever, dengue and dengue haemorrhagic fever [4]. These acute diseases affect millions of people and result in substantial mortality. *Aedes aegypti* is therefore an important disease carrying vector and, coupled with its suitability for laboratory research, has great potential for studies in molecular genetics [5].

In order to be able to carry out the genetic manipulation of mosquito vectors, a number of areas of basic research need to be developed. The areas to be considered include the analysis of genome complexity and organization, the methods for introducing DNA into mosquito embryos and cells, and the search for a mosquito DNA transformation system. Each of these areas is considered in turn in order to indicate what has been achieved in relation to what still needs to be done.

3.1. Genome organization and complexity

If the genome of mosquito vectors is to be genetically manipulated in a controlled and directed fashion, it is important to understand the size of the genome which is to be manipulated. Genome organization, i.e. the nature and dispersion pattern of repetitive sequences and how they are organized in relation to the coding sequences, is also important. This is because it will have a profound influence on the types of manipulation which can be envisaged and the approaches to be adopted to identify and clone sequences of interest.

Until recently, very little was known about the size and organization of mosquito genomes. Black and Rai [6] have analysed the genomic DNA from four species of mosquito, *An. quadrimaculatus, C. pipiens, Ae. albopictus* and *Ae. triseriatus*. More recently, Cockburn and Mitchell [7] have shown that the genomes of Anopheline mosquitoes are generally relatively small and exhibit what is called a long period interspersion (LPI) pattern of repetitive sequences (the euchromatic DNA consists of long, single copy sequences interrupted by a few

moderately repeated sequences). This LPI type of genome organization in Anopheline mosquitoes is in marked contrast to that found in the *Ae. aegypti* genome, which is both large and much more complex in its organization [8]. The fact that *Anopheles* mosquitoes have genomes which are relatively small [9] and an LPI organization means that, in molecular terms, they are simpler to work with and that many of the approaches used for analysis of the *Drosophila* genome (which also has an LPI organization pattern) are directly applicable to *Anopheles*.

3.2. Methods for introducing DNA into mosquito cells and embryos

The generation of transgenic mosquitoes requires a means for introducing DNA into the germ line of the insect as well as an efficient DNA vector system which will allow stable integration of the introduced DNA. Microinjection systems have now been developed for a number of mosquitoes including *An. gambiae* [10], *Ae. triseriatus* [11] and *Ae. aegypti* [12]. The systems which have been developed are derived from that employed for *Drosophila* transformation modified to take account of differences in the physiology and development times between *Drosophila* and the mosquito species studied.

The DNA which was introduced into the mosquito embryos in these experiments was the pUChsneo vector/helper system based on the *P* transposable element from *D. melanogaster* [13]. An antibiotic resistance gene (*neo*) forms part of this DNA vector system. The presence of this gene, if it becomes integrated into the mosquito genome, confers resistance to the synthetic antibiotic G418. Progeny of those individuals which survive microinjection are exposed to G418, so that only those mosquitoes which have integrated the injected DNA may be selected. Experiments with this vector/helper system have served to demonstrate that DNA may be injected into mosquito embryos with survival rates comparable to those in work with *D. melanogaster* [14]. Integrations of the introduced *P* element DNA have been observed in both *Anopheles* and *Aedes* mosquitoes and the integration events appear, in some cases, to be heritable and clearly involve the germ line of the mosquitoes involved. Although these events did not result from normal *P* element transposition, some functional role of the *P* sequence cannot be excluded.

As indicated above, embryonic transformation has shown some limited success. However, the transformation of cultured cells has, thus far, provided greater information regarding mosquito gene expression and its control. This is due, in part, to the simpler methods of introducing DNA into cells in culture and the more efficient screening of transformed lines. Several transfection methods are successful for mosquito cell cultures [15, 16]. These include the use of polybrene, a divalent cation, together with a glycerol shock treatment [17], and Lipofectin® , a patented lipid compound, which forms complexes with DNA and fuses with cell membranes [18].

Development of this transfection technology has allowed recent advances in mosquito molecular biology. Transient expression assays, with a DNA vector comprising the *Drosophila* heat shock protein promoter (*hsp70*) fused to the chloramphenicol acetyl transferase (*CAT*) gene, have elucidated various aspects of the heat shock response of mosquitoes. Similar studies have shown that other heterologous promoters, including the constitutive rat actin promoter, and the inducible *Drosophila* metallothionein promoter [19, 20] are recognized by the mosquito. Further work will define control sequences from cloned mosquito genes, such as those of the tubulin and heat shock genes.

More recently, we have identified a vector construct that stably transforms mosquito cells to antibiotic resistance. Southern blot analysis and in situ hybridization of transformed lines have revealed that the vector becomes integrated, both singly and in tandem arrays. In addition, the construct remains in an extremely stable chromosomal position during prolonged culture in the absence of selection. In an extension of this work, we have demonstrated that separate non-selected DNA can be stably transferred and expressed in mosquito cells along with the selectable marker gene of the vector DNA. Thus, it is possible to introduce virtually any gene into a mosquito cell. In the near future, such gene expression studies will be used to overproduce useful proteins in culture [20].

It is clear from this work, however, and the experiments involving microinjection of the *P* element into embryos that the *P* element system in its present form is not suitable for routine use in the mosquito. Thus, while the means are currently available for introducing DNA into both mosquito embryos and cultured cells, the major stumbling block is currently the lack of an appropriate DNA vector system for manipulating the mosquito genome.

3.3. Search for a mosquito DNA transformation vector system

As indicated above, the DNA vector systems currently available are not ideal for creating transgenic mosquitoes. Attempts are being made to modify the *P* system for more general use [21] but alternative elements, such as *hobo* from *Drosophila* and the *Ac* element from maize, are also being investigated for their usefulness in the mosquito system. In addition, there is increasing interest in identifying mosquito elements with the properties of mobile or transposable genetic elements (TGEs). Such an element could form the core of a mosquito transformation vector. Development of this system first requires the identification, isolation and characterization of such endogenous mosquito TGEs.

A number of approaches have been taken to identify such mobile elements in mosquitoes. One of these is to analyse specific gene systems, such as the ribosomal DNA of mosquitoes, in order to detect variants of these genes arising from the insertion of a mobile element. No such insertions have, as yet, been detected in *Ae. aegypti* rDNA [22] but insertion events have been detected in the rDNA of

An. gambiae and these elements are being fully defined [23]. The elements appear to resemble a particular class of TGEs, non-viral retroposons. It is unlikely, however, that these elements will prove ideal as transformation vectors because of the ill defined nature of their mode of transposition.

We have recently adopted an alternative strategy to directly identifying a specific class of TGEs, known as retrotransposons, in the mosquito DNA. The approach relies on utilizing the characteristic biochemical and structural properties of these elements to identify them. This has led to the successful isolation of several retrotransposon like elements from the *Ae. aegypti* genome [24, 25]. More recently, we have used the polymerase chain reaction (PCR) to develop a particularly rapid methodology for identifying endogenous retrotransposon like elements in mosquito DNA [8, 26]. Once such elements have been isolated, fully characterized and their ability to transpose autonomously established, they may be engineered to form the core of a transformation vector system.

Any DNA vector system which is to be of practical value will have to incorporate a number of features in addition to its ability to transpose. Most important of these are a selectable marker system and specific promoter or enhancer sequences. As indicated above, current DNA vectors incorporate an antibiotic resistance gene which allows for selection of transformed individuals by exposure to G418. However, this system is less than satisfactory because different mosquitoes exhibit a spectrum of sensitivity to G418 and, more importantly, because it depends on high levels of expression of the resistance gene in the transformed mosquitoes. The method of choice would be the use of a phenotypic marker, such as eye colour, so that transformed individuals may be identified by direct visual inspection. Such a method requires mutant strains of mosquitoes and the corresponding cloned gene coding for an easily scored phenotype which can be incorporated into a transformation vector. Although eye colour mutants of both *Ae. aegypti* and *An. gambiae* are available, cloning of the genes responsible for these phenotypes has not yet been completed.

Finally, a considerable body of work using the pUChsneo transformation vector, both in cultured mosquito cells and injected embryos, has indicated that the *Drosophila* heat shock promoter sequence is not entirely satisfactory for driving the expression of genes in mosquitoes because of its low efficiency and the high temperatures required to induce expression [20]. In addition, at some stage it will be desirable to express defined genes in mosquitoes in a tissue or stage specific fashion. For this to be envisaged, stage and tissue specific promoters that function in the mosquito have to be defined. None are, as yet, available but attempts to characterize the DNA sequences responsible for regulating the expression of genes encoding abundant proteins in mosquitoes are well under way [27].

All of these different aspects of basic research need to be pursued in parallel and eventually amalgamated to provide a mosquito DNA vector system of real practical value.

4. POTENTIAL APPLICATION OF TRANSGENIC TECHNOLOGY IN INSECT SYSTEMS

Once the systems necessary to create transgenic insects have been developed, how can this technology be applied? Two aspects are discussed in order to illustrate the potential of the technology. The first deals with the use of the technique for analytical purposes and the second with applying transgenic technology to medically significant mosquito populations.

4.1. Transgenic technology as an analytical tool

The introduction and insertion of a mobile genetic element at or near a particular locus can cause that allele to mutate, producing a structural or developmental effect. In *Drosophila*, in particular, TGEs have been used as mutagens in order to clone genes or gene clusters of interest via transposon tagging [28]. In essence, the TGE is introduced into the germ line of the insect by microinjection of the embryo, and the progeny scored for mutants in the phenotype of interest. Subsequently, cloned TGE probes are used for in situ hybridization to chromosomes of mutant and wild type individuals. This identifies a TGE 'newly' integrated at or near the genetic locus of interest. DNA clones are then retrieved from a genomic library prepared from the mutant stock using the TGE DNA as a probe. DNA sequences adjacent to the TGE in such clones represent the gene for the locus of interest. This approach is an extremely powerful application of the technology, since it allows the cloning of genes purely on the basis of their function.

4.2. Transgenic technology in insect populations of medical significance

As discussed above, insects are important vectors of disease to both man and agricultural animals. In this way, vector populations may have a profound impact on the health and economy of a region which in many cases, particularly in the tropics, are the most fertile and potentially productive areas. Transgenic technology may eventually have a role to play in controlling vector borne disease by providing the means to suppress vector populations by rendering them vulnerable to subsequent control measures, such as insecticide susceptibility, temperature sensitivity or ability to survive diapause. A second possibility and, perhaps, a more exciting approach, would be to alter the ability of the insect to transmit the disease. Clearly, such possibilities are for the future, but in the shorter term it is quite feasible to consider genetic manipulation of insect populations of direct commercial value, such as the honey bee or silk moth. Here, transgenic technology may be employed to confer a number of beneficial characteristics to these insects to create novel and highly productive strains. For example, the insect may be manipulated to increase the yield of the product by increasing the growth rate or by enhancing the resistance of the insect to infection, temperature shock or other detrimental factors.

4.2.1. *Potential target genes for manipulation*

Having discussed the types of manipulation of insect genomes which may be beneficial in economic or health terms, it is worth considering the types of gene system which may be potential targets for manipulation to achieve these aims. In this respect, there are a number of obvious targets for manipulation, including the genes involved in the insect immune system, developmental control genes and insecticide resistance genes. Genes influencing all of these factors have now been characterized at the molecular level for a number of different insects and it is now feasible to consider manipulating them in the germ line of these insects. In addition, a number of genes are of particular interest because they are directly implicated in the ability of insects to transmit disease causing organisms. Examples include the filarial susceptibility (f^m) and Plasmodium susceptibility (*pls*) loci of the mosquito, *Ae. aegypti*. The f^m locus is genetically well defined and there is good data on its linkage relationships. Refractoriness to infection is due to a partially sex linked, dominant gene [29]. There is marked variation in the susceptibility of *Ae. aegypti* to different filarial worms, although all of the alleles concerned map at about the same place on the sex determining chromosome. Also of particular interest is a strain of *An. gambiae* which has been selected for refractoriness to the malaria parasite and characterized genetically [30]. Attempts are currently under way to clone these genes, but it is difficult to undertake such a cloning exercise in the absence of any knowledge of the gene product. Clearly, the use of transgenic technology through transposon tagging will assist in the characterization of refractory genes and their products.

4.2.2. *Creating an 'incompetent' mosquito*

An important genotypic characteristic not met by the majority of genes encoding refractoriness is that any such gene introduced into the insect would have to be capable of altering the phenotype through the expression of a single gene copy. Unfortunately, at present there is no gene or gene product defined at the molecular level which is known to directly affect phenotype in relation to pathogen development in, or transmission by, any insect. However, in the mosquito system a number of molecules are known to affect the transmission of malaria by Anophelines. Foremost among these are the so called transmission blocking vaccines, which can achieve a total transmission blockade [31]. These vaccines attack antigens present on the gametes and ookinetes of the malaria parasite, and antibodies which recognize these antigens are able to block the development of the parasite in the mosquito midgut. A very exciting possibility therefore is to introduce the genes coding for such antibodies into the mosquito genome, thus directly conferring the transmission blocking phenotype to the insect. In this case, a transgenic mosquito would be created incorporating an antibody gene expressed in the insect midgut in response to a blood meal and which therefore blocks transmission of malaria.

This type of approach is an attractive one for a number of reasons. It eliminates the need for detailed molecular analysis of refractory mechanisms in mosquitoes and it would be a 'dominant' gene system (i.e. one gene copy only would be needed in each cell of the mosquito). The antigen target on the stage of the malaria parasite present in the mosquito may not be under the same selection pressure as other malarial antigens because it is not normally exposed to the human immune system. One implication of this may be that the transmission blocking antigens are less likely to exhibit extensive variation, so the parasite may be less able to avoid this type of transmission control mechanism. To date, evidence suggests that the ookinete antigens are, indeed, highly conserved. Another advantage of the approach is that the antibody molecule would only be expressed by the mosquito midgut following a blood meal, so that such a genetic manipulation may not reduce the overall fitness of the individual mosquito to any great extent. Finally, use of transgenic insects incorporating an antibody gene could be applied to any vector transmitted pathogen (parasite or viral) where a target antigen can be identified as being inhibited by the expressed molecule.

We are currently involved in creating a transgenic mosquito incorporating a transmission blocking antibody gene under the control of a blood meal induced, gut specific promoter sequence. This project is being undertaken with funding from the European Economic Community by R. Sinden (Imperial College, United Kingdom), A. Crisanti (University of Rome, Italy) and R. Galler (Fiocruz, Brasil); Fig. 1 illustrates the steps involved in generating this type of transgenic mosquito. Figure 2 illustrates how such an 'incompetent' transgenic mosquito would disrupt the transmission of malaria. If successful, transgenic mosquitoes expressing antimalarial antibodies may represent a potential strategy for controlling malaria and establish a precedent for a wide range of new antidisease strategies in many vector borne diseases.

4.2.3. Transgenic mosquitoes in natural populations

Once transgenic insects with the necessary characteristics have been created, there remains the question, what next? Clearly, if the manipulated insects are themselves to be cultivated for production, it may be possible to directly apply novel strains created by transgenic means. However, where this is not the case, it is necessary to consider the problems likely to be faced in applying the technology in experimental and natural populations. It may well be that such a situation would disrupt the normal adaptive process and therefore be opposed by natural selection. If this is so, then some form of drive mechanism may be needed to force the desired gene through the population. This is not an alien concept to those who have worked on the genetic control of insect populations. However, testing of such mechanisms has been limited since, in reality, they have awaited the advent of recombinant DNA technology to provide the necessary raw material.

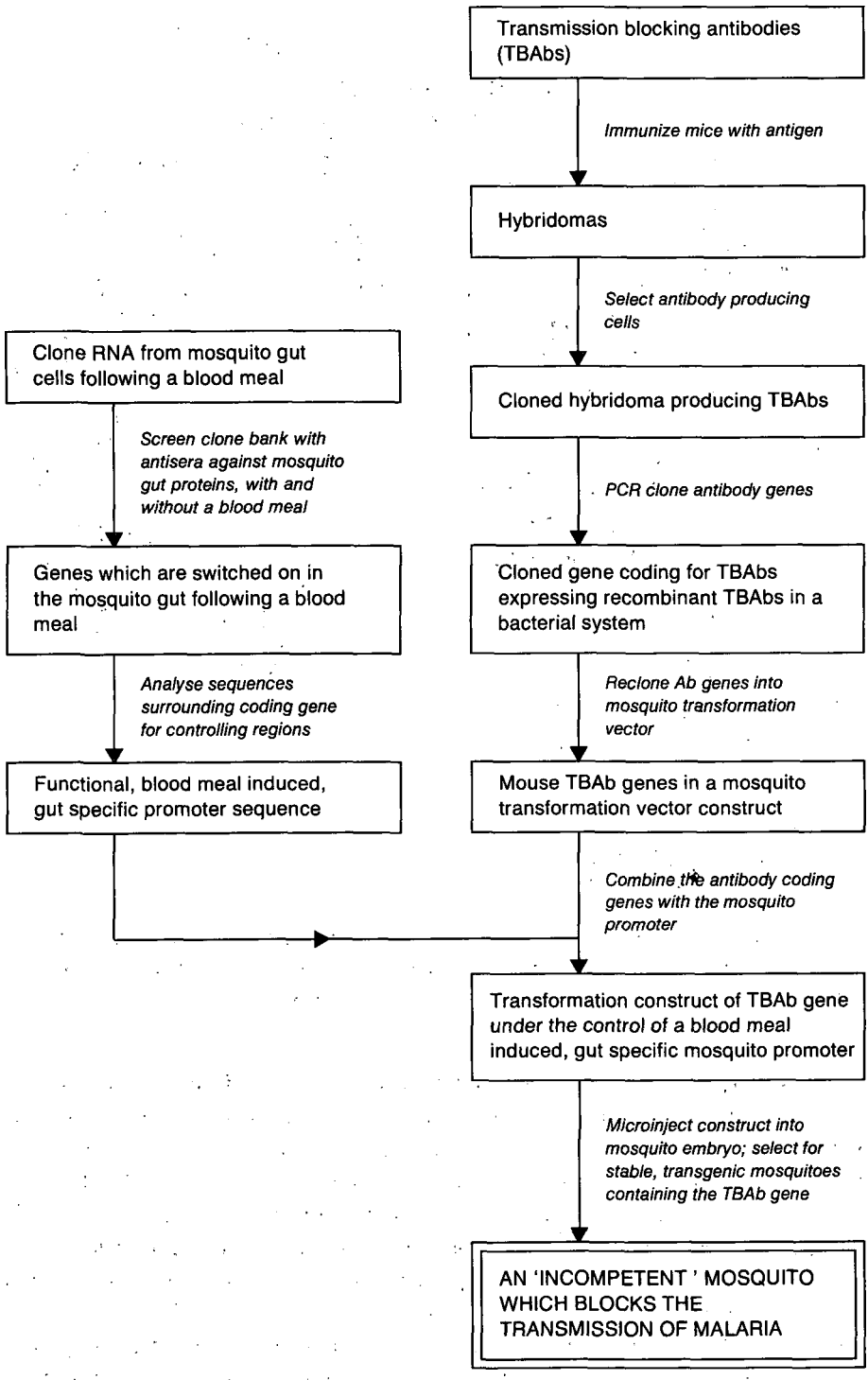

FIG. 1. Creation of a transmission blocking or 'incompetent' mosquito.

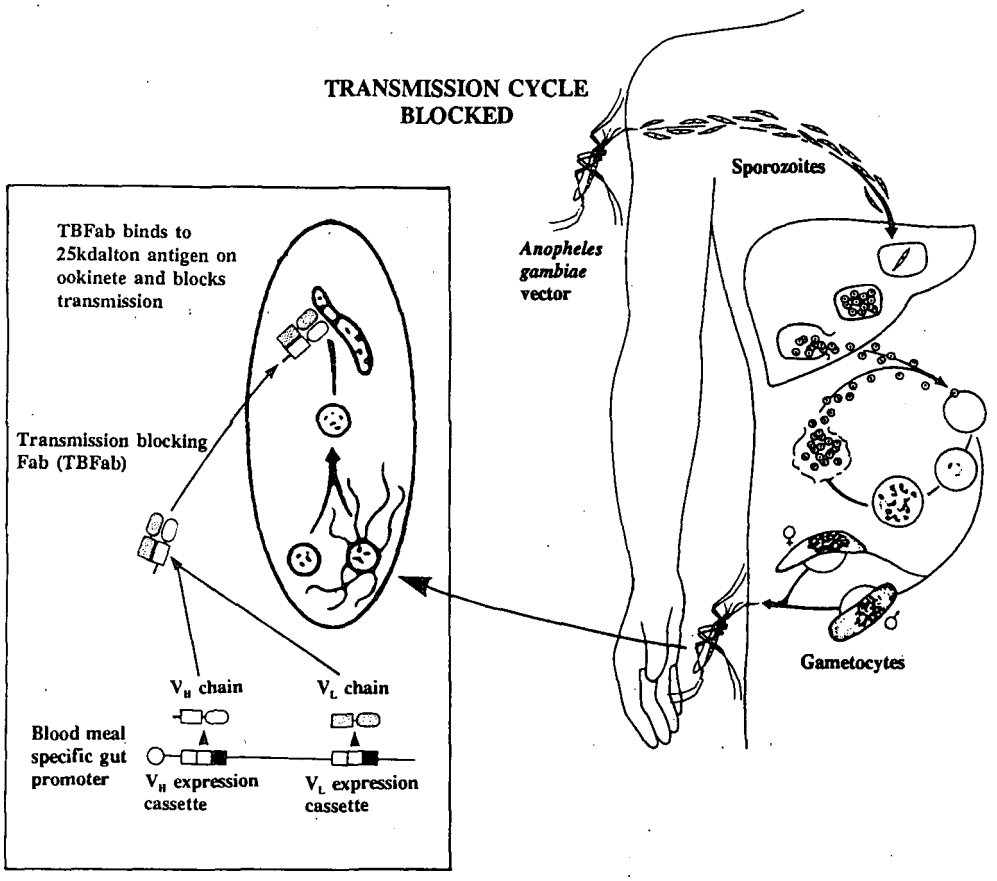

FIG. 2. Life-cycle of the malaria parasite and how transmission may be disrupted by generating a mosquito which expresses transmission blocking antibodies in the gut when the mosquito takes a blood meal infected with the malaria parasite.

Two types of drive mechanism have been suggested. One is meiotic drive, where a given chromosome is transmitted to more than the expected 50% of offspring. Any desirable genes linked to the driven chromosome would eventually approach fixation, even with the release of relatively few individuals. There is experimental evidence to support the use of meiotic drive in *Ae. aegypti*. This mechanism, driven by the M^D locus, has been used to force the marker gene *re* (red eye) into a laboratory cage population [32]. Interestingly, meiotic drive also occurs during hybrid dysgenesis and it might, therefore, also be possible to exploit this phenomenon by using either the *P* element itself, or a mobile element with properties similar to *P*, as an efficient mechanism to drive a specific gene construct through

an insect population. The second type of drive mechanism is the exploitation of genetic traits that reduce heterozygote fitness [33]. For example, the gene to be driven could be introduced into a translocation chromosome such that viable and fertile homozygotes were formed, whereas heterozygotes would display reduced fertility or viability. In this way, translocations, pericentric inversions, inter-racial hybrid sterility, cytoplasmic incompatibility and compound chromosomes all have potential since, in each case, hybrids have reduced fitness. Such mechanisms require larger release numbers, since there is no exponential increase in the frequency of the driven chromosome as with meiotic drive. However, fixation of desirable genes would occur more quickly than with meiotic drive because of the reduced fitness of the heterozygous combinations. Certainly in the case of vector populations, the most useful end result of such programmes would be the progressive replacement rather than the eradication of disease transmitting populations since an emptied ecological niche might be colonized rapidly by the migration of wild type insects.

5. TRANSGENIC INSECTS: THE FUTURE

Eventually, embryo transformation will provide the raw material to test the proposed drive mechanisms in laboratory and natural populations. The questions posed by considering the release of transgenic insects emphasize the need to assess the biological consequences of such a release. It is, however, difficult to gauge the possible hazards of such a release in the absence of experimental evidence and these ethical and safety considerations need to be faced at an early stage. In order to undertake an informed appraisal where the possible net benefits may be balanced against the potential hazards, considerable effort will have to be devoted to utilizing caged populations and the controlled release of molecularly tagged individuals together with mathematical modelling of these populations. There is clearly some way to go before any release of transgenic insects can be considered. The power of the technology is, however, so enormous that it must be explored and there is every indication that over the next few years the potential of transgenic technology in insects will be fully exploited.

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ERADICATION OF THE MELON FLY FROM OKINAWA, JAPAN, BY MEANS OF THE STERILE INSECT TECHNIQUE

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Abstract

ERADICATION OF THE MELON FLY FROM OKINAWA, JAPAN, BY MEANS OF THE STERILE INSECT TECHNIQUE.

A national project to eradicate an introduced pest, the melon fly, from Okinawa was carried out from 1972 to 1992 using the sterile insect technique. After the release of about 50 000 million sterile flies, involving about ¥. 10 000 million as investment, the melon fly was completely eradicated. The most important conditions for success were the maintenance of a quality mass reared strain and availability of precise information on the temporal and spatial distribution of wild and released flies for improvement of the release plan.

1. HISTORY

The project to eradicate the melon fly (*Bactrocera cucurbitae*) from Okinawa Prefecture was begun in 1972. There are many islands between mainland Japan and Taiwan (China) and these are called the Southwestern Islands. Okinawa Prefecture is located at the southwestern part of the island group, while the northern part belongs to Kagoshima Prefecture. Okinawa Prefecture consists of three groups of islands, the Okinawa Islands, the Miyako Islands and the Yaeyama Islands (Fig. 1). All are situated in the subtropical rain forest zone. Although most parts are now cultivated, rain forests still remain in the mountainous areas of Iriomote and Ishigaki and the northern part of Okinawa Hontô (the main island of Okinawa). The population density of the melon fly in these rain forest areas was low because host plants are not abundant. The Miyako Islands and the southern part of Okinawa Hontô are flat and the original vegetation has been destroyed and turned into crop fields and bush. The melon fly density in these areas was high.

Figure 1 also shows the spread of distribution of the melon fly to these islands. The melon fly was first discovered on Ishigaki Island in 1919, and it then invaded the Miyako Islands in 1929. It was found on Kume Island in 1970 and spread to Okinawa Hontô in 1972. After that, the distribution range widened rapidly to the northern islands belonging to Kagoshima Prefecture [1].

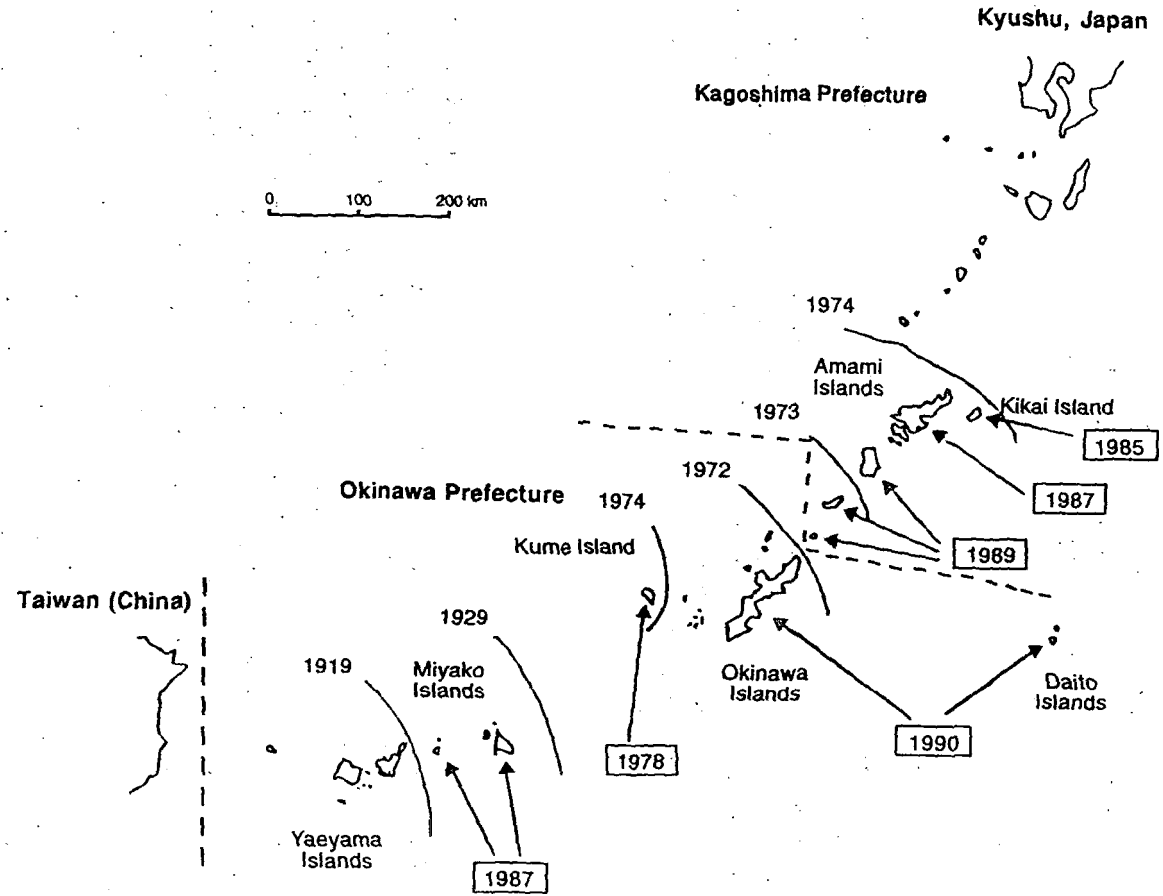

FIG. 1. Distribution and eradication of the melon fly in the Southwestern Islands, Japan. Unboxed and boxed numbers show years of distribution and eradication, respectively. The broken line shows the border of the Prefecture.

In Okinawa, a pilot eradication project was initiated on Kume Island in 1972, and the species was successfully eradicated in 1978 from this island [1, 2]. A large scale project to eradicate the melon fly from all of Okinawa was then carried out in the following sequence: Miyako Islands (from 1984 to 1987), Okinawa Islands (from 1986 to 1990) and Yaeyama Islands (from 1989 to now).

2. MASS REARING, STERILIZATION, RELEASE AND MONITORING

For this large project, the Government established large mass rearing and irradiation facilities which could produce a maximum of more than 200 million flies (males and females) per week [3]. Although the composition of larval and adult diets was basically the same as that developed in Hawaii, it must be noted that we used (a) pumpkin juice for the oviposition stimulant; (b) a round surface egging apparatus (in place of a flat surface of gauze, as in many medfly mass rearing facilities), and (c) natural day length in the adult room [1, 3, 4]. These rearing conditions are different from many mass rearing systems of fruit flies used in other countries. We decided to use them for the maintenance of natural genes in our mass rearing stock. Although the male mating competitiveness of long term mass reared fly stock is weaker than that of wild stock (see Ref. [5]), the quality of mass reared/sterilized males was kept at a reasonable level until the last stage of the project.

The pupae were irradiated by gamma rays at a dose of 70 Gy three days before adult eclosion. The pupae were mixed with fluorescent dye and sent by aircraft to the target islands. Although pupal release was accomplished in the Kume project [1, 2], we later used a chilled adult release system. Chilled adults were distributed over the islands from helicopters.

For monitoring, traps baited with cuelure and insecticide (naled, etc.) were set in the target areas. The total number of trap sites was about 550. The traps were surveyed once every two weeks and captured specimens were checked for fluorescent dye marking under an ultraviolet light; thus sterile and wild flies were counted separately. Another method used to evaluate the efficacy of SIT was to examine the infestation rate of host plants. Thousands of host fruits, mainly fruits of a wild cucurbit, *Bryonopsis laciniosa*, were collected once a month.

3. ESTIMATION OF POPULATION DENSITY

Before beginning the mass releases of sterile flies, we estimated the population density of the wild fly in various vegetations using a mark–recapture method. A modified Jackson positive method (the method of Itô and Hamada, Refs [6, 7]) and the Jackson negative method [8] were used to calculate the density and survival rate of wild males. We found that the density of the melon fly in mountainous areas was

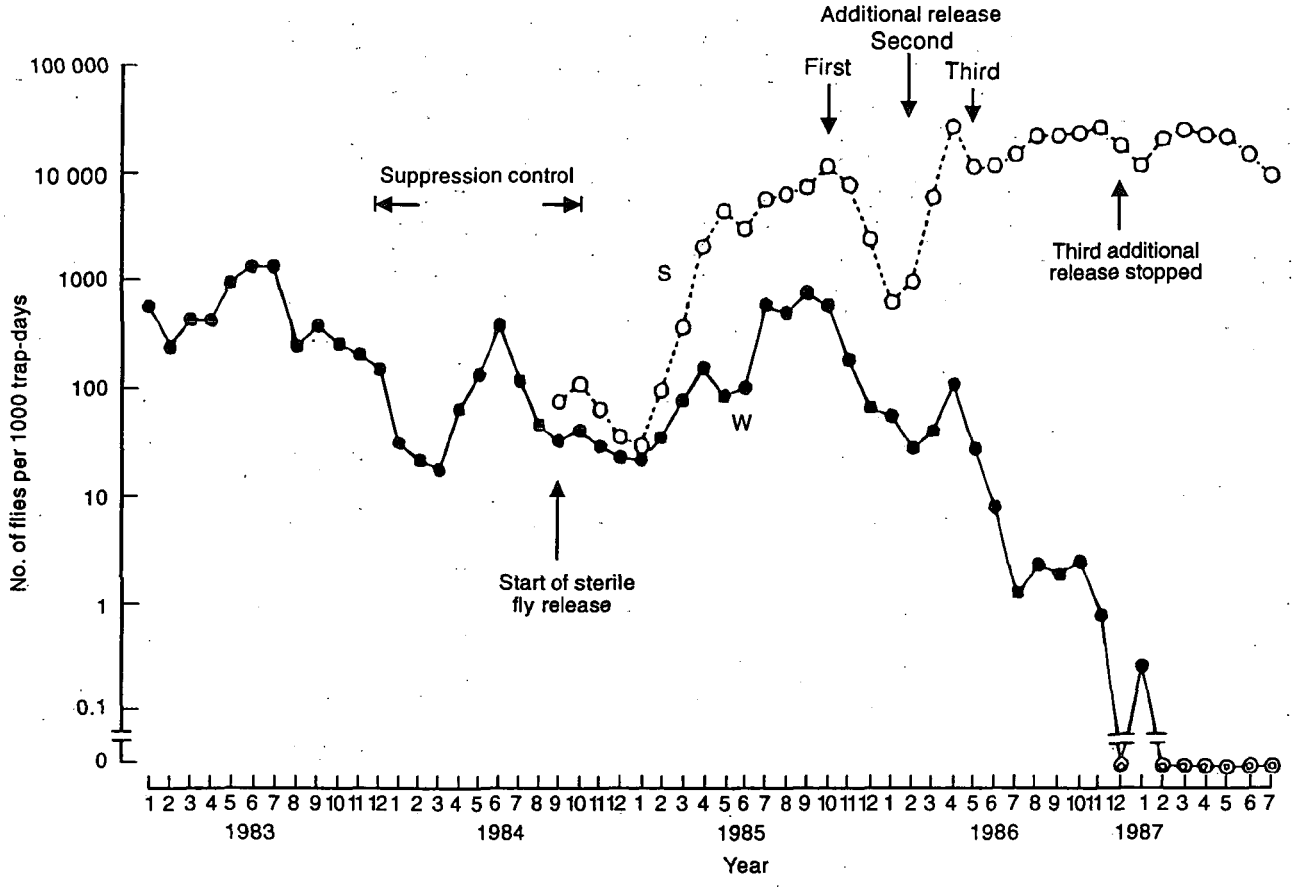

FIG. 2. Monthly change in the abundance of sterile (S) and wild (W) melon flies caught by monitor traps on the Miyako Islands.

usually less than 10/ha [9], while the density in crop fields and bushy areas sometimes reached 600/ha [7, 10]. As the maximum density in non-mountainous areas was higher than the capability of the mass production facilities, we suppressed the fly population in high density areas using the male annihilation technique before releasing sterile flies. Cotton strings soaked in cuelure and insecticide were distributed from a helicopter at a dose of 40 strings per hectare per month. We carried out this population suppression technique to decrease the density of wild flies to one tenth to one twentieth of the natural density.

4. ERADICATION FROM THE MIYAKO ISLANDS

Figure 2 shows the monthly change in the number of sterile and wild melon fly males caught by monitor traps in the Miyako Islands (total area: 227 km^2). Sterile fly release on the Miyako Islands began in August 1984; at first 30 million flies per week were released evenly in the islands. During the initial seven months, the number of sterile males caught by traps was low (about 100 per 1000 trap-days) because of low temperature and low fly quality. Early in 1985, the fly quality was improved and the number of sterile flies increased to about 10 000 per 1000 trap-days.

Figure 3 shows the local distribution of the abundance of sterile and wild melon flies in August 1985. There were high density areas in some parts of Shimoji Village, which is a well known vegetable producing area. We therefore decided to release additional flies into these high density areas. Figure 4 shows the additional release area in the Miyako Islands. At first, an additional six million flies were released per week in the Shimoji area from October 1985. The second and third additional releases were carried out in high density areas. The maximum number of flies released in the Miyako Islands was 48 million per week. Figure 5 shows the distribution and abundance of flies caught in June 1986. At this time, wild flies were caught at only a few points and the number was very small. Since February 1987, no wild flies have been caught in the Miyako Islands.

Figure 6 shows the change in infestation rate of host fruits. Until April 1986, the infestation rate lay between 0.1 and 10%, but it rapidly decreased from May 1986, and became zero in November 1986. In November 1987, the Government announced that the melon fly had been eradicated from the Miyako Islands after the release of 6340 million sterile flies.

5. ERADICATION FROM THE OKINAWA ISLANDS

The project to eradicate the melon fly from the Okinawa Islands was begun in November 1986, while the Miyako project was still under way. The release of sterile

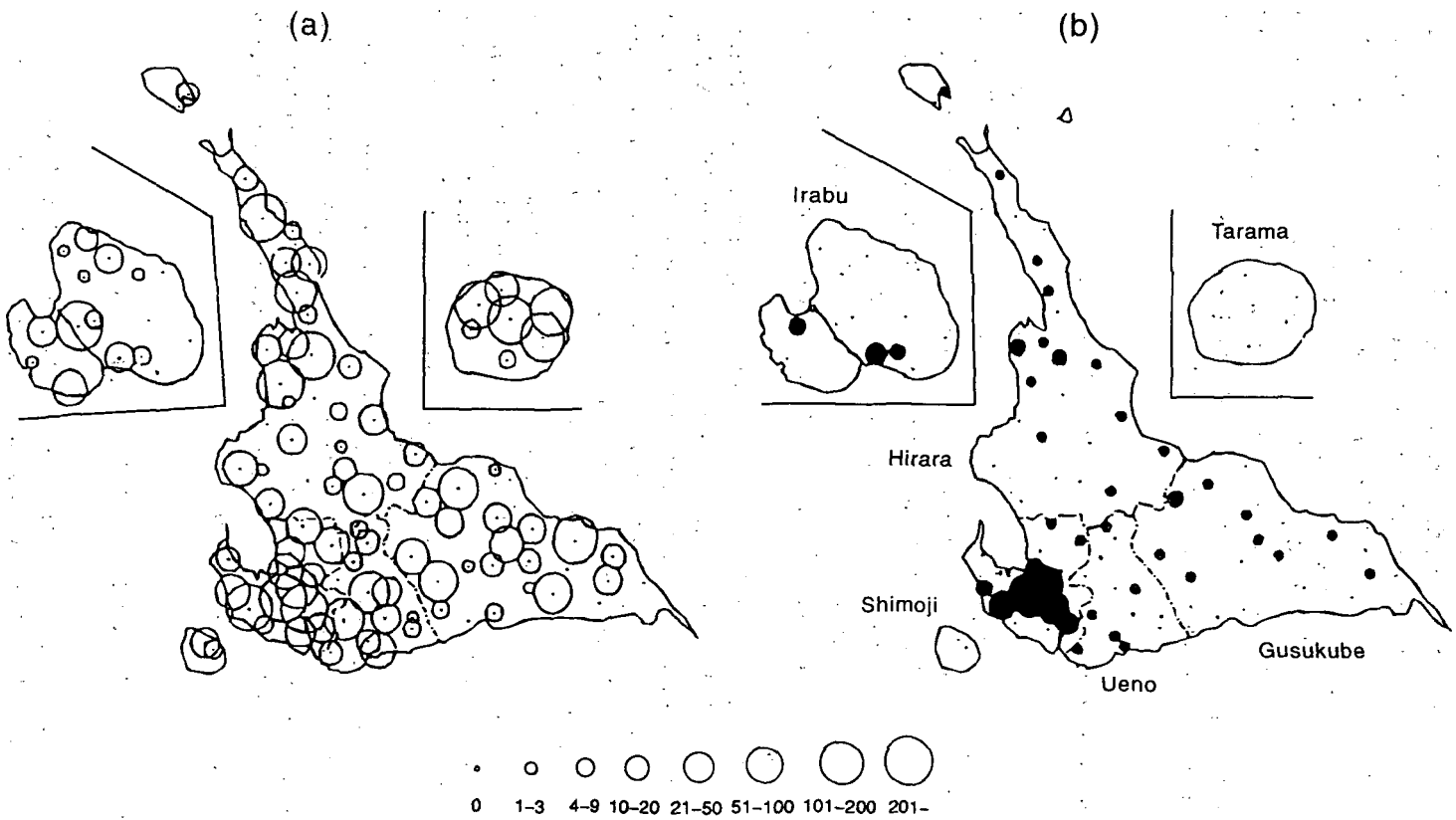

FIG. 3. Distribution and abundance of (a) sterile (○) and (b) wild (●) melon flies caught by monitor traps on the Miyako Islands in August 1985. The size of the circles indicates the number of flies trapped.

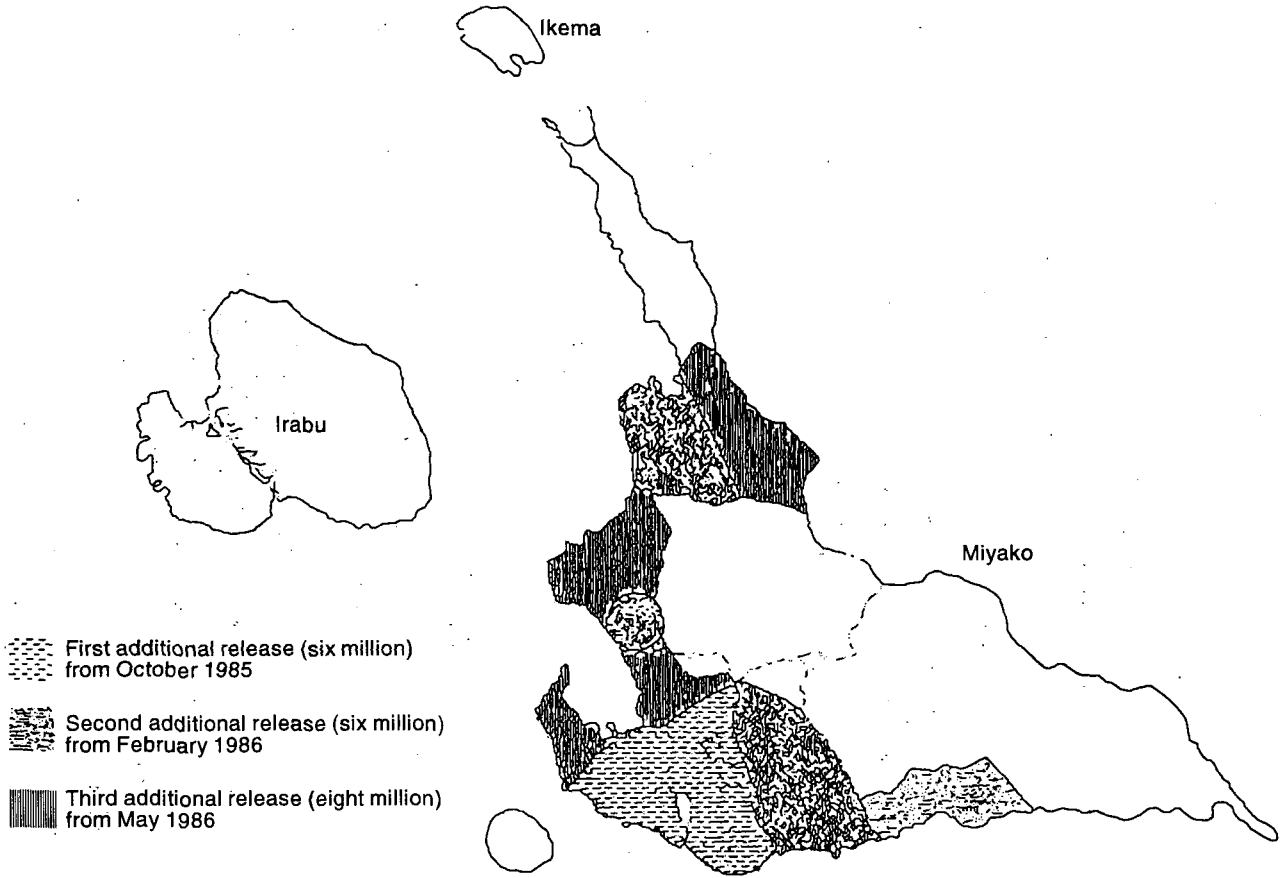

FIG. 4. The additional release areas on the Miyako Islands.

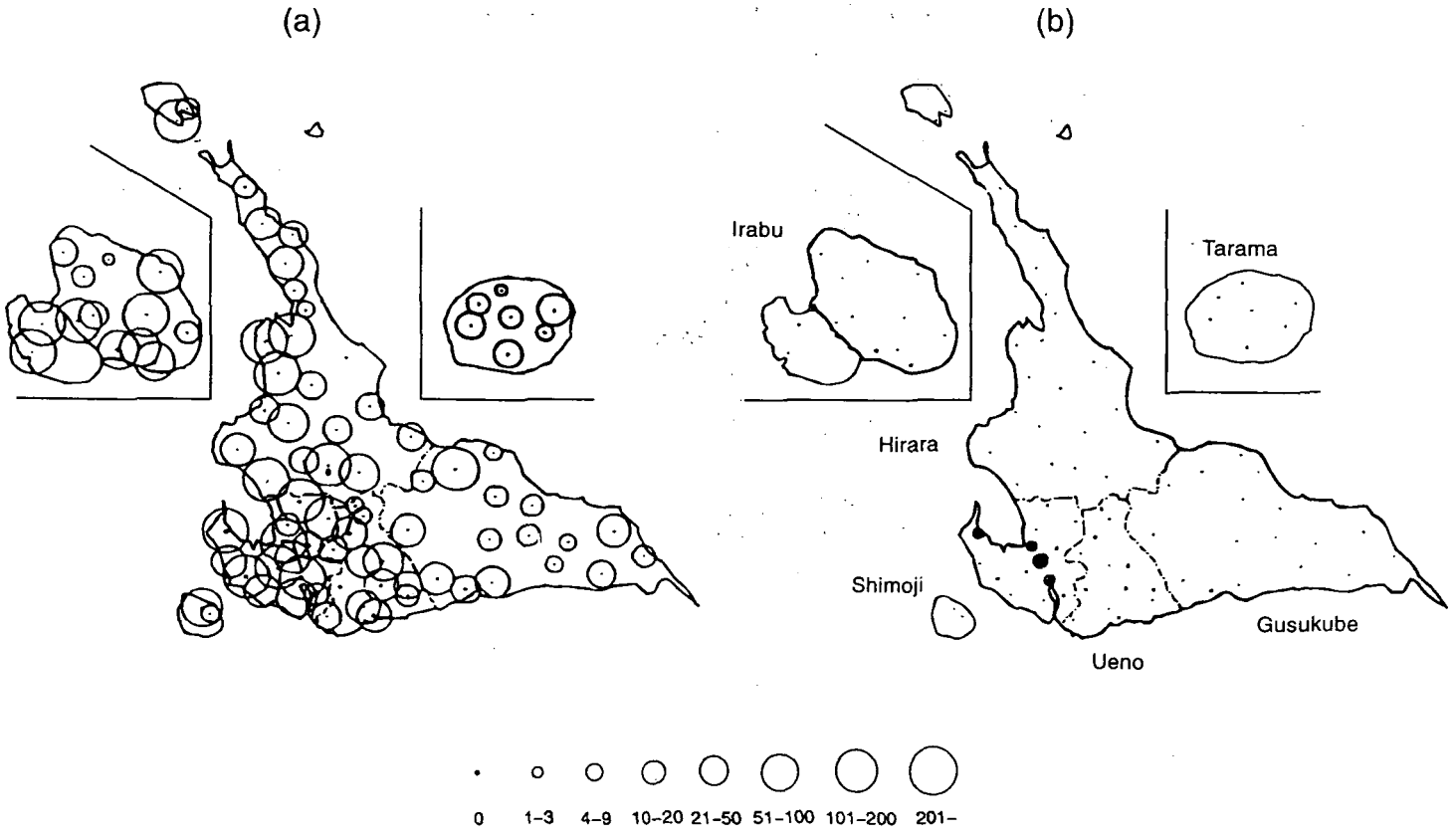

FIG. 5. Distribution and the abundance of (a) sterile (○) and (b) wild (●) melon flies caught by monitor traps in June 1986.

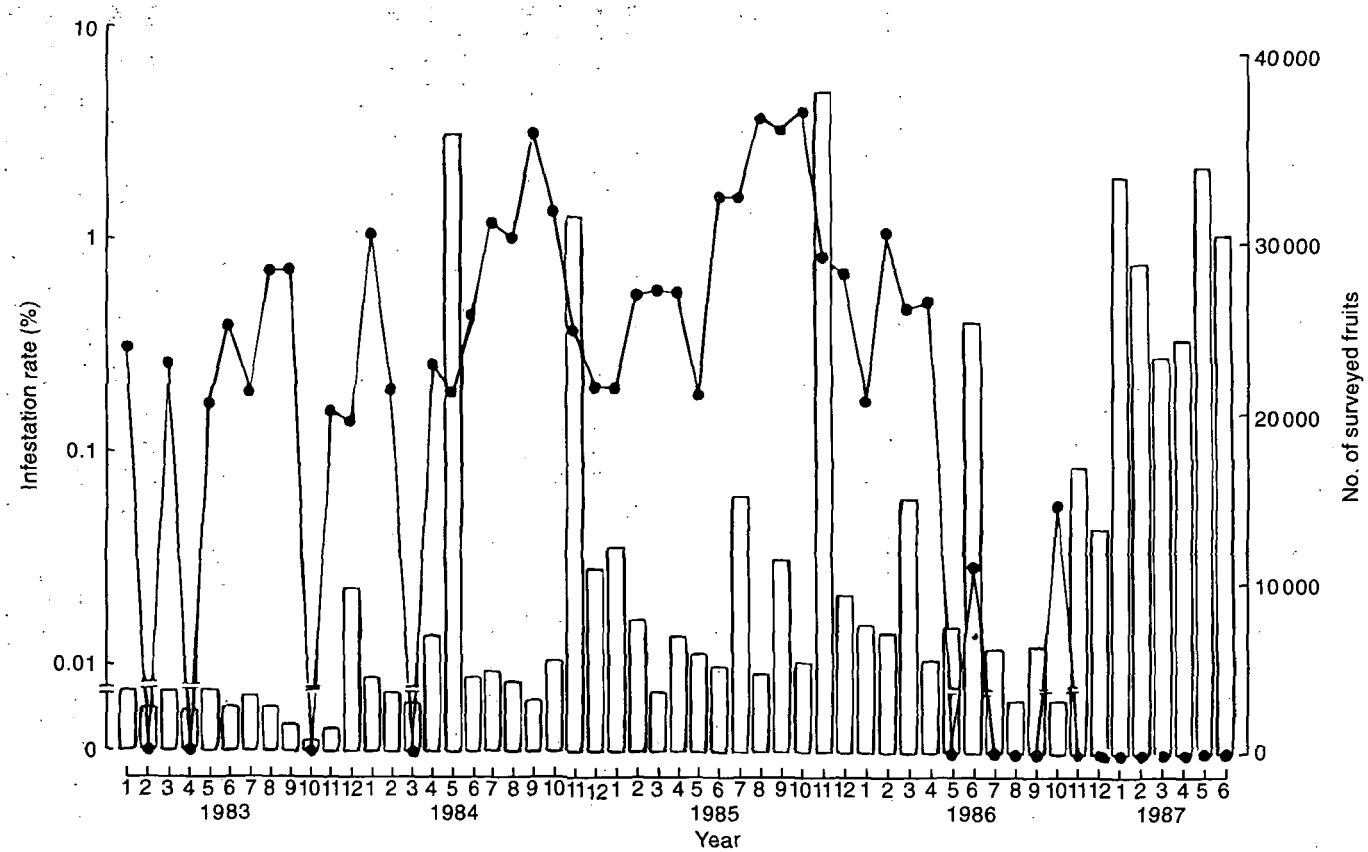

FIG. 6. Number of surveyed host fruits (histograms) and rate of melon fly infestation (●) on the Miyako Islands.

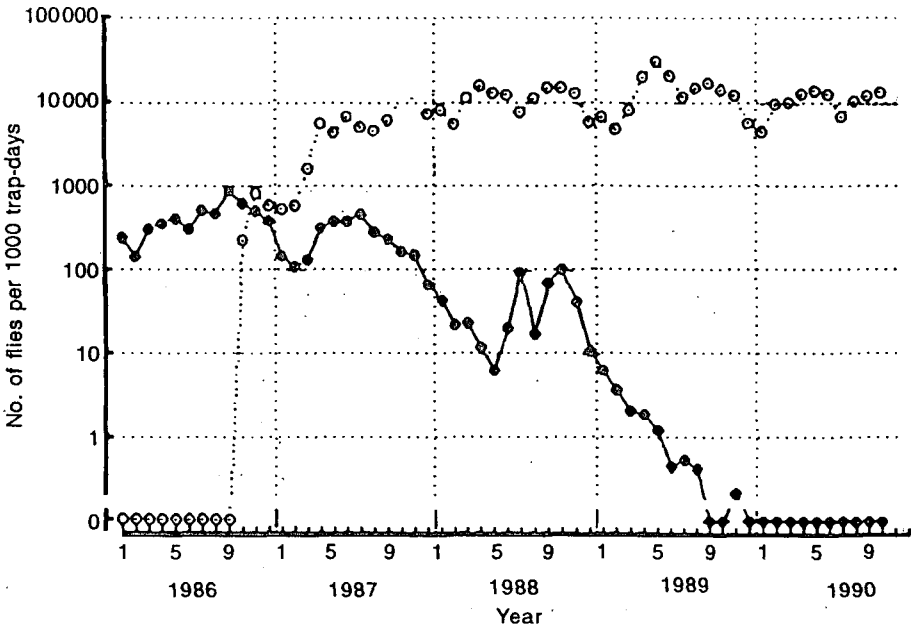

FIG. 7. Monthly change in the abundance of sterile (⊙) and wild (●) melon flies caught by monitor traps in the Okinawa Islands.

flies was begun from the southern part of Okinawa Hontô; the target areas were then expanded to the northern part and adjacent islets. As in the Miyako Islands, wild flies were caught until 1989 in the main crop producing areas and concentrated releases were made into these areas. The maximum number of sterile flies released in the Okinawa Islands was about 170 million per week.

Figure 7 shows the results of monitor trap surveys on the Okinawa Islands. Since December 1989, wild flies have not been detected. The Government announced in November 1990 that the eradication of the melon fly from the Okinawa Islands had been achieved. The total number of sterile flies released was 30 940 million.

6. ERADICATION FROM THE YAEYAMA ISLANDS

The project to eradicate the melon fly from the last target area, the Yaeyama Islands, was begun in October 1989. The number of sterile flies released was 90 million per week. From September 1991, no wild flies were caught with monitor traps (Fig. 8). We consider that melon flies from the Yaeyama Islands were eradicated. About 12 000 million sterile flies are planned to be released in this area until March 1993.

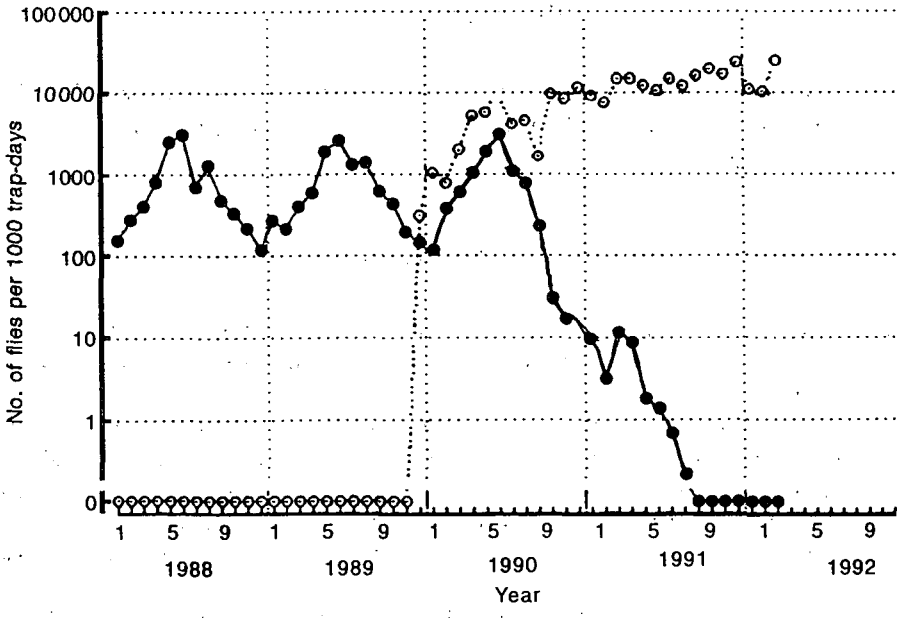

FIG. 8. Monthly change in the abundance of sterile (⊙) and wild (●) melon flies caught by monitor traps in the Yaeyama Islands.

7. CONCLUSIONS

It can be concluded that the melon fly has been completely eradicated from Okinawa. The total number of flies released (from the Kume to Yaeyama projects) was about 50 000 million, and the total investment was about ¥. 10 000, including ¥. 3600 million for the construction of facilities.

As the melon fly was also eradicated from the Amami Islands by the Kagoshima Prefectural Government by 1989 [11], Japan now has no melon flies. This is the first complete success of the sterile insect technique (SIT) eradication project after 1963 (eradication of the melon fly from Rota [12]).

Our experience, based on the success of the eradication programme using SIT, shows that the quality of mass reared, sterilized melon flies to be released is of fundamental importance and estimation of the wild population density by the mark–recapture technique and appropriate monitoring systems are necessary; the latter can detect the temporal and spatial distributions of wild and released insects. Detailed field studies on the spatial distribution pattern of the target insect are essential for improvement of the sterile insect release plan. Over the twenty years of our SIT project, we carried out research on the fundamental biology of the target insect and published more than one hundred and eighty original papers. Such basic research might have provided the conditions for our success.

ACKNOWLEDGEMENTS

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GENETIC ENGINEERING AND MOLECULAR BIOLOGY

(Session 2)

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ADVANCES IN THE PRESERVATION OF INSECT GERMPLASM

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Abstract

ADVANCES IN THE PRESERVATION OF INSECT GERMPLASM:

The current means of preserving insects that are freezing intolerant or have no dormancy capabilities for use in the laboratory or in management programmes is by continuous culture. Not only can continuous culture be a costly venture, but it can effect genetic drift and is subject to accidental loss of colonies, genetic strains and transformants. Further, the ability to be able to stockpile insects for later use in sterile insect technique and biocontrol programmes would be of tremendous benefit. Since preservation of mammalian embryos by low temperature technology has become a common procedure, researchers, insectary managers and those involved in control programmes have been looking to cryobiologists for assistance in solving the insect germplasm storage problem. The paper examines the concepts of the conventional methodology that is used for cryopreservation of cells and mammalian embryos. Also pointed out are several inherent barriers posed by embryos of insects such as muscoid flies which are incompatible with the use of the conventional techniques. Of the obstacles thus far identified, chilling intolerance and egg membrane impermeability have been given the most attention by researchers attempting to develop low temperature storage methods. Limited but promising success has been obtained using chemical dissolution of membrane waxes, infusion of embryos with multimolar cryoprotectants and avoidance of chilling injury by ultrarapid cooling and warming. The feasibility of incorporating techniques which facilitate natural insect cold hardiness into a cryopreservation protocol and alternatives to preservation of embryos are discussed.

1. INTRODUCTION

The demand for a long term storage methodology for insects has grown substantially in recent years with its successful use in sterile insect technique (SIT) and biocontrol programmes and with the advancements made in genetic engineering and molecular techniques. The need to increase the shelf-life of those insects having high genetic control or research utility relates directly to reducing costs in the maintenance of large numbers of genetic strains, combating genetic drift and catastrophic loss under mass rearing conditions while having the capability to stockpile insects

prior to release in a management system. To meet these demands, research was initiated about a decade ago by the United States Department of Agriculture in an attempt to transfer to insects the cold storage technology developed for mammalian embryos that is routinely used for preservation of domesticated and some wild zoo animals. The efforts made by Heacox et al. [1] to use these standard cryopreservation methods for preservation of *Musca domestica* embryos were largely unsuccessful, but their research identified several important barriers, unique to insects, that precluded the use of the then current 'state of the art' technology for preserving animal embryos. Since that initial study, others have joined the effort to provide researchers and those involved in insect management with long term storage methods that employ cryopreservation techniques. Thus, the objective of this paper is to review the most recent findings that relate to the preservation of insect germplasm and hopefully to give some insight to where efforts might be directed to achieve the goal of providing storage methods for certain insects. Because many insects have inherent strategies to survive subzero temperatures, this author feels that it is imperative that discussion be included of how these mechanisms could (and possibly should) be incorporated into certain preservation techniques. Further, since previous reports [2, 3] have summarized the early work on preserving insects in cold storage prior to the successful use of chemical cryoprotectants in the preservation of mouse embryos [4], mention of those studies is limited to developing the background to or an understanding of the most recent research efforts.

2. CRYOPRESERVATION: BASIC PRINCIPLES

The conventional method for cryopreservation of mammalian embryos, multicellular systems and cells requires that sufficient dehydration of the intracellular domain be achieved prior to cooling in order to avoid lethal ice formation. This is accomplished with molar concentrations of protective solutes that also protect cells from a number of potentially lethal chemical and physical changes that can occur upon freezing (Fig. 1).

2.1. Cooling rate

The cooling rate is a critical factor in conventional cryopreservation. Under slow cooling conditions, most cells and embryos tend to supercool, even in the presence of extracellular ice. Slow cooling allows cells to dehydrate osmotically through the difference in the chemical potential of the supercooled water within the cells and that of the water and ice in the surrounding medium [5]. The bulk water content of cells, as opposed to the osmotically inactive water, can be reduced to a low level via slow cooling and cryoprotectant loading and ice will either not form upon reaching the normal nucleation temperature of the cells or will be present in

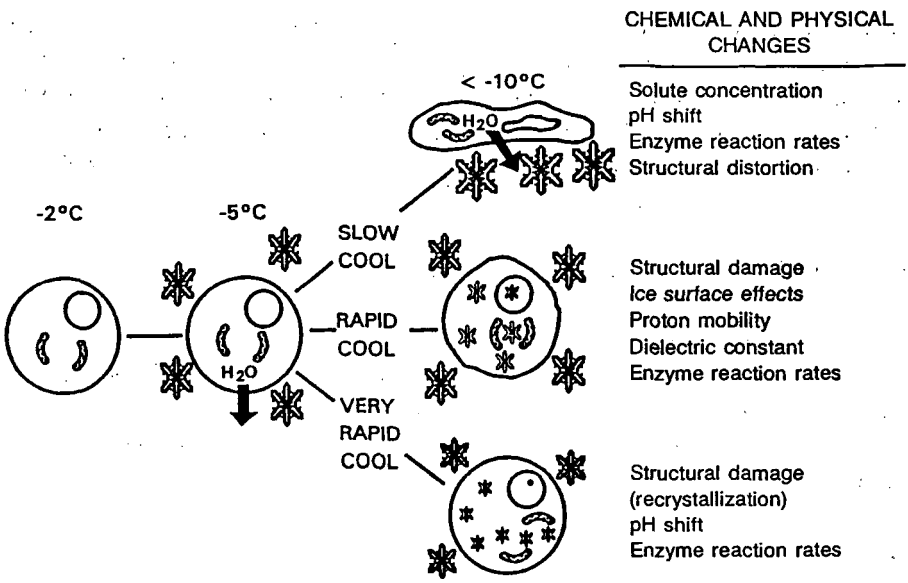

FIG. 1. Effects of three arbitrarily selected cooling rates on the movement and freezing of intracellular water in the presence of extracellular ice. Also shown are the chemical and physical changes that accompany cell dehydration and intracellular freezing.

quantities too small to become damaging. When cells are cooled at faster rates, the water efflux is limited and freezing occurs intracellularly (Fig. 1). For each system to be frozen there is an optimum cooling rate and cooling too slowly can be as harmful as cooling too rapidly. Cell shrinkage below a critical volume and severe deformation within the unfrozen fraction of water remaining in the ice channels have been suggested as the source of damage caused by a suboptimal rate of cooling [6, 7].

2.2. Warming rate

The warming process (fast versus slow) after storage at liquid nitrogen (LN_2) temperature appears to be directly related to the rate that the cells or embryos were cooled and at what subzero temperature the transfer to liquid N_2 was made [8]. Cells sensitive to slow warming are thought to have been cooled too rapidly, allowing the formation of small intracellular ice crystals, which grow by migratory recrystallization and create damage during the thaw [9]. Thus, fast warming ($>300°C$/min) is needed to prevent damaging recrystallization. Cells or embryos requiring slow warming ($<25°C$/min) have usually been cooled at a slow rate ($<1°C$/min) and may also have been transferred to LN_2 at temperatures below $-60°C$. In this case considerable dehydration has occurred, leading Leibo et al. [10] to propose that rapid warming causes injury to cells and embryos through the osmotic

stress created by rapid rehydration as the extracellular ice melts. However, others believe that neither recrystallization, melting of intracellular ice nor osmotic stress are major factors in causing injury upon warming but that it is some as yet unidentified component [8, 11]. A schematic representation of a conventional method of cryopreservation using cryoprotectant loading and slow cooling is depicted in Fig. 2.

3. INHERENT CRYOPRESERVATION BARRIERS

In general, cryopreservation procedures require that the membranes of cells and embryos be permeable to water and the solutes used for cryoprotection. Insects, except for the viviparous species and those ovipositing in protected humid environments, lay eggs which are endowed with elaborate membrane systems and protective chorions that impede water loss and entry of harmful chemicals.

3.1. Permeabilization

The egg chorions of *Drosophila melanogaster*, *M. domestica* and other muscoid flies are easily removed by immersing in dilute commercial bleach or by mechanical means. However, much effort has been spent rendering the underlying vitelline membrane permeable. A hydrophobic waxy layer is associated with the vitelline membranes surrounding *D. melanogaster* and *M. domestica* embryos and embryos of many other insects [12]. Limbourg and Zalokar [13] devised a method for permeabilizing vitelline membranes using octane. Heacox et al. [1] modified this method slightly and used it for permeabilizing the vitelline membranes of *M. domestica* embryos before treating with dimethylsulfoxide. The inconsistent results and reduced hatchability of *D. melanogaster* embryos have led researchers to make several changes in the original procedure. The apparent essential steps of the most recent methods employ either an initial rinse in isopropanol before immersion in hexane or treating the embryos with a mixture of 1-butanol and an alkane after the isopropanol rinse [14–16]. It has been reported that both methods yield >90% permeabilization and from 70–90% hatching.

3.2. The yolk system

Intuitively, the amount and/or type of yolk present in insect eggs presents a potential barrier to cryopreservation. Since the insect egg is a self-sustaining developmental system, all the materials needed for embryogenesis must be contained within the yolk nutrients. For example, the lipid content of insect eggs typically ranges from about 1.5 to 18.5% [17]. On the basis of measurements of respiratory metabolism and a decrease in the lipid content during embryogenesis, stored lipids are the main source of energy for the developing embryos of most of the insects

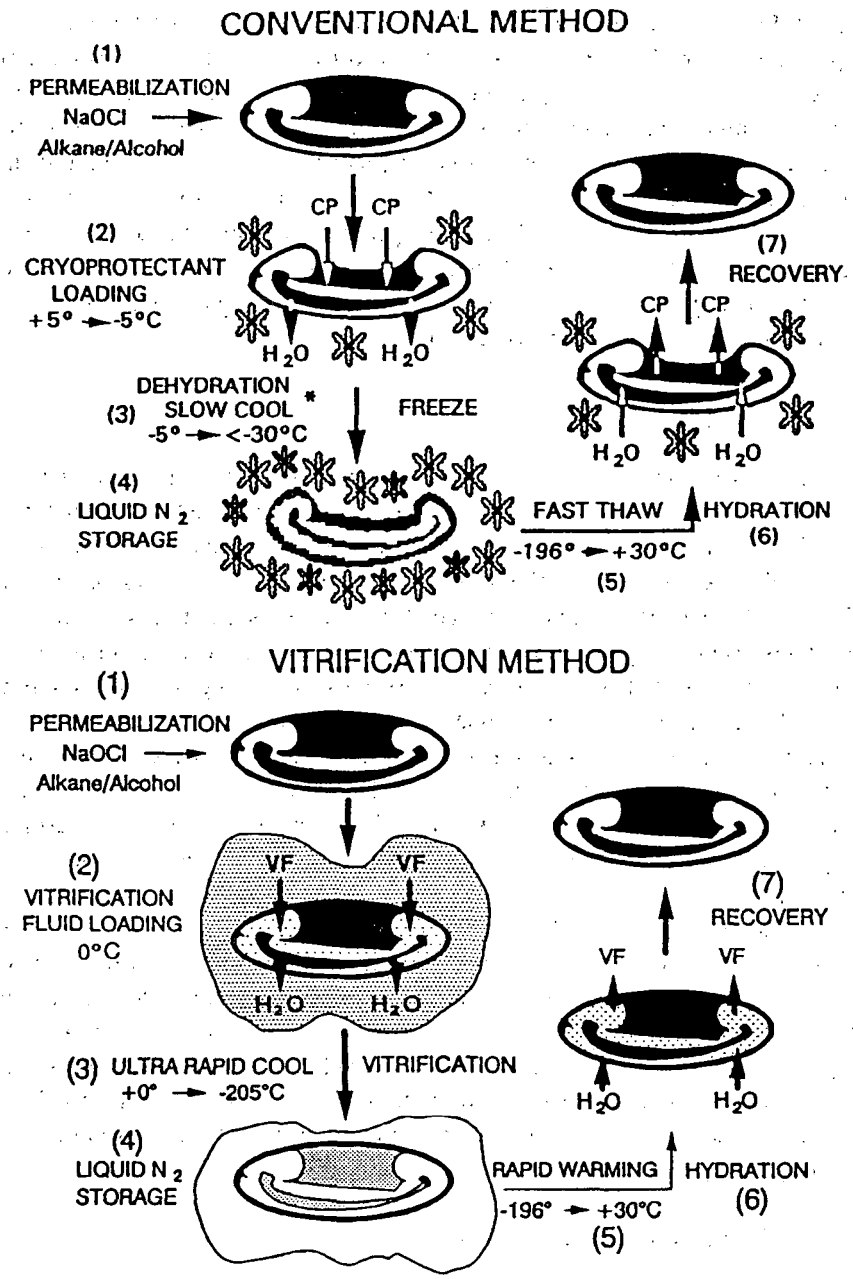

FIG. 2. Comparisons of two methods of gaining LN_2 storage for an insect embryo. The essential differences between the two methods are in the amount of cell dehydration obtained during the loading of cryoprotectants and cooling, the concentration of cryoprotectants, and the rates of cooling and warming. The asterisk at step 3 of the conventional method denotes a process which precludes use on chilling intolerant embryos (see text for details).

studied [18]. Early work by Lea and Hawke [19] has shown that the lipovitellin component produced by egg laying animals is sensitive to cold temperatures and disassociates upon freezing. Further, Lovelock [20] indicated that the protein–lipid complexes within cells are particularly susceptible to chilling and freezing because they are held together by only weak associations and not through covalent bonding. Mazur et al. [21] observed during the testing of their process for cryopreservation of *D. melanogaster* embryos that 47% developed from the time of treatment (predorsal closure) up to hatching, but hatching mostly did not occur. This may be an indication that the energy needed for larval emergence from the egg has been lost because of cold instituted damage to the yolk system.

3.3. Chilling sensitivity

The lack of success thus far in applying conventional cryopreservation procedures for *M. domestica* and *D. melanogaster* has been attributed to their prefreeze sensitivity to chilling. The theoretical low temperature at which freezing intolerant insect species such as these two flies can no longer survive is the temperature of crystallization (T_c). This is the subzero temperature at which spontaneous nucleation of body fluids occurs. Depending on the stage of development assayed, many insects can supercool to −25°C or lower before spontaneous freezing of their body fluids occurs. However, the T_c is not a good indicator of the lowest lethal temperature. Several recent studies have shown that a substantial number of insects succumb at temperatures of 10–15°C above the T_c [22–25]. These studies show that insects, like other organisms ranging from bacteria to higher plants, mammalian sperm and embryos, are sensitive to the stress caused by exposure to subzero non-freezing temperatures.

In addition to the possible membrane damage caused by chilling, we have suggested that sensitivity during the early embryogenesis of insects is probably inherent to the process of rapid cellulation [24]. During blastoderm formation, synchronous mitotic divisions of cleavage energids in *D. melanogaster* occur about every 10 min [26]. Further, Callaini and Marchini [27] have shown that during this period of cellularization of the blastoderm, chilling for 1 h at 0°C causes the formation of abnormal centrosomes and treatment of earlier embryos results in the arrest of cleavage divisions at metaphase.

3.3.1. Direct and indirect chilling injury

Direct chilling injury or 'cold shock' is injury caused by a rapid reduction in temperature, while indirect chilling injury involves damage incurred only after long exposure to low temperature, and is independent of the rate of cooling [28]. Cold shock injury in some insects has been linked to membrane failure [29, 30]. The damage caused by cold shock is thought to be due to the induction of phase transi-

tions in membrane lipids, which results in leakage and loss of the membrane function [31]. An alternative view is that chilling causes damaging thermoelastic stress through an unequal condensation of membranes relative to the cell contents [32]. Indirect chilling injury has been suggested to be associated with the formation of irreversible metabolic imbalances involving cellular energetics [33].

3.3.2. Developmental stage sensitivity

Different developmental stages possess varying capacities for tolerance to both cold shock and long term chilling. Further, there are also significant differences in the capacity for cold tolerance within a particular developmental stage of an insect. As mentioned earlier, these phenomena are important considerations when devising a cryopreservation protocol, since the conditions for cryoprotectant loading and dehydration under a conventional procedure require that the cells/embryos be chilling tolerant.

A survey of the within and between stage tolerance to long term chilling throughout the pre-adult development of the house fly showed that at 5 and 0°C, the two-day-old pupae were the most resistant to the effects of long term chilling, while at −5°C the late stage embryos were the most resistant (Fig. 3). Interestingly, three-day post-emergent adults were as sensitive as the larvae and pupae to −5°C (data not shown).

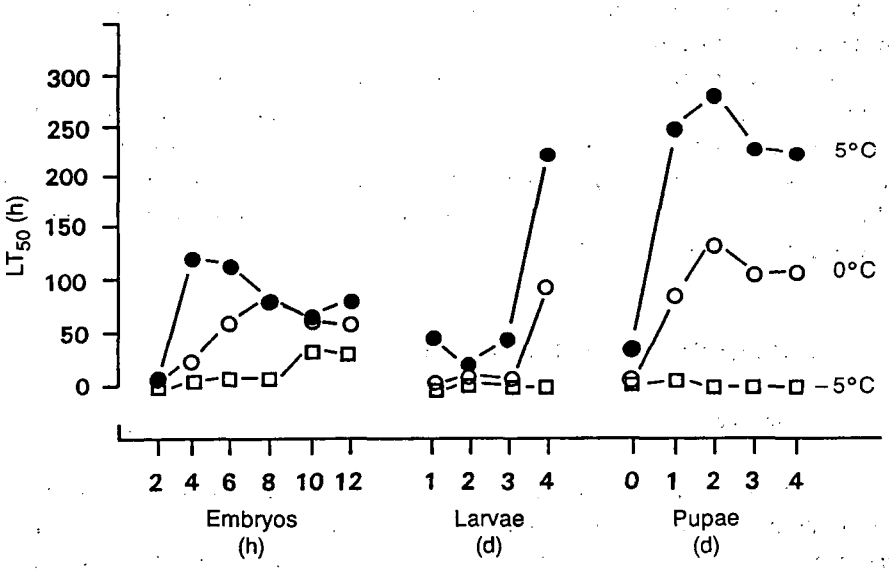

FIG. 3. Within and between stage survival to hatching (LT_{50}) of house fly embryos, larvae and pupae subjected to long term chilling at three temperatures (modified from Strong-Gunderson and Leopold [24]).

The within stage sensitivity of house fly embryos subjected to a cold shock at a temperature of −20°C is shown in Figs 4(a) and 4(b). House fly embryos supercool to a range of −26 to −34°C before freezing [24]. As indicated in Fig. 4(a), there is a rate limiting effect of cooling on survival up to and including 9 h post-oviposition. The slower cooling rate of −10°C/min was clearly better than the faster rate at all ages, except for 12 h post-oviposition, where they do not differ. Further, the younger ages are mostly more sensitive to rapid, brief chilling than are the older embryos.

When the hold time is extended from 3 to 30 min at −20°C, survival is drastically reduced (Fig. 4(b)). Only the 9-h-old embryos show any significant survival. Survival at the two faster rates of cooling, −10 and −65°C/min, does not differ, while cooling at a much slower rate, −0.5°C/min, increases survival almost four fold.

3.4. Preservation of post-embryonic stages

The foregoing discussion has assumed that the insect embryo would be the most desirable stage to use in developing a long term storage protocol. This assumption may not be applicable to a particular insect or situation. For example, preservation of embryos of a larvapositing insect such as the tsetse fly would not be attainable, since the embryo cannot be maintained outside its mother. Further, it may be more desirable to stockpile an insect to be used in a control programme in the stage closest to the one that is to be released. This would allow rapid implementation of the control programme.

Cryopreservation of the post-embryonic stages generally presents even more difficult problems than that of embryos. The post-embryonic stages are larger, more compartmentalized and often possess almost impenetrable cuticular surfaces, which would obstruct the permeabilization and even the distribution of cryoprotectants. Moreover, the older stages may be more chilling intolerant than their embryonic counterparts (see Section 3.3).

4. CIRCUMVENTING THE BARRIERS

4.1. Cryoprotectants

Avoiding or dealing with the natural barriers that interfere with the use of low temperature to effect a long term storage condition usually requires that chemical cryoprotectants be employed in addition to the reduction of freezable water. Cryoprotectants are variously classified by molecular weight, permeating ability, ice promoting or deterring and as antifreeze agents. Permeating cryoprotectants of low molecular weight can protect cells from freezing damage simply on a colligative

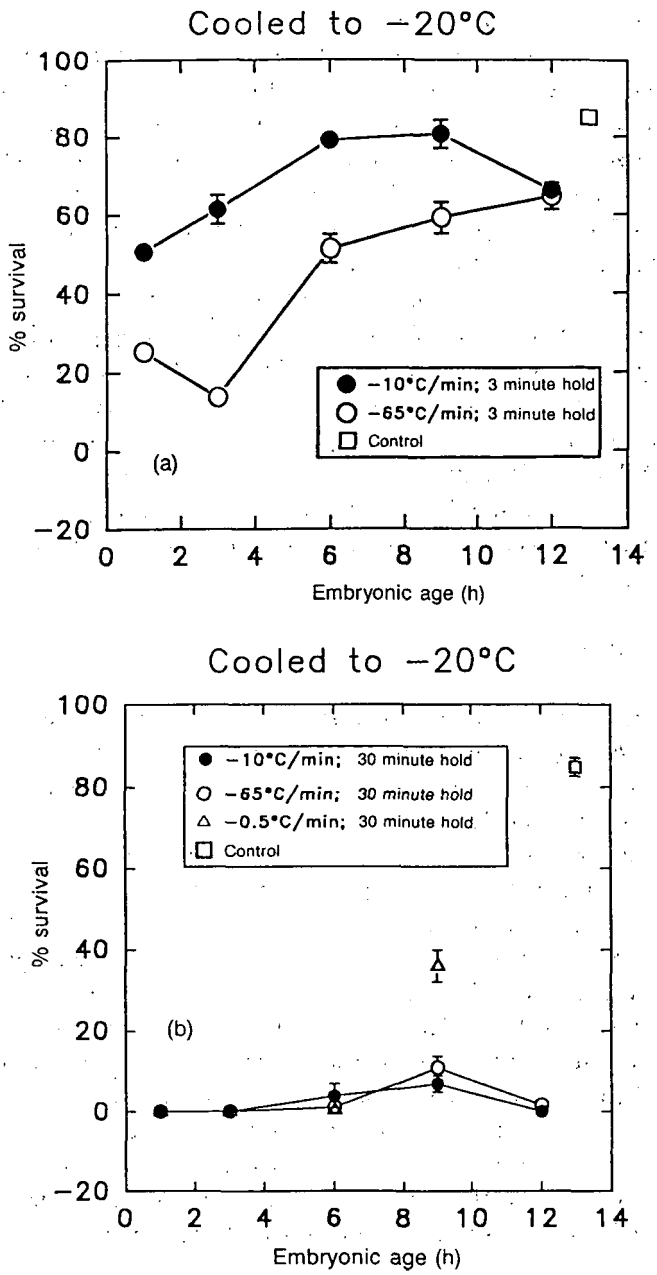

FIG. 4. (a) Survival to hatching of house fly embryos subjected to a short term cold shock and cooled to −20°C at two different rates. (b) Similar to (a) except that chilling was extended to 30 min and an additional cooling rate of 0.5°C/min was tested for the 3, 6 and 9 h (lines not connected for clarity). The warming rates for all the groups were the same as each respective cooling rate.

basis and have the capacity to keep extra- and intracellular solutes from reaching toxic levels by remaining in the aqueous phase during the freezing process. Thus, cell dehydration and shrinkage is reduced, electrolyte balance is maintained and the amount of extracellular water remaining as a liquid increases [6, 7, 34]. Others have suggested that alternative or additional actions of permeating cryoprotectants may be to stabilize the unfreezable water [34], to reduce cellular damage by changing the ice crystal configuration [35] and to resist the denaturation of the essential macromolecules during subzero dehydration [33].

Non-permeating cryoprotectants such as trehalose appear to protect cell membranes during and upon recovery from chilling and/or freezing [36]. Combining trehalose with glycerol was found to increase the viability of mouse embryos upon recovery from a freeze-thaw process [37]. The protective action of trehalose has been attributed to take the place of the H_2O molecules that normally hydrate membrane phospholipids [38]. Further, several insects have been found to accumulate large amounts of trehalose (40–70 μg/mg body weight) during the overwintering period, leading to the speculation that trehalose gives protection from chilling/freezing injury [39, 40].

4.2. Vitrification

If *M. domestica* and *D. melanogaster* were not chilling intolerant, the calculated optimum cooling rate for permeabilized embryos would be < 1°C/min [1, 41]. In theory, this rate would effect the efflux of water, the influx of a cryoprotectant and allow a conventional slow freezing strategy to be used. One means of dealing with high chilling sensitivity is to cool the embryos at extremely rapid rates. Mazur et al. [42] have calculated that a cooling rate of $\geq 20\,000$°C/min is needed to reduce the median lethal temperature of *D. melanogaster* embryos from -25 to -65°C. Further, to avoid lethal ice formation at such high cooling rates, glass forming solutes must be infused into the embryos which, upon cooling, would produce a vitrified state. Simply stated, vitrification is a process whereby a liquid through rapid cooling becomes solidified, not by the formation of ice, but by an extraordinary increase in viscosity. The way in which conventional and vitrification procedures differ is that highly concentrated solutions (>50 wt%) are used to dehydrate the embryos and to concentrate the intracellular solutes. When a concentrated cryoprotectant is present both inside and outside the embryo and cooled at ultrarapid rates, an amorphous glass is formed in the surrounding medium and the embryo that precludes the formation of ice. Also, very rapid warming of the vitrified specimen must also be employed to eliminate the possibility of devitrification, which results in the transient formation of damaging ice crystals. (Figure 2 gives a graphic comparison of the conventional and vitrification methods.)

Steponkus et al. [43] were the first to report successful use of a vitrification protocol to gain survival of *D. melanogaster* embryos after storage at -196°C.

Their two step method for infusing 12-h-old embryos with 2.1M and then 8.5M ethylene glycol (EG), followed by cooling at 54 000°C/min in liquid propane, resulted in an 18% hatch, 3% of which developed into adults. Mazur et al. [21] reported slightly better results by using 14–15-h-old embryos, altering the permeabilization solution and cooling at rates approaching 100 000°C/min in LN_2 slush. However, recent refinements made by Steponkus and Caldwell [44] have raised the hatching to near 50% and survival to adulthood to about 10%. Their refinements include optimizing the permeabilization procedure [16], choosing 13.5–14.5-h-old embryos for treatment, increasing the duration of time for the exposure to EG, using LN_2 slush as a coolant and increasing the osmotic potential of the vitrification solution.

Mazur et al. [21, 42] have made extensive studies on the cooling and warming rates required to circumvent the chilling sensitivity of *Drosophila* embryos during vitrification and they contend that very high warming rates are more important to survival than the cooling rates. It was suggested that the requirement for rapid warming was an indication that devitrification was occurring and was caused by the uneven distribution of EG in the compartmentalized embryo.

4.3. Enhancing cold tolerance for cryopreservation

Development of a cryopreservation procedure can be aided considerably by judiciously choosing the most cold tolerant stage. Many insects respond to certain environmental cues by producing natural cryoprotectants in the form of polyols, sugars and antifreeze or ice nucleating proteins, which enable them to become more cold tolerant (see reviews of Storey and Storey [33] and Duman et al. [45]). Even insects that do not exhibit overwintering strategies or experience dormancy may be induced to increase their cold tolerance. Czajka and Lee [46] found that *D. melanogaster* larvae, pupae and adults had increased tolerance to subzero chilling if they were first exposed to 0 or 5°C for 1 h. It has been suggested that this rapid cold hardening phenomenon enables insects to deal with a short term drop in the ambient temperature [47].

When house fly embryos are subjected to a cold hardening treatment at 0°C before cooling to −20°C at a rapid rate, survival was observed to increase with the 6-, 9- and 12-h-old embryos but not with the 1- and 3-h-old embryos (Table I). Slow cooling and the hold at 0°C appear to have the same cold hardening effect, since cooling of the 9-h-old embryos at a rate of −0.5°C/min from 20 to 0°C before fast cooling to −20°C produce almost identical survival rates. Mazur et al. [42], concluded that for *D. melanogaster* embryos, cold hardening cannot alleviate the need for very high cooling rates (> 100 000°C/min) to out run the accelerating effects of chilling injury at low temperatures.

Burton et al. [47] found a connection between the heat and cold shock phenomena in that a moderate heat shock could protect *D. melanogaster* larvae from cold shock injury. Exposure of house fly embryos to a heat shock before cooling to

TABLE I. PRETREATMENT OF HOUSE FLY EMBRYOS WITH COLD HARDENING OR HEAT SHOCK BEFORE COOLING TO −20°C

Embryonic age (h)	Mean per cent survival to hatching		
	No cold hardening or heat shock	Cold hardened[a]	Heat shocked[b]
1	0	0	0
3	0	0	0
6	0.2	3.7	0
9	9.7	17.7 (17.6)[c]	22.1
12	0.3	25.4	0.3

[a] 1 h at 0°C before cooling to −20°C at 65°C/min, holding for 30 min and warming at the same rate.

[b] 30 min at 38°C before cooling, holding and warming as above.

[c] Cooled at 0.5°C/min from 20° to 0°C before cooling, holding and warming as above.

−20°C imparted increased chilling tolerance only to the 9 h embryos (Table I). The mechanism of how heat shock or rapid cold hardening increases tolerance to chilling is not understood. More information on the etiology of chilling injury in insect embryos and how cold and heat stress tend to reduce its severity is needed before determining whether it can be used in a cryopreservation protocol.

Besides cold or heat stress, other factors such as changes in the photoperiod, humidity, diet quality and quantity and oxygen tension, or a combination of these factors, have been reported to enhance cold tolerance [48–51]. Even oral injections of glycerol given to the larvae of *Anagasta kuehniella* resulted in an increase in short term low temperature survival [52]. In most cases, gaining enhancement of the cold tolerance of an insect requires that its low temperature biology and physiology be thoroughly studied and understood.

5. ALTERNATIVES TO EMBRYO CRYOPRESERVATION

The alternatives to embryo cryopreservation include storage of the post-embryonic stages, gonads or primordial germ cells (pole cells) for transplantation, cleavage energids, sperm and unfertilized eggs. Except for the preservation of post-embryonic stages, the other possibilities are labour intensive and involve complex manipulative techniques such as microsurgery and injection. Techniques for the cryopreservation of gonadal imaginal discs and germ cells have been developed, so for some insects these methods offer viable options (see Ref. [3]). Also, it should

be pointed out that the preservation of gonads and germ cells allows only one-half the genome to be retained. However, recent success with the in vitro fertilization of sawflies [54], if expandable to other insects, would alleviate this problem. The current obstacle lies in the lack of knowledge on egg activation occurring at fertilization.

6. SUMMARY

The cryopreservation of insect germplasm is still largely in the developmental stages, and for those insects for which long term low temperature storage techniques have been developed, the methods are cumbersome, require considerable expertise and result in a relatively low yield of viable adults. This is not unexpected in a new area of endeavour where the research effort is being conducted by relatively few scientists on limited budgets. However, considering the progress made over the last five to six years, it can be anticipated that the technology for storage of *Drosophila* spp. and related flies will be forthcoming. The task of developing less complicated, universally utilizable methods will be made easier once effective and efficient means of dealing with chilling injury and cryoprotectant permeation of complex systems are found. There is as yet much to be learned from the study of insect cryobiology which no doubt will have application to the development of cryopreservation methods. Given the amount of diversity among insects, the only limitations to solving problems in the preservation of insect germplasm lie in the imagination of the scientists and the provision of adequate funding.

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TECHNOLOGY FOR TRANSFORMING THE GERM LINE IN ECONOMICALLY IMPORTANT INSECTS

Current status and prospects for advancements

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Abstract

TECHNOLOGY FOR TRANSFORMING THE GERM LINE IN ECONOMICALLY IMPORTANT INSECTS: CURRENT STATUS AND PROSPECTS FOR ADVANCEMENTS.

The development of a germ line transformation methodology for insects of agricultural and medical importance is critical to the implementation of novel and highly efficient means of insect management using molecular biological techniques. Currently, an efficient means of gene transfer, using the *P* transposable element based vector system, is possible in only one insect, *Drosophila melanogaster*, and this methodology has not been applicable to other insects. Progress has been made in modifying the *P* element so that it might function in non-drosophilids, although a system fully useful for a wide range of insects is still prospective. Other transposable element systems from *Drosophila* having the potential for gene vector development have been identified, specifically the *hobo* and *mariner* transposons. For both these elements, homologous genes or DNA sequences have been found in distantly related insects, suggesting that their mobility properties might be maintained in these and other insects, or that they may be used as probes to isolate related transposons. Continued studies along these lines hold the greatest promise, at present, for gene vector development. An immediate use of gene transformation in economically important insects may be for genetic sexing and male sterilization in the sterile insect technique. This is due to the understanding of sex specific gene expression and sex determination activity in *D. melanogaster*, as well as the genetic material available from this and other organisms. Model systems for highly efficient genetic sexing and sterilization based on recombinant DNA manipulation and gene transformation are discussed.

1. INTRODUCTION

The manipulation of genes and their subsequent transfer into insect host genomes offers a vast potential for improving our ability to control insects of agricultural and medical importance. Gene transfer technology might directly impact insect management programmes by facilitating: (1) genetic sexing and sterilization for sterile male release programmes; (2) insecticide resistance in beneficial insects; (3) cold hardiness or freeze tolerance to store insects for mass release or for maintaining germplasm; and (4) introduction of genetic markers into populations for monitoring gene flow, insect migration and dispersal patterns, among others. Perhaps more important than some of these immediate applications, the basic knowledge gained by gene transfer experiments will certainly enhance our understanding of insect biology relevant to insect management and control, such as resistance mechanisms, sex determination, hybrid sterility, and hormone action and metabolism. In particular, this information will be essential to the development of biological control agents, enhancement of natural enemies and developing resistance mechanisms against insect pests in plants and animals.

Despite the benefits to be derived from gene transfer methods in insects, at present the routine introduction of exogenous DNA into a host insect's genome is limited to the genus *Drosophila* using the *P* transposable element based gene vector. Non-vector mediated gene transfer has been reported sporadically for many years, although in most studies it was concluded that somatic inheritance was occurring, and in no case was chromosomal integration verified (see Ref. [1]). Quite recently, gene transformation was reported for the predatory mite, *Metaseiulus occidentalis*, at relatively high frequencies but, while stable transformation was concluded due to the presence of injected DNA after several generations, chromosomal integration was not determined [2]. Other recent efforts to achieve *P* vector mediated gene transfer in non-drosophilids, including mosquitoes, tephritids and locusts, have all been unsuccessful. However, in the mosquito studies non-*P* mediated DNA transposition was observed [3–5], suggesting that non-vector mediated transformation may be possible in this insect, albeit at low frequencies. A caveat to the reported failure of *P* mediated transformation is that all of these tests relied, at least initially, on the use of neomycin analog resistance as a selection method. For tephritids, at least, this appears to have been unreliable, since drug resistant lines were selected in which they did not contain the vector, the vector was not chromosomally integrated, or resistance was lost in succeeding generations [6–8].

While the *P* vector function in non-drosophilids remains uncertain on the basis of transformation experiments, a lack of *P* mobility in these insects is supported by our tests using transient embryonic *P* mobility assays (see Ref. [9]). Although progress has been made in defining the specific restrictions on *P* mobility and ameliorating some of them using these assays, development of a highly efficient *P* vector for non-drosophilids may be a long term effort. Given this prospect, it is

reasonable that research be continued on the discovery and analysis of other vector systems, in addition to methods to efficiently introduce DNA into germ cells and select for transformants. Potential candidates for vector development include identified transposable elements, viruses and retroviruses and symbiotic organisms. The relatively high number of transposons within middle repetitive DNA has also encouraged the search for new transposon systems for development into species specific vector systems. Of these possibilities, the best prospects for rapid development of an efficient, reliable and stable gene vector system are known transposable element systems which are in the inverted terminal repeat class, such as the *P* element. Of particular interest are the *hobo* and *mariner* elements, which were also discovered in *Drosophila* and which have subsequently been developed into gene vectors for that insect. Unlike the *P* element, these transposons have an apparent evolutionary relationship to other transposons, or other genes, in distantly related insect and non-insect organisms. This raises the possibility that the *hobo* and *mariner* elements may retain mobility properties in non-drosophilid insects, or that they may be used as probes to isolate related transposons.

2. *P* ELEMENT VECTOR

The first vector helper system for stable germ line transformation in *Drosophila melanogaster* was developed by Rubin and Spradling [10] using the transposable element *P* system (see Ref. [11] for a review of *P*). Within a few years of this development, several findings suggested that the *P* vector might similarly be effective in non-drosophilids. First, Brennan et al. [12] found evidence for *P* transposition in *Drosophila* species which are distantly related to *D. melanogaster* and in which *P* elements had not been detected. Second, the basis for a major restriction in *P* element mobility in *D. melanogaster* was discovered and ameliorated. Laski et al. [13] found that *P* mobility was restricted to the germ line due to a lack of intron 3 processing in somatic cells, resulting in a truncated non-functional transposase polypeptide. Creation of a helper (pUChs$\pi\Delta$2-3) having intron 3 DNA deleted from the transposase gene resulted in somatic production of a full length functional product which supports *P* mobility. Third, a dominant drug resistance selection system for transformants, using a vector (pUChsneo) with the bacterial neomycin phosphotransferase gene, was developed, allowing relatively easy selection of transgenic insects [14]. This finding was especially important for non-drosophilid insects in which the usual selectable markers are not available (requiring a selectable mutant allele and cloned DNA for the wild type allele).

Using the pUChsneo vector and the pUChs$\pi\Delta$2-3 helper, several laboratories attempted to create non-drosophilid transformants. Most notably, three separate

groups working with different mosquito species isolated neomycin resistant transformants, although upon molecular analysis none of them appeared to be *P* mediated [3–5]. Several laboratories working on the Mediterranean fruit fly, *Ceratitis capitata* [6,7], and our laboratory working on another tephritid, the Caribbean fruit fly, *Anastrepha suspensa* [8], obtained similar results which were unusual. Putative neomycin resistant G1 lines were selected, and in some cases vector DNA was observed in these insects [15]. However, chromosomal integrations could not be verified, and in all cases neomycin resistance was lost in subsequent generations. This suggests that neomycin resistance is not a reliable selection method, at least for tephritids. Although the basis for this has not been unequivocally determined, it may have relevance to the consideration of other selection systems. Anecdotal evidence already exists for inconsistencies in selection by neomycin resistance in *Drosophila* which are related to the amount and methods of neomycin administration, the presence of yeast, rearing conditions, etc. (see Ref. [16]). The transient nature of neomycin resistance in tephritids would suggest that extrachromosomal expression and inheritance of the vector may be occurring, perhaps borne by bacterial endosymbionts. If this is the case, then a similar situation may occur with other chemical selections, or any selection which is not defined by visible expression in insect cells (assuming that extrachromosomal expression would usually be mosaic).

Realizing that the inability to isolate transformants could be due to restrictions other than a non-functional vector, and considering vector functionality to be the primary necessity for efficient gene transfer, we developed methods to test vector functional directly, independent of chromosomal integration or dominant selection. This was achieved by modification of an assay for *P* mobility in *Drosophila* cell lines [17]. The assay consisted of an indicator plasmid, pISP, having *P* element sequences within a reporter gene, and a helper plasmid, pUChs$\pi\Delta$2-3, encoding transposase, being injected into insect embryos [18]. The functionality of the transposase gene in the host embryo was determined by *P* element excision from the indicator plasmid. After incubation and harvesting of the plasmids from the embryos, and subsequent bacterial transformation, *P* element excision could be assayed as a function of restoration of the reporter gene expression. In our initial assays, the reporter gene was β-galactosidase expression, which is monitored by blue bacterial colonies grown on the X-gal substrate. This assay only reported *P* excisions which restored the β-galactosidase reading frame, which represents approximately one third of all excisions. A subsequent new indicator plasmid, containing a gene which confers sensitivity to streptomycin inserted within the *P* element sequence, was able to report all excisions as a function of streptomycin resistance [19]. Expression of β-galactosidase further distinguished precise from imprecise excisions.

Studies with these in vivo transient expression excision assays in *D. melanogaster* indicated an approximate total excision frequency of

5.5×10^{-3}/pISP, with a precise excision frequency of about 1.7×10^{-3}/pISP [19]. In other drosophilids, the excision frequency decreased as a general function of relatedness to *D. melanogaster*, with both total and precise excisions occurring at about a 10% level in distantly related drosophilids such as *Chymomyza procnemis* and *D. virilis* [9, 18, 19]. In other dipterans, excision was not detected. These results indicated that *P* mobility is restricted in non-drosophilid insects, and that the *P* gene vector would most likely be non-functional as well in these insects.

Since a restriction in *P* transposition in somatic tissue is due to defective intron 3 splicing, we tested the possibility that splicing of the other introns might be defective in non-drosophilids [9]. Using a transposase gene lacking introns 2 and 3 (pUChsπΔ1-2-3), and a transposase cDNA (pKhsπcDNA) lacking all 3 introns, *P* mobility was tested in *D. melanogaster* and *A. suspensa*. Both intron deleted genes supported *P* excision in *Drosophila*. In *Anastrepha*, pUChsπΔ1-2-3 was not functional; however, a relatively low level of *P* mobility was supported by the cDNA representing the first observation of a significant level of *P* mobility, and thus transposase function, in a non-drosophilid. While this result suggested that transcript processing is a restriction on the transposase function in *Anastrepha*, the twenty fold lower level of excision relative to *Drosophila* indicated that other limiting factors also exist. The most straightforward possibilities are that either requisite positive acting factors are lacking or inhibiting factors exist in non-drosophilids. To test the first possibility, nuclear extracts from *D. melanogaster* embryos were co-injected with the plasmids (pISP and pKhsπcDNA) in the mobility assay [9]. Similar nuclear extract preparations were found to contain a 66 kilodalton polypeptide which binds specifically to the *P* element termini [20]. The addition of the nuclear extract further increased excision by two fold in *Anastrepha*, although, interestingly, excision was decreased in *Drosophila* by more than one third. This decreased the difference in *P* excision between the species from twenty fold using only the transposase cDNA to an eight fold difference with the addition of nuclear extract. Importantly, the negative influence of the extract in *Drosophila* raises the possibility that the action of specific positive acting factors was not fully revealed in *Anastrepha* owing to a general inhibitory effect of the extract. This should be clarified by testing more highly purified nuclear extract fractions, as well as the 66 kilodalton polypeptide, to identify specific factors. Although this objective is straightforward and has a reasonable probability for success, the protein purification and the number of mobility assays required make this a formidable task nevertheless.

P, or *P* like, elements are widely distributed in *Drosophila* [21, 22] and their presence in dipterans outside of the *Drosophila* genus has been reported [23]. While our results suggest that *P* would not be an effective vector in these insects, these *P* like elements may have vector capability in the insects in which they are found. However, if they retain the functional characteristics of *P*, their mobility properties also might be restricted to a few closely related species.

3. *Hobo*

The *hobo* transposable element was discovered in *D. melanogaster* by its association with a number of mutant alleles of cloned genes, which made its isolation and initial molecular analysis straightforward (see Ref. [24] for a review of *hobo*). The eventual identification of a complete *hobo* element revealed basic structural similarities with the *P* element in that it is a 3.0 kb element with short terminal inverted repeat sequences. A 2 kb open reading frame exists that is capable of encoding a 73 kilodalton polypeptide. Beyond these general structural similarities with *P*, there is no apparent DNA or amino acid sequence homology with *P*, nor do the elements act to mobilize one another. Like *P*, *hobo* has been developed into a vector helper transformation system in *D. melanogaster* yielding similar transformation frequencies [25].

The molecular analysis of a functional *hobo* element revealed domains of homology in the amino acid sequence between *hobo* and the plant transposons *Activator*, from maize, and *Tam3*, from *Antirrhinum* [26]. This suggested that these transposons may have a common evolutionary history and, if so, related transposons might exist in a variety of insect species, and perhaps their mobility properties are less phylogenetically restricted compared to *P*. Preliminary investigations give support to both these possibilities. Atkinson et al. [27] used *hobo* mobility assays, similar to those described for *P*, in *D. melanogaster* and *Musca domestica*. Interestingly, *hobo* mobility was detected in *Musca* without a helper plasmid, but not in a *D. melanogaster* strain known to be devoid of *hobo* elements. This suggests that the *Musca* strain tested may have *hobo* like elements capable of mobilizing *hobo*. In an effort to identify *hobo* or *hobo* like elements, we screened a wide variety of insects using polymerase chain reaction (PCR) gene amplification techniques. First, degenerate PCR primers were made to DNA sequences within the *hobo* transcriptional unit, which encode four to five amino acid sequences identical to that found in *Activator*. These primers amplified the intervening DNA sequence in both *hobo* and *Activator*. PCR amplified DNA sequences were also generated from genomic DNA from various *Drosophila* species, *Anastrepha suspensa* and *striata*, *Heliothis zea* and *virescens*, *Musca domestica* and *Stomoxys calcitrans*. Of the insects tested, it is notable that PCR fragments were not generated from *Ceratitis capitata* or *Anopheles quadrimaculatus*. Given our primary interest in tephritid insects, the single 450 bp fragment generated in *A. suspensa* was subcloned, sequenced and compared with the corresponding sequence in *hobo*. After sequence alignment, a 55% homology was determined between the sequences, which was the highest level of homology among all the transposable element sequences in the GenBank. Translation of the aligned sequences yielded an amino acid sequence homology of approximately 30%.

Transposable elements often exist as defective (non-mobile) deletion derivatives of complete elements. Thus, it is possible that transposon related

sequences exist but are missed owing to deletion of one or both of the primer sites. For both *P* and *hobo*, most of the deletion derivatives are internal, leaving the inverted terminal repeat sequences intact or with minor sequence variation. Since some homology exists between the terminal sequences of *hobo* and *Activator* (as well as other transposable elements) [28], a PCR screen was also conducted using the terminal inverted repeat sequence as a primer. Unfortunately, the PCR reaction is usually limited to efficient amplification of sequences smaller than 2 kb; thus we would not expect to generate complete sequences in the range of 3 kb. However, smaller internal deletion derivatives can be detected. As expected, numerous fragments were generated in *D. melanogaster* and closely related species, as well as drosophilids in which *hobo* had not yet been detected. Analysis of a wide range of dipterans, lepidopterans, hymenopterans and coleopterans also revealed several fragments in almost all the species. Notably, one of the only species which did not reveal *hobo* like fragments was *A. quadrimaculatus*, consistent with the internal primer result. However, several polymorphic fragments were detected in several strains of *C. capitata*.

The relatedness of these amplified sequences to *hobo* was indicated by DNA hybridization using the *hobo* Xho I fragment (a 2.6 kb internal sequence not including the termini) as a probe. A very strong signal was obtained from *D. melanogaster* strains and *Drosophila* species, such as *simulans*, known to have elements. Previous evidence from Daniels et al. [29] indicated a very limited distribution of *hobo* among drosophilids based on low stringency hybridization. Thus it was interesting to detect signals from *D. willistoni* and *D. saltans*, which were probably missed in the Daniels et al. study owing to sequence divergence or low copy number. Given the 55% homology between *hobo* and the *hobo* like internal fragment in *A. suspensa*, we were not optimistic that an unambiguous signal would occur in non-drosophilids. Among non-drosophilids, a signal was detected only from *Anticarsia gemmatalis*, the velvetbean caterpillar, suggesting a relatively high level of homology. These fragments are currently being sequenced to better define this possibility.

The apparent mobility of *hobo* in non-drosophilids, along with the discovery of DNA elements having homology with *hobo*, suggest that *hobo* based gene vectors may be functional in these insects, or that new transposable elements may be isolated on the basis of their relatedness to *hobo*. The isolation of a functional *hobo* like element, if necessary, will be a challenge, and may be pursued by testing for transcriptional activity or transient mobilization of defective elements. Despite these difficulties, these lines of investigation hold great promise for development of gene vectors for non-drosophilid insects.

4. *Mariner*

Quite recently, another transposable element from *Drosophila*, *mariner*, has been developed into a gene vector. *Mariner* was discovered in *D. mauritiana*, associated with the *white*^{peach} mutation [30]. Like *P* and *hobo*, *mariner* is a terminal inverted repeat transposon, but it is different in some significant ways (see Ref. [31] for a review of *mariner*). First, it is smaller than either *P* or *hobo*, being about 1.3 kb in length with an uninterrupted open reading frame of about 1 kb, and having inverted terminal repeats of 28 bp. Second, in contrast to both *P* and *hobo*, *mariner* exhibits a significant level of somatic mobility. This is evidenced in the *white*^{peach} strain where somatic excision of the element from the wild type gene results in a mosaic phenotype of patches of wild type red eye pigmentation in a mutant peach colour background.

Like *hobo*, but unlike *P* thus far, *mariner* like elements have been discovered recently in non-dipteran insects. Somewhat serendipitously, Lidholm et al. [32] discovered a *mariner* like element (MLE) inserted within an intron of an antibacterial protein gene, *cecropin*, in *Hyalophora cecropia*. The MLE is 1255 bp in length with 38 bp terminal inverted repeats. The internal sequence has 48% homology with *mariner*, with the terminal repeats having 68% homology. Interestingly, it was estimated that the *Cecropia* species has approximately 1000 copies of the MLE, while *mariner* has about 20–30 copies in *D. mauritiana*. Including conservative replacements, the MLE reading frame has a 56% similarity with *mariner*. Stretches of an identical amino acid sequence for six and seven residues indicates that gene amplification techniques can be used to reveal additional MLEs in a variety of insects, as we have done with *hobo*. Clearly, this should be a high priority.

MLE transcripts have not been detected in *Cecropia*, and the reading frames sequenced apparently have mutations capable of limiting full length or functional translation products [32]. However, if the inverted repeats represent true terminal sequences, then it might be relatively simple to attempt the mobilization of the MLEs from either chromosomes or plasmid constructs, by transient expression of *mariner*. Similarly, the mobilization of *mariner* itself should be tested by excision or transposition assays. If neither of these approaches is successful, then an MLE vector would require the isolation of an autonomous functional element, or an element encoding functional transposase. As with *hobo* like elements, identification of an autonomous element may not be trivial. However, the somatic expression of *mariner* presents the possibility that the initial identification of transcriptionally active elements may be considerably easier than with the germ line restricted *P* and *hobo*. Since transposase from other systems is normally produced at very low levels, more extensive and sensitive tests are required to rule out MLE transcripts in the *Cecropia* strain from which it was discovered. In addition, screening by PCR and reverse transcriptase-PCR may reveal transcripts in other *Cecropia* strains, and in other insects in which MLEs are discovered. Similar to *hobo*, the development of a gene

vector from *mariner* or *mariner* like elements may be difficult, but it also has one of the best potentials for success.

5. APPLICATION OF GENE TRANSFER TO GENETIC SEXING TECHNIQUES

Of the many potential uses of gene transfer technology for the management of economically and medically important insects, an important application with potential for immediate use is for genetic sexing and male sterilization utilized in the sterile male release technique (see Ref. [33]). The knowledge and techniques available from recombinant DNA, and transformation studies in *Drosophila*, suggest a variety of means to genetically sex and sterilize male insects. The rapid implementation of these techniques may be easier relative to other applications requiring the release of transgenic insects into the environment, since release would be limited to sterile males.

The basis for genetic sexing using molecular techniques would, most simply, follow methods already being developed with classical genetic techniques. These include the sex specific expression of a selectable gene product. A useful system for fruit flies is alcohol dehydrogenase (*adh*) gene activity [34], since it can be used with both positive and negative selections [35]. A lack of activity, as in the null mutant *adh*, is selected against with ethanol, while normal activity from the wild type gene is selected against with secondary alcohols such as pentenol. Thus, female specific expression of adh^+, when driven by a promoter such as the female specific yolk protein (YP) gene promoter [36], could be selected against by pentenol. However, while female specific YP promoters exist in almost all insects, they are only active in adults or late pupae, thereby limiting male selection after rearing costs have been incurred. A more highly efficient selection system which acts early in development and without toxic chemicals is based on temperature sensitive lethal (*tsl*) gene activity [37]. Organisms having *tsl* mutations die at elevated temperature owing to gene products which are necessary for survival becoming non-functional. The male specific expression of a wild type allele of a *tsl* gene, in an otherwise *tsl* mutant background, would allow males to survive, while females would die at an elevated temperature. Most simply, this system would require a male specific promoter which acts in most tissues or those critical to survival. Unfortunately, the identified male specific promoters in *D. melanogaster* are all limited to tissues necessary for reproduction, but which are not critical to survival [38]. Nevertheless, such male specific promoters could find use in causing male sterility by linking them to toxin genes. An alternative to the recessive *tsl* genes are mutations in genes such as a dominant temperature sensitive lethal (*DTS*) gene, which has recently been cloned [39]. This mutation acts as a neomorph or antimorph to cause lethality even

in the presence of wild type alleles. If expressed female specifically, the mutant gene product would kill only females at elevated temperatures.

Although the recombinant DNA molecules for the systems described are available or can be created, the most effective genetic sexing and sterilization strains, similarly based upon genetic information from *D. melanogaster*, are much more speculative. For example, several sex determination genes act female specifically throughout development owing to female specific intron splicing [40]. In females, the spliced intron removes a translational stop signal, allowing production of a functional product. In males, use of an upstream 3′ splice site allows the stop codon to remain in the transcript, resulting in a truncated non-functional product. If the female specific splice site were inserted in front of (5′) to a gene which encodes a selectable or toxic product, in conjunction with a conditional promoter, expression would be limited to females, allowing their selection early in development.

Interestingly, the most efficient model system for genetic sexing and male sterilization, also from *D. melanogaster*, does not directly require recombinant DNA or gene transfer techniques. This model is based upon the *transformer-2* temperature sensitive (*tra*-2^{ts}) allele [41]. The *tra-2* gene is required in chromosomal females for female differentiation. In the absence of *tra-2* (as in a *tra-2* homozygous mutant), chromosomal females develop as fully differentiated males, but they have rudimentary testes and are sterile. Although mutant XY males are not somatically affected, they are also sterile. Therefore, for the temperature sensitive allele, XX females are fertile (although weakly) at the permissive 16°C temperature, but develop as morphological sterile males at 29°C. Mutant XY males are morphologically normal at both temperatures, but sterile at 29°C. Thus, an idealized extrapolation of this homozygous mutant strain would yield breeding parentals at 16°C, while genetically identical offspring reared at 29°C would all develop as sterile males. In this situation, there would be no treatment necessary other than elevated temperature for sexing and sterilization. Indeed, genetic sexing would not be required, since all the zygotes would be usable for release, thereby reducing by half the number of parental insects needed for breeding. Although, as noted, molecular genetic manipulations would not necessarily be required to develop an analogous system, molecular analysis using gene transformation would clearly be essential to discover sex determination genes in non-drosophilid insects and to create conditional mutations by site directed mutagenesis. This system well illustrates that while gene transfer techniques may have some immediate uses for insect management, their greatest potential will only be realized as a result of their use in more basic genetic and biological studies.

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PROSPECTS FOR NON-DROSOPHILID GERM LINE TRANSFORMATION

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Abstract

PROSPECTS FOR NON-DROSOPHILID GERM LINE TRANSFORMATION.

The *hobo* transposable element of *Drosophila melanogaster* has been used to achieve stable germ line transformation of this insect. It is examined whether this element, or related transposable elements, can be used as vectors for achieving the germ line transformation of two non-drosophilids, the Australian sheep blowfly, *Lucilia cuprina*, and the housefly, *Musca domestica*. Through the use of excision assays designed to measure the mobility of *hobo* in preblastoderm embryos, it is demonstrated that this transposable element is capable of movement in these species and that this mobility is independent of the supplied *hobo* transposase, suggesting the presence of host encoded factors which are capable of interacting with *hobo* DNA. The presence of *hobo* like elements in the genomes of each of these insects is supported by the cloning of *hobo* like sequences from both these species. Analysis of these sequences is discussed, as is the possible role of *hobo* and *hobo* like elements in achieving non-drosophilid germ line transformation.

1. INTRODUCTION

The *hobo* transposable element of *Drosophila melanogaster* is approximately 3 kb in length and is a member of a class of transposable elements that contains short, inverted repeat sequences at their termini. In some respects, *hobo* elements resemble the *P* transposable elements of *D. melanogaster*. They are of similar size, they transpose through a 'DNA only' mechanism, their presence or absence can result in a phenotype characterized as hybrid dysgenesis, both have been proposed to have invaded *D. melanogaster* wild populations within the past 50 years, and they create duplications of flanking sequences upon insertion into the genome [1, 2]. Furthermore, as for *P*, *hobo* elements have been used to genetically transform *D. melanogaster* [3]. However, there are a number of significant differences between these two *Drosophila* transposable elements. There is no sequence similarity between *hobo* and *P* and the internal organization of each element is also quite different [4, 5]. The *P* element transposase is encoded by four exons and differential splicing of the last intron is the basis of the tissue specific expression of *P* transposase. While expression of the putative *hobo* transposase is not yet fully understood, the presence of one long open reading frame encoding *hobo* transposase would seem to preclude differential splicing playing a role in *hobo* transposase expression.

The ability of *hobo* elements to act as vectors of *Drosophila* transformation suggests that they may be also capable of achieving non-drosophilid insect transformation. To explore this further, we developed an excision assay which allows the detection of *hobo* element excision in insect embryos [6]. Using these assays, we demonstrated that *hobo* can be mobilized in the embryos of *D. melanogaster*, the Australian sheep blowfly, *Lucilia cuprina*, and the housefly, *Musca domestica*. Moreover, in both *Lucilia* and *Musca*, excision appears to be independent of *hobo* transposase, suggesting that endogenous genes are involved in *hobo* mobility. In support of this, we have cloned sequences from both of these non-drosophilids which display strong similarity to the *hobo* element of *D. melanogaster*.

2. MATERIALS AND METHODS

2.1. Strains

The strain of *L. cuprina* used for both microinjections and cloning was the standard wild type (SWT) strain maintained at the Division of Entomology, Commonwealth Scientific and Industrial Research Organization (CSIRO), Canberra. Three different populations of *M. domestica* were used. One had been maintained at the United States Department of Agriculture (USDA) in Beltsville, Maryland, another at the USDA in Gainesville, Florida, and the third at the CSIRO Division of Entomology, Canberra.

2.2. Plasmid constructions and *hobo* excision assays

The *hobo* excision assay, together with the construction of the plasmids used and the techniques used for the sequencing of excision events, have been described previously [6].

2.3. Cloning of *hobo* like sequences from *L. cuprina*

A size fractionated genomic library cloned into the EMBL3 derived lambda vector GEM11 (Promega) was transferred to Hybond N+ membranes (Amersham) and screened with radiolabelled DNA containing the *hobo 108* element [4] under standard conditions of low stringency (5X SSC, 54°C, 0% formamide) [7]. Positively hybridizing plaques were rescreened with the same DNA and subsequent hybridizing plaques were purified for further analysis. One of these was found, through conventional techniques of cloning and DNA sequencing [8], to contain sequences similar to the *hobo* element. DNA sequence analysis was performed using the GCG database program of the University of Wisconsin, Madison, USA.

3. RESULTS

3.1. *Hobo* excision assays in *L. cuprina* and *M. domestica*

The *hobo* excision assay is similar in concept to the *P* element excision assay described previously [9]. An indicator plasmid containing a defective *hobo* element was co-injected into pre-blastoderm embryos together with a helper plasmid which encodes *hobo* transposase. In some experiments, the indicator plasmid is injected alone. When injected alone into embryos of a strain of *D. melanogaster* that lacks autonomous elements, no excision events are recovered [6]. Co-injection of the *hobo* transposase encoding plasmid results in the recovery of excision events at a frequency of at least 0.04% [6]. Sequence analysis of these excision events revealed that, in the majority of cases, all of the *hobo* element had been removed, together with a small amount of flanking DNA sequence. Significantly, additional DNA was present at the empty excision site. This additional DNA was not from the *hobo* element but was an inverted duplication of flanking genomic DNA. In some excision events, this additional DNA was duplicated several times. Injection of a plasmid lacking *hobo* sequences did not result in the recovery of excision events, indicating that excision was dependent on the presence of *hobo* sequences in the indicator plasmid.

The pattern of *hobo* excision observed in *D. melanogaster* was found to be distinctly different to the pattern of *P* element excision events recovered from this species under identical experimental conditions [10]. Specifically, following *P* exci-

TABLE I. *hobo* EXCISION IN *L. cuprina* (SWT) AND *M. domestica*

	With *hobo* excision indicator plasmid					−*hobo* sequences
	L. cuprina (SWT)		*M. domestica*		*E. coli*	*M. domestica*
+ *hsp*70 *hobo* transposase helper plasmid	−	+	−	+	−	−
No. of independent experiments	9	5	9	7	5	6
No. of plasmids screened	75 713	44 157	42 142	47 437	1×10^5	55 722
No. of $tet^R lacZ^-$ colonies recovered	40 (0.053%)	25 (0.057%)	95 (0.225%)	14 (0.029%)	7 (0.007%)	16 (0.029%)
No. with deletions	19 (0.025%)	11 (0.025%)	55 (0.130%)	11 (0.023%)	0	2 (0.003%)
No. with deletions involving *hobo* sequences[a]	4 (0.005%)	2 (0.004%)	20 (0.047%)	11 (0.023%)	0	−

[a] We have not as yet examined the break points of the remaining 34 and 35 deletions recovered from *L. cuprina* and *M. domestica*, respectively. Agarose gel electrophoresis revealed that none of these deletions co-migrated with a known precise excision event recovered from *D. melanogaster* in which *hobo* excision involved both termini, rather that they contained deletions which were either larger or smaller in size. We therefore suggest that it is unlikely that these deletions have resulted from excisions in which both *hobo* termini have been involved. We are currently examining these plasmids in order to determine the structure of the excision events.

sion no additional DNA is found at the excision site, not all of the *P* element is excised and there is no duplication of flanking genomic DNA. The pattern of *hobo* excision in *D. melanogaster* resembled the excision of *Ac* elements and *Tam3* elements from maize and snapdragon, respectively, suggesting that these transposable elements are members of a single family of eukaryotic transposable elements. Sequence similarities between these transposable elements have previously been identified [5]. Given the wide phylogenetic range of the *Ac* element in particular, we were interested to determine whether *hobo* could be excised in non-drosophilids. Demonstration of *hobo* excision in non-drosophilids would indicate that *hobo*, or *hobo* like, elements could be useful as transformation vectors in these insects. Preblastoderm embryos of *L. cuprina* and *M. domestica* were injected with indicator plasmid in the presence or absence of *hobo* transposase encoding plasmid. Excision was observed in both species, however, at a lower frequency than that observed in *D. melanogaster* (Table I). Significantly, injection of indicator plasmid alone resulted in the recovery of excision events from both species. This suggests that *L. cuprina* and *M. domestica* contain endogenous factors which are capable of recognizing the *hobo* element and removing it from the indicator plasmid.

Sequence analysis of the excision events recovered from *L. cuprina* and *M. domestica* revealed that they did not resemble the excision events recovered from *D. melanogaster*. Rather, the excision events appeared to be imprecise. In many cases, large parts of *hobo* remained at the empty excision site and, in addition, deletions often extended several hundred base pairs into the flanking plasmid sequence. In those excision events so far characterized from *M. domestica* and *L. cuprina*, in only four and two cases, respectively, did at least one of the break points occur within *hobo* element terminal sequences. Excision assays conducted in *M. domestica* using an indicator plasmid lacking *hobo* sequences resulted in the recovery of *lacZ* plasmids at a frequency forty-three fold lower than that observed in assays using the *hobo* containing indicator plasmid, indicating that, even though imprecise, excision was dependent on the presence of the *hobo* sequences in the indicator plasmid.

3.2. Cloning of *hobo* like sequences from *L. cuprina*

A library constructed from size fractionated *L. cuprina* genomic DNA was screened with *hobo* element DNA under low stringency conditions [7]. One clone that consistently hybridized to this probe was selected for further analysis. Partial sequence analysis revealed this clone to contain sequences which were 50% similar overall to the *hobo* element HFL1. Moreover, sequence similarity increased in three regions previously identified as being conserved both at the nucleic acid and the amino acid levels between *hobo*, *Ac* and *Tam3*. While this clone is still to be entirely sequenced, the presence of these conserved regions, together with a putative terminal repeat sequence which is 83% similar to the *hobo* terminal sequences, suggests that it is closely related to the *hobo* transposable element. In addition, translation of

the putative *hobo* like transposase from this clone shows 39% identity and 59% similarity over the entire region that has been sequenced.

Further evidence for the presence of *hobo* like sequences in the genome of *L. cuprina* has been gained through the use of the polymerase chain reaction (PCR). Degenerate oligonucleotide primers based on the three regions of similarity between *hobo*, *Ac* and *Tam3* were synthesized and used to amplify the intervening sequences. Using primers from regions 1 and 2, an expected fragment of 450 bp was amplified from *D. melanogaster* DNA and, in addition, fragments of similar size were amplified from *L. cuprina* and *M. domestica*. All the fragments hybridized strongly to a radiolabelled *hobo* DNA probe. The sequence of the 264 bp *M. domestica* clone revealed it to have 66% similarity to *hobo* element HFL1. Moreover, translation of the putative open reading frame of this clone showed an 81% similarity to *hobo* transposase [11].

4. DISCUSSION

Through the use of *hobo* excision assays we had previously demonstrated that the *hobo* element can be mobilized in the soma of *D. melanogaster* embryos and that this excision was dependent on the presence of *hobo* sequences in the indicator plasmid. Moreover, the structure of empty excision sites following excision of the *hobo* element resembled the structure of empty sites following the excision of the *Ac* element of maize and the *Tam3* element of snapdragon in that all of the *hobo* element was removed and additional DNA, which was an inverted duplication of flanking genomic DNA, was left at the excision site. This supported the hypothesis proposed by Calvi et al. [5] that these three transposable elements were members of the one family of transposable elements. They identified three regions of strong nucleic acid and amino acid similarity between *Ac* and *hobo*, one of which was also common to *Ac*, *Tam3* and *hobo*.

Given the broad phylogenetic range of species in which *Ac* can be mobilized, we were interested in determining whether *hobo* could also be mobilized in a number of distantly related species to *D. melanogaster*. Previous studies based on the low stringency hybridization of *hobo* element DNA to genomic DNA prepared from 134 dipteran species suggested that *hobo* had a very narrow host distribution within dipterans, confined to the *melanogaster* and *montium* subgroups of the *melanogaster* species group [12].

We chose two independent strategies to examine for the presence of *hobo*, or *hobo* like, elements in two non-drosophilids, *L. cuprina* and *M. domestica*. The first, based on a genetic approach, assayed for the mobilization of the *hobo* element HFL1 in pre-blastoderm embryos. Excision events were recovered from both species. However, sequence analysis of these excision products revealed significant differences between their structure and the structure of excision events recovered

from *D. melanogaster*. Excision in both *L. cuprina* and *M. domestica* was imprecise, only part of *hobo* was excised, and deletions extended into flanking genomic DNA. That these excisions were dependent on *hobo* sequences in the indicator plasmid was shown by the significant reduction in the recovery of *lacZ*$^-$ plasmids when excision assays were carried out with an indicator plasmid lacking *hobo* sequences. Significantly, the presence or absence of helper *hobo* encoded transposase had little effect on either the frequency or the structure of these excision events.

The recovery of *hobo* excision events from *L. cuprina* and *M. domestica* in experiments in which *hobo* encoded transposase was absent suggests that both of these non-drosophilids possess factors functionally related to *hobo* transposase which are capable of recognizing and excising, albeit imperfectly, *hobo* elements. It is clear, however, based on the structure of the empty excision sites recovered from *L. cuprina* and *M. domestica*, that these factors differ from *hobo* transposase in their interactions with *hobo* element DNA. Whether this difference in the structure of these sites reflects differences in the excision process itself or in the subsequent repair of these sites remains to be examined.

Further support for the existence of *hobo* like elements in the genomes of these non-drosophilids was obtained by the cloning of *L. cuprina* and *M. domestica* sequences which have significant similarity to the *hobo* element. Partial nucleic acid and amino acid sequence analysis of the *L. cuprina* clone in particular has shown that the three regions identified by Calvi et al. [5] as being conserved between *hobo, Ac* and *Tam3* are also present in this clone. The question as to whether or not each of these clones represents a transposable element and, if so, whether they are functional and autonomous, should become clear once sequence analysis is complete. Excision and transposition assays using these elements in *L. cuprina*, *M. domestica* and *D. melanogaster* should provide information on the mobility of these elements in each of these species.

The presence of *hobo* like sequences in *L. cuprina* and *M. domestica*, members of two families within the Diptera, suggests that *hobo* like sequences, and perhaps *hobo* like transposable elements, may be more widely distributed throughout the Insecta than previously thought. As for the *P* element, the origin of *hobo* elements and the basis for their dispersal amongst drosophilids remain largely unknown, although there is strong evidence for both elements being recent invaders of *D. melanogaster* [2]. The contribution, if any, of horizontal transfer in the distribution of *hobo* is unknown. However, it has recently been suggested that the parasitic mite, *Proctolaelaps regalis,* may be responsible for the physical movement of *P* elements between some species of the family Drosophilidae [13]. Given the diversity of organisms possessing the *hobo/Ac/Tam3* family of transposable elements, it seems possible that horizontal transfer may have also once played a role in the distribution of these elements in extant species. Distribution of this family of elements within the Insecta is currently the focus of much activity within our laboratories. Isolation and

characterization of *hobo* like elements in selected non-drosophilids might be expected to be of some use in the extension of the techniques of modern molecular biology to commercially and medically important insects.

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DEVELOPMENT AND USE OF DNA PROBES IN MAPPING INSECT GENOMES

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Abstract

DEVELOPMENT AND USE OF DNA PROBES IN MAPPING INSECT GENOMES.

Classical genetic mapping has relied on the use of morphological, biochemical and DNA variation (restriction fragment length polymorphisms) to establish linkage relationships between loci in order to construct genetic maps. This genetic analysis has been complemented by a cytological component. Recent advances in molecular biological techniques will revolutionize the manner and the speed with which genetic maps can be constructed. The 'motor' of this revolution is the polymerase chain reaction. The paper concentrates on two techniques which are now available, namely, microsatellites and random amplified polymorphic DNA (RAPD), by providing an explanation of the principles involved together with examples of their use. A comparative assessment is made of the two approaches and some emphasis given to their use as tools for mapping specific genes.

1. HISTORICAL PERSPECTIVE

Recent advances in molecular biology have dramatically increased the accessibility of insect genomes to genetic analysis. Given certain assumptions, it is possible to generate a detailed genetic map of any insect species within an extremely short time.

Analysis and mapping of insect genomes has in the past relied on the availability of spontaneous or induced mutations at specific loci, which were then used to establish linkage groups and recombination distances between the loci. Using this approach, linkage maps can be assembled and when combined with cytological techniques, linkage group chromosome correlations can be established. This is a laborious procedure as it relies on the availability of visible mutations, by definition rare events, and the collaboration of many laboratories working on a single species. This approach is typified in the example of *Drosophila melanogaster*, where an extremely detailed map has been assembled over 80 years by a large number of geneticists and cytogeneticists. The use of protein variation, i.e. isozymes, improved dramatically the analysis of insect genomes as variation at this level is much more

widespread and is readily accessible for analysis. However, there are real limits to the number of loci that can be studied. Nevertheless, using a combination of biochemical and morphological markers, detailed genetic maps have been constructed for several important pest or beneficial species, e.g. *Lucilia cuprina* [1], *Aedes aegypti* [2], *Musca domestica* [3], *Bombyx mori* [4] and *Ceratitis capitata* [5].

For many years it has been recognized that variation at the DNA level can be accessed using cloned DNA sequences and looking for restriction fragment length polymorphisms (RFLPs), either within the cloned regions or in the flanking sequences. Variations are monitored as changes in the length of the defined DNA fragments produced by digestion of the DNA with restriction endonucleases. This technology has been extensively used to produce detailed genetic maps in many species [6]. Although this variation is ubiquitous, exhibits co-dominance and is stably inherited, serious practical limitations have been acknowledged: it is laborious, the quality of the DNA has to be high and the amount of DNA required is such that only a few RFLPs can be scored for any individual. This paper will not deal further with RFLPs.

1.1. Importance of cytology

As indicated above, cytology has been a key element in the development of genetic maps; this is especially so in many dipteran species where polytene chromosomes are available. In polyteny, multiple rounds of replication of each chromosome occur within a single nucleus, and the chromosome copies pair precisely, leading to very long and thick chromosomes characterized by a banding pattern that identifies a particular chromosome and its various sections. In *D. melanogaster*, the combination of a large number of morphological markers and extremely fine cytological mapping has enabled a wonderfully precise genetic map to be assembled, encompassing close to four thousand loci. The usefulness of polytene chromosomes for genome mapping cannot be overemphasized as any cloned sequence can be mapped precisely to its cytological location(s) using in situ hybridization.

1.2. Current approaches

The key to being able to map any particular genome is the availability of variation at the DNA level and the accessibility of that variation to analysis. The sequence is the key, with variation occurring as specific changes in the nucleotide order or as changes in the number of times a particular sequence is repeated. Both these types of variation are well known, but up until now they have not been routinely accessible to molecular analysis. This situation has now changed with the introduction of the polymerase chain reaction (PCR) [7].

The PCR utilizes in vitro enzymatic amplification of a specific segment of DNA. Three nucleic acid segments are used, the double stranded DNA template and

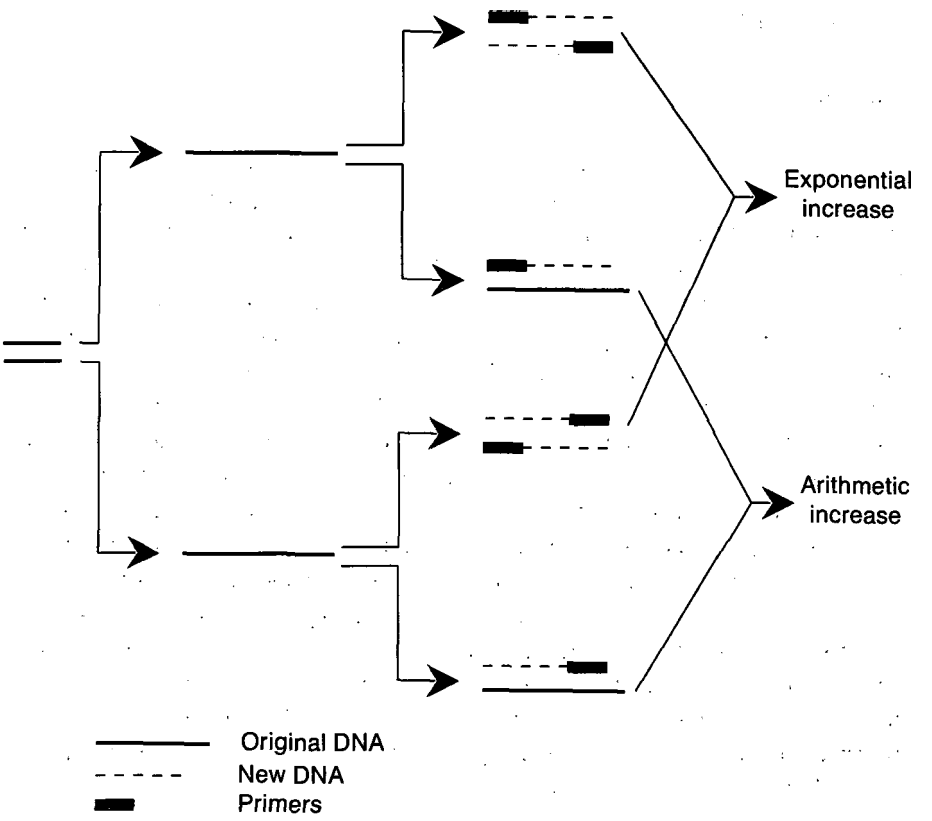

FIG. 1. Schematic of the polymerase chain reaction.

two single stranded oligonucleotide primers that are complementary to the two strands, at opposite ends of the DNA segment. By adding a thermostable DNA polymerase, dNTPs and the appropriate buffer, the DNA segment between the primers can be exponentially amplified (Fig. 1). This is done by multiple cycles of denaturing the double stranded DNA, annealing the primers and synthesizing a new double strand of DNA between the primers. This amplification product can be visualized on slab gels following ethidium bromide staining. The power of this technique is illustrated by the fact that if 30 of these cycles were repeated, then a 27 million fold amplification can be obtained from a specific segment. This means of course that very small amounts of template DNA are needed and conversely many assays could be carried out from a single individual. Most of the approaches outlined below can be carried out on insect species where no genetic information is available.

2. ANALYTICAL TOOLS USING PCR

2.1. Microsatellites

Microsatellites are dinucleotide or other simple repeat sequences, e.g. (CA)n, and they have proved to be highly polymorphic and widely distributed in diverse genomes, including the mouse and human. For example, there are 10^5 sequences containing (CA)n repeats in the human genome, mainly in the range of 9–30 repeat units, and they seem to be homogeneously distributed, approximately one every 50–150 kb [8]. When assayed using PCR, they define sequence tagged sites [9]. They are differentiated from minisatellites only by the size of the repeat unit which, in minisatellites, can be much larger. The principle (Fig. 2) is to screen a small insert genomic library with an end labelled (CA)n oligonucleotide. Once clones containing these repeats are identified, then the unique sequences flanking them can be sequenced and specific oligonucleotide primers designed which will then amplify the repeat sequence. Following amplification, the products can be visualized on non-denaturing acrylamide sequencing gels by ethidium bromide staining or autoradiography (if amplification is performed in the presence of radioactive dNTP) and differences as small as one repeat can be resolved. To generate a high density map using microsatellites, hundreds of markers are necessary. Using conventional libraries, this presents some technical problems but techniques are now available to construct libraries which contain up to 50% of clones with long microsatellite repeats [10].

In the mouse, this technique has been extensively used to generate a high resolution genetic map [11, 12]. For example, of 50 microsatellites tested, 78% were polymorphic between inbred strains and 92% were polymorphic between a wild mouse strain and the inbred lines. The authors showed that the larger the number of repeats, the more alleles per locus were available for analysis. In the mouse, there are about 10^5 copies in the genome and, if approximately 70% are polymorphic, then a marker can be generated every 20 kb. Recently, the approach has been extended to a mosquito, *Anopheles gambiae* [13], and here, out of 34 (GT)n markers tested, 27 revealed polymorphism. The presence of polytene chromosomes in this species enabled in situ hybridization to be carried out to identify the exact location of specific markers. In general, clones with large numbers of repeats hybridized to many sites on the chromosomes. When clones with less than 12 repeats were used, a single hybridization site could be identified [13].

2.2. Arbitrarily primed DNA (AP-PCR) and random amplified polymorphic DNA (RAPD)

This is the amplification of genomic DNA with single primers of arbitrary nucleotide sequence. These primers will detect polymorphisms in the absence of any

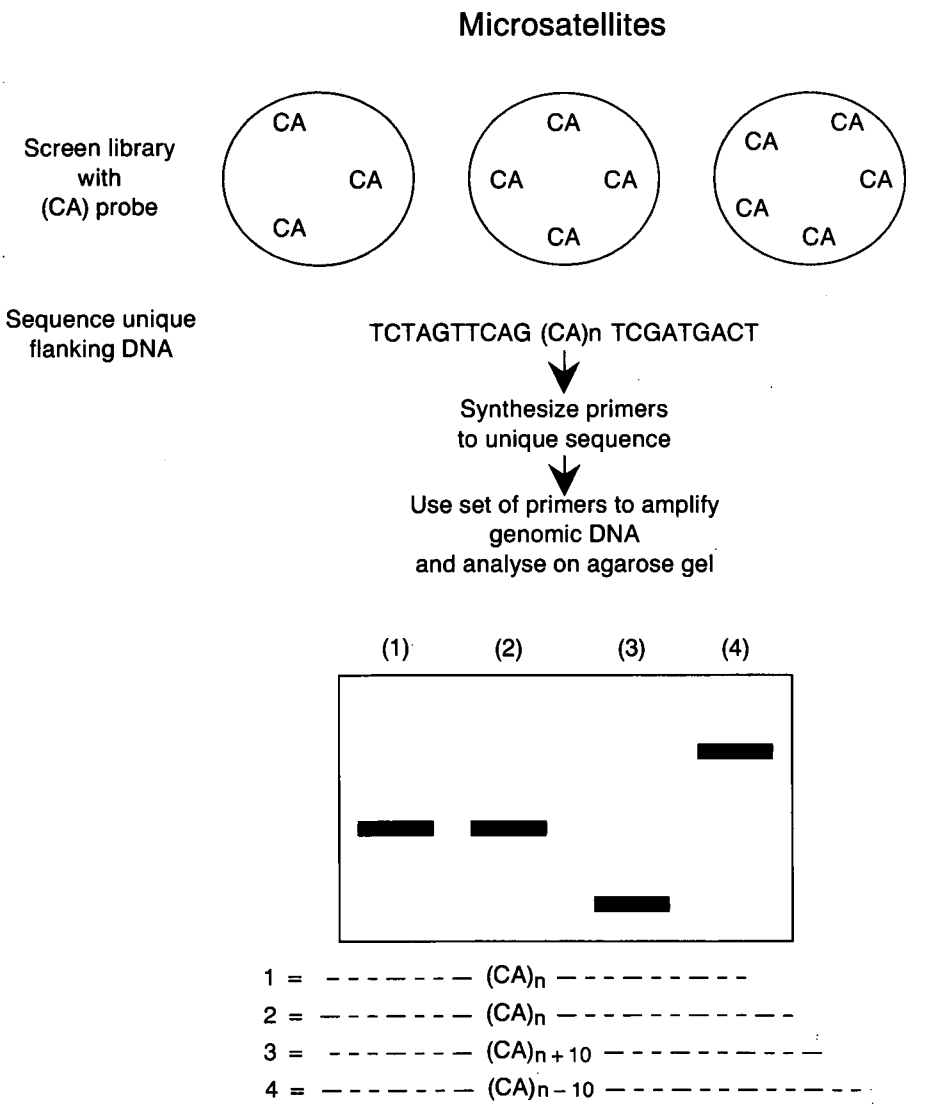

FIG. 2. Isolation and use of microsatellites to reveal polymorphisms.

sequence information and they can be used as genetic markers to construct genetic maps. The polymorphisms are visualized on ethidium stained agarose gels as the presence or absence of bands. The difference between the two approaches, AP-PCR and RAPD, is quantitative, with RAPD being generated by 10 mer oligonucleotides, while AP-PCR products are primed by much larger primers, up to 34 mers. In general, there are more amplified bands using AP-PCR. Primers for RAPD analysis

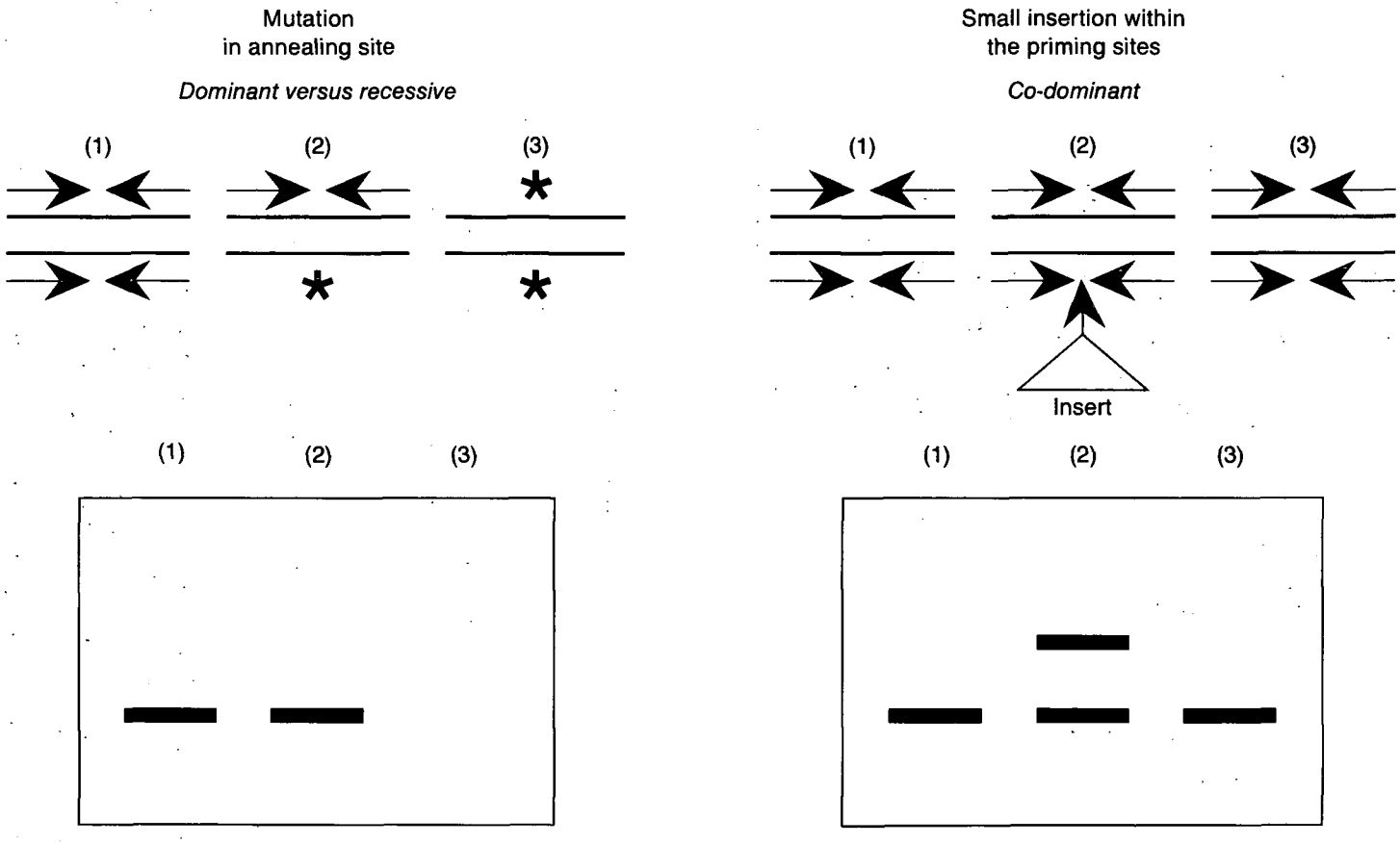

FIG. 3. RAPD polymorphisms illustrating both dominant and co-dominant phenotypes from a single priming site. An asterisk represents a mutation in an annealing site and an arrow the RAPD primers.

are 9–10 nucleotides in length, are between 50–80% GC and contain no palindromic sequences. Using mammalian, plant and prokaryotic DNA, it was shown that these primers can amplify polymorphic DNA which can be used for genetic mapping [14, 15]. The following are some characteristics of RAPD and AP-PCR products: they are generally dominant, they can contain repetitive sequences, they can be used for population studies [16] and those with repeat sequences cannot be used for correlation of cytological and genetic maps.

Figure 3 shows how RAPD can be visualized if a single site on the chromosome is being amplified. If there is a mutation in the annealing site, then both homozygote 1 and the heterozygote will produce a single band, homozygote 3 will produce no band and hence the RAPD is dominant. If a small insertion is present between the primer sites, then a co-dominant phenotype can be visualized. If the primer anneals to multiple sites, a much more complex pattern of amplified products can be produced. In Fig. 4, four different individuals are depicted which have the same mutation in different priming sites. In this case, the cryptic variation is not revealed, with all four individuals producing an identical band on the agarose gel. In Fig. 5, with insertions and deletions in multiple sites, a much more complex pattern can be produced. For genetic mapping, the occurrence of multiple sites is not a problem. However, it is impossible to be specific about the relationships of the bands, i.e. are they alleles or loci, until individual families are examined.

In the *An. gambiae* complex, RAPD has been used to develop genetic markers for differentiation of the sibling species. So far, about fifty markers have been identified. However, in situ hybridization to polytene chromosomes revealed that many of them hybridized to multiple sites [17]. In the Mediterranean fruit fly (medfly), initial results using 20 primers have clearly shown that differences in banding pattern occur between populations [18] and a more detailed genetic analysis has enabled RAPD to be assigned to the five autosomes [19]. However, the reproducibility of the results has to be improved.

In the mouse, AP-PCR primers have been used very successfully to identify strains and for genetic mapping [20, 21] and in *Arabidopsis thaliana,* RAPD was used to contruct a detailed genetic map in a very short time [22].

3. MAPPING STRATEGY

Before embarking on a genome mapping exercise in an insect where there is little formal genetics, it is important to consider the strains to be used and the strategy for their use. As already discussed, variation is the key to being able to map loci, so the question is, what is the best way to maximize such variation (differences) between the strains?

In plants, near isogenic lines and recombinant inbred lines have been extensively used in conventional RFLP analysis to map genes of economic importance and

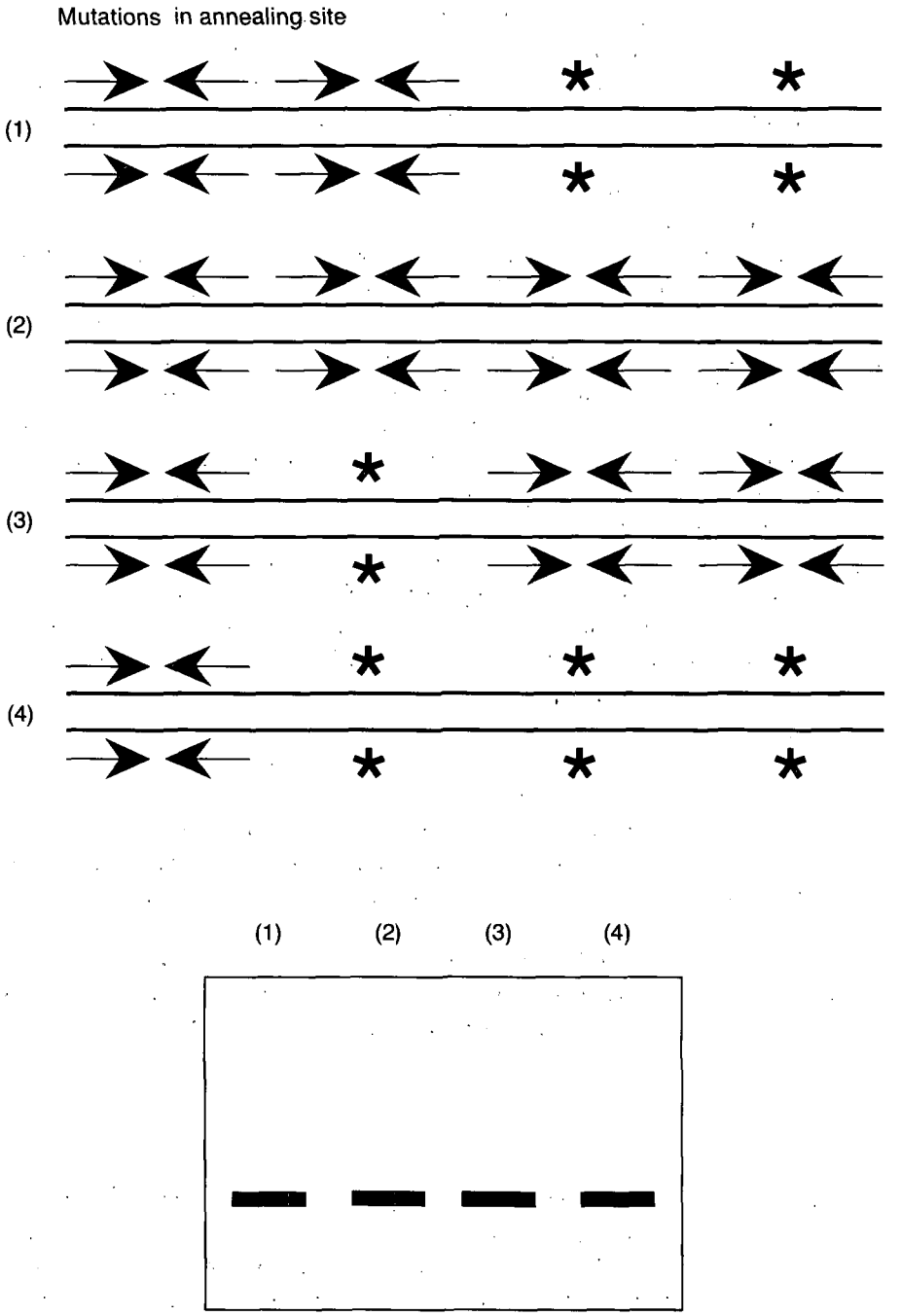

FIG. 4. RAPD amplification at multiple loci where cryptic variation is not revealed. An asterisk represents a mutation in an annealing site and an arrow the RAPD primers.

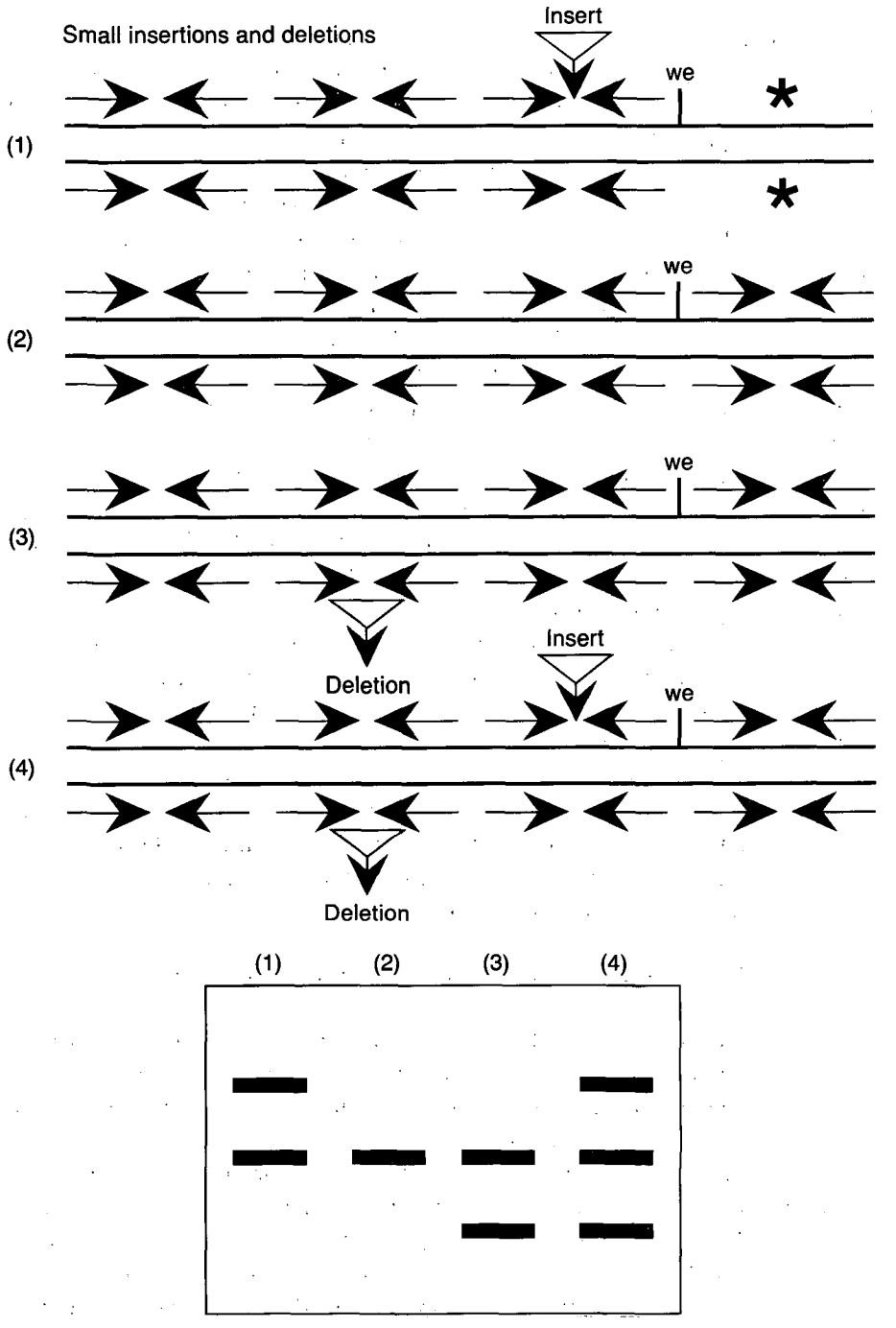

FIG. 5. RAPD amplification at multiple loci. Inserts and deletions lead to different sized bands which can be from different loci or the same locus. An asterisk represents a mutation in an annealing site and an arrow the RAPD primers.

to assemble genetic maps. These lines are now being very efficiently exploited using RAPD. For example, in one month three RAPD markers were identified as being closely linked to a *Pseudomonas* resistance gene in the tomato, whereas using the conventional approach of RFLP mapping, a period of two years was needed [23]. In *A. thaliana,* 225 loci were identified and mapped in 8 person-months [22]. In most insect species, however, such lines are not available and in many cases cannot be constructed or, even if they could, would be extremely time consuming to produce; therefore other strategies are necessary. To demonstrate an alternative approach, the medfly, *Ceratitis capitata*, can be used as an example. In this species, a collection of mutations, both biochemical and morphological, is available and multiple marker strains can be synthesized. Initially, one can construct a strain which is homozygous for at least one marker per autosome and different wild type strains can be crossed with this strain to produce an F_1 generation. Following backcrossing of individual F_1s to the multiple marker strain, a segregating F_2 generation is produced which can then be used to assign molecular markers, either RAPD or microsatellites, to their autosomes. As these techniques are PCR driven, very small amounts of DNA are necessary and up to 40 different molecular markers can be assayed from a single pair mating in a day.

The type of strategy adopted will depend on the species being investigated and the availability of markers, inbred lines, etc.; however, an investment of time in the construction of the relevant strains will increase significantly the speed with which a genetic map can be assembled.

A note of caution is necessary, however, when using RAPD. As the DNA being amplified is random and of unknown sequence, it is essential that molecular analysis be performed on a defined cross, preferably with markers. This is to ensure that the polymorphism observed on the gel can be shown to demonstrate Mendelian inheritance and that it is not simply a PCR artefact.

3.1. Identification of molecular markers linked to specific genes

In some insect vectors of disease, genes have been identified which interfere with the ability of the insect to transmit the disease, the so-called refractory genes [24]. A study of such genes will lead to a better understanding of the disease and perhaps to new methods of control.

In general, the cloning of such genes requires some knowledge of the transcript or polypeptide and in most insect vectors that knowledge is unavailable. If molecular markers can be identified which are tightly linked, they can be used to initiate a chromosomal 'walk' to the gene of interest. To use this approach it is necessary to saturate the genomic area of interest with molecular markers and assess linkage relationships. A new technique called 'bulked segregant analysis' will be an extremely valuable asset to this approach [25]. Bulked segregant analysis involves the screening of two pools of DNA in a segregating population which has been generated from a

single cross. Screening is done on DNA from the parental lines and from the segregating F_2 populations. Using this approach with RAPD, it is possible to differentiate between polymorphisms resulting from variation between the parental lines and polymorphisms linked to the genomic area of interest.

3.2. Correlation of a molecular map with a cytological map

Polytene chromosomes offer a unique possibility of combining a recombinational map with a cytological map. Once cloned or amplified sequences have been mapped recombinationally, they can be assigned to their chromosomal location using in situ hybridization to polytene chromosomes. Not only does this enable genetic distances to be correlated with cytological distances, it also enables the recombinational map of a linkage group to be correctly orientated on the polytene chromosome; this approach has been successfully demonstrated in the medfly [26].

4. CONCLUSIONS

Before concluding with a presentation of the relative merits of microsatellites versus RAPD for genome mapping, it is important to stress the significance of both these methods for the genome analysis of insect pests:

- For neither of the approaches is radioactivity necessary;
- Many assays can be performed per individual;
- The quantity and quality of the DNA need not be high;
- Southern blotting is not used, so polymorphisms can be detected in fragments containing highly repeated sequences;
- Both approaches can be carried out with low technology input;
- No prior genetic or molecular information is required;
- An almost unlimited number of markers is available.

It is clear that molecular markers of the type outlined in this paper can provide a rapid entry point into the genomes of insect pests; there are, however, important differences between RAPD and microsatellites. First, technical differences: for RAPD no libraries are necessary, only DNA and primers, which are commercially available, whereas for microsatellites a library must first be constructed, screened for microsatellites, followed by sequencing of the unique flanking region and the synthesis of specific primers. The reproducibility of results using RAPD can be a problem due to PCR artefacts occurring. This might lead to difficulties in comparing results between different laboratories, whereas for microsatellites, a collection of specific primers will be a very powerful tool for genome analysis with high repeatability. Second, genetic differences: the most important is the fact that RAPD is usually a dominant marker, whereas microsatellites are co-dominant. However, for

mapping this is not a serious problem as backcross populations map dominant and co-dominant markers with equal efficiency [22]. This difference does, however, become important when these two systems are applied to population genetic problems [16]. With microsatellites, loci with high numbers of repeats frequently have multiple alleles/loci and in general the larger the number of repeats the more alleles/loci; RAPD does not in general exhibit multi-allelism. An important factor if in situ hybridization is to be included in the genome analysis is the occurrence of repeat sequences in the probe used for the hybridization. Data from plants have indicated that up to 50% of RAPD loci hybridize to single copy DNA [14]; this has now been supported by data from the mosquito [17]. For microsatellites, the situation is clearly different and without further modification only a small proportion of the microsatellite clones will hybridize to a single polytene site, i.e. those containing low numbers of repeats.

It is at the moment unwise to be dogmatic as to which of the two approaches is best; it all depends on the question being asked and the species under consideration. However, independent of the choice of the method, the ready availability of these molecular tools will open up pest insect genomes to sophisticated analysis.

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ARE IMMEDIATE EARLY BACULOVIRUS GENE PROMOTERS USEFUL TOOLS IN INSECT CONTROL?

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Abstract

ARE IMMEDIATE EARLY BACULOVIRUS GENE PROMOTERS USEFUL TOOLS IN INSECT CONTROL?

Research on the genetic manipulation of baculoviruses for insect pest control has raised two problems. The first concerns the choice of viral promoter used to direct the expression of an introduced insecticidal gene. The second points towards the kind of insecticidal gene which should be preferentially used in order to select maximum effect on the target insect but the minimum impact on the environment. A summary is given of the major arguments in favour of the use of immediate early baculovirus gene promoters which direct the expression of insect derived peptide genes. It is thought that a correct combination will affect the insect at the first site of infection, the insect gut.

1. INTRODUCTION

Nuclear polyhedrosis viruses (NPVs) bear no morphological resemblance to any known plant or vertebrate viruses. They are fairly host specific. They do not affect beneficial non-target insects and have successfully passed extensive safety tests on mammals, birds and fish. Thus, baculoviruses can be considered safe and ecologically acceptable for the regulation of insect pest populations [1]. However, field experiments have shown that baculovirus epizootics frequently terminate infestations of insect pests, but that collapse of the target populations usually occurs after severe crop damage has been inflicted [2]. This phenomenon may limit the use of baculoviruses to the biocontrol of the forest ecosystem, rather than to an agricultural

environment in which either a more straightforward 'knock down' effect or at least a direct block in food intake is envisaged.

At the laboratory level, this inconvenience can be overcome by the introduction of an extra gene, bearing insecticidal potentials, into the baculovirus genome [3]. Several models of genetically improved recombinant baculoviruses have been proposed. The use of either an extra polyhedrin promoter or the authentic P10 promoter, or a combination of both, was favoured in order to ensure production of the occluded virus necessary for oral infestation [4]. Depending on the insecticidal activity spectrum of the expressed recombinant protein, a more or less pronounced reduction in survival time following a single application has been reported. However, both the polyhedrin promoter and the P10 promoter are ascribed to the very late phase of viral gene regulation and even appear not to become activated in insect gut cells, the first site of attack by the virus. A minimal latency time before observing any reduction in crop damage is unavoidable for these types of control vector. Already in 1988, Crawford and Miller [5] suggested the potential of early baculovirus genes for the expression of foreign genes in the early stages of infection. In analogy to the situation in *Autographa californica*, we cloned a sequentially related immediate early gene of the *Bombyx mori* NPV [6], studied the promoter characteristics of the corresponding upstream sequence by using transient transfection assays, and are now trying to create an insect control vector based on the expression of insect derived myo-active peptide genes under immediate early gene promoter control.

2. THE MODEL SYSTEM: BmNPVIEG

The 3.8 kb CLa1 fragment, spanning the 94–96% map unit (mu) region of the *Bombyx mori* NPV physical map [7], includes an open reading frame of 1572 bp as well as a 631 bp upstream regulatory region showing a high degree of sequence homology with AcMNPVIE-1 [6]. Using a polymerase chain reaction, the existence of an extra upstream regulatory region, responsible for an extended spliced expression product, corresponding to AcMNPVIE-0 was also evidenced. Apart from demonstrating that the BmNPVIEG gene product has transactivating capabilities towards viral delayed early genes [6], it became clear that the conditions needed for promoter activation of the cloned leader sequence were fulfilled in permissive as well as non-permissive insect cells [8]. On the other hand, transfection of a fusion construct in which the bacterial beta-galactosidase gene was inserted in frame downstream of the BmNPVIEG promoter allowed us to conclude that neither Vero cells (monkey kidney cells) nor developing zebra fish embryo cells were suitable hosts because beta-galactosidase activity was never observed. Although further testing of the transcriptional activation of the BmNPVIEG-*lacZ* construct by vertebrate cell type transcriptional machinery will be needed, present results point to the assumption that

the BmNPVIEG promoter could be safely used in the field. In vitro, this BmNPVIEG promoter, being isolated from any other cis or transacting viral sequence, remains operational on a continuous, prolonged base [9]. On the basis of the pronounced sequence and the functional similarity to AcMNPVIE-1, transcription of a gene inserted downstream of the BmNPVIEG promoter might also be expected to occur throughout the entire infection cycle [10]. We also tried a gene knock out experiment in which the endogenous BmNPVIEG coding sequence was replaced, through homologous recombination, by a fused *lacZ*-IEG sequence. To our surprise, apart from a wild type virus, the resulting inoculum also contained a virus that produced blue plaques after reinfection and addition of an X-gal substrate. Unfortunately, with respect to the wild type virus, the recombinant virus titre was decreased some 1000 times, making it quite impossible to purify the plaque. At this point, it remains open whether the original BmNPVIEG was indeed knocked out or whether the blue plaques were rather a result of a random integration into a site, effecting optimal viral replication. In any case, in view of the wide range of viral transactivating activity of this immediate early gene, its functional presence in the viral genome will most probably be obligatory. In our model, we are considering duplicating the BmNPVIEG promoter and inserting the second promoter in tandem downstream of the P10 promoter.

3. CHOICE OF INSECTICIDAL GENE

As pointed out in Section 1, the insecticidal activity spectrum of the gene used for the realization of a recombinant baculovirus will be a major determinant for success. It might appear obvious to use well known toxin genes from which the resulting toxin has already shown its potential for insect control. A good example is *Bacillus thuringiensis* toxins. However, one should take into consideration that these proteins exert their effect following proteolytic activation and subsequent binding to a receptor on the luminal side of the gut. A first report on experiments using an occluded recombinant virus carrying the *Bt kurstaki* HD-73 delta-endotoxin was not encouraging [11]. Neither were those experiments in which a baculovirus expressing juvenile hormone esterase, a naturally occurring enzyme responsible for juvenile hormone (JH) inactivation, was used [12]. So far, only the baculovirus carrying a gene encoding the insect specific toxin, AaIT, isolated from the venom of the scorpion *Androctonus autralis*, reduced the killing time of the test insect by 40% [13]. Although these examples might still be somewhat disappointing, one should not forget that the exploration of this field has just begun and that a major breakthrough has still to be made.

In this context, we decided to explore further the potentials of typical insect neuropeptides that participate in the normal physiology of the insect. Recently, our research group [14], as well as several others, realized a major breakthrough by

isolating and sequencing whole families of bioactive neuropeptides. However, so far only a very limited success in cloning the corresponding genes has been reported. In view of our approach, and until the original genes become available, we can only rely on the construction of synthetic genes. In a first attempt, the genes encoding two *Locusta migratoria* myotropins and one myo-inhibiting peptide were expressed in a prokaryotic system and tested for bioactivity in a midgut contraction assay. Final conclusions cannot yet be drawn, but we consider it appropriate to test these genes for correct expression and processing in our eucaryotic, BmNPVIEG based stable expression system [9]. This, in combination with practical in vitro hindgut contraction assay, will enable us to screen for the correct genes that might be worth while testing in vivo by a recombinant baculovirus in which the peptide gene will become expressed by a doubled immediate early gene promoter.

4. CONCLUSIONS

It is generally accepted that recombinant NPVs expressing insect selective toxins, hormones or enzymes could enhance their insecticidal properties. However, since a straightforward approach is not yet available, we started to develop an alternative model system: the immediate early gene promoter directed expression of a synthetic peptide gene where the expression product interacts with the muscle layer surrounding the intestinal tract. From the explanation given it is clear that we are still quite far from the ready-to-use realization of an effective recombinant virus. However, we are convinced that experimental incorporation of an easy in vitro screening system will help in choosing the right insecticidal gene. On the other hand, we are quite aware of the many difficulties we still have to circumvent.

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DEVELOPMENT OF AN ALTERNATIVE BACULOVIRUS EXPRESSION SYSTEM USING AN IMMEDIATE EARLY GENE OF THE BACULOVIRUS OF *Bombyx mori*

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Abstract

DEVELOPMENT OF AN ALTERNATIVE BACULOVIRUS EXPRESSION SYSTEM USING AN IMMEDIATE EARLY GENE OF THE BACULOVIRUS OF *Bombyx mori*.

The classic baculovirus expression system makes use of recombinant viruses to infect cells. This infection can, by the lytic activity of the virus, only result in transient expression of the recombinant protein. In this study, an alternative baculovirus expression system is demonstrated which is based on the property of viral immediate early genes, to be transcribed by uninfected cells in the absence of other viral gene products. An immediate early gene of the baculovirus of *Bombyx mori* (BmNPVIEG) has been isolated and sequenced. By using an expression vector in which the *lacZ* reporter gene is placed under BmNPVIEG promoter control, expression of the *lacZ* reported gene in insect cells in a transient way was found. When the same expression vector was co-transfected with a construct containing the hygromycin B resistance gene, transformed *Drosophila* S2 cells were able to express the reporter gene continuously.

1. INTRODUCTION

Baculoviruses are insect pathogenic viruses with a circular, double stranded DNA genome. In recent years, a subgroup of these baculoviruses, the nuclear polyhedrosis viruses (NPVs), and particularly the *Autographa californica* nuclear polyhedrosis viruses (AcNPV), has received considerable attention because of its potential use as expression vector. Baculovirus genes, based on their temporal expression during infection, can be divided into four classes: immediate early, delayed early, late and very late genes.

The classic baculovirus expression system makes use of the hyperexpressed late viral polyhedrin gene. Insect cells are infected with recombinant viruses in which the desired coding sequence is placed under the control of the polyhedrin promoter. Because of the strength of this promoter, there is hypertranscription of the foreign gene insert at a late stage in the infection cycle.

A disadvantage of this classic baculovirus expression system is the lytic activity of the recombinant virus. Recombinant proteins can only be transiently expressed and at the late phase of infection, when this hyperexpression occurs, the ability of the host cells to process newly synthesized proteins might be compromised [1].

In order to avoid the use of these lytic viruses, we decided to follow the approach used by Jarvis et al. [2]. Instead of using the polyhedrin promoter, they constructed an expression vector based on the promoter of a viral immediate early gene isolated from the *Autographa californica* nuclear polyhedrosis virus (AcMNPVIE-1 gene).

Immediate early genes are defined as those genes that can be transcribed without earlier expressed viral gene products in transient assays.

By using an immediate early gene of the baculovirus of *Bombyx mori* (BmNPVIEG), we seek to develop an alternative baculovirus expression system.

2. RESULTS AND DISCUSSION

2.1. Isolation and sequencing of BmNPVIEG

A 3823 bp long ClaI fragment, cross-hybridizing to the AcMNPVIE-1 sequence, has been cloned, subcloned and entirely sequenced by Huybrechts and co-workers [3]. This fragment contains an open reading frame of 1752 bp, encoding a protein with a molecular weight of 67 kilodalton, flanked by a 631 bp leader sequence and 1440 downstream bases. Comparison of this gene sequence with that of the AcMNPVIE-1 gene shows 95.3% homology.

The 'CAGT' motif, the transcription initiation sequence which is perfectly conserved for a number of early baculovirus genes [4], even as putative CAAT and TATA like sequences, was found in the leader sequence.

Experiments already performed with the AcMNPVIE-1 gene revealed that the gene product is involved in the transactivation of a number of delayed early genes [5–7] and at least one late viral gene [8].

A co-transfection of Sf9 cells with a plasmid containing the BmNPVIEG sequence and a construct in which the chloramphenicol acetyl transferase (CAT) gene was placed under control of the delayed AcMNPV 39K gene promoter demonstrated the cross-transactivating properties of BmNPVIEG. The activity of the delayed early gene promoter was dependent on the presence of the BmNPVIEG product.

2.2. Use of the BmNPVIEG promoter for foreign gene expression in uninfected insect cells

Because immediate early genes are defined as those genes that can be transcribed by uninfected cells in the absence of other viral gene products, we wanted to prove that the 631 bp leader sequence contains the regulatory sequences necessary for transcription of the transactivating gene in transient assays.

For this, we used the commercial pCH 110 promoter screening vector (Pharmacia) in which a functional *E. coli lacZ* gene is cloned. We inserted the 631 bp leader sequence in such a way that expression of the *lacZ* gene was dependent on the promoter activity of the BmNPVIEG leader sequence. For all the transfection experiments, we used the transfection agent DOTAP (Boehringer Mannheim GmbH, Germany) and 5–10 μg circular supercoiled plasmid DNA.

After transfection of *Bombyx mori* Bm-5 cells, we found cells expressing the *lacZ* reporter gene. Even when a BmNPV semi-permissive cell line such as *Spodoptera frugiperda* Sf9 cells, or a BmNPV non-permissive cell line such as *Drosophila* S2 cells was transformed, expression of the reporter gene was clearly found. In this way, we concluded that the BmNPVIEG leader sequence contains promoter activity by which foreign genes can be expressed in uninfected insect cells.

So far, the *lacZ* reporter gene is only transiently expressed under BmNPVIEG promoter control. A further objective of our study was to produce permanent transformants. For this, we had to extend our transforming vector with an antibiotic resistance gene. pUChshyg (kindly provided by J. Carlson, Colorado State University, Fort Collins, Colorado, United States of America) contains the hygromycin B resistance gene under control of the *Drosophila hsp*70 promoter, which can be activated in the available *Drosophila* S2 cells.

In the coding sequence of BmNPVIEG, cloned into pUC19, the *lacZ* gene is inserted in a frame 54 bp downstream of the BmNPVIEG transcriptional start site. Transformation of S2 cells with this construct (pBmNPVIEG*lacZ*) is also successful.

This prompted us to co-transfect S2 cells with pBmNPVIEGlacZ and pUChshyg. The heterogeneous mixtures of the transformed cells are cultured in selective medium containing 300 μg of hygromycin B and those cells that even contain pBmNPVIEG*lacZ* can be microscopically detected by adding X-Gal as substrate for β-galactosidase.

We followed the transformed cells for over one hundred passages and still found β-galactosidase expressing cells. Even when they are cultured, for over 30 passages, in non-selective medium they retain their transformed state.

Total cellular DNA, extracted from the transformed cells, was digested with XbaI and XhoI. XbaI has one restriction site in pBmNPVIEG*lacZ*, and pUChshyg is cleaved once by XhoI. Southern blots were probed for plasmid specific sequences. The hybridization pattern of digested total cellular DNA showed a fragment with the same length as that of digested plasmid DNA. It seems that the transforming

plasmids are maintained as stable extrachromosomal DNA. On Southern blots of non-cleaved genomic DNA, hybridized with the same probe, this extrachromosomal DNA appears as a high molecular weight molecule.

For the moment, it is still unclear in which conformation these multicopy plasmids are maintained in the transformed cells. Phenomena such as concatamerization or chromosomal linkage remain possibilities.

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MOLECULAR CLONING OF RESTRICTION ENDONUCLEASE FRAGMENTS OF DNA ISOLATED FROM THE NUCLEAR POLYHEDROSIS VIRUS OF *Heliothis armigera*

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Abstract

MOLECULAR CLONING OF RESTRICTION ENDONUCLEASE FRAGMENTS OF DNA ISOLATED FROM THE NUCLEAR POLYHEDROSIS VIRUS OF *Heliothis armigera*.

Restriction fragments of *Heliothis armigera* nuclear polyhedrosis virus (HaNPV) DNA double digested with EcoRI and BamHI were cloned in *E. coli* DH 5αF′ using the Bluescript plasmid as a vector. The inserted DNAs were estimated to be 1.5–2.0 kb in size. The DNA probe was constructed from a 1.5 kb DNA fragment labelled with digoxigenin, i.e. non-radioactive DNA labelling. The probe hybridized well with DNA isolated from positive colonies, but not with the Bluescript plasmid. Dot blot hybridization data indicated that this digoxigenin labelled DNA probe can detect viral DNA in the infected larva two days after inoculation. This probe can also be used to detect viral DNA isolated from a single NPV infected *H. armigera* larva.

1. INTRODUCTION

The nuclear polyhedrosis virus (NPV) is considered to be a potential bio-insecticide for the control of several lepidopterous insects. Among these pests, the cotton bollworm, *Heliothis armigera*, is the most destructive to crop plants because of its resistance to a wide range of chemical insecticides.

The NPV belongs to subgroup A of the Baculoviridae family of insect viruses. This virus has an envelope, rod shaped nucleocapsid, and a large, covalently closed, circular, double stranded DNA [1, 2]. The molecular weight of the viral genome was estimated at 50–100 million dalton [3]. Physical mapping of the restriction endonuclease fragments of some viruses in this group has been constructed [4–7].

In Thailand, an isolate of *H. armigera* NPV (HaNPV) was isolated from infected cotton bollworms from the field and is now being developed as a bioinsecticide [8]. Preliminary characterization of HaNPV was done by determining the specific restriction endonuclease cleavage pattern, and its molecular weight of DNA was estimated to be 65×10^6 dalton [8]. Molecular characterization of HaNPV is needed to better understand the genome organization and its functions for further improvement of HaNPV as a bioinsecticide.

In this study, we report on the cloning of the restriction endonuclease fragments of the HaNPV genome and the construction of a DNA probe for NPV diagnosis in order to study the distribution and transmission of this virus in nature. This is part of the effort to understand the molecular biology and ecology of the Thai NPV isolate.

2. MATERIALS AND METHODS

2.1. Virus purification and viral DNA isolation

The nuclear polyhedrosis virus of the cotton bollworm, *H. armigera*, was isolated from diseased larvae collected from cotton fields in Utong District, Supanburi Province, Thailand. The virus was propagated in host larvae that were mass reared on an artificial diet. Virus purification and viral DNA isolation followed the method described elsewhere [8]. Briefly, virus particles were extracted by dissolving the polyhedra in 0.1M Na_2CO_3, pH11.2, for 20 min. After removing undissolved polyhedra and debris by low speed centrifugation, the virus particles were pelleted by centrifugation at 36 000 rev./min for 2 h. Viral DNA was isolated from the purified virus by phenol:chloroform extraction, precipitated by ethanol and resuspended in TE buffer for further analysis.

2.2. Construction of recombinant plasmids

Bluescript (Stratagene) and HaNPV-DNA were each double digested to completion with EcoRI and BamHI. Enzyme treated Bluescript was separated by 0.7% agarose gel electrophoresis [9] and eluted from the gel by using a Geneclean kit (BIO, 101, La Jolla, CA, United States of America). The restriction fragments of viral DNA were collected by phenol-chloroform:isoamyl alcohol (24:1) extraction and ethanol precipitation. The restriction fragments of HaNPV-DNA and Bluescript were then ligated at 14°C for 16 h. The ligation reaction mixture consisted of 10 μL of 100 ng digested NPV-DNA, 5 μL of 100 ng digested Bluescript, 1 μL of T4 DNA ligase (Biolabs, Inc., USA), 2 μL of 10X ligation buffer and 2 μL of 100mM ATP.

2.3. Cloning of the NPV-DNA fragments

Recombinant plasmids were used to transform *E. coli* DH 5αF′ by mixing 10 μL of ligated vector and 100 μL of competent cell suspension. The mixture was maintained on ice for 30 min, incubated at 42°C for 50 s and immediately chilled for 5 min. After supplementation with 900 μL of growth medium, the cells were incubated for 1 h at 37°C and then plated on selective medium. Positive colonies were selected and the recombinant plasmids were purified as outlined by Maniatis et al. [10]. The DNA inserts were analysed by EcoRI and BamHI double digestion of the purified plasmids, followed by agarose gel electrophoresis.

2.4. Probe preparation

A fragment of EcoRI and BamHI double digested HaNPV-DNA was used to construct the probe. An eluted DNA fragment was labelled with digoxigenin-11-dUTP, i.e. non-radioactive DNA labelling (Boehringer Mannheim GmbH, Germany). The reaction mixture of 20 μL was incubated for 16 h at 37°C and stopped with 2 μL of 0.2M EDTA, pH8.0. The probe was precipitated with 2.5 μL of 4M LiCl and 75 μL of prechilled ethanol and kept overnight at −20°C. After centrifugation, the digoxigenin labelled probe was vacuum dried and resuspended in 50 μL of TE buffer, pH8.0.

2.5. Sample preparation

To prepare the DNA samples for hybridization tests, each healthy and NPV infected *H. armigera* larva was homogenized in 1 mL of ddH_2O and 1 mL of 2X lysis buffer (0.02M tris, pH7.5, 0.3M NaCl and 4% SDS), centrifuged at 14 000 rev./min for 5 min and the supernatant collected. The DNA was precipitated from the supernatant by cold absolute ethanol, vacuum dried and dissolved in ddH_2O for hybridization.

2.6. Dot blot hybridization

The DNA samples were dotted on a nitrocellulose membrane. After pre-hybridization at 68°C for 1 h in a sealed plastic bag, the samples were hybridized overnight at 68°C in a shaker bath with a hybridized solution containing 5 μL/mL of denatured DNA probe. Detection of the target DNA with a digoxigenin labelled probe was performed according to the standard experimental procedure (Boehringer Mannheim GmbH).

3. RESULTS

3.1. Cloning of the NPV-DNA fragments

Of the over two thousand colonies screened, 23 colonies were shown to be positive to the ampicillin resistant test. The plasmids isolated from colonies 3, 5, 9, 12, 15, 18 and 20 revealed distinct bands under agarose gel electrophoresis, with the sizes ranging from 3.0 to 3.5 kb (Fig. 1). These bands migrated more slowly than the 2.9 kb Bluescript plasmid, indicating that they contained inserts.

3.2. Analysis of the NPV-DNA inserts

Double digestion of the isolated plasmids with EcoRI and BamHI restriction endonucleases showed inserted DNA fragments of similar size (Fig. 2). Colonies 3, 9 and 18 contained inserted DNA with the sizes of 1.3–1.5 kb. The digestion product of colonies 5, 12 and 20 showed DNA sizes of 2.0 kb. The DNA fragment of 1.3 kb from colony 3 was used for probe construction.

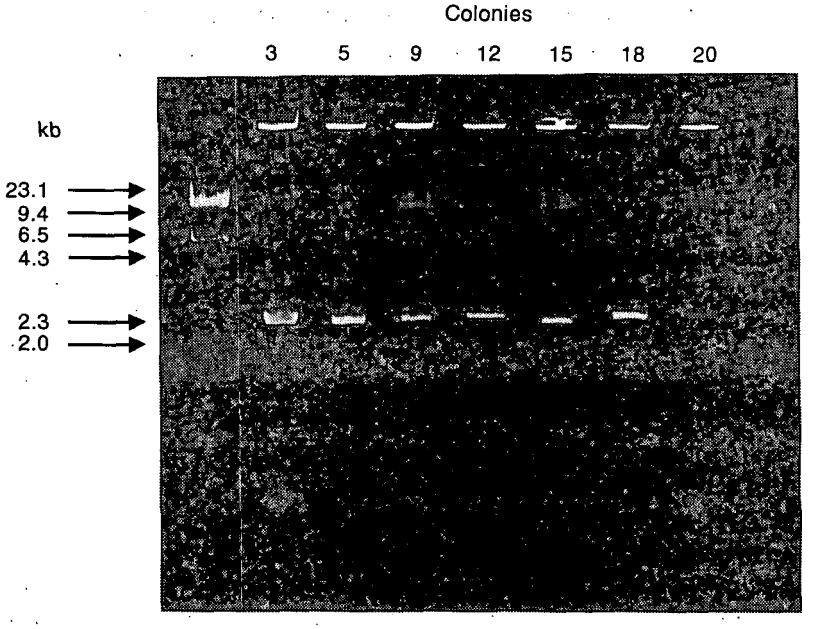

FIG. 1. Agarose gel electrophoresis of recombinant Bluescript plasmids contained HaNPV DNA EcoRI and BamHI double digested fragments. The plasmids were isolated from seven selected colonies of ampicillin resistant E. coli *DH 5α'. The HindIII fragments of lambda DNA were used as the standards for size determination.*

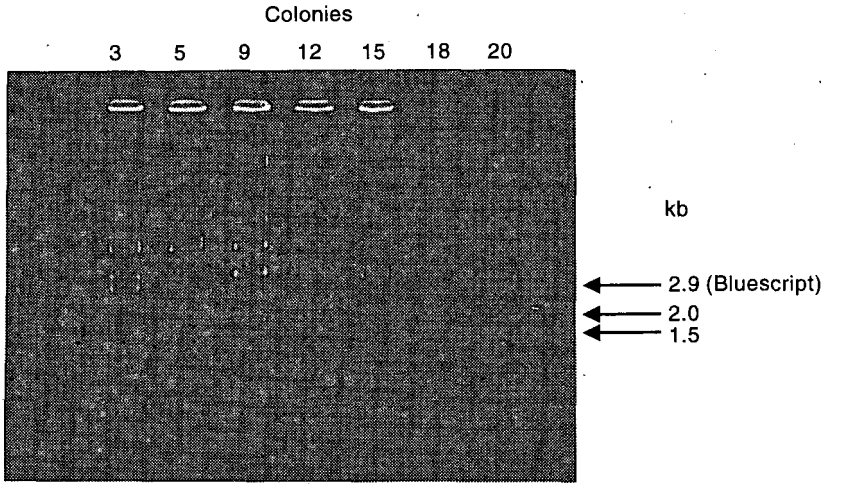

FIG. 2. Agarose gel electrophoresis of recombinant Bluescript plasmids after double digestion with EcoRI and BamHI restriction endonucleases. The plasmids were isolated from seven selected colonies of ampicillin resistant E. coli *DH 5αF'.*

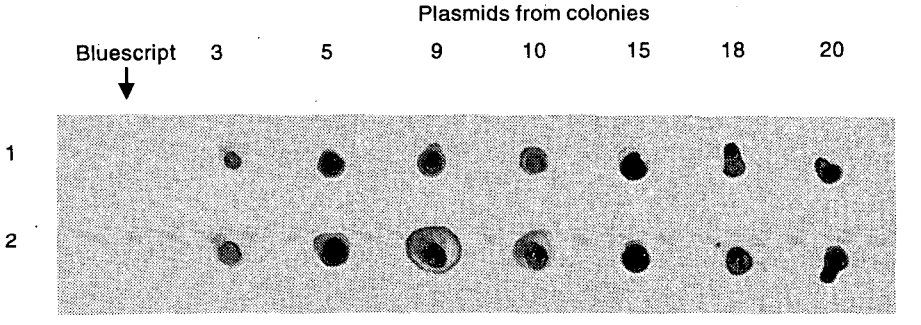

FIG. 3. Dot blot hybridization of plasmids isolated from ampicillin resistant colonies to a digoxigenin labelled NPV-cDNA probe. The DNA loaded per dot was 5 ng (row 1) and 10 ng (row 2). The non-insert Bluescript showed negative reaction to the probe.

3.3. DNA probe and dot blot hybridization

The digoxigenin labelled DNA probe constructed from the 1.3 kb inserted DNA from colony 3 reacted positively with all plasmids isolated from colonies 3, 5, 9, 15, 18 and 20. No reaction was observed when the probe was hybridized with non-insert Bluescript (Fig. 3). It is suggested that these DNA inserts may derive from the same region of the HaNPV genome.

When the probe was dot blot hybridized with DNA isolated from healthy and NPV infected *H. armigera*, positive reactions were observed only with diseased samples (Fig. 4). The viral DNA isolated from one, two and three infected larvae gave the same result when hybridized with the probe. The intensity of colour development showed the same level of hybridization, independent of the amount of DNA loaded per dot. This result indicated that DNA isolated from a single NPV infected larva was sufficient for dot blot hybridization with the 1.3 kb fragment NPV-DNA probe.

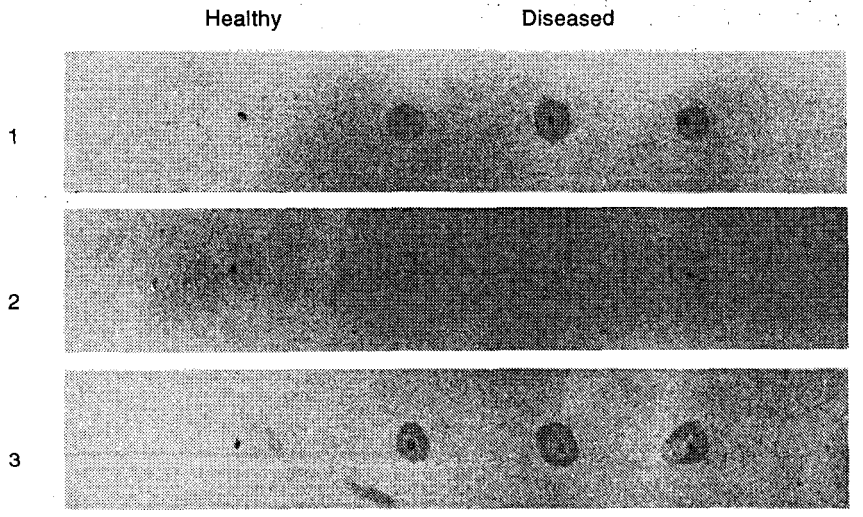

FIG. 4. Dot blot hybridization of DNA isolated from healthy and NPV infected (diseased) Heliothis armigera *larvae to a digoxigenin labelled NPV-cDNA probe. The NPV-DNA was isolated from one, two or three larvae, as indicated in rows 1, 2 and 3, respectively.*

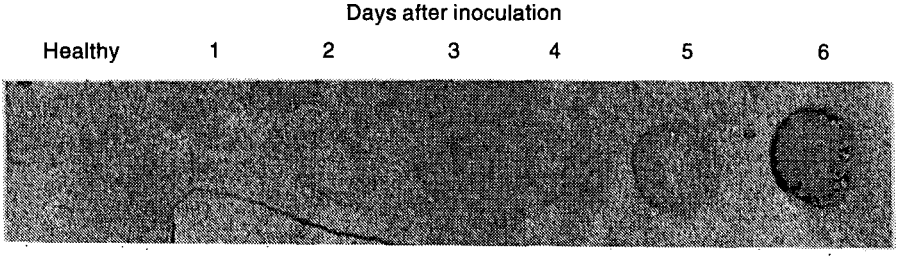

FIG. 5. Dot blot hybridization of DNA isolated from healthy and NPV infected Heliothis armigera *larvae to a digoxigenin labelled NPV-DNA probe.*

This study also demonstrated that the concentration of NPV-DNA increased in infected larvae after inoculation (Fig. 5). When NPV-DNA was isolated from *H. armigera* larvae 1, 2, 3, 4, 5 and 6 d after inoculation and hybridized with the probe, colour development was observed with the increasing intensity. The results indicated that NPV-DNA was first detected on the second day after inoculation. The viral DNA concentration increased up to the sixth day after inoculation. By that time, most of the infected larvae had died because of the viral infection.

4. DISCUSSION

The restriction endonuclease pattern has proved to be useful for the analysis of DNA because the restriction enzymes cleave DNA at specific nucleotide sequences. Its usefulness for identifying strains and genomic variants of NPV has been well recognized [11, 12]. Our previous work has shown the difference in the restriction endonuclease pattern of HaNPV-DNA after being digested with EcoRI, HindIII, PstI, SacII, HpaI and ScaI [8]. However, application of this technique to differentiate the strains of NPV found in Thailand is very limited. This is due to the fact that there is no rapid and reliable method for detecting viruses in the field so that more strains can be collected and studied. Molecular cloning of restriction endonuclease fragments of NPV–DNA will prove to be of great value in the search for and development of more effective strains of NPV in Thailand. We selected the EcoRI and BamHI fragments because of their constant 1.3–2.0 kb products, which are suitable for cloning. Although this work is at the initial stage of constructing genome mapping and full length sequence analysis, the probe derived from a cloned DNA fragment has a high potential for application. Using this probe and the digoxigenin labelling technique, NPV can be detected even in a single infected larva. It is hoped that more naturally occurring strains of NPV will be detected by this method, thereby accomplishing the selection and development of an effective strain of NPV for the control of *H. armigera*.

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CONTROLE GENETIQUE DES INSECTES: CARACTERISATION D'ELEMENTS GENETIQUES MOBILES CHEZ LES MOUSTIQUES

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Abstract-Résumé

GENETIC CONTROL OF INSECTS: CHARACTERIZATION OF MOBILE GENETIC ELEMENTS FROM MOSQUITO GENOMES.

The Juan elements constitute a family of LINE's retroposons which are dispersed in the genome of many strains, if not all, of the three mosquito species *A. aegypti, C. pipiens* and *C. tarsalis*. A specific Juan subfamily is amplified and dispersed in the genome of each of these species. They have been designated respectively as Juan A in *A. aegypti*, Juan C in *C. pipiens* and Juan Ct in *C. tarsalis*. The distribution of Juan retroposons among mosquito species does not reflect the phylogeny of these species. Furthermore, the Juan retroposons form homogeneous subfamilies: full-length copies which are reiterated in strains collected from regions covering different continents are nearly identical. These data are interpreted to indicate that the Juan retroposons have spread recently in the mosquito genomes harbouring them, perhaps upon horizontal transfer from other species. Juan A elements have been found in two isolates of *A. albopictus* and one isolate of *A. polynesiensis*, whereas numerous other isolates of these two species are devoided of any Juan-like sequence. The unfrequent presence of Juan A elements in some strains of *A. albopictus* and *A. polynesiensis* can be the result of an horizontal invasion, but more probably from cross-hybridizations which have been reported sometimes to occur between the former species and *A. aegypti*. No progeny is obtained when *A. albopictus* males containing Juan A retroposons are crossed with females lacking these elements, whereas the reciprocal cross is fertile. Such results suggest that the Juan retroposons may be responsible for incompatibilities between strains.

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Les éléments Juan constituent une famille de rétroposons de type LINE, répétés et dispersés dans le génome des moustiques *Aedes aegypti, Culex pipiens* et *Culex tarsalis*. Une sous-famille Juan particulière, désignée respectivement Juan A, Juan C ou Juan Ct, est amplifiée et dispersée dans le génome de chacune des souches de ces trois espèces analysées à ce jour. La distribution des éléments Juan parmi les différentes espèces de moustiques ne reflète pas la phylogénie de celles-ci. De plus, les copies complètes des rétroposons Juan réitérées dans des souches de différents continents forment des sous-familles homogènes. Ces observations indiquent que ces éléments ont envahi récemment les génomes qui les hébergent, peut-être à l'issue d'un transfert horizontal, de façon infectieuse. Des éléments Juan A ont été

trouvés dans seulement deux isolats d'*A. albopictus* et un isolat d'*A. polynesiensis*, alors que de nombreux autres isolats de ces espèces sont dépourvus de tels éléments. Cette contamination de rares isolats d'*A. albopictus* et d'*A. polynesiensis* par des éléments Juan A peut être le résultat d'une invasion horizontale, mais plus probablement de croisements fertiles peu fréquents entre souches de ces deux espèces et *A. aegypti*. Aucune descendance n'est obtenue quand des mâles d'une souche d'*A. albopictus* hébergeant les rétroposons Juan A sont croisés avec des femelles dépourvues de ces éléments, alors que le croisement réciproque est fertile. Ces résultats préliminaires suggèrent que les rétroposons Juan pourraient être responsables d'incompatibilités ou de dysgénésies entre souches de moustiques.

1. INTRODUCTION

La stimulation, par certains facteurs génétiques ou environnementaux, de l'activité d'un élément génétique mobile peut augmenter la fréquence des mutations et causer des variations alléliques. Ces composants instables du génome eucaryote jouent donc très certainement un rôle majeur dans la plasticité de l'information génétique et les remarquables facultés d'adaptation des insectes des populations naturelles. Dès lors, la caractérisation de tels éléments est importante pour comprendre leur rôle dans le polymorphisme et les processus d'adaptation des espèces des populations naturelles qui les hébergent et leur origine évolutive.

Surtout, des éléments transposables peuvent permettre le développement de méthodes plus efficaces de lutte génétique chez les insectes d'importance agronomique ou médicale. La lutte génétique utilise plusieurs stratégies: introduction dans la population d'insectes visés de mâles stériles (lâchers d'insectes stériles ou Sterile Insect Technique), d'une espèce proche, ou contenant des gènes défavorables pour l'espèce (allèles de stérilités, d'incompatibilités et de dysgénésies ou modifiant le rapport de sexe). Parce qu'elle a une spécificité absolue, ne sélectionne théoriquement pas d'individus résistants et permet en principe d'éradiquer une population sans pollution, la lutte génétique devrait occuper une place privilégiée dans les programmes de lutte intégrée. L'utilisation des transposons peut permettre de produire, à des coûts raisonnables, des insectes mâles stériles ou porteurs d'informations létales compétitifs dans les milieux naturels. D'abord, des transposons peuvent constituer des vecteurs de gènes [1] pour modifier par transgénèse les chromosomes des insectes afin de disposer de méthodes simples de sélection des mâles (sexage par un gène de résistance à l'alcool sur le chromosome Y) ou de transférer des gènes de stérilisation, modifiant le rapport de sexe ou létaux pour la descendance (gènes de thermosensibilité, de dérive méiotique, de dysgénésies, ...) ou les parasites véhiculés (gène de diphénoloxydase nocive pour les plasmodiums du paludisme). En outre, beaucoup d'entre eux appartiennent à la catégorie de séquences d'ADN dites 'égoïstes', capables d'envahir le génome grâce à leur propre machinerie enzymatique, responsable de leur amplification et leur dispersion. Il serait donc

particulièrement intéressant de leur associer des informations que l'on veut répandre dans les populations naturelles. Enfin, certains transposons peuvent être des agents d'incompatibilités ou de dysgénésies hybrides entre souches d'une espèce [1, 2]. Dans la mesure où des espèces particulièrement nuisibles pourraient être contrôlées, voire éradiquées par des procédés faisant appel aux techniques moléculaires, le bénéfice pour l'environnement serait considérable, malgré les problèmes éthiques soulevés.

Enfin, certains éléments mobiles peuvent constituer des outils remarquables pour identifier par mutagénèse par transposons les gènes impliqués chez les insectes dans les phénomènes complexes d'adaptation, d'apprentissage, comportementaux ou de vection d'agents pathogènes. Ainsi, la microinjection du transposon P dans la lignée germinale de *Drosophila melanogaster* a permis de muter et d'étiqueter, puis d'isoler, des gènes difficiles à étudier par les approches biologiques traditionnelles.

2. RESULTATS ET DISCUSSION

2.1. Stratégies d'identification d'éléments génétiques mobiles de moustiques

Plusieurs approches sont possibles pour identifier des éléments génétiques mobiles. La plus simple consiste à sonder le génome à l'aide d'éléments déjà identifiés chez d'autres espèces, en espérant des hybridations grâce aux analogies de séquences qui se présentent assez fréquemment. Mais cette recherche n'est envisageable en principe qu'entre groupes phylogénétiquement voisins et ne permet pas forcément d'accéder aux éléments les plus intéressants. L'analyse de mutants phénotypiques est probablement la stratégie la plus rigoureuse pour détecter des séquences mobiles; en effet, plus de la moitié des mutations dans certains gènes de *D. melanogaster* sont dues à l'insertion dans ces gènes, ou dans leurs régions régulatrices, de transposons. Des phénomènes de dysgénésies hybrides, qui ont été à l'origine de la découverte des transposons chez les invertébrés, peuvent aussi révéler leur présence; ils se manifestent par des stérilités et mutations lors de croisements entre femelles et mâles d'une même espèce, en particulier d'origines géographiques différentes [1, 2]. Les éléments mobiles constituent également des ensembles d'ADN moyennement ou très répétés et on peut les rechercher dans ce type de séquence. Enfin, des molécules d'acides nucléiques, ADN ou ARN, extrachromosomiques et des particules pseudovirales susceptibles de représenter des intermédiaires de transposition spécifiques de diverses séquences mobiles peuvent s'accumuler à certains stades du développement de l'insecte, en particulier ceux correspondant à des étapes précoces, ou dans des cellules en culture. Nous avons mis en route ces diverses stratégies pour identifier et isoler des éléments génétiques mobiles spécifiques des moustiques *Aedes* et *Culex*. Nous avons en particulier analysé des mutations dans

lesquelles des transposons pourraient être impliqués, mutations de résistances aux insecticides ou d'autres toxiques ou pour la coloration de l'œil.

Chez une population de moustiques *C. pipiens quinquefasciatus* de Californie, la résistance aux insecticides organophosphorés est due à l'amplification du gène de l'estérase de détoxication B1 [3, 4]. Cette amplification résulte de remaniements chromosomiques auxquels sont associés des éléments génétiques mobiles: des séquences appartenant à au moins trois familles différentes d'éléments génétiques mobiles sont coamplifiées, dans l'amplicon, avec le gène de l'estérase B1 [5, 6, MOUCHES, résultats non publiés].

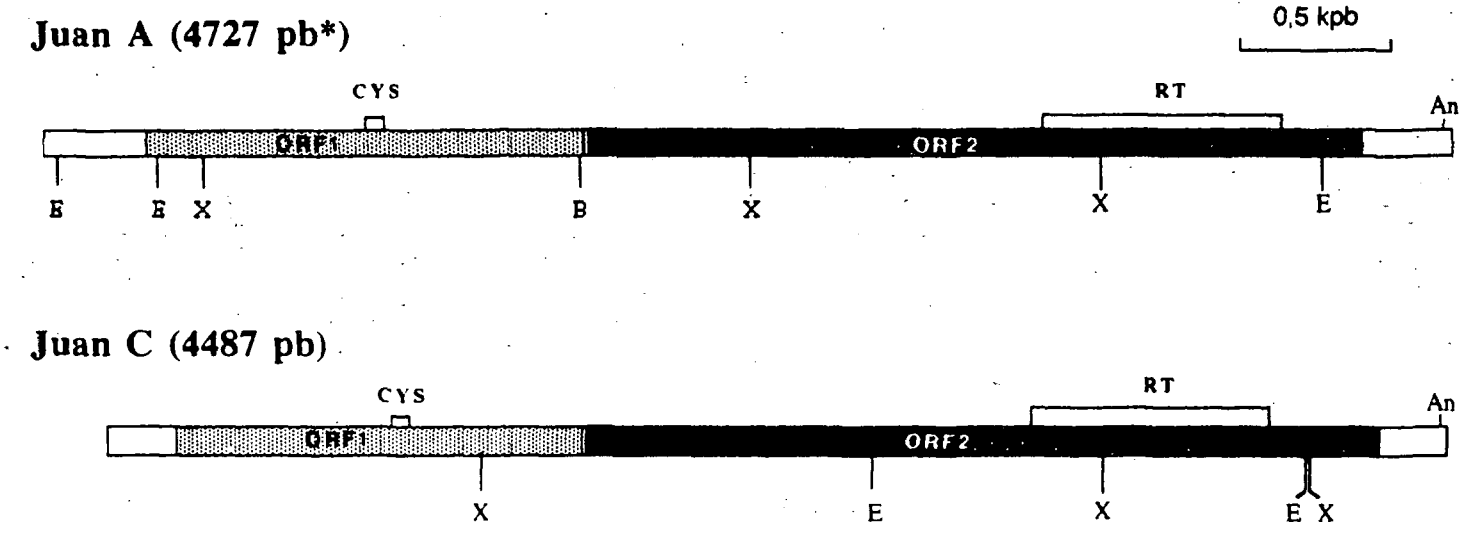
La première famille de transposons ayant été ainsi identifiée est celle des éléments désignés Juan C [5, 6]. Une copie tronquée de cette famille est coamplifiée avec le gène de l'estérase B1 et de nombreuses autres séquences de ce type sont dispersées dans tout le génome du moustique. Le séquençage de ces copies tronquées de la famille Juan C a révélé qu'il s'agissait de rétroposons de type LINE («long interspersed repetitive elements») [6], c'est à dire de transposons dont l'amplification et la dispersion dans le génome résultent d'un processus de transcription réverse d'un intermédiaire ARN [7]. En outre, l'hybridation moléculaire nous a indiqué que des séquences homologues à Juan C existent chez d'autres populations ou espèces de moustiques: *C. pipiens, C. tarsalis, Aedes aegypti,* etc.

Les deux autres familles d'éléments génétiques mobiles qui ont été identifiées dans l'amplicon du gène d'estérase B1 sont la famille des transposons CE1 d'une part et celle des éléments Pau d'autre part, ces derniers contenant en outre une séquence de type minisatellite. Les copies de ces transposons coamplifiées dans l'amplicon de l'estérase B1 sont des copies semble-t-il incomplètes et qui ne sont probablement pas responsables du processus d'amplification lui-même; des représentants complets de ces séquences mobiles sont en cours d'isolement.

2.2. Caractérisation de la famille d'éléments génétiques mobiles Juan chez les moustiques *A. aegypti* et *C. pipiens*

Des copies complètes (4487 pb) du rétroposon Juan C des moustiques *C. pipiens* ont été clonées et séquencées (accès à Genbank: M 91082) [8]. Des fragments de ce rétroposon ont été utilisés comme sondes pour purifier, par hybridation hétérologue, des copies complètes d'une sous-famille de rétroposons amplifiés et dispersés dans le génome des moustiques *Aedes* et désignés Juan A (4727 pb) (accès à Genbank: M 95171) [9]. Le degré d'amplification dans le génome des éléments Juan complets est compris entre 200 et 500 copies. Ces valeurs sont voisines de celles de certaines séquences LINE de mammifères, mais supérieures à celles observées pour les rétroposons complets de *Drosophila melanogaster* qui sont présents seulement à moins de 50 copies par génome.

Les rétroposons Juan A et Juan C contiennent deux phases de lecture ouvertes (Open reading frame ou ORF) parfaites, codant potentiellement la première une



* pb: paire de base; 1 kb d'ADN double brin a une masse moléculaire de $6,6 \times 10^5$.

*FIG. 1. Structure et organisation génétique des rétroposons Juan A d'*A. aegypti *et Juan C de* C. pipiens. *Les domaines hautement conservés sont indiqués par CYS (domaine riche en cystéine typique des protéines de liaison aux acides nucléiques) dans la phase de lecture ouverte ORF 1 et RT (domaines typiques des transcriptases réverses) dans la phase de lecture ouverte ORF 2.* An *indique une séquence riche en adénosine à l'extrémité 3' des éléments. Les sites de coupure par des enzymes de restriction sont indiqués par: B, BamHI; E, EcoRI; X, XbaI.*

protéine de liaison aux acides nucléiques (nucleic acid binding ou «gag» protein), la seconde une transcriptase réverse (Fig. 1). Ils présentent entre eux de fortes homologies au niveau de leurs séquences nucléotidiques et de celles des polypeptides potentiellement codés (37% d'homologies pour la protéine «gag», 67% d'homologies au niveau de la transcriptase réverse).

Les deux familles ont la même organisation génétique que les éléments Jockey, F, G et I de *D. melanogaster* [10, 11, 12]. En outre, les séquences nucléotidiques des rétroposons Juan A et Juan C présentent des régions d'homologies avec les éléments Jockey, F et G, mais non pas avec les rétroposons I de *D. melanogaster* et *D. teissieri*, ou T1 d'*Anopheles gambiae* [13]. Ces résultats indiquent que les rétroposons Juan et certains rétroposons de drosophiles dérivent d'un même élément ancestral.

2.3. Ecologie des rétroposons Juan chez les moustiques *Culex* et *Aedes*

Des copies des rétroposons Juan C et Juan A, ou d'éléments voisins, ont été recherchées dans des souches ou des individus issus des populations naturelles, en analysant les ADN génomiques extraits de ces insectes par hybridation avec les éléments clonés, détermination ou polymorphisme de longueur des fragments de restriction hybridés et par amplification génique in vitro par la polymérase (polymerase chain reaction ou PCR), en utilisant comme amorces des oligonucléotides spécifiques de ces éléments (Tableau I).

Approximativement le même nombre de copies du rétroposon Juan C sont amplifiées dans le génome les souches de *C. pipiens* de différents continents. Une sous-famille d'éléments très proche de celle des rétroposons Juan C, désignée Juan Ct, est amplifiée chez *C. tarsalis*. Ces éléments Juan Ct présentent un polymorphisme de restriction différent de celui des rétroposons Juan C, montrant que chacune des deux sous-familles a été amplifiée à partir d'un variant d'un ancêtre commun.

Des rétroposons Juan A sont amplifiés dans toutes les souches de moustiques *A. aegypti*. Des éléments identiques ont également été trouvés dans deux isolats de l'espèce *A. albopictus* et un isolat d'*A. polynesiensis*, mais de nombreux autres isolats de ces deux espèces sont dépourvus de tout élément de type Juan, quelle que soit la stringence des conditions d'hybridations utilisées.

2.4. Histoire évolutive des rétroposons Juan A et Juan C

Toutes les souches de moustiques appartenant aux espèces *C. pipiens*, *C. tarsalis* et *A. aegypti* que nous avons analysées jusqu'à présent hébergent, sous une forme répétée et dispersée, une sous-famille de rétroposons Juan, respectivement Juan C, Juan Ct et Juan A. Cependant, la distribution des rétroposons Juan ne réflète pas la phylogénie des espèces de moustiques. En effet, ces éléments semblent être

TABLEAU I. DISTRIBUTION DES RETROPOSONS JUAN CHEZ DES MOUSTIQUES D'ESPECES ET D'ORIGINES GEOGRAPHIQUES DIFFERENTES

Espèce	Isolat	Origine	Rétroposon
C. pipiens Say	Tem-R	Californie	Juan C
C. pipiens Say	S-Lab	Californie	Juan C
C. pipiens Say	S54	Montpellier	Juan C
C. pipiens Say	MSE	Montpellier	Juan C
C. pipiens Say	Bleuet	Montpellier	Juan C
C. pipiens Say	Lahore	Pakistan	Juan C
C. pipiens Say	Selax	Californie	Juan C
C. pipiens Say	Bouaké	Côte d'Ivoire	Juan C
C. tarsalis	NR	Californie	Juan Ct
C. tarsalis	Sus	Californie	Juan Ct
A. aegypti L.	Bobo-Dioulasso	Afrique	Juan A
A. aegypti L.	Meya	Pacifique	Juan A
A. aegypti L.	MD	Trinidad	Juan A
A. albopictus Skuse	Oahu 1971	Hawaii	—
A. albopictus Skuse	Honolulu 1986-01	Hawaii	— (a)
A. albopictus Skuse	Honolulu 1986-97	Hawaii	Juan A
A. albopictus Skuse	Poona 1988-01	Inde	— (b)
A. albopictus Skuse	Poona 1988-109	Inde	Juan A
A. polynesiensis Marks	Pap 1987-01	Tahiti	— (c)
A. polynesiensis Marks	Pap 1987-132	Tahiti	Juan A

(a), (b), (c): cent dix-sept autres isolats d'*A. albopictus* prélevés en 1986 a Hawaii, cent vingt-deux autres collectés en 1988 à Poona et cent quatre-vingt-sept autres isolats d'*A. polynesiensis* prélevés en 1987 à Tahiti ne contenaient aucun rétroposon Juan.

absents chez la plupart des isolats d'autres espèces d'*Aedes*, pourtant plus proches d'*A. aegypti* que des *Culex*. Comme il est peu vraisemblable qu'un élément amplifié et dispersé à plusieurs centaines de copies dans un génome puisse avoir été éliminé totalement sans laisser de traces, les rétroposons Juan ont donc envahi le génome des espèces qui les hébergent après la divergence évolutive des différentes espèces de moustiques.

En outre, les copies complètes d'une sous-famille Juan amplifiées dans une espèce sont remarquablement homogènes, non seulement au sein d'une souche

donnée, mais aussi entre souches d'origines géographiques différentes. Ce résultat est surprenant pour une famille d'éléments dont l'amplification et la dispersion procèdent via des étapes de transcription en ARN, puis de réverse-transcription en ADN. En effet, et contrairement aux ADN polymérases, les ARN polymérases sont dépourvues d'activités éditrices correctrices des erreurs survenant lors de la polymérisation de leurs produits. Une replication via de tels processus devrait donc conduire à des copies très hétérogènes des éléments Juan.

La distribution particulière des rétroposons Juan et l'homogénéité de chacune des sous-familles amplifiée au sein d'une espèce donnée impliquent que: (1) l'amplification d'une sous-famille Juan s'est faite chez une population unique d'une espèce donnée à partir d'une seule copie du rétroposon ou seulement de quelques exemplaires tous identiques de cet élément; (2) cette amplification a conféré un avantage sélectif à l'hôte, puisque les individus dans lesquels les rétroposons Juan ont été amplifiés ont été capables d'envahir une grande partie de l'aire géographique des espèces en cause; (3) l'amplification de ces éléments s'est produite très récemment dans les populations de moustiques. Ces données nous conduisent à émettre l'hypothèse que les rétroposons Juan ont envahi très récemment le génome des souches ou espèces de moustiques qui les hébergent, à l'issue d'un transfert horizontal entre espèces, c'est-à-dire que des copies du rétroposon Juan ont une capacité «infectieuse» à se répandre.

Des copies complètes du rétroposon Juan A semblent avoir envahi très récemment des populations prélevées récemment dans la nature des espèces *A. albopictus* et *A. polynesiensis*, alors que la plupart des populations de ces espèces, et en particulier les plus anciennes, en sont dépourvues (Tableau I). Cette invasion peut résulter d'un processus de transfert horizontal comme évoqué ci-dessus; plus probablement, il est le résultat de croisements entre ces deux espèces et *A. aegypti*. En effet, si des croisements entre *A. aegypti*, *A. albopictus* et *A. polynesiensis* sont généralement considérés comme infertiles, plusieurs auteurs ont montré que des croisements entre individus de certaines souches de ces trois espèces pouvaient être interfertiles [14,15, 16, 17].

2.5. Etude de la transposition des éléments Juan et interactions avec le génome de l'hôte: ces rétroposons sont-ils responsables d'incompatibilités entre populations de moustiques?

La transposition des éléments mobiles est habituellement réprimée chez les hôtes qui les hébergent. Les transposons peuvent être mobilisés à l'issu de différents stress subis par l'hôte, par exemples des chocs thermiques ou des croisements entre souches «éloignées» [18, 19]. Dans ce dernier cas peut survenir le phénomène de dysgénésie hybride qui, avec trois transposons différents de drosophiles, P, I et Hobo, survient en particulier lorsqu'un de ces éléments est introduit, par croisement sexuel ou par injection, dans de jeunes embryons issus de femelles elles-mêmes

dépourvues de la séquence considérée. La ressemblance de l'organisation génétique des rétroposons Juan avec celle des LINE de *D. melanogaster* suggère que, comme le facteur I, ils pourraient être responsables de phénomènes de dysgénésie hybride entre souches géographiques différentes de moustiques.

Nous avons croisé des individus des souches d'*A. albopictus* contenant le rétroposon Juan A avec l'une des nombreuses souches qui sont dépourvues de ce transposon. Les croisements sont féconds seulement de façon unidirectionnelle: les mâles sans rétroposons Juan A sont compatibles avec les femelles hébergeant l'élément, alors que le croisement réciproque est infertile. Ce résultat est du même ordre que celui obtenu dans les cas de dysgénésie hybride dus à des transposons chez les drosophiles. Il suggère que les rétroposons Juan sont facteurs d'incompatibilités entre souches d'une même espèce de moustiques. Cependant, de telles incompatibilités unidirectionnelles entre souches peuvent aussi résulter de la présence de bactéries intraovariennes de type rickettsies ou *Wolbachia.* Des expériences sont en cours pour introduire directement, par transgénèse dans des œufs embryonnés, des copies complètes du rétroposon Juan A dans les souches d'*A. albopictus* qui en sont dépourvues.

Les souches de *C. pipiens, C. tarsalis* et *A. aegypti* que nous avons analysées jusqu'à présent ont été prélevées au cours de ces vingt-cinq dernières années dans des populations naturelles géographiquement différentes. La recherche des rétroposons Juan dans des souches géographiquement isolées et des collections anciennes a été entreprise dans le but d'identifier des individus dépourvus de ces éléments, ce qui nous permettrait d'évaluer l'impact des rétroposons Juan sur le génome de ces trois espèces.

3. CONCLUSIONS

Depuis leur découverte, les éléments génétiques mobiles ont été étudiés chez un nombre limité d'espèces. Dès lors, leur histoire évolutive et les stratégies qui leur permettent d'évoluer apparemment de concert avec le génome de leurs hôtes respectifs sont peu connues. La caractérisation de nouveaux éléments à partir de moustiques permettra de mieux connaître l'évolution de ces séquences, de comprendre les mécanismes de leur interaction avec le génome hôte et d'apprécier leur importance dans les mutations adaptatives. Il sera en particulier intéressant de vérifier l'éventualité que ces éléments puissent être «infectieux», c'est-à-dire transmis de façon horizontale, entre espèces différentes. Chez les mammifères et la drosophile, certains éléments mobiles semblent avoir été fréquemment perdus et gagnés par les diverses espèces, et il a été suggéré (et illustré chez la drosophile) que certains d'entre eux ont envahi une espèce donnée à l'issue d'un transfert horizontal à partir d'une autre espèce.

L'étude plus approfondie des éléments Juan nous permettra également de déterminer s'ils peuvent être utilisés comme transposons pour muter des fonctions importantes des insectes, servir de vecteurs de gènes ou comme agents d'incompatibilités entre souches de moustiques.

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USE OF THE BIOLISTIC TECHNIQUE FOR GENE TRANSFER TO MOSQUITO EMBRYOS

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Abstract

USE OF THE BIOLISTIC TECHNIQUE FOR GENE TRANSFER TO MOSQUITO EMBRYOS.

Gene transformation for the control of mosquito transmitted diseases is currently limited by the lack of an efficient method for the transfection and integration of exogenous DNA. The biolistic technique, which uses high velocity DNA coated microprojectiles, has been developed to introduce DNA into embryos of *Anopheles gambiae*. The biolistic parameters have been characterized and optimized on the basis of transient expression of the luciferase reporter gene placed under the control of the heat shock protein 70 promoter of *Drosophila*. High luciferase activities were observed for biolistic DNA introduction performed in the early embryonic stages. Because of the very large number of embryos which can easily be transfected biolistically, and because DNA is probably delivered directly into the nuclei, the biolistic method could be useful for obtaining insect genetic transformation, despite the low frequency of exogenous DNA integration. Moreover, this technique has already been proved suitable for embryo transfection in other invertebrates, such as crustaceans.

The introduction of genes into mosquitoes that would make them refractory to pathogens is a possible strategy for controlling vector borne diseases [1-5]. However, techniques are not available to efficiently introduce genes into mosquitoes, as DNA injected into embryos rarely integrates into the host genome. This barrier was overcome in *Drosophila* by identifying the *P* transposable element that efficiently introduce genes into the germ line. Because of the lack of identified high

efficiency integration elements in mosquitoes, alternative approaches need to be developed. To compensate for the low frequency of integration, we have explored use of the biolistic technique [6] to introduce DNA into a large number of *Anopheles gambiae* embryos. This technique, which uses high velocity DNA coated microprojectiles to deliver DNA directly into the nuclei, has been successfully applied to introduce DNA into a large number of fish eggs [7] and dechorionated *Drosophila* embryos [8].

Our experiments were performed with the Biolistic PDS-1000/He particle delivery system (Bio-Rad). The experimental parameters were optimized for three different embryonic stages:

(1) *0 to 20-min-old embryos:* At this stage, the chorion is soft, which may facilitate penetration by the microprojectile. Moreover, because pronuclei fusion is in progress, delivery of DNA coated particles at this time may lead to early integration of exogenous DNA. Consequently, some fully transgenic mosquitoes could be obtained as soon as the G_0 generation.

(2) *60 to 80-min-old embryos:* At this stage, the chorion has not fully hardened. Numerous syncytial nuclei are scattered inside the ooplasm and, consequently, there is a higher probability of microprojectiles penetrating the nuclei. Moreover, some nuclei may have migrated to the germinal pole. Thus, bombardment of such embryos may lead to partially transformed G_0 embryos, fully transgenics being eventually obtained only at the G_1 generation.

(3) *150 to 180-min-old embryos:* At this stage, hardening of the chorion and vitelline membrane has been completed. The nuclei are numerous and distributed homogeneously along the embryo surface. The pole cells begin to form.

The biolistic parameters were optimized on the basis of the transient expression of a luciferase gene placed under the control of the *Drosophila* heat shock protein *(hsp)* promoter. This reporter gene was selected because of the ease of sample treatment of luminometry analyses, which are quantitative and sensitive for samples containing as few as a thousand embryos. For all the preliminary comparative experiments carried out, the transient expression of luciferase was analysed in 24-h-old embryos.

For each biolistic operation, the embryos (3000–5000) were placed immediately in a monolayer inside a circle (2 cm diameter) corresponding to the microprojectile beam size. After bombardment, the embryos were maintained in an antibiotic solution to prevent the development of microorganisms transformed by the DNA construct, which would have a luciferase activity.

Relatively low levels of luciferase transient expression were observed with 0 to 20-min-old embryos, possibly due to the small number of nuclei. Higher levels of luciferase activity were observed in the 60 to 80 and 150 to 180-min-old embryos. The biolistic parameters were then progressively optimized, coating of particles with DNA and their subsequent loading on to the macroprojectile being the most variable

operations. The best ratio of DNA/particles was about 10 μg/1 mg. The higher ratios resulted in the formation of particle aggregates. Using 900 psi rupture discs, the embryo survival rates were similar to those of the controls. The transient expression of luciferase was also analysed in 48-h-old embryos and in 1-d-old larvae. The highest luciferase activities were found in 24-h-old embryos. Both circular and linear DNA induced transient expression efficiently. The biolistic method led to transient expression in about one hundred experiments.

Experiments are now in progress, using the biolistic conditions developed from transient expression, to introduce DNA with a selectable marker into large numbers of embryos in order to select for stable integration. The biolistic technique has already been proved to be suitable for embryo transfection in other invertebrates, such as crustaceans.

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POLYMERASE CHAIN REACTION AMPLIFICATION OF RAPD MARKERS TO DISTINGUISH POPULATIONS OF THE MEDITERRANEAN FRUIT FLY, *Ceratitis capitata*

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Abstract

POLYMERASE CHAIN REACTION AMPLIFICATION OF RAPD MARKERS TO DISTINGUISH POPULATIONS OF THE MEDITERRANEAN FRUIT FLY, *Ceratitis capitata.*

Polymerase chain reaction amplification of RAPD markers was used to document the existence of molecular markers that can distinguish different populations of the Mediterranean fruit fly, *Ceratitis capitata.* The populations used include strains which differ primarily in terms of geographical origin, but, in addition, strains that have been in long term laboratory culture can also be distinguished from wild caught strains. The molecular markers identified can be used for a variety of purposes, including documentation of new forms of genetic variation, identification of strain specific molecular markers and establishment of linkage relationships between anonymous DNA sequences and desirable genes or traits of interest.

1. INTRODUCTION

Many of the techniques recently developed in molecular genetics and biology hold great promise for answering questions of importance in research on insect pests. We have initiated several molecular genetic studies of the Mediterranean fruit fly (medfly), *Ceratitis capitata*, as well as other Tephritid pest species found here in Hawaii [1–3]. In this paper, we describe our work showing that in the medfly, so called randomly amplified polymorphic DNA (RAPD) markers can be amplified using the polymerase chain reaction (PCR), and that these markers can be used to assess genetic variation within and between populations of this pest species. The populations surveyed include geographical strains originating from different

islands within the Hawaiian archipelago, various countries in Latin America, in Israel and limited amounts of material captured in recent infestations in California, United States of America.

Beyond simply identifying new forms of genetic variation, the markers identified here have many other potential uses. For example, once suitable markers have been described that identify populations of different geographical origin, it may be possible to determine the source of origin of pest species moving into a new habitat. In addition, these markers can be used to establish linkage relationships between anonymous DNA sequences and genes or traits of interest in pest species. Once such linkage relationships have been established, whole new approaches to augmenting genetically based control programmes may become practical by making it possible to clone specific genes or by more easily monitoring the introgression of desirable traits into strains through classical genetic crossing schemes.

2. MATERIALS AND METHODS

The PCR procedure we have used here has the great advantage that it can be carried out on limited amounts of material, such as dessicated or ethanol preserved material, even that which has been rendered unusable for most other types of analysis. In our procedure, DNA is first extracted from material such as a single fly or from body parts of a fly according to established protocols [1]. Typically, from a single medfly we dilute an aliquot of the extracted DNA to approximately 10 ng/μL. This small fraction of the extracted DNA serves as a template for the amplification of specific regions of the genome in the PCR procedure. The regions of the genome that are amplified can serve as molecular markers which are specific to an individual, strain, population, or species, whatever the case may be.

The particular regions of the genome that will be amplified are determined by 'primers' added to the PCR reaction. These primers are short, synthetic strands of DNA that serve as the initiation point in the amplification of a specific region of the genome. In the RAPD approach, primers are chosen at random, and they consist of strings of DNA 10 nucleotides in length [4]. After PCR amplification, the DNA fragments are visualized using ethidium bromide staining. The number and sizes of these fragments can differ between individuals or strains. The specific pattern of the DNA fragments observed are representative of the genetic make-up of the individual. Identically sized bands observed between different individuals are considered to represent genetic relatedness or similarity. To quantify these relationships, we calculated band sharing percentages for different medfly populations. This number represents the proportion of bands generated by the RAPD PCR procedure that are common, or shared, between individuals within or between populations.

3. RESULTS AND DISCUSSION

To date, fairly extensive analyses have been made of medfly populations using three different primers. These three primers are those considered to be most informative at a variety of levels of genetic differentiation. These primers were identified from tests carried out on approximately 14 different 10-mers (primers 10 nucleotides in length) synthesized for the PCR reactions. One of these primers (referred to as primer 67) is extremely sensitive to differences in the genetic make-up of individuals. We used this primer to distinguish between laboratory reared and wild caught strains of the medfly derived from the same geographical locality. The other two primers (14 and 74) identified appear to correspond to more conserved regions of the genome. However, these primers are extremely useful in terms of detecting the differences between strains of different geographical origin.

Using primers such as these, we can identify RAPD markers which discriminate between medflies originating from different geographical localities. However, in many cases where individuals from different populations are compared, some RAPD marker bands produced by the PCR method appear to be common between populations. We used this commonality to devise a measurement of similarity based on the number of RAPD marker bands which are shared within or between samples. We infer that this 'band sharing' reflects similarities in the genetic make-up of these populations.

Table I presents band sharing calculations made for samples from several different geographical localities, including a small sample of flies from a recent infestation in California. The numbers shown represent band sharing values between the different populations compared here. As is shown, the flies from California share

TABLE I. PROPORTION OF RAPD MARKER BANDS COMMON TO DIFFERENT GEOGRAPHICAL SAMPLES

	California	Hawaii	Guatemala	Costa Rica	Brazil	Argentina	Israel
California	—	0.27	0.18	0.18	0.09	0	0
Hawaii	—	—	0.18	0.18	0.09	0	0
Guatemala	—	—	—	0.18	0.09	0	0
Costa Rica	—	—	—	—	0.09	0	0.09
Brazil	—	—	—	—	—	0.09	0
Argentina	—	—	—	—	—	—	0.09
Israel	—	—	—	—	—	—	—

a greater proportion of bands in common with samples from Hawaii and Central America as opposed to other geographical localities. Similar results have been obtained with other primers as well. Assuming that these bands represent DNA polymorphisms which are common to different populations, these genetic markers can be indicative of the origin and genetic relatedness of populations.

We are attempting to obtain additional material from California to examine this further. In addition we need to examine other, presumably unrelated, geographical populations to better understand these results. We are encouraged, however, that we can produce these DNA polymorphisms to be used as population specific molecular markers using the PCR technology.

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RIBOSOMAL DNA OF *Aphidius* (HYMENOPTERA: BRACONIDAE) NEES

Structure and intraspecific variations

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Abstract

RIBOSOMAL DNA OF *Aphidius* (HYMENOPTERA: BRACONIDAE) NEES: STRUCTURE AND INTRASPECIFIC VARIATIONS.

An investigation is being carried out on the ribosomal DNA (rDNA) molecular organization of closely related Aphidiinae species belonging to the genus *Aphidius* Nees, of relevant interest in biological control, with the aim of evaluating the variability within and between species. After construction of a restriction map of the most represented *A. ervi* rDNA cistrons, the molecular organization of the rDNA repeating units of single individuals and populations was studied in Southern blot analyses. Heterogeneity within the *A. ervi* rDNA cistrons and between populations of different geographical origin was identified. This approach allowed the conclusion to be reached that differences in the rDNA cistrons can be diagnostic of species and populations, therefore providing a useful tool in biological control programmes.

1. INTRODUCTION

Among the insects, most of the detailed studies on rDNA using the well developed recombinant DNA technologies for DNA structural analysis have been performed on the dipteran fly *Drosophila melanogaster* [1]. More recently, the study of rDNA has been extended to several insect species of medical and agricultural importance in order to start the molecular characterization of the genome and to investigate the molecular systematics of an ever-growing number of species: Diptera, Orthoptera, Lepidoptera and Hymenoptera [2–4].

The study of the rDNA molecular structure of insects takes advantage of the well described characteristics of these genes in *D. melanogaster*, mainly their high copy number and the presence in each repeated unit of a highly conserved coding region and of less conserved sequences: non-transcribed spacers (NTS) and introns [5–8]. Restriction analyses of the spacer structure of cloned rDNA gene units have indicated that the spacers are internally repetitious and that the observed heterogeneity in length derives in part from a different number of internal repeated sequences [9–12]. Another particular feature of *D. melanogaster* rDNA is that the 28S coding region sequences are interrupted by non-coding DNA sequences (rDNA introns) in about 40% of the repeated units [13]. Almost all the dipteran species so far investigated contain introns in some fraction of their 28S rRNA coding regions [2].

We are investigating the rDNA molecular organization of closely related Aphidiinae species, taking special interest in *Aphidius ervi* Haliday. We have already demonstrated the real potentialities of this approach, both in the discrimination of species and in the determination of the phyletic relationships between taxa. The results reported in this paper show that the rDNA structure and organization of *A. ervi* is complex and heterogeneity exists both within the rDNA cluster and between individuals and populations of different geographical origin.

2. MATERIALS AND METHODS

Aphidius ervi Haliday cultures were partially laboratory reared on their natural hosts: *Acyrthosiphon pisum* (Harris), starting from field collections of mummies in different areas of Campania. *Aphidius ervi* was also continuously reared on *A. pisum* on potted broad bean plants. Both the aphids and the parasitoids were kept in two separate climatic cabinets at 22 $\pm$ 1°C, 70 $\pm$ 10% relative humidity and under long day photoperiodic conditions (18 h light: 6 h dark).

Only adults were treated according to the methodologies described below for DNA extraction and subsequent processing. All the specimens utilized were individually anaesthetized with CO_2 for species identification.

(1) *DNA extraction*: The DNA from single individual and from pooled samples was extracted according to Endow and Glover [14].

(2) *Clones*: A complete, uninterrupted genetic ribosomal unit (cDm Y22) inserted into pMB9 was obtained from P.K. Wellauer (Institut Suisse de Recherches Expérimentales sur le Cancer, Lausanne, Switzerland) and its different fragments were used as probes in Southern blot experiments. Cloned DNA was prepared according to Dawid et al. [6]. Nick translation was performed according to Rigby et al. [15].

(3) *Restriction analysis, electrophoresis, transfer and hybridization of DNA*: Digestions with restriction enzymes were carried out under the conditions recommended by the manufacturers (Amersham, Biolabs, Boehringer Mannheim GmbH). Restriction maps were determined by analysing single and double digestions on 0.6–1.5% agarose gels in Tris–borate EDTA buffer [16]. The DNA was transferred on to nitrocellulose filters, hybridized as outlined by Southern [17] and washed four times in 2 × SSC, 0.1% SDS for 15 min at 56°C.

3. RESULTS AND DISCUSSION

3.1. The *A. ervi* rDNA restriction map

To complete the *A. ervi* rDNA restriction map reported on in our previous paper [18], total DNA was extracted from pooled samples of adult specimens and digested with several restriction enzymes in single and double digestions. Southern blot experiments were performed using as probes the labelled *D. melanogaster* complete genetic ribosomal unit (cDm Y22) or its different parts: 18S, 28Sα and 28Sβ. An example of the numerous experiments performed is given in Fig. 1, where we chose conditions under which only the main bands, corresponding to the most represented rDNA cistrons, were observed. The restriction map of *A. ervi* rDNA is shown in Fig. 2. The restriction enzymes BglII, PstI and KpnI have only one site in the complete repeating unit, giving the first indication of the length of most of the rDNA cistrons, which is approximately 12 kb.

Many of the sites conserved between the different species of insects were present also in *Aphidius* rDNA. The highly conserved RI site in 18S, the BglII and HindIII sites in 28S and PstI site in the internal transcribed spacer (ITS) between 18S and 28S were also present in this gene, which enabled us to determine the position of these regions in the *A. ervi* repeating unit. On the basis of the length of the main bands obtained with enzymes with only one site in the repeating units and the fact that the transcribed region was almost identical in length in all the species so far examined (about 7 kb), the spacer length was found to be 5.5–6 kb. This calculation

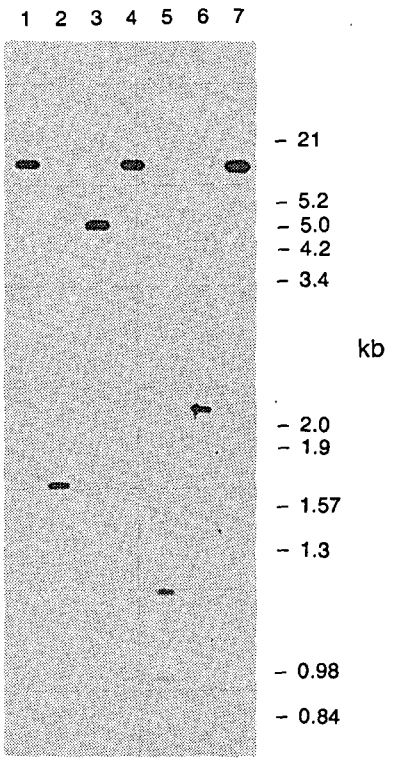

FIG. 1. Southern blot analysis of genomic DNA from A. ervi *digested with (1) HindIII/SacI; (2) HindIII/PvuII; (3) HindIII/PstI; (4) HindIII/KpnI; (5) HindIII/DraI; (6) HindIII/accI; and (7) HindIII and hybridized with labelled* D. melanogaster *28α.*

does not take into account the possible occurrence of other sources of variability, such as the insertion sequences in the 28S region.

3.2. Differences in length of the rDNA cistrons within and between *A. ervi* individuals

We have demonstrated in a previous paper [18] that differences exist between four *Aphidius* species (*A. ervi* and *A. microlophii* belonging to the *ervi* species group; *A. urticae* and *A. smithi* belonging to the *urticae* species group) that could be a means of distinguishing some species. We have now focused our attention on *A. ervi* to investigate whether the differences were detectable within the cluster of rDNA of each single individual and between populations of different geographical origin. In this set of experiments, we used conditions and restriction enzymes that allow maximum detection of heterogeneity in an attempt to also identify less represented repeating units.

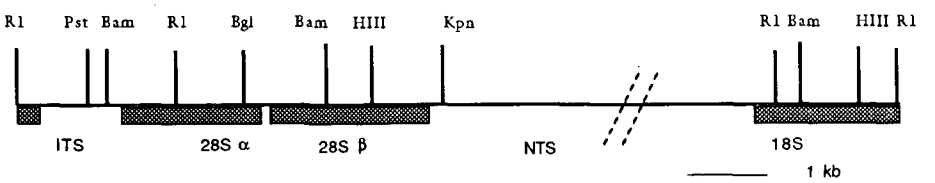

FIG. 2. Restriction map of A. ervi *rDNA. As stressed in the text, the most represented rDNA cistrons are about 12 kb long. Without taking into account the possible occurrence of other sources of variability, their spacer length would be 5.5–6 kb.*

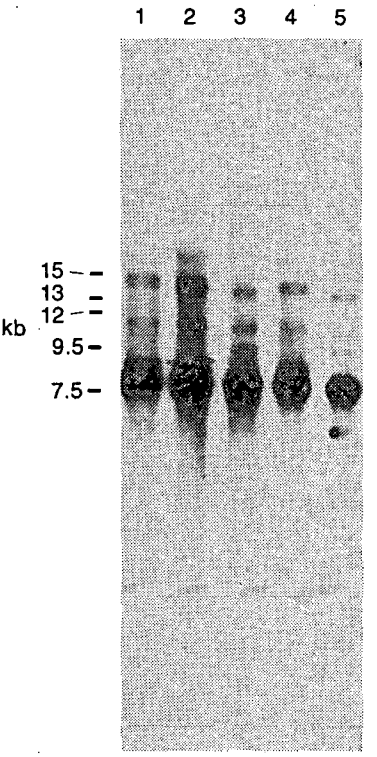

FIG. 3. Southern blot of genomic DNA extracted from single laboratory reared A. ervi *individuals digested with the restriction enzyme BamHI and hybridized with the labelled 28Sβ2 fragment of* D. melanogaster *rDNA.*

As can be seen in the map (Fig. 2), the BamHI enzyme sites in the 18S and 28Sβ2 coding regions identify a fragment which, from comparison with *Drosophila*, should contain the NTS as well as the site where 28S insertions occur in many insects. We therefore used this enzyme in Southern blot experiments (Fig. 3) of genomic DNAs extracted from single laboratory reared *A. ervi* individuals hybridized with the labelled 28Sβ2 fragment of *D. melanogaster* rDNA as probe.

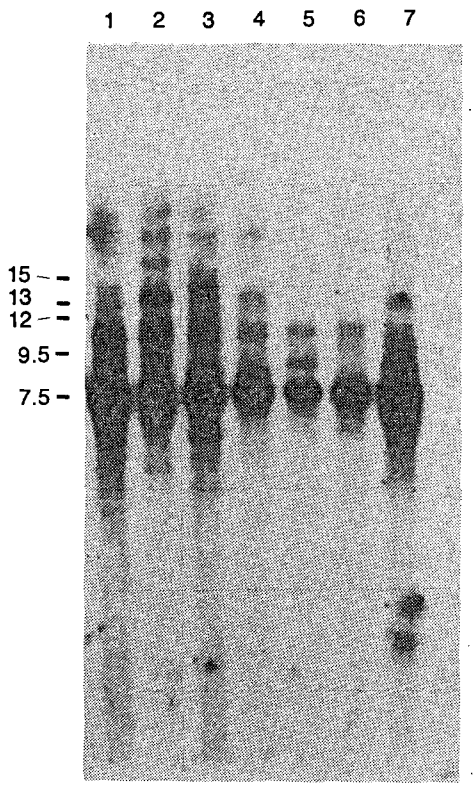

FIG. 4. Southern blot of genomic DNA from A. ervi *single individuals collected from different areas (lanes 1–2, Nola; lane 3, Sarno; lanes 4–6, Ischitella; and lane 7, Castelvolturno). The enzyme and probe are the same as those used in Fig. 3.*

Numerous bands appear in each lane, indicating that a considerable amount of variability exists within the rDNA repeating units. A main band of about 7.5 kb appears in all the individuals, indicating that this is the most represented class in single individuals, but this band seems to differ slightly between individuals (either 7.5 or 8 kb). The other most represented bands (11 and 13 kb) are present in most of the individuals, sometimes split as doublets, even if in different relative amounts. Faint bands with lengths up to 20 kb also appear, indicating that very long repeating units exist in *A. ervi*.

It can be concluded that there is heterogeneity in this species within the rDNA cistrons. In addition, differences at quantitative and qualitative levels also exist between single individuals reared in the laboratory starting from heterogeneous wild populations.

3.3. Heterogeneity of the rDNA structure between different populations of *A. ervi*

To investigate the rDNA population structure further, we analysed single individuals collected from wild populations of different geographical origin. Figure 4 shows the restriction patterns of samples collected in four different areas of Campania, corresponding to Lanes 1–2, 3, 4–6 and 7. Differences are visible between samples of the same and of different origin. Also in these experiments, the main band changes between individuals (7.5–8 kb), but strong bands are also present at a higher and/or a smaller molecular weight. There are bands present only in single individuals; however, the variability within a single population is as high as between populations. Therefore, more individuals have to be analysed to define the population rDNA restriction profiles.

In conclusion, we have identified differences in the rDNA structure both within and between *A. ervi* individuals and populations. However, to characterize better the origin of the identified differences and to understand their evolutionary importance, it is necessary to isolate the rDNA genes of *A. ervi* and to proceed through their sequence analysis, an effort which has already started in our laboratory.

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MITOCHONDRIAL DNA VARIABILITY IN GEOGRAPHICAL POPULATIONS OF THE BRAZILIAN SCREWWORM FLY

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Abstract

MITOCHONDRIAL DNA VARIABILITY IN GEOGRAPHICAL POPULATIONS OF THE BRAZILIAN SCREWWORM FLY.

Restriction endonucleases analysis of mitochondrial DNA (mtDNA) was used to examine the genetic variability of the screwworm in twelve populations collected in seven locations of southern and northern Brazil. In two distinct areas of São Paulo State, seasonal sampling was performed to detect any possible variation in mtDNA. A group of five enzymes, ClaI, HaeIII, HindIII, MspI and PvuII, was sufficient to differentiate some of the 12 screwworm sample populations tested. The results of this study support theoretical predictions that mtDNA analysis is a highly sensitive method for examining the population structure of *Cochliomyia hominivorax* mtDNA. Variability in *C. hominivorax* mtDNA should prove useful in the efforts to trace the origin and dispersion of the species in Brazil.

1. INTRODUCTION

The screwworm fly, *Cochliomyia hominivorax*, is an important pest which parasitizes livestock and other warm blooded animals of the New World. Adult females lay eggs on open wounds or in active myiases; this results in injuries containing hundreds to thousands of larvae at different stages of development. If the myiases are not treated in time, aggravation through reinfections may become fatal to the host. Many cases are reported in the literature of human beings infected by screwworm larvae.

The main host for this pest in Brazil is cattle, but other domestic and wild animals may be parasitized.

A basic knowledge of the genetic variability of *C. hominivorax* is necessary to understand the population structure and evolution of this species in order to develop efficient methods of pest control in Brazil.

The molecular approach to the study of genetic variability has been shown to be very effective and to generate fast and reliable data in cases where other types of analysis may be time consuming or impractical. In the case of insect populations,

one of the preferred methods is mitochondrial DNA analysis, which has been shown to represent a powerful tool for obtaining information on the gene flow, population structure, biogeographical hybridization zones and phylogenetic relationships. Mitochondrial DNA variation has also become an important tool for detailed studies of the intraspecific population structure because of its considerable polymorphism and strong sensitivity to founder events or population subdivision. The main reasons for this are the maternal mode of inheritance and the lack of recombination.

In this study, mtDNA variability was analysed in twelve screwworm samples collected from seven localities of southern and northern Brazil. In two different areas of São Paulo State, seasonal sampling was performed to detect any possible variation in mtDNA. The specific objectives of this work are to answer the following questions: (1) Can mtDNA restriction site analyses provide a sensitive method for examining genetic variability in these natural populations; and (2) Can this analysis reveal convenient genetic markers to trace the maternal lineages in these natural populations?

2. MATERIALS AND METHODS

Live screwworm samples (larvae and adults) were obtained from wounds in infested cattle at the following localities: Adamantina (Ad-1, Ad-2, Ad-3), Caraguatatuba (Ca-1, Ca-2, Ca-3, Ca-4), Botucatu (Bot-1), Paulinia (Pa-1), Amparo (Amp-1), Alfenas (Alf-1) and Morro do Chapeu (Ba-1). MtDNA was purified from individual larvae, pupae or adults, as described in Ref. [1]. The screwworm larvae were reared in the laboratory in a medium of fresh cattle groundmeat supplemented with blood and water (2:1). The larvae were maintained at approximately 30°C. Mature larvae were allowed to pupate in sawdust. Adults were maintained in screen cages at 25°C, with dried milk, sugar and ferment.

MtDNA was purified from individual larvae, pupae or adults as described in Ref. [1]. The following 15 restriction enzymes were used: AccI, BamHI, ClaI, EcoRI, ECoRV, HaeIII, HindIII, HpaI, MspI, PvuII, PstI, SppI, SttI, XbaI and XhoI. The purified mtDNA was nick translated to yield a ^{32}P labelled probe. Hybridization was carried out under standard conditions [1]. Visualization of the mtDNA fragments was conducted using autoradiography and the fragment sizes were estimated from autoradiographs using a digitizer and Bioscan software.

3. RESULTS AND DISCUSSION

The initial screening survey revealed that five of the fifteen enzymes used were suitable for detecting mtDNA variation among the sampled populations. Three different restriction patterns were obtained for ClaI and PvuII, four for HindIII and

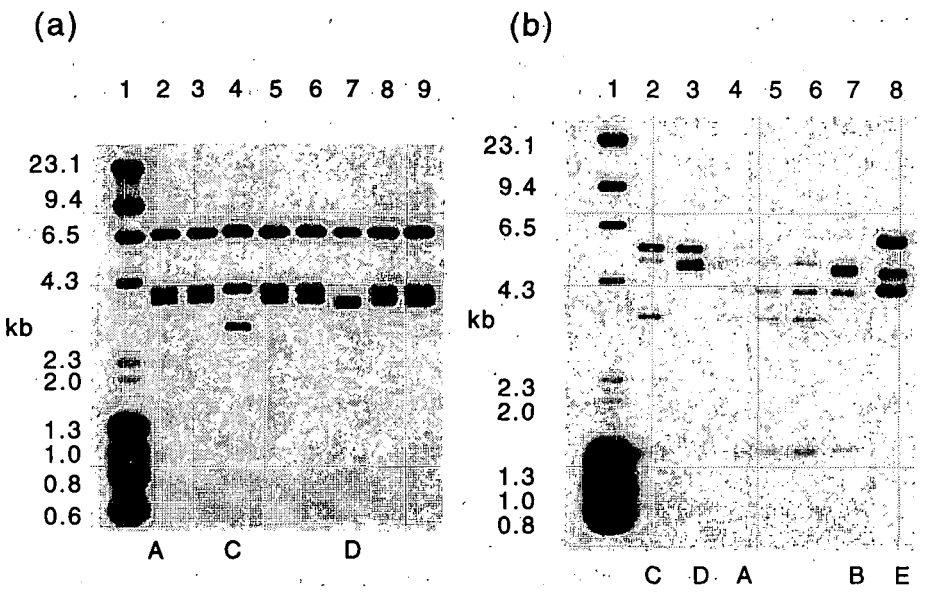

FIG. 1. Autoradiographs of a Southern blot showing C. hominivorax *mtDNA from distinct populations. (a) Digested with HindIII: lane 1 = λDNA/HindIII plus φX 174 DNA/HaeIII standards. Fragment sizes in kilobase pairs on the left; lane 2 = Caraguatatuba (São Paulo), summer 1991; lane 3 = Botucatu (São Paulo); lanes 4 and 5 = Caraguatatuba (São Paulo), winter 1991; lane 6 = Alfenas (Minas Gerais); lane 7 = Caraguatatuba (São Paulo), summer 1991; lane 8 = Paulinia (São Paulo). (b) Digested with MspI; lane 1 = λDNA/HindIII plus φX 174 DNA/HaeIII standards; lanes 2 and 3 = Caraguatatuba (São Paulo), winter 1991; lane 4 = Paulinia (São Paulo); lane 5 = Botucatu (São Paulo); lane 6 = Caraguatatuba (São Paulo), summer 1991; lane 7 = Caraguatatuba (São Paulo), winter 1991; lane 8 = Caraguatatuba (São Paulo), summer 1991.*

five for HaeIII and MspI. The different restriction patterns for each enzyme were designated by capital letters in the order that they happened to be discovered. As seen in Fig. 1, the enzyme HindIII showed three patterns, designated A (lanes 2, 3, 5, 6, 8 and 9), B (lane 4) and C (lanes 4, 5 and 6); MspI showed A (lanes 4, 5 and 7), B (lane 7), C (lane 2), D (lane 3) and E (lane 8).

The estimated mtDNA length was similar to that reported by Roehrdanz and Johnson and Roehrdanz [2, 3] for screwworm populations from North and Central America and has approximately 16.3 kilobase pairs.

On the basis of the fragment patterns of four enzymes, HaeIII, HindIII, MspI and PvuII, 14 different haplotypes were defined for the sampled populations. The interpopulation analysis has shown high variability in mtDNA. Three geographical populations exhibited different single haplotypes. Two haplotypes were found in three other localities. In one specific locality, Caraguatatuba (São Paulo State), nine

different haplotypes were found. The occurrence of shared haplotypes was in some cases independent of the geographical distance. In the populations that showed more than one haplotype, the frequencies varied and in most cases one type was prevalent. The results indicated that different geographical populations have different haplotype compositions and therefore different maternal lineages for mtDNA.

High variability in mtDNA was also found by Roehrdanz and Johnson and Roehrdanz [2, 3] in 30 screwworm lines from North and Central America. Sixteen haplotypes found individually in only two screwworm lines in Mexico showed ample distribution, while the remaining haplotypes were found individually in only one location. A comparison of these results with our data showed that no common haplotypes were found on both the areas analysed. Taylor et al. [4] described a single haplotype in a population found in Tripoli, Libyan Arab Jamahiriya, but there was no similarity to any of the haplotypes found in Brazil. This sample was collected from a recent introduction of the screwworm, probably from South America. A further screening from other regions of Brazil and other South American countries could perhaps indicate its possible origin.

The seasonal analysis also indicated genetic heterogeneity. In one of the areas (Caraguatatuba), two predominant haplotypes were found; these were the predominant types found in all four samples taken in this locality. However, seven other types were found in the samples collected during the summer (Ca-2) and in autumn (Ca-4), but they were not detected in other samples at this locality. This variability, plus the fact that large numbers of cattle are being taken in and out of the area, could be the reasons for the presence or absence of flies of these haplotypes. Only further temporal analysis of the mtDNA in this locality will provide more conclusive data on the screwworm population in this area. In another locality (Adamantina, São Paulo State), no significant variation was observed during the year.

These preliminary data on the high variability observed on the mtDNA of *C. hominivorax* samples collected in seven localities have indicated the existence of different maternal lineages in Brazil. Despite the high dispersal capacity of the screwworm flies and the movement of livestock in some regions, screening of some of the samples has suggested local differentiation in mtDNA and unique haplotypes.

Only further analysis of mtDNA, involving more samples from different areas of Brazil, is needed, especially concerning the spreading and introduction of this pest in other areas of the world, with special concern for Africa.

It has become increasingly clear that genetic variability among and within populations of insect pests affects the success of biological control agents and insecticide resistance management. The high interpopulation mtDNA variation found in 12 screwworm populations from 7 locations suggests that further studies of mtDNA will be valuable to address the evolutionary, genetic and pest management questions of this insect in South America.

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GENETIC FINGERPRINTING APPLIED TO TSETSE FLY SPECIES

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Abstract

GENETIC FINGERPRINTING APPLIED TO TSETSE FLY SPECIES.

The bacteriophage M13 was used as a probe to detect DNA fingerprinting (DNAfp) profiles in adults from laboratory colonies of the three subgenera of tsetse flies (*Austenina*, *Nemorhina* and *Glossina*). In all three subgenera, the probe revealed profiles of multiple components similar to those found in other organisms. The general complexity of the profiles varied between subgenera and between species and subspecies. A common overall DNAfp pattern was observed within a subspecies but variations occurred at the intrapopulation level. Evidence is presented that DNAfp provides a means for population biology studies, such as comparisons between field collected flies and those from established laboratory colonies. Pedigree analysis was performed in the context of further development of a genetic linkage map using DNAfp markers and studies related to the molecular basis of hybrid sterility in tsetse flies. A pedigree established by mating a male and a female from different lines ('RUCA' × 'Cent') of *G. m. centralis* showed, in addition to a Mendelian inheritance of DNAfp fragments, an amplification of the intensity of certain bands in the offspring. It is suggested that DNAfp offers a tool for analysing the molecular genetic aspects of mating flies from different geographical areas.

1. INTRODUCTION

The information accumulated in the fields of biology and genetics has now made tsetse flies, the vectors of African trypanosomiases, interesting and amenable models for studies at the molecular level [1]. There are 31 species and subspecies within the genus *Glossina* (Diptera: Glossinidae) and they are arranged in three subgenera or species groups [2–5]: subgenus *Austenina* Townsend (= *fusca* group, 15 taxa); subgenus *Nemorhina* Robineau-Desvoidy (= *palpalis* group, 9 taxa); and

subgenus *Glossina s. str.* (= *morsitans* group, 7 taxa). The divisions are based mainly on structural characters, but are supported also by ecological information [1].

DNA probes would be useful in research on tsetse flies, particularly in the study of population genetics, expansion of the existing linkage map and in the search for new methods of genetic control. Tandemly repeated sequences are well known for their ability to detect, by DNA hybridization, high levels of variation between individuals and to generate profiles commonly termed DNA fingerprinting (DNAfp). DNAfp has found a wide range of applications in population biology studies, linkage analysis studies, genetic relationships in vertebrate species and for individual identification in forensic science [6]. The objective of this paper is to demonstrate some potentials of DNAfp for developing the molecular biology of tsetse flies.

The experimental procedures to perform DNAfp from individual insects have been described previously [7]. In the present studies, the bacteriophage M13, known to reveal DNAfp profiles in many organisms, including insects, was used as a probe [7, 8].

2. RESULTS

2.1. DNAfp variability in tsetse colonies

Adult flies from laboratory colonies of tsetse flies of the three subgenera *(Austenina, Nemorhina* and *Glossina)* were analysed by DNAfp (Fig. 1). The M13 sequence, detecting multiple hybridizing components in all three subgenera, acted as a multilocus probe. Comparison of DNAfp profiles between subgenera and between species and subspecies showed a great deal of variation in terms of the number and position of the bands detected by the probe. Except for *G. brevipalpis,* the DNAfp profiles of each subspecies were characterized by a conserved overall pattern. In addition, the DNAfp profiles revealed intrapopulation variation within a subspecies. Except for the 'RUCA' and 'Cent' colonies of *G. m. centralis,* each species of tsetse was represented, in the present study, by a laboratory colony that originated from a single geographical area. Therefore, it is not possible for us to generalize on the geographical or ecological variations in DNAfp within a subspecies. However, the DNAfp profiles of flies from the 'RUCA' and 'Cent' colonies have a conserved DNAfp pattern. Although these colonies descended from puparia collected in Zambia and the United Republic of Tanzania, they originate from within the same main fly belt. The similarity of the DNAfp in the two colonies suggests that along this fly belt, which is several hundred kilometres in length, a continuous gene flow has occurred between the Zambian and Tanzanian populations,

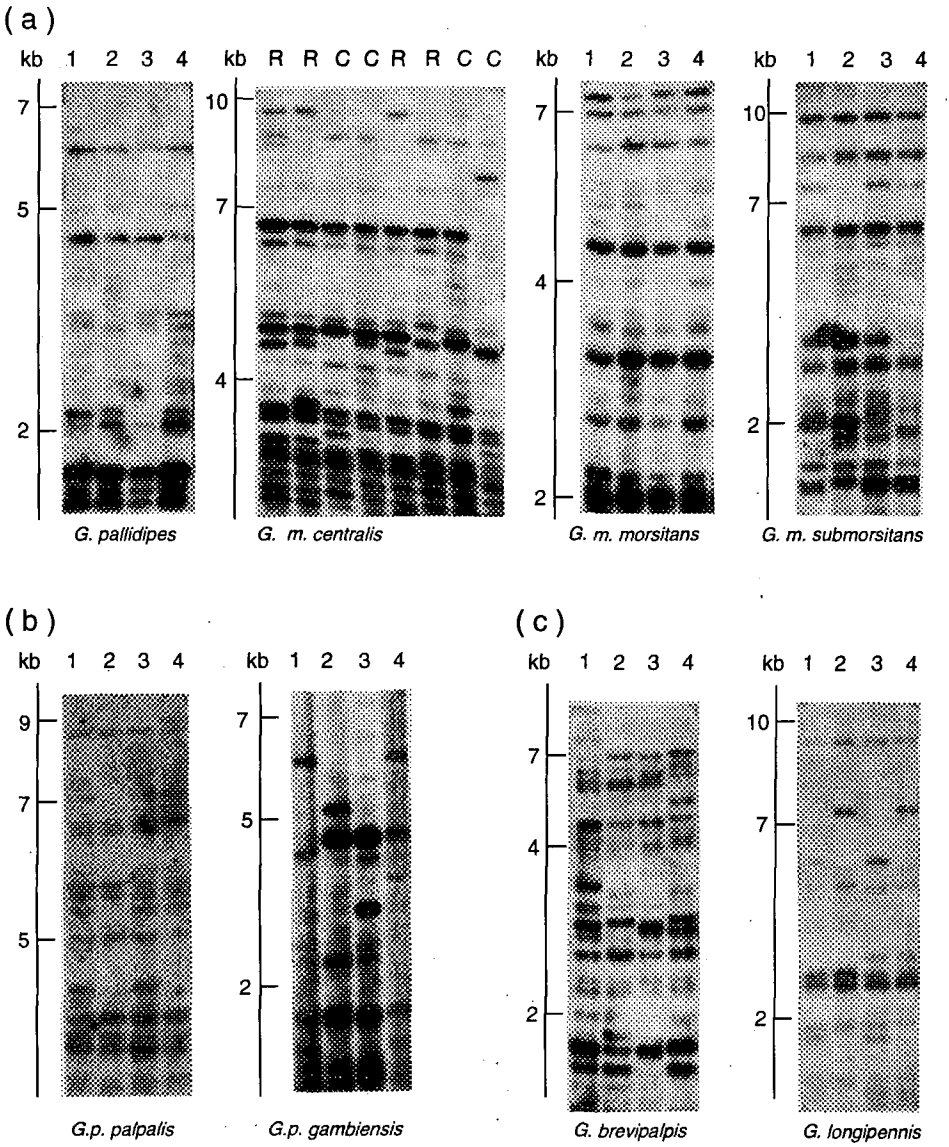

FIG. 1. DNA fingerprinting of adult tsetse flies. (a) Subgenus Glossina: G. pallidipes, G. m. centralis *(lines: R = 'RUCA', C = 'Cent')*, G. m. morsitans *(line: 190)*, G. m. submorsitans *(line: 'Brist'); (b) subgenus* Nemorhina: G. p. palpalis, G. p. gambiensis; *(c) subgenus* Austenina: G. brevipalpis, G. longipennis.

TABLE I. GENETIC VARIABILITY IN ADULT TSETSE FLIES FROM DIFFERENT SUBGENERA

Subgenus	Species	Fragment size (kb)	Mean No. of bands (N) (No. of individuals)	Band share x ± SD	Probability (P)
Austenina	*G. brevipalpis*	10–1	21.2 ± 0.3 (20)	0.31 ± 0.01	1.6×10^{-11}
	G. longipennis	10–1	10.2 ± 1.7 (14)	0.77 ± 0.17	6.9×10^{-12}
Nemorhina	*G. p. palpalis*	10–1	13 ± 2.2 (8)	0.85 ± 0.6	0.12
	G. p. gambiensis	7–1	6 ± 3.4 (6)	0.3 ± 0.23	7.2×10^{-4}
Glossina	*G. pallidipes*	7–1	13.0 ± 2.7 (11)	0.75 ± 0.08	2.3×10^{-2}
	G. m. centralis				
	Line: 'RUCA'	10–1	22.2 ± 0.9 (4)	0.76 ± 0.07	2.2×10^{-3}
	Line: 'Cent'	10–1	20.0 ± 2 (12)	0.84 ± 0.1	3×10^{-2}
	G. m. morsitans				
	line: 190	10–1	10 ± 0.7 (12)	0.60 ± 0.03	6×10^{-3}
	G. m. submorsitans				
	line: 'Brist'	10–1	12.0 ± 0.8 (13)	0.93 ± 0.06	0.28

$x = ((Nab/Na) + (Nab/Nb))/2$, where Na and Nb represent the number of bands in the individuals a and b and Nab is the number shared by both [9].
$P \simeq x^N$: Probability of two individuals having identical DNAfp.

making them indistinguishable by DNAfp. Alternatively, it is also possible that the results suggest that colonization has selected for markedly similar DNAfp patterns.

The average number of bands detected by the M13 probe and band sharing estimates, x, between individuals were used to quantify the amount of intrapopulation variation within the different colonies (Table I) [9]. *Glossina brevipalpis* and *G. p. gambiensis* display a large amount of intrapopulation variation, comparable to that found in other species [10] and indicating that individual specific DNAfp can be obtained in tsetse flies. Allozyme frequency data in *G. brevipalpis* colonies indicated that the mean level of heterozygosity per locus is among the highest recorded in any tsetse population [11]. Except for *G. brevipalpis* and *G. p. gambiensis*, the amount of intrapopulation variation in the laboratory colonies is notably reduced (Table I). There is a dramatic reduction in the general complexity of the profile and in the appearance of bands that are fixed in the population. Several factors may have contributed to this: (1) the relatively small size of the initial breeding population in some colonies (known to be the case in the *G. m. submorsitans* colony); (2) during laboratory maintenance each colony may

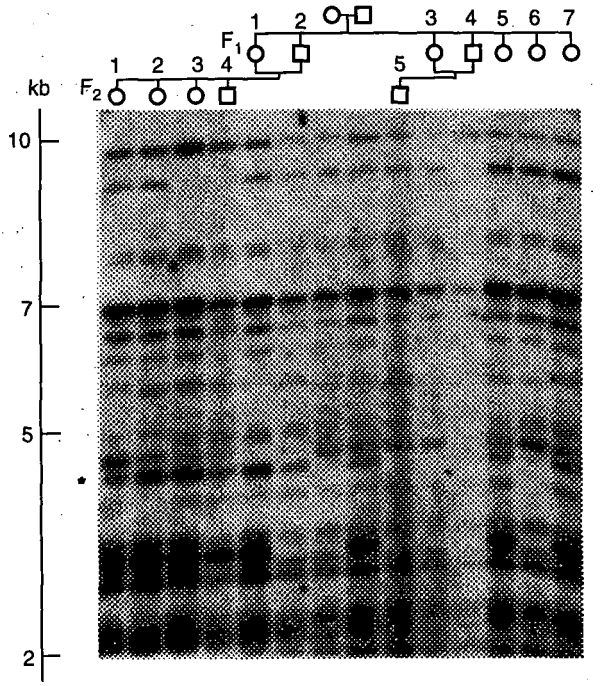

FIG. 2. DNA fingerprinting in pedigree analysis of tsetse flies. Analysis of some of the individuals resulting from an extensive G. m. centralis *pedigree which originated by mating a 'RUCA' virgin female with a 'Cent' male. Males are represented by squares and females by circles and the offspring are numbered consecutively. The star indicates the position of the band showing an amplification of intensity in offspring females F_{11} and F_{17}.*

have gone through genetic bottlenecks (almost certainly true for all of the small colonies); and (3) all the colonies (except *G. brevipalpis* and *G. longipennis*) were subjected to inbreeding and selection to increase the frequency of specific alleles. Nonetheless, the amount of variation in DNAfp provides a means for population genetic comparisons between field collected and colonized tsetse flies. In this context, one can propose that DNAfp be used as a routine technique to monitor genetic variability and genetic changes in tsetse colonies.

2.2. Genetic inheritance in pedigree analysis

A pedigree analysis was performed in anticipation of the establishment of a genetic linkage map using DNAfp and of studies of the molecular basis of hybrid sterility. Figure 2 represents the M13 hybridization of part of a large pedigree that resulted from mating a virgin female from the 'RUCA' line (*G. m. centralis* from Zambia) with a male from the 'Cent' line (*G. m. centralis*, originating from the

United Republic of Tanzania). For the reasons explained above, the DNAfp profiles showed little variation between individuals and a large majority of bands are common to both parents. In this two generation pedigree, neither the parents nor the different offspring have an identical profile and therefore each DNAfp is unique to an individual. The pedigree demonstrates that there is a germ line stability of inherited fragments, that the transmission of polymorphic fragments of maternal and paternal origin can be followed in the progeny, and that each inherited fragment has a counterpart in at least one parent. In this particular pedigree, there was no specific linkage of any fragment to a sex chromosome, nor did any mutated bands appear. Some fragments showed a dramatic increase in intensity in the segregation profile of offspring, revealing that they are inherited as a homozygous allele. Two offspring, females F_{11} and F_{17}, showed an amplification in intensity of a fragment that cannot be explained through normal Mendelian inheritance. Similar observations were made during the analysis of other pedigrees involving the same intercolony cross. Although it is rather speculative at this stage of the study, this observation may suggest the mobilization of repeated sequences such as transposable elements.

ACKNOWLEDGEMENTS

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ISOLATION AND PRELIMINARY RESTRICTION SITE MAP OF MITOCHONDRIAL DNA FROM *Ceratitis capitata* IN BRAZIL

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Abstract

ISOLATION AND PRELIMINARY RESTRICTION SITE MAP OF MITOCHONDRIAL DNA FROM *Ceratitis capitata* IN BRAZIL.

Ceratitis capitata, as other tephritid fruit fly species, is a fresh fruit parasite during its larval stages. A pest introduced to Brazil, it has now spread over large areas of the country. Unfortunately, this species has shown very few genetic markers at the protein level that can be used to track population scattering. Therefore, a search was started for suitable genetic markers, the initial goal being to find genetic variation in the mitochondrial genome. Mitochondria were isolated from pupae reared in the laboratory for several years by the usual cell fractionation. The mitochondrial pellet was lysate; its DNA was further purified by the CsCl equilibrium gradient in an ultracentrifuge. The purified mtDNA was cut with restriction endonucleases and analysed by agarose gel electrophoresis, followed by direct visualization with ethydium bromide or by Southern blot hybridization with biotinylated probes, revealed by the histochemical reactions with streptavidin alkaline phosphatase conjugates. Eleven sites were detected: one with BamHI, four with EcoRI, four with HindIII, two with EcoRV and none with PstI. Small fragments were detected with silver stain after polyacrylamide gel electrophoresis. The sites were mapped with multiple restriction enzyme digestions. The total size of the mitochondrial genome was estimated to be about 16 kb.

1. INTRODUCTION

The Mediterranean fruit fly (medfly), *Ceratitis capitata*, was first recorded in Brazil at the beginning of the century [1]. Until the last decade, it had not been detected in the northern and northeastern regions of Brazil, the Reconcavo Baiano being its northernmost limit [2]. However, some populations have recently been recorded in Maranhão State (in the 1990s) in previously unrecorded hosts. These populations could have resulted from early introductions, with subsequent spreading, or they could have originated from more recent introduction events. The study of genetic markers can be useful in deciding between these alternative hypotheses.

It is known from studies made at the phenotypic level that Brazilian populations of *C. capitata* have sufficient genetic plasticity to adapt to new environments [3]. However, previous studies performed with the use of isozyme markers have shown little variation in the sampled populations [4].

Over the past decade, analysis of genetic variability at the mitochondrial genome has proved to be a powerful tool for tracking dispersion and for studying the population structure of animal species [5–6]. Maternal inheritance and the high degree of variation were believed to be the cause of this success [7]. To characterize the mitochondrial genome of Brazilian populations, we started a programme that began with the determination of the restriction sites of a long term laboratory population.

2. MATERIALS AND METHODS

2.1. Isolation of mtDNA

All the flies were obtained from a long term, laboratory reared colony of *C. capitata* first collected in Jundiaí in 1975 [3]. MtDNA from pupae at least 3 d old were isolated according to Ref. [8] (typically 10–20 g). We used TES buffer (Tris-HCl 30mM, pH7.6, EDTA 10mM, Saccharose 0.25M) instead of the original MIM buffer for mitochondria isolation in order to improve the yield. We also used a single ultracentrifugation step in a caesium chloride–ethidium bromide equilibrium gradient.

2.2. Restriction digestion of the mtDNA samples

The two isolated mtDNA samples were either digested with the restriction enzymes or used to prepare biotinylated probes. In order to make precise measurements of the electrophoretic migration rates, all the digestions were carried out in a single buffer ('One-phor-all' Pharmacia). Typically, two units of each enzyme were used to digest 50–200 ng of DNA for each slot.

2.3. Agarose gel electrophoresis

Gels of 0.4–0.8% agarose were prepared in TBE 0.5X buffer according to Ref. [9]. Low voltages were employed (20–30 V) to ensure better resolution. The gels were stained with ethidium bromide after running and then photographed. Fragments of lambda DNA obtained from restriction nuclease digestion were used as standards.

2.4. Polyacrylamide gel electrophoresis

To analyse small DNA fragments, vertical slab gels were also cast in TBE 0.5X buffer. A thickness of 0.7 mm was used for the 5% polyacrylamide gels. After running, the gels were fixed and silver stained.

2.5. Detection of DNA fragments with biotinylated probes

Biotinylated probes were prepared according to Ref. [10] by means of a nick translation reaction using 14 biotin dATP as labelled precursor. After electrophoretic running, the gels (with samples of 5 ng of mtDNA or less if pure, or more than 100 ng if total) were blotted against nitrocellulose membranes. The membranes were hybridized with probes in formamide 45% at 42°C overnight (further details can be found in Ref. [10]). Detection after membrane washing was performed by a reaction with streptavidin–alkaline phosphatase conjugates and BCIP–NBT histochemical staining.

3. RESULTS

3.1. Estimation of restriction fragment lengths

After careful measurement of the migration distances of the bands in photographs of the gels (Fig. 1), we performed a polynomial regression analysis between migration distances and the logarithm of the size from the known fragments of lambda phage DNA. Table I shows length estimates of fragments obtained after digestion with single and double restriction enzymes. The overall size of the mtDNA from *C. capitata* was calculated to be 16 209 bp, with 15 930 to 16 488 bp as the 95% confidence interval.

Some bands that appeared to be the result of a single fragment in the agarose gels could be resolved into two bands with polyacrylamide gel electrophoresis (Fig. 2).

3.2. Map construction

A tentative map is shown in Fig. 3. The site distances used to construct the map were those obtained from the length estimates of the presumptive fragments themselves (Table II). The map shown is therefore one of minimum distances. Double digestions with HindIII and EcoRI once showed a slightly increased distance between the two smaller bands than the distance observed in HindIII digestion. We therefore concluded that there was an EcoRI site located in the smaller HindIII fragment.

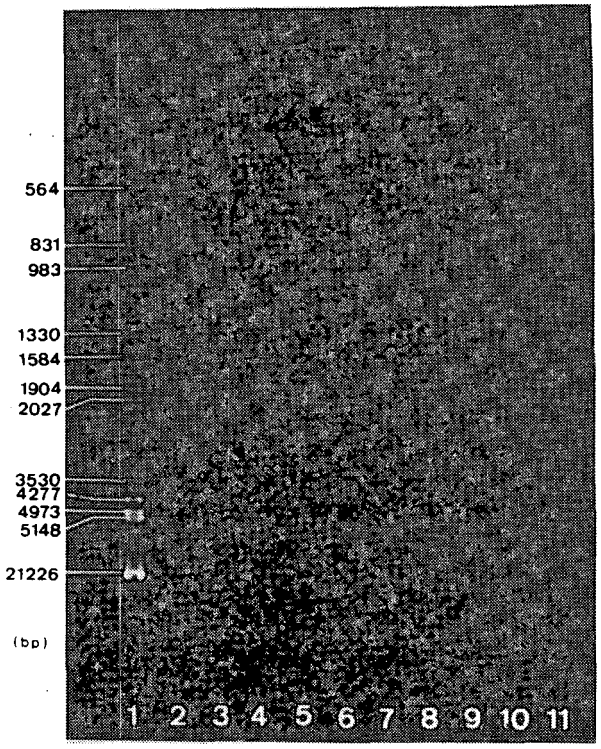

FIG. 1. Ethidium bromide stained 0.8% agarose gel showing DNA fragments after restriction enzyme digestion. Lane 1, lambda phage, and lanes 2–11, C. capitata *mtDNA. The enzymes used were: 1, EcoRI and HindIII; 2, HindIII; 3, HindIII, BamHI and EcoRV; 4, EcoRI; 5, EcoRI and BamHI; 6, EcoRI and HindIII; 7, EcoRI and HindIII; 8, EcoRI and EcoRV; 9, EcoRV; 10, EcoRV and HindIII; and 11, EcoRV and BamHI.*

3.3. Detection of the biotinylated probes

Figure 4 shows a nitrocellulose membrane after biotin detection. In this membrane, pure digested mtDNA fragments were analysed. In the PstI digestion, a very high molecular weight DNA can be observed.

4. DISCUSSION

The mitochondrial DNA from *C. capitata* has a length that is a model value in animals [11]. The incompatibility that existed among the overall size estimates from different digestions can be explained by poor estimates in the longer fragments,

TABLE I. LENGTH ESTIMATES OF SOME FRAGMENTS OF DIGESTED *C. capitata* mtDNA. THE VALUES ARE THE MEANS OF TWO DIFFERENT MEASUREMENTS. FOR DOUBLE DIGESTIONS, ONLY THE DATA FROM NEW GENERATED FRAGMENTS ARE PRESENTED

Digestion	No. of fragment	Length (bp)	95% confidence interval	
BamHI	1	16 543	16 019	17 085
EcoRI	1	7 444	7 230	7 664
	2	6 172	6 006	6 343
	3	1 636	1 603	1 670
	4	1 078	1 050	1 107
HindIII	1	8 262	8 018	8 514
	2	6 849	6 659	7 048
	3	473	448	501
	4	467	445	490
EcoRV	1	11 563	11 225	11 912
	2	4 858	4 753	4 965
EcoRI + BamHI	2	4 290	4 192	4 393
	3	3 029	2 969	3 092
HindIII + BamHI	2	4 318	4 230	44 087
	3	2 102	2 059	2 146
EcoRV + BamHI	1	9 436	9 144	9 737
	3	1 998	1 960	2 039

or by the undetected loss of very small fragments, or by a combination of both factors. None of the data obtained are compatible with the hypothesis of polymorphic sites in the strain studied.

The observation of very high molecular weight mtDNA in undigested samples (as the PstI digest in Fig. 3) can be explained by the existence of chained quaternary mtDNA structures caused by topoisomerases [12].

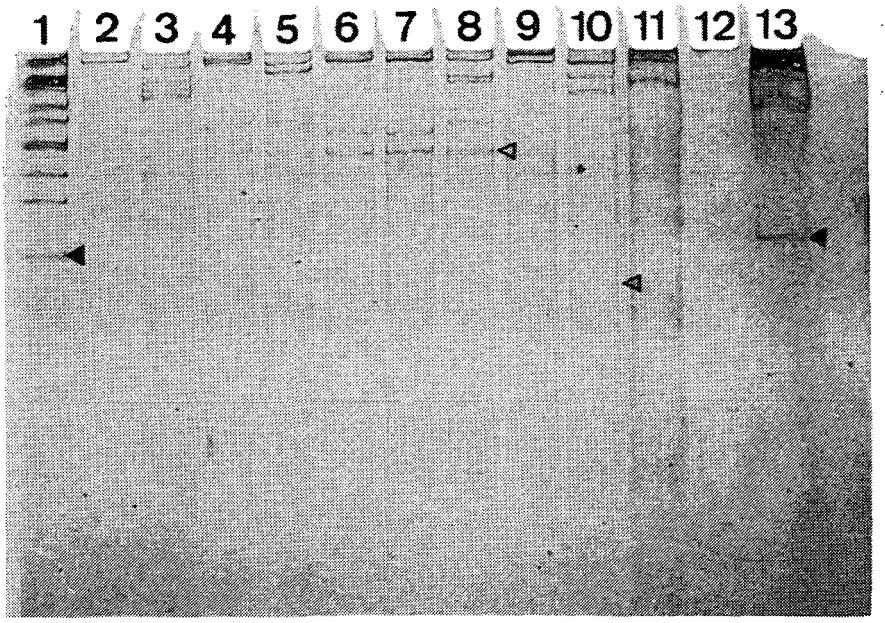

FIG. 2. Silver stained 5% polyacrylamide gel. The samples are the same as in Fig. 1, with the addition of lane 13, which is lambda phage DNA cut with HindIII. The open arrowheads indicate the positions of double bands that were detected as single bands in the agarose gels. The filled arrowheads indicate the 564 bp lambda DNA fragment.

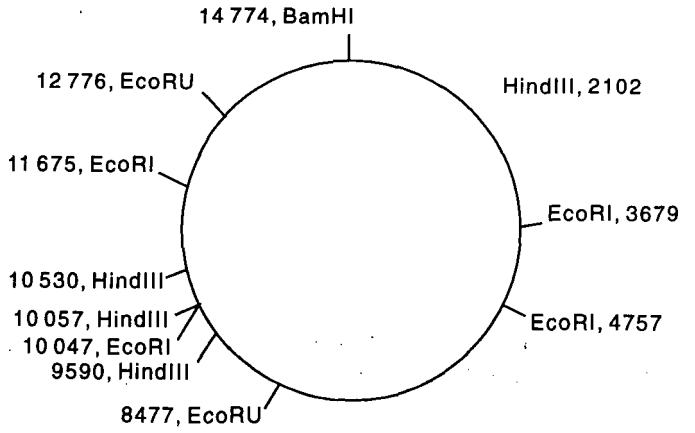

FIG. 3. Ceratitis capitata *restriction site map of the enzymes studied. The distances between the sites are minima (in bp). The starting point was chosen to be the only BamHI site observed.*

TABLE II. MAP DISTANCES BETWEEN SUCCESSIVE RESTRICTION SITES (CLOCKWISE)

Sites	Distance (bp)	95% confidence interval	
BamHI and HindIII	2102	2059	2146
HindIII and EcoRI	1577	1540	1615
EcoRI and EcoRI	1078	1050	1107
EcoRI and EcoRV	3720	3630	3811
EcoRV and HindIII	1113	1088	1139
HindIII and EcoRI	102[a]	Not determined	
EcoRI and HindIII	473	448	501
HindIII and HindIII	457	435	480
HindIII and EcoRI	1145	1120	1170
EcoRI and EcoRV	1101	1069	1133
EcoRV and BamHI	1998	1960	2039

[a] Estimated by difference between digestion, as shown in lanes 2 and 6 of Fig. 2.

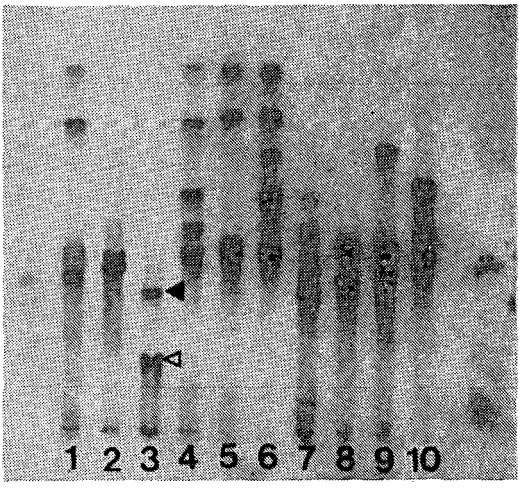

FIG. 4. Detection of C. capitata *mtDNA by hybridization after transfer to a nitrocellulose membrane. The digestions were: 1, EcoRI; 2, HindIII; 3, PstI; 4, EcoRI and BamHI; 5, EcoRI and HindIII; 6, EcoRI, HindIII and BamHI; 7, BamHI; 8, EcoRV; 9, EcoRV and BamHI; and 10, EcoRV and HindIII. The open arrowhead indicates the position of the quaternary forms of mtDNA. The filled arrowhead indicates whole mtDNA molecules.*

The procedures used to construct the map are suitable for scaling up and use in population studies. The use of non-radioactive probes provides a safer research environment. The map obtained here can also be used for comparisons and the construction of phylogenetic trees if different patterns are to be obtained in the future.

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GENETICS

(Session 3)

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RADIATION INDUCED CHROMOSOME ABERRATIONS FOR THE GENETIC ANALYSIS AND MANIPULATION OF THE MEDITERRANEAN FRUIT FLY, *Ceratitis capitata*

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Abstract

RADIATION INDUCED CHROMOSOME ABERRATIONS FOR THE GENETIC ANALYSIS AND MANIPULATION OF THE MEDITERRANEAN FRUIT FLY, *Ceratitis capitata*.

Gamma radiation was used to induce chromosome aberrations for the analysis and genetic manipulation of the Mediterranean fruit fly (medfly). Several new translocations linking different autosomes to the male determining Y chromosome were detected. In three separate experiments, a total of 34 translocation strains were recovered. Sixteen of these strains were subjected to more detailed analysis. The results concerning their overall viability are presented. In addition, an attempt has been made to determine the cytological location of the white pupa mutation. For this purpose, deletions were induced. Of the over 8000 pupae screened, four strains with visible chromosome aberrations were found.

1. INTRODUCTION

The Mediterranean fruit fly (medfly), *Ceratitis capitata*, is a serious agricultural pest in tropical and subtropical areas. To eliminate the damage caused by this fly, the sterile insect technique (SIT) has been applied as an environmentally safe alternative to insecticides (for a review, see Ref. [1]).

However, the cost effectiveness and application range of this technique could be improved if methods for male-only releases were developed. The released sterile males are the primary active agent in SIT and, consequently, the cost of mass rearing could be reduced if procedures to eliminate the females as early as possible were available. Furthermore, the number of flies required to achieve control can be reduced, since the problem of preferential mating among the released flies no longer exists [2]. Additional savings in programme costs result from the reduction in monitoring, which is greatly simplified if the only females trapped are those from the wild population [3].

The application range of the SIT is extended in two respects if only males are released. First, those countries that did not allow use of this technique because of the damage caused by the sterile females attempting to deposit their eggs into the fruit can now also apply SIT. Second, SIT can also be used to control rather than to eradicate the pest.

To be able to release only males, genetic manipulation of the medfly is necessary. In all existing genetic sexing systems, the wild type allele of a selectable marker is linked to the male sex through translocations between the respective autosome and the Y chromosome. This type of chromosome aberration is usually induced by radiation. The selection of the appropriate sexing gene determines primarily the cost effectiveness of the sex separation (i.e. it should be as early and as accurate as possible and should not require expensive equipment), while choosing which translocation to use in the strain determines the genetic stability of the sexing system during mass rearing. It has been shown that recombination in the males occurs [4, 5] and that genetic exchange in the chromosomal region between the sexing gene and the translocation break point leads to the breakdown of the sexing system [6].

2. RESULTS AND DISCUSSION

2.1. Induction of Y autosome translocations

In the first experiment (T7-1) (see Table I) aimed at the induction of Y autosome translocations, pupae of the wild type strain Egypt II (EgII) were irradiated with 50 Gy in a ^{60}Co source 1 d before adult emergence. Single males, 61 in total, were mated with females homozygous for the mutation white pupa (*wp*) [7]. F_1 males were also crossed with females from the *wp* strain. The F_2 progeny of these families, originating from a single irradiated male, were scored for pseudolinkage between *wp* and sex, i.e. for the appearance of only mutant females and only wild type males. Seven families were found. In parallel, the remaining families were analysed for translocations linking either chromosome 3 or chromosome 4 to the Y chromosome, i.e. the same crossing scheme as that described for *wp* was used, but in this case the mutations dark pupa (*dp*) [8] and apricot eye (*ap*) [8] were used [9, 10].

Further genetic and cytological analyses of these 13 new translocation strains showed that seven (11.5% of 61 families) displayed pseudolinkage with chromosome 5 and six (9.8% of 61 families) with chromosome 3 or 4, respectively. This means that under these experimental conditions approximately 10% of all the scored families carry at least one Y autosome translocation for any of the five autosomes, assuming that the autosomes not tested here (chromosomes 2 and 6) exhibit the same characteristics.

TABLE I. INDUCTION OF Y AUTOSOME TRANSLOCATIONS

Experiment	Dose (Gy)	No. of single pair families			
		Screened	With pseudolinkage (marker used)	With more than 1 autosome involved	Producing adjacent-1 adults
T7-1	50	61	7 (*wp*)	5	3
		(52)	3 (*ap*)	1	0
		(52)	3 (*dp*)	1	1
T7-2	50	150	8 (*wp*)	6	4
T7-3	40	300	12 (*we wp*)	5	5–8

Note: Numbers in parentheses denote remaining families after screening for pseudolinkage with *wp*.

In the second experiment (T7-2), 150 irradiated EgII males were scored for pseudolinkage between *wp* and sex. In this case, eight (5.3% of 150 families) new translocations were detected. In both experiments (T7-1 and T7-2), a high frequency of complex translocations was observed, i.e. of 21 translocations, 13 involved more than one autosome. In the most extreme case, three autosomes were indirectly linked via autosome–autosome translocations to the Y chromosome. The presence and the complexity of the translocation has a severe impact on the viability of these strains (Table II) [10–12]. Strains with simple Y autosome translocations produce approximately half the number of males per 1000 fertilized eggs as the standard non-translocation control (Fig. 1). If two autosomes are involved, this value is again reduced by 50% and in the case of translocations linking three autosomes to the Y chromosome, male production is reduced to approximately 13% of the control.

Consequently, the most suitable strains for mass rearing carry translocations where only one autosome is involved. Experiments T7-1 and T7-2 resulted in four strains where only chromosome 5 and the Y chromosome were linked. To increase this number, a third experiment (T7-3) was initiated. This time, a dose of 40 Gy was used and 300 single pair families were analysed (Table I). Of 13 families with pseudolinkage, eight contained simple T(Y;5) translocations.

To construct genetic sexing strains, the position of the Y autosome break point has to be known. Genetic and cytological experiments were performed to determine the break points in the existing strains [10, 11] and to analyse their stability using either *wp* or a temperature sensitive lethal (*tsl*) [13] as the selectable marker [11].

TABLE II. VIABILITY OF Y AUTOSOME TRANSLOCATIONS INVOLVING ONE, TWO OR THREE AUTOSOMES [10–12]

Strains	No. of eggs	% hatch	% pupae	% males	No. of males/1000	Refs
T(Y;5)30C	5 380	74.7	83.5	46.7	291	[11]
T(Y;5)1-61	9 210	64.2	82.3	44.5	235	[12]
T(Y;5)2-22	2 700	69.8	80.9	41.4	234	[12]
T(Y;5)3-129	1 800	69.7	67.2	40.0	187	
T(Y;5)3-245	1 800	84.6	69.1	36.6	214	
T(Y;5)1-59	5 600	67.8	79.5	37.0	200	[12]
T(Y;5)2-40	500	56.6	66.4	41.5	156	[12]
T(Y;3)1-5	300	62.0	75.3	34.3	160	[10]
T(Y;3)1-30	300	85.7	66.9	30.8	177	[10]
T(Y;4)1-9	300	74.7	82.6	36.2	223	[10]
T(Y;4)1-33	300	61.7	87.0	36.6	197	[10]
Total T(Y;A)	28 640	68.2	79.7	42.0	227	
T(Y;2;5)1-15	500	39.4	56.3	42.3	94	[12]
T(Y;3;5)1-56	4 900	48.6	60.4	39.0	114	[11]
T(Y;2;5)2-82	5 100	46.2	65.6	34.6	105	[11]
T(Y;3;4)1-43	300	47.7	83.2	43.7	173	[10]
Total T(Y;A;A)	10 800	47.0	63.3	37.2	111	
T(Y;2;4;5)2-54	500	36.2	39.8	26.4	38	[12]
T(Y;3;4;6)1-50	300	31.0	49.5	43.5	67	[10]
Total T(Y;A;A;A)	800	34.3	43.1	33.1	49	

2.2. Induction of deletions

The second essential prerequisite for the construction of stable genetic sexing strains is to determine the chromosomal location of the selectable marker. In several experiments we have attempted to induce deletions of the *wp* locus (Table III) [14]. Because no balancer chromosomes exist in the medfly, the deletions were induced in a Y autosome translocation (T(Y;5)30C, Refs [14, 15]), i.e. the deletions remained linked to the Y chromosome and, therefore, to the male sex. The irradiated males (T(Y;5)*wp*+/*wp*+) were crossed in large cages with *wp* females and the F_1 generation was screened for the occurrence of white pupae. Males emerging

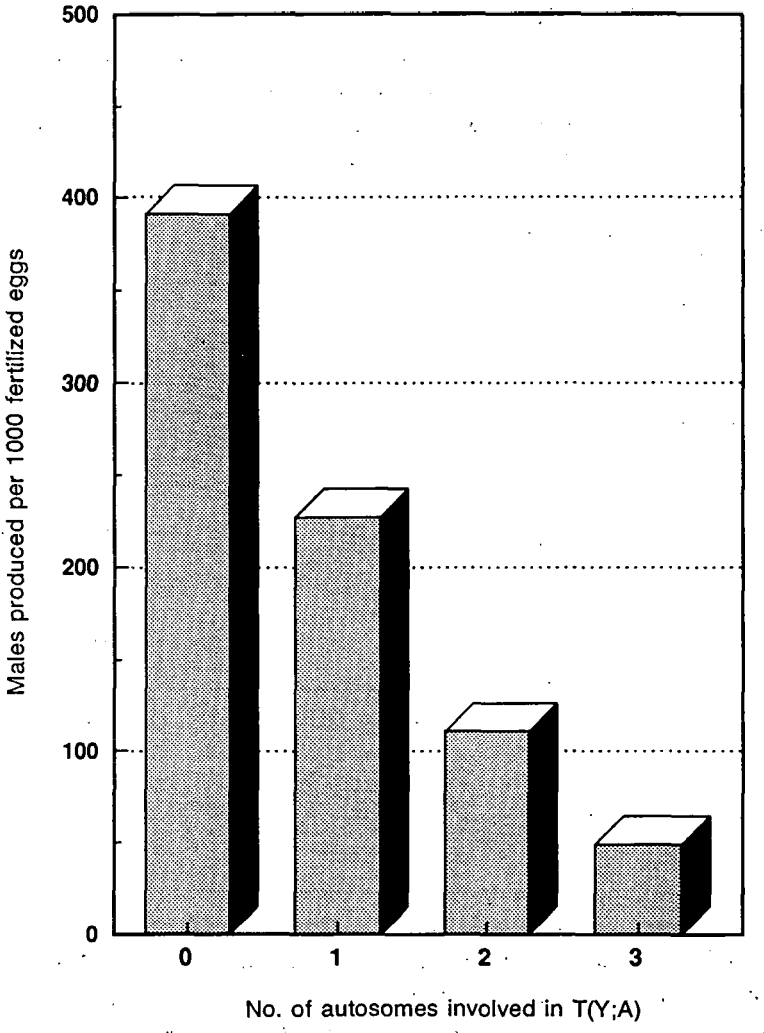

FIG. 1. Production of males in strains with no, one, two or three autosomes linked to the Y chromosome. Male survival is calculated per 1000 fertilized eggs.

from these pupae were used to establish single pair families and their offspring were analysed cytologically. In the first three experiments (T6-1, T6-2 and T6-3) (see Table III), five *wp* males were recovered. However, analysis of the trichogen polytene chromosomes revealed a chromosome aberration in only one of them, and this was a transposition and not a deletion. In the fourth experiment, the dose was reduced to 30 Gy. This resulted in the recovery of four *wp* males, three of which showed a deletion and were used in combination with the transposition to locate the *wp* locus on chromosome 5 [12].

TABLE III. INDUCTION OF DELETIONS IN T(Y;5)30C [12]

Experiment	Dose (Gy)	No. of brown pupae	No. of white pupae	No. of *wp* females	No. of *wp* males	Strain [Ref.]	Aberration
T6-1	50	Not counted	3	0	2	T6-1-9	No
						T6-1-12 [12]	Transposition
T6-2	50	1540	7	0	3	T6-2-1	No
						T6-2-5	No
						T6-2-6	No
T6-3	50	1338	2	1	0		
T6-4	50	639	0				
	40	1052	0				
	30	3483	6	1	4	T6-4-1 [12]	No
						T6-4-2 [12]	Deletion?
						T6-4-3 [12]	Deletion
						T6-4-4 [12]	Deletion
Total		>8052	18	2	9		4

3. CONCLUSIONS

Many of the genetic and cytogenetic tools required to analyse and manipulate the medfly genome are now available [16]. Approximately ninety mutations have been identified and the polytene chromosomes have been mapped [17–20]. In addition, many Y autosome translocations have been induced. These can be used either to locate mutations or to construct genetic sexing strains. Mapping through radiation induced deletion has also been established in the medfly. The first case was the mapping of the alcohol dehydrogenase (*Adh*) loci on chromosome 2 [21–23]. We used this technique to locate the *wp* gene on the right arm of chromosome 5 [15] and, recently, we isolated three potential deletions for the mutation white eye (*we*) [24] (data not shown).

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INHERITANCE OF REFRACTORINESS TO TRYPANOSOME INFECTION IN TSETSE

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Abstract

INHERITANCE OF REFRACTORINESS TO TRYPANOSOME INFECTION IN TSETSE.

Differences in susceptibility to midgut infection between teneral flies from susceptible and outbred stocks disappear in non-teneral flies, showing that maternally inherited susceptibility to midgut infection is a condition expressed only in teneral flies. The increased susceptibility of colonized *Glossina morsitans morsitans* to trypanosome infection compared with wild flies can be related to the spread of flies carrying rickettsia like organisms through colonization. The relative refractoriness of non-teneral flies suggests that flies fed prior to release in a sterile insect technique programme would not play a significant part in the spread of *Trypanozoon* or *Trypanosoma congolense* infections.

1. INTRODUCTION

The enhanced susceptibility to infection of teneral flies has long been recognized. Duke [1] found that repeatedly infecting *Glossina fuscipes* with *Trypanosoma brucei* sensu lato (sl) did not significantly increase the infection rates over flies infected only at the first feed. Wijers [2] and Harley [3] later confirmed the superior susceptibility of teneral flies to *Trypanozoon* infections. It was later shown [4, 5] that teneral flies are also more susceptible to *T. congolense* infections than fed flies.

The present work examines the relationship between the heritability of refractoriness to trypanosome infection in tsetse and the teneral state of the fly.

2. MATERIALS AND METHODS

2.1. Teneral and non-teneral infection rates

Glossina morsitans morsitans from two stocks were used: (1) outbred, and (2) susceptible — an iso-female line selected for susceptibility to trypanosome infection [6]. Flies were infected [7] with bloodstream form *T. congolense*

(stock 1/148) [8] either when teneral or at the second to seventh subsequent feed. All the flies were starved for three days before infection.

2.2. Comparison of laboratory and wild flies

Glossina morsitans morsitans puparia were collected in Zimbabwe from the same population from which the original Langford colony was derived in 1967.

Haemolymph was collected from flies emerging from wild collected puparia and from a control group taken from the Langford *G. m. morsitans* colony. Haemocytes were stained and examined by light microscopy for the presence or absence of rickettsia like organisms (RLO) infections.

The emergent female flies from wild collected puparia and a control group of females from the Langford *G. m. morsitans* colony were also infected with *T. b. rhodesiense* (EATRO 2340) [9].

2.3. Dissection

Mouthparts (labrum and hypopharynx) and midguts were dissected and examined for trypanosomes 21 d post-infection (*T. congolense* infections), and salivary glands and midguts were dissected and examined 28 d post-infection (*T. b. rhodesiense* infections).

3. RESULTS

3.1. Teneral and non-teneral infection rates

Table I shows the infection rates in two lines of *G. m. morsitans* infected with *T. congolense* at increasing ages. The non-teneral flies of both lines had significantly lower infection rates than the teneral flies. While all the midgut infections matured when the flies were infected as tenerals, the non-teneral transmission index (% hypopharynx/midgut) was significantly reduced at all stages compared with the tenerals.

The midgut infection rates in the susceptible line of teneral *G. m. morsitans* were significantly greater than in the outbred teneral flies ($P < 0.001$). A comparison with non-teneral flies showed no significant differences in the midgut infection rates between the lines of flies.

3.2. Comparison of laboratory and wild flies

Significantly less (21.8%, n = 202) haemolymph samples from wild *G. m. morsitans* were found to be infected with RLO than Langford colony flies (76.0%;

TABLE I. FLY AGE AND *T. congolense* INFECTION RATES IN SUSCEPTIBLE AND OUTBRED LINES OF *G. m. morsitans*. THE TENERAL FLIES WERE INFECTED ON THE DAY AFTER EMERGENCE FROM THE PUPARIUM (GROUP 0). THE NON-TENERAL FLIES WERE INFECTED AT SUBSEQUENT BLOODMEALS (GROUPS 1-6). THE INTERVAL BETWEEN BLOODMEALS WAS THREE DAYS

(N : number of flies dissected; MG : % midgut; Hyp : % hypopharynx infections)

No. of feeds	Susceptible			Outbred		
	N	MG	Hyp	N	MG	Hyp
Teneral	44	95.5	95.5	77	57.1	57.1
1	74	16.2	8.1	103	20.4	16.5
2	80	16.3	2.5	117	5.1	3.4
3	81	3.7	1.2	111	3.6	1.8
4	76	11.8	5.3	195	6.7	3.1
5	83	8.4	3.6	96	9.4	6.3
6	37	2.7	0.0	115	13.0	4.3

n = 105; $P < 0.001$). Wild flies also showed significantly lower midgut infection rates (22%, n = 46) than colonized flies (82%, n = 32) ($P < 0.001$); the salivary gland infection rates were 0.0% and 6.5%, respectively.

4. DISCUSSION

The results presented here show that tsetse flies, once fed, remain highly refractory to infection throughout their lives. The differences in susceptibility to midgut infection observed between outbred *G. m. morsitans* and a line from the same population selected for susceptibility to infection disappeared in the non-teneral flies. This suggests that enhanced susceptibility to *T. congolense* and *Trypanozoon* midgut infection in *G. m. morsitans*, which has been shown to be maternally inherited [10], is expressed only in tenerals and is abolished after the second feed.

We have previously shown that susceptibility to trypanosome infection is related to RLO infection in teneral *G. m. morsitans* [11]. The increased susceptibility to midgut infection of laboratory reared *G. m. morsitans* compared with wild flies shown here may be attributed to the accumulation of RLO in the absence of selection to which the field population would normally be exposed.

Refractoriness to trypanosome infection has been shown to be due to trypanosome killing by tsetse midgut lectin [12]. Teneral flies would be expected to produce less midgut lectin or to produce inhibitors which would disable the lectin prior to contact with the parasites. Two such inhibitors, D glucosamine and N-acetyl-D-glucosamine, when added to the tsetse bloodmeal, have been shown to permit fed flies to become superinfected; this could be generated by the enzyme digestion of chitin [13]. Tsetse RLO produce chitinases in vitro [14] and their action in the puparium could increase the susceptibility of the teneral fly. This effect would, however, be abolished by the large amounts of lectin produced in response to feeding. The fact that older flies can become superinfected by the addition of lectin inhibitory sugars [13] suggests that tsetse midgut lectin normally prevents parasitism in non-teneral flies and that there is no physical barrier (for example, the peritrophic membrane) to infection in older flies.

The significance of non-teneral flies in the transmission of trypanosomiases remains controversial. Harley [3] found that 3.0% of the non-teneral *G. f. fuscipes* acquired mature infections of *T. b. rhodesiense* compared with 14.3% of the teneral flies, challenging the opinion of Wijers [2] that flies could only be infected with *Trypanozoon* at the teneral feed. Wijers [2] was, however, working with *T. b. gambiense*, which produces few mature infections, even in teneral flies [15]. Recent related experiments suggesting that non-teneral flies may be of epidemiological significance [16–19] have often been conducted with colonized *G. m. morsitans* which, as we have shown, have lost much of their innate refractoriness to midgut infection.

The present work shows that a laboratory colony of tsetse susceptible to trypanosome infection when teneral becomes highly refractory to midgut infection when infected as non-teneral. If flies are to be released in a sterile insect technique programme, then it is important to ensure that such flies are fed prior to release in order to minimize disease transmission. However, logistically it would be advantageous to breed stocks of tsetse which are as intrinsically refractory to trypanosome infection as tenerals.

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ACTIVE QUALITY CONTROL IN MASS REARED MELON FLIES
Quantitative genetic aspects

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Abstract

ACTIVE QUALITY CONTROL IN MASS REARED MELON FLIES: QUANTITATIVE GENETIC ASPECTS.

It is important to maintain the quality of mass reared flies throughout many generations in establishing the sterile insect technique. In practical mass rearing of the melon fly, *Bactrocera cucurbitae*, many traits have already been differentiated between mass reared and wild flies. First, the differing traits of wild and mass reared melon flies are reviewed and the factors which have caused these differences are considered. In the mass rearing procedure, some artificial selection pressures have been attributed to many traits of the flies. Next, an attempt is made to predict the change in larval period in mass rearing. As a result, a quantitative genetic model successfully predicts the change in traits. Finally, consideration is given to some correlated responses to artificial selection in mass rearing. Many such responses to artificial selection in the mass rearing process are explored. The survival rate that correlated to reproduction was successfully controlled by artificial selection for reproduction in mass rearing. On the basis of these results, active quality control for mass reared melon flies in the future is discussed.

1. INTRODUCTION

The complete eradication of the melon fly, *Bactrocera cucurbitae* Coquillett, from Japan, using the sterile insect technique (SIT) will be announced in 1993 [1]. After eradication, we must make efforts to prevent reinvasion by the melon fly from foreign countries. Should reinvasion occur, we will also make an effort to eradicate the invading population from the location where reinfestation originated, immediately after detection. Thus, we have to continue mass rearing of the melon fly for SIT even after eradication. There is no other valid method for eradication except SIT [2]. In this context, it is important to maintain mass reared flies over many

generations continuously. However, a method to maintain the high quality of mass reared flies that are suitable for use with SIT has not been established. Our goal is to establish a method to control and improve the quality of mass reared flies. In this paper, we review the differences in traits between wild and mass reared melon flies and estimate the change in these traits caused by mass rearing. Two questions are addressed. First, are there any correlated responses among these traits? Second, can we control the changes in these traits by intentional selection in mass rearing?

2. DIFFERENCES IN TRAITS BETWEEN WILD AND MASS REARED MELON FLIES

The differences in some characteristics between wild and mass reared melon flies have been extensively studied in Okinawa, Japan. We summarize the results of these reports in Table I [3–17]. In the table, the traits are divided into five categories: reproductive activity (RA), duration and timing of the life history (LH), dispersal ability (DA), activity rhythm (AR) and variation within individuals (VI). Forty-six comparisons were conducted independently for 18 traits in both wild and mass reared melon flies. For 'RA', mass reared flies were always more prolific than wild flies in all ten comparisons. For 'LH', mass reared flies always had shorter life history stages than did wild flies in all 20 cases. The dispersal ability of mass reared flies was not superior to wild flies. Diurnal rhythm, except for the initiation time of mating in the day, was not affected by mass rearing in two studies, while the initiation time of mating in the day for mass reared flies was earlier than that for wild flies in three studies. Variations within individuals in the pre-oviposition period, reproduction, oviposition, longevity, pre-mating period and the number of matings in mass reared flies were smaller than those in wild flies (Table I) [6].

In addition to these traits, sensitivity to space during copulation [18] and the mating behaviour of wild flies [19] also differed from mass reared flies, while the mating site of wild flies in a field cage (i.e. on leaves or fruits or stems) did not differ from that of mass reared flies [16].

What factors have caused these differences between wild and mass reared flies? In a laboratory reared insect strain, in-breeding depression by random drift is the important problem when effective population size, Ne, is small, while artificial selection pressures are important when it is large [20]. Artificial selection pressures are significant in SIT, which requires mass rearing of the insect.

In our mass rearing process, three types of artificial selection are in operation. The first process for mass rearing was selection for flies that displayed a high fitness value, considering the abnormally high density in an adult cage. These mass reared flies mated about 1 h earlier than wild flies ('AR') [9, 15, 16] and had somewhat decreased flight ability compared with wild flies ('DA') [12]. Also, the mating behaviour of mass reared flies differed from that of wild flies [18, 19]. This could

TABLE I. COMPARISON OF TRAITS OF WILD AND MASS REARED MELON FLIES IN THE MASS REARING FACILITIES OF OKINAWA [3–17]

Traits[a]	Results[b]			References
	W > M	W = M	W < M	
Type 'RA'				
Fecundity	0	0	5	[3–6]
% of females that laid eggs	0	0	1	[7]
Hatchability of eggs	0	0	1	[7]
Frequency of oviposition[c]	0	0	1	[6]
Number of matings[c]	0	0	2	[6, 7]
Type 'LH'				
Development time[d]	1	0	0	[8]
Pre-oviposition period	6	0	0	[3–6]
Age of peak fecundity	1	0	0	[5]
Ovarian development time	1	0	0	[9]
Post-ovipositional life span	1	0	0	[6]
Longevity	4	0	0	[5–7, 10]
Pre-mating period	5	0	0	[5–7, 9, 11]
Remating interval	1	0	0	[7]
Type 'DA'				
Flight ability	1	0	0	[12]
Dispersal ability in the field	3	1	0	[10, 13, 14]
Type 'AR'				
Initiation of mating in a day	3	0	0	[9, 15, 16]
Diurnal rhythm	0	2	0	[9, 17]
Type 'VI'				
Variation within individuals[e]	4	2	0	[6]

[a] See text for classification of types.

[b] W and M indicate wild and mass reared populations, respectively. Wild flies were maintained on pumpkin as a larval diet, and were used in experiments of less than three generations after introduction from the field.

[c] Per lifetime.

[d] Wild flies used were six generations after introduction from the field.

[e] Pre-oviposition period, fecundity, frequency of oviposition, longevity, pre-mating period and number of matings observed in a lifetime.

have been caused by the first type of selection. In the second selection process, flies that adapted well to the artificial diet and the artificial egging devices were selected for the next generation. The difference in reproduction ('RA') between wild and mass reared flies may be a result of adaptation to the artificial egging device and the artificial diet. In the third process, flies that developed earlier in each stage were selected for the next generation. The SIT requires a high level of efficiency in the production of mass reared flies. The mass reared flies actually had a shorter development time [8] and shorter pre-mating and pre-oviposition periods, but they also had shorter longevity compared with wild flies ('LH') [6].

3. PREDICTION OF CHANGE IN THE LARVAL PERIOD

The genetic bases of the traits mentioned above (see Table I) must be clarified in order to obtain high quality mass reared flies. If the selection intensities in the mass rearing process and the heritability for a trait are obtained, we can predict the degree of change in the trait using a quantitative genetic method [21]. We predicted the amount of genetic change in the larval period as depicted in our study [22]. If a selection is by truncation, the response to selection per generation is expected to be $R = ih^2\sigma_p$ [21]. About 3% of the larvae that developed most slowly were selected out artificially for each generation in the mass rearing procedure for the melon fly in Okinawa, i.e. 3% was the selection intensity (i) [23]. The heritability (h^2) of the larval period for the melon fly was estimated to be 0.2704 [24]. The phenotypic standard deviation, σ_p, in the wild strain ranged from 1.20 to 1.48 d [25]. The value calculated by the equation closely corresponded with the observed value at the 40th generation. The results showed that the larval period of the melon fly was getting shorter as generations passed through our mass rearing system. We analysed all the records of the mass rearing of the melon fly and found similar changes in larval periods to our prediction in the actual mass rearing process (Table II).

4. SELECTION EXPERIMENT FOR DEVELOPMENT TIME

The quality of mass reared flies might be greatly influenced by characters correlated to the shortened larval period resulting from mass rearing. We tried a selection experiment for development time (from egg to adult) and then examined some correlated responses to the selection for development time.

4.1. Selection response

Eggs were collected from the mass reared strain of the 41st generation and about 1600 eggs (0.1 mL in volume) were placed on 110 g of the larval medium.

TABLE II. CHANGE IN THE LARVAL PERIOD IN MASS REARED MELON FLIES AFTER INTRODUCTION FROM THE FIELD

Generation	Larval period (d) (mean (SD))
2	7.47 (0.18)
4	7.20 (0.31)
6	7.01 (0.10)
8	6.80 (0.15)
10	6.09 (0.15)
12	6.39 (0.23)
14	6.33 (0.10)
16	6.40 (0.11)
18	6.28 (0.10
20	6.27 (0.14)

The first 50 males and 50 females that emerged were selected to propagate the short development time lines (S line) and the last 50 males and females were selected to propagate the long development time lines (L line). Two replicates were conducted. One set of short and long lines (designated S-1 and L-1) was tested and maintained together, as was the second replicate (S-2 and L-2). The two replicates were initiated at the same time.

There was a clear response to selection for long development time, but no response for the short one (Fig. 1). Realized heritabilities were calculated for the first ten generations of selection as the regression of the population mean on the cumulative selection differential. The significance of each regression was determined by analysis of variance. The realized heritabilities of two replicates were significantly different from zero for the L lines and divergence (Table III). The response to selection for development time was mainly caused by the change in the larval period rather than the pupal period [8].

4.2. Correlated responses

Correlated responses to selection for development time were measured (Table IV) [22, 26, 27]. Head width and wing length were measured in generation 13

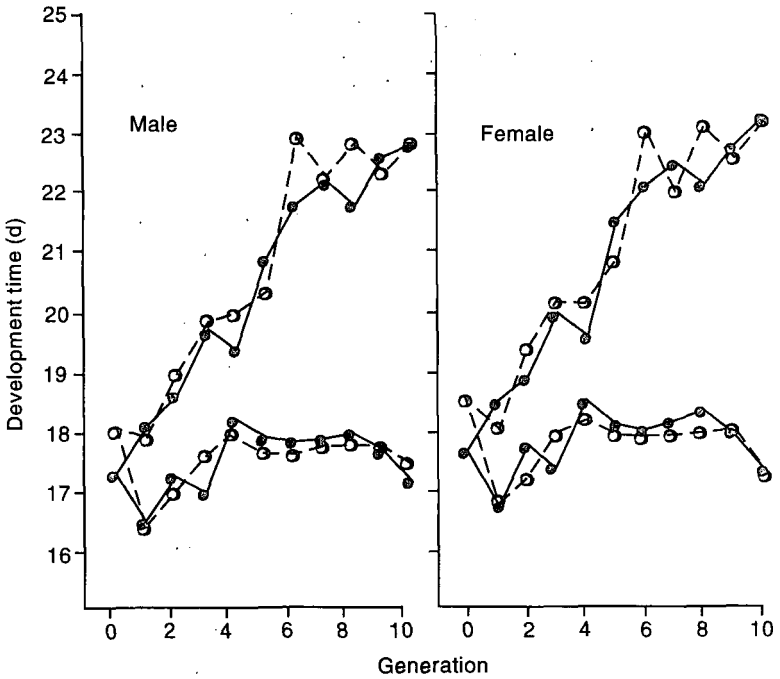

FIG. 1. Response to selection for short and long development times (● : first replicate; ○ : second replicate).

for the S line and in generation 11 for the L line. We found significant correlated responses for head width and wing length to selection for development time. The size of the L lines was larger than the size of the S lines ($P < 0.01$, ANOVA[1]). In *Drosophila melanogaster,* the line selected for larger flies always took longer to develop [28]. Total fecundity, peak fecundity, longevity, number of matings and the pre-mating periods were measured in generation 6 for the S line and in 5 for the L line. Total fecundity, peak fecundity and longevity did not correlate with development time, in spite of the size differences in the flies of both lines (not significant, two way ANOVA; see Ref. [29]). The number of matings and the pre-mating period did correlate with the development time, but the interaction between the selection regime and replicates was also significant ($P < 0.01$, two way ANOVA). The mating time in the day was measured in generation 19 for the S line and in 15 for the L line. The mating time in the day of the S lines was earlier than in the L lines ($P < 0.05$, Kruskal-Wallis test).

[1] ANOVA: Analysis of variance.

TABLE III. REALIZED HERITABILITIES CALCULATED AS THE REGRESSION OF THE POPULATION MEAN ON THE CUMULATIVE SELECTION DIFFERENTIAL FOR THE FIRST TEN GENERATIONS

	Short line	Long line	Divergence
Replicate 1			
Male	−0.0761	0.3057***	0.1716***
Female	−0.0610	0.3257***	0.1822***
Replicate 2			
Male	−0.0424	0.3781***	0.2072***
Female	−0.0041	0.3719***	0.2126***

*** $P < 0.001$ (ANOVA).

5. CORRELATED RESPONSES BETWEEN TRAITS

The correlated responses of the melon fly to artificial two way selection have been studied in four cases (Table IV). In two selection experiments conducted for early and late reproduction, there were correlated responses to the traits in early and late fecundity, sexual maturation and mating time in the day. There were no correlated responses to the traits in total fecundity, flight ability, flight velocity and the beginning of wing vibration by males in the day. Different results for longevity were obtained from these two experiments [26, 27].

In two selection experiments conducted for pre-adult developmental time, there were correlated responses to the traits of size and mating time in the day. There were no correlated responses to the trait in fecundity, number of lifetime matings, the pre-mating period, or longevity [22, 27]. The evidence that mass reared flies mated about 1 h earlier than wild flies [8, 14, 15] should be related to this correlation. The difference in the change in mating time may be a mixed result of the abnormally high adult density in the egg collection cage and the correlated response to artificial selections for early reproduction or short developmental periods in mass rearing. These three selection experiments, except for development time, have no replications. Thus Nakamori [26] and Suenaga [27] cannot rule out the possibility that the results were affected by mutation or genetic drift. More systematic selection experiments are needed to elucidate the nature of the correlated responses between traits in the melon fly.

TABLE IV. CORRELATED RESPONSES TO TWO WAY SELECTED TRAITS IN THE MELON FLY

Selected traits	Reference	Assayed traits	Correlated response
Reproduction, early versus late	[26]	1. Total fecundity	No
		2. Male longevity	No
		3. Female longevity	No
		4. Initiation time in sexual maturation	Early < late
		5. Flight ability	No
		6. Flight velocity	No
		7. Beginning of wing vibration by male in the day	No
Reproduction, early versus late	[27]	1. Early fecundity	Early > late
		2. Late fecundity	Early < late
		3. Longevity	Early < late
		4. Mating time in the day	Early < late
Larval period, short versus long	[27]	1. Mating time in the day	Fast ≤ slow[a]
Development time, short versus long	[22]	1. Fecundity	No
		2. Number of matings	No
		3. Pre-mating period	No
		4. Male longevity	No
		5. Female longevity	No
		6. Head width	Fast < slow
		7. Wing length	Fast < slow
		8. Mating time in the day	Fast < slow

[a] A symbol of ≤ shows that there was only a little difference between both strains.

6. APPLICATION OF CORRELATED RESPONSES TO QUALITY CONTROL

The longevity of the mass reared melon flies has gradually decreased [30]. However, in *Drosophila melanogaster*, the decreased longevity, which has a genetic correlation with the onset of reproduction, can be recovered by selection for late

TABLE V. DIFFERENCE IN SURVIVAL RATE OF THE STRAIN SELECTED FOR LATE REPRODUCTION AND THE NON-SELECTED STRAIN OF MASS PRODUCED MELON FLIES

Generation	Strains (mean ± standard deviation)		Mann–Whitney U test
	Non-selected	Selected for late reproduction	
34	0.30 ± 0.11 (5)[a]	0.43 ± 0.09 (6)	$P < 0.05$
35	0.42 ± 0.04 (5)	0.51 ± 0.06 (6)	$P < 0.05$
36	0.40 ± 0.08 (5)	0.48 ± 0.09 (11)	$P = 0.07$
37	0.32 ± 0.06 (5)	0.49 ± 0.11 (6)	$P < 0.05$
38	0.29 ± 0.01 (5)	0.38 ± 0.07 (6)	$P < 0.05$
39	0.25 ± 0.08 (5)	0.45 ± 0.04 (6)	$P < 0.01$
40	0.27 ± 0.09 (3)	0.40 ± 0.07 (6)	$P < 0.05$

[a] Number of populations assayed for the survival rate.

fecundity (Refs [31, 32], but see Ref. [33]). To introduce such a selection technique to our mass rearing of the melon fly, we divided the mass reared flies into two strains after 34 generations. In the first strain, flies were derived from eggs collected from the second to the sixth week after adult emergence (non-selected strain). In the second strain, flies were derived from eggs collected from older adults only five to six weeks after emergence (selected for late reproduction strain) [30]. The survival rate at the tenth week in the selected strain was significantly longer than the non-selected one, except at the 36th generation (Table V). Thus, we can easily control one of the life history traits by changing the timing of egg collection.

7. ACTIVE QUALITY CONTROL

What is the important trait of mass reared insects for successful SIT? It is the ability of the mass reared males to mate with wild female flies in the field. In order to accomplish this, we must select for males with increased flight ability, providing the capability of dispersing and attracting wild female flies. If wild females will mate more frequently with our mass reared males, rather than with wild males, the quality control of the mass reared flies based on artificial selection will become 'active'. The actual selection process is, however, complicated.

We should search for the genetic correlation between the traits controlling mating behaviour and the traits that can be easily selectable in mass rearing. An example of these traits is life history. The correlations between longevity and flight ability found in *Drosophila melanogaster* [34] may be a valuable candidate for the SIT process.

We were able to control change in a trait by using the response of a correlated trait. As of now, there is only one successful case in our mass rearing of the melon fly. To use the direct and correlated responses to artificial selection in mass rearing for active quality control, we must accumulate information on many genetic parameters, i.e. selection intensity, additive variance, heritability, genetic correlation, genotype environment correlation, etc. There are two main tools to obtain these parameters: parent/offspring regression analysis and family analysis [21]. We can predict the change caused by selection in any trait using these parameters.

We hope this report will be the first step in active quality control based on quantitative genetic aspects and that this technique can be easily incorporated into future SIT projects.

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SEARCH FOR SEX SPECIFIC GENES IN THE MEDFLY, *Ceratitis capitata* *Preliminary data on* Sxl

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Abstract

SEARCH FOR SEX SPECIFIC GENES IN THE MEDFLY, *Ceratitis capitata:* PRELIMINARY DATA ON *Sxl.*

The genes involved in sex determination seem to be among the most valuable for developing efficient biological control strategies based on the sterile insect technique (SIT) in *Ceratitis capitata* and in other insects of economic importance. In addition, these genes are of primary interest in studies of the basic biology and genetics of systems such as *C. capitata* that are relatively little characterized. To examine both of these topics, *Drosophila* was chosen as the model system and the strategy was to utilize probes derived from *Drosophila melanogaster* genes to isolate by cross-hybridization the homologous *Ceratitis* genes. The first intent was to develop rapidly a good basis of the molecular genetics of the sex determining genes, as a preliminary step in achieving the proposed goals. To start, sex lethal (*Sxl*) was chosen, the key control gene involved in sex differentiation and dosage compensation of the X linked genes. In a second set of experiments, a female specific gene, recently identified in *Drosophila,* was used to probe *C. capitata.* Preliminary data are reported here on the identification of a *C. capitata* gene homologous to the *Drosophila Sxl.* Using as a probe a DNA subfragment derived from the *Drosophila* female *Sxl* cDNA clone, an adult *C. capitata* female cDNA library was screened and a clone isolated that, on the basis of nucleotide and amino acid sequence analysis, was found to have originated from a *C. capitata* gene homologous to the *Drosophila Sxl.* Some observations are also presented on the molecular characterization in *C. capitata* of the function corresponding to the *Drosophila* female specific gene.

1. INTRODUCTION

The Mediterranean fruit fly (medfly), *Ceratitis capitata,* is an extremely important pest of fruit and vegetable crops. Control of this pest has been historically based on the use of insecticidal sprays and, more recently, on the sterile insect technique (SIT).

To overcome some limitations connected with SIT application, namely to obtain better biological efficiency and reduce costs, it is necessary to release only sterilized males. To attain these results, the development of suitable genetic sexing strains is required, and there are two potential approaches to achieve elimination at the early developmental stages of females from a mass reared medfly culture: the first involves classical genetics [1] and the second involves the application of recombinant DNA technology [2].

Our group is participating in an international project focused on the development of a germ line transformation system for the medfly and is presently engaged in the search for sex specific genes in *C. capitata* [3].

The isolation of such genes is, in our project, a preliminary but essential step to building up hybrid DNA constructs that, once the transformation is operating in this organism, could allow female specific lethality [2]. On the other hand, these genes are also intrinsically of primary interest for studies of the basic biology and genetics of systems which have been characterized relatively little, such as *C. capitata*. The impressive recent advances in the molecular genetics of another fruit fly, *Drosophila melanogaster,* where the genetic and molecular bases of sex determination have been largely elucidated, make this insect a perfect model system from which to transfer the know-how to insects of economic importance such as *C. capitata*, which diverged from *D. melanogaster* about 125 million years ago. For all these reasons, and to isolate *C. capitata* sex specific genes, we chose the strategy of cross-hybridization with probes derived from *Drosophila* genes. The first gene used was sex lethal (*Sxl*), the key control gene in sex differentiation (reviewed in Ref. [4]) and dosage compensation in *D. melanogaster* (reviewed in Ref. [5]).

The process of sex determination in *Drosophila* involves a cascade of regulatory genes that connects the primary sex determining signal (the X:A ratio) to the terminal differentiation genes whose products are responsible for the sexual dimorphic characteristics of the adult. The special feature of this regulatory cascade is that most of the regulatory interactions are based on sex specific splicing of the primary transcripts common to both sexes. The most recent molecular findings are concerned with four genes in this regulatory pathway: sex lethal (*Sxl*), transformer (*tra*), transformer-2 (*tra-2*) and doublesex (*dsx*). Sxl is the gene that responds directly to the primary sex determining signal and is active only in females. It has been shown that the *Sxl* transcripts are different in the two sexes. Without entering into details, the male specific transcript carries an exon that is absent in the female specific one, and this extra exon contains an in-frame stop codon which leads to the production of a truncated, 'function-less' polypeptide. Moreover, the *Sxl* female protein has RNA binding properties. This protein has recently been shown to bind specifically to its own transcript, thus autocatalysing the female specific pattern of splicing and maintaining the gene in the active female mode [6, 7]. In addition to its own regulation, *Sxl* controls the activity of the somatic sex determining genes entirely through its control on the *tra* gene. As with *Sxl,* the *tra* gene is active only

in females and its activity is similarly regulated at the level of RNA splicing. Analogous mechanisms based on sex specific alternative splicing ensure the production and function of the other two relevant genes in the sex cascade, *tra-2* [8] and *dsx* [9].

The genetic programme specifying the sexual fate is then achieved through the activation, mediated by the *dsx* gene products, of different sets of structural genes in males and females.

In this paper, we mainly report the isolation, by cross-hybridization with *Drosophila* probes, of a *C. capitata* gene homologous to *Sxl*.

2. MATERIALS AND METHODS

All methodologies and buffers not specifically described were carried out according to Sambrook et al. [10].

2.1. Labelling of DNA probes

For Northern and Southern analyses and for screening of libraries, the DNA probes consisted of DNA fragments isolated from 5% acrylamide gel and labelled to a specific activity of 5×10^8 (counts/min)/μg with a Multiprime labelling kit (Amersham).

2.2. cDNA library

The total *C. capitata* RNA was extracted from female adults according to the guanidinium/CsCl method. The poly $(A)^+$ RNA was purified with the Stratagene poly $(A)^+$ Quick^{TM} mRNA purification kit.

A cDNA library from *C. capitata* female adults poly $(A)^+$ RNA was prepared in Uni-Zap^{TM} XR using the Stratagene Zap-cDNA synthesis kit. The titre of the non-amplified library was 2.8×10^6 plaque forming units (PFU)/mL. The titre of the amplified library was 1×10^{10} PFU/mL.

2.3. Screening of cDNA libraries and Southern analyses

In screening an embryonic genomic library (kindly provided by the colleagues of the Insect Group of the University of Crete) of the cDNA library and in Southern analyses, we utilized low stringency conditions: 55°C in 5X SSPE, 5X Denhardt's solution, 0.5% SDS and salmon sperm DNA, with washes at 55°C in 2X SSC, 0.1% SDS.

2.4. Northern analyses

The RNA was fractionated on 1.2% agarose formaldehyde gel, transferred to a nylon membrane (Hybond N, Amersham), hybridized and washed following the protocols suggested by Amersham. Low stringency hybridizations were conducted at 37°C in 50% formamide, 5X SSPE, 5X Denhardt's solution, 0.5% SDS and salmon sperm DNA, with washes at 42°C in 2X SSC, 0.1% SDS.

2.5. DNA sequencing and analysis

The nucleotide sequences were determined by the dideoxy method. Sequencing data were analysed using the FASTA analysis program [11].

3. RESULTS

Two *C. capitata* libraries, an embryonic genomic library and a cDNA library from female adults, constructed in the phage vectors EMBL4 and Uni-Zap^{TM} XR, respectively, were screened at low stringency for homologues of the *Sxl* gene of *D. melanogaster* using as a probe a subfragment derived from the *Drosophila* female cDNA (fragment A in Fig. 1, panel (b)) [6]. Several recombinant clones were isolated from the genomic library, but were all 'false positive' to the final control of the sequence analysis, and all of them were shown to be relatively within reach of TA or CA stretches. Similar results were obtained in another screening carried out utilizing the entire cDNA as a probe [12]. Actually, the choice to utilize the subfragment was made on the basis of the observation that the *Sxl* cDNA itself contains some $(TA)_n$ or $(CA)_n$ repeats and because of the notion that working at low stringency could be responsible for the false positive signals. The subfragment, however, is relatively free of such sequences. In the cDNA screening, approximately 1×10^6 phage plaques were plated and four positive independent clones were isolated. The four clones were restricted with the enzymes EcoRI and XhoI to extract the inserts and subjected to Southern hybridization with the same probe. Three clones contain an insert of 1.2 kb and the fourth of only 0.6 kb. At the moment, we have characterized one of these clones, *Sxl*Cf1 at 1.2 kb. A restriction map was constructed and sequence analysis was conducted; the nucleotide and amino acid sequences were compared with the corresponding sequences of the male and female cDNA *Drosophila* clones utilizing the FASTA program for scoring similarities and constructing alignments (data not shown) [11].

In Fig. 1, we show a schematic representation of the *Ceratitis* clone and the female and male *Sxl*/*Drosophila* cDNAs.

The major difference between the two *Drosophila* cDNA clones is the presence of an extra 190 bp exon (exon 3) in the male cDNA. The structure and the nucleotide

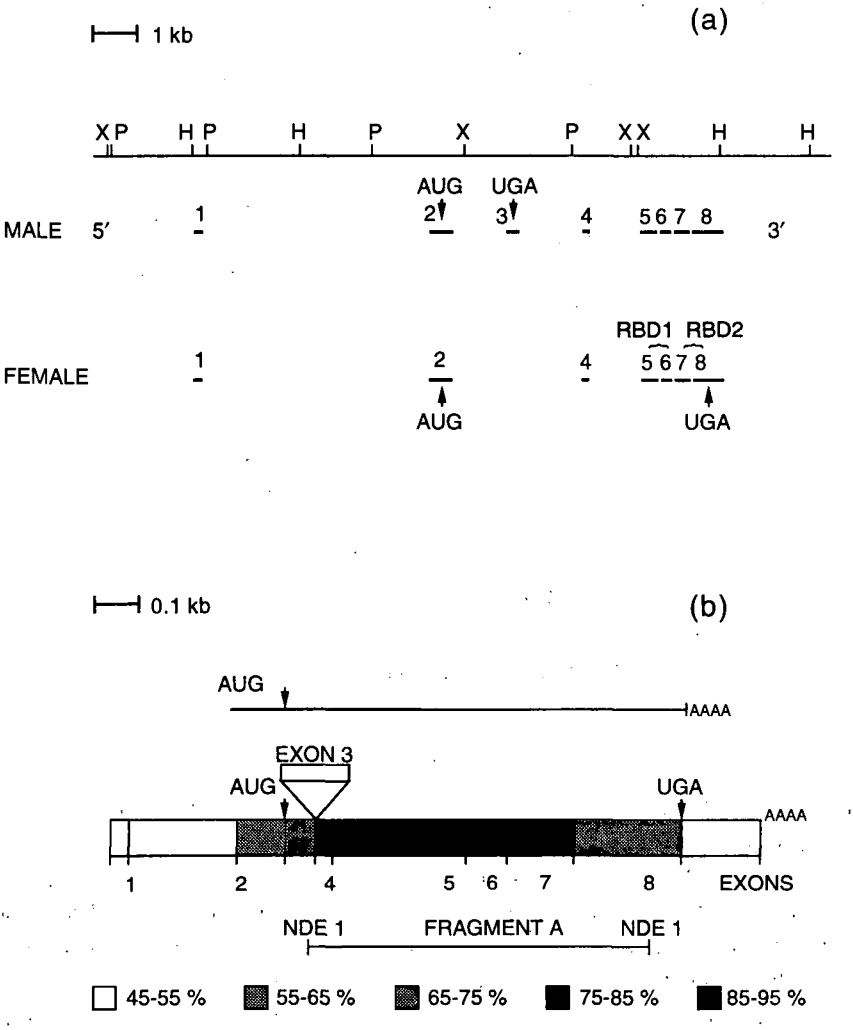

FIG. 1. (a) Genomic map of Sxl *in* D. melanogaster, *with a schematic diagram illustrating the approximate positions of the exons present in adult male and female specific transcripts [6]. The open reading frames are indicated by arrows. RBD1 and RBD2 indicate the two RNA binding domains present in the* Sxl Drosophila *protein; they extend from exon 5 to exon 8; the brackets limit the first and the second domain, respectively. The restriction sites are: X, Xho, I; H, HindIII; P, PstI. (b) A comparison between the* D. melanogaster Sxl *cDNA (middle) and the homologous cDNA in* C. capitata *(top). The boxes on the* Drosophila *cDNA illustrate the different percentages of homology. Fragment A (bottom) was used to screen the adult female cDNA library.*

TABLE I. BASE UTILIZATION IN RNA BINDING DOMAIN 2

Drosophila	*Ceratitis*
207 bp	
48 A	72 A
58 C	47 C
59 G	44 G
42 T	44 T
Base at position III[a]	
8.5% A	36.1% A
33.1% C	24.3% C
36% G	15.7% G
21.4% T	23% T
G + C contents[a]	
C + G 69.1%	C + G 40%

[a] The percentages are approximated to the first decimal value.

and amino acid sequences of the *Ceratitis* cDNA clone appear to be highly conserved with respect to the *Drosophila* female cDNA.

The overall nucleotide sequence homology is about 58%, while the amino acid homology is about 62%; these are, in absolute, very significant values. The sequence corresponding to the male exon 3 is not present in the *capitata* cDNA that, as in the *Drosophila* female, contains a single long open reading frame that starts at an AUG codon in a region corresponding to the *Drosophila* exon 2. Sequences homologous to exons 4, 5, 6, 7 and 8 are present in the same order and are more or less the same length. The codon usage in this open reading frame agrees with the *C. capitata* codon bias [13].

Table I shows the base composition in one of the two RNA binding domains, RBD2, present in the *D. melanogaster* female *Sxl* cDNA and in the corresponding region of the *C. capitata* clone (Fig. 1, panel (a)).

In the latter clone, the coding region is preceded by a 5′ untranslated leader of 108 bp, instead of the 482 bp present in both the female and male *Drosophila* clones. In the *Drosophila* cDNA clone, the termination codon UGA is in exon 8 and is followed by 217 bp, ending with 3 A [6]. In our clone, after the stop codon there are only 6 bp followed by 25 A.

4. DISCUSSION AND CONCLUSIONS

The evidence presented here indicates that we have isolated a cDNA derived from the *C. capitata* gene homologous to the *Drosophila Sxl* gene. This clone was selected from an adult female cDNA library and appears to be related to the *Drosophila* female specific transcript, since any sequence corresponding to the *Drosophila* male specific exon 3 is absent.

As reported in Section 3, the overall nucleotide and amino acid sequence homologies are significantly high (58 and 62%, respectively), orders of magnitude that prove an identity of function in the two organisms. Or, more precisely, indicate a very conserved function in the two species, even though they have been separated, as we said, by more or less 125 million years. Indeed, a recent analysis of the evolutionary divergence of the *transformer* gene in *Drosophila* gives lower values of nucleotide and amino acid homologies between the sequences in *D. melanogaster* and *D. hydei,* even though they diverged only 60 million years ago [14].

We do not yet have any idea of the genomic organization of the *Sxl* gene in *Ceratitis,* but the fact that the dimensions of the inserts of three out of four clones we have isolated are 1.2 kb seems to us to indicate, with a good probability, that these are the dimensions of the transcript in *C. capitata.*

If this hypothesis is true, then the main differences between the female *Drosophila* and *Ceratitis* transcripts are in the 5′ and 3′ untranslated regions.

As regards the 5′ end, and again on the basis of the insert dimension, we think that our clone is complete and that in *Capitata* there is a shorter untranslated leader. With respect to the 3′ end, the clone ends with a relatively long poly A tail and there is a clear polyadenylation consensus signal.

A very important point concerns the topological analysis of the homology patterns. The reported values for the nucleotide and amino acid sequences are average values but, as shown in Fig. 1, panel (b), there is basically a symmetric distribution in the value of nucleotide contents.

As regards the nucleotide sequences, from the 5′ to 3′ ends of the clone the values range from 45–55%, to a maximum of 85–95%, more or less in the middle of the clone, and end at a range between 55 and 65%. The same pattern, with higher values, is obtained for the amino acid sequence values, i.e. in the region of the two RNA binding domains there are long stretches with more than 95% homology.

It is interesting to note that the codon bias in the two genomes is very different. As given in Table I, even in a region so highly conserved in terms of amino acid sequences as RBD2, the usage of bases in position III strongly differs, while the G + C content is 69.1% in *D. melanogaster* and only 40% in *C. capitata* [13].

The cDNA sequences and a more detailed analysis, together with a comparison of the genomic organization of the *Sxl* in the two insects, will be published elsewhere (manuscript in preparation), but we would like to stress here the very high degree of homology between the *Drosophila* and *Ceratitis Sxl* gene and its topological distribution.

In the products of the *Drosophila Sxl* gene, two RNA binding domains, RBD1 and RBD2, are present. These domains are crucial in the process of sex specific splicing of *Sxl* itself [6] and *tra* [8]. In conclusion, the fact that this part of the gene and the gene product are so highly homologous in *Drosophila* and *Ceratitis* is confirmation of the functional relevance in evolutionary terms. In addition, all these observations strongly suggest that, even if the chromosomal basis of sex determination differs between these two insects [3, 15], the molecular regulatory pathways should be extensively conserved.

The cloning of the *C. capitata Sxl* gene will therefore provide the immediate possibility of studying the molecular bases of sex determination in this organism and checking if here also the sex regulatory cascade is based on the differential splicing of primary transcripts common to both sexes.

Once transformation in *C. capitata* is achieved, the introduction of the homologous *Sxl* minigene in a construct that allows its inducible expression as antisense RNA should permit selective elimination of females from the mass reared culture at an early stage of development [2].

The antisense RNA will in fact interact with the *Sxl* transcripts both in female and male insects, inducing loss of function, [2], but because only the females need it to maintain the sex pathway of the *Sxl* gene, they will be the only ones affected [4, 6, 7].

At the same time, for the reasons presented in Section 1, we are also interested in genes involved in a more terminal sex differentiation event, i.e. oocyte development. Nucleotide and amino acid sequences of genes involved in the ovarian specific functions are also more likely to be conserved, and, in fact, *D. melanogaster* and *C. capitata* chorion [16] and vitellogenin [13] gene sequences show good homology.

Recently, our group reported in *D. melanogaster* the identification and the initial molecular characterization of a function expressed at high levels during embryogenesis and oogenesis [17], as visualized by in situ hybridization to whole mount preparation of ovaries and embryos [18]. In Northern blots of these stages a single transcript of 2.6 kb was revealed. From the patterns of expression we concluded that this gene is involved in establishing the egg morphology and in germ line formation. Again, using a subfragment derived from a *D. melanogaster* cDNA as probe, we identified and partially characterized, at the molecular level, a corresponding function in *Ceratitis*.

On a Southern blot of genomic *Ceratitis* DNA, restricted with EcoRI and HindIII, two bands of 3.5 and 2.7 kb are visible in the EcoRI track and four (3.4, 2.9, 2.1 and 1.8 kb) in the HindIII track.

At the RNA level, the *Drosophila* probe identifies at least two transcripts of about 1.3 and 2.2 kb, respectively, present at different stages of *Ceratitis* development.

As a direct approach towards the isolation of *C. capitata* sex specific genes, we have prepared *Ceratitis* cDNA libraries from adult males and females, and

ovaries and testes, and we will proceed to identify, by subtractive analysis, clones expressed in a sex specific manner.

From the data we have shown, we think it is possible to conclude that the cross-hybridization approach, *Drosophila* versus *Ceratitis*, to isolate genes of the latter organism is still in certain contexts (i.e. highly conserved genes utilizing cDNA libraries) a valid, if not general, strategy.

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TRANSFECTIONS OF DNA INTO CULTURED INSECT CELLS AND EMBRYOS
*Progress and prospects**

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Abstract

TRANSFECTIONS OF DNA INTO CULTURED INSECT CELLS AND EMBRYOS: PROGRESS AND PROSPECTS.

The ability to manipulate insect genomes to develop or enhance methods of control for pest and beneficial species has been a goal of geneticists working in the areas of medical and agricultural entomology. In vitro gene manipulation techniques combined with methods that enable integration of exogenous DNA into a host genome have become routine procedures for many organisms. Except for the drosophilid species, these advancements have not been realized for other insects. A lack of functional gene vectors and the means to introduce foreign DNA into non-drosophilid species have limited most studies to the analysis of transient expression of manipulated genes in cells and embryos of relatively few insects. The paper reviews the current technology used to deliver DNA to cultured cells and embryos of insects, examines the mechanisms effecting transfection and compares the relative transfection frequency of the various methods. A discussion of possible modifications and combinations of transfection methodology that could aid in the integration of DNA into a host embryo's genome is presented.

1. INTRODUCTION

The introduction of exogenous DNA into cultured cells of insects has been accomplished by a variety of methods [1], while introduction into embryos has been done mostly by microinjection [2]. The search for alternative DNA delivery methods has become a goal of many researchers, since the transfection frequency elicited by a particular technique may vary widely among cell lines [1, 3] and the microinjection of embryos tends to be labour intensive and inefficient [2]. While the prospects of

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producing transgenic insects of economically important species for use in genetic control programmes are encouraging, the lack of efficient DNA vector systems such as the *P* element transposon of *Drosophila* [4, 5] prevents their development.

Insect cell culture provides an easy and economical system for testing the effectiveness of transfection methods and evaluating the activity of various promoters combined with cloned genetic material of interest. Since cultured cells are not exposed to potential regulators such as hormones, electrolytes and the positional effects when cells are organized into tissues and organs, gene expression can be more easily analysed. Further, cell cultures provide a sufficient number of cells when vigorous selection schemes are used in conjunction with transformation systems where integration occurs only rarely (e.g. gene replacement).

After gaining success in cultured cells, the DNA constructs can then be tested in embryos using DNA delivery systems developed for whole organisms. While microinjection of individual insect embryos is the commonly used procedure, development of methods allowing concurrent delivery of a DNA construct into large numbers of embryos is needed. Such methods would permit the use of gene transfer systems that are typically employed only in cell cultures.

Thus, the intent of this paper is to examine the current methods being used for the transfection of insect cells and, using this information as background, to suggest approaches that could be used to increase the frequency of transfection in insect embryos and to decrease the amount of labour or handling required to achieve transfection.

2. CHEMICALLY MEDIATED TRANSFECTION OF INSECT CELL LINES

The most common technique used to transfer foreign DNA to cultured insect cells from the culture medium has been the calcium phosphate co-precipitation method. Calcium phosphate is thought to aid uptake by the cells through precipitating the DNA on the cell membrane, inducing endocytosis and protecting the internalized DNA from degradation by endogenous nucleases [6]. Calcium phosphate co-precipitation has largely been used to transfer purified DNA to a variety of *Drosophila* cell lines [7–12], but it has also been used successfully in cultured mosquito and lepidopteran cells [3, 13]. Further, the temperature at which the DNA and calcium phosphate are co-precipitated can also be an important factor in gaining higher transfection frequencies. Preparation of the DNA–calcium phosphate co-precipitate at 50°C instead of at ambient temperature gave a tenfold higher transfection frequency in mosquito cells [3].

Polycations such as DEAE–dextran, polybrene, polyornithine and polylysine have been used to transfect dipteran, coleopteran and lepidopteran cell lines [1, 14–18]. The negatively charged DNA molecule forms a complex with the posi-

TABLE I. COMPARISON OF THE TRANSFECTION EFFICIENCY OF THREE CHEMICAL MEDIATORS WITH ELECTROPORATION IN THE BOLL WEEVIL CELL LINE[a] BRL-AG-3C, WHEN USING THE *hsp70–CAT* VECTOR

Transfection mediator	Mediator concentration (μg/mL)	DNA concentration (μg/mL)	% conversion of chloramphenicol[b]
Polybrene	12.5	16	< 1
Polylysine	12.5	8	11.0
Lipofectin	12.5	4	75.1
Electroporation[c]	—	30	2.6

[a] Cell concentration = 1×10^7 cells/mL.

[b] Measured as per cent conversion of available chloramphenicol to the acetylated products.

[c] Cells given three pulses at 0.6 ms each, 175 V, field strength 0.9 kV/cm (data from Stiles et al. [17]).

tively charged polycation molecule and together they bind irreversibly to the cell membrane and are apparently internalized by phagocytosis. Occasionally, DMSO is used to facilitate entry of the polycation–DNA complex into the cells, but it was found to be of no advantage when used in transfecting *Aedes albopictus* or *Anthonomus grandis* cells [10, 17].

Felgner et al. [19] developed a transfection procedure using the synthetic cationic lipid, N-[1-(2,3-dioleyoxy)propyl]-N,N,N-trimethylammonium chloride. Transfection is accomplished through a fusion of cells with liposomes containing the DNA. To this author's knowledge, this chemical mediator, marketed under the label Lipofectin™, has not been used extensively in the transfection of insect cells. Stiles et al. [17] recently compared the transfection efficiency of the cationic liposome method with that of polylysine, polybrene and electroporation and found it to be far superior in gaining transient expression of the chloramphenicol acetyltransferase *(CAT)* gene in a boll weevil cell line (Table I).

3. NON-CHEMICAL METHODS OF CELL CULTURE TRANSFECTION

The formation of somatic cell hybrids by controlled fusion of cells derived from two different parental lines can serve as a method of introducing exogenous DNA into a cell culture. Somatic cell fusion has been performed by treating with polyethylene glycol, inactivated Sendai virus, lectins and electrical stimuli [20, 21].

While cell fusion has been accomplished in *Drosophila* and mosquito cell lines [21–23], the lack of selective media needed for transformant recovery by complementation of a mutation in each parental line and problems deriving cell lines from mutant insect strains have limited the use of this method.

Another method that uses electric current to effect transfection of cell cultures is electroporation. This is a rapid procedure in which a suspension of cells and DNA is placed between two electrodes and given a high voltage pulse. Transient holes or pores in the cell membrane are produced through which the DNA is able to enter the cell. After a short period of time, the holes reseal and the cells resume normal functions. Mann and King [24] used electroporation to aid entry of a baculovirus vector into a *Spodoptera frugiperda* cell line and Stiles et al. [17] obtained a low transfection frequency in *A. grandis* cells when using the heat shock protein 70 (*hsp70*)–*CAT* plasmid. One of the advantages of this method is that internalization of the exogenous DNA is not dependent on the phagocytic capacity of cells. Other advantages include the ability to obtain integration of low gene copy numbers, as opposed to the large tandem arrays seen in the chemical methods [25], and to use either plasmid or genomic DNA [26].

One of the most effective methods of transferring genes to lepidopteran cell lines is by using insect viruses. The most commonly used of the insect viruses is the nuclear polyhedrosis baculovirus, *Autographa californica* (AcNPV). For a detailed description of how baculoviruses infect cells, enter nuclei and replicate, see the review of Faulkner and Carstens [27]. Baculovirus expression vectors (BEVs) are recombinant insect viruses that have foreign genes inserted into the viral genome under the regulation of a strong promoter for the viral gene that produces the polyhedrin protein. While BEVs have been used to transfect lepidopteran cell lines with a variety of foreign genes [28–30], they function poorly in most other insect cell lines. However, some success was gained in the expression of the CAT gene linked with a retroviral promoter that was transferred by a BEV to *Drosophila* and *Ae. aegypti* cells [31].

For the most part, transfection by a BEV results in transient gene expression, since the host cells are eventually killed by the viral infection. Jarvis et al. [32] were able to overcome this obstacle by placing foreign DNA under an early viral promoter (IE1) and eliminating that part of the viral genome which produces the cytopathic effects occurring late in the infective cycle. With this method, they were able to obtain stable transformation of a *S. frugiperda* cell line. Some of the advantages of using BEVs are that very high levels of gene expression can be obtained, large amounts of DNA can be inserted into the host genome and the virus is non-infectious for vertebrates [33, 34].

Except for the recent reports by Kjer and Fallon [3], Stiles et al. [17] and Hartig et al. [35], little attention has been given to comparing the effectiveness of the various DNA delivery systems that are available for transfection of the insect cell lines. Cell lines can differ widely in their susceptibility to the often toxic effects of

a chemical or physical facilitator for transfection. It is often essential to balance the toxic effects of a method with the optimal transfection frequency. Some methods employing chemical mediators or electroporation typically produce >50% cell death at the chemical concentration or field strength where the transfection frequency is the highest. Other factors contained in a particular transfection protocol which affect the fate of the transfected DNA include the type of plasmid vector used, linearized versus circular DNA, the cell line, the selection regime, the DNA concentration and the suitability of the regulatory sequence (see the review of Walker [1]). Thus, except for the dipteran and coleopteran cell lines previously mentioned, there have been few studies where effective transfection protocols have been optimized for the cell lines derived from other medically and agriculturally important insects.

4. TRANSFECTION OF INSECT EMBRYOS BY MICROINJECTION

The transfer of DNA to *Drosophila* embryos by microinjection has become a routine procedure where the preliminary steps involve the chorion removal of recently oviposited eggs in a dilute commercial bleaching agent or by mechanical means, desiccation of about 1–5% of the egg volume and covering with a halocarbon oil [36]. The injection site is usually at the posterior end of the egg where the pole cells are located and injection is done before cellularization of the blastoderm. Injected embryos are then returned to a humid environment until hatching. This technique has been modified variously to accommodate the insect embryos of other species. A growing number of insect embryos, including two tephritid species [37, 38], the silkworm moth [39–41], the migratory locust [42], the sheep blowfly [43], mosquitos [44, 45] and the house fly [46], have all been transfected successfully with various DNA constructs using microinjection procedures. Apparently, removal of the egg chorion was not possible in several species, but some embryos survived injection through the intact chorion regardless.

It has been estimated that, under the best of conditions, about 80% of the injected *Drosophila* embryos hatch, 40–50% of these survive to adulthood and then 10–90% of the surviving adults are sterile because of their genetic background or have injection induced abnormalities [47]. Further, the survival to adulthood from injected embryos of three mosquito species ranged from 6 to 15% [44, 48, 49] and for the silkworm moth embryos it was 3.2% [40].

It should be mentioned that the physical manipulations required to facilitate transfection by microinjection may influence expression of a heterologous gene when it is linked to the *Drosophila hsp70* promoter. Eberlein and Mitchell [50] suggested that the stress of dechorionation, desiccation, injection and incubation under halocarbon oil was responsible for inducing expression of *hsp70–CAT* activity in *Drosophila* embryos, which was above that expected to be expressed constitutively in the absence of a heat shock. A microinjection procedure involving injection of an

egg within the gravid female mite, *Metaseiulus occidentalis,* has provided a means of gaining stable transformation for this species [51]. A transformation frequency of 0.5% was obtained without benefit of a transposable element system and survival of the injected females was described as an order of magnitude higher than that gained with injection of mosquito embryos. It is not known whether the stable transformation achieved by this method is related to the reproductive biology of this phytoseiid mite, or to the introduction of exogenous DNA prior to fertilization of the egg. Regardless, the potential of this method for transforming other arthropods certainly deserves further study. It may be the only method whereby insects having a unique reproductive cycle like that of the tsetse fly can be transformed.

5. OTHER TYPES OF DNA DELIVERY METHODS

The search for methods that can deliver DNA simultaneously to large numbers of embryos rather than treating them individually by microinjection has been encouraged by recent studies. Baldarelli and Lengyel [52] used a bombardment technique whereby particles 1.2 μm in diameter were coated with a DNA construct. The particles were blasted shotgun like into samples containing 10 000–20 000 dechorionated *Drosophila* embryos using a commercially available biolistic bioparticle delivery system. The survival to adulthood was not reported in this study nor was the transfection frequency, but it was described as 'high'.

Another method which has the capability of delivering exogenous DNA simultaneously to samples containing hundreds of embryos involves the use of electroporation. Kamdar et al. [53] were able to obtain a 95% frequency of transient expression of the aldehyde oxidase gene transfected into *ma-l* mutant *Drosophila* embryos. These results were obtained *without dechorionation* and about 75% of the treated insects survived to third instar larvae. Dechorionation reduced survival to 10% at the third instar, but frequency of expression was 100%.

6. ENHANCEMENT OF DNA DELIVERY TO GERM CELLS

Since it is essential to integrate DNA into the germ line when stable transformation of an organism is desired, manipulations which have the potential to selectively increase the likelihood of uptake by germ cells would benefit any scheme devised for gene integration. For example, injection of gene constructs conjugated to yolk proteins into the haemocoel of female insects during egg development has been suggested as a means of gaining selective uptake by oocytes [2]. Another strategy could involve transfection of sperm cells, either by direct injection of the testes or by transfecting sperm before artificial insemination (e.g. the honey bee) or before in vitro fertilization [54]. Lipofection of murine sperm was found to be an

efficient method for transfering DNA but, unlike an earlier report [55], no transgenic mice were obtained upon testing by in vitro fertilization [56].

Pole cell transplants between mutant lines of *Drosophila* have been routinely used to produce germ line mosaics for developmental and genetic studies. While it may not be beneficial to transfect these primordial germ cells outside the embryo, combining the exogenous DNA with a chemical mediator such as a cationic liposome or polybrene before injection into the pole cell region of the egg may increase the efficiency of transfection. Extending the life of an introduced DNA construct through several cycles of cell division or in the case of the preblastoderm embryo, cleavage divisions, could also enhance the chances for integration by a vector or by random integration. This, too, could be accomplished by combining the construct with a chemical mediator before transfecting by injection, electroporation or bombardment of the embryos.

7. SUMMARY

There is a definite need for development and refinement of DNA delivery systems for cells, embryos and perhaps gonads to realize the potential that genetic engineering by gene transformation presents for enhancing insect management programmes. Progress in this area is clearly indicated by the encouraging results gained with optimizing cell culture transfection procedures and the recent successes achieved in the transfection of embryos obtained by electroporation and particle bombardment. However, with the development of new gene vectors to accommodate different species of economically important and biologically diverse insects, continued attention to optimization and modification of DNA delivery systems will no doubt be required. Further, the development of new gene vectors, which depends in part on gaining reproducible transfection efficiencies and, consequently, creating reliable and reproducible DNA delivery systems, increases the probability that transgenic insects important to the fields of medicine and agriculture will be obtained.

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USE OF Y LINKED TRANSLOCATIONS IN LOCATING MUTANT LOCI *(Bl, dp)* ON POLYTENE SALIVARY GLAND CHROMOSOMES OF *Anopheles stephensi* LISTON

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Abstract

USE OF Y LINKED TRANSLOCATIONS IN LOCATING MUTANT LOCI (*Bl, dp*) ON POLYTENE SALIVARY GLAND CHROMOSOMES OF *Anopheles stephensi* LISTON.

Using two Y linked translocations, in which the break points were tightly linked to the morphological mutants (*Bl, dp*), the location of mutant loci on polytene salivary gland chromosomes of *Anopheles stephensi* was determined. In searching for discontinuities in the polytene chromosomes of male larvae from the T(Y-3)20 translocation involving a black larva mutant, a single break point was found in region 36D/37 of 3R. Analysing the polytene chromosomes of male larvae from the T(Y-3)12 translocation involving the diamond palpus, the translocation break point was determined at position 34A of 3R. Because the Y/autosome breakpoint in T(Y-3)20 was tightly linked to the black larva mutant (*Bl*), and the break point in T(Y-3)12 was tightly linked to the diamond palpus mutant *dp*, it was concluded that gene *Bl* is located very close to the map reference 36D/37 of 3R and that the gene *dp* is located at position 34A of 3R. The mitotic chromosomes of these Y linked translocations are described.

1. INTRODUCTION

Anopheles stephensi Liston is an important malaria vector in the Indian subcontinent and has been the subject of genetic and cytogenetic studies aimed at the development of genetic control strategies [1–5]. These strategies are mainly involved

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in the release of sterile mosquitoes carrying chromosomal rearrangements into field populations with the aim of population suppression or replacement. In the isolation of chromosomal rearrangements or other genetic studies, it is very necessary to employ mutant markers in order to make the crosses manageable, simple and efficient. Further, Y/autosome translocations and deficiencies have been used to determine the location of some mutant loci in different mosquito species [6, 7]. In *An. stephensi*, a genetic sexing system has been developed that is based on the translocation of dieldrin resistance to the male determining chromosome [5]. Analysing the salivary gland chromosomes of this genetic sexing line, Robinson and Pham van Lap [8] were able to determine that the location of the dieldrin locus in 3L was very close to map reference 43/44. Sakai et al. [1], in studying different translocations, suggested that the diamond palpus (*dp*) and black larva (*Bl*) mutants are associated with the shorter of the mitotic autosomes and with the 3R element in polytene configurations. During the isolation of the genetic sexing system in *An. stephensi*, Robinson et al. [9] isolated two male linked translocations in which the translocation break points were tightly linked to *Bl* and *dp*. These Y linked translocation strains provided us with an ideal opportunity for localizing the above-mentioned genes on the polytene chromosomes of *An. stephensi*. The results of our study represent another step towards a better understanding of the genetics of *An. stephensi* and may be useful in future investigations relating to the development of genetic control strategies.

2. MATERIALS AND METHODS

2.1. Strains

The following Y linked translocation strains isolated by Robinson et al. [9] were used:

— *T(Y-3)20*: A translocation between the male determining chromosome and chromosome 3. The Bl^+ allele was completely linked to the translocation break point. *Bl* is co-dominant and *Bl*/+ has an intermediate colour between +/+ and *Bl/Bl*.

— *T(Y-3)12*: A Y linked translocation in which *dp*, an autosomal recessive on chromosome 3 [10], was tightly linked to the translocation break point.

2.2. Rearing

All the mosquitoes were held at 28°C and 80% relative humidity (RH). The larvae were fed on fish food (Tetramicromin) and the adult blood meals were provided by anaesthetized mice. Since the translocation strains were very stable, they were maintained generation by generation without any selection.

2.3. Cytology

The mitotic and meiotic chromosomes were prepared from the testes of fourth instar larvae, as described by French et al. [11], to confirm the presence of the translocations. The salivary glands of early fourth instar larvae were dissected in a droplet of 5% diluted Carnoy's fixative and then transferred to a small drop of concentrated Carnoy (3 ethylalcohol:1 acetic acid) on a siliconized coverslip. This tissue was stained with 2% lacto-aceto-orcein. The squashed preparations were sealed using photo glue and stored at 2°C. The chromosomes were investigated under a Carl Zeiss microscope. Before dissection of the salivary glands, the late third instar larvae or early fourth instar larvae were kept overnight in an insectary set at 20°C. According to Seawright et al. [7], low temperature treatment enhances the preparation of readable polytene chromosomes. The salivary gland chromosome map of Sharma et al. [12] was used to identify the break points.

3. RESULTS AND DISCUSSION

3.1. Standard mitotic and salivary gland chromosome complements

The Lahor wild type line (+/+) described by Robinson et al. [9] was used for making the preparations of the standard mitotic and polytene salivary gland chromosomes. The mitotic chromosome complement of *An. stephensi* consists of one pair of short subtelocentric sex chromosomes and two pairs of longer autosomes, one metacentric and the other submetacentric. The longer and shorter autosomes are designated as chromosomes 2 and 3, respectively. The sex chromosomes are morphologically distinguishable in males. The shorter sex chromosome has been designated as the Y and the longer one the X chromosome. The short arms of the Y and X chromosomes are equal in length, but the long arm of X is longer than that of Y.

Analysis of the polytene salivary gland chromosomes of the wild type line showed a complement composed of five arms, the shortest being X, which was often separated from the chromocentre, and four autosomal arms. The Y chromosome is heterochromatic and does not polytenize [1]. The longest arm in the whole complement is designated as 2R, which is characterized by a big puff in zone 7. The shortest autosomal arm is designated 2L, which can easily be recognized by its length and by other characteristics. The centromere part of 2L (region 20) always formed a loop, as described by Mittal and Dev [13]. The 3L and 3R arms are nearly equal in length. They can be recognized by several characteristic landmarks. The free end of 3R is flared, with two dark bands and some dot line bands in region 29A. The free end of 3L is characterized by several light bands. In general, the banding pattern in the polytene salivary gland chromosomes of our *An. stephensi* specimen was

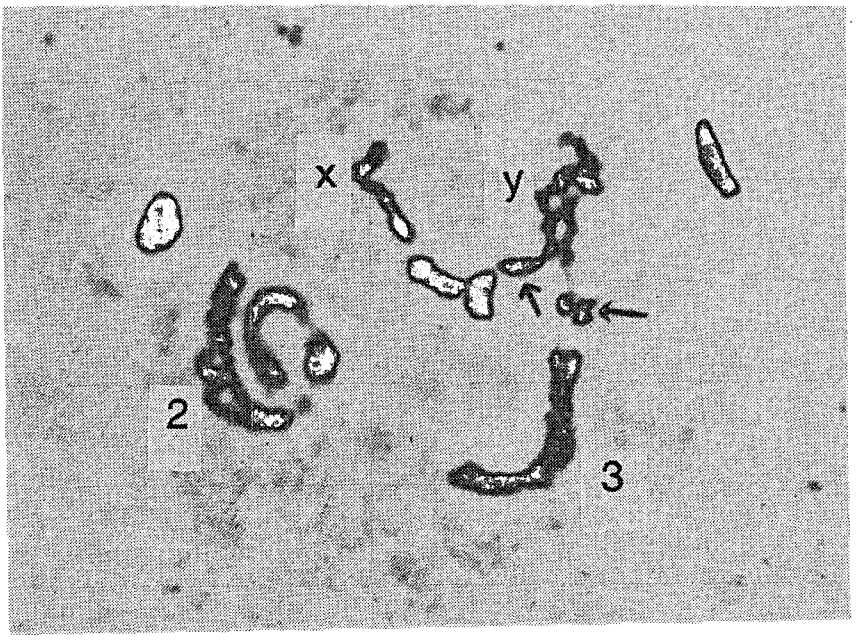

FIG. 1. Mitotic chromosome complement of the T(Y-3)20 translocation heterozygote. The arrows denote the breaks.

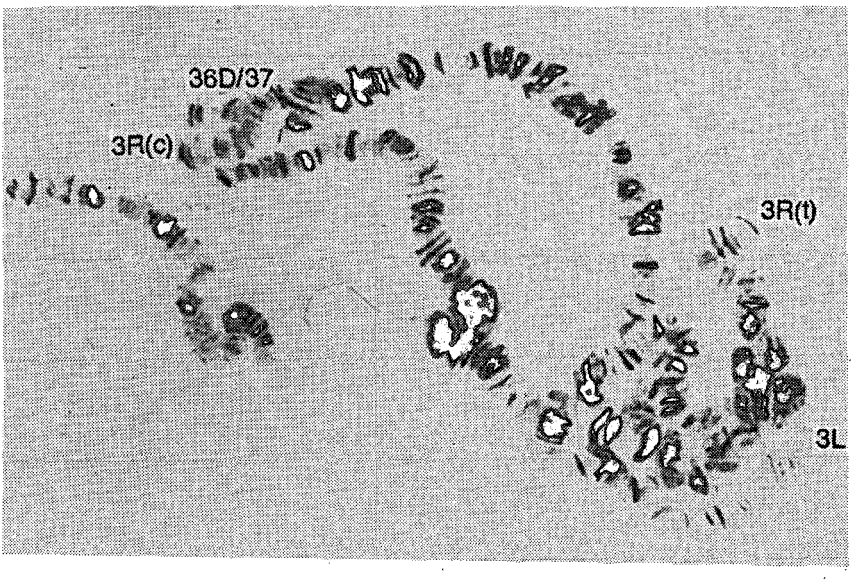

FIG. 2. Discontinuity in region 36D/37 of 3R.

similar to that described in the map of Sharma et al. [12] and to that shown in the photomap of Mittal and Dev [13]. Therefore, the polytene salivary gland chromosome map of Sharma et al. [12] was used to designate the chromosome breaks in our translocation strains. By analysing the mitotic and polytene chromosomes in different translocations of *An. stephensi*, in which visible alterations occurred, Sakai et al. [1] showed that the 2R–2L arms in the polytene chromosome complement are associated with the longer of the mitotic autosomes and that the 3R–3L polytene elements are associated with the shorter of the mitotic autosomes.

3.2. Cytological characterization of T(Y-3)20

Analysis of the mitotic chromosomes of the male larvae of translocation T(Y-3)20 revealed an unusual karyotype in all cases. There was unequal exchange between 3 and Y, with chromosome 3 losing a very long segment and receiving a short one from the long arm of Y. Therefore, the translocated chromosome 3 was nearly one arm shorter than its normal homologue and the Y chromosome became longer than normal. In Fig. 1, the synapsis was clearly observed between the long arm of Y and the normal 3. Therefore, the break points can be seen in the middle of the Y chromosome and in the region very near to the centromere of chromosome 3 in the mitotic chromosomes.

Analysis of the polytene salivary gland chromosomes of the male larvae of the translocation strain showed a complement composed of five arms as normal. But by searching for discontinuities in the autosomal arms, the position of the Y/autosome break was determined. In Fig. 2, the 3R and 3L arms can be seen, the break point being localized in region 36D/37 of 3R. The Y chromosome is heterochromatic and was not observed in the polytene chromosome complement, but Fig. 2 shows that it has a strong affinity to the chromocentre. As the black larva locus must be tightly linked to the translocation breakpoint [9], it was concluded that the gene coding for larva colour (*Bl*) is located in 3R, very close to map reference 36D/37. Sakai et al. [1], using different translocations, showed that the gene *Bl* is located in 3R. Robinson et al. [9] suggested that the *Bl* locus is located near to the centromere. Our results now confirm that it is located on the polytene chromosome.

3.3. Cytological characterization of T(Y-3)12

Analysis of the mitotic chromosomes of the male larvae of this strain showed an abnormal karyotype (Fig. 3). Mitotic configurations showed an unequal exchange between chromosome 3 and the long arm of the Y chromosome. Chromosome 3 lost a long segment and received a short one from the Y chromosome, so becoming about half an arm shorter than its normal homologue. Analysing the polytene salivary gland chromosomes of male larvae showed that a single break point was found in region 34A of 3R (Fig. 4). As the *dp* locus must be tightly linked to the break point [9],

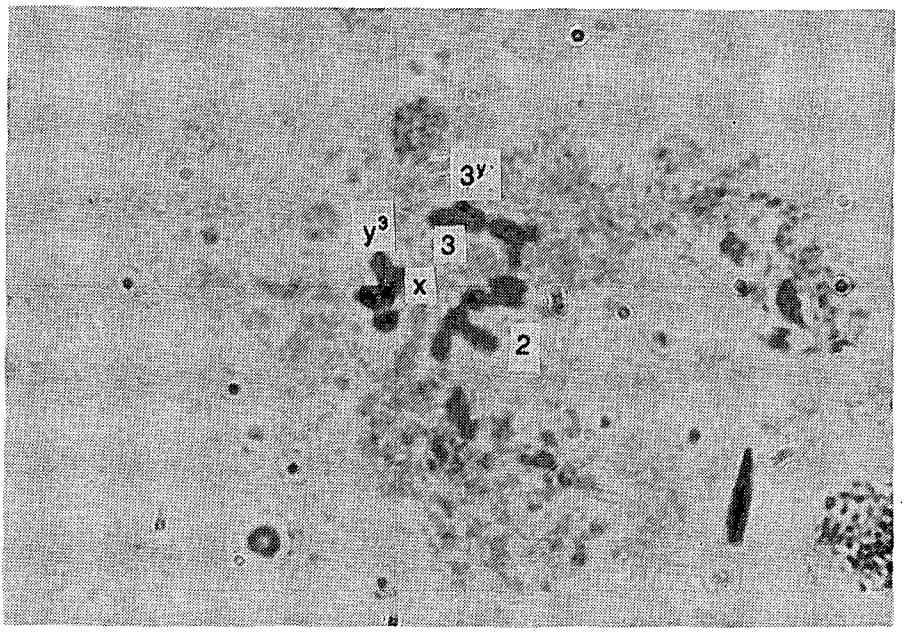

FIG. 3. Mitotic chromosome complement of the T(Y-3)12 translocation heterozygote.

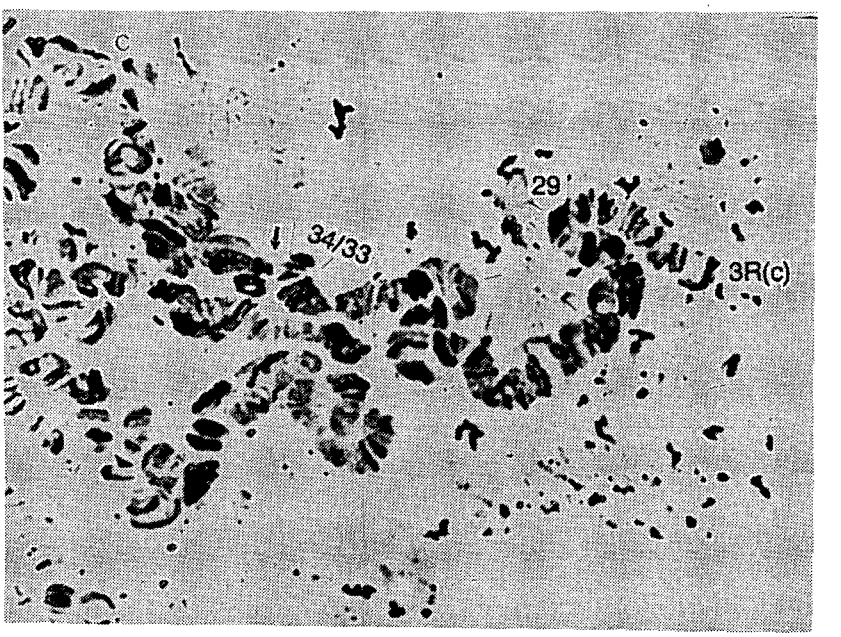

FIG. 4. Translocation break of T(Y-3)12 in region 34A of 3R. The arrow denotes the break.

it was concluded that the *dp* locus was located in 3R, very near to region 34A. By analysing several translocations in which the *dp* locus is involved, Sakai et al. [1] showed that the diamond palpus mutant is associated with the shorter of the mitotic autosomes and with the 3R element in polytene configurations. The break points in the 3R aberrations studied in their experiment were distributed from zones 30–35 and all showed moderately strong linkage with the *dp* locus. Our T(Y-3)12 translocation showed strong linkage to *dp*, therefore it should be located very near to the above mentioned region.

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ANALYSIS OF STRUCTURAL REARRANGEMENTS OF LEPIDOPTERA CHROMOSOMES USING THE CENTRIFUGATION SPREADING TECHNIQUE

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Abstract

ANALYSIS OF STRUCTURAL REARRANGEMENTS OF LEPIDOPTERA CHROMOSOMES USING THE CENTRIFUGATION SPREADING TECHNIQUE.

A centrifugation spreading technique (CST) was used to analyse 12 sex chromosome mutant female lines of the Mediterranean flour moth, *Ephestia kuehniella*. The method permitted visualization of the long synaptonemal complexes of microspread pachytene nuclei in the electron microscope. In four lines, T(W;Z)1–4, a stable translocation of a Z chromosome segment on the W chromosome was confirmed. In the other lines, designated ASF (abnormal segregating females), unexpected phenotypes segregated in atypical ratios. A quadrivalent with both sex chromosomes and an autosome bivalent was observed in females of the ASF-3 line, indicating that part of the Z chromosome was translocated on to one of the autosomes. In seven ASF lines, an additional fragment of the Z chromosome was found to be responsible for the segregation of unexpected phenotypes. It was shown that CST is useful for the study of chromosome aberrations in species with extremely small metaphase chromosomes.

1. INTRODUCTION

Concerning the use of genetic methods for the suppression of lepidopteran species, there is a great need for additional data on Lepidoptera cytogenetics. For instance, very little is known about the cytogenetic basis for the high resistance of Lepidoptera to ionizing radiation; little is also known about the chromosomal aberrations leading to the inherited sterility phenomenon. However, the chromosomes of Lepidoptera are difficult to investigate using standard cytogenetic methods because they are too small, too numerous and uniform in shape during metaphase. These

technical problems can partially be overcome when a modified centrifugation spreading technique (CST) [1] is used. The method, also called the microspreading technique [2], permits visualization of the long synaptonemal complexes (SCs) of spread pachytene nuclei in the electron microscope (EM).

We used CST to analyse chromosome aberrations in females of 12 sex chromosome mutant lines of the Mediterranean flour moth, *Ephestia kuehniella* Zeller. These structural rearrangements of the sex chromosomes were induced by gamma rays in wild type females and isolated using the sex linked recessive marker *dz* [3]. *Ephestia kuehniella* offers the advantage that a great deal of cytogenetic work has already been done on it. The moth possesses a WZ/ZZ sex chromosome mechanism, which is typical for most lepidopteran species, and females are the heterogametic sex. Although the diploid chromosome set of this moth is large (2n = 60), the WZ bivalent can frequently be recognized in spread pachytene oocytes from remnants of the compact heterochromatin associated with the W chromosome [1, 2, 4].

2. MATERIALS AND METHODS

2.1. Insects

In the present study, we used wild type strain C (kept in České Budějovice) [5] and 12 sex chromosome mutant lines [3] of *E. kuehniella*. The sex linked recessive mutation *dz* (dark central area of the forewings) [6] served as a genetic marker. In each mutant line, all structurally normal Z chromosomes were marked with the *dz* allele. All the mutant females were heterozygous for the *dz* locus and, therefore, were phenotypically of the wild type. To maintain these lines, the sex chromosome mutant (but wild type) females were mated to *dz* males in each generation. In four lines, T(W;Z)1–4, females were proved by segregation data to possess part of the Z chromosome, including the dz^+ allele translocated on to the W chromosome. In the other lines, designated ASF (abnormal segregating females), unexpected phenotypes segregated in atypical ratios; the genetic mechanism for this is unknown (Table I).

The insects were reared on flaked oats at 20–21 °C. For EM preparations, the ovaries were dissected from both the feeding and wandering larvae of fifth instar or from prepupae; the testes of third and fourth instar larvae were used.

2.2. EM microspreading of pachytene nuclei

The CST was performed according to the following protocol [1]. Gonads, dissected in a physiological solution, were transferred to a hypotonic solution (83 mM KCl and 17 mM NaCl) and disrupted using fine tungsten needles. After

TABLE I. CHARACTERISTICS OF SEX CHROMOSOME MUTANT LINES OF *E. kuehniella* ACCORDING TO PREVIOUSLY OBTAINED SEGREGATION DATA [3]. IN EACH MUTANT LINE, AS WELL AS IN THE CONTROL CROSSES, THE PROGENY ORIGINATED FROM CROSSES BETWEEN PHENOTYPICALLY WILD TYPE FEMALES (+) AND *dz* MALES

Line code	Segregated progeny (%)			
	Females		Males	
	+	*dz*	+	*dz*
T(W;Z)1	46	—	—	54
T(W;Z)2	48	—	—	52
T(W;Z)3	45	—	—	55
T(W;Z)4	50	—	—	50
ASF-1	40	12	—	48
ASF-2	39	17	—	44
ASF-3	32	34	—	34
ASF-4	37	9	11	43
ASF-5	33	16	14	37
ASF-6	33	14	15	38
ASF-7	33	16	14	37
ASF-8	30	9	11	50
Control	—	52	48	—

10–20 min, the swollen material was transferred to a spreading solution (0.02% Joy detergent, Procter and Gamble, United States of America, adjusted to pH8.6–8.9 with a 0.01M borate buffer solution of pH9.22, Merck, Germany) and allowed to disperse for 5–10 min. Then the material was centrifuged in a Teflon microcentrifugation chamber on to a hydrophilized EM grid through a 0.1M sucrose cushion containing 1% formaldehyde (pH8.2–8.5). The centrifugation was performed at 4°C in a minifuge 2 (Heraeus, Germany) at 2200 rev./min for 10 min. Then the specimen was fixed for 2 min with a fresh formaldehyde–sucrose solution, rinsed for 30 s in 0.4% Kodak Foto Flo detergent (pH8.0–8.2), air dried and stained for 30 s in 1% ethanolic phosphotungstic acid. Micrographs were taken in a Philips EM 400 operated at 80 kV.

In each line, at least 30 well spread nuclei of pachytene oocytes were examined for the configuration of sex chromosomes and their structural changes. A digitizer tablet and two computer programs, MESSCHRO and EVALCHRO (written by W. Traut), were used to measure chromosomes on the EM micrographs.

3. RESULTS

3.1. Wild type females

In the oocyte pachytene nuclei, the complete set of 30 bivalents was observed. Synaptonemal complexes formed by 29 autosomal homologues consisted of two completely paired lateral elements of equal length with chromatin loops radiating from them. In some preparations, a central element was visible in the free space between the two lateral elements. In most of the pachytene complements, the WZ bivalent could be identified as the only SC with both lateral elements of unequal length and from the remnants of compact heterochromatin tangles decorating the W chromosome axis [1]. Measurement of 47 WZ bivalents revealed that the W chromosome axis was, on average, 20% shorter than that of the Z chromosome. Only 60% of the WZ bivalents were completely paired, often showing characteristic twisting of the longer Z lateral element along the shorter W lateral element (see WT in Fig. 1). In the remaining cases, the WZ bivalents displayed partial pairing, and in a few complements the sex chromosomes were not paired at all. This may indicate delayed pairing of these non-homologous chromosomes in the early pachytene.

3.2. Wild type males

The male pachytene complements were composed of 30 well paired bivalents. The sex chromosomes (ZZ) could not be recognized because of homologous pairing and the absence of any morphological marker. In contrast to the female SCs, almost every SC in the male complements had one recombination nodule, usually seen in the free space between the lateral elements. This finding is in accordance with the fact that male meiosis is chiasmatic, in contrast to the achiasmatic meiosis of *Ephestia* females [7]. Recombination nodules were also found in three dimensional reconstructions of *Bombyx mori* male SCs [8].

3.3. T(W;Z) females

In the females of four T(W;Z) lines, part of the Z chromosome, genetically marked by dz^+, is linked to the W chromosome [3]. Therefore, we used the symbol W^+ for this neo-W chromosome. The W^+Z bivalents in all four lines resembled,

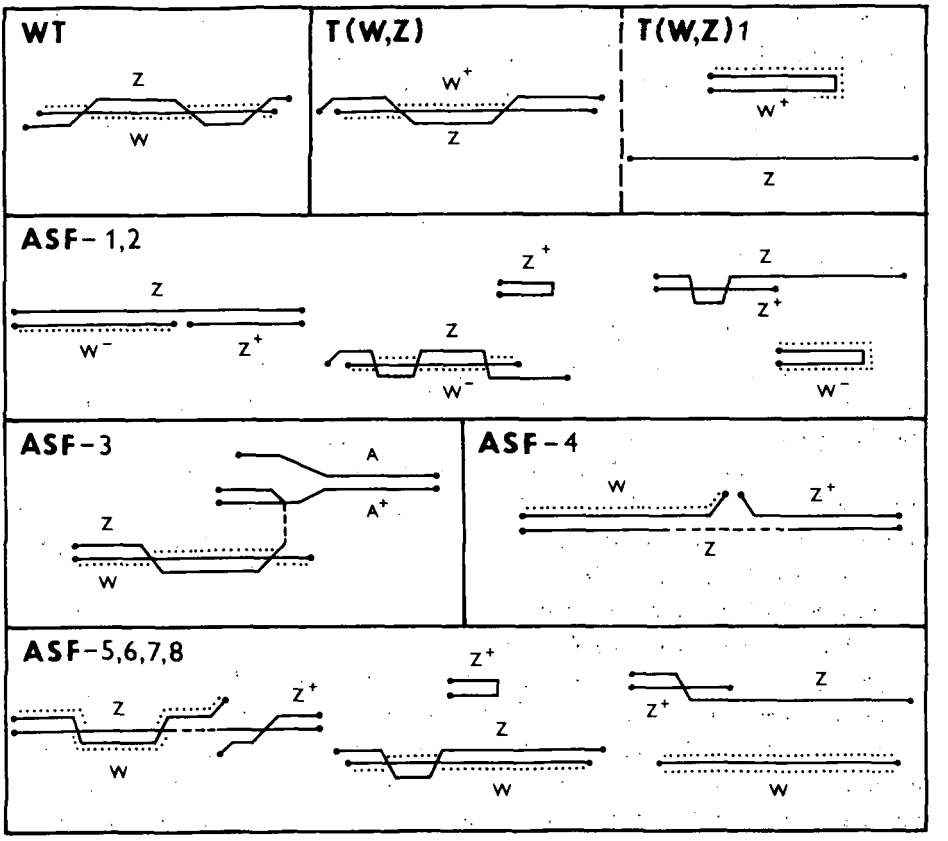

FIG. 1. Schematic drawing of the most frequent pachytene configurations of sex chromosomes in WT, T(W;Z) and ASF females.

with small differences, the WZ bivalent of wild type females. No heterochromatin tangles were observed at one end of the W^+ lateral element documenting the presence of the translocated segment of the Z chromosome (Fig. 1). This segment was relatively long, especially in T(W;Z)1 and T(W;Z)3 females, in which its length represented 30–45% of the whole W^+ axis. In accordance with previously obtained data [9], the shortest segment without heterochromatin tangles was observed in T(W;Z)4 females (about 25% of the W^+ axis). In all the lines, the W^+ lateral element was shorter than the Z lateral element in spite of the translocation presence. This indicated that the original W chromosome had been shortened by gamma rays. In contrast to the wild type WZ bivalent, most of the sex chromosome SCs in T(W;Z) females displayed complete synapsis, probably due to homologous pairing between the translocated segment and the corresponding part of the normal

Z chromosome. Concerning W^+Z pairing, exceptions were only observed in T(W;Z)1 females. In 15% of the nuclei of these females, the W^+ chromosome was autosynapsed, while the Z chromosome remained unpaired.

3.4. ASF females

In most ASF-1 and ASF-2 females, the pachytene complements displayed a fragment of the Z chromosome (Z^+) in addition to the normal Z chromosome. The length of the Z^+ axis was about 40% that of the Z axis. The W chromosome was most probably deleted because its axis (w^-) was evidently shorter than that of the wild type chromosome W. Three main types of configuration of sex chromosomes were observed (Fig. 1). In about 60% of the nuclei, a W^-ZZ^+ trivalent was formed. The remaining nuclei showed either a W^-Z bivalent and an autosynapsed Z^+ fragment, or a ZZ^+ bivalent with very unequal lateral elements and an autosynapsed (or free) W^- chromosome.

A quadrivalent with the WZ bivalent and an autosome bivalent (AA^+) was frequently found in ASF-3 females (Fig. 1). This indicated that a part of the Z chromosome marked with the *dz*$^+$ allele had been translocated on to one of the autosomes. Besides the quadrivalent, other configurations of WZ and AA^+ were observed. For example, both the bivalents were separated, and AA^+ could be recognized as an SC with unequal lateral elements. The unpaired end of this SC might represent the translocation. However, investigations of a number of ASF-3 female larvae did not reveal any structural changes in their SC complements. These larvae were regarded as *dz* females, which are segregated in the progeny of the ASF-3 line with a high frequency.

More than 70% of the microspread pachytene nuclei in ASF-4 females showed a well synapsed WZZ^+ trivalent (Fig. 1). The Z^+ fragment was significantly longer than fragments occurring in the other ASF lines. It was typical that the Z chromosome axis was considerably extended and appeared to be thinner in the middle part. Thus, the Z lateral element was adjusted to synapse with both the W and Z^+ lateral elements.

A shorter Z^+ fragment, representing about 30–40% of the length of the Z chromosome axis, was regularly observed in the pachytene complements of ASF-5, ASF-6, ASF-7 and ASF-8 lines. Similar to the ASF-1 and ASF-2 lines, three types of sex chromosome pairing were recorded. WZZ^+ trivalents were less frequent than those of the ASF-1 and ASF-2 lines; they were found in half of the nuclei. Many complements displayed a WZ bivalent and a Z^+ fragment, which was usually autosynapsed. In the remaining complements, a ZZ^+ bivalent with very unequal lateral elements and a free W univalent were observed.

4. DISCUSSION

With the CST used it was possible not only to describe the structural rearrangements in the 12 sex chromosome mutants of *E. kuehniella*, but also to correlate the previously obtained cross-breeding data [3] with the pairing behaviour of sex chromosomes.

In the T(W;Z) lines, a stable translocation of a Z chromosome segment on the W chromosome was confirmed. Owing to the homology between this segment and the corresponding part of the Z chromosome, the translocation seemed to increase the pairing affinity of the W and Z chromosomes, which are largely — if not completely — non-homologous in females of the wild type strain [2].

In the ASF-3 line, another type of translocation was found to be responsible for the segregation of wild type and *dz* females at a ratio of 1:1 (see Table I). The dz^+ allele was translocated on to an autosome and, therefore, exhibited autosomal inheritance. However, this finding did not explain the total absence of wild type males in the progeny. One may only speculate that these males were lethal because of trisomy for some important genes of the Z chromosome.

The atypical phenotypic ratios in the other ASF lines were due to the presence and pairing behaviour of those Z chromosome fragments possessing the dz^+ allele. In WZZ^+ trivalents, which appeared to be the predominating configuration of sex chromosomes in the females of these lines, the Z^+ fragments were synapsed with their structurally normal homologues. In such cases, they should segregate together with the W chromosome, simulating a W linkage. On the other hand, free or autosynapsed fragments could segregate randomly, resulting in the occurrence of exceptional phenotypes of both sexes in the progeny. However, only exceptional *dz* females segregated in the ASF-1 and ASF-2 lines but, apart from the deleted W chromosome, we did not find any substantial difference between these and the remaining five ASF lines in the configuration of sex chromosomes. Thus, the absence of exceptional wild type males in the ASF-1 and ASF-2 lines remains to be clarified.

In conclusion, the present paper shows that CST is a suitable method for chromosome analysis in species with extremely small metaphase chromosomes. Therefore, this method may be useful in investigating the effects of ionizing radiation on the chromosomes of any lepidopteran species that are candidates for genetic pest control.

ACKNOWLEDGEMENT

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Ceratitis capitata: SUITABLE MARKERS FOR POPULATION GENETICS AND GENOME ORGANIZATION ANALYSIS

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Abstract

Ceratitis capitata: SUITABLE MARKERS FOR POPULATION GENETICS AND GENOME ORGANIZATION ANALYSIS.

Biochemical and molecular markers (RAPD) can be used for population genetics and genome analysis of the Mediterranean fruit fly (medfly), *Ceratitis capitata.* At the population level, biochemical markers allowed recognition of: (a) the presence of geographical genetic heterogeneity, and (b) provided information on the relative contribution of different types of evolutionary forces during the process of colonization. The polymorphisms in genomic fingerprints, generated by the RAPD approach, provide a tool to improve the significance of the estimates of genetic relatedness between medfly populations, as well as being able to distinguish between slightly divergent flies. For genome organization analysis, biochemical markers provided information on the relationship of genome structure to genome function. The RAPD approach may provide a new tool to explore and completely map the medfly genome.

1. INTRODUCTION

The Mediterranean fruit fly (medfly), *Ceratitis capitata* Wied., is one of the major fruit crop pests in the world. It is a polyphagous and multivoltine tropical species which in the last hundred years has spread from its supposed origin in Africa to a number of countries, including the Mediterranean basin, parts of South and Central America and Australia [1].

The use of biochemical markers [2] has broadened the available knowledge on the genetic features of this species at two main levels:

(a) Population genetics and taxonomic relationships [3–5],
(b) Genome organization [6, 7].

In addition, the study of the genetic control of enzyme systems, such as ADH [8, 9], and of proteins typical of critical developmental stages (LSP) [10] has permitted study of the evolution of protein patterns and facilitated the approach to gene duplication and gene expression problems during the developmental cycle of the medfly.

The extension of the analyses of genetic variability in the medfly at the DNA level will provide opportunities for delving deeper into knowledge on the genetic features of the medfly, as well as for integrating new ideas and new tools for a many-faceted approach. Among the recent molecular advances, random amplified polymorphic DNA (RAPD) analysis [11, 12] seems to be an extraordinarily powerful approach to generating very quickly a large number of markers that are useful in population genetics analysis and in genome mapping of the medfly.

2. POPULATION GENETICS

The use of biochemical markers permits examination, at different levels of analysis, of the genetic consequences of the geographical spread of this species. At a first level, geographical genetic heterogeneity has been recognized in this species range. At the second level, the study of enzyme variability provided information on the relative contribution of different types of evolutionary forces during the process of colonization (selection, genetic drift and gene flow).

Intraspecific genetic differentiation is believed to be associated with the colonization history of the medfly. From the point of view of the colonization pattern, world populations of *C. capitata* can be divided into three main categories: (a) ancestral populations from sub-Saharan Africa; (b) ancient populations from the Mediterranean basin and (c) new populations from South and Central America. Genetic parameter estimates, such as the average number of alleles per locus, the proportion of polymorphic loci and the average number of heterozygous individuals, seem to be correlated with the population's evolutionary history. In fact, they reveal a trend of decreasing genetic variability from the putative source area of the species towards the periphery of its geographical range. These estimates, together with the results of multivariate and cluster analyses of allele frequencies, confirmed the above mentioned subdivision of world medfly populations [3, 4].

Data on the spatial distribution of allelic variation show that the pattern is highly heterogeneous for different loci. Parameters of variability or distances involving gene frequencies enlightened the dynamic aspects of evolutionary processes,

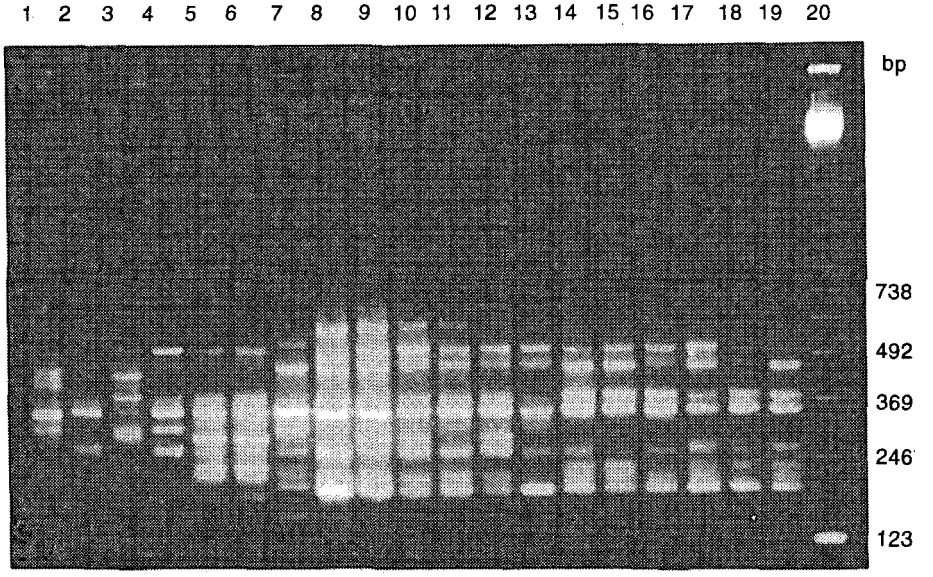

FIG. 1. Polymorphic amplification patterns obtained from genomic DNA of Ceratitis capitata *flies from Kenya (lanes 1–7), from the Metapa laboratory strain (lanes 8–13) and from the M84 line (lanes 14–19). Lane 20 shows the molecular size markers. NP4 primer was used for the amplification reaction.*

such as selection, drift and gene flow. In fact (i) comparison of F_{ST} distribution among loci led to the identification of groups of loci probably affected by selection; (ii) genetic distance analysis permitted inference of the amount of drift between geographical populations (this factor seems to have played a major role in the dispersion processes of the peripheral populations); and (iii) gene flow estimates, in terms of the number of immigrants per generation, are significant between the original and the derived Mediterranean populations and support the hypothesis of recent colonization [13]. The sub-Saharan Kenyan population has all the properties of a native population and could belong to the centre of diffusion of the species. It has probably maintained a very large size, as deduced by the high number of rare alleles present only in this population [14].

A parallel study of protein and DNA variation by means of allozyme and RAPD markers is in progress on samples of wild medfly populations and on laboratory strains. Although such markers may be influenced by different types and degrees of selection pressure, they would be affected similarly by such factors as population size and gene flow. An unexpectedly high degree of individual variability in the genomic DNA is evident when the wild flies from Kenya are screened for RAPD polymorphisms. The DNA of the majority of the Kenyan flies exhibits a

unique pattern, differing in several fragments from that of any other. The observed profiles are typical DNA fingerprints and can be generated using different single primers of arbitrary sequence. A lesser degree of genomic variability is detected in the colonized laboratory strains which, however, maintain a certain degree of DNA polymorphism. An example is illustrated in Fig. 1, in which the polymorphic RAPD profiles from African flies are compared with the ones derived from the Metapa laboratory strain and from the M84 line. These data, as expected, support the previous results obtained with allozyme markers on the role of drift in the colonization process and on the high degree of genetic variation present in the medfly African ancestral populations. However, RAPD analysis, compared with the allozyme approach, detects a larger amount of hidden variation both in wild and laboratory medfly samples.

The polymorphisms in genomic fingerprints, generated by the RAPD approach, provide a tool to improve the significance of the estimates of genetic relatedness between medfly populations, as well as to distinguish between slightly divergent flies. A great deal of information data will be available for the evolutionary population genetics of the medfly. From the point of view of application, the availability of markers which can unambiguously discriminate between strains as well as samples of *C. capitata* is of utmost importance for the biological approach to medfly control, such as the sterile insect technique (SIT).

3. LINKAGE MAPS AND COMPARATIVE GENETICS

The available genetic maps of *C. capitata*, which rely heavily on isozyme markers [6, 7], provide useful information on the relationship between the genome structure and genome function in this insect. To date, a total of 30 biochemical markers and 12 morphological and functional genes have been linked, linearly arranged and mapped on five out of six linkage groups of *C. capitata*. These groups include structural gene coding for enzyme functions, lethal and sex ratio distorter genes and developmental proteins. Map distances on the five linkage groups show that the marked loci are distributed over wide map intervals. Therefore, sufficient loci are now available for extending the physical chromosome maps to also include chromosome characters. Because many of the biochemical loci considered have been mapped in other Tephritidae flies [15], in *Drosophila melanogaster* [16] and in *Musca domestica* [17], it is possible to compare the linkage arrangements of horthologous genes in these different species. This type of analysis provides the basis for establishing chromosomal homologies, and for examining the overall organization of the genome in these dipteran species.

For high resolution linkage mapping in *C. capitata*, the availability of molecular markers is essential. High density genetic maps, comprised of molecular markers, may lead to the identification of several previously unidentified loci of bio-

logical importance in *C. capitata* that can be exploited also to improve autocidal control of this insect by genetic manipulation. From the methodological point of view, the RAPD approach is easier and faster than other established molecular methods used for genetic mapping. Using only one primer, in one multilocus cross, it is possible to study the linkage relationships among several markers and to recognize their linkage groups. Other advantages in utilizing RAPD polymorphisms are that map planning can be approached without having to first identify RFLP probes and, subsequently, RAPD generated DNA polymorphic fragments can be isolated directly from gels and reamplified for use of probes in restriction mapping strategies and for mapping on polytene chromosomes by in situ hybridization.

4. CONCLUSIONS

Significant work remains to be done to examine the geographical distribution of DNA variation, particularly in light of the contrasting distribution of protein electrophoretic variation observed at particular biochemical loci across medfly populations. As concerns the study of medfly genome organization, the approach may provide a new tool to explore and to completely map the genome of this insect.

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COMPARISON OF *Bacillus thuringiensis* AND *Bacillus cereus*

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Abstract

COMPARISON OF *Bacillus thuringiensis* AND *Bacillus cereus.*

Bacillus cereus and *Bacillus thuringiensis* are closely related, spore forming soil bacteria. *B. thuringiensis* produces insecticidal crystal proteins during sporulation and these toxins are the most important biopesticide in the world today. Genomes of the *B. thuringiensis* and *B. cereus* strains were analysed by pulsed field gel electrophoresis after treatment of the DNA with the restriction enzyme NotI. The NotI fingerprint patterns varied both within the *B. thuringiensis* and the *B. cereus* strains. The size of the fragments varied between 15 and 1350 kb. When physical maps of the *B. thuringiensis* and *B. cereus* strains were compared, *B. thuringiensis* appeared to be as closely related to *B. cereus* as the *B. cereus* strains were to each other. Nine out of 12 *B. thuringiensis* strains and 18 out of 25 *B. cereus* strains produced enterotoxins. The close relationship between *B. thuringiensis* and *B. cereus* should be taken into consideration when *B. thuringiensis* is used as a biopesticide.

1. INTRODUCTION

Bacillus cereus and *Bacillus thuringiensis* are spore forming, common soil bacteria. They are very closely related, and in some taxonomic studies *B. thuringiensis* is regarded as a variant of *B. cereus*. This implies that the differences between the strains may not be of taxonomic importance — yet the differences may be of vast practical and medical importance.

B. thuringiensis is well known for its insecticidal activity, producing toxins active against lepidopteran, dipteran or coleopteran larvae [1, 2]. The toxin genes are usually localized on large plasmids, and the protoxins are found inside the bacteria during sporulation as crystalline inclusion bodies. Four classes of *cry* genes are characterized and one bacterial strain may produce several toxins. Today, *B. thuringiensis* is the most widely used biopesticide in the world, and several companies produce toxin crystals/spores with different insect specificities. Hundreds of tonnes of spores/toxins are used in various parts of the world every year.

Like other members of the Bacillaceae family, *B. cereus* secretes proteins very efficiently. Some strains produce and secrete enterotoxins, and may cause food poisoning in humans [3]. *B. cereus* is the most common contaminant in dairies [4].

B. cereus and *B. thuringiensis* are very closely related, yet one is regarded as a contaminant while the other is useful and of great commercial interest. We wanted to compare strains of the two bacteria by analysing their DNA fingerprint patterns, looking for characteristic patterns.

2. MATERIALS AND METHODS

2.1. Bacteria strains

The bacteria strains were obtained from the American Type Culture Collection (ATCC), from the Bacillus Genetic Stock Center (BGSC), Ohio, or through H. Nissen of Ås, Norway, from the Public Health Laboratory (PHLS), United Kingdom. One strain (*B. cereus* 45) was isolated and provided by the National Institute for Public Health, Oslo, Norway.

2.2. Preparation of the DNA

The bacteria were grown on liquid cultures, harvested by centrifugation and embedded in low melting agarose plugs of 100 μL each, about 10^6 bacteria per plug [5, 6]. The plugs were then treated with detergents and proteolytic enzymes that dissolved and degraded all the cellular components of the bacteria, except the DNA. The purified chromosomal (and large plasmid) DNA thus obtained was protected within the agarose plug.

2.3. Analysis of the DNA by pulsed field gel electrophoresis

A DNA plug was incubated with a restriction enzyme that cut infrequently into the genome, like NotI, which recognizes the sequence GCGGCCGC. The DNA fragments were then separated by pulsed field gel electrophoresis, where the direction of the electric field changes, thus allowing for separation of DNA fragments of more than 1500 kb [7]. The fragment patterns from individual strains after staining with ethidium bromide were analysed at pulse times ranging from 2 to 90 s; the electrophoresis was routinely run for 18–24 h in a Beckman Gene Line apparatus.

2.4. Hybridization

After staining the gel, the DNA was denatured and blotted on to supported nitrocellulose filters [8]. The filters were baked at 80°C for 2 h and hybridized to ^{32}P labelled DNA probes as described previously [6, 9]. The probes used were either genes or gene fragments from *B. cereus* (phospholipase C genes, beta-lactamase genes), *B. thuringiensis* (the *cryI* gene), or *B. subtilis* (*AbrB*, the pyruvate

dehydrogenase gene), or they were random fragments from a library of *B. cereus* ATCC 10987 prepared in our laboratory (BC probes). Hybridization was performed in 3 × SSC/0.1% SDS/10% dextran sulphate (1 × SSC is 0.3M NaCl plus 0.03M sodium citrate). The filters were washed for 2 × 30 min in each of the solutions: (1) 3 × SSC/0.1% SDS; (2) 1 × SSC/0.1% SDS; and (3) 0.3 × SSC/0.1% SDS.

2.5. Analysis of enterotoxin

Bacteria were grown overnight at 30°C, centrifuged (10 000g, 20 min, 4°C), sterile filtered and the enterotoxin was measured in the filtrate using a kit for *B. cereus* enterotoxin, BCET-RPLA, obtained from Oxoid, UK.

2.6. Haemolytic activity

Bacteria were spread on agar plates containing 1% human erythrocytes. Zones of haemolysis around the colonies were observed after incubation overnight at 37°C.

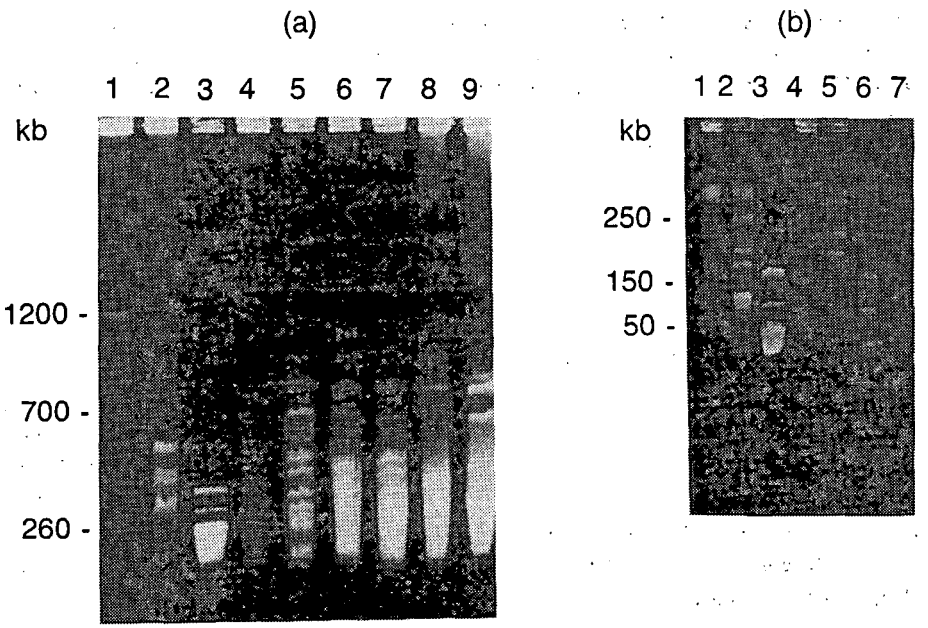

FIG. 1. Pulsed field gel electrophoresis of DNA from Bt strains after digestion with NotI. The electrophoresis was run (a) at 60 s pulses at 150 mA for 22 h, and (b) at 30 s pulses at 150 mA for 18 h. (a) (1) Size markers, yeast chromosomes (Bio-Labs); (2) B. t. kurstaki; *(3)* B. t. kenya; *(4)* B. t. canadensis; *(5)* B. t. entomocidus; *(6)* B. t. dendrolimus; *(7)* B. t. subtoxicus; *(8)* B. t. morrisoni; *and (9)* B.t. darmstadiensis. *(b) (1) Yeast chromosomes; (2)* B. t. kurstaki; *(3)* B. t. kenya; *(4)* B. t. canadensis; *(6)* B. t. morrisoni; *and (7)* B. t. darmstadiensis.

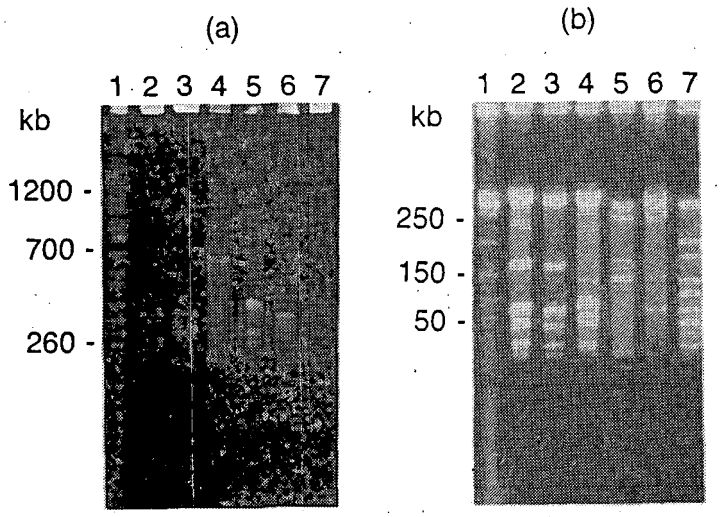

FIG. 2. Pulsed field gel electrophoresis of DNA from B. cereus *strains after digestion with NotI. The electrophoresis was run (a) at 60 s pulses at 150 mA for 20 h, and (b) at 30 s pulses at 150 mA for 18 h. (a) (1) Yeast chromosomes; (2)* B. cereus *ATCC 11778; (3)* B. cereus *ATCC 1457; (4)* B. cereus *ATCC 10876; (5)* B. cereus *ATCC 10987; and (6)* B. cereus *F 4810/72; (7) F837/76. (b) (1) Size marker, lambda concatemers; (2)* B. cereus *6EI; (3)* B. cereus *6E2; (4)* B. cereus *45; (5)* B. cereus *ATCC 21281; (6) B. cereus ATCC 27877; and (7)* B. cereus *ATCC 38018.*

3. RESULTS

3.1. NotI fingerprint patterns

Both the *B. cereus* and *B. thuringiensis* strains contained NotI fragments ranging in size from 15 to 1350 kb (Figs 1 and 2). The NotI fingerprint patterns of eight Bt strains (Fig. 1) were fairly similar in some strains (*B. t. entomocidus, B. t. dendrolimus* and *B. t. subtoxicus*), while the fragment patterns were very different in other strains (*B. t. kurstaki, B. t. kenya, B. t. canadensis, B. t. morrisoni* and *B. t. darmstadiensis*). A similar variation among the *B. cereus* strains was observed (Fig. 2(a), (b)).

3.2. Physical maps

Probes were assigned to individual NotI fragments by hybridization (Fig. 3). The sizes of the neighbouring fragments were obtained when partial NotI digests were analysed. We used more than 50 probes and aligned all the NotI fragments of

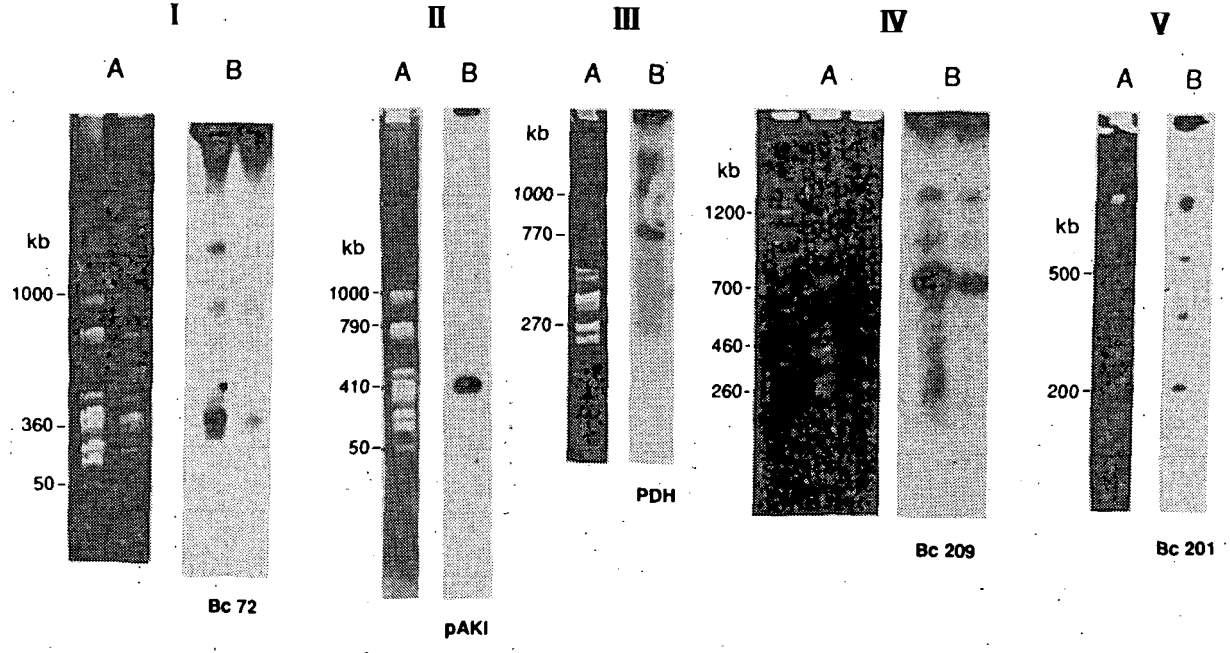

FIG. 3. (A) Pulsed field gel electrophoresis of B. cereus *ATCC 11778 NotI fragments, and (B) autoradiography of blotted NotI fragments. The fragments were separated at 60 s pulse time at 150 mA for 20 h (I, II, III and IV), or at 30 s pulses (V). The probes used for hybridization were as indicated in (B).*

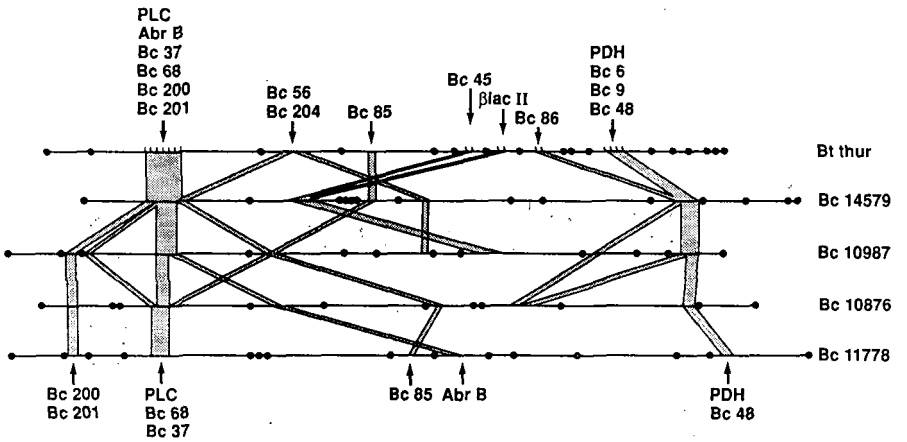

FIG. 4. Comparison of maps of one B. thuringiensis *and four* B. cereus *strains.*

four *B. cereus* strains [6, 9], as well as one *B. thuringiensis* strain [10]. When these maps were compared (Fig. 4), it was observed that: (1) the *B. thuringiensis* strain appeared to be as closely related to the four *B. cereus* strains as the four *B. cereus* strains were to each other; and (2) the differences between the strains may be explained by inversions/deletions/insertions.

3.3. Haemolytic activity and enterotoxin production

When the haemolytic activity and the enterotoxin production were measured in 12 *B. thuringiensis* strains and 25 *B. cereus* strains, most of the strains were haemolytic, and more than half of both the strains were positive for enterotoxin (Table I).

TABLE I. HAEMOLYTIC ACTIVITY AND ENTEROTOXIN PRODUCTION BY THE *B. cereus* AND *B. thuringiensis* STRAINS

	B. cereus		*B. thuringiensis*	
	Positive	Negative	Positive	Negative
Haemolytic activity	23	2	10	2
Enterotoxin production	18	7	9	3

4. CONCLUSIONS

From these studies it was concluded that:

(1) The DNA from strains of *B. thuringiensis* and *B. cereus* shows a characteristic fingerprint pattern when their NotI fragments are analysed by pulsed field gel electrophoresis. The pattern appears to be specific for the individual strains. Although a few strains were closely related, no typical *B. thuringiensis* or *B. cereus* pattern was discovered.

(2) *B. thuringiensis* strains cannot be distinguished from *B. cereus* strains by DNA fingerprint analysis. Our results are in keeping with data from other laboratories, indicating that *B. thuringiensis* strains are insecticidal variants of *B. cereus*.

(3) Nine out of 12 *B. thuringiensis* strains produce enterotoxins that may cause diarrhoea in humans. This information should be taken into consideration when *B. thuringiensis* is used as a biopesticide.

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POSTER PRESENTATION

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MOLECULAR GENETICS OF INSECTS
Oogenesis and maternal control of early development

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The central interest of our research concerns the molecular bases of maternal control of development in *Drosophila melanogaster* and other insect species. We are studying a group of female semi-sterile and maternal effect mutants, located in region 32 of the standard salivary gland map of the second chromosome of *Drosophila*. Besides detailed genetic analyses to define the phenotypic aspects of the mutation, we analysed the structure of ovaries and early embryos produced by homozygous mutant females using DAPI or propidium iodide staining. At the molecular level we isolated by chromosome walking 250 kb of DNA from region 32D-E, performed a restriction enzyme site polymorphism analysis in *wild type* and mutant stocks and identified a copia like *blood* transposon inserted in region 32E of the *abo* chromosome. We demonstrated that the *abo* phenotype is strictly correlated with the presence of the *blood* transposon. We identified transcripts on the two sides of the transposon insertion site and isolated corresponding cDNA clones. From sequence analysis of the cDNAs and the 10 kb genomic region, we identified a gene whose putative product has an average 60% identity with the guanylate cyclase/atrial natriuretic factor receptors (sea urchin, rat, mouse, human). The data obtained by all the different approaches appear to indicate that this group of genes is involved in general processes such as cell proliferation and/or cell migration. As part of the study of the maternal effect mutants in region 32, we identified molecularly a vitelline membrane protein (VMP) gene, called *VMP32E*, whose regulatory sequences were dissected by using in vitro mutagenesis coupled with *P* element mediated germ line transformation.

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OPERATIONAL PROGRAMMES

(Session 4)

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GENETIC CONTROL OF COTTON INSECTS
The pink bollworm as a working programme

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Abstract

GENETIC CONTROL OF COTTON INSECTS: THE PINK BOLLWORM AS A WORKING PROGRAMME.

Establishment of a continuous population has been prevented over a 24 year period in the San Joaquin Valley, USA, through continuous, daily in-season release of sterile pink bollworms based on an extensive trap monitoring programme. A post-harvest crop destruction ordinance and occasional use of pheromones as disruptants were the only other factors used by programme management, except in 1990. In 1990, the programme used a conventional insecticide on 280 acres (113 ha) out of 1.18 million acres (477 546 ha) of cotton. During the four year period 1986–1989, a management system was explored using a high rate pheromone disruption system and sterile insects. Major reductions in conventional insecticide usage, while maintaining extremely low populations, were evident in this semi-isolated valley of southern California. It is hoped that this will provide a model for a future large scale test on up to 20 000 acres (8100 ha) of cotton.

1. INTRODUCTION

The use of genetic control mechanisms for cotton insects has been explored to varying degrees with several insects, including the tobacco budworm, *Heliothis virescens* (F.), and the boll weevil, *Anthonomis grandis* Boheman. Their use and

actual application on a large scale have been limited, however, to the pink bollworm, *Pectinophora gossypiella* (Saunders). This paper covers that usage and its potential for expansion.

It is important to have a historical perspective of the pink bollworm problem in the United States of America. At the present time, it is a problem in the western USA, restricted to the irrigated desert areas. Pink bollworms are occasionally recovered from states east of Texas, with only one endemic population on wild cotton in the southern extreme of Florida. The Floridian population has total geographical isolation from any commercial cotton growing area in the USA.

The pink bollworm is most destructive in Arizona, southern California and the adjacent northwestern Mexican desert cotton growing areas. Its introduction and establishment in central Arizona in the mid-1950s and into the Colorado River Basin of western Arizona, southern California and northwestern Mexico in 1965 have led to the development of a major pest problem with extreme side effects on the whole ecosystem. This is, of course, exacerbated by a long growing season which, when pushed for maximum production, results in crop longevity, giving pink bollworm tremendous population potential. The extensive use of broad spectrum conventional pesticides, including the newer synthetic pyrethroid compounds, has resulted in expensive control systems with secondary pest problems. The only major area in the western cotton belt where the impact of the pink bollworm has not been felt by the grower is in the central California San Joaquin Valley. In this area, the establishment of the pink bollworm has been prevented through the use of sterile insect release technology, minor use of pheromones as a disruptant and adequate cultural control. This paper briefly outlines the details and provides programme statistics for that project. In addition, it discusses efforts to integrate sterile release technology with pheromone systems and optimum cultural practices in the heavily infested southern California desert.

2. THE SAN JOAQUIN EXCLUSION PROGRAMME

The San Joaquin Valley has never had a known continuous endemic population of the pink bollworm, even after the spread of this insect into the Colorado River desert growing areas in 1965 and 1966. The San Joaquin Valley is approximately 175 miles (282 km) (direct line) from any generally infested areas of southern California. The entire 175 miles is a host free zone. This expanse of desert and mountains has formed a *partial* barrier to pink bollworm movement. Movement within the desert region between the generally infested southern California deserts and the San Joaquin Valley has been documented by Stern and Savacherian [1] and by trap lines maintained by programme personnel in the mountain passes between the desert cotton growing areas and the San Joaquin Valley. Extensive movement into the desert area has been documented early in the spring, late in the summer and

TABLE I. ANNUAL SUMMARY (1985–1989) OF THE MATING PROPENSITY OF STERILE PINK BOLLWORM FEMALES SHIPPED TO THE SAN JOAQUIN VALLEY, AS DETERMINED IN THE LABORATORY

Year	% mated (48 h)
1985	43
1986	70
1987	81
1988	76
1989	86
1990	85
1991	87

throughout the fall. Movement over mountain passes into the adjacent San Joaquin Valley has been documented in the fall. Programme trap data further provide evidence of movement within the San Joaquin Valley, since late season increases in detection have coincided with captures in the desert trap lines. All of these data have been gathered by using gossyplure baited traps placed along highways in the deserts between cotton growing areas and in field traps.

In 1968, a programme was initiated to protect the 500 000 ha of cotton in the San Joaquin Valley by releasing irradiated pink bollworm moths. Initially, the released moths were irradiated with 25 krad of gamma rays, but since 1973 the dose has been lowered to 20 krad. When sterile insects are treated at this dosage, they will produce eggs but most of these progeny do not survive to reach the adult stage. These few adults are infertile. Thus, the main purpose of lowering the dosage to 20 krad was to obtain more competitive males rather than to employ F_1 sterility.

Sterile insects are now reared in Phoenix, Arizona, using techniques or modifications of techniques described by Stewart [2]. Attention to detail and constant refinements of these rearing processes since 1971 have improved the quantity and quality of insects available for shipment.

The increase in quality is illustrated in Table I, which summarizes quality control mating tests carried out by programme personnel. These data come from samples taken each day of shipment (seven days a week) throughout the season.

TABLE II. ANNUAL PRODUCTION OF THE PHOENIX PINK BOLLWORM REARING FACILITY, 1970–1991

Year	Total moths × 10^6
1970	135
1971	120
1972	113
1973	99
1974	41
1975	154
1976	177
1977	192
1978	301
1979	385
1980	372
1981	401
1982	497
1983	594
1984	579
1985	489
1986	702
1987	735
1988	804
1989	826
1990	809
1991	813

Three samples of 20 pairs are held in cages in the laboratory for 48 h before dissection. An additional set of samples are checked for mating on arrival in the release zone. Those females which contain spermatophores are considered mated. Table I shows an increased mating propensity between 1985 and 1991 after small procedural improvements were made in shipping. Mean mating levels of samples taken directly from shipment containers (0 h) did not exceed 3.3% for any year.

Laboratory space was increased by approximately 15% during the 1970–1989 period. All other production increases were technical. The increases in production from 1973 to 1991 are illustrated in Table II.

The Phoenix United States Department of Agriculture (USDA)/California Cotton Growers funded facility was the only provider of sterile insects for the San Joaquin Valley from 1970 to 1977 and from 1983 to the present. It has also provided significant numbers of pink bollworms for research work, including sterile insect research. These trials included those in the Moapa Valley of Nevada [3], St. Croix [4] and in the Coachella Valley of California (reported here). Pheromone isolation and identification projects [5, 6], were also supported.

From 1977 to 1982, a commercial 'satellite' pink bollworm rearing facility was operated for eight months of each year. This facility also provided moths for the San Joaquin Valley.

Sterile insects are shipped at 40 ± 5°F (4.44 ± −15°C) from Phoenix, Arizona, to Bakersfield, California, where they are transferred (still in a chilled condition) to aircraft and released at approximately 500 ft (152 m) above the cotton fields. The time of collection from eclosion chambers to the time of release is from 24 to 48 h. Insects are held in an immobile state by chilling during this time.

The sterile and native populations are currently monitored with pheromone traps baited with gossyplure in a controlled release formulation. The Delta trap [7] has been used since 1974.

With the advent of gossyplure and its availability in 1974, the conduct of this programme has become much more precise. For this reason, this paper discusses programme operations since that time. A summary of pertinent information is given in Table III.

The number of traps has varied from 12 850 to 33 711. Traps have been placed at average densities varying between one trap per 39.5 acres (15.99 ha) to one trap per 79.8 acres (32.3 ha). Traps are serviced weekly. The data produced by the traps is used to schedule the release of sterile insects. From 1974 to 1991, the area requiring release of sterile insects ranged from 4.6 to 29.5% of the total San Joaquin cotton production area.

Each year the season starts with releases on cotton in sections (640 acres (259 ha)) which had moth finds from the previous year. Thus, the percentage of the total acres released varies. As appropriate (depending upon native moth capture rates for the previous year), buffer zones are also released around these sections. Moth releases are controlled at the section level (640 acres). As new, native (unmarked) pink bollworms are found, the releases are adjusted accordingly.

Moth release typically begins before the first flower buds are present on the cotton plants (pin square) and continue with daily shipments of moths throughout the season. Releases of sterile insects are normally terminated each year during the last two weeks of October or the first two weeks of November.

TABLE III. GENERAL STATISTICS FOR THE SAN JOAQUIN PROGRAMME, 1974–1991

Year	No. of acres[a] mapped × 1000	No. of traps maintained × 1000	Acres[a] per trap × 100	Maximum acres[a] released × 100	Per cent acres[a] released	Mean No. of steriles per acre[a] released
1974	1275	32	39.5	101	7.9	366
1975	866	22	40.0	117	13.1	1 287
1976	1130	27	41.0	90	7.9	2 141
1977	1353	33.7	40.0	400	29.5	1 030
1978	1501	19.6	76.3	355	26.6	1 286
1979	1625	20.6	78.8	254	15.6	2 512
1980	1463	20.8	70.2	177	12.1	2 880
1981	1451	19.4	74.8	352	24.3	2 251
1982	1326	16.9	78.3	147	11.0	5 260
1983	1005	12.9	78.2	132	13.1	4 452
1984	1392	17.6	79.0	42	6.1	13 329
1985	1336	17.2	77.9	59	4.6	8 167
1986	1061	13.3	79.8	44	4.1	14 930
1987	1212	19.9	60.8	63	4.6	10 864
1988	1368	22.7	60.4	71	5.2	10 655
1989	1128	19.2	58.8	104	9.2	7 258
1990	1184	19.5	60.7	129	10.8	5 438
1991	1103	18.4	59.9	140	12.6	5 806

[a] 1 acre = 0.405 ha.

TABLE IV. STERILE MOTH RELEASE AND TOTAL MOTH CAPTURE DATA IN THE SAN JOAQUIN VALLEY

Year	Sterile moths released × 10^6	Per cent sterile moths recovered	No. of sterile moths recovered	Non-sterile moths recovered (native)	No. of sterile insects captured per native
1974	37.01	0.76	282 897	437	647
1975	150.11	1.01	1 528 260	245	623.8
1976	173.64	0.63	1 229 742	1474	834
1977	412.17	0.40	1 677 900	7402	22
1978	455.99	0.09	429 063	69	621.8
1979	636.98	0.09	545 295	754	723
1980	510.49	0.11	566 170	4492[a]	126
1981	794.26	0.11	864 861	677	127.7
1982	772.12	0.13	1 041 280	120	8 677.3
1983	586.77	0.18	1 057 735	863[a]	1 225.6
1984	571.50	0.38	2 144 018	351[a]	6 108.3
1985	483.74	0.09	434 739	160	2 717.1
1986	660.35	0.05	352 825	62	5 690.7
1987	670.07	0.15	1 057 925	294	3 598.7
1988	754.87	0.16	1 188 335	891	1 335.7
1990	701.50	0.27	1 943 863	3239[b]	600.14
1991	812.97	0.45	3 668 337	263	10 105.6

[a] Larvae finds of four larvae: four larvae found in respective years.

[b] In 1990, two fields had a cumulative average peak of 15.2% boll infestation.

The number of sterile moths released has increased steadily since 1974 (Table IV). Major improvements in rearing technology and management systems in 1975 represented a significant improvement in programme operations. A very dramatic degree of improvement in rearing is seen when one compares the numbers of moths shipped to the San Joaquin Valley in 1975 compared with 1974. Shipments increased from 37.01×10^6 moths in 1974 to 150.11×10^5 in 1975, and 173.6×10^0 in 1976. Although native unmarked populations were low early in 1974, it became apparent that a major problem existed as the season progressed. The total sterile moth captures of 282 897 was 647 times higher than the non-sterile recovery, but an acceptable ratio[1] of 60 sterile:1 native moth at the individual field level could not be maintained when native moth capture rates became very great in August and September. It was very fortunate that improvements were brought on line before the 1975 season. The production in 1975 was able to cover the entire 'infested' area adequately from the beginning of the season.

Relatively high native moth capture (1474) late in the season in 1976 followed tropical storms originating off Baja California, Mexico and resulted in a decision to augment sterile moth production at the USDA facility by outside contract production for the 1977 season. This contract provided 28% of the 412 million moths used in the San Joaquin Valley in 1977 and provided an average of 39% of all production until the 1983 season. In 1983, the 586 million moths shipped were provided exclusively by the USDA facility in Phoenix.

In 1980, we captured the highest number of native moths in programme history. Unlike 1974, however, coverage of the entire area where moths were detected was achievable throughout the season. Major efforts were also made through grower meetings throughout this area to optimize cultural control through rapid crop destruction after harvest and early plough down of host material.

The following year, 24% of the Valley received sterile insects. This rate is high in comparison with the preceding few years of release, but with good winter mortality between the 1980 and 1981 season, it was apparently adequate.

In 1990, following a warm dry winter in which those cotton plants left standing were not frozen, populations developed predominantly in two areas within the Valley. For the first and only time, a conventional insecticide was used against a larval population. This population was large enough in two fields to be measured with conventional sampling procedures. Guthion was applied on 28 September and 3 October on 280 acres (113 ha). The crop in these fields was chemically terminated rapidly following these treatments and cultural measures of the harshest nature relative to the pink bollworm were taken. It must be emphasized that this was the only time in the 24 years that sterile insects have been released in the San Joaquin Valley that a conventional insecticide was used against the pink bollworm. The only other

[1] Through experience, the ratio of 60 sterile moths:1 native moth has been established as a working, safe ratio.

in-season treatment available to programme personnel has been the use of gossyplure as a mating disruptant. Its use has never exceeded 0.2% of the total acreage, or 4.6% of any year's release area. Our problem was not repeated in 1991. To date in 1992, native moth capture rates are at a very low level.

When one tries to assess this programme, with its massive number of traps and the extensive amount of data they generate, it is apparent that any analysis would be highly speculative in nature. There are no check plots and/or adjacent identical infested areas.

It should also be noted that until the present time, we have used resources (the sterile moth) on an as-available basis. We are now constructing in the Animal and Plant Health Inspection Service (APHIS), with the co-operation of Texas A&M University, a heuristics based expert system to help the decision maker utilize the extensive database that this project generates. We acknowledge that although the project has its limitations, two pertinent points have not been missed by the cotton industry. The first is that there is no detectable ongoing pink bollworm population in the San Joaquin Valley, and second, that our rearing technology has improved significantly and consistently over the years.

3. INTEGRATION OF THE STERILE INSECT SYSTEM WITH PHEROMONE DISRUPTION

Once the pink bollworm spread into the Colorado River Basin, the entire cotton pest management system changed drastically. Cotton growers were determined to continue maximizing yields and were quick to utilize regularly scheduled insecticide application. This philosophy continued, essentially unabated, for approximately twenty years. In the most severely infested areas (such as in the Imperial Valley), grower leadership is now seeking other remedies. In addition to working with sterile insect technology, our laboratory, the Agricultural Research Service and industrial co-operators have worked extensively in the development of pheromone communication disruption. The large scale field testing of the Mitsubishi pink bollworm rope in 1985 [8] in Imperial Valley provided a breakthrough. This breakthrough led to the carrying out of an 'area wide' management project in the Coachella Valley on its restricted acreage.

The Coachella Valley is reasonably isolated during the main growing season and is measurably affected by migration only during storm conditions that include surface winds from the southeast, usually late in the season. In 1986, we started working in a co-operative venture with the growers of the area and their pest control advisor (M. Grummet of Foster Gardner Chemical Co.). Our first step in 1986 was to treat the entire valley with the high rate pink bollworm rope system, at 30 g of active ingredient (AI) per acre in one application at the six to eight leaf cotton development stage. Our results were so encouraging that between 1987 and 1989 we

TABLE V. CONTROL STRATEGIES AND CONVENTIONAL INSECTICIDE USE PATTERNS FOR PRE-MANAGEMENT AND AREA WIDE MANAGEMENT SYSTEMS IN THE COACHELLA VALLEY

Treatment system	Fields treated with pheromone	Mean No. of conventional insecticides	No. of fields treated with insecticides	Cumulative conventional insecticide treatments
1985 insecticide only (pre-trial data)		7.27	56/57	414
1986 pheromone and insecticides	31/31	1.8	17/31	56
1987 sterile insects, pheromone and insecticides	17/21	1.03	7/27	28
1988 sterile insects and pheromone	4/31	0	0/31	0
1989 sterile insects, pheromone and insecticides	3/23	1.9	18/23	44

TABLE VI. SUMMARY OF BOLL DATA FROM THE COACHELLA VALLEY PINK BOLLWORM MANAGEMENT STUDY, SHOWING TOTAL LARVAE PER SAMPLE AND NUMBER OF BOLLS SAMPLED

	1986		1987		1988		1989	
Sample week	No. of bolls sampled	Total larvae	No. of bolls sampled	Total larvae	No. of bolls sampled	Total larvae	No. of bolls sampled	Total larvae
1	50	3			370	0		
2	500	3	500	0	726	0	560	0
3	960	2	1700	0	1200	0	720	0
4	1180	0.9	2400	7	1600	1	960	0
5	1520	1	2400	33	2000	0	1520	0
6	1520	3	2500	34	2160	0	1600	1
7	1600	0	2700	11	2320	0	1760	0
8	1520	1	2700	6	2480	5	1564	18
9	1600	1	2700	5	2480	5	1860	39
10	1760	43	1350	25	2480	2	1960	39
11	2200	138	1350	41	2480	0	1240	47

TABLE VII. DATA FROM BOLL SURVEYS SELECTED RANDOMLY FROM FIELDS IN IMPERIAL VALLEY, CA, PARKER VALLEY, AZ, AND ALL COACHELLA FIELDS

Date	No. of larvae per 100 bolls		
	Imperial Valley[a]	Coachella	Parker
1–5 July	—	0	—
6–14 July	7.84	0	—
17–21 July	8.84	0	—
24–28 July	9.5	0.06	3.6
31 July - 4 August	11.40	0	5.4
7–11 August	15.34	1.15	11.05
14–18 August	25.10	1.99	16.2
21–25 August	30.7	1.98	18.86
28 August - 1 September	34.34	3.8	21.89
3–6 September	—	—	23.02
10–13 September	—	—	16.98
24–27 September	—	—	14.15

[a] Data adjusted from Boll Box Survey to equate with standard cutting procedures [4].

started releases of 500 sterile pink bollworms per day. This was done in connection with a minimum shortening of the growing season. Under this system, sterile insect release started at the three to four leaf cotton stage. If a ratio of 60:1 sterile to native insects was not achieved in a given field at a six leaf stage, that field received a pheromone treatment. Thus, while not all fields would receive pheromone, all fields received sterile insects. If the chemical control thresholds were exceeded at any time in the season, the grower and his pest control advisor were expected to utilize chemical control to complete the growing season. This treatment was on a field by field basis, as needed.

Pink bollworm sterile and native populations were monitored with Delta traps at 2 traps per 40 acres (16.19 ha) and with samples of 80–100 bolls collected per field each week. The bolls were then returned to the laboratory for examination under large lighted 3× magnification lenses. Samples were taken from the second week in July through August.

Management trial results: The overall treatment strategies, pheromone usage and insecticide use patterns are shown in Table V. Most important, we achieved major reductions in conventional pesticide usage in the four years that we operated this programme with pheromone and combined pheromone and sterile insect strategies, as shown in the table.

Before 1986, the majority of cotton growers in the Valley used expensive multiple chemical pesticide applications for control of the pink bollworm even though other crops, such as grapes, dates, citrus and vegetables, were considered to be more important and were planted on the better land. Cotton is usually grown on more marginal ground at as low a cost as possible. In the most successful year, 1988, no conventional organic insecticides were used. In 1988, pink bollworm movement from Imperial Valley to Coachella Valley was not significant until the last weeks of August, as detected by traps in the desert between the two valleys. In 1989, Imperial Valley terminated its cotton by ceasing all irrigation in early to mid-August. All the 130 fields were defoliated chemically by 1 September, with only 12 exceptions. Movement was very apparent across the desert from the last weeks of July and throughout August. Our control with minimal insecticide in Coachella Valley was still acceptable, as illustrated by both the pesticide use patterns (Table V) and by low larval populations (Table VI).

It should be noted that, although use of conventional insecticides was being restricted significantly, the overall population levels were held at drastically reduced levels. This is particularly true in comparison to the larval populations in Imperial Valley and Parker Valley (Arizona) in 1989. This should be noted when comparing our data with those from co-operative monitoring projects in Imperial Valley [9] and a Parker, Arizona, project (Table VI) [10].

The larvae population in Table VII are from boll samples taken all season long in 45 fields in Parker, Arizona, 55 fields in the Imperial Valley, California, and in our test fields in Coachella. In Imperial and Parker, all fields were treated heavily with conventional insecticides during this time.

4. SUMMARY AND FUTURE PROSPECTS

The twenty-five year history of the San Joaquin Valley Exclusion Project is considered by the California cotton industry to be a major success. The industry funds their programme at more than 85% of its total cost and has opted to spend US $2.1 million for two new rearing buildings, with options for major expansion in order to continue this programme.

In addition, these same growers and the USDA are pursuing an expanded trial most likely to be in the Imperial Valley. This valley is ideal as a target for expanding these techniques because it normally has much larger acreage than Coachella and is, as a cotton growing area, under extreme duress. Normal cotton production of

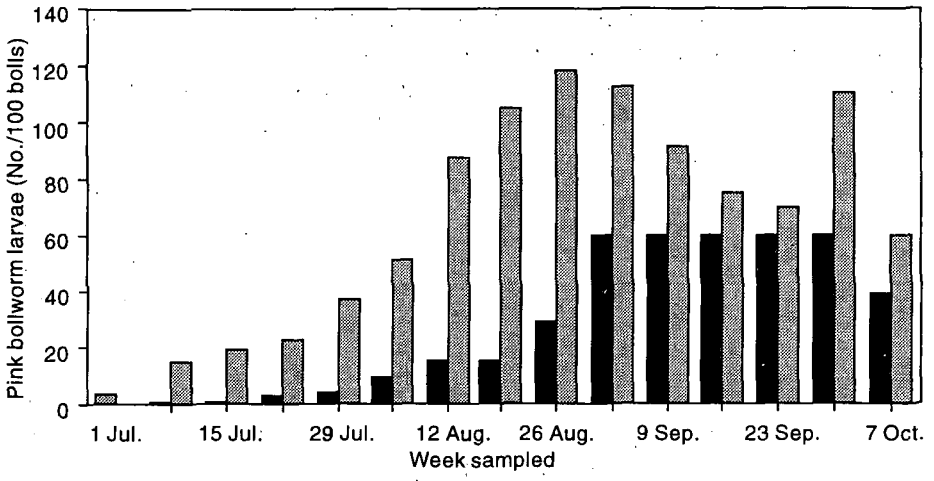

FIG. 1. Comprehensive larval infestation in the Imperial Valley before and after reduction in season length expressed as larvae per 100 bolls (■ : 1990; ▨ : 1989).

40 000–80 000 acres (16 188–32 376 ha) has been the rule over past years. Cotton production has slipped to approximately 4000 acres (1618 ha) largely due to the extreme cost of treating pink bollworm infestations and a new biotype of the whitefly, Bemisia. In addition, growers in this community have opted to drastically shorten the growing season, which has had a marked impact on the pink bollworm population. Our laboratory, in co-operation with the Imperial County Commissioner of Agriculture and the Agricultural Research Service, monitored the pink bollworm population extensively in 1989 before any winter cultural programme could affect the population of that year. Populations were thus mentioned in an identical fashion in the season of 1990, following a mandatory fall termination in 1989. The populations have been drastically reduced, as evidenced by adult moth counts in Delta traps and boll infestation data (Fig. 1) [11]. In addition, test releases in 1990 clearly indicated that a 500 sterile moth per acre per day release was feasible.

The importance of these approaches and the necessity of finding a better management scenario are imperative in today's ecologically concerned society. We can no longer live with major insecticide usage for pink bollworm and its associated secondary pest problems. The introduction of a new biotype of whitefly has further drastically affected the entire agricultural ecosystem. In addition, we cannot keep ahead of insecticide resistance for key pests such as the pink bollworm and the whitefly now found in cotton. We must reduce environmental hazards from pesticide use, including drift and residue. The pink bollworm problem, with today's knowledge, can only be dealt with on an organized, area wide, rational basis.

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IMPLEMENTATION OF THE STERILE INSECT RELEASE PROGRAMME TO ERADICATE THE CODLING MOTH, *Cydia pomonella* (L.) (LEPIDOPTERA: OLETHREUTIDAE), IN BRITISH COLUMBIA, CANADA

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Abstract

IMPLEMENTATION OF THE STERILE INSECT RELEASE PROGRAMME TO ERADICATE THE CODLING MOTH, *Cydia pomonella* (L.) (LEPIDOPTERA: OLETHREUTIDAE), IN BRITISH COLUMBIA, CANADA.

The sterile insect release (SIR) programme to eradicate the codling moth, *Cydia pomonella* (L.), from the Okanagan region by the year 2000 has begun. The SIR programme includes about 8000 ha of apple and pear trees. In many orchards, the cessation of insecticidal sprays for codling moth control should permit apples to be grown without pesticide applications during the fruit development period, a major environmental and economic benefit. Research done by M.D. Proverbs and colleagues over twenty years has established techniques for rearing, sterilizing and releasing codling moths. However, the SIR costs estimated from a pilot project were more than twice those of chemical sprays to control the pest. Nevertheless, recent studies have shown that a programme would be economical if only the minimum required number of moths was released, if the eradication area was treated in steps and if reinfestation was prevented. The British Columbia Fruit Growers' Association helped to develop an implementation plan which included a budget, a revenue scheme and a political and administrative framework. The plan was approved by the municipal governing bodies in the region, as well as the Provincial and Federal Governments. Enabling legislation was passed in 1989. Funds for equipment and a rearing facility to produce about five million moths per week were provided by the two senior governments, and the municipal governing bodies will collect property taxes and parcel taxes based on the orchard area to cover the operational costs. The first phase of the programme, wild population reduction, started in 1992; the second, sterile moth release, will begin in 1994, and the third, prevention of reinfestation, will start in 1997. Recent improvements in the rearing procedures will increase efficiency and production security, and reduce worker health hazards. The integrated pest management systems in apples and pears may require some changes when the moth release phase of SIR begins.

1. INTRODUCTION

Thirty years of research and planning have finally developed into the sterile insect release (SIR) programme to eradicate the codling moth, *Cydia pomonella* (L.), by the year 2000 from the Okanagan region of south-central British Columbia, Canada. This region includes about 8000 ha of apple and pear trees, mostly in commercial orchards. Besides the Okanagan Valley, portions of other valleys and districts are included, e.g. the Similkameen Valley, Salmon Arm and Creston, to incorporate all the areas which grow commercial fruit and exchange fruit and fruit containers.

The codling moth is regarded in this region as the most important pest of apples, and as a major pest of pears. Avoiding codling moth damage, and reducing the use of expensive hazardous insecticides, are of great monetary and environmental benefit to growers and consumers. There is potential for the development of pesticide resistance in the pest population, and restrictions on pesticide use are becoming more common. In many orchards, it is expected that the cessation of chemical sprays for codling moth control will permit apples to be grown without spraying any pesticides during the fruit development period, and such apples could have a marketing advantage. A SIR programme will reduce the need for increasingly costly, foreign made chemical insecticides, while encouraging local employment.

2. BACKGROUND RESEARCH

Research done over twenty years by M.D. Proverbs and colleagues at Agriculture Canada's research station in Summerland has established the techniques for rearing, sterilizing and releasing codling moths [1, 2]. Related research has been conducted in the Yakima Agricultural Research Laboratory (United States Department of Agriculture) in the neighbouring state of Washington, USA [3]. In the pilot project (1976–1978) [2] that was the culmination of Proverbs' research, about 500 ha of apple and pear orchards in the Similkameen Valley were treated, first with insecticides to reduce the wild moth population, and then with reared sterile moths to eradicate the pest. The technology was effective in rearing and releasing the sterile moths, which appeared to eradicate the wild population, at least in some local areas. The project was considered to be a field success, and received strong grower support.

In the pilot project, about 1 000 000 moths were reared each week on a sawdust based artificial diet [2, 4]. About 10% of the moths were put in oviposition cages to mate and lay eggs for the next production cycle, while the remaining 90%, both sexes in approximately equal numbers, were released after exposure to

35 krad[1] gamma radiation in a ^{60}Co irradiator. This dose induced about 92% sterility in the male moths and complete sterility in the females. The diet included Calco red dye, which made the internal organs turn red. (The organs of the wild moths are yellow in colour, and therefore the origin of the moths caught in field traps can be determined by this colour difference.) Chilled moths were released via a specially designed dispenser mounted on dune buggies that were driven through the orchards. The desired sterile:wild ratio of 40:1 was, on average, exceeded.

3. ECONOMIC STUDIES

After the pilot project was completed, analysis of the annual costs showed that these were more than twice those of chemical sprays to control the pest [2]. Such a high price for SIR, despite its environmental benefits, was considered unacceptable to growers and precluded implementation. However, there were those in research and amongst the growers (especially organic growers in the Similkameen Valley) who hoped that another review of SIR, which would include a wide range of economic options and a long term view, might yield a positive result. Agriculture Canada, notwithstanding the unfavourable economic implications of the pilot project, decided to fund several studies by agricultural economists to review codling moth SIR and reassess its commercial feasibility [5–7].

3.1. The Holm reports [5, 6]

A complete review of the strategy, methodology and costs of SIR was made. Various scenarios for a possible future programme were considered. The concept of three years of moth release followed by three years of monitoring [2], with the expectation that the cycle would be repeated, was abandoned in favour of assuming that eradication would occur after three release years and that post-release protection activities against reinfestation could maintain the pest free status. To reduce capital costs, a small rearing facility was proposed which could produce enough moths to service only one-sixth of the total area. At three years of release for each of the six zones, 18 years would be required to eradicate the moth from the whole area. To further reduce costs, only the minimum required number of moths estimated by Proverbs [8], plus 25% to cover the rearing risks, would be produced. The SIR costs were compared with the chemical control costs, and no environmental costs or savings were considered per se because of the difficulty of estimating such figures. The encouraging conclusion of this study was that a commercial eradication programme would be feasible economically in the long run if changes in the strategy and operations were made.

[1] 1 rad = 1.00×10^{-2} Gy.

The second report [6] described sensitivity analyses of the various risks and uncertainties in a SIR programme, assessing what impact changes in the various components of the programme would have on its final economic feasibility. The major aspects considered were the risk of contamination or disease reducing moth production, the release ratio and, therefore, the number of moths to be reared in relation to the somewhat unknown size of the wild population, the method of moth release, the cost of chemical sprays, the risk of other pests requiring chemical sprays, and the risk of reinfestation after eradication. The results of the analyses indicated that the number of moths required for field release, and the cost of chemical sprays, were the most important aspects in assessing the cost benefits of SIR over insecticidal treatments. Ground release of moths was more cost effective than aerial release with rented helicopters. Other aspects had only a minor impact on SIR economics, but the programme would become uneconomical if the apple maggot became established and required frequent sprays for control, since the codling moth would be controlled partially by these sprays.

3.2. The Hansen report [7]

This report continued the analysis of a commercial SIR programme. A larger production facility and larger zones (three zones instead of six) provided a more economical result than in the previous model, and also shortened the time period needed to treat all the orchards. A benefit–cost ratio between 1.1 and 1.4 was expected. The cost of chemical sprays, and the average number of sprays applied per year, were reduced. Motorcycles with sidecars were chosen as the most cost effective vehicles for ground release.

4. IMPLEMENTATION PLAN — THE DEBIASIO REPORT [9]

Since the effectiveness of SIR technology had been demonstrated in the pilot project [2], the economic studies had given positive predictions about the financial robustness of a codling moth SIR programme, and the benefits to apple and pear growers were both monetary and environmental, the British Columbia Fruit Growers' Association decided to investigate the feasibility of implementing a programme over the whole area represented by its members. The growers obtained a grant from the Federal and Provincial Governments to hire a consultant to prepare an implementation plan. The plan was to recommend practical operating procedures that included economies of scale, to show the time relationships between the different activities, to review programme costs, to develop a revenue scheme, to prepare a budget for each year of operation, and to propose a political and administrative framework for the programme.

A committee of growers, government entomologists, economists and horticulturalists and municipal government officials met frequently with DeBiasio to guide the development of the implementation plan.

DeBiasio found that the proposed programme was very strong economically, with a benefit–cost ratio of 1.88 and an internal rate of return of 22%.

The key recommendations in the implementation plan were as follows:

(1) *To request funds from Federal and Provincial agencies for capital expenditures.*

(2) *To obtain revenue for operational costs through local property and parcel taxes.* Since all the members of the communities would benefit somehow from a SIR programme, it was considered appropriate to tax all the properties in the SIR programme area as well as to tax all apple and pear orchards through the parcel tax.

(3) *To collect property taxes for eight years only* to help cover the costs in the initial years of the programme, especially the first five years, when costs would be high and parcel taxes would not yet have been collected in all the areas. The property tax rate would vary from year to year according to the need for revenue, but it would be rather low — possibly ranging from Can. $0.01 to Can. $0.26 per Can. $1000 of property value (not including improvements).

(4) *To base parcel taxes on the number of hectares planted to apples and pears.* The parcel tax would commence when the sterile moths were being released, and continue indefinitely. Through the parcel tax, the growers would pay by far the largest part of the operational cost. The proposed parcel tax rate (Can. $100/ha in 1988 dollars) was intentionally set lower than the average cost of spraying chemicals (Can. $126/ha) so that growers would realize an economic advantage. (The spray cost was based on 2.5 sprays per season for the codling moth, and included the cost of insecticide, the operating cost of application equipment and owner labour cost.) DeBiasio proposed that the moths be released for three years in one-half of the area (Zone 1), and then in the second half (Zone 2) for the next three years, each zone containing about 4000 ha of orchard. (A natural break between orchard areas was identified as the line between zones.) The Can. $100/ha parcel tax should be levied for six years to growers receiving the moths first (Zone 1, the area where the codling moth causes the most damage), and for three years to growers receiving the moths last (Zone 2). After that, when eradication would have been achieved, the rate would be reduced progressively to pay indefinitely the cost of maintaining the area free of codling moth.

(5) *To obtain special legislation from the Provincial Government to establish the Okanagan–Kootenay Sterile Insect Release Board within the political and administrative framework of regional districts*, units of local municipal govern-

ment covering both the rural and urban areas. Five regional districts (Okanagan–Similkameen, Central Okanagan, North Okanagan, Columbia–Shuswap and Central Kootenay) would work together, each district appointing one member of its board of elected officials to represent it on the SIR Board. Other people representing various interested parties, such as grower organizations and Federal, Provincial and municipal agricultural departments, should be included as non-voting Board Members. The SIR Board would be empowered to levy and collect the property and parcel taxes through the regional district taxation system. Also, the Board should have authority to operate and administer the SIR programme, to enter property as required, and to enforce the programme activities.

(6) *To construct a rearing facility to produce moths for 4000 ha at a time.* Two release zones, instead of three or six, would enable all the growers to receive the benefits of the programme within a reasonable time. However, making two zones instead of one would still allow for a relatively small, and therefore relatively inexpensive, rearing facility. The facility design provided was only a sketch based on enlargement of the existing research facilities, with some rearrangements to improve the sanitation and material flow and to reduce the costs.

5. SIR PROGRAMME PHASES

There are three distinct phases of the programme: prerelease sanitation, rearing and release of sterile moths, and protection against reinfestation. For a given zone, the three phases follow each other in time, and operations in one zone are independent of those in the other zone. The current programme schedule is as follows:

Zone 1: 1992–1993, prerelease sanitation; 1994–1996, rearing and release of sterile moths; 1997, protection against reinfestation.

Zone 2: 1995–1996, prerelease sanitation; 1997–1999, rearing and release of sterile moths; 2000, protection against reinfestation.

5.1. Prerelease sanitation (two years)

Since the efficiency of SIR technology increases when the number of sterile moths becomes much greater than that of wild moths, the goal of prerelease sanitation is to reduce the wild moth population as much as possible using other control methods, primarily the application of insecticides.

Apple and pear trees are located, mapped and then categorized as abandoned, non-commercial or commercial orchard trees. Abandoned trees provide a refuge for

codling moths and must be cut down. Few abandoned trees exist in Zone 1 because of the low rainfall, but in the wetter Zone 2 there is a significant number of abandoned trees.

Non-commercial trees are found primarily in urban areas, in backyards and in very small orchards. It is difficult to monitor the wild population size in these trees, so control actions need to be prophylactic rather than remedial. The recommended methods of control include well timed chemical sprays, blossom or fruit removal early in the season, and placement of corrugated cardboard strips around the tree trunks and limbs to trap the mature larvae searching for cocooning sites.

Commercial orchard trees are monitored for moth density by using sex pheromone traps. The SIR programme employs monitors travelling in all terrain vehicles (ATVs) to count the moths caught in the traps each week. Trap data and control recommendations are given weekly to orchardists to enable them to apply appropriate and timely control tactics.

Release of marked sterile male moths into orchards followed by sex pheromone trap counts of marked and wild moths, especially if done at the peak emergence period of a brood, provide a measure of the sterile:wild moth ratio. This ratio indicates how low the wild population density is, and is a measure of the readiness of an orchard for full scale sterile moth release. Observations in 1990 and 1991 in several Similkameen Valley orchards using this method showed that some orchards were ready, but many were not.

At harvest, field samples of fruit can estimate the level of damage incurred, especially damage that indicates mature larvae have exited and will overwinter in the orchard. Such estimates permit a projection of the number of moths that will emerge in the first brood the following year. Again, such observations in the Similkameen Valley showed that some orchards had low codling moth populations, but others not. On the basis of the number of sterile moths scheduled for release in the SIR programme, to maintain a sterile:wild ratio of $\geq 40:1$, an overwintering larval density of ≤ 100 larvae/ha the previous winter may be acceptable. The damage level that would generate such a larval density is hard to determine because of the variable crop sizes and the kind of control tactics used, but codling moth damage levels of 0.1–0.3% may be acceptable, depending on the particular orchard. Many growers who spray insecticides appear to achieve codling moth damage levels of 0.2–0.3% [10]. Proverbs advocated a very low prerelease damage level of 0.05% [2]; such a level is desirable if it can be achieved with existing control technology.

5.2. Rearing and release of sterile moths (three years)

The production goal of the SIR programme during peak production is 5.2 million moths per week derived from 10 500 trays of diet. After emergence, the male and female moths will be collected and then released from ATVs within one or two days in orchards. Moths for 1 ha will be released while the ATVs travel 300 m

between the tree rows. Releases will be carried out twice a week in each orchard, and continue for about 20 weeks to cover both codling moth broods. Sex pheromone traps, set up periodically for one night only in selected orchards, will catch both sterile and wild male moths and provide an estimate of the sterile:wild ratio.

The new rearing facility will produce 4 000 000 moths per week for release. If 4000 ha are treated twice a week, then each hectare of orchard will receive 500 moths at each release, and over a 20 to 25 week season up to 25 000 moths. Since up to 36 500 moths/ha were released in the pilot project [2], admittedly achieving high sterile:wild ratios, it is very important that the current SIR programme emphasizes prerelease sanitation as well as moth production and quality in order to achieve the goal of eradication within the targeted time period.

If 100 larvae/ha overwinter in an orchard, only about 50 larvae will survive the winter and emerge as adults during the spring brood. If temperatures are hot, up to 20 moths (10 males, since the sex ratio is 1:1) might emerge within one week. If 500 sterile male moths are released per hectare per week, then the sterile:wild ratio is a satisfactory 50:1 in the week of the brood when keeping a high ratio would be the most difficult; in other weeks, the ratio should be even higher.

A mathematical model [11], based on the sterile insect technique work of E.F. Knipling, indicates that an average sterile:wild ratio of 40:1 in the first brood leads to extinction of the wild population in a 4000 ha zone by the end of the third brood (1.5 years) if certain assumptions are true. These assumptions are: that the natural population growth rate in the first brood each year is 5, and in the second brood 10; that sterile male competitiveness is the same as in wild males; that male and female sterility are both 100%; and that the sex ratio is 1:1. In the SIR programme, the radiation dose that may be used, 35 krad, causes only 92% sterility in male moths. As a result, there will be more surviving eggs and larvae in each brood than expected, and this will cause some fruit damage but, since the mortality of these larvae will be higher than usual and most will be males [12], the number of female moths produced in each brood will be similar to what the model predicts. Also, F_1 sterility of the few adult progeny from sterile male matings with fertile females will reduce the threat to population growth, since the progeny will be virtually sterile [12]; they will behave like released sterile moths.

Releases in urban areas will be done from special release boxes that allow the moths to escape but prevent birds from entering the boxes. Experimental releases in the city of Penticton indicated that placement of boxes every two blocks should provide a good distribution of moths.

5.3. Protection against reinfestation (indefinitely)

Three major activities will be required after eradication — monitoring for the presence of wild moths, releasing sterile moths at border sites to prevent invasion of wild moths, and controlling the transfer of infested fruit containers.

Monitoring with sex pheromone traps must be continued indefinitely in the whole eradication area. Should even one wild moth be trapped, immediate action could be needed to eradicate the possible new infestation. Therefore, the colony would have to be maintained so that sterile moths could be released at any time.

There is one major corridor of reinvasion into the Okanagan region, i.e. the border area at Osoyoos, where US orchards lie adjacent to Canadian orchards. To prevent fertile moths in the USA from flying into Canada, the programme will release sterile moths on the border, or even in the nearby US orchards, to create a zone that is free of wild moths. There may be other corridors of reinvasion at the periphery of the SIR area, and if wild moths are found entering the eradication zone through these corridors, they would also have to be treated with sterile moths. One mitigating factor that will slow down any reinvasion process is the sedentary habit of female moths. While males fly some distance, perhaps kilometres, females usually fly only short distances, perhaps metres, in their lifetime. Reinvasion is unlikely in the Okanagan region because of the narrow host range of the codling moth (virtually only found on apples and pears, which are irrigated crops in a dry region), and the narrow, deep valleys in which these crops are grown.

Considerable transfer of large fruit bins, used to transport fruit from the orchard to the packing house, occurs in the Okanagan region. This transfer could involve long distances, since fruit may be moved from one valley to another. If codling moth larvae emerge from infested apples after harvest, the larvae may spin cocoons in the bin crevices and remain there in diapause all winter. Bins are placed in orchards during the spring and summer after they have been emptied to await harvest, and any larvae would then continue development and adults emerge, ready to infest trees. This threat of reinfestation via bin transfer can be eliminated if the original source of the bins is identified and if they are returned to their source. Heat treatment of bins to kill any resident larvae may be necessary if the transfer of infested bins cannot be prevented, but the cost of treatment could be high.

6. GETTING STARTED (1988–1992)

Following the completion of DeBiasio's report [9] on SIR programme implementation, the leaders of the British Columbia Fruit Growers' Association met several times to obtain clarification and to discuss the implications of the programme. After careful consideration, they agreed that the proposed implementation plan was acceptable.

In the next step the concurrence of all local governments (in the SIR programme area) in the five regional districts was obtained. In 1989, the Provincial Government passed Bill 75, which provided a legal basis for the regional districts to collect taxes for and operate the programme. The SIR Board was formed. A request for capital funds was made to the Federal and Provincial Governments.

In 1990, the Provincial Government requested three consultants to evaluate the programme plans. Two consultants, W.H. Sudlow with experience in screwworm eradication and G.G. Rohwer with experience in Mediterranean fruit fly eradication, reviewed the SIR field operations plans. Both scientists generally approved of the plans. The third consultant, G.G. Hartley, who had experience in rearing many different insect species, reviewed the plans for the rearing facility. Hartley's report recommended significant changes to the building design and rearing operations, especially in relation to sanitation, contamination control, production security, quality control and worker safety and health. A redesign of the building by members of the SIR Board indicated that the cost of the new design would be much greater than that of the old, and that this could create problems in obtaining capital funds.

In 1991, the Federal and Provincial Governments agreed to provide funds for preliminary work on the building design so that more accurate estimates of the construction costs could be made. An engineering firm was contracted to do the work. The preliminary cost estimate for the facility was about Can. $8 million, a good deal more than DeBiasio's [9] estimate of Can. $3 million. The SIR Board and the two governments eventually set the capital budget at Can. $7.7 million. By the end of 1991, the capital funds were assured. Construction of the rearing facility began in 1992 in Osoyoos, at the extreme southern end of the Okanagan Valley in Canada; it should be operational by early 1993. Additionally, in 1992 some staff were hired and the first year of prerelease sanitation was initiated in Zone 1.

7. IMPROVEMENTS IN SIR TECHNOLOGY

The technology used during the pilot project [2] is the basis of the commercial SIR programme. However, some modifications in the technology have been made to improve prerelease sanitation, quality control, production security and the working environment, and to reduce costs.

7.1. Prerelease sanitation

To assist in controlling the codling moth, pheromone mating disruption was tested on an experimental basis in some, especially organic, orchards; the moth populations were usually greatly reduced [13].

Considerable automation and use of electronic equipment have been introduced into programme operations. Trap data are recorded in the field with electronic data collectors and transmitted to the computer at programme headquarters by modem. Staff can then immediately analyse the field data, both through database software and a geographical information system that permits mapping of the moth infestations.

7.2. Rearing

To improve the quality and competitiveness of the reared sterile moths, Hutt [14] reared codling moths under daily fluctuating temperatures. He found that more male moths reared under fluctuating temperatures than under constant temperatures were caught in the sex pheromone traps during cool evenings. We confirmed this finding in a similar experiment in the Okanagan region, and therefore moths destined for release in spring or autumn should be reared under fluctuating temperatures. In another of our experiments during warm weather, however, just as many constant temperature reared sterile moths as released wild moths were caught in the traps, indicating that constant temperature rearing should be acceptable in the summer. This last experiment also showed that reared moths responded to the traps as well as wild moths, indicating that the flight and pheromone responses of the reared males should be satisfactory for a SIR programme.

To maintain vigour in the laboratory colony, and prior to starting a colony in the new SIR rearing facility, infested apples were collected from commercial orchards in the Okanagan region in 1992. For about six weeks, the male moths reared out of these apples were added to oviposition cages, and the same number of colony males removed, to increase the heterogeneity of the colony.

The moths that will form the nucleus of the SIR colony should be free of the codling moth granulosis virus. Efforts are being made to create a virus free colony which will be transferred to the rearing facility.

The competitiveness of reared sterile male moths with wild males in mating wild females appears to be satisfactory in laboratory tests.

A modification made to the existing oviposition cages [15] reduced by 50% the time needed to remove the paper egg sheets each day. Water wicks with reservoirs inside the cages to provide the moths with water for the duration of oviposition took less time to install than inserting wet wicks into the cages every day. A series of experiments revealed the appropriate number of moths to place in these cages in order to obtain the number of eggs that would lead to optimum production in the next brood.

Two methods of rearing will probably be used at the SIR facility: (1) the usual sawdust diet held under long day length for continuous rearing, and (2) a soft diet held under short day length for larvae that will spin cocoons outside the diet, enter diapause and then be stored in the cold until a time of year when large numbers of moths are required.

In the pilot project [2], moths that were emerging at large in an emergence room were collected by attracting them to UV lighted cages on the floor. In an attempt to avoid the health hazards of entering a room with moth scales in the air, to make moth collection less labour intensive and to collect high quality moths, the concepts of this collection system and that used by Stewart [16] for pink bollworm

moths were combined in a prototype automated moth collector that was built and tested, and then further developed for the new facility.

In collaboration with the engineering firm, the new rearing facility was designed to incorporate many features that have improved the quality control, rearing efficiency and production security, and reduced the rearing costs and health and safety hazards to workers.

8. INTEGRATED INSECT PEST MANAGEMENT SYSTEMS

The big change for these systems in apples and pears will be the elimination of chemical insecticides for codling moth control. The most commonly sprayed chemical at present is azinphosmethyl, a broad spectrum organophosphate. Cessation of these sprays, applied after petal fall until near harvest, will have an impact on the control of other insect pests. Also, the application of insecticides for other insects could kill the released sterile moths.

It is expected that the major pests in apple that could require sprays in the summer are the eyespotted bud moth, *Spilonota ocellana* (D. & S.), the white apple leafhopper, *Typhlocyba pomaria* McAtee, and two species of two generations-a-year leaf rollers. Reports from the pilot project [2], work done by Madsen and Downing [17] and Madsen [18], and recent experiments at Summerland indicated that few summer pest problems are likely to require sprays in SIR apple orchards, especially if spring sprays have been applied. Should sprays be needed to save the crop, chemicals such as azinphosmethyl, diazinon and endosulfan will kill some released codling moths, and therefore releases should be stopped for a few days after treatment. This will have little effect on SIR, since wild moths will also be killed by the sprays. An approach to controlling other pests would be to apply chemical treatments in the spring as a first priority and, if needed, biological control agents that would not harm the released moths, such as *Bacillus thuringiensis* (*Bt*), during the summer.

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ADVANCES IN SHEEP BLOWFLY GENETIC CONTROL IN AUSTRALIA

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Abstract

ADVANCES IN SHEEP BLOWFLY GENETIC CONTROL IN AUSTRALIA.

Economic analysis indicates that the benefit:cost ratio of eradicating the sheep blowfly (SBF), *Lucilia cuprina*, from Australia would be highly favourable. One strategy would be to combine trapping with either the genetically impaired female technique (GIFT) or the sterile male technique (SMT), both of which use genetic sexing systems. GIFT strains are complex genetic sexing systems that contain chromosome rearrangements and other genetic mutations. Mass reared females are killed or debilitated because they are homozygous for recessive mutations. Released males transmit the mutations and rearrangements by mating with field females. The genetic death caused by partial sterility of the rearrangements and homozygosis for the mutations in the field is sufficient to cause population collapse, leading to eradication. GIFT strains currently held contain sex linked translocations, eye colour mutations, homozygous-viable pericentric inversions and temperature sensitive lethal mutations. Field trials of GIFT have demonstrated the successful suppression of target populations. However, the mass rearing methods used during the trials are not suitable for large scale use. There is a need for collaboration by entomologists and engineers on R&D to develop cost effective rearing systems. An idea under consideration is a modular facility capable of simultaneously rearing more than one pest species. This could initially be used for SBF eradication, then for the sterile insect technique (SIT) against other major pests such as fruit flies, codling moth and heliothis, and be available for SIT campaigns against any incursions by exotic pests.

1. INTRODUCTION

The sheep blowfly (SBF), *Lucilia cuprina*, is an important myiasis pest of sheep in Australia. The annual costs of prevention and production losses average A $135 million [1].

Insecticide usage against SBF has led to the evolution of resistance to cyclodienes, organophosphates and carbamates [2]. Resistance to the currently used triazines has not yet been reported.

The spectre of insecticide resistance and the success of the sterile insect technique (SIT) against the screwworm in the United States of America led to the initiation of genetic and ecological research into the genetic control of SBF [3].

A review of the ecological studies is beyond the scope of this paper. Nevertheless, their importance in the development of genetic control must be emphasized. They have established a quantitative basis for planning and assessing genetic and other control measures [4, 5]. They have permitted the use of ecological approximations [6] in the assessment of trials, the design of release strains and the evaluation of tactics. Models based on these studies should save many millions of dollars in large scale control programmes.

Until recently, the genetic control programme was guided by the assumption that, while eradication would be feasible in isolated areas, this goal could be excessively ambitious for the entire continent. Therefore, we concentrated on developing systems suitable for long term suppression of SBF populations rather than eradication.

Recently, simulations combining genetic and ecological models suggested that these suppression systems were also capable of eradication [7]. Moreover, at low release rates they could be much more effective than SIT. Thus, both long term suppression and eradication are currently being considered as feasible options.

The paper summarizes these and other developments which have made the genetic control of SBF more commercially attractive.

2. THE GENETIC METHODS AVAILABLE

The genetic methods available for SBF include SIT, the sterile male technique (SMT) and the genetically impaired female technique (GIFT). Genetic sexing systems form the basis of both SMT and GIFT.

GIFT was formerly called the field female killing (FK) system [7]. This system combines chromosome rearrangements with eye colour and other mutations, such that mass reared females are killed or debilitated. The mutations and rearrangements are inherited in the offspring of field females mated by released males (Fig. 1), causing genetic death, leading to population collapse [7, 8].

The systems constructed in SBF contain homozygous-viable pericentric inversions on the same chromosomes as the eye colour mutations, opposite the sex linked translocation which carries the wild type eye colour genes [8]. One strain also contains a temperature sensitive lethal (*tsl*) mutation.

The inversions stabilize the strain against genetic breakdown and contribute to genetic death in GIFT systems because crossing over within heterozygotes generates inviable products [8].

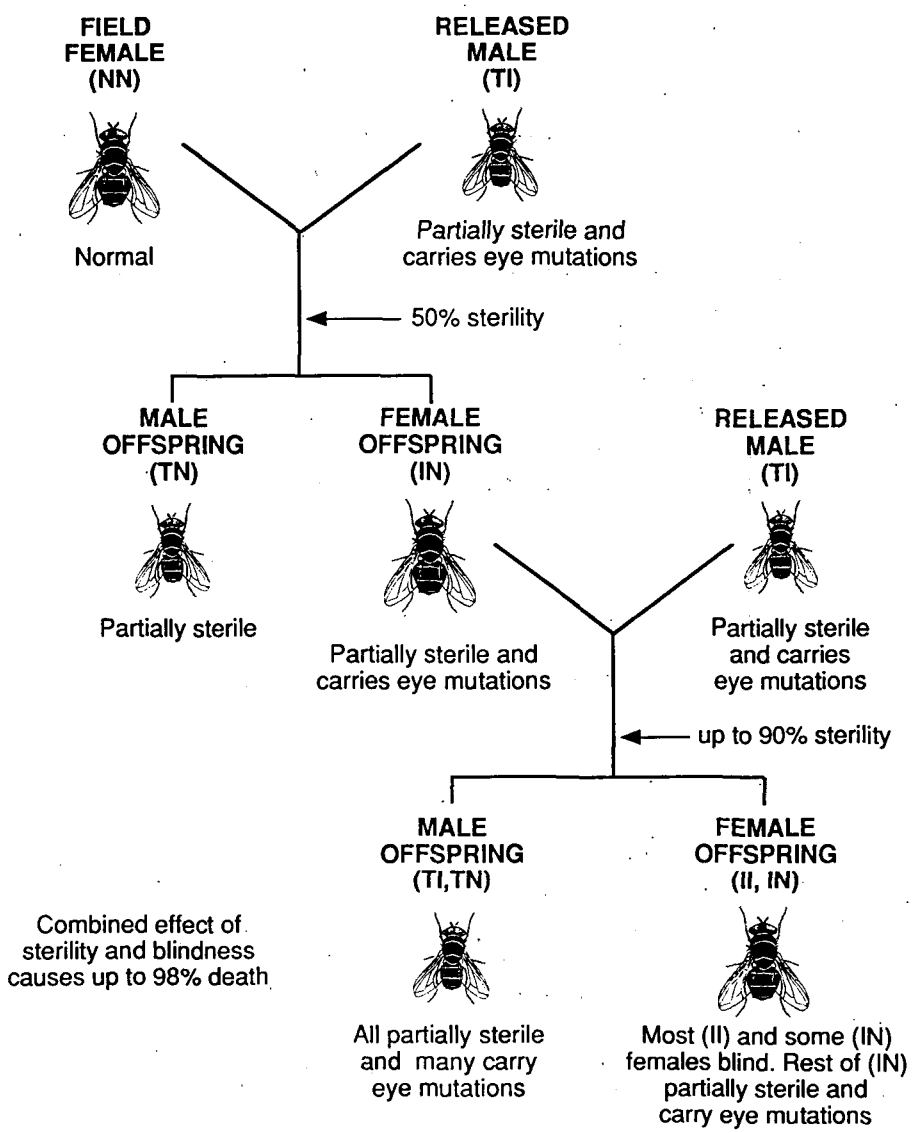

FIG. 1. Proposed GIFT genetic control system: transfer of genes and chromosomes into a field population. N represents the normal autosome set; I represents the autosome set containing homozygous-viable inversions and eye mutations on both chromosomes 3 and 5 (heterozygotes fertile in males, semi-sterile in females), passed to the daughters of released males; T represents the sex linked (Y;3;5) translocation (semi-sterile), passed to the sons.

The SMT would use a simpler genetic sexing system, involving a translocation between the Y chromosome and only one autosome, plus a single eye colour mutation, a *tsl* mutation and a pericentric inversion.

Both SIT and SMT would involve the sterilization of males by irradiation before release, whereas GIFT males cause genetic death without irradiation. It is possible that with SIT or SMT, released males would suffer from radiation induced loss of competitiveness, whereas GIFT males would not. This question has not been investigated adequately in SBF.

SMT and GIFT should be cheaper to implement than SIT because females can be eliminated using the *tsl* mutations at an early stage of mass rearing, thereby reducing both capital outlays and operating costs.

In addition, stringent safeguards against the escape of fertile flies from a rearing operation, as required with SIT, would be less necessary with GIFT or SMT. The female flies in these cases would be debilitated and unable to survive in the wild. Any males escaping the factory would be either identical to those being released (GIFT) or at least generate partial sterility in their daughters (SMT).

3. SIMULATION OF GENETIC CONTROL

The GENCON simulation programs [6–8] were developed for a variety of uses.

3.1. Comparison of genetic and sterile male alternatives

Simulations predict that at high release rates SIT should cause more rapid suppression than GIFT [7]. On the other hand, at low release rates GIFT should give more rapid suppression and earlier eradication than SIT (Fig. 2).

However, if releases cease before eradication, the predicted recovery of target populations is more rapid following SIT than with GIFT [7].

The better performance of GIFT strains at low release rates or when releases are interrupted is due to generation in the target population of males similar to the mass reared GIFT males (Fig. 1).

3.2. Assessment of the potential of inversions for genetic control

One edition of GENCON [8] was designed to evaluate GIFT systems containing pericentric inversions before investing the resources necessary to make these rearrangements [9]. The simulations predicted that inversions would give both faster suppression initially and greater persistence of suppression if the releases were interrupted than GIFT systems without inversions.

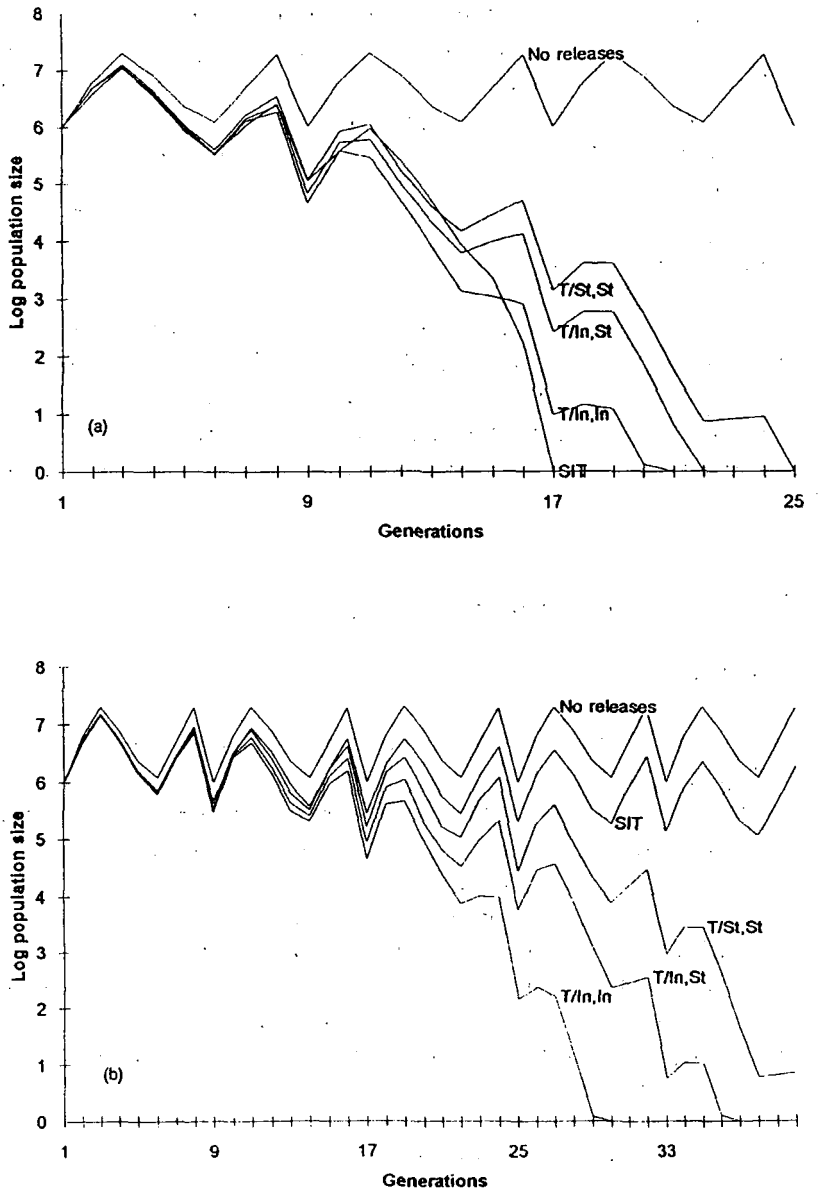

FIG. 2. Simulations of autocidal control using SIT or GIFT strains carrying a T(Y;3;5) translocation. Initial population (one million) flies of each sex, with the rate of increase in each generation (eight per year) influenced by simulated seasonal conditions and density [8]. GIFT strains: T/St,St — standard (non-inversion) chromosomes, heterozygous for three eye colour mutations; T/In,St — chromosome 3 inversion only, heterozygous for two eye mutations; T/In,In — chromosome 3 and 5 inversions, heterozygous for two eye mutations. Release rates: (a) 0.6 million males per generation; and (b) 0.3 million males per generation.

Simulations to illustrate the different potentials of SIT (or SMT) and GIFT are presented in Fig. 2. At a release rate of 0.6 million males per generation (Fig. 2(a)), SIT results in eradication within two years; GIFT strains with two, one or no inversions take progressively longer. At half this release rate (Fig. 2(b)), SIT does not give eradication but the GIFT strains do, with the rate of suppression increasing with the number of inversions.

3.3. Analysis of field trial data

Field trials of GIFT carried out in the 1970s did not achieve suppression of target populations. Initially, this was attributed to an inappropriate release method [10], but simulations using an analytical edition of GENCON suggested that these trials were also influenced by the immigration of wild flies, density dependent effects [6] and lower survival of the sons of released males in the field, possibly due to the dieldrin resistance mutation they carried [11].

4. GENETIC STUDIES AND STRAIN DEVELOPMENT

4.1. Male recombination

Genetic sexing strains deteriorate following genetic exchange in males and selection favouring recombinant genotypes. High male recombination frequencies in two Y linked translocation strains led to the theory that breakage of the Y chromosome caused increased male recombination [12]. However, studies in other species did not appear to follow this rule [13].

A reinvestigation of this problem in SBF showed that the frequency of male recombination was independent of the presence or type of translocations [14]. Variability in genetic backgrounds seems to be the most likely cause of the differences in the male recombination frequencies seen in some crosses. We concluded that strain stability in our sexing systems could not be improved by the selection of particular types of translocation.

4.2. Use of chromosomal inversions to stabilize strains

The best cure for male recombination appears to be that adopted by mosquito geneticists, i.e. including inversions in genetic sexing systems [15].

Inversions can be used in several ways, depending on the specific use intended for the sexing system [16]. We chose homozygous-viable pericentric inversions, since these have several advantages over other types of inversion and can be used in both GIFT and SMT strains. One advantage is that the inversions and translocation can be on separate chromosomes, and can readily be changed if necessary.

The products of recombination within heterozygous, pericentric inversions are usually inviable. Thus, including such an inversion in a release strain ensures the selective elimination of the recombinant products in males.

In heterozygous females, recombination within a pericentric inversion can cause up to 50% semi-sterility. In GIFT systems, all the daughters of released males are heterozygous for the inversions and are therefore partially sterile, increasing the level of genetic death available.

Including a pericentric inversion on both of the non-translocated autosomes in a GIFT strain causes up to 75% semi-sterility in the daughters of released males, giving maximum genetic death rates of approximately 98% when combined with two eye colour mutations and the translocation (Fig. 1).

The fertility of mass reared strains is not affected by the inversions. In heterozygous males, the frequency of recombination is too low to cause observable sterility. In homozygous females, fertility is not affected because crossing over does not generate inviable products.

4.3. Isolation of homozygous viable inversions

A search for homozygous-viable pericentric inversions on chromosome 3 yielded four, one of which is suitable for inclusion in GIFT systems [9].

Small scale field trials demonstrated that released males carrying this inversion were as competitive at mating released sentinel females as released non-inversion males [17].

A similar search on chromosome 5 yielded four large pericentric inversions from 6139 screened chromosomes, of which one is homozygous viable [17].

4.4. Isolation of new genetic sexing translocations

All the sexing systems tested so far have descended from a translocation constructed to carry the dieldrin resistance mutation *Rdl* to permit early killing of females in mass rearing [18].

However, dieldrin treatment proved harmful to the released males [19]; the *Rdl* gene also reduces fitness in the field [11]. Hence, a search was undertaken for suitable translocations without this gene.

GIFT translocations involve the Y chromosome and two autosomes, each carrying the wild type allele of a selected eye colour mutation: chromosome 3 (white) plus either chromosome 5 (topaz) or 6 (yellow). They must have a higher fertility than the 25% usually associated with three chromosome translocations (a fertility of approximately 50% gives a reasonable compromise between suppressive potential in the field and fecundity in mass rearing) [7].

For SMT genetic sexing systems, selection of translocations with a fertility approaching 100% should be the goal.

More than two hundred translocations were screened over a three year period [17]. First, two chromosome translocations were screened for near centromeric break points. Experience suggests that these are more likely to be highly fertile than other translocations. We assume that this is related to the positions of the break points with respect to the euchromatic male meiotic pairing sites [20, 21].

These were then screened for high fertility (70–100%). Two highly fertile translocations have been retained for possible use in SMT systems. These and several other translocations were also reirradiated and screened genetically for inclusion of a third chromosome in the rearrangement. These were then screened as above, first cytologically and then for 40–60% fertility.

Four translocations which fit the selection criteria for GIFT were saved. One has given encouraging results in preliminary field mating tests [17].

4.5. Isolation of *tsl* mutations

Temperature sensitive lethal mutations, which die at one temperature but survive at another, can be used to eliminate females from mass reared strains prior to release.

The mutagen ethyl methanesulphonate (EMS) was used to treat flies, since this chemical gives a higher proportion of *tsl* mutations than mutagens such as radiation or alkylating agents [22].

Treated males homozygous for the chromosome 3 white eye mutation were crossed to a balancer chromosome [9] in a scheme designed to make the treated chromosomes homozygous. In the final generation of this scheme, eggs were incubated for 24 h at 35°C and then reared at 27°C. If no lethal mutation had been generated on the treated chromosome, 1/3 of the offspring would be white eyed and 2/3 wild type. The absence of white eyed offspring indicated a lethal gene on chromosome 3. Such families were then screened for survival when hatched at 27°C.

Of the 318 chromosomes tested, 62 lethals were found, two of which were *tsl*. One *tsl* was too weak to be useful, and the remaining one was saved for incorporation with a Y autosome translocation into a genetic sexing strain [17].

This strain gives 99% males when the eggs are incubated for 24 h at 35°C and 67% males at 27°C, compared with 57% males in a non-*tsl* strain with this translocation. Male production averages 27% less at 35°C than at 27°C, which may indicate a dominant effect of the *tsl* [17]. This is being investigated further.

We are mapping the *tsl* mutation genetically and will try to cross it on to an inversion bearing chromosome. If this is not possible, it may be necessary to isolate a new *tsl*, starting with an existing homozygous viable inversion.

5. FIELD TRIALS

In the 1980s, two field trials demonstrated the suppression of SBF populations using GIFT. In these trials, migration was reduced by better geographical barriers than in previous trials, and suppression of target populations to low levels was achieved [23, 24].

In a mainland trial (240 km^2) conducted in 1984–1985, a genetic death rate averaged 50% suppressed populations to below the spring emergence level for the entire season, unlike nearby areas where the fly densities increased by factors of 50–80 [23, 24].

In an island trial (40 km^2) carried out in 1985–1986, genetic death rose to 90% after six months of releases [23, 24]. The population was suppressed to undetectable levels after eight months and remained at very low levels for nearly one year after releases ceased [23].

A larger island trial (1990 km^2) was conducted in 1989–1991 in the Bass Strait. A substantial reduction in the target population occurred in 1989–1990 [25], but problems in the mass rearing programme [26] reduced the release numbers at critical times, preventing useful suppression in 1990–1991.

The main causes of irregular mass rearing production included inadequate facilities and equipment and lack of appropriate criteria for controlling the critical processes. This has led to recognition of the need for collaboration by entomologists and engineers to develop large scale rearing systems.

6. ECONOMIC CONSIDERATIONS AND COMMERCIAL PROSPECTS

Economic analysis indicates that when the technical problems with mass rearing are solved, the benefit:cost ratio of genetic control of SBF will be highly favourable [1]. Analysis of genetic control as a potential business enterprise suggests that it could be attractive to private investors, who would be paid a fee for the SBF eradication service [27].

Considerable interest has been shown by government and private enterprise in development of genetic methods for insect eradication commercially. One approach under discussion would be to first eradicate SBF from Tasmania. This is currently seen as an economically justifiable operation on its own, but it could also be used to develop improved rearing technology suitable for Australia wide eradication.

6.1. Tactical and technical considerations

Broadly speaking, there are three potentially cost effective approaches to the genetic control of SBF: (1) suppression without eradication as a specific goal; (2) local eradication protected by a barrier zone and quarantine measures; and (3) total eradication from Australia.

The above strategies are not mutually exclusive. For example, total eradication would involve a series of local eradications, with a moving barrier between the eradicated and infested areas. Moreover, if a suppression campaign achieved local eradication, the goals could then be modified, either to maintain the eradicated status with a barrier or to proceed to total eradication.

The initial approach adopted will influence decisions such as the choice between SIT/SMT or GIFT, and whether genetic control is centrally organized or decentralized.

GIFT was designed as a low cost suppression system, requiring fewer released males than SIT or SMT. Because GIFT does not require radiation equipment, it could be implemented either as a single large scale operation (e.g. 500–1000 million males per week) or several small scale local operations.

The SIT/SMT options, on the other hand, would require irradiation equipment, which could be prohibitively expensive if installed in a large number of smaller scale operations. These options would necessitate centrally organized, large scale rearing and release operations.

6.2. Economic considerations

A GIFT suppression campaign using dispersed small scale rearing facilities could be implemented with relatively low capital costs for each plant, but lost opportunities for economies of scale would mean greater operating costs than with a central high technology system.

Economic sensitivity assessments suggest that eradication in as short a time as possible, while initially more capital intensive, would provide a more cost effective result than an indefinite suppression programme [1]. In this scenario, SMT would probably be the most profitable genetic control system to adopt.

However, under the current economic circumstances in Australia, the large amount of money (e.g. A $20 million [27]) required to build suitable rearing facilities before any benefit is obtained is seen as too adventurous by some politicians and rural industry groups.

Therefore, it may be expedient to begin with small scale suppression programmes using GIFT strains, possibly at the local government or farmer co-operative level. This approach could transfer significant economic and environmental benefits to the rural community, until such time as improved technology and investor confidence permit the implementation of a large scale eradication programme.

6.3. Combining trapping with genetic control

It is possible that combining trapping with an autocidal technique could be highly cost effective. Trapping is effective in suppressing SBF populations [28]. It

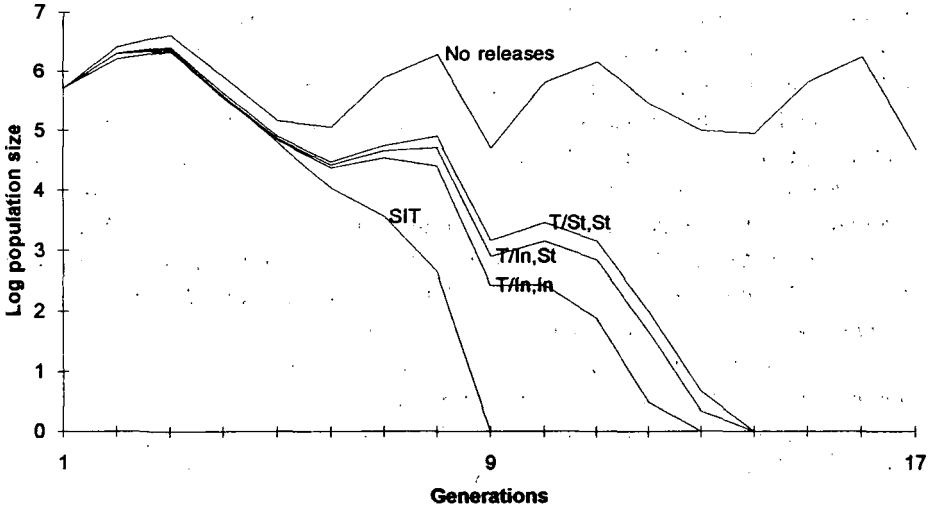

FIG. 3. Simulations of autocidal control with concurrent trapping removing 50% of the flies per generation. The strains and ecological parameters are as in Fig. 2, with a release rate of 0.3 million males per generation.

should be possible to remove half of the SBF population each generation [29], but traditional trapping methods are too costly. However, recent progress in developing a synthetic lure for SBF [30] may change this situation.

Simulations suggest that trapping would not eliminate a population by itself, but would greatly accelerate the rate of eradication by autocidal methods (Fig. 3). In these simulations, the strains and numbers released are identical to those used in Fig. 2(b). Trapping + SIT predicts eradication within one year, compared with no eradication without trapping. Trapping + GIFT predicts eradication times of about 1.5 years, down from 3.5–5 years without trapping.

6.4. Multispecies mass rearing facilities

An idea currently receiving consideration is a mass rearing facility capable of simultaneous rearing of more than one pest species. If this concept is technically feasible, it should be economically more cost effective than a single species rearing plant whose utility would cease on achievement of eradication. The costs of and benefits accruing from its construction could be spread over several sectors of the Australian rural industry. Moreover, an operational facility with trained personnel would be able to respond quickly if an eradicated pest were to reinvade Australia.

This concept has arisen partly out of concern for ensuring Australia's preparedness against possible incursions by the Old World screwworm fly,

Chrysomya bezziana. This species and the New World screwworm fly, *Cochliomyia hominivorax*, have each been detected in Australia once within the last five years, although neither incursion has resulted in establishment [31, 32].

Both the Mediterranean fruit fly, *Ceratitis capitata* (in Western Australia) [33] and the Queensland fruit fly, *Bactrocera tryoni* (mainly in eastern Australia) [34], have been or are currently the subject of SIT programmes aimed at local eradication. An outbreak of *B. tryoni* in Western Australia was eradicated in 1990 [35].

Other potential candidates for autocidal control in Australia are the codling moth, *Cydia pomonella*, and the heliothis species, *Helicoverpa armigera* and *H. punctigera*, both of which attract heavy insecticide usage on various crops.

A multispecies rearing facility would probably be used for SBF eradication in the first instance, then for SIT against other major pests. At the same time it would be available for rapid implementation of a SIT campaign if either screwworm species became established in this country.

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ERRADICACION DEL GUSANO BARRENADOR DEL NUEVO MUNDO

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Abstract-Resumen

ERADICATION OF THE NEW WORLD SCREWWORM.

Historically, the New World Screwworm has been one of the most destructive and costly pests, attacking both domestic and wild warm blooded animals, including humans. Its original habitat was the tropical and subtropical areas of the southern United States of America, Mexico, Central America, two thirds of South America and the islands of the Caribbean. In 1972, the Governments of Mexico and the United States of America set up a commission to eradicate the parasite from northern and western Mexico, down to the Isthmus of Tehuantepec, and to establish a "sterile fly barrier" in that zone. For the purpose of eradication the commission used the combined action of two basic systems — production and dispersion of sterile flies, and field operations. In 1986, in view of the progress made, it extended its screwworm eradication activities to the Yucatán Peninsula and the countries of Central America. Thus, more than 90% of Guatemala's territory is now free from this pest. Belize was declared free from it in June 1992. Eradication operations are in progress in Honduras and El Salvador. In addition, it is planned to conclude agreements with Costa Rica, Nicaragua and Panama. Mexico had officially been declared screwworm-free in February 1991, but there was an outbreak which it is hoped will have been brought under control by the end of 1992. Mention should also be made of the activities which have been carried out to eradicate the screwworm in the Libyan Arab Jamahiriya. As a result of the joint eradication efforts in that region, the Libyan Arab Jamahiriya was declared free from this pest in June 1991. Hence the New World Screwworm eradication programme has basically been successful in the United States of America, Mexico, Central America and North Africa.

ERRADICACION DEL GUSANO BARRENADOR DEL NUEVO MUNDO.

El gusano barrenador del Nuevo Mundo ha sido históricamente una de las plagas más destructivas y costosas que atacan a los animales de sangre caliente, tanto domésticos como silvestres, e incluso al hombre. Originalmente se encontraba distribuído en las áreas tropicales y subtropicales del sur de los Estados Unidos de América, México, Centroamérica, dos terceras partes de Sudamérica y las islas del Caribe. En 1972, los gobiernos de México y de los Estados Unidos de América crearon una comisión con el objeto de erradicar el parásito del norte y oeste de México, hasta el Istmo de Tehuantepec y de establecer una "barrera de moscas estériles" en esa zona. Para la erradicación, la comisión ha utilizado la acción conjunta de dos sistemas fundamentales: la producción y dispersión de moscas estériles y las operaciones de campo. Debido a los progresos conseguidos, en 1986 extendió sus actividades de

erradicación del gusano barrenador a la Península de Yucatán y a los países centroamericanos. Así, Guatemala está libre de la plaga en más del 90% de su territorio. Belice se declaró libre de la plaga en junio de 1992. En Honduras y El Salvador ya se están efectuando operaciones de erradicación. También se proyecta establecer convenios con Costa Rica, Nicaragua y Panamá. México fue declarado oficialmente libre del gusano barrenador en febrero de 1991, pero se produjo un brote que se espera haber controlado antes de concluir 1992. Cabe destacar también las actividades que se realizaron para erradicar el brote del gusano barrenador en la Jamahiriya Arabe Libia. Los esfuerzos conjuntos para la erradicación en esa región dieron como resultado que en junio de 1991 se declaró al país libre de la plaga. Así pués, el Programa de erradicación del gusano barrenador del Nuevo Mundo ha logrado progresos fundamentales en los Estados Unidos de América, México, Centroamérica y norte de Africa.

1. INTRODUCCION

El gusano barrenador del Nuevo Mundo (GBNM) es producto del ciclo biológico de la mosca cuyo nombre científico es *Cochiliomyia hominivorax* (Coquerel). El ciclo de vida del parásito dura aproximadamente 21 días. La mosca hembra ya fecundada pone sus huevecillos en las heridas abiertas de animales o seres humanos, donde después eclosionan y las larvas de ellos emergidas penetran desgarrando el tejido, y se alimentan del líquido que se forma. A los siete días, las larvas caen al suelo, donde se entierran para convertirse en pupas. Después de siete días a dos meses, las moscas adultas emergen para completar el ciclo biológico.

Originalmente este parásito se encontraba distribuído en las áreas tropicales y subtropicales del sur de los Estados Unidos de América, México, Centroamérica, dos terceras partes de Sudamérica y las Islas del Caribe.

Para que el parásito pueda infestar, es indispensable la existencia de una herida, siendo las más comunes el ombligo de los recién nacidos y las producidas por castración, trasquila y marcaje. Los daños causados por el GBNM son enormes, siendo los principales la baja de producción, retardo en el crecimiento, deterioro de las pieles, susceptibilidad a infecciones secundarias e incluso la muerte de los animales. Además de los animales domésticos, también se ven afectados los silvestres de sangre caliente, así como el hombre.

La manera de eliminar el parásito fue descubierta en los años treinta, cuando se observó que la mosca podía ser esterilizada sexualmente utilizando radiaciones, ya que la hembra se aparea una sola vez en su vida y al hacerlo con un macho estéril los huevecillos depositados por ella no nacen, haciendo posible la erradicación.

En 1961 quedó eliminado el parásito del sureste de los Estados Unidos de América. A pesar de los esfuerzos desarrollados, el GBNM continuaba causando daños en las regiones de los Estados Unidos de América y de México, por las constantes migraciones de insectos fértiles a lo ancho de la frontera. Ante esto, los

ganaderos de ambos países solicitaron a sus respectivos gobiernos el apoyo para eliminar la plaga, por lo cual el 28 de agosto de 1972 se formó la Comisión México-Americana para la Erradicación del Gusano Barrenador del Ganado.

2. ERRADICACION DEL GBNM

Para la erradicación del parásito, la Comisión ha utilizado la acción conjunta de los dos sistemas fundamentales siguientes.

2.1. Producción y dispersión de moscas estériles

En 1976 inició su producción la Planta de Moscas Estériles de Chiapa de Corzo, Chiapas (México), que sustituyó a la anteriormente ubicada en Mission, Texas (Estados Unidos de América).

El proceso implantado ha sido diseñado para asegurar una eficiente producción y esterilización de insectos en la cantidad y calidad requeridas. Actualmente la Planta produce 300 millones de insectos por semana, ya que así lo requiere técnicamente el Programa.

Una vez llevada a cabo la esterilización sexual por radiación con ^{137}Cs durante un tiempo comprendido entre 1'49" y 1'52" a 7000 rads, se procede al empaque y envío de los insectos a los Centros de distribución, para su dispersión en las zonas infestadas por medio de aviones, siguiendo patrones previamente establecidos.

Actualmente, debido a la magnitud del Programa, se tiene ubicado el Centro de distribución en el Aeropuerto de Tuxtla Gutiérrez, Chiapas (México), así como Centros de dispersión en Tenosique, Tabasco (México) y en Retalhuleu y Puerto Barrios (Guatemala).

2.2. Operaciones de campo

Las operaciones de campo consisten en la inspección de los animales, a fin de detectar y curar todo tipo de heridas en ellos producidas, así como en el reporte y envío para su diagnóstico de las larvas colectadas, con el consiguiente tratamiento de moscas estériles.

Las rutas de inspección se establecen basándose en la biología del parásito, ya que deben realizarse en circuitos con una duración de hasta 21 días, con el propósito de eliminar el riesgo de la presentación de "reciclajes" de la plaga.

Para proteger de reinfestaciones las zonas ya libres, es necesario mantener un control sobre las movilizaciones de los animales; debido a lo anterior, el Programa estableció en 1984 un sistema de estaciones de inspección y cuarentena, consistente en tres estaciones permanentes localizadas en las rutas pecuarias del Istmo de

Tehuantepec, y apoyadas por otros puntos de inspección temporales dentro de las áreas aún infestadas.

Todos los animales que cruzaban por las estaciones permanentes en dirección este-oeste eran sometidos a inspección minuciosa para detectar la presencia del GBNM, y bañados con una solución larvicida. Cuando se detectaba algún animal infestado o con heridas que podían infestarse, se ponía en cuarentena hasta lograr su curación.

En los ocho años de actividad de las Estaciones cuarentenarias se inspeccionaron 2 688 829 animales, de los cuales se pusieron en cuarentena 1063, detectándose 175 parasitados con el GBNM. Cabe mencionar que en ese período se inspeccionaron 10 703 animales provenientes de otros países, detectándose 6 infestados, a los que se les impidió la entrada en el país.

La implantación de las Estaciones cuarentenarias contribuyó definitivamente a evitar la presencia de brotes del GBNM en las zonas ya libres.

Para el mejor desarrollo de las operaciones antes señaladas, ha sido necesario utilizar como apoyo los medios masivos de comunicación, y establecer lazos de coordinación con los organismos oficiales y privados relacionados con la actividad pecuaria.

3. PROGRESOS Y RESULTADOS

Debido a los progresos del Programa, en 1986 se modificó el convenio que dio origen a la Comisión México-Americana para la Erradicación del Gusano Barrenador del Ganado, con la finalidad de extender las actividades de erradicación a la Península de Yucatán y a los países centroamericanos.

Como resultado de todas las acciones citadas, en julio de 1990 se presentó el último caso positivo en México, por lo cual el 25 de febrero de 1991, México fue declarado libre del GBNM.

La erradicación de la plaga en territorio mexicano se obtuvo después de 19 años de intenso trabajo y con una inversión de 600 millones de dólares de los Estados Unidos, lográndose como beneficio un ahorro para la ganadería de México del órden de 82,6 millones de dólares anuales.

Como consecuencia de la erradicación del parásito en México, durante todo 1991 se realizaron actividades de sobrevigilancia epizootiológica, con el propósito de seguir contando con el apoyo de los productores; así también, se estrechó la coordinación con las diferentes dependencias del sector agropecuario.

De esta manera, el 31 de diciembre de 1991, la Comisión terminó las actividades de campo y de cuarentena en México, quedando a cargo de la sobrevigilancia la Comisión México-Estados Unidos para la Prevención de la Fiebre Aftosa y Otras Enfermedades Exóticas.

Sin embargo, después de 18 meses del último caso, el 22 de enero de 1992 se presentó una muestra positiva en el Estado de Campeche, por lo cual se ha aplicado un programa operativo de emergencia para su control.

Actualmente se han comunicado 41 casos positivos, localizados en los Estados de Campeche, Chiapas, Tabasco, Veracruz y Tamaulipas; en este último Estado se detectó el último caso, el 30 de septiembre, en el Municipio de Aldama.

El origen del brote viene causado posiblemente por la introducción ilegal de animales desde países centroamericanos aún infestados.

Las acciones en el operativo se han repartido, siendo el SINESA (Sistema Nacional de Emergencia en Salud Animal) el responsable de la inspección, detección, muestreo, diagnóstico y control de las movilizaciones de los animales, mientras que la Comisión México-Americana para la Erradicación del Gusano Barrenador del Ganado se ocupa de la producción y dispersión de las moscas estériles requeridas, del monitoreo y tampeo entomológico en las zonas afectadas y de los apoyos técnicos colaterales.

Actualmente la Comisión está dispersando un promedio de 100 millones de insectos por semana sobre las áreas reinfestadas de México.

Con las actividades señaladas se espera establecer un pronto control y erradicación del brote en beneficio de la salud y producción pecuaria nacional.

En lo que respecta a las operaciones en los países centroamericanos, se han logrado avances sustanciales. Así, Guatemala está libre de la plaga en más del 90% de su territorio y se considera que para fines de 1992 lo estará en su totalidad.

En cuanto a Belice, no se han registrado casos positivos desde octubre de 1991, por lo que también se declaró libre de la plaga el 21 de junio de 1992.

De la misma manera, en Honduras y El Salvador ya se están efectuando operaciones de erradicación, con los correspondientes envíos de moscas estériles desde la Planta de Tuxtla Gutiérrez, Chiapas (México).

Tanto en el Programa para Belice como en el de Honduras y El Salvador, se utiliza la técnica de la "mosca aletargada", consistente en transportar y dispersar a granel el insecto, a temperaturas bajas y con control de la humedad; esta técnica elimina la caja y su empaque tradicionales, reduciendo los costos de manejo y dispersión consecuentemente.

El Programa tiene proyectado establecer convenios con el resto de los países centroamericanos, tal como se ha hecho en 1992 con Nicaragua, y se hará con Costa Rica y Panamá en 1993. En este último país se establecerá una barrera de moscas estériles en el Tapón del Darien para evitar migraciones de insectos fértiles desde Sudamérica.

También se tiene planeada la construcción de una nueva planta productora de moscas estériles, la cual podría ubicarse en Costa Rica o en Panamá, de acuerdo con las facilidades que se otorguen. Dicha planta sustituiría a la que actualmente se tiene en Chiapas (México).

Es importante destacar las actividades que se realizaron para erradicar el brote del GBNM presentado en la Jamahiriya Arabe Libia, causado por la introducción de animales infestados. Para la erradicación, la FAO solicitó a la Comisión los apoyos técnicos correspondientes, consistentes en asesoría, capacitación, estudios entomológicos y suministro de moscas estériles.

En consecuencia, entre diciembre de 1990 y octubre de 1991 se enviaron a la Jamahiriya Arabe Libia un total de 1322 millones de moscas estériles, por vía aérea directa de Tuxtla Gutiérrez a Trípoli. Se empezó por dispersar 3,5 millones por semana, pasando después a 28 millones, para llegar en su última etapa a 40 millones semanalmente. Los esfuerzos conjuntos dieron como resultado que el último caso en este país se presentara en abril de 1991, por lo cual se le declaró libre de la plaga el 22 de junio de ese año.

El Programa de erradicación del gusano barrenador del Nuevo Mundo ha logrado progresos fundamentales en la lucha contra el parásito en los Estados Unidos de América, México, Centroamérica y norte de Africa. Sin embargo, para alcanzar la meta contemplada, es necesaria la continua participación de todas las instituciones y personas involucradas, lo que deberá redundar en beneficio de la salud y de la economía pecuarias.

ERADICATION OF THE NEW WORLD SCREWWORM FROM THE LIBYAN ARAB JAMAHIRIYA

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Abstract

ERADICATION OF THE NEW WORLD SCREWWORM FROM THE LIBYAN ARAB JAMAHIRIYA.

The New World screwworm, *Cochliomyia hominivorax* (Coquerel), invaded North Africa in the late 1980s. It became established in the Libyan Arab Jamahiriya, where it was first detected in 1988 and confirmed in 1989. This devastating pest of livestock became the target of a major eradication programme costing approximately US $80 million. The basis of the eradication programme was the sterile insect technique. A total of 1400 million sterile screwworm flies from Mexico were released over a 40 000 km^2 area between December 1990 and October 1991. Total eradication was achieved. The eradication programme included a very strong quarantine effort to prevent expansion of the infested area in the Libyan Arab Jamahiriya. Animals in the infested area were inspected and wounds treated about every three or four weeks. Trapping of adults determined the presence or absence of adult screwworm flies. A major information programme supported the eradication effort.

1. INTRODUCTION

The New World screwworm (NWS), *Cochliomyia hominivorax* (Coquerel), is a native animal parasite of the New World. It is the most serious livestock insect pest in the New World and has been the target of eradication programmes in Mexico, the United States of America, some of the Caribbean islands and, currently, Central America. [1, 2].

The NWS is an obligate parasite of warm blooded animals. The larval stage attacks only living flesh. A gravid female NWS fly is attracted to the wounds of

warm blooded animals, where she deposits eggs in clutches of about 20–200 (Fig. 1). The eggs are laid along the edge of the wounds, which can be as small as a tick bite. The navel of newborn is a favourite site for deposition. Also, management practices such as dehorning, castration and branding are excellent NWS oviposition sites. In NWS endemic areas, management practices are to a large extent regulated by NWS seasonal populations. Thus, births, castration, dehorning, branding, etc., are timed for when the NWS population is at its seasonal low period.

Individual livestock owners can effectively reduce or eliminate losses resulting from NWS attack. However the cost is very high, particularly for labour. During the season of the year when NWS populations are sufficiently large to result in losses of livestock, each animal must be inspected individually at least two times per week and all wounds, whether NWS infested or not, must be treated with the appropriate insecticide to kill the worms in the infested wound and to prevent an infestation (Figs 2 and 3).

The eggs that are laid on the wound hatch within 24 h and the larvae feed on the living flesh and exudate from the damaged flesh. Feeding within the host continues for five to six days during which time the larvae enlarge the wound significantly. The odours emanating from the wound are attractive to gravid females, who may lay additional eggs and thus greatly aggravate the situation. When mature, the larvae leave the wound and burrow into the ground and pupate. During warm

FIG. 1. A wound which is susceptible to screwworm attack.

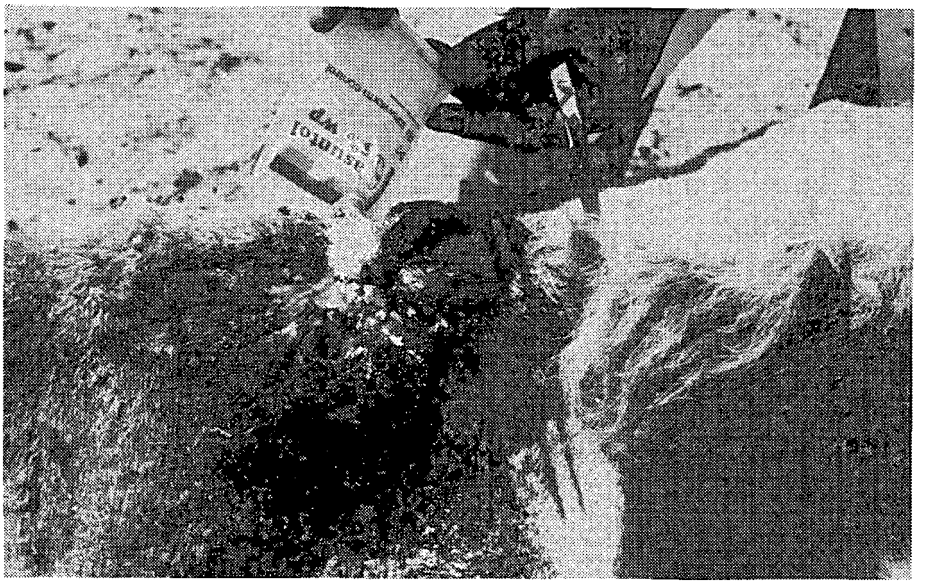

FIG. 2. Treating a screwworm infested wound with an insecticide.

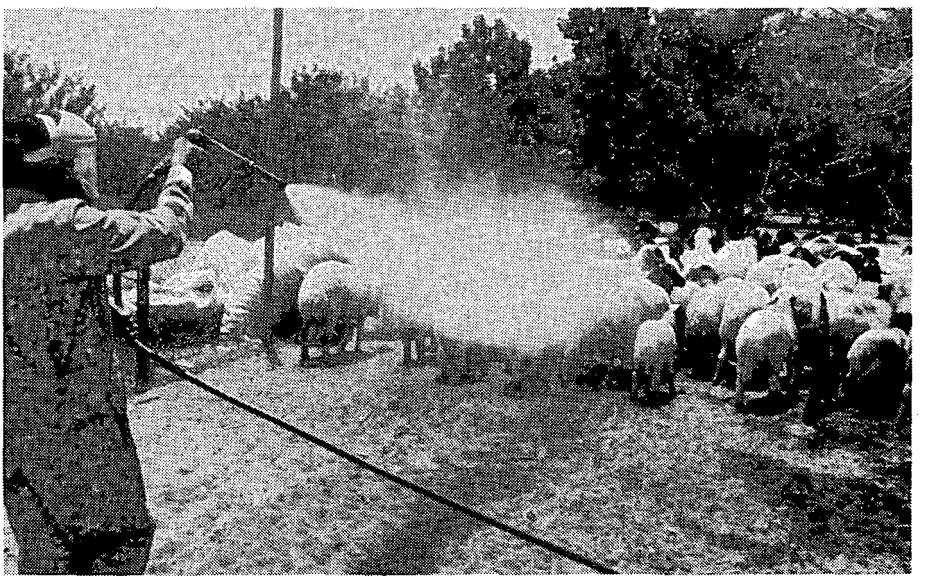

FIG. 3. Spraying a flock of sheep with an insecticide to control the screwworm.

weather (20°C or higher), the pupal period is about 8 d. The adult flies which emerge from the pupae will then mate within a few days and the females deposit eggs on wounds. The total life-cycle requires about 21 d during good weather conditions.

The cost of controlling the NWS by individual livestock producers has been estimated at US $5–10 per animal per year. As stated previously, the major part of this cost is associated with labour; however, the cost of insecticides is also significant. Although there are a number of insecticides available which kill screwworm larvae, Coumaphos is widely used because of its effectiveness against larvae, some residual activity and lack of repellent activity against the adult female fly. This latter feature can be quite important, since if the adult fly is repelled by the insecticide she will deposit her eggs on another wound which has not been treated with insecticide.

The NWS attacks wildlife and in south Texas the mortality rate of newborn white tailed deer ranges from 25–80% each year prior to the eradication of the pest. People living in endemic NWS areas have learned to live with the pest by protecting wounds and by frequent inspection of wounds, primarily of children. The use of insecticide is widespread for this purpose.

2. ERADICATION OF THE SCREWWORM FROM THE LIBYAN ARAB JAMAHIRIYA

The NWS was discovered in the area around Tripoli, Libyan Arab Jamahiriya, in early 1988 when unusually severe myiasis cases in sheep were reported. The pest was identified in late 1988, confirmed by the Food and Agriculture Organization of the United Nations (FAO) in early 1989 and an eradication programme was planned and implemented [3–8].

It is not known exactly when the NWS was introduced into the Libyan Arab Jamahiriya, but it probably occurred prior to 1988; it could have been 1987, or perhaps it occurred as early as 1986. The confirmation that the NWS was established in the Libyan Arab Jamahiriya resulted in termination of livestock imports (primarily sheep) from the New World to that country. It also resulted in the termination of most livestock movement from this country into neighbouring countries.

The NWS infestation in the Libyan Arab Jamahiriya undoubtedly resulted from immature stages being transported in live animals, or viable pupae being transported in association with live animals. Sheep are the most commonly imported animal and thus the probable carrier of the disease. However, a NWS infected dog, zoo animal or even a human could have been the source of the infestation.

Nearly all of the live animal imports into the Libyan Arab Jamahiriya are by ship. Air transport has been rarely used, but would certainly be the most likely mode of import because of the short time needed for transport and the relatively short time of the egg and larvae stages in the host animal, about seven to eight days. The short time that the parasite is in the egg or larvae stage and on and in the host animal

precludes an infested animal being placed on a ship in the New World and the animal being removed from the ship in the Libyan Arab Jamahiriya with the same infestation. The transit time by ship exceeds the seven to eight days that the immature stages are in or on the host animal. Thus, there seem to be two possibilities: (1) the adults from the parent generation mated and infested wounded animals on board ship and it was the F_1 generation which arrived in the Libyan Arab Jamahiriya; (2) pupae of the parent generation were in a suitable pupation media on board ship and this media, with the NWS pupae, was removed in that country. Since the infestation was originally centred around Tripoli, it is reasonable to assume that the infestation originated from the Tripoli port area.

Mating and genetic studies were conducted in the USA to ensure that the Mexican sterile NWS was sexually compatible with the Libyan NWS strain. These data showed that the two strains would readily mate and produce viable offspring and that the mitochondrial DNA of the two strains was similar [9]. Further, the mitochondrial DNA of the Libyan strain was quite similar to other strains available in the laboratory which originated from some of the Caribbean islands and Central American countries. Studies to determine the geographical origin of NWS strains have not been conducted and thus it is not possible to pinpoint the country of origin for the Libyan NWS infestation.

Prior to the establishment of the NWS in the Libyan Arab Jamahiriya, the myiasis resulting from NWS attack was not an International Office of Epizootics reportable disease. This has changed and NWS myiasis is now a category B reportable disease.

The establishment of a NWS population in North Africa was a major concern to national and international organizations because of the fear that it would spread throughout North Africa, migrate down the Nile River to other parts of Africa and spread throughout the Middle East as well as southern Europe. Because of this concern, a major eradication campaign was determined to be the only feasible approach to solving the problem. Fortunately, extensive knowledge was available from the New World on NWS eradication. The NWS had been eradicated first from southeast USA and then from the southwest. This was followed by eradication from Mexico and some of the Caribbean islands. The current programme has the objective of eradicating the pest from Central America and Panama and the establishment of a barrier at the Darien Gap to prevent reinfestation. In addition, plans are being developed to eradicate the pest from those Caribbean islands which are still infested.

The cost of NWS eradication in the Libyan Arab Jamahiriya can be rather easily calculated. The cost of loss of exports from the New World to North Africa is much more difficult to calculate and at present no data are available. Furthermore, because of the wide publicity about NWS infestation in this country it is likely that exports of live animals from NWS endemic countries in the New World have been reduced because of fear of a repeat infestation in other parts of the world. Again, no data are available.

The decision to initiate a NWS eradication campaign in the Libyan Arab Jamahiriya was made jointly by FAO and the Government of the Libyan Arab Jamahiriya. Once this decision was made, intensive planning was undertaken in order to estimate the costs of the eradication campaign and to prepare the technical details required [10]. The only eradication technology available was that used for the past twenty-five or more years in the New World and involved the use of the sterile insect technique (SIT) in combination with surveillance and quarantine and supported by an intensive information campaign. It was fortunate that there was extensive knowledge and there were people available with experience in NWS eradication. By utilizing these resources, the eradication campaign was implemented late in 1990, completed in October of 1991 and confirmed in June of 1992. The speed with which the eradication was achieved and the fact that the spread of the NWS did not occur as was originally feared, at least partially because of effective quarantine activities, greatly reduced the cost from the original planning figures. The programme took advantage of the 1990–1991 cool winter in that country and sterile flies were dispersed prior to the time when the overwintering generation would emerge and start the spring NWS population increase. Only six NWS cases were reported during 1991 as compared with over 12 000 during 1990 [3–5].

During the period mid-December 1990 to mid-October 1991, 1300 million sterile NWSs were dispersed in the NWS infested area of the Libyan Arab Jamahiriya, which was approximately 28 000 km^2. During the actual eradication campaign, two dispersals of sterile NWSs were made each week over the entire infested area. This resulted in a high ratio of sterile to wild NWSs. One release per week is not effective for NWS eradication. The sterile flies were released in predetermined grid patterns with weekly effective flight lanes 2 km apart [3, 6, 7].

The sterile NWSs were reared in Tuxtla Gutierrez, Mexico, sterilized in the late pupal stage (Fig. 4), packaged in adult emergence/dispersal boxes (1600 sterile pupae per box) and sent by air freight to the Libyan Arab Jamahiriya. During the early part of the programme, the boxes of sterile NWSs were sent by temperature controlled truck from Tuxtla Gutierrez to Mexico City where they were placed on a scheduled airline flight to Frankfurt, Germany, and then by charter aircraft to the Libyan Arab Jamahiriya. Later in the programme, direct charter flights were flown from Tuxtla Gutierrez to Tripoli (Figs 5–7).

Appropriate temperature control space was provided in the country, as well as staff to handle the incoming sterile NWSs. The release of the sterile NWSs was by Twin Otter aircraft flying at about 250 km/h at an elevation of 500 m. Releases were made during the early morning to avoid excessive heat during the other parts of the day (Fig. 8).

About one hundred teams, each consisting of two individuals with a vehicle and the necessary treatment and sampling supplies, conducted extensive surveillance (Fig. 9). These teams were assigned specific areas and inspected all livestock within that area every 21–28 d. Larvae samples collected were sent to the Central Veteri-

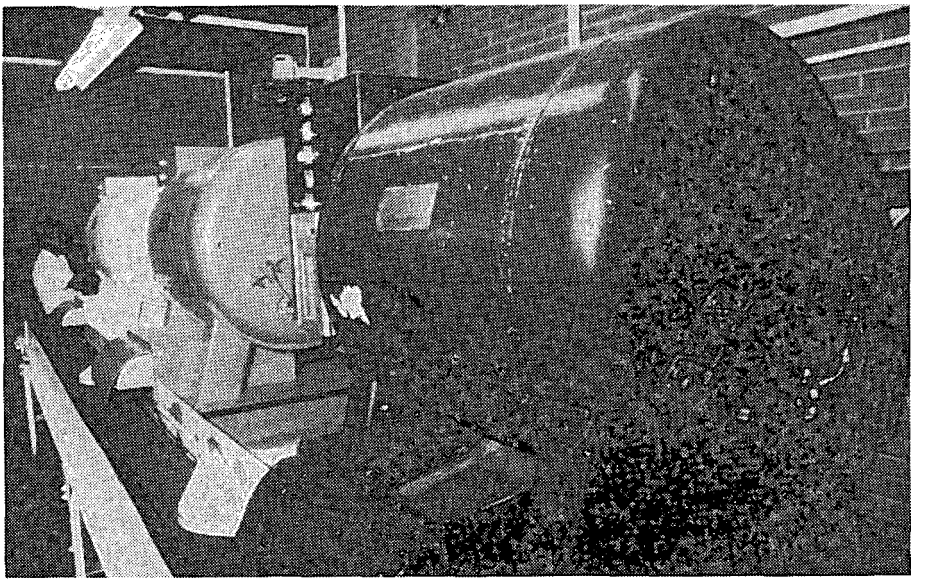

FIG. 4. Radiation units (^{137}Cs) used in Tuxtla Gutierrez, Mexico, to sterilize screwworm pupae.

FIG. 5. Loading boxed and palletized sterile flies onto a charter aircraft at Tuxtla Gutierrez, Mexico. The plane will fly directly to Tripoli, with a stop in Bermuda for fuel and a crew change. The flight takes about 14 h.

FIG. 6. Unloading boxed, palletized, sterile screwworm flies at Tripoli International Airport.

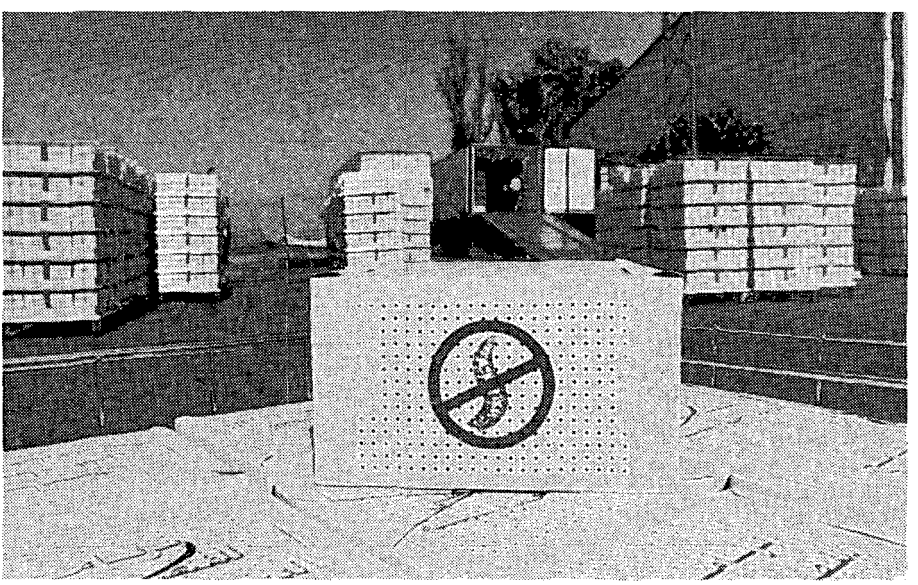

FIG. 7. Sterile Fly Distribution Centre, Tripoli International Airport, showing boxed sterile screwworm flies which had just arrived from Mexico and were being placed in temperature controlled trailers.

FIG. 8. Loading boxed sterile screwworm flies onto dispersal aircraft at Tripoli International Airport. Each box contains 1600 sterile flies which will be released in the predetermined grid over the infested area.

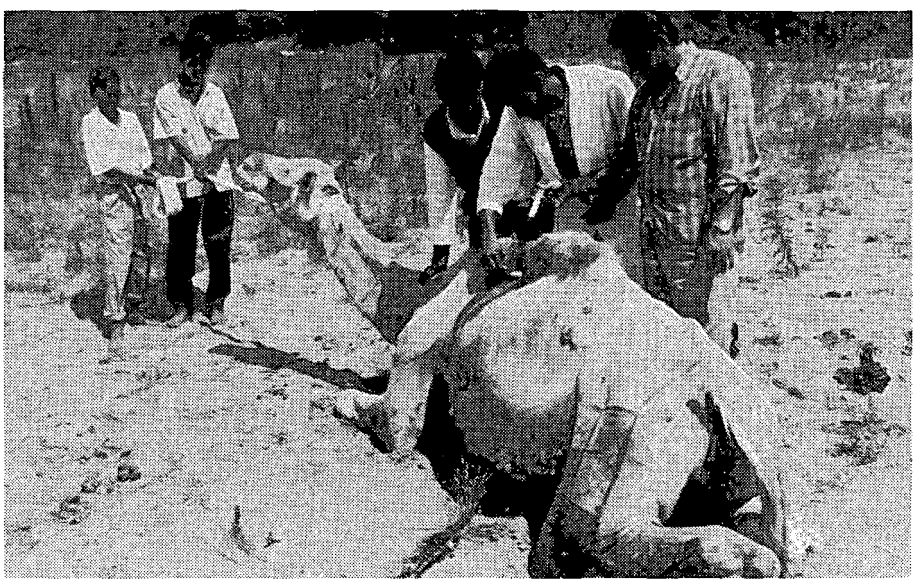

FIG. 9. A surveillance team treating the wound of a camel with insecticide to prevent screwworm infestation.

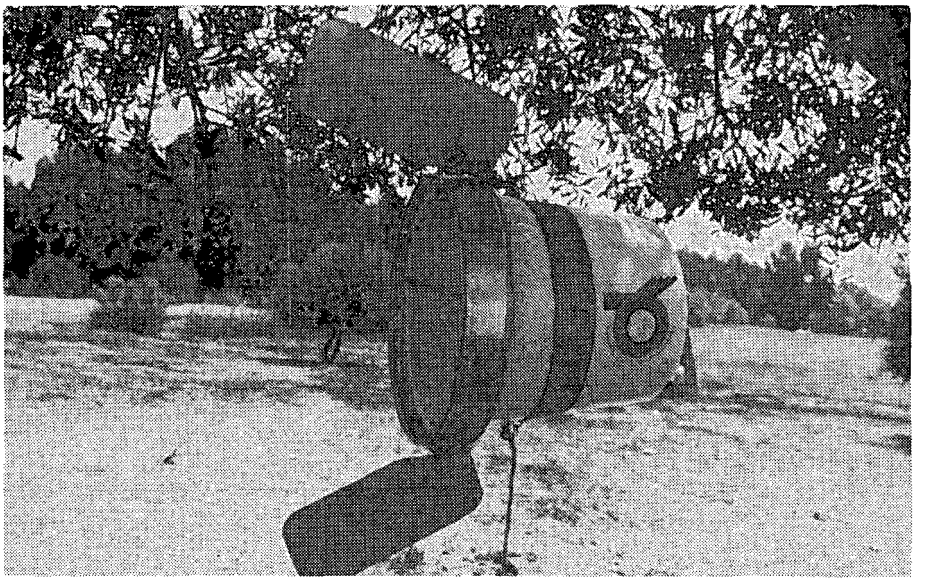

FIG. 10. A screwworm fly trap.

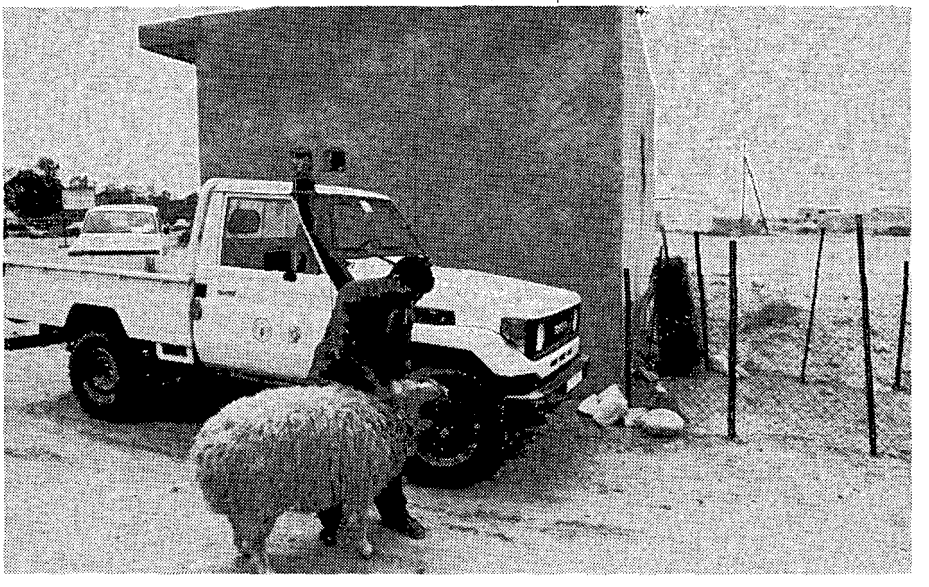

FIG. 11. Inspecting a sheep for screwworm infestation at a quarantine station.

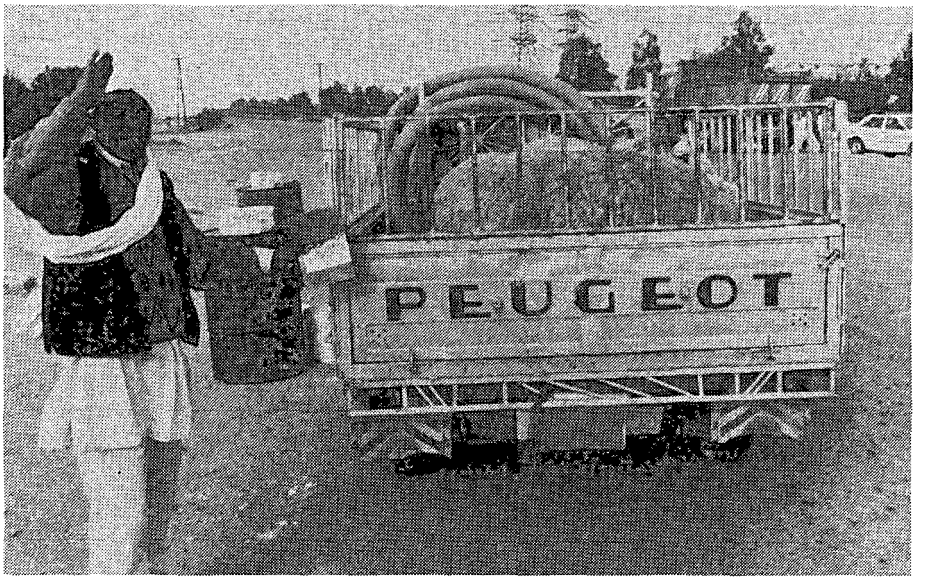

FIG. 12. After passing through the quarantine station, a farmer is given literature describing the programme and the reasons for the quarantine stations.

nary Laboratory in Tripoli for identification. Approximately eighty traps were utilized to confirm the dispersal of sterile NWSs throughout the infested area, as well as to estimate the ratio of sterile to wild NWSs (Fig. 10).

Eleven quarantine stations were located at the periphery of the infested area to prevent the spread of the disease into neighbouring uninfested areas (Figs 11 and 12). Fortunately, the infested area was semi-isolated by the Mediterranean Sea in the north, the desert in the south and quite dry and harsh areas with little livestock to the east and west. This semi-isolation and the effectiveness of the quarantine prevented a significant spread of the disease.

Climatic conditions in a belt about 15–25 km wide along the coast were such that though the NWS could easily overwinter, south of this area the climatic conditions did not permit NWS overwintering. Again this helped in preventing the spread of the disease.

3. CONCLUSIONS

The NWS has been successfully eradicated from the Libyan Arab Jamahiriya. A large part of the credit for the success of this campaign should go to the effective support provided by the Government of the Libyan Arab Jamahiriya, which included cash paid on time, the assignment of a technically competent and dedicated staff (a total of about four hundred), the very high priority that the Government placed on

successful NWS eradication, which resulted in the many usual bureaucratic problems being solved instantly and the fact that all concerned set aside politics so that the programme could succeed.

The eradication programme which was successfully implemented in this country cost a total of about US $80 000 000. An economic analysis clearly demonstrated the benefits of this expenditure [11]. Approximately half of this was paid for by the Government of the Libyan Arab Jamahiriya in cash and in kind, while the remainder was from a number of donors. The eradication programme was planned and implemented by FAO and was supported by the IAEA, International Fund for Agricultural Development and the United Nations Development Programme. In addition, major support was received from the Mexican–American Commission for the Eradication of Screwworm, the organization which is responsible for screwworm eradication in Mexico and is the only source of sterile NWSs.

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EFFECTIVE CONTROL OF THE MEDITERRANEAN FRUIT FLY BY GENETIC SEXING MALE ONLY STERILE INSECT TECHNIQUE RELEASES DURING 1989–1990*

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Abstract

EFFECTIVE CONTROL OF THE MEDITERRANEAN FRUIT FLY BY GENETIC SEXING MALE ONLY STERILE INSECT TECHNIQUE RELEASES DURING 1989–1990.

For two successive years, the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), was effectively controlled by a combination of bait spraying onto boards suspended on host trees in May, followed by weekly releases of about one million gamma sterilized genetic sexing males till the end of the year. The treated area, Kibbutz Gvulot in southern Israel, included about 500 ha of citrus, mango and backyard fruit trees. In both years, the female/male ratio declined to extremely low levels in November–December, suggesting that the wild population had been driven to near extinction. This was apparent from fruit infestation as well, which was kept to levels below 0.1% in both years as compared with similar or even higher levels in the control area, which was treated by air bait spraying about weekly from September till the end of the year.

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1. INTRODUCTION

The Mediterranean fruit fly (medfly), *Ceratitis capitata*, is the major fruit pest in many countries around the world, including Israel. The only effective control relies on the wide and indiscriminate use of pesticides (such as poisoned bait sprays). Sterile insect releases of the medfly were tested in various countries, including Israel [1], and relied always on the joint release of sterilized males and females due to the lack of a sound method of genetic sexing. The construction of 'genetic sexing' strains of the medfly [2–5] permitted the testing of an all male release programme, which should reduce the cost of handling and release, eliminate oviposition punctures by the released females [6] and enhance the efficacy of the released males [7].

The all male release method was previously tested on the island of Procida (Italy) in a field cage [7] and in a limited field test [8]. It was also tested in a field cage in Hawaii [9].

The present test lasted for two entire seasons. Its main objective was to evaluate the feasibility of the all male release method as a practical control technique in the field and compare it with currently used malathion bait aerial applications.

2. MATERIALS AND METHODS

2.1. Test site

The test was carried out in the northern Negev. This is a semi-arid region in the south of Israel. The groves were relatively isolated from neighbouring cultivated groves by a savannah-like plain. Releases were made over a 500 ha area in the groves of the Kibbutz Gvulot on citrus, mango orchards and backyard trees (apricots, fejoa, etc.). The control area, in the Kibbutz Zeelim, located approximately 6 km from the test area, included similar sized groves of citrus and mango (Fig. 1).

The conventional medfly control method was used in Zeelim (aerial sprays of malathion baits). These were applied, on the basis of trimedlure (TML) trap surveys, almost every ten days in 1989. In 1990, the malathion bait applications were governed by LADD® trap[1] surveys (see Section 2.2). The citrus orchards in Zeelim received 12 applications (from September to December) and the mango orchards 15 applications (from July to October), i.e. an average interval of eight days between applications.

The sterile insect technique (SIT) test area in Gvulot received in both test seasons two applications of malathion bait sprays, at the end of April and May, one and two weeks before the beginning of the releases to reduce the density of the wild population of medflies.

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[1] LADD, Inc., Burlington, Vermont, USA.

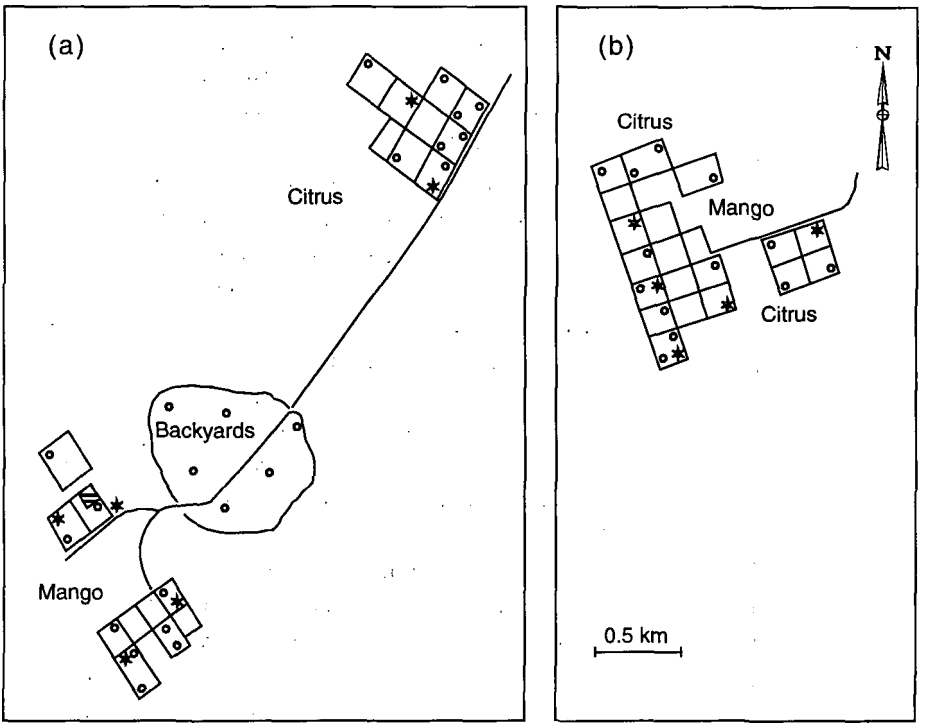

*FIG. 1. (a) Test area, Kibbutz Gvulot and (b) control area, Kibbutz Zeelim (o : trimedlure trap; *: LADD trap).*

2.2. Genetic sexing strain and releases

The T:Y(wp)30c strain (also called the 'Robinson' strain) was used. The strain was originally constructed by A.S. Robinson and stabilized during its mass rearing in the Agency's Laboratory at Seibersdorf, near Vienna [1]. The strain had brown (wild type) pupae males and white pupae females. The flies used in the test were mass reared at Seibersdorf [10], where the pupae were photoelectrically sexed and gamma sterilized (95 Gy) before being shipped to Israel. Weekly shipments of approximately two million male pupae, one to two days before adult eclosion, were air freighted under anoxia (in plastic bags) in cooled, insulated cardboard containers and delivered directly to the SIT test area within 24 h from irradiation–packing. A small sample of pupae was sent to the Rehovot Laboratory upon arrival for quality control tests. In 1989, the pupae were distributed in regular 53 cm × 37 cm paper bags, with 5000 pupae per bag. We inserted crumpled newspaper sheets sprayed with a 10% sugar solution and two pieces of rubber sponge (10 cm × 8 cm × 1 cm)

soaked with the same sugar solution into each bag. The emerging adult flies were kept in the bags for three days at a temperature of 25–27.5°C for sexual maturation. The flies were then released at approximately 400 sites, uniformly spread in the test area, and the bags were torn and hanged on the trees. Thirty-five consecutive weekly releases from May to December 1989 were carried out.

In 1990, the paper bags were replaced with 32 cm × 17 cm × 40 cm flat-bottom paper bags and the total number of pupae per bag was reduced to 4500. The number of release sites was increased to 450. The releases were delayed for five weeks as compared with 1989 and only 30 weekly releases were carried out from June to December 1990. Quality control tests were carried out, both at Seibersdorf and Rehovot, for pupal size, adult emergence, adult flight ability and strain breakdown (the occurrence of brown pupae females and white pupae males).

The survival of the released flies in the groves was checked by the mark–release–recapture method after the first release in the citrus grove in 1990. Following first release, all the flies were collected weekly from the LADD traps and checked for dye traces (as mentioned in Section 2.3).

Aerial release (by helicopter) was carried out once in 1990, in the first release, in order to compare it with the ground release. Fluorescent dyes were used to differentiate between the flies from the two release methods, which were carried out simultaneously. 'Arc Yellow' dye (for aerial release) and 'Neon Red' dye (for ground release) (Day-Glo Colour, Cleveland, Ohio, USA) were used, with 2 g of dye per 1 L of pupae, prior to the release. Equal numbers of flies were released by each method at 75 sites, uniformly spread in an isolated group of groves (4500 flies per site). As the test was preliminary in nature, we may only conclude that flies released from the air reached the traps and were not less abundant than ground released flies. It seemed that air release did not harm the flies.

2.3. Monitoring the medfly population

Adult male and female flies were counted in LADD traps and the TML conventional traps, throughout the test period. The LADD traps consist of red plastic spheres located in the middle of a yellow rectangular plastic sheet and covered with Tanglefoot. The trap attracts both sexes, whereas the TML trap attracts males only. In 1989, a total of 21 LADD traps and 10 TML traps were placed in the SIT test area (Gvulot) and 13 LADD traps and 5 TML traps were placed in the control area (Zeelim). Four additional TML traps were placed outside the test area, at varying distances, between Gvulot and the nearest cultivated areas. In 1990, two traps were also placed in the area between the backyards and the citrus groves to intercept migrating medflies.

In the 1989 test season, the TML traps captured large numbers of released males and interfered with the SIT test. It was therefore decided not to use these traps in 1990 and to rely only on the LADD traps, which had been very effective in attract-

ing both sexes of the medfly. Thus, in the 1990 test period, 19 LADD traps were placed in the SIT test area (Gvulot) and 10 LADD traps were placed in the control area (Zeelim). All the traps (in 1989 and 1990) were inspected weekly with a binocular magnifier (2.5X) (Temom Hessen, Germany). The flies were sexed, counted and removed from the trap.

Two methods were used to distinguish between natural and released flies:

(1) The released flies were marked with fluorescent "Neon Red" dye, with 2 g of dye per 1 L of pupae, prior to release. In 1989, this was done four times during the release period: in May a single release; in July, September–October and in December four successive releases were marked. In 1990, such marking was done three times during the release period: in June a single release; in September–October and in November–December four successive releases were marked. The objective of the successive markings was to eliminate the possibility of capturing released unmarked flies when the flies were checked for colour marking. Flies were therefore recaptured for examination only four days after the last release of marked flies. The recaptured flies were sexed and inspected under ultraviolet light. No more than 50 specimens of each sex were examined from each trap.

(2) It was assumed that released (irradiated) females can be distinguished from normal and sexually mature females by their undeveloped ovaries. (The method will not distinguish irradiated from one-day-old normal females.) The ovaries of all the live and dead females (where possible) caught by the LADD traps were dissected. Such dissections were carried out routinely every week during the last three and a half months of the test in 1989 and throughout the test period in 1990.

2.4. Fruit infestation

In both release seasons we searched intentionally for fruits which seemed infested by the medfly. The fruits were either picked from the trees or collected from under the trees. They were inspected in the laboratory under magnification. When oviposition punctures were observed, the fruits were maintained for the emergence of larvae and pupation.

Inspections of harvested fruits were carried out in 1989, during five picking days in November 1989, December 1989 and the beginning of January 1990. During the 1990 test season, inspections were carried out in December 1990 and January 1991. We checked both picked fruits from the picking bins and collected dropped fruit from under the trees on the same dates.

Picking bins. We screened the upper layer of fruit in 10–20 bins (each had a surface area of 1 m^2 and contained approximately 100–220 fruits). Fruits with suspected ovipunctures were taken to the laboratory to verify the nature of the 'ovipunc-

ture'. These fruits were then kept for possible emergence of medfly larvae. The 'sterile stings', where no larvae emerged, and the fruit with live medfly infestation were counted separately. Ten thousand individual fruits were checked in the test area as well as in the control in both test seasons.

Dropped fruits. All the dropped fruits were collected from under ten randomly selected trees on each picking day. Sterile stings and the emergence of live larvae were recorded. A yield of approximately 500 fruits per tree was assumed and our calculation of the percentage of infested fruits was based on that figure.

3. RESULTS AND DISCUSSION

3.1. Quality of the irradiated and released flies

The quality of the released flies was checked thoroughly in the mass rearing facility at the Agency's Laboratory at Seibersdorf. For technical reasons, only part of these data was examined for 1989, while for 1990 all data were examined. Certain aspects of quality such as adult eclosion, the percentage of white pupae and the percentage of females among the shipped flies, were also checked in Rehovot (following the air journey of the flies from Vienna to Israel).

Adult eclosion in the shipments arriving at Rehovot was a major concern during both the 1989 and 1990 periods. It seemed to fluctuate greatly between shipments (Figs 2 and 3). It dropped often to lows of 30% and less. The data from Seibersdorf (Figs 2 and 3) suggested that the air journey and handling contributed most to pupal mortality and the decrease in adult eclosion (Fig. 4). In nine shipments (two in 1989 and seven in 1990), the differences between the Rehovot and Seibersdorf data were between 50% and 70%! Our conclusion is that in spite of detailed instructions given to airport and shipment company personnel, on certain dates the pupal shipments were not handled correctly, mainly because of ignorance as a result of airport personnel changes.

The sorting of the genetic sexing line was carried out at Seibersdorf using an electro-optical sorting machine. Such sorting left between 2 and 5% of white pupae among the wild type batch, with occasional exceptions of 10% white pupae early in 1989 (Fig. 4), and fluctuated between 1 and 7% in 1990 (Fig. 5). The inclusion of such a proportion of white pupae (assuming that these are females) meant that at least 100–250 females were released at each release site, or a total of 20 000–100 000 females were released in the whole test area each week. These were all sterilized females, of course, but their interference with the activity of the released males, as well as their interference with our checking method, was obvious.

The occurrence of females among the wild type pupae was a measure of the stability of the genetic sexing line T:Y(wp)30c. In 1989, there seemed to be a rather accelerated decline and breakdown of the line (Fig. 4) and the proportion of females

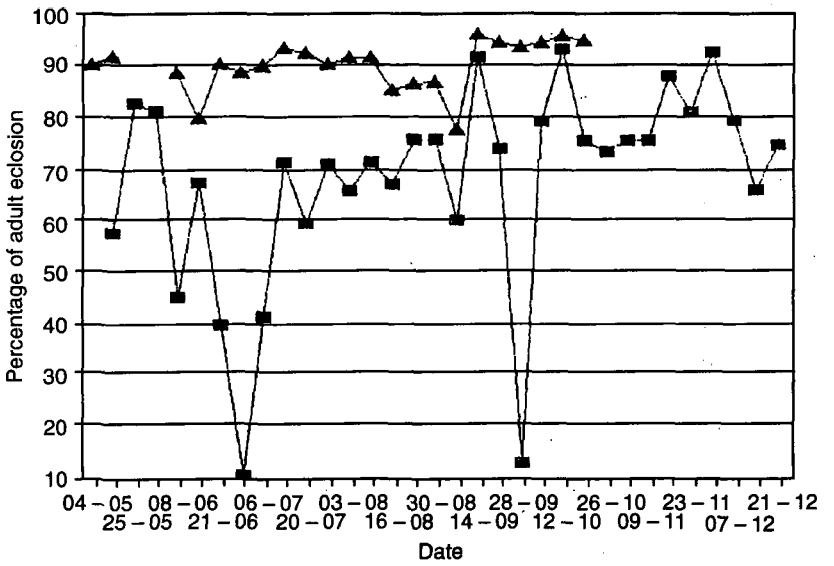

FIG. 2. Effect of air freighting on adult eclosion of the medfly, 1989 (■ : Rehovot laboratory; ▲ : Agency's Laboratory Seibersdorf).

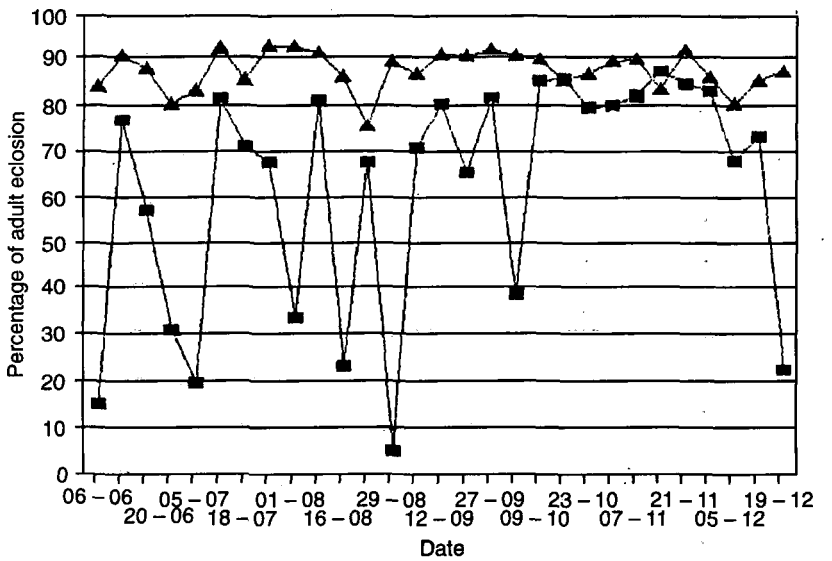

FIG. 3. Effect of air freighting on adult eclosion of the medfly, 1990 (■ : Rehovot laboratory; ▲ : Agency's Laboratory Seibersdorf).

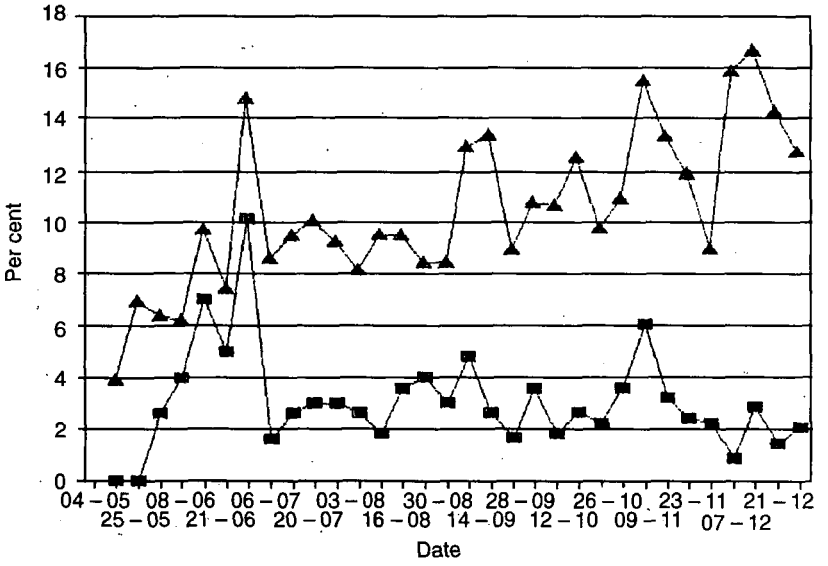

FIG. 4. Percentage of white pupae and females in released medflies, 1989 (■ : white pupae; ▲ : females).

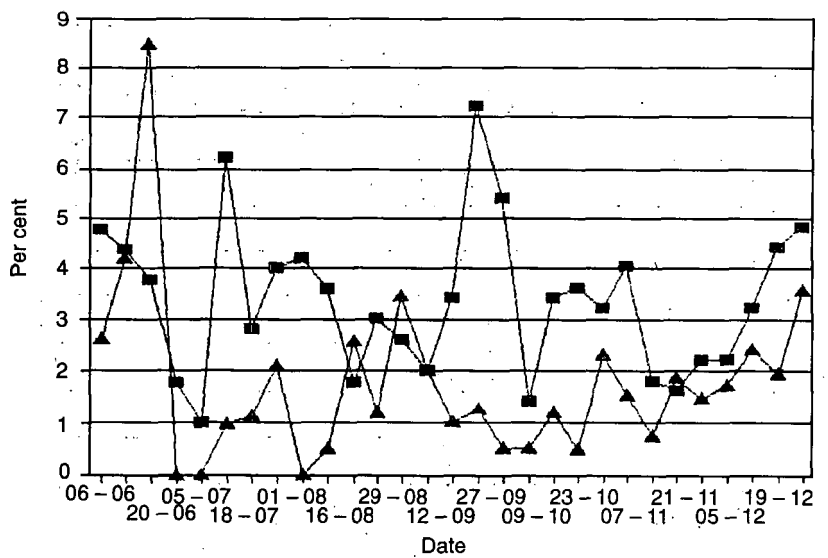

FIG. 5. Percentage of white pupae and females in released medflies, 1990 (■ : white pupae; ▲ : females).

among the wild type flies reached 7% and higher early in the test period. The line used in 1990 was much more stable and the proportion of females fluctuated between 2 and 7% throughout the season, with an exception of 8% at the beginning of the test (Fig. 5). It should be noted that 1% of the flies meant that at least 10 000 additional (though sterilized) females were released with the males every week.

The survival of released flies was also monitored and it was found that these flies survived for no more than three weeks in the orchard. The number of marked flies in the trap dropped from 297 to 21 to 2 in the three consecutive weeks following the release.

3.2. Trends of the medfly population

The trend of the female population in the control (Zeelim) and test (Gvulot) areas is presented in Fig. 6 for 1989 and in Fig. 7 for 1990. We transformed the LADD data to display the number of females per trap per day. The transformation was direct for Zeelim, where all the females were native. In Gvulot, we had to distinguish between the released and native females. The ratio between released and native females was calculated from the data obtained by the mark–release–recapture method, as well as the routine dissections of ovaries. The data were transformed into the total number of females and the number of native females per trap per day.

In 1989, there was a sharp increase in the number of female catches in the control orchard in June and July, followed by a sharp decrease (owing to conventional control operations) towards the end of the test season (Fig. 6). In the SIT orchards in Gvulot, a similar but much smaller increase was observed. This increase continued to the middle of August and declined later, but did not reach the 'zero' level as in the Zeelim control area. The occurrence of females in the SIT orchard (Gvulot) could not be attributed only to the presence of females in the released flies, but also to the continuous presence of native females in the orchard (Fig. 6).

In 1990, the female population in the control orchard seemed to be much better controlled and showed a steady and continuous decline throughout the season until it disappeared completely in December. The SIT orchard also harboured a relatively small population of females, with some increase towards the end of October and November (Fig. 7). The female population nevertheless consisted in these instances largely of released females.

The decline in the female population due to the release of sterile males can also be viewed through the change in the sex ratio (females/males) during the release period (Table I). The number of males per trap increased during the season (due to the presence of a large population of released males). At the same time the proportion of females among the captured flies declined to a level which was close to the 'impurity' of the released line, or even lower (Figs 4 and 5).

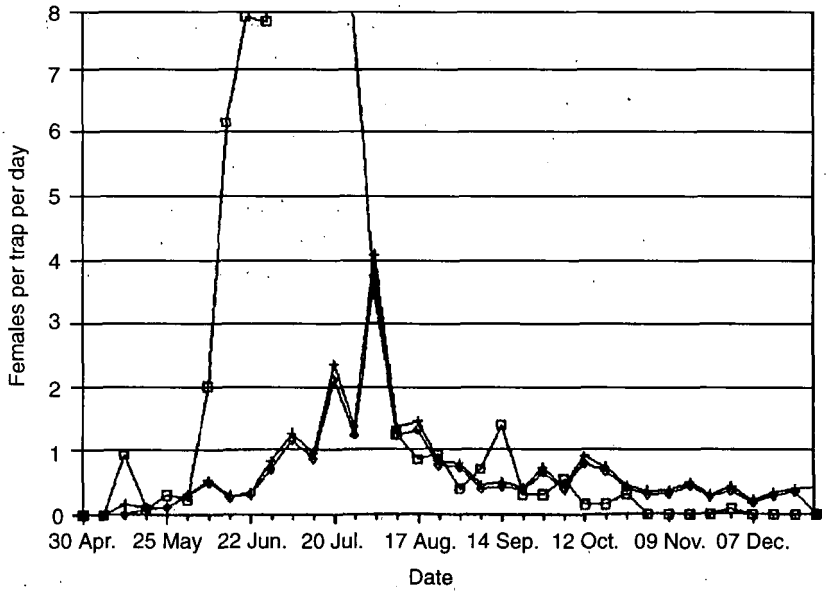

FIG. 6. Sterile insect technique test — Kibbutz Gvulot, 1989 (trend of the female population) (□: Kibbutz Zeelim; +: total, Gvulot; ◇: native, Gvulot).

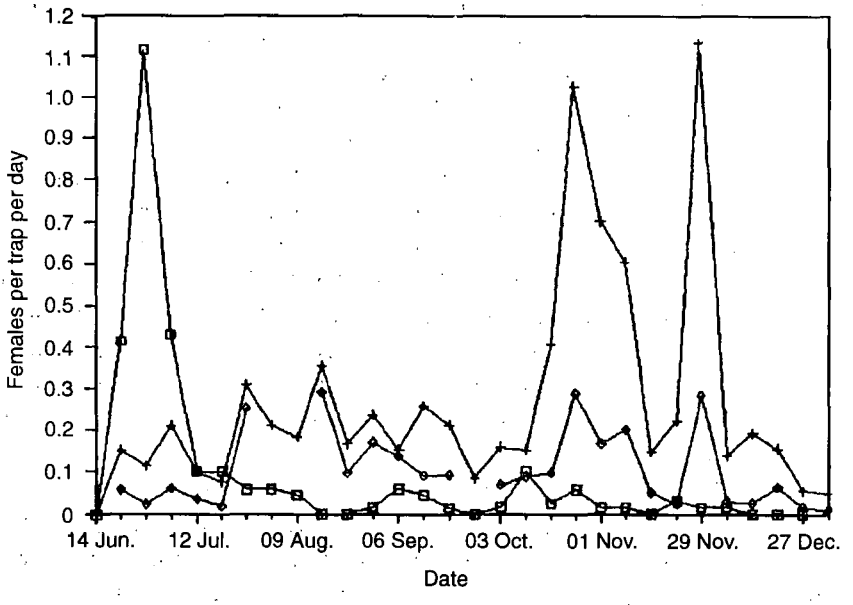

FIG. 7. Sterile insect technique test — Kibbutz Gvulot, 1990 (trend of the female population) (□: Kibbutz Zeelim; +: total, Gvulot; ◇: native, Gvulot).

TABLE I. AVERAGE NUMBER (PER MONTH) OF MALES PER LADD TRAP AND THE SEX RATIO IN CITRUS ORCHARDS IN KIBBUTZ GVULOT

Month	No. of males/trap	Sex ratio, females/males
September 1989	12.0	0.18
October 1989	15.9	0.16
November 1989	56.2	0.03
December 1989	61.0	0.02
June 1990	8.8	0.20
July 1990	15.4	0.07
August 1990	36.8	0.03
September 1990	46.8	0.03
October 1990	33.3	0.05
November 1990	33.0	0.03
December 1990	77.9	0.01

3.3. Fruit infestation in 1989

Four thousand grapefruits were checked, in the SIT test area in Gvulot, in picking bins on 19 and 30 November 1989. Of these, 16 were found with sterile stings and only 5 fruits with live medfly maggots. On the same dates, and also on 19 December 1989, 176 grapefruits were collected from under 30 trees and inspected for medfly damage. Two fruits were found with sterile stings and 33 with live medfly maggots. (Of the 33 fruits, 29 were collected from a single tree on 19 December 1989.) Six thousand oranges of the variety 'Shamouti' were inspected on 28 December 1989, 31 December 1989 and 1 January 1990. Of these, 92 fruits were found with sterile stings and only 2 fruits with live medfly maggots. On the same dates, 222 oranges were collected from under 30 trees and inspected for medfly damage. Of these, four were found with sterile stings and six fruits with live medfly maggots.

The control orchard in Zeelim included only grapefruit and Mineola orange hybrids. Eight thousand grapefruits were inspected on 13 November, 20 November and 3 December 1989 and also on 2 January 1990. Ten fruits were found with sterile stings and four with live medfly maggots. A total of 329 fruits were collected from

TABLE II. FRUIT INFESTATION IN THE SIT AND CONTROL ORCHARDS

Season		Harvested fruit		Tree infestation[a]	
		Per cent sterile stings	Per cent live maggots	Per cent sterile stings	Per cent live maggots
1989	SIT area	1.1	0.07	0.06	0.130
	Control	0.1	0.04	0.00	0.004
1990	SIT area	0.05	0.40	0.10	0.076
	Control	0.21	0.59	0.15	0.210

[a] Based on an average number of 500 fruits per tree.

under 40 trees and only 1 was found with live medfly maggots. The 2000 Mineola fruits which were inspected on 12 December 1989 and the 91 fruits collected from under 10 trees on the same date showed no sign of medfly damage.

The infestation rates in 1989 are presented in Table II. The harvested fruit (grapefruit and oranges) at the SIT site (Gvulot) included 1.1% fruits with sterile stings and 0.07% fruits with live maggots. In the control orchard in Zeelim, only 0.1% of the harvested fruit had sterile stings and 0.04% had live medfly maggots. The infestation found in the dropped fruit was transformed to an estimated infestation per tree. The average yield per tree consisted of 500 fruits and the infested fruits found on the ground under the trees was calculated to be part of these 500 fruits. Thus, the SIT site had an infestation rate of 0.06% of sterile stings per tree and 0.13% of fruits with live medfly maggots per tree. If the 29 fruits found under a single tree are excluded, infestation of 0.024 and 0.04%, respectively, are reached. Similarly, the control orchard in Zeelim showed 0.1% sterile stings and 0.04% live medfly maggots in the harvested fruits, and no sterile stings and 0.004% live medfly maggots in the fruit collected from under the trees. The conclusion was that the SIT method was effective and produced very good medfly control.

3.4. Fruit infestation in 1990

Monitoring for medfly infestation in citrus fruit throughout the test, when fruits that seemed infected were intentionally searched, showed a low rate of infestation. The infestation started in October (5.0%) and declined in November (3.7%) and December (1.5%).

Ten thousand grapefruits were checked in the SIT test area in Gvulot in picking bins on 20 December 1989 and on 9–12 January 1991. Of these, five were found with sterile stings and 40 fruits with live medfly maggots. On the same dates, 353

grapefruits were collected from under 50 trees and inspected for medfly damage. Twenty-five fruits were found with sterile stings and 19 with live medfly maggots.

A total of 10 000 grapefruits were inspected in the control orchard in Zeelim on 12, 26 and 31 December 1990 and on 6 and 11 January 1991. Ten fruits were found with sterile stings and four with live medfly maggots. A total of 310 fruits were collected from under 50 trees. Thirty-seven fruits were found with sterile stings and 52 fruits with live medfly maggots.

The total infestation rate in 1990 is presented in Table II. The harvested fruit (grapefruit and oranges) at the SIT site (Gvulot) consisted of 0.05% fruits with sterile stings and 0.4% fruits with live maggots. In the control orchard in Zeelim, 0.21% of the harvested fruit had sterile stings and 0.59% live medfly maggots. The infestation found in the dropped fruit was transformed to an estimated infestation per tree. The average yield per tree consists of 500 fruits and the infested fruits found on the ground under the trees were calculated to be part of these 500 fruits. Thus, the SIT site had an infestation rate of 0.1% sterile stings per tree and 0.076% of fruits with live medfly maggots per tree. Similarly, the control area in Zeelim had an infestation rate of 0.15% sterile stings and 0.21% live medfly maggots per tree. The SIT method in 1990 was as effective, if not more so, as in 1989.

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TSETSE STERILE INSECT TECHNIQUE PROGRAMMES IN AFRICA
Review and analysis of future prospects

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Abstract

TSETSE STERILE INSECT TECHNIQUE PROGRAMMES IN AFRICA: REVIEW AND ANALYSIS OF FUTURE PROSPECTS.

Following the successful eradication of the screwworm, *Cochliomyia hominivorax* (Coql.), from the island of Curaçao and the southeastern United States of America, the sterile insect technique (SIT) was tested against several other noxious insects, including the tsetse fly. Between 1967 and 1987, experiments were conducted in Zimbabwe and the United Republic of Tanzania (East Africa) and in Burkina Faso and Nigeria (West Africa) to test the feasibility of the new technique in eradicating selected species of tsetse fly. For the Zimbabwe programme, sterile flies were obtained from field collected pupae treated with the chemosterilant tepa^{R}. Complete eradication was not achieved, primarily because of insufficient sterile males emerging from the wild pupae. In later programmes in the United Republic of Tanzania, Burkina Faso and Nigeria, flies were obtained from laboratory bred mass reared colonies. Males were sterilized either as pupae (Tanzanian project) or as young adults using gamma irradiation from a ^{60}Co or ^{137}Cs source. Efforts currently in progress to apply the technique to eradicate *Glossina austeni* from Zanzibar Island, United Republic of Tanzania, have attracted considerable interest and funding from several international organizations. Past and current tsetse SIT programmes are reviewed and future prospects of the technique in large scale tsetse/trypanosomiasis programmes are discussed.

1. INTRODUCTION

A little over three decades ago, the screwworm, *Cochliomyia hominivorax* (Coql.) was eradicated from the island of Curaçao and the southeastern United States of America [1], using a novel approach to pest management. Since then, the sterile insect technique (SIT) has been shown to be effective against many tropical fruit flies, several destructive moths, the bollweevil, the stable fly, certain mosquitoes and the tsetse fly, *Glossina* species [1].

The method of choice for controlling tsetse flies has been the use of insecticides, applied over large areas of tsetse infested country, using ground or aerial spraying techniques. In northern Nigeria, for example, approximately 250 000 km^2

of savannah land, equivalent to about 30% of the tsetse infested area, were cleared of tsetse by ground and limited aerial spraying of insecticides [2].

The use of SIT in tsetse control is a recent introduction. Over the past 25 years, the technique has been introduced into several tsetse infested African countries through the training of personnel already involved in national tsetse control activities, through scientists participating in IAEA Co-ordinated Research Programmes and, more practically, through Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture field operational technical co-operation projects, or through bilateral or similar projects funded with donor contributions. Four examples are described briefly to illustrate the approach adopted in each case, the progress achieved and the lessons learned. It is important to stress that all the tsetse SIT programmes undertaken to date have been, essentially, research and development projects aimed, primarily, at testing the feasibility of the new concept in a practical field situation.

2. THE EARLY YEARS

The story begins in Zimbabwe in 1964, on an island in Lake Kariba. At that time, the sterility principle for pest control had been tested, with positive results on a number of insects; much curiosity had been aroused with regard to its applicability to the tsetse fly because of the fly's low reproductive capacity and its relatively low population density in nature. Because very little information was available on tsetse rearing and radiation sterilization, and the behaviour of sterile flies released into the natural environment, a research programme was called for [3].

Sponsored by the United States Agency for International Development (USAID), the project was conducted jointly by the Agricultural Research Service of the United States Department of Agriculture, the then Agricultural Research Council of Central Africa and the then University of Rhodesia. The target species was *Glossina morsitans morsitans*, the most important transmitter of trypanosomiasis in eastern Africa. The species was most abundant in the selected field station in the valley of the Zambezi River near Lake Kariba. The objectives of the project were to develop methods of rearing and sterilizing the target species, and to evaluate SIT against the species in the field.

In the initial stages, attempts were made to rear the flies in large screen cages covering as much as 10 000 ft^2 of natural habitat.[1] Flies were allowed to feed at will on cattle and bushpigs. Smaller cages of 8 in × 8 in × 11 in (22 cm × 22 cm × 28 cm) were also used. Both types of cage were stocked with pupae and adults collected from the field in the project area. The large field cage

[1] 1 $ft^2 = 9.290 \times 10^{-2}$ m^2.

design and the general rearing conditions meant that there was no climatic or other control of the rearing environment. In the end, the attempt to rear *G. m. morsitans* in its natural habitat was unsuccessful. The next stage of the experiment therefore relied upon the use of field collected materials.

Sterility was induced in the males by dipping field collected pupae in a 5% solution of the chemosterilant tepa[R]. Irradiation was not used because only pupae of unknown ages were available, which meant that mortality from the over-exposure of younger pupae to high doses of gamma radiation could be excessive [3].

Prior to the release of sterile flies, the original population (estimated at 3000 flies/mile^2) was reduced by two aerial applications of dieldrin.[2] Trays of chemosterilant treated pupae were placed at selected points in the test area. Within 20 months of the operation, the population of *G. m. morsitans* was reduced to below the detectable level [3].

Complete eradication was not achieved for several reasons. The number of field collected pupae available for sterilization was limited. Because of the limited number of pupae, only a small number (1.2% of any batch) of treated pupae emerged as sterile males each day. It was, therefore, necessary to leave the pupae in the field for a long time in order to obtain an adequate number of released sterile males. The long exposure, in turn, meant that the chemosterilant became unstable and, therefore, failed to produce complete sterility in the flies emerging later [3]. In addition, the pupae were of unknown ages, therefore rather high mortality resulted from exposing 'under age' pupae to the chemosterilant. The test results emphasized the need to obtain known age pupae and/or adults for sterilization. Uncertainties about the reliability of chemosterilization would require that gamma irradiation be used as the preferred method of inducing sterility.

3. THE PROJECT AT MKWANJA RANCH AND TANGA, UNITED REPUBLIC OF TANZANIA

The first large scale SIT field project involving the use of laboratory reared radiation sterilized tsetse flies was initiated towards the end of 1971 in the United Republic of Tanzania. The Tanga based project was, in fact, the continuation of studies begun four years earlier in Zimbabwe. The objective was to integrate the release of sterile males with limited applications of insecticides to control the tsetse population. The research would also demonstrate whether good quality flies could be produced under African conditions for use in a SIT programme. The project was jointly funded by the USAID and the Government of the United Republic of Tanzania.

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[2] 1 mile^2 = 2.590 km^2.

The target species, *G. m. morsitans*, was colonized in the laboratory using flies from pupae collected in the field (Handeni), about 100 km from Tanga, the project headquarters [4]. The colony, held in three separate facilities, was fed daily on goats purchased locally and another group on rabbits imported from Europe. The newly acquired goats were quarantined and treated for trypanosomiasis and worm infections before being used for fly feeding. From the 60 000 fly colony (45 000 females and 15 000 males), approximately 6000 surplus males were available weekly for sterilization and release [4]. A back-up colony of some 10 000 females was maintained in the Agency's Laboratory at Seibersdorf.

The target area, Mkwanja Ranch, 195 km^2 in size and located 100 km by road from the production centre in Tanga, proved ideal all round for the study, except that it was not strictly 'isolated'. Therefore, an artificial barrier (1 km in width) was constructed on the perimeter of the project area in an attempt to ensure isolation and prevent fly immigration from the adjoining vegetation [5].

Detailed studies of the natural tsetse fly population on the Mkwanja Ranch were followed by two aerial applications of endosulfan at 28-d intervals in order to suppress the native population of *G. m. morsitans* before the release of sterile males. The flies were sterilized by exposing late stage pupae to 11.8 krad of gamma radiation from a ^{137}Cs source.[3] Pupae collected over a three or four day period were irradiated as a group. Sterilized pupae were held refrigerated at 10°C while being transported by road to the release site. Refrigeration ensured synchronous emergence, so that a large number of sterile males became available within a very short time following pupal release in the field. Releases were made twice a week, giving, on average, 135 sterile males per square kilometre per month. During the 15 months of fly releases, a total of 510 000 pupae were utilized at a cost of US $0.22 per pupa [4].

The main achievements of this programme can be summarized as follows:

(1) For the first time, a large, self sustaining colony of tsetse flies was raised and maintained under African conditions. The use of local animals (goats) to mass rear *G. m. morsitans* was clearly demonstrated, and the flies produced from such a system were of high quality. A major objective of the experiment was thus realized.

(2) With a combination of limited application of insecticides and release of sterile males, the natural population of the target species of tsetse was reduced, albeit by only 81%. Total eradication was not achieved because the target area was reinvaded by flies from the adjoining vegetation. The barrier clearing became overgrown with bush because of unseasonably heavy rains during the project period.

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[3] 1 rad = 1.00×10^{-2} Gy.

4. SIT AGAINST RIVERINE AND SAVANNAH TSETSE IN BURKINA FASO

Experiments carried out in Burkina Faso between 1976 and 1984 aimed at testing and then applying SIT for eradicating riverine and savannah tsetse flies.

In a five year research programme, *Glossina palpalis gambiensis* was eradicated in a 32 km (100 km^2 area) riverine forest through combined application of a non-persistent insecticide, thiodan and the release of laboratory reared sterile males of the target species [6]. The research involved testing the effectiveness of Challier–Laveissière traps, mechanical barriers (total clearing and burning) and application of persistent insecticides for re-enforcing the barriers.

A large scale programme initiated in 1981 was directed against two riverine species of tsetse, *G. p. gambiensis* and *G. tachinoides*, and one savannah species, *G. m. submorsitans*. Jointly sponsored by the German Gesellschaft für Technische Zusammenarbeit (GTZ) and the French Institut d'élevage et de médecine vétérinaire des pays tropicaux (IEMVT) and supported by the Government of Burkina Faso, this was the first large scale tsetse SIT project directed against eradication of more than one species at the same time. The project covered 3500 km^2 of agropastoral land, including approximately 500 linear kilometres of riverine forest. Clearing tsetse flies in the area would permit the development of livestock in a 240 000 ha area of the Sideradougou agropastoral zone. The project headquarters and mass rearing facility were located at Bobo Dioulasso [7].

The flies were fed in vivo (on rabbits) or in vitro (on defibrinated or heparinized bovine blood collected from the local abattoir). To prevent bacterial infection, the blood was treated with 50 krad gamma radiation in a ^{60}Co source. At the peak of production, the colonies contained approximately 150 000 *G. p. gambiensis,* 115 000 *G. tachinoides* and 40 000 *G. m. submorsitans* [7].

Prior to fly release, a thorough entomological survey was undertaken over the entire Sideradougou area. Access roads were first provided, and Challier–Laveissière traps were placed at 100 m intervals for a full day. In the savannah areas, parallel transects were cut every 4–5 km, and surveys similarly undertaken. To isolate the experimental zone, the entire drainage system was protected by three permanent barriers comprising traps and insecticide (deltamethrin) impregnated screens placed 100 m apart; some were hung from tree branches. In dense forests, the screens were placed 20–30 m apart. In the more open country to the southeast, as many as 1700 traps were deployed [7].

A total of 7204 screens deployed over a two month period reduced the fly population by 88% for *G. p. gambiensis* and 92% in the case of *G. tachinoides.*

Adult males sterilized with 11—13 krad gamma radiation were fed one blood meal before being transported for release at the experimental site. Releases were done during the rainy season, first on a weekly basis but later fortnightly. In all, more than 900 000 sterile males (713 000 *G. p. gambiensis* and

225 000 *G. tachinoides*) were released. Complete eradication was achieved within four months.

The Burkina Faso project demonstrated for the first time the use of insecticide impregnated screens for tsetse population suppression and as strategic barriers against reinvasion. Also, for the first time sterile flies were fed in the laboratory before being released. Both practices were later adopted and used with positive results in the Nigerian project, BICOT.

5. BICOT, NIGERIA

The project Biological Control of Tsetse Using the Sterile Insect Technique (BICOT) was initiated in 1979 following several preparatory meetings between the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture and officials of the Government of Nigeria. With financial contributions by Belgium, Germany, Italy and Sweden, and in-kind contributions from the United Kingdom, the experiment was successfully concluded in 1986. The objective was to demonstrate the use of SIT in eradicating *Glossina palpalis palpalis*, and to investigate ways of integrating the technology into the regular tsetse control operations in Nigeria.

The target species, *G. p. palpalis*, was mass reared at the project headquarters in Vom, using both in vivo (guinea pigs) and in vitro (fresh bovine and freeze dried porcine blood) feeding techniques. The bovine blood was obtained from the local abattoir, but the freeze dried porcine blood was imported from the Agency's Laboratory at Seibersdorf. At peak production, the colony contained 180 000 females. A back-up colony of about 80 000 females was maintained at the Agency's Laboratory on an in vitro feeding system [8].

The project field area, located approximately 250 km by road south of Vom, is part of the Lafia Agricultural Development Project, which included provision for crop farming and livestock grazing. The target area, 1500 km^2 in extent, was characterized by three major river systems: Akuni, Achiba and Ehula-Ganye. These were treated as separate operational areas. Extensive cultivation by subsistence farmers provided an effective barrier to the south of the project area. Otherwise, no physical barriers such as vegetation clearing and bush burning were instituted; however, the western boundary zone was secured by limited ground spraying with dieldrin. The native fly population was suppressed using biconical traps and deltamethrin impregnated blue screens. This approach, already demonstrated in Burkina Faso, proved effective in reducing the population to as low as 5% of the original.

Males sterilized as 2 to 4-d-old adults with 120 Gy of gamma radiation in a ^{60}Co source were fed at least two blood meals before being released. Giving them at least one blood meal lowered the probability of their becoming infected with trypanosomes. Fly releases were done at weekly intervals from predetermined points

2 km apart. Up to 800 sterile males were released at each point. Initially, a 3:1 ratio of sterile to native males was used. Later, the ratio was increased to 10:1. Survival of the sterile males was excellent in spite of the 3 h transportation by car from Vom to the release site. Over 25% of the males were still alive two weeks after release. Using the release ratio of 10:1, eradication was achieved in 8–12 months, i.e. by the end of 1986, in all the river systems within the 1500 km^2 project area.

To prevent reinfestation of the cleared area, insecticide impregnated blue screens were placed at critical areas 100 m apart, on the project boundaries. The interval was decreased to 50 m or less in areas of denser vegetation [9]. Screens were reimpregnated every three to four months. Frequent (fortnightly) visits were made to inspect the screens and to replace the lost ones.

6. GENERAL COMMENTS

From the foregoing review, several lessons emerge that should benefit and guide future tsetse SIT programmes.

6.1. Mass rearing

The difficulty in mass rearing tsetse flies was one of the main obstacles to applying SIT for tsetse eradication in the past. Since the days of the Zimbabwe programme, tsetse rearing on a large scale has become a routine activity. Fly colony maintenance is now done using artificial feeding systems, with little or no reliance on live animals. Thus, the expense involved in maintaining large colonies of host animals can be eliminated. This approach was demonstrated effectively in Burkina Faso and Nigeria. The project in Burkina Faso also demonstrated the possibility of mass rearing and releasing more than one species of tsetse at the same time. Since 1988, varying numbers of six different species of *Glossina* have been maintained successfully on an artificial rearing system at the Agency's Laboratory at Seibersdorf. Clearly, the basic obstacle to application of SIT for tsetse control or eradication has been overcome. What is needed is to refine rearing operations and to introduce procedures that would further reduce operational costs.

6.2. Sterilization

With field collected pupae of unknown age (as was the case in the Zimbabwe programme), chemosterilization provides a means of inducing sterility in emerging adults without the high mortality associated with the gamma irradiation of young pupae. However, gamma radiation sterilization is safer, more accurate and much simpler to administer than chemosterilization. Moreover, the programme in Burkina Faso and Nigeria demonstrated that gamma sterilization of the adult does not produce

any deleterious effect on the survival, behaviour or sexual performance of sterile males released in the field.

6.3. Prerelease population suppression

Suppression of the native fly population was a major undertaking in all the programmes. While limited spraying of insecticides was used in the initial programmes to achieve suppression, the same result was obtained in later programmes using insecticide impregnated targets or screens placed at vantage locations for a limited time, prior to release of the sterile males. In all cases, the screens/targets were effective in reducing the tsetse population by as much as 95%.

6.4. Barriers against reinvasion and reintroduction

Maintenance of barriers is an essential part of any tsetse eradication programme, especially if the target area is relatively small, as was the case in the four programmes described. Without a secure barrier system, reinvasion of the cleared area is almost a certainty. Thus, creating and maintaining barriers was considered important in all these programmes. In an island situation, a barrier as such would not be necessary; however, reintroduction of flies from outside sources must be prevented through the imposition of strict quarantine measures. In the vast open savannah type of situation, an artificial barrier may have to be created through complete removal of trees and bushes, as was done in the Tanzanian (Mkwanja Ranch) project. On the other hand, in the two West African programmes (Burkina Faso and Nigeria) insecticide impregnated screens served both for suppressing the native fly population prior to the release of sterile males and for preventing reinvasion from outside. In addition, in the Nigerian programme sterile males were used as a 'biological' barrier to secure the various river systems following eradication of the target species.

Whatever the method of choice, the cost of creating, maintaining and monitoring the effectiveness of the barriers can be high. Such a cost must be considered an integral part of the total cost of a tsetse SIT programme.

7. CURRENT ACTIVITIES

The tsetse SIT programmes concluded to date were supported financially, either through bilateral type arrangements (Zimbabwe, the United Republic of Tanzania and Burkina Faso) or through extrabudgetary funds provided through an international organization (Nigeria). Considering the total inputs for each of the projects described, it is obvious that similar funding would be necessary for future programmes. Unfortunately, very few, if any, bilaterally assisted tsetse SIT pro-

grammes have been initiated since the USAID project in the United Republic of Tanzania and the successful German–French project in Burkina Faso. As far as international organizations are concerned, it is pertinent to note that the FAO is supporting tsetse/trypanosomiasis control projects in several countries in East, Central and West Africa. However, none of these has an SIT component, except the one on Zanzibar, United Republic of Tanzania, where the IAEA is also involved.

The IAEA is currently supporting small to medium scale tsetse SIT projects through its technical co-operation programme in several African countries. Activities in Ghana, Mali, Nigeria, Uganda, the United Republic of Tanzania and Zambia include ecological studies of the target species in a given country or locality, establishment of tsetse colonies and experimental feeding in vitro or, in some cases, mass rearing in anticipation of large field programmes.

In Zanzibar, the United Republic of Tanzania, a major SIT programme is planned, starting in 1993, to eradicate *G. austeni*, the only tsetse species on the island and which is the sole transmitter of animal trypanosomiasis on Zanzibar. For this programme, the facility in Tanga is already being used to produce sterile flies for release on Zanzibar. Ecological studies have been initiated that involve the testing of various devices for trapping the target species, *G. austeni*, which has eluded detection for many years. In the Jozani forest, one of the main habitats of the species, baseline data on fly abundance and population structure are currently being assembled. Both sticky panels and insecticide impregnated blue screens are being evaluated for population sampling and population suppression. At the same time, an FAO field team, supported by staff of the Zanzibar Department of Livestock Development, is engaged in fly population suppression using insecticide (deltamethrin) 'spot-on' treatment of animals. Monitoring of animal trypanosomiasis in the island's cattle population is also in progress. Pilot releases have been initiated using sterile male flies from the Tanga mass rearing facility. Results to date indicate that the sterile flies adapt and disperse well within the forest. It is anticipated that a full scale SIT programme will be in place in early 1993 and that eradication of *G. austeni* will be completed within two years. The programme is already benefitting from a substantial financial input from FAO. Both the Organization of Petroleum Exporting Countries (OPEC) Fund and the International Fund for Agricultural Development (IFAD) have pledged financial support. Additional funding is being solicited from the Government of Belgium and other sources.

8. FUTURE PROSPECTS FOR TSETSE SIT

It has been shown that when integrated with other methods, SIT could be an effective tool for eradicating tsetse flies or for reducing tsetse populations to manageable levels if control is the objective. The difficulty of maintaining viable laboratory colonies of tsetse has been overcome, and mass rearing is now a routine operation.

A number of African scientists and technicians have been trained in the use of the technology, and several administrators in Africa have been made aware of the potential of the technology in solving the tsetse/trypanosomiasis problem. Therefore, it is reasonable to expect that requests for assistance will increase in the future to apply SIT for tsetse/trypanosomiasis control. However, application of SIT on a routine basis for large scale tsetse population management will only be possible when certain additional conditions have been satisfied.

First, governments of the affected African countries must decide whether and under what conditions to utilize the technique as part of their tsetse/trypanosomiasis control programme. In making this decision, governments will depend upon tsetse specialists, preferably their own nationals, who are knowledgeable in all aspects of the problem. Therefore, it is important that tsetse scientists in Africa become more than familiar with SIT.

Second, governments, having made the decision to utilize the technique, must be willing to commit resources, unreservedly, for implementing the programme. This commitment is even more important should it become necessary to conduct a regional tsetse control programme embracing several countries. In this connection, it must be conceded that although 38 African countries are listed as 'affected by tsetse', the priority rating of the problem is not the same in all countries, for various reasons.

Third, external (donor) support must be assured because of the inability of some national governments to cope with the financial requirements of tsetse eradication programmes. Unfortunately, donor countries have their own preferences for countries to assist in Africa, and sometimes their own preferred tsetse control/eradication approaches. Therefore, it is necessary to work towards the harmonization of the attitudes, approaches and objectives of donors.

Fourth, it will be necessary to keep the cost of SIT operations as low as possible. (Knipling — quoted by Dame and Schmidt [3] — suggested that tsetse pupae produced at US $0.05 per pupa would be economically acceptable for SIT programmes.) Since one of the main sources of the high cost is fly production and handling, labour requirements for mass rearing must be reduced. Therefore, serious consideration should be given to establishing regional rearing facilities. Such an approach would take away the financial burden from individual countries setting up, equipping and running mass production facilities. A well designed, robust production facility, one for western and another to serve eastern African countries, would be used by several countries on a cost sharing basis. It was recently recommended by a group of consultants meeting in Vienna that, in designing regional rearing facilities, a 'modular system' should be considered as it has the advantage that it could be expanded or contracted, depending upon circumstances. Furthermore, construction of these mass rearing facilities has been adopted as a goal in the IAEA medium term plan.

9. CONCLUSIONS

Until the scourge of trypanosomiasis is totally eliminated, tsetse control or eradication will continue to be a major concern for all affected countries in Africa. In the fight against tsetse flies, insecticide application will continue to feature prominently until other proven, more effective, alternatives become available.

Currently, traps and insecticide impregnated targets and screens are being promoted by several organizations, including the FAO; village communities and individual stock farmers are being encouraged to utilize these simple and inexpensive devices to control tsetse flies. Although traps and targets have been shown to be effective in reducing tsetse populations to a very low level, the evidence to date is that these methods are not effective for eradicating tsetse populations [8]. Therefore, where eradication is the objective, a more effective approach would be required. Also, it should be borne in mind that tsetse populations could, possibly, develop resistance to insecticides and even targets and screens.

The effectiveness of SIT in eradicating tsetse populations has been demonstrated. It is an environmentally acceptable approach for tsetse population management. The technique can be used most effectively in combination with other population reduction methods and, more importantly, in area wide tsetse management programmes as opposed to field by field operations that individual farmers could undertake using tsetse traps and targets. In the field by field approach, there is always the danger of flies from an adjoining untreated farm or plot invading a 'treated' area. Using the area wide approach, an entire tsetse infested area covering several livestock farms, for example, could be effectively covered. Therefore, in addition to being an effective tool for tsetse eradication, SIT should be considered the method of choice for eradicating tsetse populations in a given area, with little or no deleterious effect on the environment. Currently, a major concern to address in order to make the technique routinely applicable is to keep costs to the minimum. With improvements in mass rearing, including the introduction of automation plus detailed planning of field programmes, the cost of producing sterile flies and of the entire operation of tsetse SIT programmes should be brought down to within financially acceptable limits.

The number of countries opting to apply SIT for tsetse control may be expected to increase in the future. Therefore, it would be more practical and less expensive to plan and execute SIT programmes on a regional basis than on an individual country basis. The approach could be modelled on the recently concluded Onchocerciasis Control Programme in West Africa, which involved several countries of the subregion. Regional tsetse SIT programmes would indeed give a more realistic and true meaning to the idea of area wide control, for which SIT is suited.

The importance of controlling or eradicating tsetse in order to eliminate trypanosomiasis cannot be overemphasized. Since 1974, when the World Food Conference of the United Nations mandated the FAO "to launch ... a long-term

programme for the control of African animal trypanosomiasis as a project of high priority", considerable emphasis has been placed on the fact that tsetse flies occupy approximately 10 million km^2 of the land surface in tropical and subtropical Africa. The point has been stressed repeatedly in official FAO documents that over 70% of this area could be used to produce an additional 120 million head of cattle if freed of tsetse. In other words, the presence of tsetse in 70 million km^2 of land in Africa is effectively robbing the continent of 1.5 million tonnes of meat per year [10].

The FAO Special Action Programme that was initiated following the United Nations mandate makes provision for drug treatment of animal trypanosomiasis cases, breeding of trypanotolerant cattle and control of the tsetse vector. In addition, emphasis is placed on area development following tsetse control or eradication. Therefore, in some situations, for example, when area development means establishing a cattle ranch in a tsetse infested area, tsetse eradication would not only be justified but would be the only means of ensuring protection of livestock that could be at risk from trypanosomiasis.

Finally, a word on the role of international organizations. The primary reason for controlling or eradicating tsetse in vast areas of the African continent is to eliminate trypanosomiasis and thus create conditions for increased agricultural production and sustainable rural development. This noble ideal can only be attained with external financial support from individual donor countries and the international community, because of the generally poor economies of most of the affected countries. The experiments carried out over the past 25 years to test the feasibility of SIT tsetse control/eradication were possible only because donor countries were willing to assist recipient countries in Africa. It is true that two United Nations organizations, FAO and the World Health Organization, are already involved in activities aimed at controlling animal and human trypanosomiasis, respectively. Since vector eradication can lead to total elimination of trypanosomiasis, perhaps the programmes and approaches adopted by the FAO and WHO would be more effective in eliminating trypanosomisis if some aspects of their respective programmes were to be harmonized with those of other organizations, especially the IAEA, which is responsible for the research, development and application of SIT. The WHO played a key role in the successful implementation of the Onchocerciasis Control Programme in West Africa. Perhaps it is not too much to expect other UN bodies to play a leading role, not only in identifying regional tsetse SIT projects but also in assisting the affected African countries in their planning, funding and execution.

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POSTER PRESENTATION

IAEA-SM-327/16P

EVALUATION OF ORIENTAL FRUIT FLY CONTROL USING THE STERILE INSECT TECHNIQUE AT DOI ANG KHANG

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The Oriental fruit fly, *Dacus dorsalis* Hendel, is a major pest of fruits in Thailand. Its attack on fruits not only reduces the yield but also affects international trade because of the quarantine restrictions imposed by major importing countries. Heavy reliance on insecticide applications has led to new problems such as a resurgence of secondary pests, undesirable chemical residues and environmental contamination. The sterile insect technique (SIT) is one of the few highly specific control methods which can be used to overcome these problems. Doi Ang Khang was selected for the pilot test as a model for larger operations. The 20 km^2 Doi Ang Khang area located at the northwest tip of the Chiang Mai provincial area (northern Thailand) is highland country (1400 m above sea level) comprising semi-isolated areas surrounded by hills and slopes. It is covered by regular plantations of peach, pear, persimmon and other fruit crops. The Office of Atomic Energy for Peace, which has pioneered SIT activities against fruit flies at Doi Ang Khang since 1982, has extended these activities until 1996.

Fruit fly pupae to be sterilized and released were reared in the mass rearing facility in Bangkok. Before irradiation, the pupae were marked with a fluorescent dye two days before emergence. The marked pupae were packed into 800 mL narrow polyethylene bags, which were then closed with rubber bands. The pupae were sterilized at 90 Gy with gamma rays from a ^{60}Co source. The bags of pupae were packed into polystyrene containers and kept cool with ice packs. Five million sterilized puae were transported to Doi Ang Khang by train and vehicle. During the period February to September, sterilized pupae were sent to the area and released every week in 15 release huts. After the release of the sterilized flies, the percentage of infested peaches decreased from 54.7% in 1984 to 17.1% in 1991. In 1991, the cost of fruit fly control using SIT was US $30 397, while the net return from the increasing value of the fruits was US $121 558.

In addition, the public has benefited from some of the advantages accrued from SIT, namely, reduced risks of toxic damage and pollution.

F_1 STERILITY AND INSECT BEHAVIOUR

(Session 5)

Chairman

V.A. DYCK
Canada

INTEGRATION OF INHERITED STERILITY AND OTHER PEST MANAGEMENT STRATEGIES FOR *Helicoverpa zea* *Status and potential*

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Abstract

INTEGRATION OF INHERITED STERILITY AND OTHER PEST MANAGEMENT STRATEGIES FOR *Helicoverpa zea:* STATUS AND POTENTIAL.

The corn earworm, *Helicoverpa zea* (Boddie), is one of the most destructive pests of field crops in the United States of America. Currently, control of *H. zea* is achieved almost entirely through the use of synthetic insecticides. However, successful application of the inherited sterility principle to a wild population of *H. zea* during a pilot test has encouraged further development of this pest control strategy. Data from recent studies and population models suggest that the full potential of inherited sterility as an area wide control strategy for *H. zea* and other lepidopteran pests may be realized only when inherited sterility is integrated with other suppression methods. Applications of singular, non-insecticidal methods for area wide suppression of *H. zea* have had limited success and have been vulnerable to temporary programme failures (e.g. inclement weather, reduced insect rearing output, etc.). An integrated approach would be horizontally diversified through the use of several strategies which use different modes of action and, therefore, would be less vulnerable to temporary programme failures than would a single strategy. Combining two or more control strategies with additive effects may increase programme efficiency. However, the greatest benefit in combining inherited sterility with other control strategies is the dynamic synergistic effects. The demonstrated potential of inherited sterility to reduce the reproductive ability of *H. zea* and the demonstrated capacity of inherited sterility to perform compatibly and synergistically with other control strategies suggest that inherited sterility should be a major component of future integrated approaches to managing *H. zea*.

1. INTRODUCTION

The corn earworm, *Helicoverpa zea* (Boddie), is one of the most destructive pests of field crops in the United States of America. Currently, control of *H. zea* is achieved almost entirely through the use of synthetic insecticides on a crop to crop basis. Such a management strategy is costly, non-selective and only effective at the

target site for a short period of time. Insecticide resistance, the mounting concern over pesticide pollution and the desire to effectively manage *H. zea* both within fields and on an area wide basis have encouraged scientists to identify and develop new methods of control.

The potential for using inherited sterility as a component of the area wide management of *H. zea* has been discussed by LaChance [1]. Knipling [2] demonstrated the potential advantage of inherited sterility over the sterile insect technique (SIT) through the use of population models. Carpenter et al. [3] found that substerilizing doses of radiation induced deleterious effects in *H. zea* that were inherited through the F_2 generation. Subsequently, a series of investigations was conducted to further define and understand the effects of inherited sterility in *H. zea*. Irradiated (100 Gy), laboratory reared moths were competitive with non-irradiated, laboratory reared moths in attracting and securing mates under field conditions [4]. Females that mated with irradiated males, and females that mated with non-irradiated males had the same mating propensity and the same refractory period [5]. Although there was a difference in mortality between *H. zea* larvae from irradiated and non-irradiated parents when they were reared in the laboratory, this mortality differential was reduced when larvae were reared under natural conditions in the field [6]. Other studies have revealed no interaction between inherited sterility and diapause in *H. zea* [7].

As a result of these studies, a pilot test was conducted in small mountain valleys in North Carolina (USA) to assess the influence of released, substerilized (100 Gy) males on wild *H. zea* populations and to measure the infusion rate of inherited sterility into the wild population. Results from this study revealed that the number of wild males captured per hectare was positively correlated with the distance from the release site of irradiated males. Analyses of the seasonal population curves of wild *H. zea* males calculated from mark–recapture data suggested that seasonal increases of wild *H. zea* males were delayed and/or reduced in the mountain valleys where the irradiated males were released. The incidence of larvae with chromosomal aberrations (progeny of irradiated, released *H. zea* males [8]) collected from the test sites during the growing seasons indicated that irradiated males were very competitive in mating with wild females and were successful in producing F_1 progeny, which further reduced the wild population.

Successful application of the inherited sterility principle to a wild population of *H. zea* during this pilot test has encouraged further development of this pest control strategy. However, because *H. zea* is a highly mobile, polyphagous pest with a wide geographical distribution, and because the technology required to rear *H. zea* economically is incomplete, the conventional methodology employed in SIT programmes may benefit from certain modifications. Therefore, the development of a pest management approach in which inherited sterility is integrated as a primary component with other pest control strategies is to be encouraged.

2. CURRENT STATUS

Knipling [9] demonstrated the potential benefit of combining sterile insects with conventional control methods. Recently, laboratory studies have revealed that inherited sterility is compatible with other methods of pest control. In a study using two insect strains and two insecticides, Carpenter and Young [10] found that progeny from irradiated parents and progeny from non-irradiated parents demonstrated the same level of insecticide resistance. Because the ratio between irradiated and non-irradiated insects is the most critical factor in regulating the efficacy of SIT or inherited sterility release programmes, insecticide applications during a continuous release of irradiated insects would benefit the release programme. Although the insecticide would reduce the number of both irradiated and non-irradiated insects, it would not change the ratio. Therefore, subsequent to insecticide applications the wild population would be at a lower level and the ratio of irradiated to non-irradiated insects would be increased by continual releases of irradiated insects. Host plant resistance is another method of pest control that would reduce the rate of increase for both irradiated and non-irradiated insects, while simultaneously allowing the continual release of irradiated insects to increase the ratio of irradiated to non-irradiated insects. Carpenter and Wiseman [11] investigated the effects of inherited sterility and host plant resistance on *H. zea* development, and found that larvae resulting from irradiated males by non-irradiated female crosses were equally competitive with normal larvae for all the parameters measured.

Additional laboratory and field studies have been initiated to measure the compatibility of inherited sterility and augmented natural enemies such as parasitoids and viruses. Preliminary data indicate that parasitoids and viruses are compatible with the inherited sterility method.

3. PERSPECTIVE

Integration of inherited sterility and other pest control strategies into a successful management approach is predicated upon the validity of the following: (1) inherited sterility is effective at low release ratios; (2) inherited sterility is effective at different rates of increase for the pest population; (3) other pest control strategies do not negatively impact irradiated insects and their progeny more than that of the wild population; and (4) the inherited sterility component is flexible in its application.

3.1. Influence of low release ratios and rates of increase

A computer model using data from Carpenter et al. [3] quantified the influence of different parameters on the predicted efficacy of the inherited sterility method.

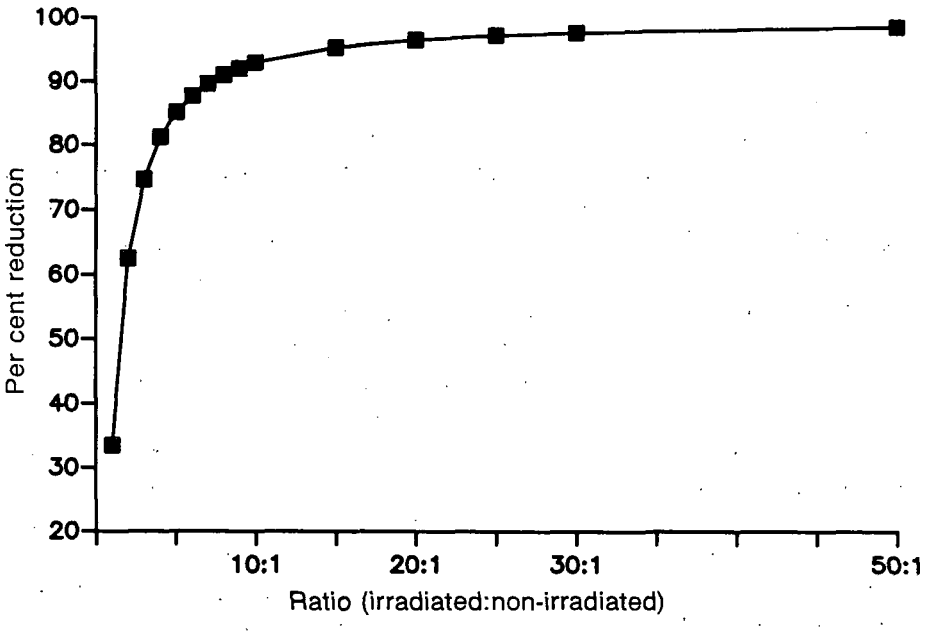

FIG. 1. Per cent reduction in normal population growth at different release ratios of irradiated (100 Gy) males.

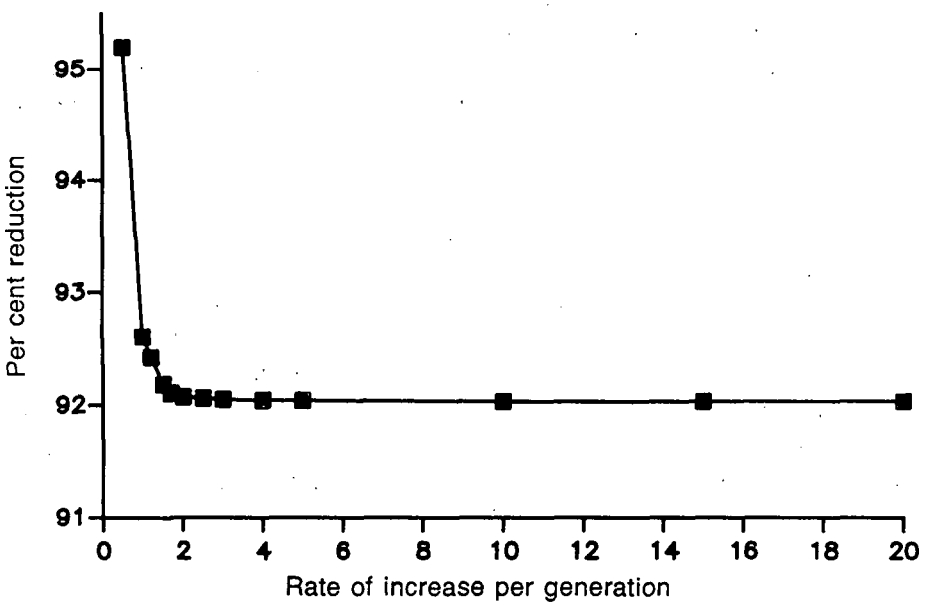

FIG. 2. Relationship between the per cent reduction in normal population growth and the rate of increase per generation.

Increases in the release ratio had the greatest effect on the percentage reduction in normal population growth when the release ratio was less than 10:1 (Fig. 1). Even a release ratio as low as 1:1 reduced the normal population growth by 30%. These model predictions were corroborated in a pilot test (unpublished data) in which release ratios averaging less than 5:1 reduced the wild population of *H. zea* by an average ($\pm$ SE) of 73.5 $\pm$ 14.9%.

The population model suggests that the efficacy of inherited sterility is almost constant when the rate of increase per generation is twofold or higher (Fig. 2). This is in contrast to the efficacy of SIT which declines as the intrinsic rate of increase rises. The model also suggests that a substantial increase (3%) in treatment efficacy of inherited sterility occurs when the normal population is in decline (0.5 fold per generation). Because the *H. zea* population normally declines following the emergence of the overwintering generation, the increase in treatment efficacy of inherited sterility revealed in the model provides an additional potential for managing this pest population.

3.2. Impact of other pest control strategies

Available data from insecticide resistance and host plant resistance studies suggest that the impact of other pest control strategies will not be greater for irradiated insects and their progeny than for wild insects. As explained earlier, conventional uses of both insecticides and host plant resistance would increase the ratio of irradiated to non-irradiated insects, thus producing synergistic effects. Furthermore, models developed by Knipling [12, 13] depicting different integration scenarios suggest that combining inundative releases of parasites with sterile insects will yield both additive and synergistic effects. Although the parasite technique and SIT have different modes of action, the effectiveness of SIT increases the ratio of adult parasites to adult hosts, and the effectiveness of the parasites increases the ratio of sterile to fertile insects. Knipling [13] calculated that combining sterile insects with parasites theoretically could be 10 000 times more effective than if each technique were used alone. While these figures are impressive, even greater suppression could be expected if parasite releases were combined with the inherited sterility technique. Not only is inherited sterility in *H. zea* more effective than full sterility in reducing population increases, but the inherited sterility technique produces sterile F_1 larvae that would provide an increased number of hosts for the parasites. Therefore, the number of parasites produced would increase even if the rate of parasitism remained the same (host density independent), and whether or not additional parasites were released.

3.3. Flexibility of inherited sterility application

Flexibility in application of the inherited sterility method has been described by LaChance [1]. The level of sterility and the number of individuals in the F_1 and F_2 generations are controlled by the dose of radiation administered to the P_1 generation, and by whether only irradiated males or both irradiated males and females are released. This flexibility should be enhanced by the integration of other pest control methods, thus allowing management strategies to be modified as seasonal changes occur in the pest status of *H. zea* and the host plant availability.

There are many different scenarios in which an integrated approach employing inherited sterility could be envisioned to control *H. zea* populations. The release of partially sterile male and female *H. zea* would produce large numbers of sterile larvae that could be field reared on early season weeds or possibly whorl stage corn. Parasites (native and/or released) could use these sterile larvae as hosts and, thereby, substantially increase the parasite population for the next *H. zea* generation. Also, surviving sterile larvae would produce sterile *H. zea* adults for the next generation. If the economic injury level of cultivated host plants indicated that additional larvae were undesirable, then the dose of radiation administered to *H. zea* could be increased to a level that would reduce or eliminate the number of progeny from irradiated females, or releases could be limited to irradiated males.

Although inherited sterility is compatible with synthetic organic insecticides, parasites and/or predators are not generally compatible with these insecticides. If insecticides are needed to reduce *H. zea* infestations, insect growth regulators, biologicals, or other formulations compatible with natural enemies should be considered. An alternative management scheme could be to establish *H. zea* host plants in insecticide free areas adjacent to insecticide treated crops. Host plants could be artificially infested with *H. zea* larvae to provide parasites (native and/or released) with an adequate supply of hosts. If the *H. zea* larvae used in the artificial infestations were sterile (progeny of irradiated parents), then non-parasitized larvae would not contribute to the increase of the wild population, but would produce sterile *H. zea* adults for the next generation.

Resistant plant varieties, if available, would play an important role in an integrated control programme involving inherited sterility. Resistant varieties with antibiosis as the mode of action would not only provide some plant protection, but also would reduce the number of *H. zea* entering the next generation. Therefore, in an irradiated *H. zea* release programme, resistant varieties could effectively increase the ratio of irradiated to non-irradiated *H. zea*.

4. DISCUSSION

Employment of an integrated management approach for *H. zea* will no doubt require consideration of numerous economic, ecological, behavioural and logistical

factors. Currently, this knowledge is incomplete. However, the model presented in Fig. 1 provides some unique insights into how different control strategies could be combined for greatest efficiency. Although the effectiveness of inherited sterility continues to increase as the ratio of irradiated to non-irradiated insects increases, the efficiency decreases quickly once a 10:1 ratio has been obtained. A similar loss of efficiency occurs in the parasite release technique when the parasite to host ratio increases above 5:1. According to these models, the economic feasibility of combining inherited sterility and parasite release techniques would be greatest when the ratio of irradiated to non-irradiated *H. zea* is $\leq 10:1$ and the ratio of parasite to host is $\leq 5:1$. Access to reliable efficiency curves for all the control strategies involved in an integrated control programme would facilitate the optimal use of programme funds.

The full potential of inherited sterility as an area wide control strategy for *H. zea* and other lepidopteran pests may be realized only when inherited sterility is integrated with other suppression methods. Applications of singular, non-insecticidal methods for area wide suppression of *H. zea* have had limited success and have been vulnerable to temporary programme failures (e.g. inclement weather, reduced insect rearing output, etc.). An integrated approach would be horizontally diversified through the use of several strategies which used different modes of action and, therefore, would be less vulnerable to temporary programme failures than would a single strategy. Combining two or more control strategies with additive effects may increase programme efficiency. However, the greatest benefit in combining inherited sterility with other control strategies is the dynamic synergistic effects. The demonstrated potential of inherited sterility to reduce the reproductive ability of *H. zea* and the demonstrated capacity of inherited sterility to perform compatibly and synergistically with other control strategies suggest that inherited sterility should be a major component of future integrated approaches to managing *H. zea*.

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EVALUATION OF THE F_1 STERILITY TECHNIQUE FOR POPULATION SUPPRESSION OF THE PINK BOLLWORM, *Pectinophora gossypiella* (SAUNDERS)

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Abstract

EVALUATION OF THE F_1 STERILITY TECHNIQUE FOR POPULATION SUPPRESSION OF THE PINK BOLLWORM, *Pectinophora gossypiella* (SAUNDERS).

Field cage studies were carried out to evaluate the F_1 sterility technique for population suppression of the pink bollworm, *Pectinophora gossypiella* (Saunders), in cotton. For this purpose, six field cages (1.8 m × 1.8 m × 1.8 m) were placed over cotton seedlings in the field. Five pairs of laboratory reared, untreated adult moths were released in all six cages during the first week of August. In addition, on the same date 100 pairs of adults, following irradiation of the mature pupae at 100 Gy, were released in two cages and 250 such pairs of irradiated adults in two other cages in ratios of 20:1 and 50:1 (irradiated:normal), respectively. The results of per cent larval infestation in the rosette blooms in different field cages indicated that larval infestation in the flowers was significantly lower (4.93%) in cages where the moths were released at the 50:1 ratio than in cages where moths were released at the 20:1 ratio (6.71%). Larval infestation in the flowers was significantly highest (21.87%) in the control cages. Similarly, larval infestation in the green bolls was significantly reduced to the subeconomic level (5.23%) in the 50:1 cages compared with the 20:1 cages (8.10%). However, larval infestation in the control cages exceeded 20%. These preliminary studies indicate the potential of the F_1 sterility technique for effective control of the pink bollworm.

1. INTRODUCTION

The feasibility of using induced sterility for the control of numerous economically important lepidopteran pests has been investigated [1]. The pink bollworm, *Pectinophora gossypiella* (Saunders), demonstrates typical characteristic lepidopteran responses to gamma radiation [2, 3].

The possibility of achieving insect population suppression by introducing irradiated insects of the same species was suggested by Serebrovskij [4]. Subsequently, Proverbs [5] demonstrated that the F_1 progeny of the irradiated codling moth was sterile. Since then, a number of investigators have reported the same phenomenon in other lepidopteran pests [2, 3, 6]. The theoretical models of

Knipling [1] for population suppression of Lepidoptera by releasing partially sterile males were confirmed by Flint et al. [7], who reported that pink bollworm moths irradiated at 10 krad and released in field cages were more effective in population suppression than moths treated with higher doses of gamma radiation.[1] Bartlett and Butler [8] found that the F_1 female progeny of irradiated females (15 and 20 krad) paired with untreated males were more than 90% sterile. Furthermore, releases of either sex sterilized at 10, 15 or 20 krad or both sexes sterilized at 10 krad suppressed the pink bollworm population from 82.6 to 99.9% over two generations. Henneberry and Clayton [9] found that the number of progeny produced by laboratory reared moths exposed to 15 krad or higher doses and paired with untreated females was reduced by over 80% compared with those produced by untreated pairs. The reproduction rate of male and female pink bollworm moths from 10 or 15 krad irradiated pupae and mated to untreated females or males was reduced by 88% or more [10]. In lepidopterous species, partial sterility in the treated P_1 males is associated with high levels of sterility in the F_1 progeny. Thus, fairly low doses of gamma radiation should probably be administered to the released males in order to achieve an effective combination of induced partial sterility and conditional lethal mutations capable of suppressing a native population and, simultaneously, of changing its genotype. This paper reports on the evaluation of the F_1 sterility technique for population suppression of the pink bollworm in field cages.

2. MATERIALS AND METHODS

Field cage studies were carried out to evaluate the efficiency of irradiated moths and their F_1 progeny for population suppression of the pink bollworm. For this purpose, six field cages (1.8 m × 1.8 m × 1.8 m) were placed over cotton seedlings in the field. The native population of pink bollworm, if any, from diapausing larvae in the cages was monitored by gossyplure baited traps and by inspecting the cotton fruiting bodies weekly. After confirming that there was no infestation in the cages, five pairs of laboratory reared, untreated adult moths were released in all six cages during the first week of August. In addition, on the same date 100 pairs of adults, following irradiation of the mature pupae at 100 Gy, were released in two cages and 250 such pairs of irradiated adults in two other cages at ratios of 20:1 and 50:1 (irradiated:normal), respectively. The remaining two cages were kept as the control, with no irradiated moths. Subsequently, three releases of moths at the above mentioned ratios were made in the respective cages at three week intervals. The observations made on the rosette blooms and larval infestation in the green bolls were recorded at weekly intervals.

[1] 1 rad = 1.00×10^{-2} Gy.

TABLE I. MEAN PER CENT LARVAL INFESTATION OF PINK BOLLWORM IN THE FLOWERS AND GREEN BOLLS OF COTTON IN FIELD CAGES

Release ratio	Per cent larval infestation in
Normal:irradiated	Flowers:green bolls
1:50	4.93b:5.24b
1:20	6.71b:8.57b
Control	21.87a:21.35a

Note: Means with the same letters are statistically non-significant (P = 0.05).

3. RESULTS

3.1. Flower infestation (rosette bloom)

The results of the per cent larval infestation in rosette blooms in different field cages (Table I) indicated that larval infestation in the flowers was significantly lower (4.93%) in cages where the moths were released at the 50:1 ratio compared with cages where they were released at the 20:1 ratio (6.71%). Larval infestation in the flowers was significantly the highest (21.87%) in the control cages.

The results of the larval population buildup in the different field cages, as recorded for flowers by inspecting the rosette blooms (Fig. 1), showed that the first rosette bloom was recorded two weeks after the release of normal moths in the control cages. However, rosette blooms appeared after the third week of release in cages where the ratio of irradiated:normal moths was maintained at 20:1 and 50:1. Although the trend of larval population buildup in the flowers was almost identical in all the cages, the releases of irradiated moths resulted in reduced moth population development in the 20:1 and 50:1 cages. However, larval infestation in the 50:1 cages was significantly lower when compared with the rest of the field cages.

3.2. Boll infestation

The data on larval infestation in green bolls in different field cages (Table I) indicated that infestation was significantly reduced, to the subeconomic level (5.23%), in cages where a release ratio of 50:1 moths was maintained as compared

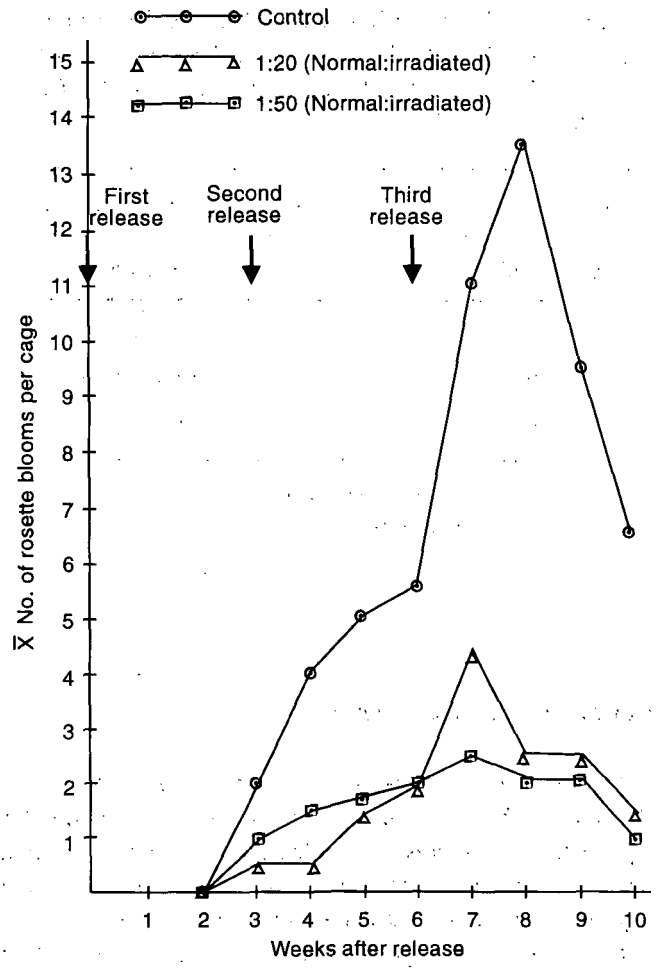

FIG. 1. Pink bollworm infestation in cotton flowers.

with the cages with a release ratio of 20:1 moths (8.10%). However, larval infestation in green bolls was significantly the highest (21.98%) in the control check cages.

The results of larval population buildup in mature green bolls in different cages (Fig. 2) indicated that in cages receiving irradiated moths, following irradiation of mature pupae, larval infestation appeared three weeks after the first release, whereas in the control cages the infestation appeared after two weeks. The increase in larval infestation was rapid in the control cages, reaching its peak seven weeks after the first release of moths. In the cages where a release ratio of 20:1 was maintained, larval infestation in green bolls was significantly lower than in the control cages. Although the larval infestation trend in the green bolls after three releases of moths

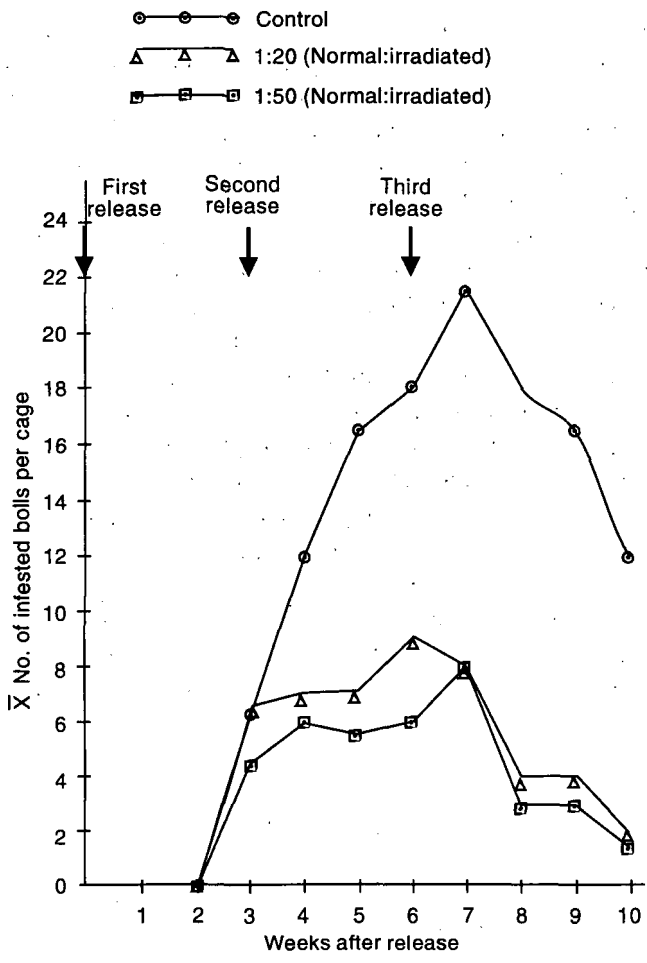

FIG. 2. Pink bollworm infestation in the green bolls of cotton.

was almost identical in all the cages, larval infestation in the 50:1 cages was significantly lower than in the 20:1 cages.

4. DISCUSSION

The greatest disadvantages of using radiation sterilized male moths to control lepidopteran populations have been the costs of rearing and the failure of the irradiated males to compete successfully with the wild males [11]. However, data on delayed larval infestation in field cages receiving irradiated moths suggest that the irradiated male and female pink bollworm moths were competitive in mating with

non-irradiated, laboratory reared pink bollworm moths. Furthermore, male moths treated with 100 Gy mated effectively and prevented the females from remating. The males and females that emerged from the irradiated pupae elicited lower oviposition responses when mated with either untreated or treated males. It could, therefore, be inferred that radiation sterility in moths that emerged from the irradiated pupae was comparable to F_1 sterility [2, 3, 9, 12].

The release of partially sterilized moths at a ratio of 50:1 at three week intervals reduced larval infestation to the subeconomic level (5%) compared with the cages where a release ratio of 20:1 was maintained (9%). In the control cages, larval infestation exceeded 20%. The relatively reduced infestation in bolls in field cages demonstrated that the number of progeny from partially sterile moths was limited and failed to cause significant damage to the crop. These results are in close conformity with those of Bariola et al. [13] and Flint et al. [7]. The reduced larval infestation in both the flowers and the bolls indicated the additive effect of induced and inherent sterility in pink bollworm moths. Therefore, the releases of irradiated moths should be carried out more frequently than at three week intervals to ensure that sexually active sterile or partially sterile males are always present to mate with the native females. These preliminary studies indicate the potential of the F_1 sterility technique for the effective control of the pink bollworm. However, further detailed investigations are needed to evaluate the efficiency of the F_1 sterility technique under field conditions.

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EVALUATION OF THE POTENTIAL CONTROL OF THE EUROPEAN CORN BORER (*Ostrinia nubilalis* Hb.) IN THE FIELD BY RADIATION INDUCED F_1 STERILITY

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Abstract

EVALUATION OF THE POTENTIAL CONTROL OF THE EUROPEAN CORN BORER (*Ostrinia nubilalis* Hb.) IN THE FIELD BY RADIATION INDUCED F_1 STERILITY.

The European corn borer (*Ostrinia nubilalis* Hb.) is considered to be the most important pest of maize in Romania after panicle emergence. It covers the entire cropping area of the country. Data collected over a five year period have indicated an average of 44% plants infested, 1.1 larvae per plant, 23 180 larvae/ha and 550 kg/ha or 7.5% losses in yield. Depending on the geographical area, the climatic conditions, the structure of the culture and the corn hybrids cultivated in spring, the ECB population can theoretically be estimated at 263–5845 larvae/ha. Of a total area of 3×10^6 ha corn cultivated in Romania, it can reach levels of between 789 and $17\,535 \times 10^6$ larvae. A mass rearing technique suitable for Romanian conditions has been developed. In Romania, the European corn borer is attacked by many natural enemies throughout its life; the role of parasites and predators is presented in the paper. It is now possible to apply biological control by *Trichogramma* spp. but the results differ depending on the region in which the control is applied. Sex pheromone formulation developed in Romania is less efficient, but it can be used to record the flight of the corn borer males. Control of the flies in small areas (1–2 ha) was attempted by mating disruption and mass capture of males in sticky traps. The results show that under these conditions it is not possible to control the pest. Experiments performed in field cages have demonstrated a theoretical possibility of controlling the pest by releases of flies having inherited F_1 sterility. Our data have demonstrated that the percentage of stems attacked by the pest and the number of overwintering larvae per stem were correlated with the hatching percentage. Releases of the F_1 generation with inherited sterility, in field cages, at different ratio of males with inherited F_1 sterility to wild males demonstrate that this method of control can be used as part of an integrated control system.

1. INTRODUCTION

The European corn borer (*Ostrinia nubilalis* Hb.) (ECB) is one of the most damaging pests of maize. It covers an extensive area in almost all parts of the world, especially in the Northern Hemisphere, and is able to thrive in a wide range of climates, from equatorial to temperate zones. Its particular economic significance has determined the intensive research being carried out on this insect, especially in North America.

In Romania, ECB is most harmful after panicle emergence, and is encountered in all corn culture areas. The losses induced have risen to 40% of the grain yield [1]. Data collected over a five-year period indicated averages of 44% attacked plants, 1.1 larvae per plant, 23 180 larvae/ha and 550 kg/ha (or 7.5%) loss in yield [2] (Table I).

Under the ecological conditions prevailing in Romania, the insect develops one generation per year, except for areas in the south, where a second partial generation has been recorded with a population that is less than 20% that of the previous generation. The mass flight of the overwintering generation of moths commonly takes place a few days before panicle emergence, the main attack taking place in tunnels inside the stems. The attack on corn is economically important, but the pest also feeds on hemp and sorghum crops, as well as on various species of spontaneous flora.

Because of the particular significance of ECB for the corn crop, a series of investigations has been undertaken in Romania with a view to preventing outbreaks of this pest using chemicals [3], biological means [4–8] and resistant hybrids [9–15].

In recent years, particular attention has been paid to the study of synthetic sex pheromones [16–19] and, since 1988, investigations have been carried out on male sterilization using radiation [20–23].

TABLE I. IMPORTANCE OF EUROPEAN CORN BORER FOR MAIZE CROPS IN ROMANIA

	Limiting values	Average
Plants attacked	13.0–70.0%	44.0%
Larvae/plant	0.2–2.2	1.1
Larvae/ha	2630–58 455	23 180
Yield reduction/ha	80–1326 kg	550 kg
Loss/larva reaching diapause	13.9–57.0 g	34.0 g
Increase in tunnelling in the stalk	— stalk breakage	
	— incidence of stalk rot	

2. LIFE HISTORY

The life history of ECB has been studied in detail by many workers and its biology is well known. The developmental rates are influenced by many factors, including temperature and moisture.

3. MIGRATION

The ECB is a poor flyer and until now large migrations have not been reported. The moths leave the emergence sites in corn field debris and fly to places with dense vegetation, usually grasses, where a good microclimate exists for feeding, resting and mating.

After mating, the female moth leaves the mating site and deposits her eggs in corn fields. This movement is possibly repeated. Not enough experimental data are available on the flying capacity of the moths.

4. POPULATION

Depending on the geographical area, the climatic conditions, the structure of the cultures and the corn hybrids cultivated in spring, the ECB population has theoretically been estimated at 263–5845 larvae/ha (on average, 2318). In all corn cultivated areas (3×10^6 ha) it can rise to 789–17 535 $\times$ 10^6/larvae. Such large populations can be reduced by weather conditions and soil tillage.

5. MASS REARING OF ECB

Since all investigations aimed at preventing ECB attack are conditioned by the availability of large numbers of insects, mass rearing of this insect is essential.

Consequently, a series of experiments were performed on various diets, using bean meal as the basic ingredient because it affords satisfactory growth of the ECB and because the evolution of ECB is not influenced negatively by any of the ingredients added. The diet ingredients shown in Table II are of practical importance because they were used in all the investigations carried out on male sterilization. These ingredients are common, readily available and inexpensive. Depending on the extent of the rearing facilities, the cost of 1000 pupae ranged from 751 to 4130 lei (US $1 = lei 430 (Oct. 1992)) (Table III).

When evaluating the performance of *O. nubilalis* moths reared for a varying number of generations on an artificial diet, it was found that the fecundity and longevity values did not exhibit significant differences between generations. The

TABLE II. INGREDIENTS USED IN THE DIET FOR MASS ARTIFICIAL REARING OF *Ostrinia nubilalis* Hb (ONE BATCH = 4500 kg)

Ingredient	Amount	%
Bean meal	372.0 g	8.20
Wheat bran	160.0 g	3.53
Brewers' yeast	136.0 g	3.00
Milk powder substitute for calves	106.0 g	2.34
Salt mixture for poultry	40.0 g	0.88
Sugar	133.0 g	2.93
Ascorbic acid	13.6 g	0.30
Sorbic acid	10.0 g	0.22
Acetic acid glacial	14.8 mL	0.33
Formaldehyde	8.4 mL	0.19
Agar	40.0 g	0.88
Water	3500.0 mL	77.20

results of experiments carried out on generations 81 and 106 were similar to those obtained in generations 8 and 9, respectively [24].

It is worth mentioning that mass rearing of ECB has been running for 161 successive generations and is still in progress, unlike individual rearing, which will be discontinued after nine successive generations because of a degeneration in induced sterility.

A mass rearing technique has been developed that fits the Romanian conditions. On the basis of this technique, using a diet composed essentially of indigenous ingredients, insects can be reared continuously, so satisfying the increasing requirements.

Since sufficient biological material is available, it was possible to perform experiments on the sterilization of *O. nubilalis* males by irradiation.

6. DAMAGE LEVELS

Corn plants do not yield well as a result of ECB damage. The damage resulting from leaf feeding is of no relevance, but stalk tunnelling is of great importance. In Romania, the reduction in yield ranges between 80 and 1326 kg/ha (on average, 550 kg/ha).

TABLE III. COST OF ECB (*Ostrinia nubilalis* Hb.) REARED AT FUNDULEA, ROMANIA (US $1 = lei 430 (OCT. 1992))

	12 000 pupae/ month (lei)	300 000 pupae/ month (lei)
Artificial diet	4 230	105 750
Wages	35 100	70 200
General expenses	8 115	39 145
Common expenses	2 110	10 185
Total	49 555	225 280
Cost/1000 pupae	4 130 (US $9.6)	751 (US $1.75)

7. CONTROL

Integrated control of this pest is now being explored. New pest management strategies should include the economic threshold levels, regular monitoring surveys, biological control opportunities, etc.

8. CHEMICAL CONTROL

Because the mass flight of moths usually takes place a few days before panicle emergence, the corn is generally too tall when the decision is made to control ECB. Equipment availability and the time required to complete application over large areas often necessitate use of aerial treatment, but this is very expensive. For this reason, no chemical control of ECB is carried out in Romania, as a result of which no insecticide resistance exists.

9. NATURAL CONTROL BY BIOLOGICAL AGENTS

In Romania, ECB is attacked by many natural enemies throughout its life. The parasites and predators are presented in Table IV and Table V shows the most important biological factors affecting populations of *O. nubilalis* Hb.

Biological control of ECB by *Trichogramma* spp. is theoretically possible in Romania, but Table VI shows that control differs, depending on the region in which

TABLE IV. PARASITES AND PREDATORS OF *Ostrinia nubilalis* Hb. IN ROMANIA

Host stage	Family	No. of species	Importance
Egg	Trichogrammatidae	4	0;+++
	Anthochoridae	2	0;+
	Nabidae	4	0;+
	Chrrysopidae	2	0;+
Larva	Chalcididae	8	0;+
	Ichneumonidae	14	+;++
	Tachinidae	5	+;++
	Chloropidae	8	0;+
Pupa	Ichneumonidae	1	0

Note: 0: Unimportant.
+: Of small account.
++: Important.
+++: Of the first importance.

TABLE V. PRINCIPAL BIOLOGICAL FACTORS AFFECTING POPULATIONS OF *Ostrinia nubilalis* Hb. IN ROMANIA

Biological factor	Affected stage of host	Limiting value (%)	Average (%)
Trichogramma spp.	Egg	0–75.0	7.8
Lydella thomsoni Hert.	Larva	0–15.6	4.6
Sinophorus crassifemur Thom.	Larva	0–5.0	1.8
Fungi	Larva	13–47.2	31.6
Bacteria	Larva	11.7–35.2	19.1

TABLE VI. CONTROL OF *Ostrinia nubilalis* Hb. BY *Trichogramma maidis* PINT IN FUNDULEA AND TURDA

Fundulea

	Control			Five weekly treatments (200 000/ha)		
	1989	1990	1991	1989	1990	1991
% attacked plants	49.7	28.1	57.5	37.9	28.0	40.8
Larvae/plant	0.61	0.21	1.1	0.52	0.19	0.8

Turda

	Control		One treatment (200 000/ha)		Two treatments (200 000/ha)	
	1988	1989	1988	1989	1988	1989
% attacked plants	93.3	74.6	49.0	24.1	34.3	10.1

it is applied. *Trichogramma* species has weak flying and searching activity and a short period of life. For these reasons, it is necessary to repeat the releases in corn fields (7 m–7 m) and no results are achieved when the temperature is over 31°C in the fields. The parasites must be applied manually; this type of control is expensive and the results are not precise.

10. PLANT RESISTANCE

Selections of inbred corn lines resistant to ECB have been made in Romania over the past two decades. Today, many types of hybrid are available that show an intermediate resistance tolerance to *O. nubilalis*.

At the Research Institute for Cereals and Industrial Crops at Fundulea, there is an extensive breeding project in which germplasm resistant to ECB is used as genetic stock for breeding purposes.

11. PHEROMONES

Components of various ratios and amounts of the synthetic ECB sex pheromone were evaluated in the field. The results showed a relatively high number of males captured in the sticky traps throughout the flying period; this varied with the location and the pheromone variant used. It is worth mentioning that in Romania both *cis (Z)* and *trans (E)* pherotypes of this species are found, but the latter are missing in the central and northeastern parts of the country.

The pherotype *trans (E)* is generally found in less numerous populations, but the problem of ECB in Romania lies in the *cis (Z)* pherotype.

Even though the synthetic sex pheromone formulation developed in Romania is less efficient, it can be used to record the flight of ECB males; thus flight curves can be drawn for generations 1 and 2 (Fig. 1).

We should stress that in Romania the first generation is particularly important, occurring in the second half of June; the second generation, specific to the southern part of the country, appears in August and is practically devoid of damage potential.

There are a series of theoretical models for using sex attractants for insect control. In 1990 and 1991 at Fundulea, we tried to control ECB in small areas (1–2 ha) isolated in the forest by mating disruption and mass capture of males in sticky traps.

The results presented in Tables VII and VIII showed that it is not possible to control ECB in small areas because we presume that mating of the females did not take place in the corn fields.

Sex pheromones are now used in ecological studies, mainly for migration and evaluation or monitoring of pest populations.

12. LABELLING ADULTS

At present, 'Calco Red Dye' is used to investigate pest dynamics and to estimate natural populations. This marker behaved very well in our trials by labelling adults obtained from larval rearing on a regular diet to which the dye had been added.

13. USE OF IONIZING RADIATION FOR PARTIAL STERILIZATION OF ECB

In an attempt to develop the fundamentals of genetic control through F_1 sterility as a component of an integrated control programme for ECB at the Research Institute for Cereals and Industrial Plants at Fundulea, investigations related to the sterile insect release technique have been carried out within the framework of an IAEA Co-ordinated Research Programme, 'Radiation Induced F_1 Sterility in Lepidoptera for Area-Wide Control'.

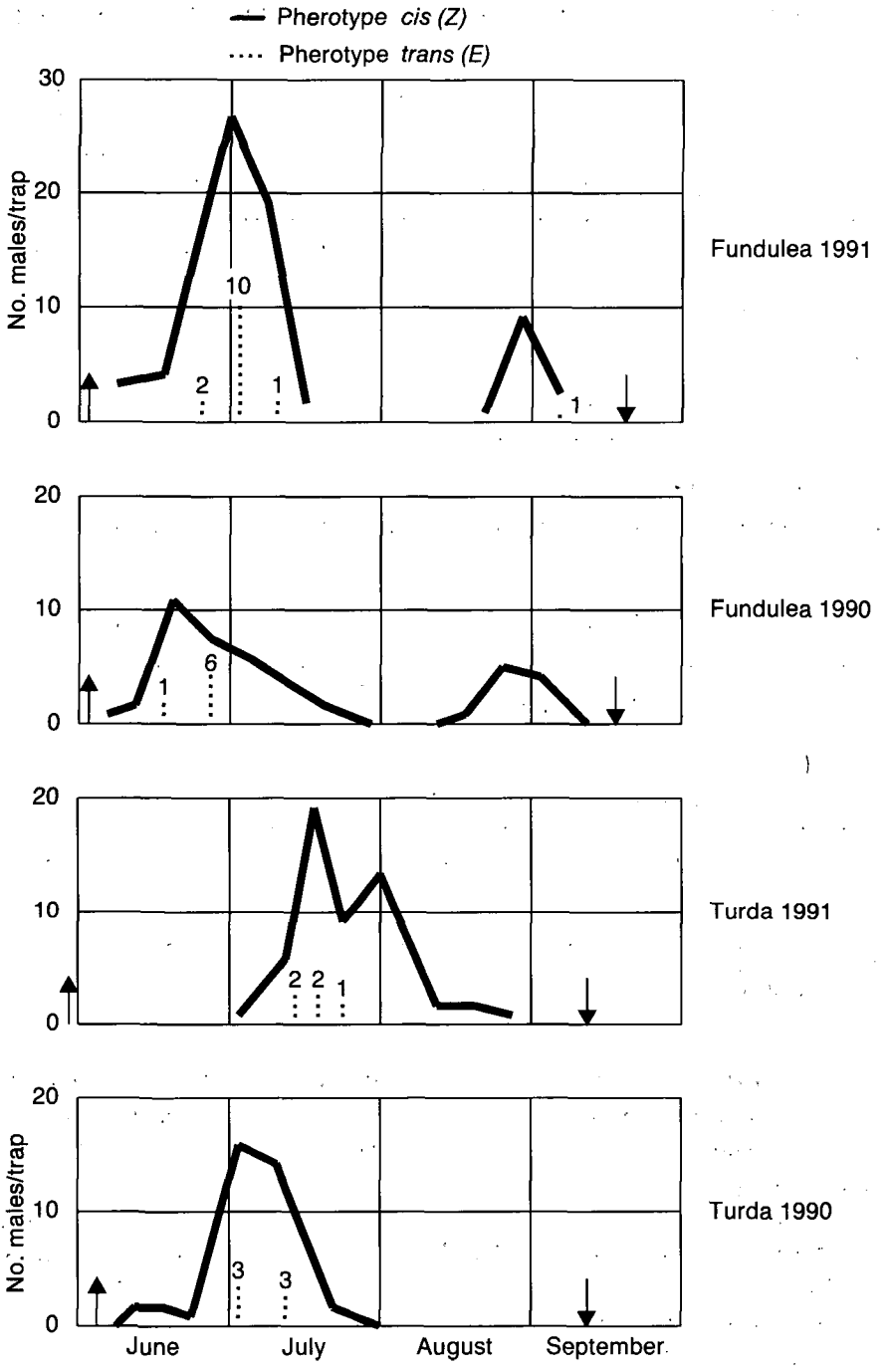

FIG. 1. Flight curves of the ECB, registered by number of males captured in sticky traps.

TABLE VII. CONTROL OF *Ostrinia nubilalis* Hb. — MASS TRAPPING (MT)

Year	No. of males/trap (total males)		Attacked plants (%)		Larvae/plant	
	Control	MT[a]	Control	MT[a]	Control	MT[a]
1990	30.2	11.4 (183)	23.3	22.8	0.17	0.23
1991	50.0	18.8 (301)	62.0	55.3	0.55	0.47

[a] Sixteen sticky traps/ha.

Note: Numerals in parentheses denote total number of males captured per year.

TABLE VIII. CONTROL OF *Ostrinia nubilalis* Hb. — MATING DISRUPTION

Year	Attacked plants (%)		Larvae/plant	
	Control	MD[a]	Control	MD[a]
1990	18.8	18.0	0.2	0.25
1991	33.4	30.6	0.45	0.5

[a] One hundred lures/ha.

Application of this technique requires knowledge of the method, radiation dose and biological parameters of the specimens being exposed to radiation in order to ensure the required degree of substerility of the pest with the minimum detrimental effect on the irradiated insects.

The insects used in experiments were derived from a laboratory strain and have been reared according to individual and mass rearing techniques elaborated at Fundulea.

Preliminary trials have established the role of pupae age and radiation dose on the emergence rate of moths.

The experiment with irradiated pupae revealed that hatching was dependent on their age at treatment and the radiation dose: the older the pupae, the less affected

their hatching in comparison with the unirradiated check pupae. It should be noted that adult emergence was negatively influenced in direct proportion to the radiation dose [21].

As a result of these preliminary trials, subsequent investigations used 6-d-old pupae, from which the adults emerged within the next 48 h.

The two sexes had different levels of sterility at the same radiation dose; males were more resistant to radiation than females, which had a higher degree of sensitivity. Complete sterility of the females was obtained with 30 krad, a dose[1] at which the males had only 86.5% sterility, i.e. partial sterility [22].

It is well known that pupal sexing is particularly difficult. Therefore, for sterile insect releases it is necessary to irradiate both the male and female pupae together. It is necessary to use partially sterile males because their competitiveness is close to that of hormale ones, on one hand, and completely sterile females, unable to generate larvae which subsequently would attack corn plants, on the other. To increase the degree of sterility at a lower rate of radiation, the F_1 generation had to be used for releases instead of the P generation.

To decrease the rate of radiation, the P male genitors were irradiated with 10 or 15 krad as 6-d-old pupae. The males that emerged were crossed with normal females. The offspring of these crossings were inbred or backcrossed; F_1 observations were carried out on the number of eggs/female, the percentage egg hatch, the adult lifespan and the percentage sterile couples.

The data in Tables IX and X showed no significant decrease in the number of eggs laid by one female in the P, F_1 or F_2 generations after irradiation with 10 and 15 krad. Generally, the number of eggs was a little lower when adults from the P_0 I × N were inbred.

We failed to obtain a sufficiently large population to test in F_2 populations from F_1 I × I because hatching rose to 25.4% at 10 krad and 9.1% at 15 krad; however, the larvae obtained did not reach the adult stage.

In all the combinations tested in F_1, hatching decreased with the increasing radiation dose. The hatching percentage in F_1 was lower in the three variants from P_0 I × N, when compared with the parent generation. Egg viability in F_1 was significantly reduced in comparison with the control for both the doses applied, indicating the existence of recessive lethal genes within these populations. In F_2, a decrease in the percentage egg hatching was also noted in all the combinations tested, indicating the inheritance of harmful effects induced by males irradiated in the parent generation. It is also remarkable that the variants analysed for F_2, although more fertile than those in F_1, still exhibited a noticeable effect of lethal genes. No significant reduction in the adult lifespan was noted in P, F_1 or F_2.

The percentage sterile couples increased in descendences derived from irradiated males, reaching the highest values in inbred variants.

[1] 1 rad = 1.00×10^{-2} Gy.

TABLE IX. TREATMENT OF *Ostrinia nubilalis* Hb. MALES WITH A SUBSTERILIZING DOSE OF 10 krad

Generation	Cross variant		Egg masses/ female	Hatchability (%)	Longevity of adults		Sterile pairs (%)
	Male	Female			Male	Female	
P_0	N × N(C)		7.6	90.5	8.9	7.7	4.2
	I × N		7.7	67.5	7.5	6.7	6.7
F_1	N × N (C)		7.4	88.0	6.9	7.0	5.7
	A × N		7.6	53.6	8.1	8.3	8.5
	N × A		7.2	59.2	7.2	7.8	3.2
	A × A		6.0	25.4	6.1	6.8	8.2
F_2	N × N (C)		6.8	85.3	8.7	7.9	6.8
	N × (A × N)		7.2	70.1	8.2	8.1	8.1
	N × (N × A)		6.9	80.1	8.3	7.8	8.4
	(A × N) × N		6.7	64.0	7.9	8.0	8.9
	(A × N) × (N × A)		7.1	45.2	8.1	7.6	7.1
	(A × N) × (A × N)		6.5	38.3	7.8	7.9	12.8
	(N × A) × N		7.3	68.4	8.5	8.1	7.5
	(N × A) × (A × N)		7.0	40.3	8.7	8.2	8.6
	(N × A) × (N × A)		6.7	32.8	8.1	8.2	14.3

N: non-irradiated or normal; I: irradiated; A: progeny from irradiated male × normal female (I × N); C: control.

This experiment showed that the F_1 sterility obtained at 10 krad was lower than that at 15 krad; consequently, for field cage releases 15 krad were used.

Experiments performed in 1990–1991 and in 1992, and carried out in 3 m × 2 m × 2 m field cages, have demonstrated a theoretical possibility for controlling the pest by the release of insects with an inherited F_1 sterility (Tables XI and XII).

TABLE X. TREATMENT OF *Ostrinia nubilalis* Hb. MALES WITH A SUBSTERILIZING DOSE OF 15 krad

Generation	Cross-variant		Egg masses/ female	Hatchability (%)	Longevity of adults (d)		Sterile pairs (%)
	Male	Female			Male	Female	
P	N × N (C)		7.6	90.5	8.9	7.7	4.2
	I × N		8.1	42.3	6.9	7.5	10.2
F_1	N × N (C)		7.4	88.0	7.5	7.0	5.7
	A × N		7.5	42.8	7.2	7.5	18.7
	N × A		8.0	40.7	8.2	6.7	15.2
	A × A		7.1	9.1	6.0	5.9	22.5
F_2	N × N (C)		7.7	88.7	7.4	7.2	4.3
	N × (A × N)		6.9	60.3	8.5	7.9	10.5
	N × (N × A)		7.3	64.7	7.7	7.8	11.2
	(A × N) × N		8.0	55.9	8.2	8.0	10.1
	(A × N) × (N × A)		7.5	59.2	8.1	8.0	12.3
	(A × N) × (A × N)		7.8	33.5	7.9	7.3	18.7
	(N × A) × N		6.9	60.8	8.9	7.9	8.0
	(N × A) × (A × N)		8.7	53.2	7.5	7.8	10.8
	(N × A) × (N × A)		7.3	29.9	7.8	7.3	21.1

N: normal; I: irradiated; A: progeny from irradiated male × normal female (I × N).

The data in these tables show no significant reduction in the number of eggs laid by irradiated females, except for variant $F_1 \times F_1$. The hatching percentage was lower in all the variants using F_1 moths; this effect still persisted in F_2 and disappeared in F_4.

The percentage attacked stems and the number of overwintering larvae per stem were correlated with the hatching percentage.

TABLE XI. FIELD CAGE RELEASE TESTS — 1990

Cross-variant variant		No. egg batches/ female	Egg hatch (%)	Stems damaged (%)	No. larvae/ stem	Larvae/ ha (%)
Male	Female					
Isolated	Control	2.48	42.5	76	1.92	100.00
F_1*	N	2.7	20.3	4	0.04	2.08
N	F_1*	2.6	19.5	8	0.08	4.17
F_1*	F_1*	1.9	3.7	0	0	0
F_2*	N	2.8	25.7	36	0.6	31.25
F_2*	F_2*	2.3	14.2	16	0.28	14.58
F_4*	F_4*	2.5	40.3	80	1.8	93.75

* : progeny from irradiated male (15 krad) × normal female; N : normal.

TABLE XII. FIELD CAGE RELEASE TESTS — 1991

Cross-variant variant		No. egg batches/ female	Egg hatch (%)	Stems damaged (%)	No. larvae/ stem	Larvae/ ha (%)
Male	Female					
Isolated	Control	3.4	60.5	84	1.88	100.00
F_1*	N	3.5	28.7	8	0.12	6.3
N	F_1*	3.2	28.0	16	0.24	12.8
F_1*	F_1*	2.5	4.1	0	0	0
$12F_1$*+12N	N	3.7	99.5	68	1.16	61.7
$16F_1$*+ 8N	N	3.4	47.4	44	0.72	38.3
$18F_1$*+ 6N	N	3.5	20.5	32	0.6	31.9
$20F_1$*+ 5N	N	3.0	8.7	16	0.44	23.4

* : progeny from irradiated male (15 krad) × normal female; N : normal.

Releases of the ECB F_1 generation in field cages demonstrated that F_1 sterility can be used as a part of an integrated control system along with other means of control.

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THE STERILE INSECT TECHNIQUE AND TRANSMISSION OF INHERITED STERILITY TO CONTROL THE DIAMONDBACK MOTH, *Plutella xylostella* (L.), AND THE CABBAGE WEBWORM, *Crocidolomia binotalis* ZELL.

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Abstract

THE STERILE INSECT TECHNIQUE AND TRANSMISSION OF INHERITED STERILITY TO CONTROL THE DIAMONDBACK MOTH, *Plutella xylostella* (L.), AND THE CABBAGE WEBWORM, *Crocidolomia binotalis* ZELL.

Two major cabbage insect pests, the diamondback moth (DBM), *Plutella xylostella* (L.), and the cabbage webworm (CWW), *Crocidolomia binotalis* Zell., have been studied intensively in relation to the possibility of insect control, either by using the sterile insect technique (SIT) or by transmission of inherited sterility. Four hundred and fifty moths each of *P. xylostella* and *C. binotalis* were sterilized with 0.30 and 0.40 kGy of gamma radiation, respectively. The irradiated insects, released into two laboratory cages (90 cm × 60 cm × 60 cm) containing 50 unirradiated moths, reduced the F_1 population of *P. xylostella* and *C. binotalis* by 61.1 and 65.3%, respectively. In the field cage (2 m × 2 m × 2 m), the F_1 population reductions in the respective insect species were 55.6 and 50.5%. Release of about 4500 irradiated *C. binotalis* into an experimental plot (15 m × 10 m) containing 500 normal moths reduced the F_1 population by 41.02% in the dry season and 50.55% in the rainy season. Release of about 5000 irradiated *P. xylostella* into an experimental plot containing about 350 moths of a natural population resulted in a reduction in egg hatch of 85.9% in the unreleased plot and 17.0% in the released plot. Experiments on the F_1 sterility of *P. xylostella* and *C. binotalis* were also conducted to explore the possibility of controlling these pests by using inherited sterility. In DBM, a substerilizing dose of 200 Gy resulted in considerable F_1 sterility. Release of F_1 inherited sterile DBM moths into population laboratory cages caused different levels of population suppression, depending on the irradiation dose. A substerilizing dose of 200 Gy induced inherited F_1 sterility, which reduced the population by 22% in DBM. In *C. binotalis*, a substerilizing dose of 0.275 kGy was sufficient to induce inherited sterility, because the levels of sterility in the parent and in the F_1 generation were 65.82 and 84.64%, respectively.

1. INTRODUCTION

The use of insects to control populations of their own kind through the transfer of damaged genetic material represents a new approach that could prove to be useful for the control of a number of major insect pests throughout the world. This autocidal method of control is now generally referred to as the sterile insect technique (SIT). Experiments have been conducted under laboratory and field cage conditions to explore the possibility of using SIT for controlling two cabbage pests in Indonesia. The diamondback moth, *Plutella xylostella* (L.), and the cabbage webworm, *Crocidolomia binotalis* Zell., are the most important pests of cruciferous crops [1]. These insects attack many varieties of cabbage. The other important insect pest of cabbage is the cabbage looper, *Diachrysia (Plusia) orichalcea* (Z.) [2]. The highest economic losses of cabbage crops caused by *P. xylostella* and *C. binotalis* occur in the dry season whenever control measures using insecticides are not properly applied [3]. *Plutella xylostella* is found on young cabbage plants, while *C. binotalis* is found on young and old cabbage plants.

To control these insects, farmers usually apply various types of insecticide, e.g. Ambush 2 EC, Basudin 60 EC, Dipel WP and Lannate L [4]. The use of insecticides tends to result in the development of insect resistance. A strain of *P. xylostella* found at Lembang, West Java, has shown resistance to permethrine (pyrethroid insecticide) (up to eleven fold) [5]. Increasing insect resistance to insecticides stimulates farmers to apply a higher dose or more toxic insecticides. The use of the parasite *Angitia cerophaga* to control *P. maculipennis* was introduced in Indonesia, but the results were not satisfactory, particularly in regions where intensive use of insecticides was practised [6].

The purpose of this paper is to explore the possibility of using SIT and transmitted inherited sterility to control the major cabbage pests in Indonesia because of the inadequate control measures available.

2. MATERIALS AND METHODS

2.1. SIT programme in *P. xylostella* and *C. binotalis*

Unsexed, 3 to 4-d-old pupae of *P. xylostella* were irradiated with 0.30 kGy of gamma rays in a ^{60}Co Gamma Cell-220 irradiator. Two cages (90 cm × 60 cm × 60 cm) were used in the laboratory test. Fifty (25 pairs) unirradiated and 450 (225 pairs) irradiated moths (at a ratio of 1:9) were placed in one cage; only 50 (25 pairs) unirradiated moths were introduced into the second cage. Moths that emerged at the larval stage were fed cabbage leaves. The test was repeated five times.

The field cage tests were conducted at the field station of the Horticultural Research Institute in Cipanas, West Java. Two field cages (2 m × 2 m × 2 m) containing 300 two-week-old cabbage crops were used to conduct experiments that were similar to those carried out in the laboratory. The experiments were replicated three times.

The field tests were slightly different to those done in the laboratory and field cage tests. They were carried out in the agricultural production area of Cipanas, West Java. Each experimental unit (plot) (15 m × 10 m) contained 600 two-week-old cabbage crops. The natural population was developed prior to the release of irradiated moths in the plots. The irradiated moths were released into the plot at a ratio of 14:1 to the natural population. Egg hatchability was observed by collecting eggs randomly.

In the *C. binotalis* test, unsexed, 6-d-old pupae were exposed to 0.40 kGy. The conditions for the laboratory tests were the same as those used for *P. xylostella*. The F_1 generation was measured by counting the larvae in weeks 1 and 2 after release.

The field cage tests were also carried out in the Cipanas area. The conditions for these tests were the same as those used in the *P. xylostella* laboratory test, except that four-week-old cabbage crops were used.

The field tests were again conducted in the Cipanas area, but the experimental side of the field was isolated to avoid reinfestation. Two experimental plots (7 m × 7 m) containing 250 cabbage crops were used. The conditions were similar to those of both the laboratory and field cage tests, but the number of insects used was ten times higher than that used in either of the other tests.

2.2. Transmission of inherited sterility in *P. xylostella* and *C. binotalis*

Substerilizing doses of 0.10–0.2 kGy were applied to study the level of sterility of the irradiated pupae of *P. xylostella* and their subsequent generations (F_1 and F_2). Other biological parameters such as the viability of the pupae and adults and their fecundity were also observed. In addition, preliminary results of the population reduction in *P. xylostella*, as affected by the release of F_1 moths under laboratory conditions, were observed.

In *C. binotalis*, 3 to 4-d-old male pupae were exposed to substerilizing doses that ranged from 0.25 to 0.35 kGy. The 40 moths that emerged were crossed with untreated females to observe the F_1 sterility level. Female pupae were also observed in order to obtain information on their potential for developing F_1 sterility. The substerilizing doses used to obtain the inherited sterility in the females were much lower than those used for the males, ranging from 0.075 to 0.15 kGy. The inherited sterility measured in the F_1 generation was similar to that of the irradiated male pupae.

TABLE I. REDUCTION IN THE F_1 POPULATION OF *P. xylostella* AND *C. binotalis* AS AFFECTED BY THE RELEASE OF STERILE MOTHS

Condition	No. of parents (P)			No. of F_1 populations		Reduction (%)
	Control	Treated		Control	Treated	
		Unirradiated	Irradiated			
P. xylostella						
Laboratory[a]	50	50 +	450	1141	430	61.1
Field cage[b]	50	50 +	450	378	180	55.6
Field plot	315	338 +	4827	809[c]	466[c]	42.4
C. binotalis						
Laboratory[a]	50	50 +	450	556	193	65.3
Field cage[b]	50	50 +	450	275	136	50.55
Field plot:						
Dry season	500	500 +	4500	1219	719	41.02
Rainy season	500	500 +	4500	2216	1096	50.55

[a] Average of five tests.
[b] Average of three tests.
[c] First instar larvae.

3. RESULTS AND DISCUSSION

3.1. SIT in *P. xylostella* and *C. binotalis*

The effects of releasing sterile insects on the reduction in population are shown in Table I. Release of 450 irradiated (sterile) moths into the laboratory cage containing 50 unirradiated moths reduced the emerging F_1 population from 1141 in the control cage to 430 in the released cage. Release of irradiated moths into the unirradiated population at a 9:1 ratio resulted in a 61.1% reduction in the F_1 generation.

In the field cage tests (Table I), the F_1 populations of the control and released cages were 378 and 180, respectively. This indicated that the reduction in the F_1 population, as affected by the release of irradiated moths, was 55.6%. In the field tests, the release of irradiated moths to the field population at a ratio of about 14:1 resulted in a reduction in egg hatchability from 85.9 to 17%. The number of eggs collected from the control and treated plots was 942 and 2743, respectively.

However, the F_1 population was 809 larvae (85.9% of 942) in the control plot and 466 larvae (17% of 2743) in the treated plot. Therefore, the reduction in the F_1 population was 42.40%.

In *C. binotalis*, the effects of releasing irradiated (sterile) insects on the reduction in progeny were quite promising (Table I). By releasing 450 irradiated into 50 unirradiated insects under laboratory and field cage conditions, the F_1 population was reduced by 65.3 and 50.55%, respectively. Release of about 4500 irradiated pupae into an experimental plot containing 500 unirradiated pupae reduced the F_1 population by 41.12% in the dry season and 50.55% in the rainy season. The difference in F_1 population reductions obtained under laboratory, field cage and field conditions may have been due to an uncontrolled source of variation related to the different test conditions.

3.2. Inherited sterility in *P. xylostella* and *C. binotalis*

The effects of substerilizing doses of gamma irradiation on the sterility of the F_1 and F_2 generations in DBM are presented in Tables II and III. The use of inherited sterility in lepidopteran insects showed its potential for suppressing populations [7]. In *P. xylostella*, the viability of the irradiated pupae was much lower than that of the unirradiated pupae (54.66 versus 74.33%), as indicated in Table II. Similar results were also found for moth emergence. The fecundity was not affected by the doses of radiation. The average sterilities for F_1 males and females were about 64% at 175 Gy and 63% at 200 Gy. The F_2 sterility level was lower than that

TABLE II. VIABILITY, FECUNDITY AND STERILITY OF F_1 IRRADIATED DBM MALE PUPAE

Irradiation dose (Gy)	Viability (%)[a]		Fecundity[b]		Sterility (%)[c]	
	Pupae	Moths	Male	Female	Male	Female
0 (unirradiated)	74.33	71.66	1826.00	1826.00	12.21	12.21
100	58.66	57.66	1614.66	1954.00	41.52	42.86
125	44.66	41.00	1638.66	2083.66	55.01	56.18
150	54.66	52.66	1541.00	1781.33	55.19	53.58
175	46.00	38.66	1534.66	1404.00	54.55	74.09
200	54.66	49.66	1852.00	1656.00	56.81	71.74

[a] Average percentage of 100 neonatal larvae, three replications.
[b] Average of the number of eggs produced by ten pairs of each sex, three replications.
[c] Average of ten pairs of moths of each sex, three replications.

TABLE III. VIABILITY, FECUNDITY AND STERILITY OF F_2 IRRADIATED DBM MALE PUPAE

Irradiation dose (Gy)	Viability (%)[a]				Fecundity[b]				Sterility (%)[c]			
	F_1 ♀ × ui ♀		F_1 ♂ × ui ♂		F_1 ♂ × ui ♀		F_1 ♀ × ui ♂		F_1 ♂ × ui ♀		F_1 ♀ × ui ♂	
	Pupae	Moths	Pupae	Moths	Male	Female	Male	Female	Male	Female	Male	Female
0 (unirradiated)	74.33		69.66		1395		1395		4.32		4.32	
100	76.66	75.00	63.66	58.00	1186.50	1683.50	951.00	1072.00	9.47	11.92	10.79	7.51
125	61.00	51.00	56.00	50.66	1382.00	1113.00	1096.50	1222.00	19.56	9.88	15.55	19.36
150	69.66	65.00	47.66	43.33	1108.00	1147.50	977.00	1329.00	7.59	15.93	13.26	8.90
175	37.00	29.33	19.66	17.00	1139.50	1074.00	1015.00	696.50	17.06	33.65	15.55	41.85
200	62.33	56.33	68.00	60.66	1465.50	1119.00	1223.50	1376.00	8.82	13.18	9.33	14.69

[a] Average percentage of 100 neonatal larvae, three replications.

[b] Number of eggs produced by ten pairs of moths of each sex, two replications.

[c] Ten pairs of moths, two replications.

ui: unirradiated.

TABLE IV. PERCENTAGE[a] OF POPULATION REDUCTION IN DBM AS AFFECTED BY THE RELEASE OF F_1 MOTHS OF IRRADIATED MALE PUPAE

Treatment (No. of released moth pairs)	Replication				Total	Average
	1	2	3	4		
5 ui	—	—	—	—	—	—
5 ui + 45 i − 1	8.93	2.37	22.16	5.22	38.68	9.67
5 ui + 45 i − 2	14.43	16.91	17.66	29.73	77.73	19.43
5 ui + 45 i − 3	25.43	20.47	33.53	12.31	91.74	22.93

[a] Calculated $\frac{u - r}{u} \times 100\%$

where u is the offspring population (moths) in the unreleased cage/check and R is the offspring population (moths) in the released cage.

ui: unirradiated.
i − 1: irradiated with a dose of 100 Gy.
i − 2: irradiated with a dose of 150 Gy.
i − 3: irradiated with a dose of 200 Gy.

of F_1; however, it was close to the sterility level of the irradiated parents (Table III). Therefore, doses of 175 or 200 Gy can be considered for obtaining inherited sterility in the F_1 progeny of *P. xylostella*. The results of the experiment to show the effects of releasing F_1 sterile moths on population reduction in *P. xylostella* under field cage conditions are shown in Table IV. Release of F_1 moths originating from irradiated pupae at doses of 100, 150 and 200 Gy into laboratory cages containing unirradiated moths at a ratio of 9:1 caused population reductions of 9.67, 19.43 and 22.93%, respectively.

In *C. binotalis*, an experiment on the F_1 inherited sterility was conducted to explore the possibility of its application in a control programme. The relationship between the substerilizing doses and sterility are shown in Tables V and VI. A dose of 0.25 kGy resulted in 63.95% sterility in the irradiated male parents and about 81% (average) sterility in the F_1 generation (Table V). The higher the dose, the higher the level of sterility in both the parents and the F_1 generation. A dose of 0.275 kGy was sufficient to obtain inherited sterility, because the levels of sterility in the parents and in the F_1 generation were 65.82% and 84.64%, respectively.

TABLE V. EFFECTS OF SUBSTERILIZING DOSES ON THE INHERITED STERILITY OF IRRADIATED MALE PUPAE OF *C. binotalis*

Dose (kGy)	P_{σ} i × ♀ ui		F_1 ♂ × ♀ ui		F_1 ♀ × ♂ ui	
	No. of eggs	Sterility (%)	No. of eggs	Sterility (%)	No. of eggs	Sterility (%)
0	2384	4.43	1186	3.81	1180	5.21
0.250	2172	63.95	891	81.71	819	82.66
0.275	2084	65.82	819	83.78	794	84.64
0.300	1909	68.05	759	85.51	689	85.96
0.325	1870	70.86	636	88.52	607	89.46
0.350	1686	74.79	599	92.82	547	93.60
HSD: 1%	1.189		2.57		2.59	
5%	1.38		1.87		1.89	
CC	11.1		14.34		14.42	

i: irradiated.
ui: unirradiated.
HSD: high significant difference.
CC: correlation coefficient.

TABLE VI. EFFECTS OF SUBSTERILIZING DOSES ON THE INHERITED STERILITY OF IRRADIATED FEMALE PUPAE OF *C. binotalis*

Dose (kGy)	P_{φ} i × ♂ ui		F_1 ♂ × ♀ ui		F_1 ♀ × ♂ ui	
	No. of eggs	Sterility (%)	No. of eggs	Sterility (%)	No. of eggs	Sterility (%)
0	1445	3.75	720	5.4	520	4.3
0.075	1121	28.6	467	15	486	11.6
0.1	875	54.75	385	30	290	6.9
0.125	743	74.65	341	40	285	3.6
0.15	626	79.6	296	54	246	4.4
HSD: 1%	22.44		20.4		5.51	
5%	17.81		16.55		4.45	
CC	23.42		15.63		26.9	

i: irradiated.
ui: unirradiated.

The irradiated female parents showed slightly different results (Table VI). The F_1 progeny of the irradiated males were more sterile than the parents, and the F_1 progeny of the irradiated females were partially sterile, but they were as sterile as the irradiated female parents (Tables V and VI). The male progeny were still the more sterile of the two sexes. These phenomena also occur in the cabbage looper, *Trichoplusia ni* (Hubner) [8].

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ESTERILIDAD EN LA F_1 DE *Diatraea saccharalis* (Fab.), LEPIDOPTERA: CRAMBIDAE

I. Efectos de las dosis subesterilizantes sobre la reproducción y la competividad

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Abstract-Resumen

F_1 STERILITY OF *Diatraea saccharalis* (Fab.), LEPIDOPTERA: CRAMBIDAE. I. EFFECTS OF SUBSTERILIZING DOSES ON REPRODUCTION AND COMPETITIVENESS.

Female and male chrysalises of *Diatraea saccharalis* (Fab.) were irradiated at doses of 150, 200 and 300 Gy. The moths which emerged were mated with untreated individuals of the opposite sex. Their mating capacity was not affected, nor was there any effect on the mating capacity of the progeny of males irradiated at the first two doses down to the F_3 generation, or on that of the F_1 of the irradiated females. Sterility was high where one of the pair had been irradiated; the percentage of non-viable eggs was between 79.2 and 99.4 for irradiated parents and between 90.8 and 99.8 in the F_1 of irradiated males. There was recovery of fertility in the F_1 of the treated females, the percentage of dominant lethal mutations lying between 4.4 and 34.5. The F_2 adult progeny of the initially irradiated males retained significantly high percentages of non-viable eggs, but the absolute values were lower than in the preceding generation, indicating some recovery of fertility. In the F_3, the recovery of fertility was even greater, the percentage of non-viable eggs approaching the level of the control, except in the case of males whose ancestors were all from the line of irradiated males. The action of radiation continued to be evident during post-embryonic development. The percentages of adult formation in the F_1 of both irradiated males and females and in the F_2 of irradiated males were significantly lower than in the control; the F_3 larval generation exhibited adult conversion values similar to the control. The moths from male chrysalises irradiated at 200 and 300 Gy were competitive when mated in the proportion of 1:1:1 with untreated females and males. The competitiveness values calculated by the Fried method were 0.78 and 3.08, respectively, and, for the higher dose, the mating competitiveness calculated by the method of direct observation of mating adults was 1.33.

ESTERILIDAD EN LA F_1 DE *Diatraea saccharalis* (Fab.), LEPIDOPTERA: CRAMBIDAE. I. EFECTOS DE LAS DOSIS SUBESTERILIZANTES SOBRE LA REPRODUCCION Y LA COMPETIVIDAD.

Se irradiaron crisálidas hembras y machos de *Diatraea saccharalis* (Fab.), con dosis de 150, 200 y 300 Gy. Las polillas emergidas se aparearon con individuos del sexo opuesto

no tratados, no afectándose su capacidad para copular, ni la de la descendencia de los machos irradiados con las dos primeras dosis hasta la generación F_3 y tampoco en la F_1 de las hembras irradiadas. Fue alta la esterilidad de las parejas donde uno de sus miembros era irradiado; los porcentajes de huevos inviables (% HI_v) se situaron entre 79,2 y 99,4% de los parentales irradiados y entre 90,8 y 99,8% en la F_1 de los machos irradiados. La fertilidad en la F_1 de las hembras tratadas se recuperó, estando entre 4,4 y 34,5 los porcentajes de mutaciones letales dominantes. Los adultos descendientes F_2 de los machos inicialmente irradiados mantuvieron significativamente altos los porcentajes de HI_v, pero los valores absolutos fueron menores que en la generación precedente, indicando una cierta recuperación de la fertilidad. En los F_3 la recuperación de fertilidad fue aún mayor, tendiendo los porcentajes de HI_v al nivel del testigo, excepto en los machos cuyos antecesores fueron siempre de la línea de machos irradiados. Durante el desarrollo post-embrionario continuó manifestándose la acción de las radiaciones. Fueron significativamente menores que el testigo los porcentajes de formación de adultos en la F_1 tanto de machos como de hembras irradiadas y en la F_2 de los machos irradiados; la generación larval F_3 presentó similares valores de conversión en adultos que el testigo. Las polillas procedentes de crisálidas machos irradiadas con 200 y 300 Gy fueron competitivas al ser apareadas en la relación 1:1:1 con hembras y machos no tratados. Los valores de competitividad calculados por el método de Fried fueron 0,78 y 3,08 respectivamente, y en la dosis superior la competitividad para el apareamiento calculada por el método de observación directa de adultos copulando fue de 1,33.

1. INTRODUCCION

Desde el primer informe sobre la trasmisión de esterilidad a la descendencia F_1 de insectos machos irradiados aparecida en *Carpocapsa pomonella* [1] se ha observado que ocurre el mismo fenómeno en muchos otros lepidópteros y hay criterios de que la esterilidad en la F_1 puede ser inducida en todas las especies de este orden [2]. Siendo de particular interés el desarrollo de ésta como un método práctico de control de lepidópteros [3], Walker et al. [4, 5] y Sanford [6] coincidieron en que se presentaba alta esterilidad en la F_1 de los machos de *Diatraea saccharalis*, pero hay diferencias en la respuesta a las dosis evaluadas por estos autores, planteando ambos que podía haber interferencia en los resultados debido a factores genéticos, nutritivos e incluso acción de patógenos. Por otra parte, Walker et al. [7] plantearon que se podía obtener una moderada producción de adultos bastante competentes irradiando crisálidas pero que se reduce la competitividad severamente al aplicar la dosis esterilizante, y se daña la esperma. Estudios realizaos sobre otras especies de lepidópteros [2, 8, 9] han demostrado que los insectos irradiados con dosis subesterilizantes son competitivos sexualmente. Por todo lo anterior y debido a la importancia que tiene por ser la plaga más extendida y peligrosa del cultivo de la caña de azúcar, está incluida entre las que deben ser estudiadas para su control por método genético [2, 6, 10].

El objetivo de este trabajo fue determinar los efectos de la exposición de crisálidas de *Diatraea saccharalis* a las radiaciones gamma, en el rango de dosis subletales entre 150 y 300 Gy, sobre la capacidad reproductiva y competitividad de las polillas emergidas y sobre la capacidad reproductiva de su descendencia.

2. MATERIALES Y METODOS

Los insectos procedían de una cría en laboratorio, alimentándose las larvas con una dieta merídica [11].

Las crisálidas con más de cinco días de edad y previamente sexadas se colocaron en viales de vidrio de 3,0 cm de diámetro y 7,5 cm de altura tapados con tapones de algodón y gasa, y se irradiaron en una fuente de ^{60}Co. La temperatura ambiente durante la irradiación fue de 22°C y el aire de la cámara no fue renovado durante el período de irradiación. El procedimiento seguido fue aplicar primero la mitad de la dosis, esperar dos horas, y aplicar entonces la segunda mitad. Las crisálidas durante el período de recuperación se colocaban dentro de una incubadora a 27 ± 1°C.

2.1. Estudio de la capacidad reproductiva

Durante la mañana siguiente a la emersión de los adultos, se colocaban éstos individualmente en las jaulas de apareamiento y ovoposición con un individuo del sexo opuesto no irradiado como pareja; los recipientes de apareamineto eran frascos de vidrio de 10 cm de diámetro y 12,5 cm de altura con papel parafinado en su interior, cubierto el fondo con algodón y papel de filtro embebidos en agua.

Los papeles parafinados se revisaban diariamente, recortándose las porciones con puestas de huevos, las que se ponían a incubar hasta la eclosión de las larvas. Las larvas se contaban y transferían a los recipientes con dieta, además se contaban los huveos que quedaban en "cabezas negras" sin eclosionar y los huevos infértiles. Al morir cada hembra se determinaba la presencia de espermatóforos en la bursa copulatrix.

A los trece días de eclosionadas las larvas se individualizaban en recipientes de cría con dieta hasta su conversión en pupa; al emerger el adulto se formaban las parejas con individuos de la cría, repitiéndose el proceso con la descendencia hasta la F_3, según el esquema que aparece en la Fig. 1 y cuya simbología representa a los individuos parentales irradiados y las generaciones subsecuentes, donde el número indica la dosis con que se irradió la pupa que dió origen al adulto parental irradiado y la primera letra su sexo (macho: M; hembra: H); en ese orden se le sigue adicionando letras según el sexo de la generación filial que sea. Por ejemplo: $F_2$200MMH significa una hembra F_2 hija de un macho F_1 que era hijo de un macho irradiado en estado de pupa con 200 Gy.

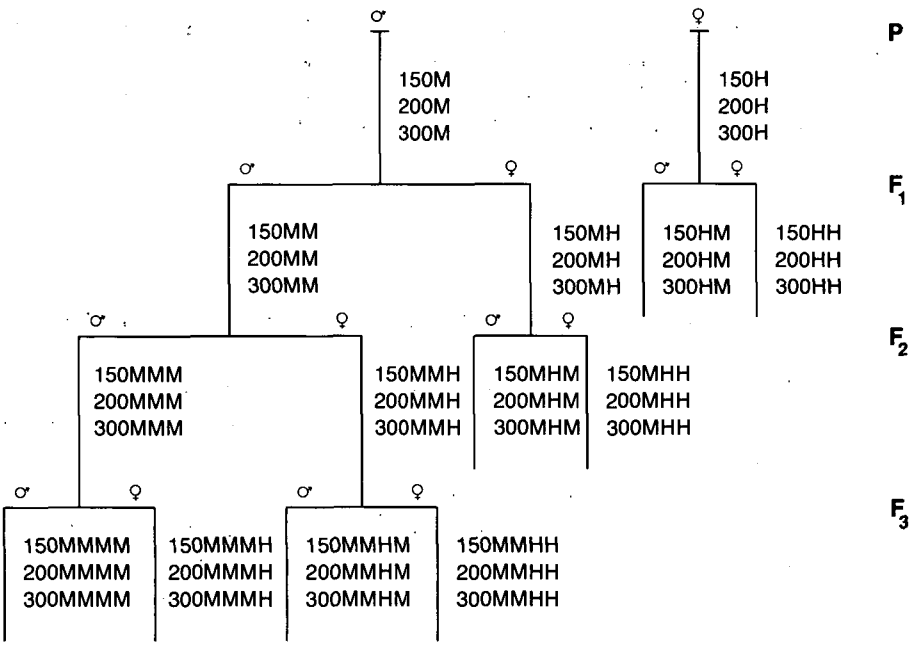

FIG. 1. Simbología y esquema utilizado para identificar las variantes evaluadas.

Las dosis de irradiación empleadas fueron 150, 200 y 300 Gy (16 Gy/min), así como un testigo no irradiado. En todos los casos se trataron machos y hembras; para los parentales se tomaron 21 parejas y para las descendencias el número dependió de la disponibilidad de individuos. Los indicadores evaluados fueron: huevos puestos por cada hembra copulada, huevos fértiles en cabezas negras, larvas eclosionadas, espermatóforos por hembra copulada, crisálidas y adultos formados. Con los primeros tres indicadores se calcularon los porcentajes de huevos con mutaciones letales dominantes (MLD), porcentajes de huevos inviables (HI_v) y promedio de larvas eclosionadas, evaluándose estadísticamente por la prueba de rangos múltiples de Duncan para el 5% de probabilidad de error, previa transformación de los porcentajes en arcsen raíz cuadrada y el número de larvas eclosionadas en $\sqrt{x + 0,5}$; con los demás indicadores se calcularon los porcentajes de cópula, de pupación, de emersión de adultos y de conversión larva-adulto, comparándose con el testigo a través del estadígrafo ts.

2.2. Estudio de la competitividad

Se usaron los adultos emergidos hasta 48 horas después de irradiadas las crisálidas macho con dosis de 200 y 300 Gy (10,14 Gy/min). Para calcular las competiti-

vidad sexual (Cf) se utilizó el método de Fried [12] evaluando la cantidad de larvas emergidas en cada una de las tres combinaciones de apareamiento siguientes:

Número de machos irradiados (♂♂I)	Número de machos normales (♂♂N)	Número de hembras normales (♀♀N)
10	10	10
10	—	10
—	10	10

Las jaulas de apareamiento fueron recipientes cilíndricos plásticos de 25 cm de alto y 24 cm de diámetro acondicionados como los del experimento anterior y el tratamiento a las puestas de huevos fue similar también. Se replicó 10 veces cada combinación y se compararon las medias de larvas emergidas por hembra copulada mediante la prueba de Duncan para un 5% de probabilidad de error, previa transformación a raíz cuadrada.

También se calculó la competitividad de los individuos irradiados con 300 Gy por el método directo de adultos copulando (Co), según la siguiente fórmula:

$$Co = \frac{i/n}{S/N}$$

donde n es el número de machos normales que copularon, i es el número de machos irradiados que copularon y N y S son las cantidades de machos normales e irradiados utilizados respectivamente [13].

Para identificar el tipo de macho se coloreaba parte de un ala con solución de verde brillante, aplicada con un pincel, unas veces a los irradiados y otras a los no irradiados. En cada jaula se ponían 30 adultos (10 machos normales, 10 machos irradiados y 10 hembras), se extraían con un vial de vidrio las parejas que se formaban durante la noche hasta el siguiente día y se registraba el tipo de macho que había copulado. El experimento se replicó 18 veces.

3. RESULTADOS Y DISCUSION

Los adultos emergidos de crisálidas de más de cinco días de edad e irradiadas con dosis de 150, 200 y 300 Gy no presentaron afectada su capacidad para copular con individuos del sexo opuesto no tratados, como se observa en el Cuadro I. Esto ha sido similar en otras especies de lepidópteros cuando son tratados con dosis subesterilizantes [14–16], no alterándose negativamente tampoco esta capacidad en la descendencia F_1 de las parejas donde las hembras fueron irradiadas y la descenden-

CUADRO I. NUMERO DE PAREJAS FORMADAS POR MACHOS O HEMBRAS IRRADIADAS CON INDIVIDUOS DEL SEXO OPUESTO CORRESPONDIENTE NO IRRADIADOS. PORCENTAJES DE COPULA Y SIGNIFICACION. EVALUACION DE SU DESCENDENCIA

Generación	Variante	Número de parejas	Copularon (%)	ts[1]	Significación[2]
Parental	testigo	21	71,4	—	—
	150M	21	66,6	0,334	NS
	200M	21	57,1	0,971	NS
	300M	21	57,1	0,971	NS
	150H	20	75,0	0,258	NS
	200H	20	65,0	0,442	NS
	300H	18	94,4	−2,030	S
F_1	testigo	23	47,8	—	—
	150MM	24	50,0	0,717	NS
	150MH	24	50,0	−0,149	NS
	200MM	24	58,3	−0.425	NS
	200MH	14	50,0	−0,128	NS
	300MM	9	0,0	—	—
	300MH	1	100,0	—	—
	150HM	15	86,6	−2,611	S
	150HH	15	73,3	−1,594	NS
	200HM	9	55,5	−0,394	NS
	200HH	13	61,5	−0,796	NS
	300HM	4	75,0	−1,047	NS
	300HH	8	62,5	−0,722	NS
F_2	testigo	24	79,1	—	—
	150MMM	10	80,0	−0,055	NS
	150MMH	7	71,4	0,419	NS
	200MMM	12	66,6	0,800	NS
	200MMH	0	—	—	—
	150MHM	4	75,0	0,184	NS
	150MHH	10	70,0	0,561	NS
	200MHM	13	69,2	0,662	NS
	200MHH	20	90,0	−1,006	NS
F_3	testigo	25	84,0	—	—
	150MMMM	21	66,6	1,378	NS
	150MMMH	25	56,0	2,218	S
	200MMMM	25	80,0	0,369	NS
	200MMMH	25	84,0	0,000	NS
	150MMHM	25	76,0	0,710	NS
	150MMHH	25	84,0	0,000	NS

[1] Significación para un 5% de probabilidad de error (t student) con respecto al testigo en cada generación.

[2] NS: no significativo. S: significativo.

CUADRO II. PROMEDIOS DE FECUNDIDAD, PORCENTAJES DE HUEVOS INVIABLES (HI_v), PORCENTAJES DE MUTACIONES LETALES DOMINANTES (MLD) Y LARVAS ECLOSIONADAS EN PAREJAS FORMADAS POR UN MACHO IRRADIADO Y UNA HEMBRA NO TRATADA Y EN SU DESCENDENCIA

Generación	Variante	Fecundidad promedio	HI_v (%)	MLD (%)	Larvas eclosionadas
Parental	testigo	190,1a	40,5b	15,7b	112,2a
	150M	172,6a	79,2a	66,7a	44,8ab
	200M	134,6a	74,0a	64,9a	46,2ab
	300M	139,6a	93,8a	89,9a	8,6b
F_1	testigo	345,5a	32,5c	7,7c	209,5a
	150MM	175,0b	98,9ab	93,3a	4,1b
	200MM	155,9b	99,8a	99,4a	1,0b
	150MH	172,8b	90,8b	85,0b	18,0b
	200MH	144,7b	91,9ab	89,3ab	15,8b
F_2	testigo	414,4a	13,3c	5,0c	331,3a
	150MMM	282,2ab	69,5ab	36,4b	90,1b
	200MMM	150,8b	86,5ab	63,8ab	20,6c
	150MMH	283,4ab	44,2bc	28,8bc	151,8ab
	200MHM	255,6ab	87,5ab	75,9ab	26,6b
	150MHH	185,8b	93,1a	92,4a	25,2b
	200MHH	415,2a	64,1ab	62,5ab	122,6b
F_3	testigo	207,2ab	1,9e	1,6e	199,1a
	150MMMM	3,5c	93,2a	89,4a	0,3c
	200MMMM	190,2ab	66,8b	59,4b	63,5b
	150MMMH	149,9b	48,8bc	46,6bc	90,6b
	200MMMH	154,7b	34,2cd	19,4cd	96,5b
	150MMHM	252,5a	6,1e	4,0de	234,6a
	206MMHH	285,2a	11,6de	7,8de	245,3a

Letras iguales en la misma columna de cada generación indican que no hay diferencias significativas para un 5% de probabilidad de error.

cia hasta la F_3 donde los machos de las parejas parentales fueron irradiados con las dos dosis inferiores; en este último caso, la diferencia significativa que apareció en $F_3$150MMMH fue para el 5% y no para menores niveles de significación, por lo que pensamos que esto no se debió a la acción de las radiaciones sobre los parentales tres generaciones antes. En el Cuadro I vemos también que las nueve parejas forma-

das por los machos $F_1$300MM no copularon y sólo una pareja de $F_1$300MH pudo formarse, no siendo posible en este caso hacer comparación estadística.

En el Cuadro II vemos que no hubo diferencias significativas con respecto al testigo en le fecundidad de las hembras normales apareadas con machos irradiados, pero la aparición de mutaciones letales dominantes y los porcentajes de huevos inviables fueron significativamente mayores. También vemos que el promedio de larvas eclosionadas fue significativamente bajo en las parejas en que los machos fueron irradiados con 300 Gy, presentándose valores intermedios y no significativamente diferentes cuando los machos estaban irradiados con 150 ó 200 Gy.

Por consiguiente, la aplicación de radiaciones gamma a las crisálidas machos produjo un aumento de la esterilidad en las parejas formadas con hembras no tratadas, lo que coincide con lo obtenido por Sanford [6] en dosis menores, siendo esto mayor en la dosis de 300 Gy. Con respecto a la F_1 descendiente de machos irradiados, el Cuadro II muestra que las fecundidades promedios tanto de las hembras $F_1$150MH y $F_1$200MH como de las hembras copuladas por los machos $F_1$150MM y F_1 200MM fueron significativamente inferiores al testigo y similares entre sí y los % de HI_v y de MLD fueron significativamente superiores a los del testigo, apareciendo los valores mayores en los provenientes de los parejas donde el macho era de la línea de los irradiados. Además, se observa que estos porcentajes fueron más altos que los de la generación anterior (P150M y P200M) y la cantidad de larvas eclosionadas promedio fue significativamente baja, incluso menor que los promedios de sus respectivos progenitores. El aumento de la esterilidad en la generación F_1 procedente de machos irradiados ocurrió como ya había sido reportado para esta especie [4–6] y para muchas otras especies de lepidópteros [8, 14–16], sobre todo la heredada por los machos [17]. La esterilidad fue más marcada en las parejas donde el macho era descendiente de irradiados.

En la F_2 de la línea de los machos inicialmente irradiados se pudieron formar por lo general pocas parejas de adultos (Cuadro II) e incluso ninguna de $F_2$200MMH por no emerger hembras. Además no se consideraron en los análisis los resultados de fecundidad, fertilidad, etc., de las tres parejas que copularon en la variante $F_2$150MHM por ser muy pequeña la muestra. En la generación F_2 se observa una recuperación de la fecundidad promedio al nivel del testigo (Cuadro II), aunque este indicador en $F_2$200MMM y en $F_2$150MHH se mantuvo significativamente diferente. Los % de HI_v y de MLD se mantuvieron significativamente altos, aunque se observa que los valores promedio fueron por lo general menores que los de la generación precedente, e incluso no apareció diferencia significativa entre los resultados de $F_2$150MMH y el testigo. En cuanto al número promedio de larvas eclosionadas, vemos que aún fue significativamente bajo en la mayoría de todas las variantes descendientes de irradiados con relación al testigo pero sus valores absolutos mayores que los de la generación precedente [8, 15], lo que indica una cierta recuperación de la fertilidad.

CUADRO III. PROMEDIOS DE FECUNDIDAD, PORCENTAJES DE HUEVOS INVIABLES (HI_v), PORCENTAJES DE MUTACIONES LETALES DOMINANTES (MLD) Y LARVAS ECLOSIONADAS EN PAREJAS FORMADAS POR UNA HEMBRA IRRADIADA Y UN MACHO NO TRATADO Y EN SU DESCENDENCIA

Generación	Variante	Fecundidad promedio	HI_v (%)	MLD (%)	Larvas eclosionadas
Parental	testigo	190,1a	40,5b	15,7b	112,1a
	150H	114,8ab	93,0a	82,1a	12,0b
	200H	120,7ab	99,4a	98,1a	2,1b
	300H	108,2b	99,4a	98,2a	1,9b
F_1	testigo	345,5a	32,5a	7,7a	209,5a
	150HM	271,1a	74,2a	34,5a	67,1a
	200HM	132,6b	40,2a	12,8a	43,3a
	150HH	280,9a	20,9a	4,4a	191,1a
	200HH	281,4a	30,7a	12,4a	123,6a
	300HH	205,5ab	58,8a	21,2a	96,4a

Letras iguales en la misma columna de cada generación indican que no hay diferencias significativas para un 5% de probabilidad de error.

En la F_3 las fecundidades promedio de las variantes de esta generación no fueron significativamente diferentes con respecto al testigo, excepto la de $F_3$150MMMM (Cuadro II) que fue anormalmente baja al ser comparada con las otras variantes de su generación y con relación a la fecundidad promedio de las generaciones precedentes. En esta generación F_3 la recuperación de fertilidad fue aún mayor, tendiendo los % de HI_v y de MLD al nivel del testigo [8], excepto en los machos cuyos antecesores fueron siempre de la línea de machos irradiados (150MMMM y 200MMMM), donde se mantuvieron niveles significativamentes altos de MLD y de huevos inviables puestos por las hembras que ellos copularon.

Con relación a la capacidad reproductiva de las hembras, vemos que su fecundidad al ser irradiadas con la dosis de 300 Gy (Cuadro III) fue significativamente baja, tomando valores intermedios no estadísticamente diferentes de la fecundidad de las hembras que recibieron dosis de 150 y 200 Gy. Los % de HI_v y de MLD en todas las variantes fueron significativamente altos y el promedio de larvas eclosionadas, por tanto, muy pequeño. La fecundidad en la generación F_1 correspondiente fue similar a la del testigo (Cuadro II) en las hembras de la cría apareadas con los machos $F_1$150HM, mientras que fue significativamente menor la de las copuladas por $F_1$200HM. No se incluyó en el análisis estadístico los resultados de $F_1$300HM

CUADRO IV. PORCENTAJES DE PUPACION, EMERSION Y CONVERSION EN ADULTOS DE LAS LARVAS PROCEDENTES DE PAREJAS PARENTALES DONDE UNO DE LOS MIEMBROS ES IRRADIADO Y DE LAS LARVAS DE LOS ADULTOS DESCENDIENTES F_1 Y F_2

Generación larval	Variante	Larvas evaluadas	Pupación		Emersión de adultos		Conversión larva-adulto	
			%	ts	%	ts	%	ts
F_1	testigo	350	90,8		93,1		84,6	
	150M	341	94,1	−1,644	73,2	7,024*	68,9	4,935*
	200M	182	81,3	3,059*	79,0	4,205*	64,2	5,183*
	300M	44	75,0	2,706*	36,3	7,188*	27,2	7,723*
	150H	235	77,8	4,329*	81,9	3,716*	63,8	5,729*
	200H	55	83,6	1,507	78,2	2,775*	65,4	3,097*
	300H	33	90,9	−0,010	60,0	4,383*	54,5	3,692*
F_2	testigo	350	98,2		96,2		94,6	
	150MM	21	90,4	1,624	84,2	−1,660	76,1	2,444*
	150MH	74	51,3	10,012*	52,6	6,591*	27,0	12,331*
	200MM	13	100,0	−0,930	92,3	0,604	92,3	0,325
	200MH	51	80,3	4,369*	87,8	1,950	70,5	4,511*
F_3	testigo	280	98,9		81,9		81,0	
	150MMM	85	96,4	1,377	75,6	1,236	72,9	1,566
	150MMH	85	98,8	0,080	94,0	−3,088*	92,9	−2,926*
	200MMM	85	98,8	0,080	88,0	−1,389	87,0	−1,326

* Significativo con respecto al testigo de esa generación y columna para un 5% de probabilidad de error (t student).

ya que fueron muy pocas las parejas que se pudieron formar. Las hembras F_1 copuladas por machos de la cría pusieron una cantidad de huevos no significativamente diferente. Los démas indicadores que aparecen en el Cuadro III no dieron resultados estadísticamente diferentes entre las variantes y el testigo. La irradiación de las crisálidas hembras dió como resultado que el porcentaje de infertilidad de las polillas emergidas al copular con machos de la cría fuese muy alto, incluso mayor que el correspondiente producido por los machos irradiados al copular con hembras de la cría, lo que indica que las hembras fueron más sensibles a las radiaciones que los

machos. Esto es similar a lo ocurrido en otros lepidópteros [15, 17], y la poca descendencia adulta F_1 que se obtuvo de estas hembras irradiadas tuvo una recuperación de su fertilidad [17], lo que también habría que tenerse en cuenta al elaborar una estrategia de liberación en la lucha contra esta plaga.

Durante el desarrollo post-embrionario (Cuadro IV) continuó manifestándose la acción de las radiaciones, de manera que los porcentajes de conversión larva-adulto fueron significativamene más bajos en la generación larval F_1, tanto de los machos como de las hembras parentales irradiadas, y continuó siendo así en la generación larval F_2 (excepto en $F_2$200MM que, por su muy escaso número, tuvo valores similares) descendiente de los adultos F_1 de machos inicialmentes irradiados, lo

CUADRO V. RELACION ENTRE LA DOSIS DE RADIACION Y LA COMPETITIVIDAD SEXUAL DE *Diatraea saccharalis* EVALUADA POR EL METODO DE FRIED

Dosis (Gy)	Larvas eclosionadas por hembra copulada[1]			Larvas esperadas	Valor de competitividad (Cf)
	Hn	Hs	Hc		
200	190,9a	69,2c	137,7b	130,0	0,78
300	165,3a	28,3c	61,8b	96,8	3,08

[1] Transformación raíz cuadrada.

Hn cantidad promedio de larvas procedentes de ♂♂N : ♀♀N.

Hs cantidad promedio de larvas procedentes de ♂♂I : ♀♀N.

Hc cantidad promedio de larvas procedentes de ♂♂N : ♂♂I : ♀♀N.

Letras diferentes en la misma fila indican que hay diferencias significativas para un 5% de probabilidad de error.

CUADRO VI. COMPETITIVIDAD EN EL APAREAMIENTO DE *Diatraea saccharalis* IRRADIADA CON 300 Gy, EVALUADA POR EL METODO DIRECTO DE ADULTOS COPULANDO

Hembras copuladas por: (media ± ES)		Valor de competitividad (Co)
Machos normales	Machos irradiados	
3,83 ± 0,25	5,11 ± 0,32	1,33

ES = error estandard.

que concuerda con los resultados para otros lepidópteros [8, 14, 15] y los obtenidos por Sanford [6] en la generación larval F_1. Los porcentajes de pupación, emersión de adultos y de conversión larva-adulto de las larvas F_3 fueron similares o superiores al testigo.

En el Cuadro V se observa que el valor de la competitividad Cf (calculada por el método de Fried) de adultos irradiados en estado de crisálida con 200 Gy fue cercano a la unidad, siendo la cantidad promedio de larvas emergidas por hembra copulada (Hc) similar a la cantidad esperada. Con la dosis de 300 Gy y el valor de competitividad superior a uno, la cantidad promedio de larvas emergidas fue aproximadamente un tercio menor que la esperada. Tambien se observa que en cada dosis la cantidad promedio de larvas emergidas fue significativamente menor cuando estaban juntos en la jaula de apareamiento machos irradiados y normales y menor aun cuando solo había machos irradiados, en todos los casos con hembras normales.

En general, estos resultados de competitividad obtenidos coinciden con los reportados en *Plodia interpunctella* (Hubner) [18], *Ostrinia furnacalis* (Guen) [11] y *Heliothis virescens* (F.) [19]. Los resultados de la observación directa de los adultos copulando, irradiados con la dosis mayor, aparecen en el Cuadro VI. El promedio de machos irradiados que copularon fue mayor que el de los normales y, correspondientemente, el valor de competitividad (Co) mayor que uno, lo que coincide con lo obtenido en el experimento anterior calculado por el número de larvas eclosionadas.

4. CONCLUSIONES

Los efectos de las radiaciones y la esterilidad heredada en la reproducción de *D. saccharalis* fueron generalmente similares a los descritos para otras especies de lepidópteros.

Los machos adultos irradiados en estado de crisálida con 200 y 300 Gy fueron competitivos sexualmente, en condiciones de laboratorio, con relación a los machos no tratados.

La presencia de igual cantidad de machos irradiados con las dosis estudiadas y de machos normales provocó una significativa reducción del promedio de larvas emergidas por hembra copulada, siendo mayor esta reducción cuando se aplicó la dosis de 300 Gy.

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ESTERILIDAD EN LA F_1 DE *Diatraea saccharalis* (Fab.), LEPIDOPTERA: CRAMBIDAE
II. Dinámica de apareamiento y efectos sobre su descendencia

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Abstract-Resumen

F_1 STERILITY OF *Diatraea saccharalis* (Fab.), LEPIDOPTERA: CRAMBIDAE. II. MATING DYNAMICS AND EFFECTS ON PROGENY.

The effect of irradiation of *Diatraea saccharalis* pupae at substerilizing gamma doses on the competitiveness of adult males emerging from the irradiated pupae and on their sterile progeny was evaluated on the basis of mating dynamics, mating duration and the length of the pre-mating period, as well as the sex ratio and the variation of pupal weights in the progeny. It is concluded that substerilizing gamma doses do not affect the indicators evaluated and that in the progeny the sex ratio is altered in favour of males and F_1 pupal weights are reduced significantly.

ESTERILIDAD EN LA F_1 DE *Diatraea saccharalis* (Fab.), LEPIDOPTERA: CRAMBIDAE. II. DINAMICA DE APAREAMIENTO Y EFECTOS SOBRE SU DESCENDENCIA.

Se evaluó el efecto de la irradiación de pupas de *Diatraea saccharalis* con dosis subesterilizantes de radiaciones gamma sobre la competitividad de los adultos machos emergidos de las pupas irradiadas y sobre su descendencia estéril, mediante la evaluación de la dinámica de apareamiento, la duración de la cópula y la duración del período de preapareamiento, así como la relación de sexos en la descendencia y la variación de los pasos pupales en ésta. Se concluye que las dosis de radiaciones gamma subesterilizantes no afectan los indicadores evaluados y que en la descendencia se produce una alteración en la relación de sexos a favor de los machos y una significativa reducción de los pesos pupales en la F_1.

1. INTRODUCCION

La inducción de esterilidad en la descendencia de lepidópteros irradiados con dosis subesterilizantes de radiaciones gamma constituye la base del método de control de plagas denominado esterilidad herada o esterilidad en la F_1 [1]. Este fenómeno ha sido comprobado en *D. saccharalis* [2–4] y a partir de estos resultados

se desarrollaron experimentos para evaluar la competitividad de los adultos irradiados con las dosis que dieron lugar a ese efecto. La finalidad de esos experimentos era evaluar la aplicabilidad de los tratamientos en un programa de control o erradicación de la plaga en cuestión.

2. MATERIALES Y METODOS

El material biológico empleado fueron pupas de más de 5 días de edad, procedentes de la cría del Instituto de Investigaciones de Sanidad Vegetal cubano, mantenida con la dieta y metodología vigentes [5, 6]. Se aplicó la dosis de 300 Gy fraccionada con dos horas de intervalo entre cada media dosis, y las irradiaciones se hicieron en una fuente de cobalto 60 con una tasa de dosis de 10 Gy/min y a una temperatura de 22 a 25°C.

Diariamente se colectaban los adultos emergidos y se colocaban en jaulas de apareamiento diseñadas y construidas para facilitar las observaciones y para colectar las parejas formadas durante las mismas. Estas consistian en armazones de alambrón soldado de 4 mm con forma cilíndrica de 40 cm de diámetro y 50 cm de altura, recubiertas con tela "marquisette" y provistas de mangas de lienzo para trabajar en su interior. Todas las observaciones se llevaron a cabo de noche entre las 20.00 y las 8.00 horas del siguiente día, a una temperatura de 22 ± 2°C, con luz roja para facilitarlas.

En las jaulas so colocaban los adultos recién emergidos en la relación 1:1:1 de machos irradiados, machos no irradiados y hembras no tratadas, empleándose entre 6 y 14 adultos de cada categoría por jaula. En total se evaluaron 164 adultos de cada tipo en las 17 réplicas del experimento. Para diferenciar los machos de una u otra categoría durante las observaciones se marcaban éstos con una disolución de verde brillante al 1%, alternándose las marcas en las diferentes réplicas del trabajo.

Con los datos tomados durante las observaciones se calcularon, tanto para los machos irradiados como para los controles, los siguientes indicadores:

— frecuencias de apareamiento en cada hora,
— duración de las cópulas,
— tiempo que tardaron los machos recién emergidos en aparearse.

Con los resultados se hicieron las distribuciones de frecuencias correspondientes, comparándose éstas mediante pruebas de Ji cuadrado ($p = 0{,}01$).

Para la evaluación de la descendencia se tomaron aleatoriamente 9 parejas donde el macho era irradiado y 7 parejas testigo no tratadas. Se recogieron las masas de huevos, se incubaron a 30 ± 1°C y 80% de humedad relativa hasta la completa emersión de las larvas, las cuales se contaron y transfirieron a viales de vidrio de 40 ml de capacidad, con 10 ml de dieta, cubiertos con tapones de algodón y gasa. Del total de larvas vivas, a los 14 días se tomó aleatoriamente el 30% y se transfirió

individualmente a viales con 10 ml de dieta fresca, que se mantuvieron en las condiciones de temperatura y humedad antes descritas hasta la formación de las pupas.

Las pupas fueron colectadas diariamente, sexadas, contadas y pesadas, comparándose los valores medios calculados mediante el estadígrafo "z" para un 1% de probabilidad de error.

3. RESULTADOS Y DISCUSIÓN

En la Fig. 1 se presentan los resultados del estudio de la sincronía de apareamiento de los machos irradiados y los normales con hembras no irradiadas. El período de mayor actividad sexual en las condiciones descritas se produjo entre las 20.00 horas y la 1.30 del siguiente día, existiendo una sincronía casi perfecta entre ambos tipos de machos. La disminución de la actividad sexual fue coincidente también, pues en ambos casos ésta se produjo pasadas las 2.00 horas del siguiente día.

Con relación a las duraciones medias de las cópulas, tampoco se manifestaron diferencias significativas entre los resultados de los machos irradiados y los no irradiados (la Fig. 2 muestra gráficamente estos resultados). Los apareamientos duraron 69,3 $\pm$ 12,1 minutos en los controles no tratados y 67,9 $\pm$ 11,9 minutos en las parejas formadas por los machos tratados y las hembras no tratadas, lo que evidencia que en este aspecto hay también un comportamiento similar.

En la Fig. 3 se presentan los resultados de la evaluación del tiempo que media entre la emersión de los adultos y el inicio de las cópulas, cuando esta emersión se produce de noche. No se encontraron diferencias significativas en las distribuciones de los apareamientos, en períodos de tiempo iguales entre los machos irradiados y los no irradiados, por lo que ambos evidentemente poseen períodos de preapareamiento iguales.

Las evaluaciones realizadas por Walker et al. en la década del 60 [7] y por Contreras Dúran en 1980 [8] a *D. saccharalis* no abarcaron los aspectos contemplados en este trabajo, ya que el primero centró su atención en las dosis esterilizantes y sólo evaluó la mortalidad y las deformaciones en los adultos emergidos de las pupas irradiadas con dosis esterilizantes, y el segundo enfocó su trabajo sólo al aspecto radiobiológico.

Por otra parte, el Cuadro I presenta los resultados de la evaluación de los efectos de las radiaciones sobre la descendencia de los adultos irradiados durante el estado de pupa. Entre éstos destaca la significativa variación de la relación de sexos a favor de los machos, indicativa de una alta tasa de mortalidad de las hembras, como producto de la acción de las mutaciones letales recesivas que afectan preferentemente a los individues de ese sexo. El efecto en cuestión minimiza las posibles consecuen-

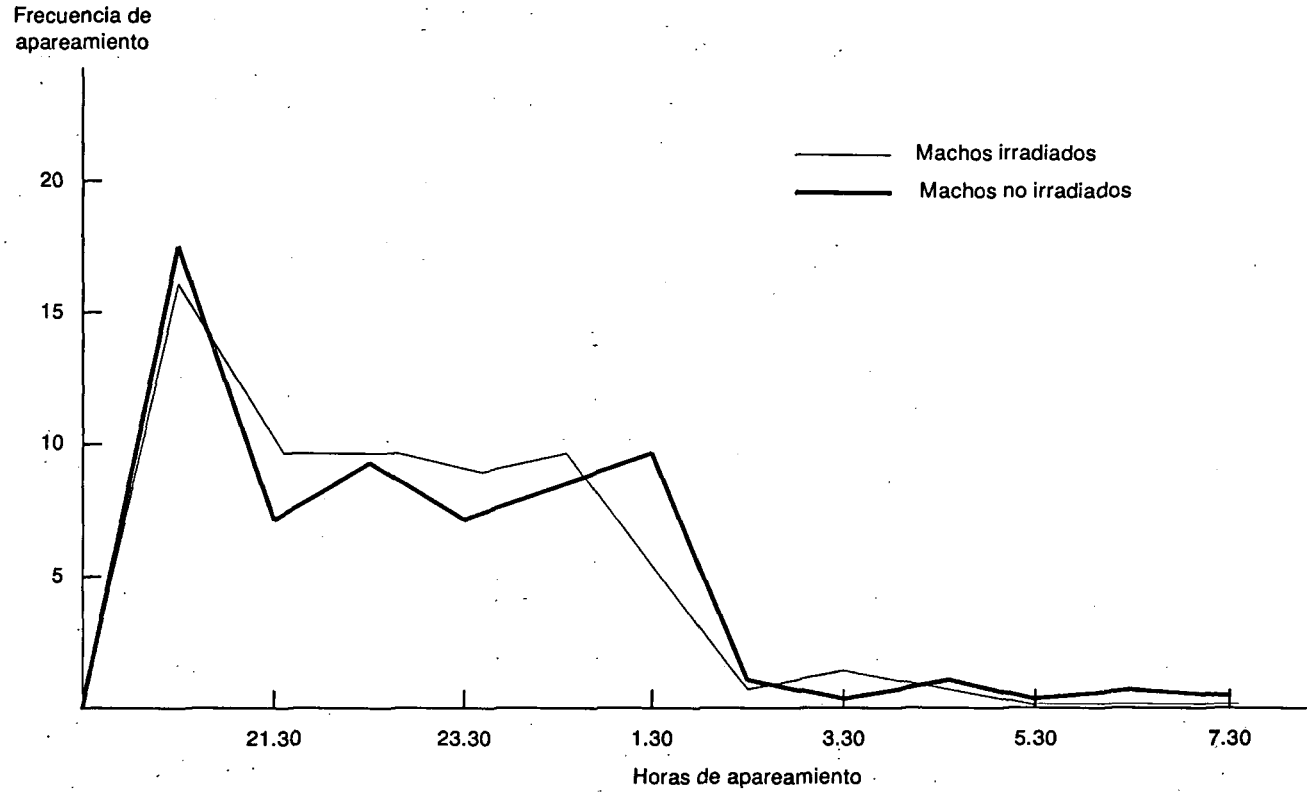

FIG. 1. Dinámica de apareamiento de los machos irradiados y no irradiados.

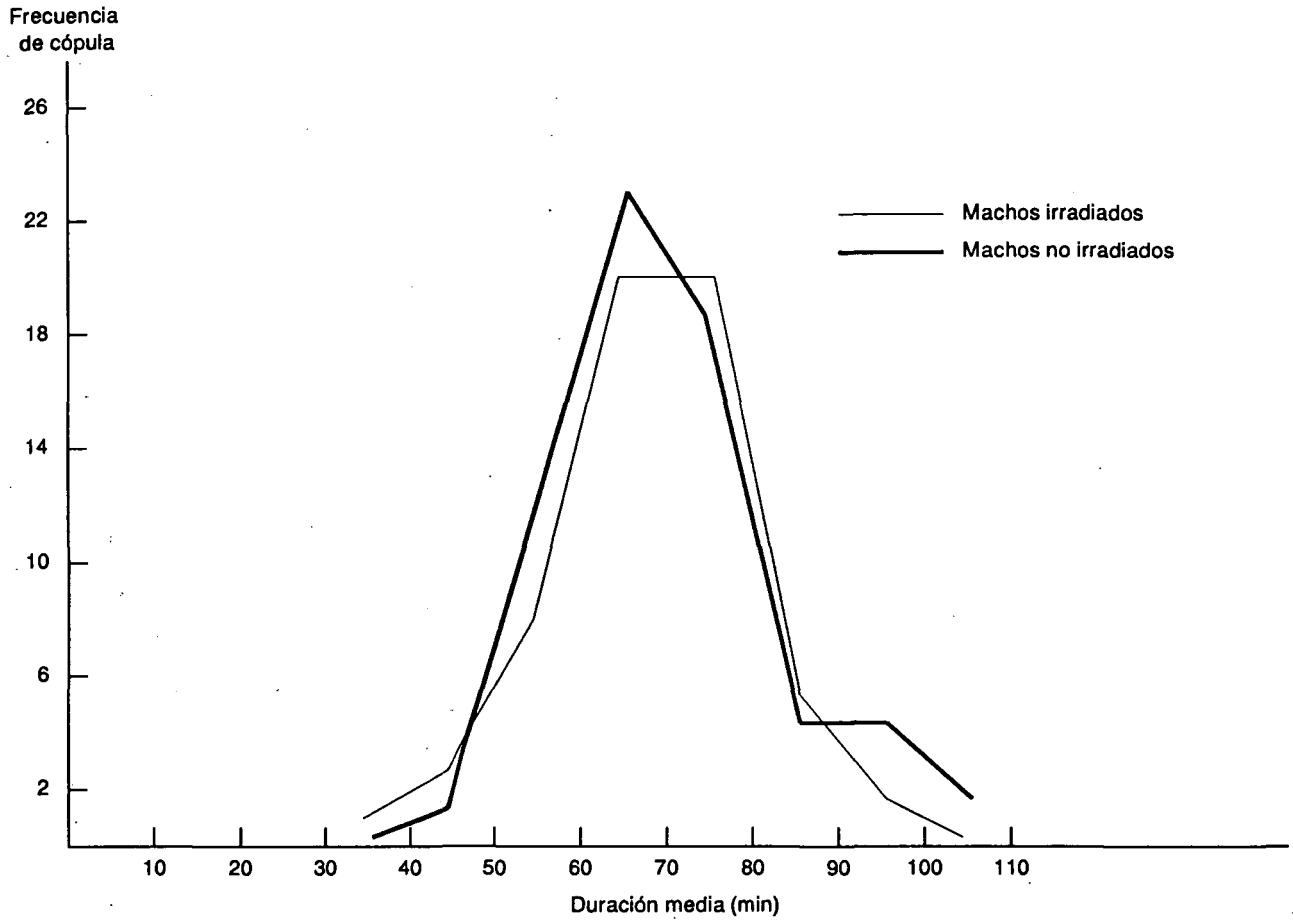

FIG. 2. Distribución de las duraciones medias de las cópulas de las parejas formadas.

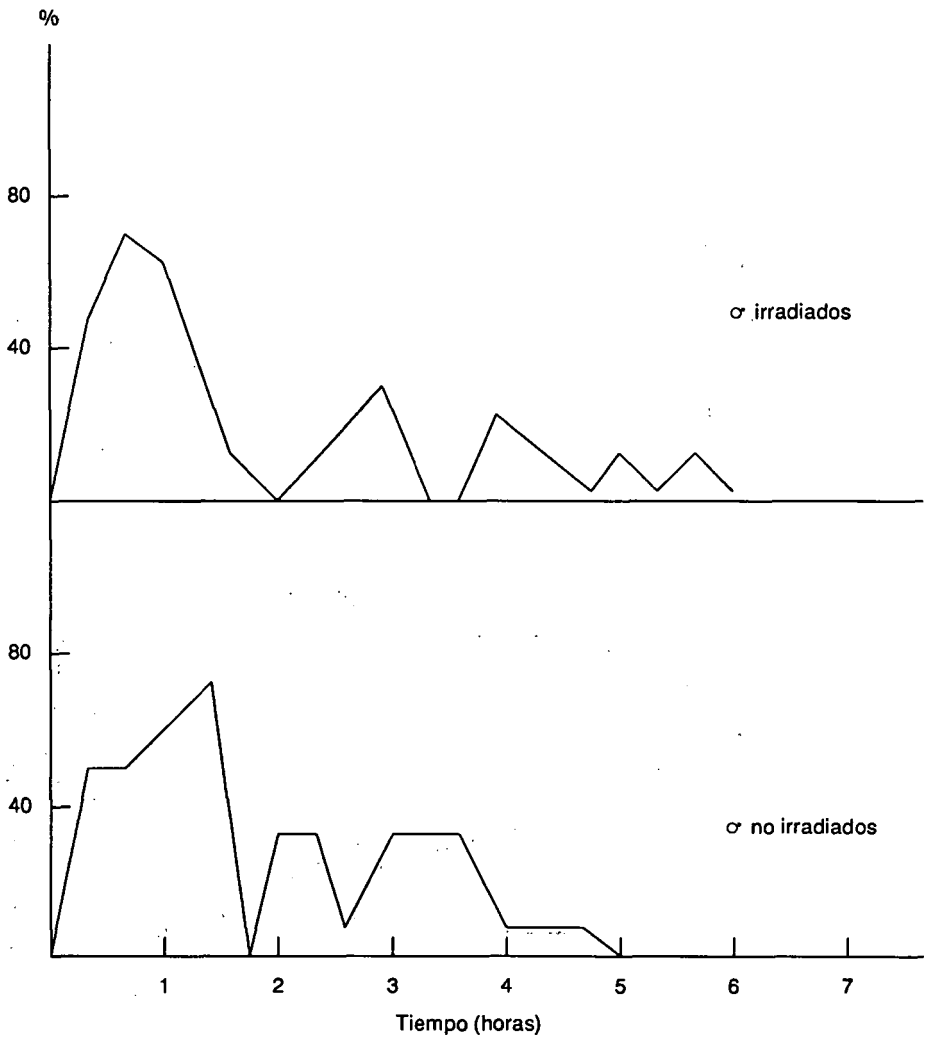

FIG. 3. Distribución de los inicios de apareamiento de los adultos machos recién emergidos con hembras no irradiadas recién emergidas.

cias negativas derivadas de la emersión de las hembras en la descendencia, en el sentido de una hipotética competencia con la población natural.

El Cuadro II presenta los valores promedio de los pesos de las pupas machos y hembras de la F_1 de los adultos machos irradiados y de los testigo. Tanto las pupas macho como las hembras de la F_1 testigo son significativamente más pesadas que las pupas de los descendientes de los machos irradiados, lo que, evidentemente, es una consecuencia del efecto de las radiaciones aplicadas a sus padres.

CUADRO I. NUMERO DE ADULTOS OBTENIDOS Y COMPOSICION SEXUAL DE LA DESCENDENCIA F_1 DE LOS ADULTOS MACHOS PROCEDENTES DE PUPAS IRRADIADAS CON 300 Gy DE RADIACIONES GAMMA

	Parejas evaluadas	Larvas evaluadas	Pupas macho	Pupas hembra	Pupas totales	Relación sexual
Machos irradiados	9	204	114	31	145	3,7
Testigo	7	309	95	103	198	0,9

CUADRO II. PESO DE LAS PUPAS MACHO Y HEMBRA DESCENDIENTES F_1 DE LOS ADULTOS MACHOS IRRADIADOS EN EL ESTADO DE PUPA

	Pupas evaluadas			Peso ($\bar{X} \pm$ D.S.) mg	
	machos	hembras	Total	machos	hembras
Parentales irradiados	106	31	137	51,56 ± 14,2	81,00 ± 24,5
Testigo	65	101	186	59,22 ± 17,7	96,31 ± 18,3

Estos resultados muestran una incidencia de los efectos de las radiaciones sobre la descendencia de los adultos machos irradiados que se manifiesta a nivel somático, lo que viene a sumarse a lo ya reportado en cuanto a su alta esterilidad.

4. CONCLUSIONES

La irradiación de pupas macho de *D. saccharalis* con dosis de 300 Gy, según el sistema de dosis fraccionadas y dos horas de intervalo entre cada media dosis, no afecta la competitividad de los machos desde el punto de vista de la conducta, y da lugar a que en la descendencia se manifiesten los siguientes efectos: mutaciones recesivas ligadas al sexo, y una disminución significativa del peso de las pupas tanto hembras como machos en la descendencia estéril.

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PARTIAL STERILIZING RADIATION DOSE EFFECT ON THE F_1 PROGENY OF *Spodoptera litura* (Fabr.)
Growth, bioenergetics and reproductive competence

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Abstract

PARTIAL STERILIZING RADIATION DOSE EFFECT ON THE F_1 PROGENY OF *Spodoptera litura* (Fabr.): GROWTH, BIOENERGETICS AND REPRODUCTIVE COMPETENCE.

Partial sterilizing gamma radiation doses (4–20 krad) were evaluated with respect to the pre-imaginal and imaginal behaviour of a serious pest in Indian tropical regions, *Spodoptera litura* (Fabr.), with a view to generating more competitive and sterile F_1 insects to be used for the control of this pest. A radiosensitivity differential between males and females was apparent in the P_1 generation, and male irradiation was considered better than female irradiation for producing behaviourally more viable, but infertile, F_1 candidates when a moderate sublethal dose of 13 krad was tested. It was pertinent to assess the viability of F_1 larvae before studying adult behaviour. Various growth and developmental characteristics of F_1 progeny of the treated moths were studied. A reduction in the growth indices and an increase in the level of malformations observed in F_1 progeny were found to be dose dependent. The nutritional profile and energy budget of F_1 progeny of the irradiated male moths were ascertained. The irradiation impact was more pronounced in F_1 females than in F_1 males, reflecting the better viability of F_1 males. Oviposition and egg viability were found to be negatively correlated with irradiation when F_1 crosses (self-crosses and out-crosses) were studied. F_1 progeny exhibited more sterility than their parent generation. Also, F_1 males inherited more sterility with better reproductive competence than F_1 females. Higher doses reduced male longevity, whereas the irradiation effect on mating frequency was not significantly evident. According to the present investigations on F_1 bioenergetics and reproductive performance, the feasibility of employing F_1 sterility by the administration of substerilizing gamma doses to parent moths might be considered with further cautious appraisal for this pest's management.

1. INTRODUCTION

The use of radiation as a non-chemical tool has an impetus for controlling lepidopteran pests, but it requires a modification strategy in the conventional sterile insect technique. Owing to the enhanced level of radiation damage and reduced

mating competitiveness caused by the high ionizing doses required for complete sterilization, the release of partially sterile insects has been suggested [1–3]. *Spodoptera litura* (Fabr.), a serious polyphagous pest in the Indian subcontinent, was reported to have a negative correlation of radiosusceptibility with age, and fully sterile insects were found to possess a great deal of somatic damage and poor reproductive performance [4, 5]. The work reported on here was undertaken to evaluate the effect of substerilizing gamma radiation doses on the biology of the tobacco caterpillar (*S. Litura*) with a view to assessing the competence of F_1 progeny and the potential of inherited sterility.

2. MATERIALS AND METHODS

The nucleus culture of *S. litura* was maintained on the leaves of castor, *Ricinus communis*, under controlled environmental conditions: 27 ± 2°C temperature, 75 ± 5% relative humidity and a 12 h light:12 h dark regimen. The irradiation facilities at the Institute of Nuclear Medicine and Allied Sciences, Delhi, were used and the required doses of gamma irradiation were applied to 0 to 24-h-old adult moths from a ^{60}Co source at a dose rate of 780 rad/min.[1] Reproductive pairing of the moths was conducted in perspex–nylon cages. Adult moths were fed a 20% honey solution and a fresh castor leaf was used as the ovipositional trap. Various reproductive characteristics such as the total number of eggs oviposited, the percentage egg hatch, the mating frequency (number of spermatophores/mated female), the percentage mating and the longevity of both sexes were observed for the treated and normal crosses. The corrected sterility and control of reproduction[2] were computed in response to the reproductive performance of the treated insects and their F_1 progeny involved in different in-crosses and out-crosses.

Growth and development were studied in terms of the percentage pupae and adult formation, the developmental period, the growth index, the degree of morphogenetic deformities and the sex ratio. The nutritional profile and energy budget of sixth instar F_1 *S. litura* larvae of the treated male moths were evaluated [6, 7].

The data were subjected to appropriate statistical analyses for comparison of the means ($\bar{x}$ ± standard error) computed from ten replicates [8, 9].

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[1] 1 rad = 1.00×10^{-2} Gy.

[2] Control of reproduction is given by $\frac{(V_c - V_t)}{V_c} \times 100$, where V_c denotes the viable number of eggs in the control and V_t the viable number of eggs in the treated insects.

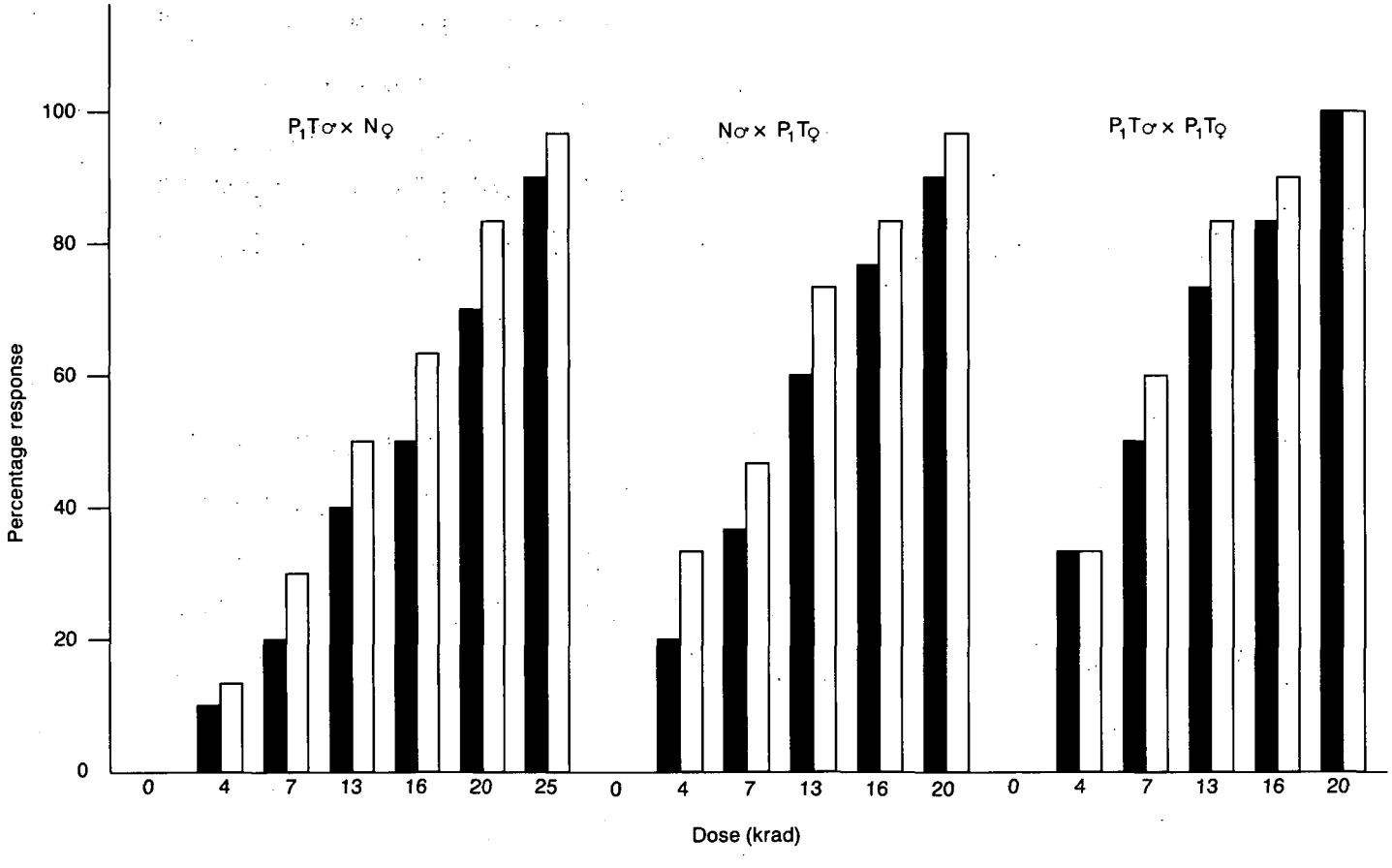

FIG. 1. Reproduction of S. litura *irradiated as* P_1 *adults (■: corrected sterility; □: control of reproduction).*

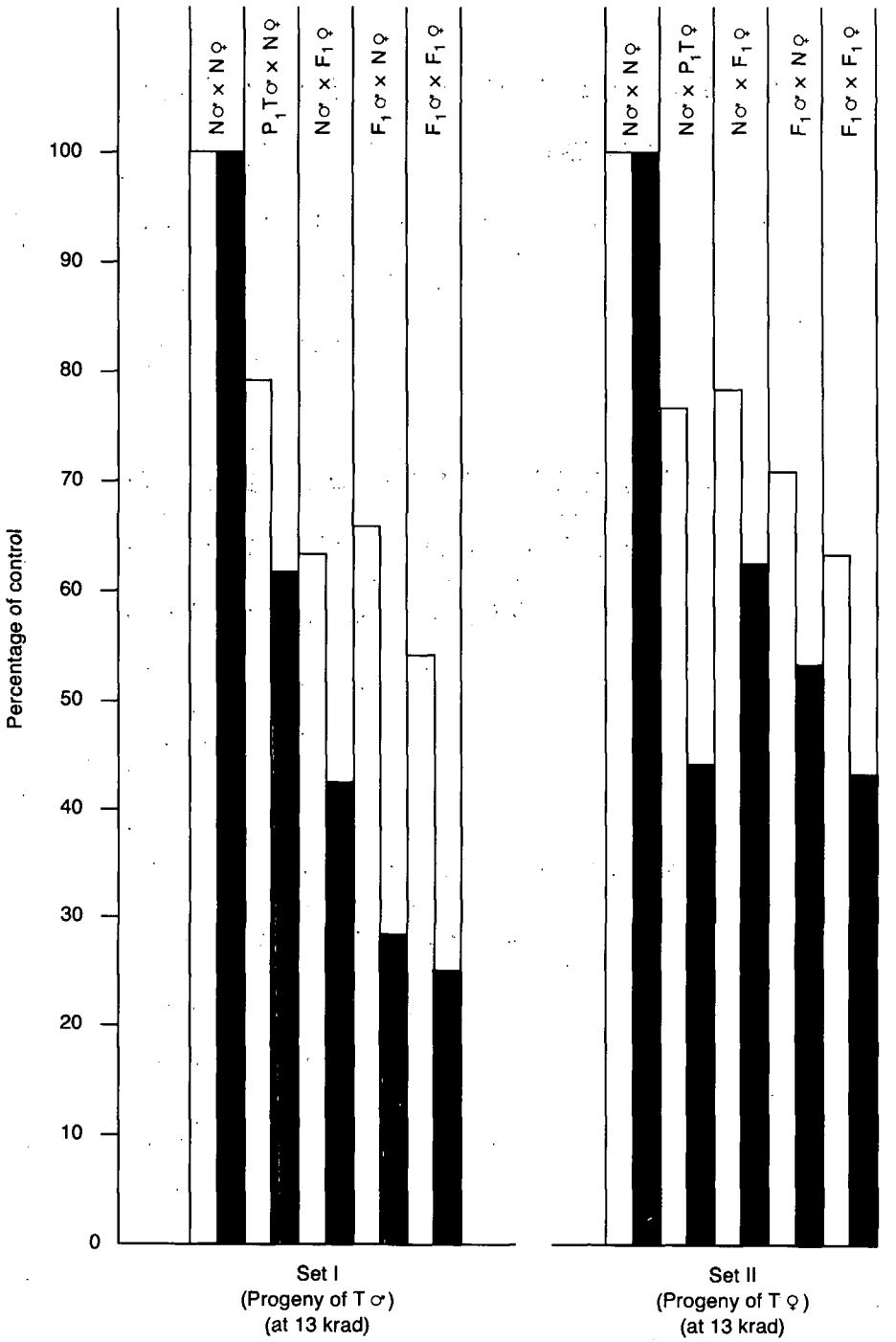

FIG. 2. Reproduction of S. litura *in the F_1 progeny of irradiated male and female adults (□ : eggs/♀ ; ■ : egg hatch).*

3. RESULTS AND DISCUSSION

The reproductive behaviour of *S. litura* treated as 0 to 24-h-old moths was observed (Fig. 1). The adverse effect of irradiation on the reproductive potential was found to be greater for females than for males, as has also been pointed out in studies of *S. frugiperda* [1, 10]. Initially, the radiosensitivity of male and female *S. litura* regarding inherited sterility was assessed with respect to one gamma dosage of 13 krad; the male moths were found to be better candidates for producing more sterile and competitive F_1 progeny (Fig. 2). Hence, studies on the substerilizing dose range (4–20 krad) were pursued for *S. litura* treated as male moths.

It was imperative to study the F_1 viability in relation to growth, food utilization and energy efficiencies of the larval stage, which might reflect the behavioural potency of F_1 adults. Table I shows the various growth and development characteristics of *S. litura* in the F_1 progeny of treated males. The percentage pupation at 4 krad was 79.2; it was reduced to 40% at 20 krad. Likewise, the effect of treatment was apparent on adult production, causing 69.1, 59.6, 49.3, 36.4 and 20.9% adult formation at 4, 7, 13, 16 and 20 krad, respectively. The post-embryonic developmental period of F_1 progeny was delayed, resulting in a reduction in the growth index, which exhibited a negative relationship with the gamma dosage. Morphogenetic deformities affecting the mouth parts, legs and wings were observed and the malformation intensity was determined for male and female F_1 moths. For instance, a gamma dose of 13 krad resulted in about 50% adult formation, with 21% malformation in F_1 males and 25% malformation in F_1 females. The sex ratio was observed to be skewed in favour of males in the F_1 progeny of treated moths; this effect was significant at 7 krad and higher doses.

The influence of substerilizing doses on the dry matter nutritional profile, as a function of the physiological response to irradiation stress, was studied (Table II). The phagoperiod (PP) was increased to 3.25 d in males and 3.47 d in females at a dose of 20 krad compared with the untreated moths (1.81 d in males and 1.94 d in females). The growth rate (GR) and consumption index (CI) values were higher in normal males than in normal females, unlike the weight gain, food balance, approximate digestibility (AD), efficiency of conversion of ingested food (ECI) and efficiency of conversion of digested food (ECD), which were found to be higher in unirradiated females than in unirradiated males. Interestingly, the impact of irradiation was more marked in F_1 females than in F_1 males for all the nutritional parameters studied. For instance, the GR was reduced from 0.580 at 0 krad to 0.322 at 20 krad in F_1 males, whereas it was 0.567 at 0 krad and decreased to 0.288 at 20 krad in F_1 females.

The radiosensitivity of F_1 males and females was also ascertained in terms of the energy budget (Table III). The gross energy in food balance of unirradiated F_1 insects was 1551 cal in males and 1765 cal in females, and showed a negative correlation with irradiation, which resulted in a reduction in energy in food balance

up to the level of 25% in F_1 males and 30% in F_1 females at 20 krad.[3] The energy stored in the body, the coefficient of metabolizable energy (CME), the efficiency of storage of ingested energy (ESI(E)) and the efficiency of storage of metabolizable energy (ESM(E)) were determined in unirradiated and irradiated F_1 insects; the adverse effects of irradiation were more pronounced in F_1 females than in F_1 males. For instance, at 20 krad the gross energy stored in the body, ESI(E)) and ESM(E), was reduced by 48, 38 and 31% in F_1 males compared with 58, 48 and 41%, respectively, in F_1 females with respect to the control.

In the progeny of treated males, reproductive competence was studied in three different crosses: $F_1♂ \times N♀$, $N♂ \times F_1♀$ and $F_1♂ \times F_1♀$ (Table IV). The fecundity and fertility were affected by irradiation and the radiosusceptibility increased at higher doses. The number of eggs oviposited by normal females (crossed with F_1 males) and F_1 females (crossed with normal males) was reduced in relation to the ovipositional response elicited in a parental cross, but the irradiation impact of these out-crosses was less than that observed in F_1 self-crosses. The fertility measured in terms of egg viability was markedly affected in the F_1 progeny in comparison to that exhibited by parents treated with the substerilizing doses. The effect of radiation on F_1 fertility was apparent in groups treated with doses as low as 4 krad. A dose of 13 krad, which caused about 50% fertility, leading to 39% sterility in the treated males, could induce 72.4% sterility in F_1 males and 56.5% sterility in F_1 females, whereas the sterility shown by F_1 self-crosses at this dose was about 75%. At a higher dose of 16 krad, the inherited sterility in F_1 males was 78.8% and in F_1 females 66.4%, whereas F_1 self-crosses at 16 krad exhibited 82.6% sterility. While comparing a particular pairing cross (e.g. $F_1♂ \times N♀$) between radiation treatments, the sterility of the F_1 progeny increased with the increase in radiation dose administered to the P_1 male parent. In other words, F_1 progeny of the 13 krad treated males were more sterile than the progeny of 7 krad treated males. However, mating was more fertile when the F_1 females were out-crossed than when the F_1 males were out-crossed. The percentage mating was higher in F_1 males than in F_1 females and a significant decrease in mating was observed at higher doses. For instance, the mating success in F_1 males was 75% at 13 krad and 52% at 20 krad. In F_1 progeny, the mating frequency was not significantly affected, although male longevity was affected from 16 krad onwards.

Generally, the proportion of non-productive F_1 pairs increased as the radiation dose increased. This could be attributed to the reduction in percentage mating or to an insufficiency or absence of sperm transfer, or failure of the sperm to fertilize the egg or a combination of all three; this remains to be determined by further sperm studies. The inherited sterility was of a greater degree in the progeny of the treated males than in the treated P_1 moths, and more competitive F_1 progeny with a sex

[3] 1 cal = 4.184 J.

Text cont. on p. 440

TABLE I. EFFECT OF GAMMA IRRADIATION ON THE GROWTH AND DEVELOPMENTAL CHARACTERISTICS OF *S. litura* IN F_1 PROGENY DERIVED FROM $P_1T\sigma^{\prime} \times N\varphi$ CROSS

Dose (krad)	Pupation (%)	Adult formations (%) (N)	Developmental period (d) (first instar–adult) (D)	Growth index (N/D)	Malformation (%)		Sex ratio male:female
					Male	Female	
0 (control)	91.66 ± 3.0	83.9a ± 2.95	22.78a ± 0.56	3.68a ± 0.05	5.6 ±0.60	6.71 ±0.71	1:1.03
4	79.2 ± 2.0	69.16b ± 2.79	23.4ab ± 0.54	2.97b ± 0.22	16.6 ±1.96	19.0 ±2.01	1:0.85
7	69.9 ± 2.4	59.65c ± 2.25	24.32ab ± 0.62	2.45bc ± 0.12	17.91 ±2.02	23.0 ±2.11	1:0.69
13	56.7 ± 3.1	49.35d ± 2.20	25.09b ± 0.37	1.96c ± 0.04	21.09 ±1.52	25.01 ±1.32	1:0.57
16	48.31 ± 2.6	36.4e ± 2.13	25.31b ± 0.11	1.43d ± 0.07	32.6 ±2.22	40.22 ±1.92	1:0.38
20	40.05 ± 2.11	20.95f ± 2.29	25.98b ± 0.58	0.80e ± 0.03	39.15 ±2.28	14.50 ±2.56	1:0.35

Note: Means followed by the same letter in columns are not significantly different ($P < 0.05$; Duncan's multiple range test).

TABLE II. EFFECT OF GAMMA IRRADIATION ON THE NUTRITIONAL PROFILE OF *S. Litura* IN F_1 PROGENY DERIVED FROM THE $P_1T\sigma' \times N\varphi$ CROSS (FOR EXPLANATION OF ABBREVIATIONS, SEE TEXT)

Dose (krad)	PP (d)	Food ingested (g)	Faeces (g)	Weight gain (g)	Food balance (g)	Mean weight (g)	GR	CI	AD (%)	ECI (%)	ECD (%)
					Males						
0	1.81 ±0.06	0.3744 ±0.020	0.1817 ±0.018	0.0807 ±0.005	0.1925 ±0.014	0.0777 ±0.004	0.580 ±0.024	2.74 ±0.18	51.77 ±2.19	21.57 ±0.77	44.31 ±3.01
4	2.47 ±0.12	0.3696 ±0.029	0.1968 ±0.011	0.0802 ±0.004	0.1727 ±0.021	0.765 ±0.002	0.432 ±0.025	1.96 ±0.12	45.41 ±1.87	22.42 ±1.05	50.97 ±3.59
7	2.78 ±0.15	0.3499 ±0.021	0.2013 ±0.019	0.769 ±0.005	0.1485 ±0.011	0.0740 ±0.005	0.385 ±0.022	1.77 ±0.12	43.04 ±2.93	21.96 ±0.72	52.92 ±3.46
13	2.87 ±0.14	0.3399 ±0.014	0.2227 ±0.012	0.0701 ±0.001	0.1170 ±0.007	0.0703 ±0.005	0.362 ±0.024	1.75 ±0.13	34.54 ±1.93	20.92 ±1.03	61.80 ±3.86
16	3.18 ±0.08	0.3395 ±0.026	0.2012 ±0.018	0.0615 ±0.003	0.1382 ±0.012	0.0567 ±0.002	0.344 ±0.020	1.85 ±0.06	40.85 ±2.76	18.73 ±1.49	47.35 ±4.80
20	3.25 ±0.10	0.3122 ±0.014	0.1693 ±0.008	0.0577 ±0.003	0.1427 ±0.011	0.0554 ±0.004	0.322 ±0.026	1.79 ±0.09	45.36 ±2.51	18.57 ±1.08	42.29 ±3.26

TABLE II. (cont.)

Dose (krad)	PP (d)	Food ingested (g)	Faeces (g)	Weight gain (g)	Food balance (g)	Mean weight (g)	GR	CI	AD (%)	ECI (%)	ECD (%)
					Females						
0	1.94 ±0.03	0.4167 ±0.024	0.1930 ±0.014	0.1018 ±0.006	0.2236 ±0.020	0.0923 ±0.005	0.567 ±0.018	2.37 ±0.18	53.20 ±2.93	25.27 ±2.13	50.11 ±3.98
4	2.64 ±0.11	0.3903 ±0.029	0.2029 ±0.014	0.0928 ±0.003	0.1873 ±0.016	0.0866 ±0.002	0.409 ±0.018	1.68 ±0.08	47.57 ±1.94	24.97 ±2.29	53.87 ±3.89
7	3.11 ±0.09	0.3614 ±0.016	0.2246 ±0.009	0.0856 ±0.005	0.1367 ±0.010	0.0830 ±0.005	0.332 ±0.014	1.41 ±0.07	37.53 ±1.62	23.95 ±1.49	65.53 ±3.12
13	3.20 ±0.12	0.3593 ±0.028	0.2073 ±0.019	0.0819 ±0.007	0.1519 ±0.011	0.0774 ±0.005	0.328 ±0.013	1.45 ±0.04	42.65 ±1.84	22.59 ±0.52	53.82 ±2.97
16	0.21 ±0.13	0.3547 ±0.032	0.2157 ±0.018	0.0625 ±0.004	0.1390 ±0.015	0.0617 ±0.004	0.321 ±0.017	1.78 ±0.06	38.89 ±1.75	18.05 ±0.95	46.76 ±2.33
20	3.47 ±0.14	0.3332 ±0.014	0.1902 ±0.006	0.0588 ±0.002	0.1429 ±0.009	0.0602 ±0.002	0.288 ±0.019	1.62 ±0.08	42.63 ±1.17	18.05 ±1.27	43.26 ±4.01

TABLE III. EFFECT OF GAMMA IRRADIATION ON THE ENERGY BUDGET OF *S. litura* IN F_1 PROGENY DERIVED FROM THE $P_1T\sigma' \times N\varphi$ CROSS (FOR EXPLANATION OF ABBREVIATIONS, SEE TEXT)

Dose (krad)	Gross energy in food ingested (cal)	Gross energy in faeces (cal)	Gross energy in food balance (cal)	Gross energy stored in body (cal)	CME (%)	ESI(E) (%)	ESM(E) (%)
			Males				
0	2301 ±80	750 ±44	1551 ±42	524 ±15	67.40 ±2.57	22.77 ±0.86	33.78 ±3.70
4	2271 ±58	840 ±22	1431 ±52	513 ±10	63.01 ±2.11	22.58 ±1.18	35.84 ±2.11
7	2150 ±52	880 ±47	1270 ±37	434 ±13	59.06 ±2.13	20.18 ±0.62	34.17 ±1.11
13	2089 ±35	997 ±31	1092 ±37	350 ±11	52.27 ±1.98	16.75 ±0.91	32.05 ±1.00
16	2086 ±49	921 ±46	1165 ±31	305 ±17	55.84 ±2.24	14.62 ±1.12	26.18 ±2.02
20	1918 ±35	760 ±26	1158 ±25	270 ±17	60.37 ±1.30	14.07 ±0.80	23.31 ±1.77

TABLE III. (cont.)

Dose (krad)	Gross energy in food ingested (cal)	Gross energy in faeces (cal)	Gross energy in food balance (cal)	Gross energy stored in body (cal)	CME (%)	ESI(E) (%)	ESM(E) (%)
			Females				
0	2561 ±89	796 ±35	1765 ±53	661 ±22	68.91 ±2.22	25.81 ±2.42	37.45 ±4.06
4	2398 ±71	866 ±35	1532 ±40	594 ±17	63.88 ±2.17	24.77 ±1.96	38.77 ±3.46
7	2221 ±40	982 ±31	1289 ±35	484 ±13	58.03 ±1.60	21.79 ±1.32	37.54 ±1.09
13	2208 ±71	928 ±49	1280 ±25	409 ±19	57.97 ±1.81	18.52 ±0.31	31.95 ±1.10
16	2179 ±40	988 ±46	1191 ±38	310 ±14	54.65 ±1.97	14.22 ±0.76	26.02 ±1.30
20	2047 ±35	808 ±53	1239 ±29	276 ±13	60.52 ±1.25	13.48 ±0.78	22.27 ±2.06

TABLE IV. EFFECT OF GAMMA IRRADIATION ON THE REPRODUCTIVE COMPETENCE OF *S. litura* (TREATED AS P_1 ADULTS) IN THE F_1 GENERATION

Dose (krad)	Cross*	Fecundity (eggs/female)	Fertility (egg hatch) (%)	Mating frequency	Mating percentage	Life span (d)		Corrected sterility (%)	Control of reproduction (%)
						Male	Female		
0 (control)	N♂ × N♀	1991 ± 76	85.4a ± 2.4	1.79 ± 0.13	94 ± 3.1	12.05 ±0.41	10.71 ±0.67	0	0
4	P_1T♂ × N♀	1915 ± 68	73.96b ± 2.3	2.02 ± 0.16	83 ± 2.9	11.47 ±0.50	10.35 ±0.34	9.8	13.10
	F_1♂ × N♀	1985 ± 94	61.27c ± 2.0	2.19 ± 0.22	85 ± 2.6	11.66 ±0.37	10.91 ±0.35	26.74	26.90
	N♂ × F_1♀	1655 ± 85	67.35bc ± 3.1	1.82 ± 0.21	78 ± 35	11.08 ±0.59	11.11 ±0.48	18.75	33.70
	F_1♂ × F_1♀	1568 ± 69	61.71c ± 1.8	1.70 ± 0.29	76 ± 9.2	11.30 ±0.61	9.75 ±0.53	27.40	39.75
7	P_1T♂ × N♀	1742 ± 69	64.90bc ± 2.6	2.29 ± 0.15	77 ± 2.5	10.70 ±0.33	10.14 ±0.44	20.85	30.64
	F_1♂ × N♀	1501 ± 70	49.22d ± 2.5	1.99 ± 0.24	76 ± 4.4	10.91 ±0.31	9.98 ±0.71	42.09	56.38
	N♂ × F_1♀	1344 ± 72	59.90c ± 2.2	1.80 ± 0.11	68 ± 1.9	11.64 ±0.74	9.91 ±0.13	27.74	51.70
	F_1♂ × F_1♀	1308 ± 70	47.11d ± 2.8	1.76 ± 0.19	62 ± 5.8	10.91 ±0.71	10.14 0.73	44.57	61.63
13	P_1T♂ × N♀	1592 ± 57	49.90d ± 2.3	1.98 ± 0.11	71 ± 2.4	10.27 ±0.53	9.68 ±0.41	39.14	51.26
	F_1♂ × N♀	1329 ± 59	23.39fg ± 1.8	1.96 ± 0.23	75 ± 3.5	10.80 ±0.40	9.29 ±0.51	72.48	81.62

TABLE IV. (cont.)

Dose (krad)	Cross*	Fecundity (eggs/female)	Fertility (egg hatch) (%)	Mating frequency	Mating percentage	Life span (d)		Corrected sterility (%)	Control of reproduction (%)
						Male	Female		
	N♂ × F_1♀	1278 ± 142	35.98e ± 2.2	1.60 ± 0.33	62 ± 5.3	10.78 ±0.35	10.04 ±0.31	56.59	72.41
	F_1♂ × F_1♀	1085 ± 100	21.26fg ± 2.1	1.85 ± 0.47	59 ± 7.6	9.85 ±0.96	10.01 ±0.44	74.98	85.63
16	P_1T♂ × N♀	1502 ± 35	40.21e ± 3.1	1.95 ± 0.18	68 ± 3.9	10.39 ±0.36	9.41 ±0.57	50.96	62.94
	F_1♂ × N♀	1311 ± 69	17.99g ± 2.1	1.89 ± 0.22	62 ± 4.4	10.50 ±0.33	9.59 ±0.52	78.85	86.06
	N♂ × F_1♀	1252 ± 67	27.80f ± 1.5	1.71 ± 0.17	54 ± 3.8	9.98 ±0.17	9.99 ±0.41	66.46	79.12
	F_1♂ × F_1♀	1014 ± 37	14.71g ± 1.2	2.01 ± 0.63	51 ± 6.6	0.09	8.96	82.69	90.71
20	P_1T♂ × N♀	1251 ± 53	25.19f ± 2.3	2.36 ± 0.17	60 ± 4.0	9.49 ±0.53	9.43 ±0.40	69.28	83.75
	F_1♂ × N♀	1215 ± 89	2.98h ± 0.4	1.61 ± 0.24	52 ± 5.1	9.90 ±0.35	9.50 ±0.28	96.49	97.86
	N♂ × F_1♀	1092 ± 113	12.25g ± 0.6	1.33 ± 0.29	40 ± 7.1	9.95 ±0.13	9.53 ±0.13	85.22	91.97
	F_1♂ × F_1♀	831 ± 94	0	1.60 ± 0.16	39 ± 5.8	9.06 ±0.50	9.22 ±0.77	100	100

Note: Means followed by the same letter in the same test are not significantly different ($P < 0.05$; Duncan's multiple range test).

[a] P_1: parental generation; F_1: first filial generation of T♂ × N♀; N: normal; T: treated.

ratio skewed in favour of males in the present findings are in accordance with the observations made in other lepidopteran species [2, 11, 12]. Interestingly, F_1 males showed better competence in terms of larval and adult behaviour than F_1 females. Further critical evaluations need to be conducted, both in the laboratory and in the field, on the competence of F_1 progeny, to select an appropriate dose and to optimize the strategy of using inherited sterility for the suppression of this lepidopteran pest.

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MATING BEHAVIOUR OF THE MELON FLY
Sexual selection and sperm competition

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Abstract

MATING BEHAVIOUR OF THE MELON FLY: SEXUAL SELECTION AND SPERM COMPETITION.

Melon fly females show strong mate choice and, after continuous mass releases of sterile males, a genetic fraction, which leads females to reject the courtship of mass reared males, will increase (a sterile insect technique (SIT) resistant strain). To prevent this increase, as large a number of sterile males as possible should be released from the start of SIT. Last male sperm precedence exists mainly due to the short lifespan of melon fly sperm. A long duration of copulation is necessary for males to inhibit female remating, which reduces the fitness of the first male. This information is important for the future use of SIT for insect species that mate many times.

1. INTRODUCTION

The melon fly, *Bactrocera* (formerly *Dacus*) *cucurbitae*, is an important pest of cucurbit vegetables and many tropical fruits. A large project using the sterile insect technique (SIT) to eradicate this species from the Southwestern Islands (Okinawa and Amani), Japan, has been under way since 1972 and, as Yamagishi et al. [1] report, almost complete success has been achieved.

Three topics are presented here which have been developed from our melon fly project:

(1) The existence of mate choice among melon fly females, and its 'evolution' in nature during mass releases;

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TABLE I. MAJOR CONDITIONS NECESSARY FOR THE SUCCESSFUL ERADICATION OF INSECT PESTS USING SIT

(1)	Mass rearing without a decrease in the vigour of released males.
	(a) *Phenotypic effects:* Decrease in vigour due to poor nutrition, high density, etc., in mass rearing facilities.
	(b) *Genetic effects:* Changes in behaviour because of genetic changes that took place during continued mass rearing ('domestication')
(2)	Sterilization without a decrease in the vigour of released males.
(3)	No economic damage due to the released adults.
(4)	Isolated distribution of the target species.

(2) Two aspects of sperm competition in the melon fly, namely, the absence of last male sperm precedence and the inhibition of female remating;
(3) The importance of mating behaviour studies for improvements in SIT and fruit fly control.

Table I shows the major conditions that are necessary for the successful eradication of a pest using SIT. For the melon fly, the second item mentioned above was achieved; the harmful effect of sterilization with gamma ray irradiation on the survival and dispersal of males was negligible.

Item 1 above consists of two aspects, phenotypic and genetic effects. In the melon fly, the latter is far more important than the former (see, for example, Ref. [2]). In the case of the melon fly, damage to crops from released adults is negligible and the target area, the Southwestern Islands, is very well isolated.

2. FEMALE MATE CHOICE AND ITS EVOLUTION

Figure 1(a) [3, 4] shows the changes in mating competitiveness of sterile melon fly males under field conditions on Kume-zima, from where the fly was eradicated in 1977.

Evaluation of the mating competitiveness of mass reared and sterilized males is usually carried out in laboratory cages. However, the results often do not reflect mating competitiveness under field conditions. Iwahashi et al. [3] developed a method which was used to measure field competitiveness in the Kume project. Before the start of mass releases, the competitiveness value was moderately high and acceptable (0.83), but the value decreased over the life of the project. Near the end of the project, after the flies had been reared for 18 generations in the mass rearing

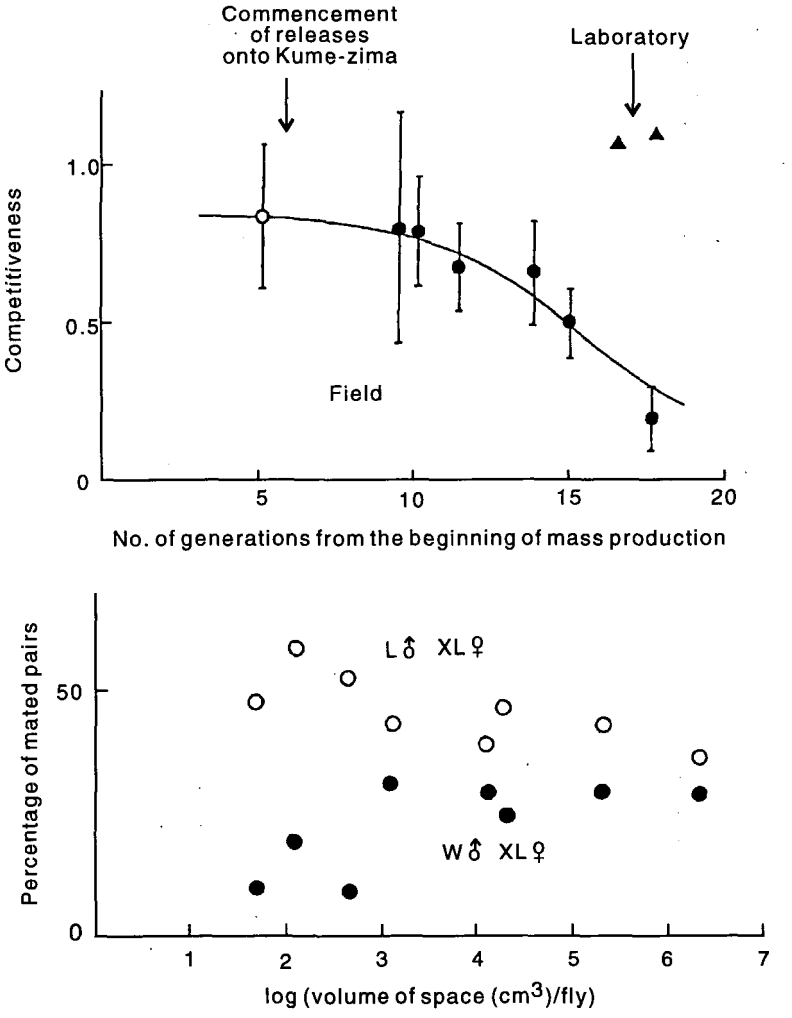

FIG. 1. (a) Mating competitiveness of mass reared and sterilized melon fly males measured under field conditions (modified from Ref. [3]) (○: data taken from Kudaka-zima; ●: data taken from Kume-zima; ▲: competitiveness values measured in laboratory cages. (b) Relationship between the size of the cage per fly and the percentage of successful mating of males of mass reared (33–34 generations (○)) and wild (●) strains when caged together with mass reared females (from Ref. [4]).

laboratory, the competitiveness value fell to only 0.2. These results possibly originate from genetic effects. However, at this time the competitiveness values obtained in laboratory cages were still high (see triangles in Fig. 1(a)).

Soemori et al. [4] reported the results of experiments that suggest an explanation for the discrepancy between laboratory and field data. They released individually marked flies into cages or rooms of different sizes and recorded the matings in the evening. Figure 1(b) shows the percentage of released males that mated in relation to the size of the experimental area. Males of the wild strain could not mate well in a small space, but males of the laboratory strain could mate.

Iwahashi and Majima [5] found that melon fly males formed leks for mating. In the evening, males aggregated on a non-host tree where each male established his

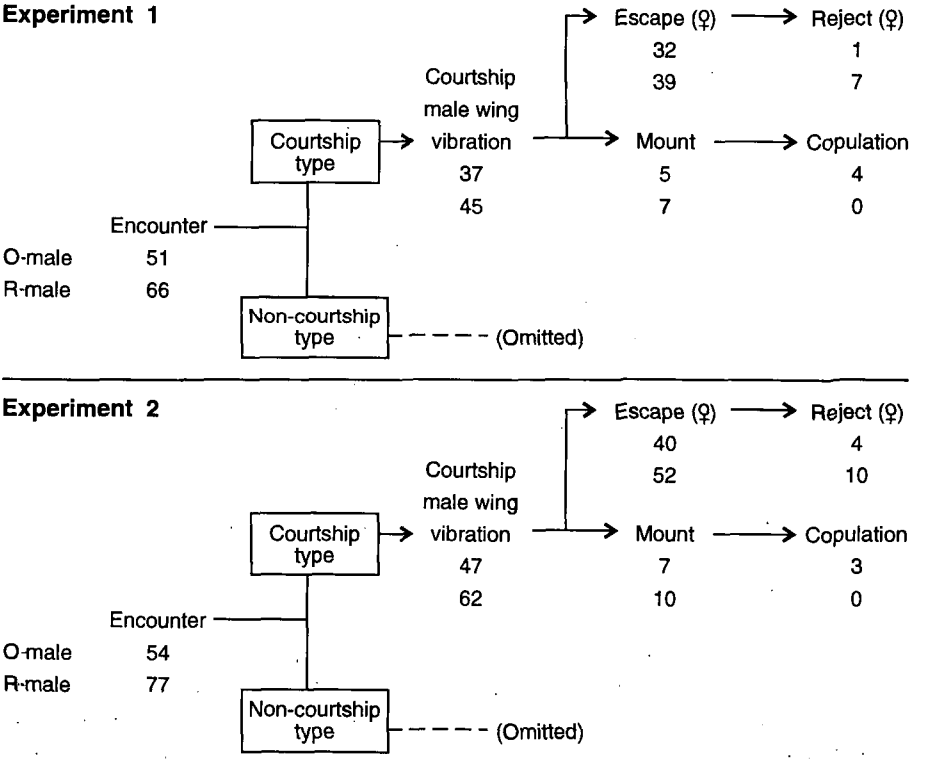

FIG. 2. Mate choice by wild melon fly females collected on Okinawa-Hontô (Okinawa Island, O females) to wild males (O males) and males of a mass reared strain (R males). The arrows indicate the direction in which the behavioural sequence proceeds. The upper numerals indicate the frequencies of the transitions when O females encountered O males, while the lower numerals indicate cases when O females encountered R males (redrawn from Refs [7, 8]).

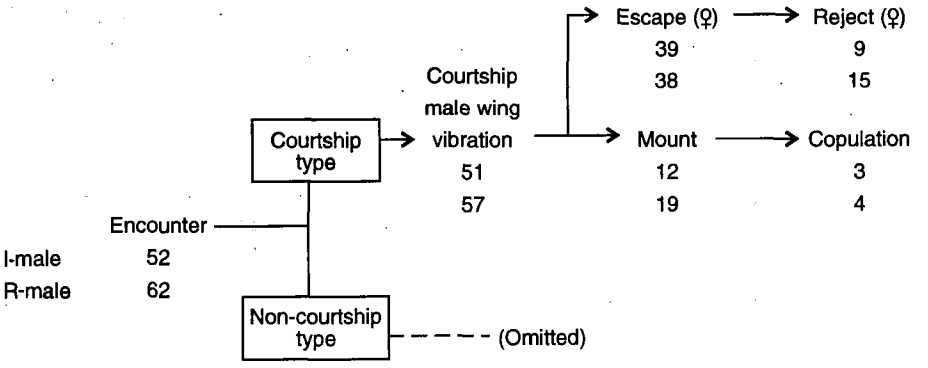

FIG. 3. Mate choice by wild melon fly females, collected on Ishigaki-zima (I females), to Ishigaki wild males (I males) and mass reared males (R males) (from Ref. [8]). For a full explanation, see Fig. 2.

territory, usually on a single leaf. They then secreted sex pheromone to attract females. When a female arrived, a territorial male courted her with special movements and sounds. However, the female often rejected the courtship and flew away; a female usually accepted copulation after visits to the territories of several males. This is evidence of female mate choice in insects, which has been demonstrated only in a few other animals, despite Darwin's [6] earlier suggestion.

Hibino and Iwahashi [7] compared the mating success of males of a wild and a mass reared strain. Figure 2 [7, 8] shows the results of field cage experiments using flies of a wild strain of Okinawa-Hontô ('O flies'). The figure shows that even when courted by wild males (O males), wild females (O females) accepted copulation in only 4 of 37 courtship trials, or 3 of 47 trials. It is notable that O females never accepted courtship from mass reared (R) males. This may be caused by differences in the courtship song [9].

When Hibino and Iwahashi carried out these experiments, the melon fly on Okinawa-Hontô was near extinction as a result of the eradication project. Therefore, O flies were subjected to strong selection pressure by the released sterile males.

Hibino and Iwahashi [8] carried out a similar experiment using wild flies taken from Ishigaki-zima, where there had been no SIT programme. Figure 3 [8] shows that females of the Ishigaki wild strain (I females) accepted courtship from R males, as well as by I males. Table II [8] shows the mean duration of wing vibration from the time of the male–female encounter to the escape of the female. In O females there was a significant difference between the duration of encounters with O males and with R males, but in I females the difference was insignificant.

There are two possible explanations for the differences in behaviour between flies from the two islands. The first is that O flies and I flies had genetically different

TABLE II. MEAN AND STANDARD DEVIATION OF DURATION (IN SECONDS) OF WING VIBRATION FROM ENCOUNTER TO ESCAPE OR MOUNT WHEN O FEMALES ENCOUNTERED O MALES OR R MALES (I), AND WHEN I FEMALES ENCOUNTERED I MALES OR R MALES (II). N INDICATES THE NUMBER OF SAMPLES [8]

Type of encounter	Wing vibration from encounter to			
	Escape	N	Mount	N
(I)				
O females to O males	60 ± 105	40	146 ± 149	4
O females to R males	25 ± 24	52	39 ± 31	10
Mann-Whitney U test	$P < 0.05$		$P < 0.05$	
(II)				
I females to I males	50 ± 45	39	93 ± 76	9
I females to R males	50 ± 47	38	101 ± 117	15
Mann-Whitney U test	NS		NS	

NS: not significant

courtship and acceptance characters and the courtship character of R males was more similar to that of I males. The second is the 'SIT resistance hypothesis', which states that although the range of the wild male courtship character was narrow, the wild female population was heterogeneous and contained individuals who accepted a broad range of male courtship characters. However, females that accepted sterilized R male courtship could not reproduce. Thus, under strong selection pressure from SIT, a female genotype that accepted the courtship of mass reared males may have become extinct.

To test the two hypotheses, Hibino and Iwahashi [8] carried out an experiment using O females and O and I males. There were no differences between the two, which suggests that the mate choice of O females changed to a rejection of the courtship of R males after being artificially selected by SIT. Thus, the authors made an interesting discovery: evolution of mate choice which had not been demonstrated before in animals.

How can this problem be overcome? Tsubaki and Bunroongsook [10] conducted a simulation experiment to estimate the effect of the change in mate choice.

Their model is an expansion of a logistic model developed by Itô [11]. They showed that the effect of a reduction in mating competitiveness of mass reared and sterile males is far more important than the effect of a change in female mate choice, and that releases of two to three times more sterile males than could be released in a non-mate choice model can eradicate the target insect.

3. LAST MALE SPERM PRECEDENCE

Because melon fly males insert free sperm into the females' spermathecae, and many females copulate more than once, sperm mixing may take place. Thus, sperm competition might be an important problem for SIT.

To examine this possibility, we used sterile males to measure the P_2 values, an index of the last male sperm precedence in the melon fly. Figure 4 shows the results obtained by Yamagishi et al. [12]. When the interval between the first and second matings was short (1–4 d), there were no significant differences in the hatchability of eggs between the first normal–second sterile (NS) and first sterile–second normal (SN) matings. However, when the interval was 16 d or more, hatchability was significantly higher when the second mating was normal than when the second mating was sterile. Thus, last male sperm precedence could be detected ($P_2 > 0.5$). Two processes, the consumption of sperm during fertilization and the outflow of sperm at the time of fertilization, or sperm mortality, could be a reason for this change.

As melon fly females do not oviposit without stimuli from host fruit, oviposition can be manipulated by either placing or not having a pumpkin slice in the rearing cups. In Fig. 4, NS1 and SN1 are experiments where females oviposited during the period between the first and second matings because we placed pumpkin slices in the cups, while NS2 and SN2 are experiments where the females could not oviposit because no pumpkin was present. There were no significant differences between the hatchability values of NS1 and NS2 or SN1 and SN2, suggesting that it was not sperm loss owing to fertilization and the related processes, but sperm mortality that was the major cause of differences in P_2 values.

Figure 5(a) is a sperm survival curve showing a decrease in the number of sperm in the spermathecae of non-ovipositing females [13]. The curve in Fig. 5(b) shows the change in P_2 values expected from the survival curve [12]. The circles and triangles are the observed P_2 values. These values were about 0.5 (no last male precedence) for short mating intervals, but increased with the increase in mating intervals. There was no significant difference between the expected and observed values (χ^2 test, $P > 0.05$).

Based mainly on knowledge of the long lifespan of sperm in the spermathecae of social insects, entomologists have often considered that sperm may survive for a

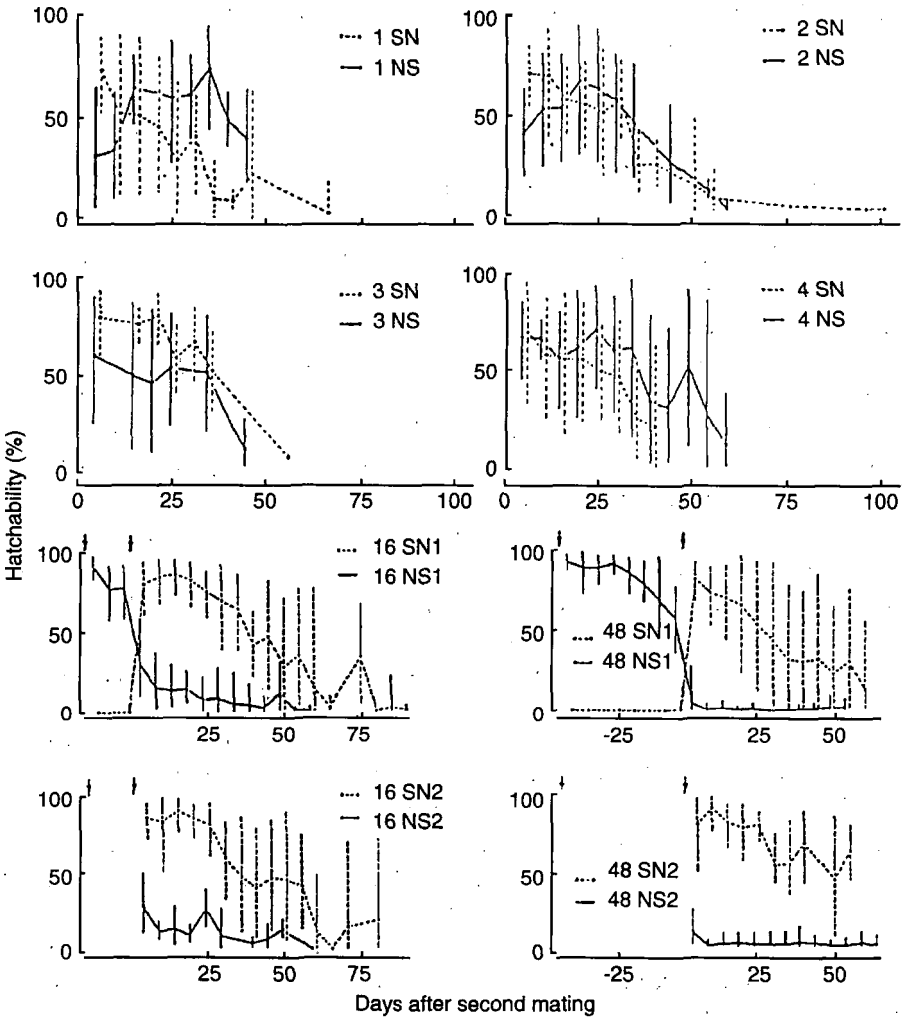

FIG. 4. Mean hatchability of eggs laid by melon fly females mated with normal (N) and sterile (S) males (from Ref. [12]). NS: Mated first with sterile and then with normal males; SN: first sterile and second normal mating. The numbers on the graphs indicate the mating interval, in days. NS1, SN1: Females that could oviposit after the first mating. NS2, SN2: Females that could oviposit only after the second mating.

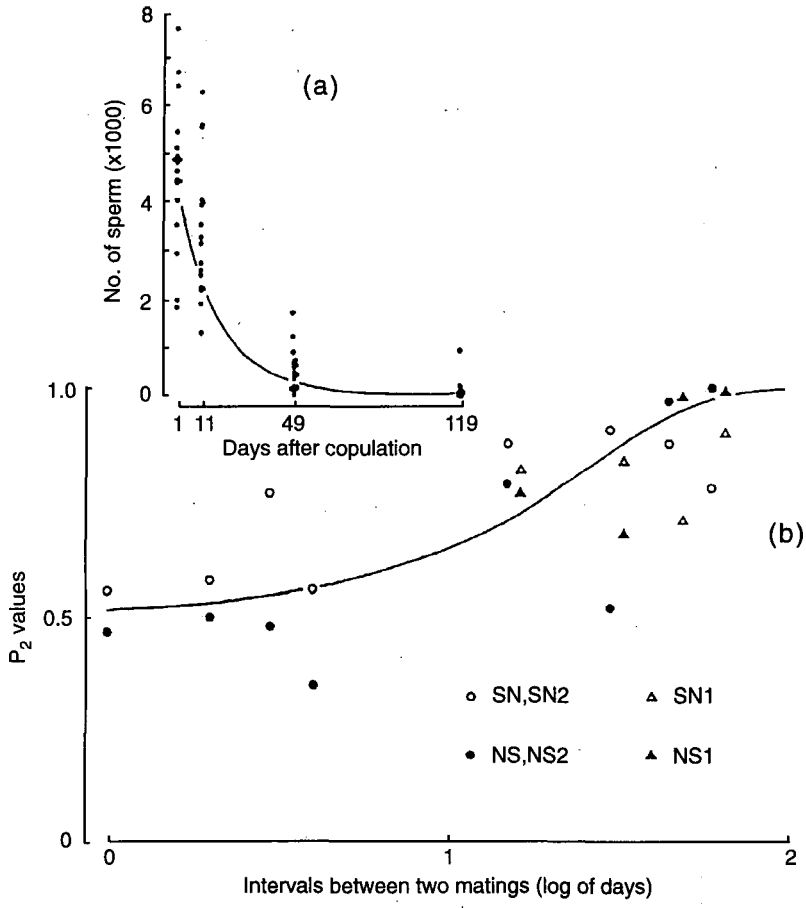

FIG. 5. (a) Change in the number of sperm in female spermathecae without oviposition (from Ref. [13]). (b) P_2 values in relation to intervals between two matings. The curve shows the expected values calculated from the sperm survival rate shown in (a) (from Ref. [12]).

long time in spermathecae (see, for example, papers in Ref. [14]). The Yamagishi et al. report [12] is the first case where the short lifespan of sperm is the main reason for the high P_2 values.

4. INHIBITION OF FEMALE REMATING

In the melon fly, sperm transfer is completed after 4 h of copulation, but melon fly copulations continue for more than 8 h. To examine the reason for this long duration of copulation, Kuba and Itô [15] studied the effects of the duration of copulation

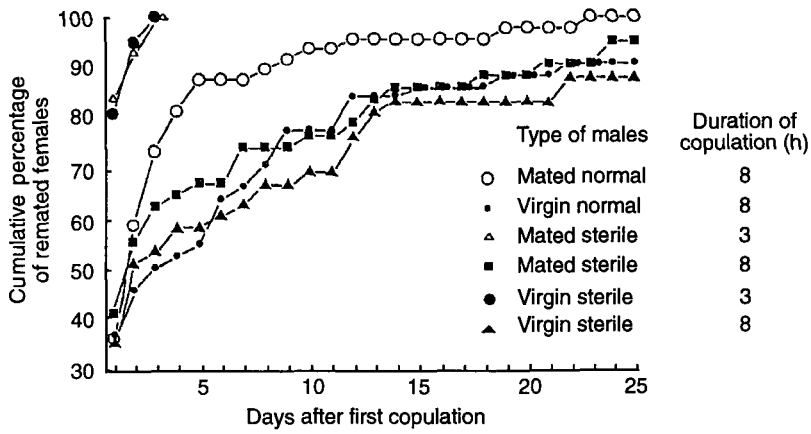

FIG. 6. Cumulative remating curves of melon fly females mated with virgin and previously mated normal and sterile males. The mated sterile males lack sperm (from Ref. [14]).

on the inhibition of female remating. Virgin normal males, virgin sterile males and normal and sterile males that had already mated four times were used. Gamma ray irradiation not only induces dominant lethal mutations in sperm which exists in the testes of melon fly pupae, but it also completely destroys spermatogonia. Therefore, sterile males that have mated three or four times have no sperm, and are good subjects for the evaluation of the effects of sperm.

Figure 6 shows changes in the cumulative percentage of remated females that copulated the first time for 3 or 8 h with different types of males. Three hours of copulation did not inhibit female remating, but 8 h did. Copulation not only with virgin sterile males, but also with spermless sterile males inhibited female remating at the same rate as normal matings. Our recent experiment showed that 4 h of copulation did not inhibit female remating, but 6 and 8 h did.

In *Drosophila*, although secretion from male accessory glands is the main factor that inhibits female remating, the existence of sperm in spermathecae also inhibits female remating. In the melon fly, however, sperm is not an important factor inhibiting remating. We suggest that the transfer of an accessory gland substance after 4 h of copulation is the main factor inhibiting female remating. Determining the importance of the accessory gland substance and its chemical structure, as Chen et al. [16] did for *Drosophila*, are the next subjects of our study.

5. CONCLUSIONS

Melon fly researchers in Japan have developed studies which are important not only for future improvement of sterile insect release programmes and in fruit fly con-

trol, but also important in understanding the evolution of sexual selection. The results given in this paper show that more basic ecological and ethological studies are necessary to improve the genetic control of insect pests.

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FIRST FIELD ASSESSMENT OF THE DISPERSAL AND SURVIVAL OF MASS REARED STERILE MEDITERRANEAN FRUIT FLY MALES OF AN EMBRYONAL, TEMPERATURE SENSITIVE GENETIC SEXING STRAIN

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Abstract

FIRST FIELD ASSESSMENT OF THE DISPERSAL AND SURVIVAL OF MASS REARED STERILE MEDITERRANEAN FRUIT FLY MALES OF AN EMBRYONAL, TEMPERATURE SENSITIVE GENETIC SEXING STRAIN.

Functional temperature sensitive lethal (*tsl*) genetic sexing strains of the Mediterranean fruit fly (medfly) have been recently developed at the Agency's Laboratory at Seibersdorf. Unlike previous medfly genetic sexing strains, these 'second generation' sexing strains are stable under mass rearing conditions and allow females to be killed at an early stage of development. Over the past year, successful efforts to mass rear these strains have shown that they are amenable to routine large scale rearing with only minor adjustments in existing production facilities. In addition, results of detailed greenhouse behavioural assessments have confirmed that the behaviour and mating activity of *tsl* males is comparable overall not only to that of males of the pupal colour sexing strain that was successfully used in the field, but also to that of wild males. Field assessment of the most promising *tsl* strains is the next step in the evaluation of these flies. Before embarking on large pilot evaluations, however, one more aspect of *tsl* male behaviour had to be tested: dispersal and survival under orchard conditions. The results of the first preliminary field assessment of mass reared sterile *tsl* males carried out in citrus orchards in Chios, Greece, in September 1992, are reported. Sterile *tsl* males were recaptured during all ten days of trapping after the initial release. Even though the numbers recaptured declined rapidly, mortality rates were comparable with similar dispersal studies of bisexual medfly strains. Furthermore, and in spite of windy weather, *tsl* males dispersed not only downwind, but also upwind more than the minimum of 100 m required to cover the 200 m distance between flight lines during conventional aerial sterile fly releases. Based on the results obtained to date, the first pilot test using *tsl* males will take place in the oasis of Tozeur, Tunisia, starting in 1993.

1. INTRODUCTION

The benefits of releasing only males in fruit fly control programmes based on the sterile insect technique (SIT) are manyfold [1]. However, pupal colour strains, the only genetic sexing strains of the Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wied.), currently available, are not applicable in mass rearing facilities because females must be reared to the pupal stage and mechanical separation of pupae by colour is inexact, slow and expensive.

Recently, temperature sensitive lethal (*tsl*) genetic sexing strains of the medfly have been developed at the Agency's Laboratory at Seibersdorf [1]. Unlike the previous sexing strains, these 'second generation' sexing strains are (a) stable under mass rearing conditions and (b) allow females to be killed at an early (embryonal) stage. This represents an important breakthrough because both of these attributes were considered indispensable for genetic sexing strains with any potential to replace bisexual strains in sterile medfly production facilities.

Over the past year, the main procedures developed to mass rear these *tsl* strains have proved amenable to routine large scale rearing, with only minor adjustments in existing facilities. Standard mating tests, as well as detailed greenhouse behavioural assessments, have confirmed that the behaviour and mating activity of *tsl* males is comparable with that of the pupal colour sexing strain (successfully used in a large pilot trial in Israel [2]), as well as with that of wild males (unpublished data).

Field assessment of the most promising *tsl* strains is the next step in the evaluation of these flies. Pilot trials of *tsl* strains are planned for various sites in the Maghreb countries of North Africa. Starting in 1993, the first of these pilot tests is planned for the oasis of Tozeur, in southern Tunisia. Before embarking on large pilot evaluations, however, one more aspect of *tsl* male behaviour had to be assessed, namely dispersal and survival under orchard conditions. We report here on the results of the first preliminary assessment of the dispersal and survival of mass reared sterile *tsl* males under field conditions.

2. MATERIALS AND METHODS

The field study was carried out during the first half of September 1992 on the island of Chios, Greece. Minimum daily temperatures ranged between 16 and 21°C and maximum temperatures between 27 and 33°C. During the short time available it was only possible to carry out one test lasting ten days. Typical of the Mediterranean region, property sizes on Chios are small and orchards are interspersed between houses and gardens. Under these conditions, the best site available for a sufficiently large dispersal area included various contiguous citrus orchards belonging to different owners (Fig. 1). These were separated from each other by 3–5 m high stone

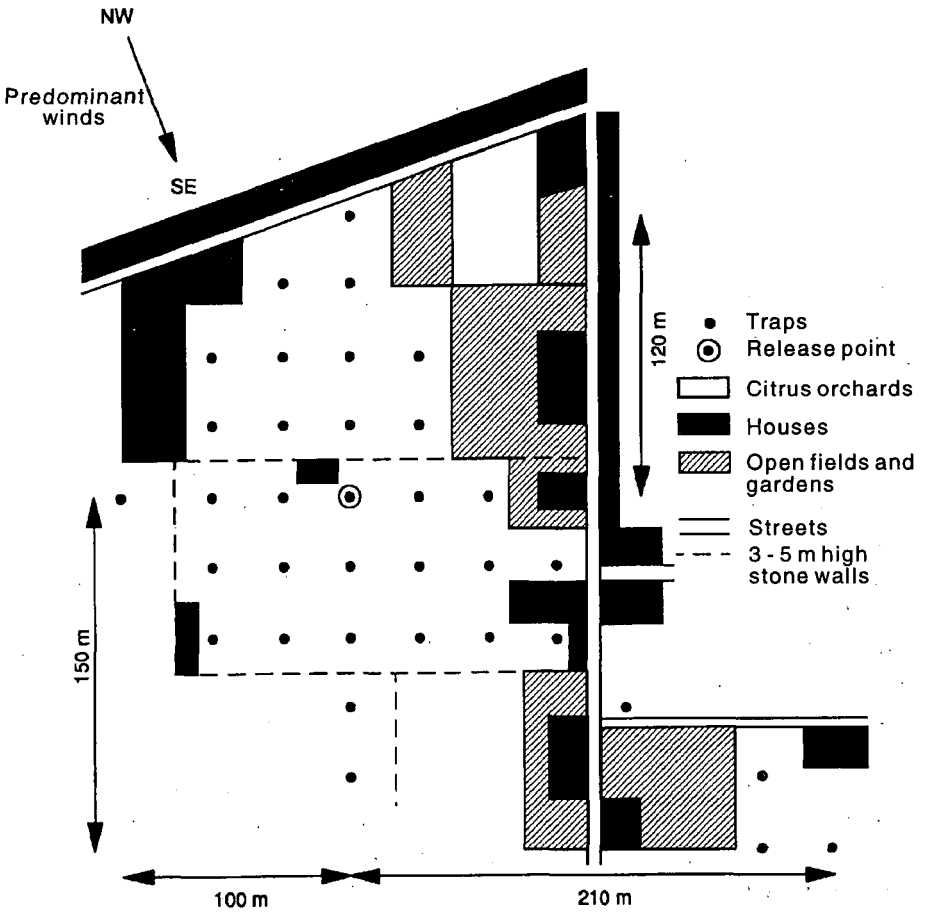

FIG. 1. Distribution of a grid of 35 Jackson traps in various contiguous citrus orchards in Chios, Greece, to assess the dispersal and survival of sterile males of tsl *strain Vienna-42 (1-61) under field conditions.*

walls, which apparently protect trees from strong winds. The citrus fruits, mostly orange and mandarin, were largely at an immature stage. A 30 m × 30 m grid of 35 Jackson traps was distributed over the central orchards and a few traps were placed inside the outer orchards (Fig. 1).

The males released originated from *tsl* strain Vienna-42 (T(Y;5)1-61), which had been held for nine generations under mass rearing conditions at the Agency's Laboratory at Seibersdorf. To eliminate female embryos, eggs were subjected to a heat treatment of 32°C for 48 h of incubation in aerated water. Thereafter, they were seeded at a rate of 1.4 mL/kg of larval diet. Pupae of the first three larval collection

days were irradiated in air one day before emergence with a dose of 90 Gy. Subsequently, they were marked with a fluorescent dye and packed in anoxia in 1.0 L plastic bags and immediately shipped in a cardboard container to Chios. The total transport time from Vienna to Chios was 12 h. Upon arrival at Chios, the pupae were placed in paper bags at a rate of approximately 25 mL of pupae/release bag. The paper bags had previously been painted internally with sugar and provided with resting surfaces for emerging males. The bags were held under field conditions and, two to three days after emergence, they were opened during the afternoon to release the sterile males from one central release point. Control paper bags were retained to determine the average per cent of emergence, which was 86.5. Based on these data, approximately 240 000 males were released.

Traps were first installed 23 h after release of the flies. Then, during each of ten days, the traps were installed and removed after 1 h between 1700 and 1800 h. As in previous dispersal studies [3], this short exposure period was an attempt to ensure that the traps themselves would not bias the movement of the males. Each trap was installed and removed sequentially in the same order in the predetermined grid pattern so that all traps were exposed for 1 h daily. Initially, traps were baited with fresh, slow release trimedlure plugs. After each trap removal, the inserts covered with Tanglefoot were observed under ultraviolet lamps. The head of each unmarked fly was squashed for signs of fluorescent dye and the number of marked and unmarked males was recorded. The traps were provided with new sticky inserts on the following day, just before installation.

Starting with the fly release and in parallel with the entire trapping period, control males were held in a rectangular open mesh field cage (1 m × 0.5 m × 0.5 m). They were held under orchard conditions and provided with water and a mixture of sucrose and yeast hydrolysate. The number of flies in the field cage corresponded to one paper bag. Dead males in the cage were counted and removed daily. At the end of the ten day trapping period, the number of males remaining in the cage was recorded.

3. RESULTS

The population of wild flies remained relatively constant throughout the trapping period, despite the near absence of mature fruit in the citrus orchards (Table I). Most of these wild flies originated apparently from sour oranges which had ripened the previous month. Only during the first trapping day were wild fly captures high, a normal situation at the initiation of trapping with a dense trap grid. The *tsl* males were recaptured in the citrus orchards during each of the ten days of trapping following the initial release (Table I). The numbers recaptured, however, declined very rapidly (Fig. 2). As a result, only on the first two days of trapping were their numbers higher than those of wild flies (Table I). The daily rate of disappearance of *tsl*

TABLE I. AVERAGE DAILY CAPTURE (±SD[a]) OVER TEN DAYS OF WILD AND STERILE MALES OF *tsl* STRAIN VIENNA-42 (1-61) CAUGHT IN A TRAP GRID OF 35 JACKSON TRAPS IN CITRUS ORCHARDS IN CHIOS, GREECE

Day	Average No. of recaptured *tsl* males per trap per hour[b]		Average No. of captured wild males per trap per hour[b]		Ratio L:W[c]
1	127.8	(171.8)	70.4	(83.2)	1.815
2	119.6	(131.7)	45.9	(40.5)	2.606
3	26.7	(23.5)	43.5	(42.0)	0.614
4	9.0	(13.8)	43.1	(48.6)	0.209
5	3.7	(4.0)	43.7	(40.5)	0.085
6	1.8	(2.0)	34.6	(32.0)	0.052
7	1.4	(1.6)	47.1	(35.2)	0.030
8	0.5	(0.7)	46.9	(30.6)	0.011
9	0.5	(0.8)	50.0	(30.3)	0.010
10	0.2	(0.4)	56.1	(37.7)	0.004

[a] SD: standard deviation.
[b] Traps were deployed only 1 h per day.
[c] L: Laboratory; W: wild.

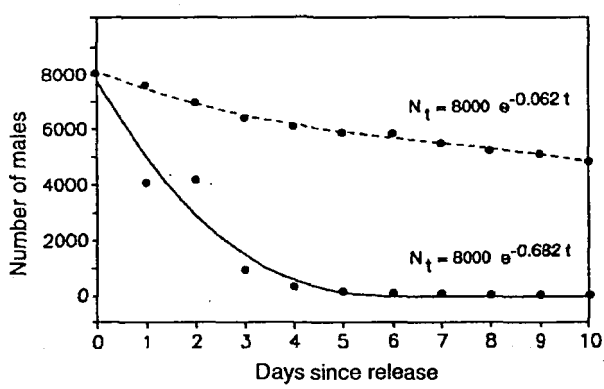

FIG. 2. Comparison of sterile males of tsl *strain Vienna-42 (1-61) recaptured in Jackson traps up to ten days after release with control males of the same strain and shipment surviving in field cages with food and water. In the equation* $N_t = N_0 e^{-\mu t}$, μ *is the rate of disappearance (mortality and emigration, or only mortality in the case of the cages), and* N_0 *the estimated initial population, i.e. not the total released population, but rather the fraction of the population equal to the fractional trapping rate (———: field trap catches; - - - -: field cage control).*

TABLE II. RATIO OF DOWNWIND TO UPWIND CAPTURES (IN RELATION TO THE RELEASE POINT) OF STERILE MALES OF *tsl* STRAIN VIENNA-42 (1-61) AND WILD MALES OVER TEN DAYS IN CITRUS ORCHARDS IN CHIOS, GREECE

Day	Ratio of downwind to upwind captures		Wind speed (Beaufort scale)
	Sterile *tsl* males[a]	Wild males[a]	
1	1.81	2.57	4
2	1.16	1.12	5
3	1.20	1.23	6
4	2.48	1.63	6
5	0.65	1.39	7
6	0.44	0.93	5
7	0.34	1.18	3
8	0.83	0.83	3
9	0.55	0.96	2
10	0.45	0.97	3

[a] No significant interaction (P = 0.64) strain versus trapside over time (two way analysis of variance (ANOVA)).

males, either due to mortality or emigration, was 0.68. This rate was at least ten times higher than that of the flies in the control field cage (0.06). However, in the latter case the rate includes only mortality and not emigration or recapture.

Continuous winds from the northwest prevailed during the whole ten day recapture period following release. Wind speeds ranged from a light or gentle breeze (2 or 3 on the maritime Beaufort scale) up to a moderate gale (7 on this scale = 50–61 km/h). The *tsl* males dispersed in spite of the wind not only downwind, but also upwind. In fact, the ratio of downwind to upwind recaptures decreased throughout the study, and from days five to ten, a majority of recaptures occurred on the upwind side of the initial central release point (Table II). The ratio of downwind to upwind captures for wild flies followed a similar pattern.

The percentage distribution of average recaptures per trap at various distances from the release point is shown in Fig. 3. Although *tsl* males were recaptured in all traps deployed starting from the first day of trapping, only 0.6% of recaptures per trap occurred initially in the most distant traps compared with 43% recaptured at the

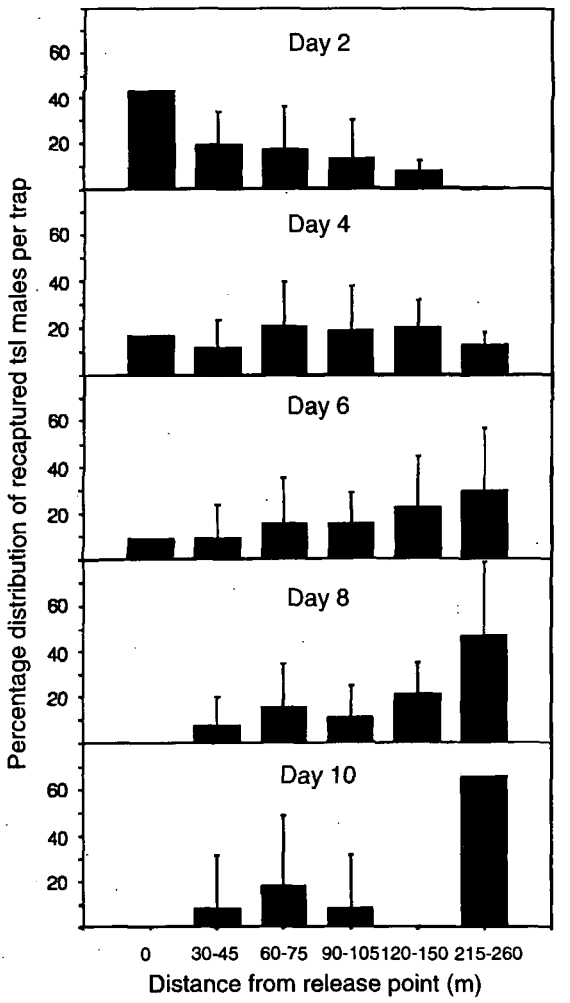

FIG. 3. Percentage distribution of recaptured sterile males of tsl *strain Vienna-42 (1-61) per trap and distance from the release point.*

central release site. However, the percentage of recapture per trap at the release site declined rapidly to 16 on day four, 9 on day six, and finally 0 on days eight and ten. At the outlying traps on the other hand, the percentage of recapture per trap increased to 13 on day four, 30 on day six, 47 on day eight and finally 66 on day ten.

4. DISCUSSION

To date, a number of very thorough field assessments of the dispersal and survival of sterile medflies have been carried out in different regions of the world [3–8].

Although these studies did not involve male only releases, they all report rapid declines in the numbers of released sterile flies. On average, the last recaptures of released medflies in these studies have been on day eight, when released populations were monitored until they had reached a practically undetectable level. This rapid disappearance of flies occurred even where the recapture area covered a 1 km^2 non-host orchard [3], and fly emigration was insignificant, as evidenced by the few flies that reached the outer perimeter of the traps.

In our dispersal study, the rate of disappearance was again high, although *tsl* males were recaptured during each of the ten trapping days. The percentage of recapture of *tsl* males was approximately 4, which is considerable, taking into account that the traps were deployed daily for only 1 h in the afternoons. Not all of the high rate of disappearance of *tsl* males from the trapping area was due to mortality within the release area. The percentage of males recaptured in the most distant traps in the outlying orchards increased throughout the study (Fig. 3). In addition, *tsl* males probably disperse farther than males of bisexual strains. In various field studies [2, 8, 9], it was shown that sterile males range much wider in search of wild females when released alone than when they are released together with females. This advantage of sexing strains, where only sterile males are released, results in considerable increases in the effectiveness of SIT [9].

Although emigration and recapture account for some fly losses, they do not account for most of the tenfold difference in survival between the *tsl* males in the orchard and those in the protected control field cage. Unlike released *tsl* males, *tsl* males in the control cage had access to food and water. The dissection of recaptured *tsl* males showed, however, that they had the same type of light green dense substance in their crops as wild males, probably an indication that sterile males were finding and feeding at the same natural food sites as wild males.

Probably a more important factor to consider here is mortality due to predation as a considerable number of cases of predations on the released flies were noted in the release area. Particularly apparent was predation by yellowjacket wasps (*Vespula germanica*) [10]. This may account to a large extent for the enormous difference in survival between the *tsl* males in the orchard and those in the protected control field cage. During observations of sterile medflies released on a mango tree, Baker and van der Valk [11] also observed rapid declines in fly numbers immediately after release. They found no evidence that the physical effect of heavy rain might reduce numbers. However, although it was not measured, they suggested that heavy predation, including by wasps, might account for the precipitous fall in fly numbers.

We quantified our observations of predation losses using a field cage (2.2 m high × 3 m diameter) with a fruit bearing citrus tree. A release bag containing *tsl* males was opened within the field cage. The entrance of the field cage remained open (approximately 0.5 m wide) to allow access to foraging wasps. On each of the three occasions this study was replicated [12], wasps entered the open cage in large numbers and within 5–7 h killed all of the more than 1000 flies that

had been released. Under natural conditions, the escape of the flies would have been less constrained than in the cage, and predation losses would probably have been less drastic. However, the study confirmed that predation losses can be very large and suggested that predaceous wasps are the most likely explanation for the much more rapid decline in the numbers of males released in the field than in the closed control field cage.

In another study within the same field cage (unpublished data), we recorded the foraging of individual wasps to compare predation on wild and sterile males. We found that although wild males suffer less, they still suffer considerable losses from wasp predation. This is also confirmed by the observation that a large majority of the wild males captured live for behaviour studies after the first trapping day were still sexually immature. However, sterile males were on average three to four times as likely as wild males to be captured by wasps [12, 13]. Particularly vulnerable to predation were young *tsl* males and those engaged in pheromone calling and lekking activities. In view of the high predation mortality, and because the reproductive behaviour of released sterile males is still increasing in intensity three days after the normal 3-d-old release, there would be advantages to releasing flies that are older than those currently released (Ref. [11]; Hendrichs et al., unpublished data).

5. CONCLUSIONS

The results of this field test should be considered to be preliminary as only one release was carried out and the recapture area was not sufficiently large to distinguish accurately between mortality and emigration. Even so, findings on the survival and dispersal of *tsl* males are comparable with the most thorough field evaluations of bisexual strains reported in the literature. Flies seemed able to locate food and water and to respond to trimedlure. Furthermore, and in spite of windy weather, they dispersed not only downwind, but also upwind more than the minimum of 100 m required to cover the 200 m distance between flight lines during conventional aerial sterile fly releases. Larger field trials using these strains seem warranted, although consideration should be given to releasing sterile males that are older than those currently released to reduce predation losses before the flies attain their full sexual maturity.

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RECENT PROGRESS IN THE DEVELOPMENT OF ATTRACTANTS FOR MONITORING THE MEDITERRANEAN FRUIT FLY AND SEVERAL *Anastrepha* SPECIES

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Abstract

RECENT PROGRESS IN THE DEVELOPMENT OF ATTRACTANTS FOR MONITORING THE MEDITERRANEAN FRUIT FLY AND SEVERAL *Anastrepha* SPECIES.

Recent developments in the analytical methods used for collecting and analysing semiochemicals that are attractive to fruit flies are reviewed. These efforts have resulted in increased knowledge of the nature of the pheromonal components released by males of the Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann), and males of several *Anastrepha* species. A recently developed bioassay system is described that permits the testing of various substrates for biological activity in a flight tunnel, while simultaneously collecting a portion of the volatiles from the attractive source for subsequent chemical identification and quantification. This system is currently being used to re-examine the attractiveness of various protein based baits for pest fruit flies. It was found, for example, that an increase in the pH of the protein bait Nulure significantly increases the attraction of female medflies to this bait. Similarly, pheromone research using the described systems for the bioassay and collection of attractive pheromonal components from the medfly and from several *Anastrepha* species are under way, and the results from this research are presented.

1. INTRODUCTION

The availability of effective lures for monitoring fruit flies is of paramount importance for control and eradication programmes. Control of the Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann), and several *Anastrepha* spp. is of considerable economic importance worldwide. Currently used lures to monitor for medfly include trimedlure [1] and hydrolysed protein baits [2]. Although trimedlure is effective in attracting male medflies, it is only weakly attractive to female medflies. Protein baits used in glass McPhail traps attract both male and female medflies and several *Anastrepha* species; however, this monitoring method is cumbersome to deploy and the effectiveness of these traps is reportedly low [3].

Thus, there is considerable interest in developing improved lures, especially synthetic lures, that would attract female fruit flies.

This paper reports on recent developments both in analytical methods used for collecting semiochemicals that are attractive to fruit flies and in bioassay methods used for determining the attractiveness of these chemicals in flight tunnel tests. Also presented are the results of recent efforts to improve protein based attractants and to develop synthetic pheromone attractants for the medfly and several *Anastrepha* spp. fruit flies.

2. METHODS TO COLLECT AND BIOASSAY FRUIT FLY ATTRACTANTS

2.1. System to collect fruit fly semiochemicals

To fully understand insect chemical communication systems and to maximize the potential development and use of volatile semiochemicals produced by insects and plants, the chemicals emitted must be collected and analysed both qualitatively and quantitatively. The collection process must be conducted in a manner that will minimize both the stress placed on the organism being studied, as well as the collection of unwanted background organic compounds from the surrounding environment. An illustration of the system recently designed by us is shown in Fig. 1 and a complete description of the system has been published [4]. Unique to this system is that inlet air is purified by passage through three 7-cm-diameter, 0.5-cm-thick charcoal filter discs. The system consists of four main components: the air filtration unit, the volatile collection unit, the collector trap and the multiport collector base. The volatile collection chamber is constructed of glass and the tubing in the system is stainless steel to minimize contamination. Manual switching for the collection of volatiles on one of the three collector traps or purge is accomplished using a four way, multiport valve. A flow meter set at 1 L/min is used to control the amount of air pulled by a vacuum pump through the collector traps or to purge the system.

Volatiles are collected on traps using Super-Q® (Altech Assoc. Inc., Deerfield, IL, USA) as the adsorbent. Super-Q traps are prepared by packing approximately 20 mg of the adsorbent in 4 cm long × 4.0 mm i.d. glass tubes and the material is contained between two stainless steel frits.

2.2. Flight tunnel bioassay system

A bioassay system has been developed in our laboratory that permits the testing of various substrates for biological activity in a flight tunnel, while simultaneously collecting a portion of the volatiles from the attractive source for subsequent chemical identification and quantification. The bioassay system consists of four main subcomponents designed for specific tasks and capable of varying certain parameters

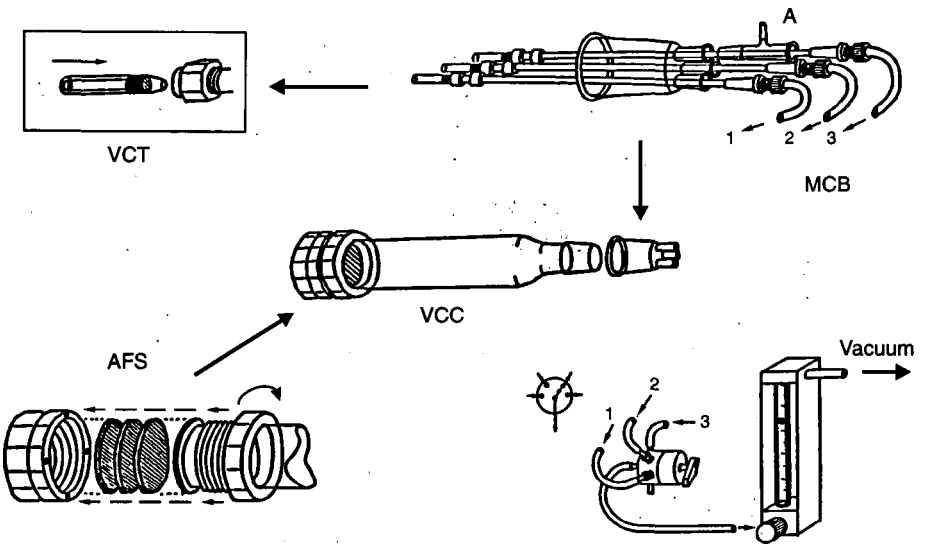


FIG. 1. A system designed for the collection of volatiles released from plants. The system components are as follows: an air filtration system (AFS), a multiport collector base (MCB), a volatile collection chamber (VCC) and a volatile collector trap (VCT).

between experiments (Fig. 2). These components include the bioassay flight tunnel, the air flow system and air delivery system, the insect isolation trap and, finally, the volatile collection system. The air flow system contains sheets of cloth that have been infused with charcoal material, as described for the volatile collection system. This material purifies the air entering the tunnel, which facilitates pheromone collection at various points inside the flight tunnel with minimal contamination.

This system was used to determine the periodicity of response of female medflies to the pheromone released by male flies. Studies conducted with female factory and feral medflies indicated that these flies were highly responsive to odours from live males in the flight tunnel bioassay, with an average of 50–75% of the tested females trapped. The periodicity of response is shown in Fig. 3. The system described should be of general utility in the determination of the attraction of pest fruit flies to suspected attractants, and is currently being used to test the response of females to synthetic pheromone blends.

3. PROTEIN BAIT ATTRACTANTS

Prior to the isolation of the 'food' attractant chemicals from various protein sources, it is paramount to determine what chemical conditions are required for attractiveness. Although considerable efforts have been made on improving protein

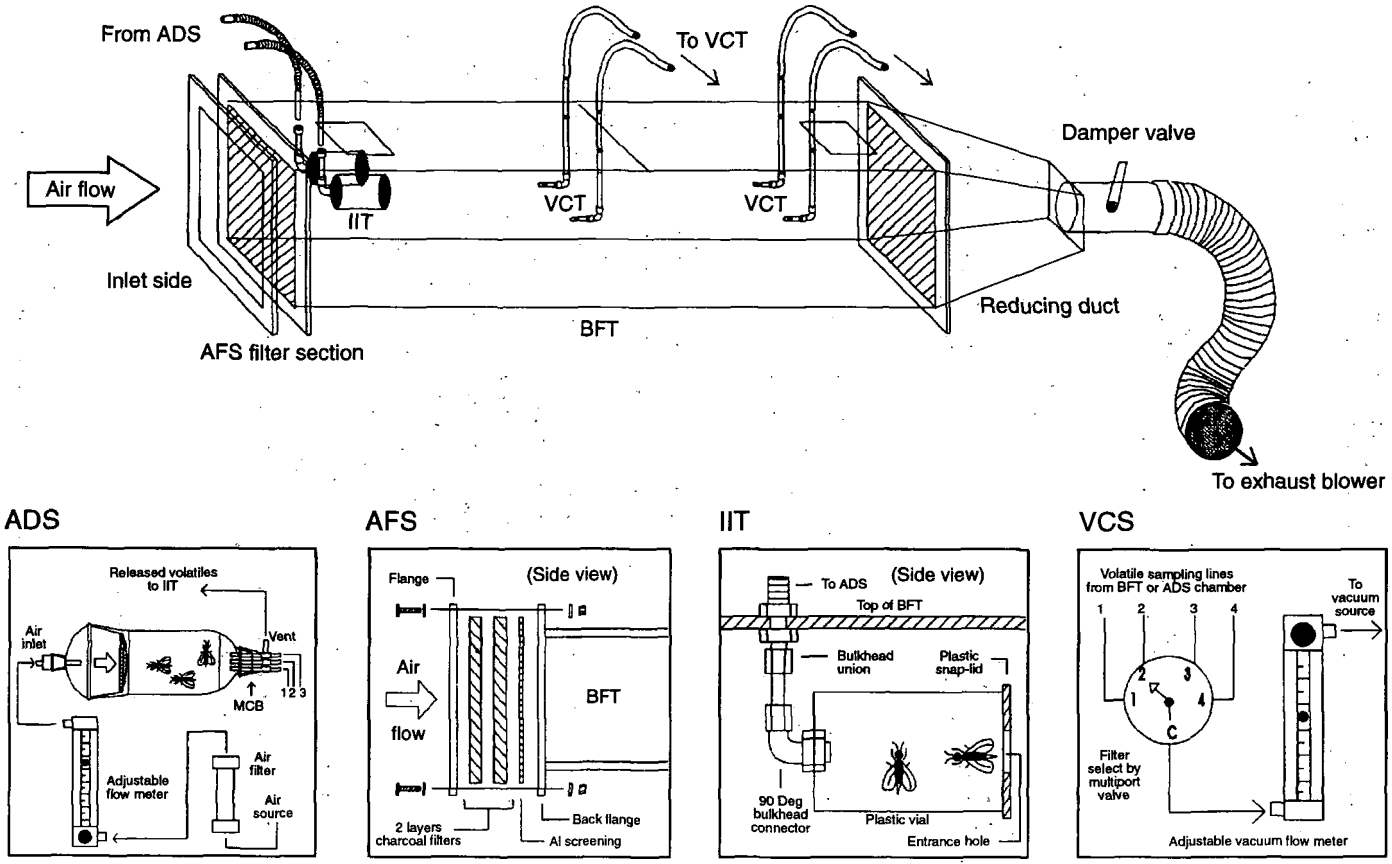


FIG. 2. The wind tunnel bioassay system, which is comprised of a bioassay flight tunnel (BFT), an air flow system (AFS), an air delivery system (ADS), two insect isolation traps (IITs), a multiport collector base (MCB), a volatile collection system (VCS) and a volatile collector trap (VCT).

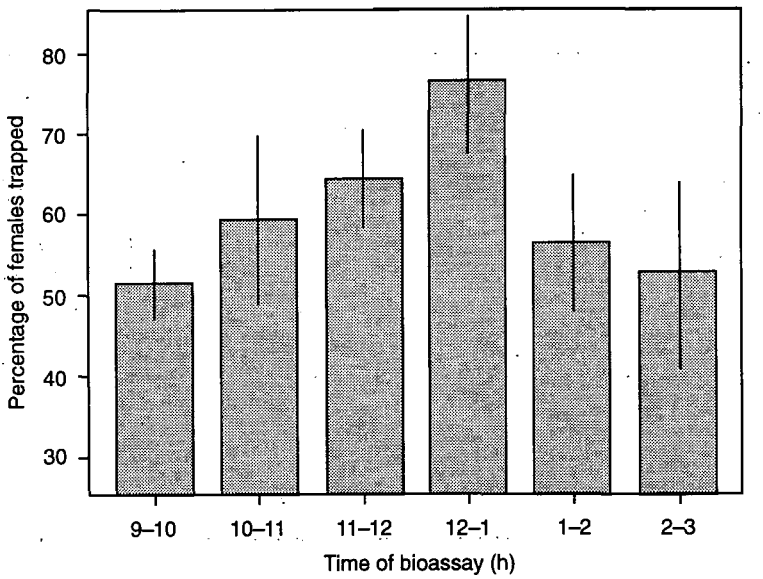


FIG. 3. The percentage of female medflies responding to volatiles from five male medflies during the day. The lines indicate the standard error (n = 12).

baits, these efforts have been largely empirical. We recently determined that the attractiveness of Nulure (Miller Chemical and Fertilizer Co., Hanover, PA, USA) for medflies can be 'programmed' on the basis of pH. In tests conducted in Guatemala, we compared solutions of torula yeast plus borax pellets, 10% Nulure plus 1, 3, 5 and 10% borax, and a control (water) in McPhail traps. Every 2–3 d, flies were removed from the traps, the pH of the bait recorded, recycled bait replaced in the traps, and the traps moved to a new position sequentially. Trapped flies were sorted by sex and species, and the number per trap recorded. The effect of bait treatment was analysed by one way analysis of variance followed by Duncan's mean separation test ($P = 0.05$). Based on five replicates, Nulure at a pH of 8.5 captures significantly more female medflies than both Nulure at a lower pH and torula yeast pellets (Fig. 4). These findings allow the determination of chemicals emitted from Nulure having various degrees of attractiveness and allow us to determine either the amount of/or ratio of differences of potentially attractive chemicals released from protein baits.

4. MEDFLY PHEROMONE

Baker et al. [5] reported the identification of nine compounds emitted by male medflies. However, no behavioural data to substantiate pheromonal activity were

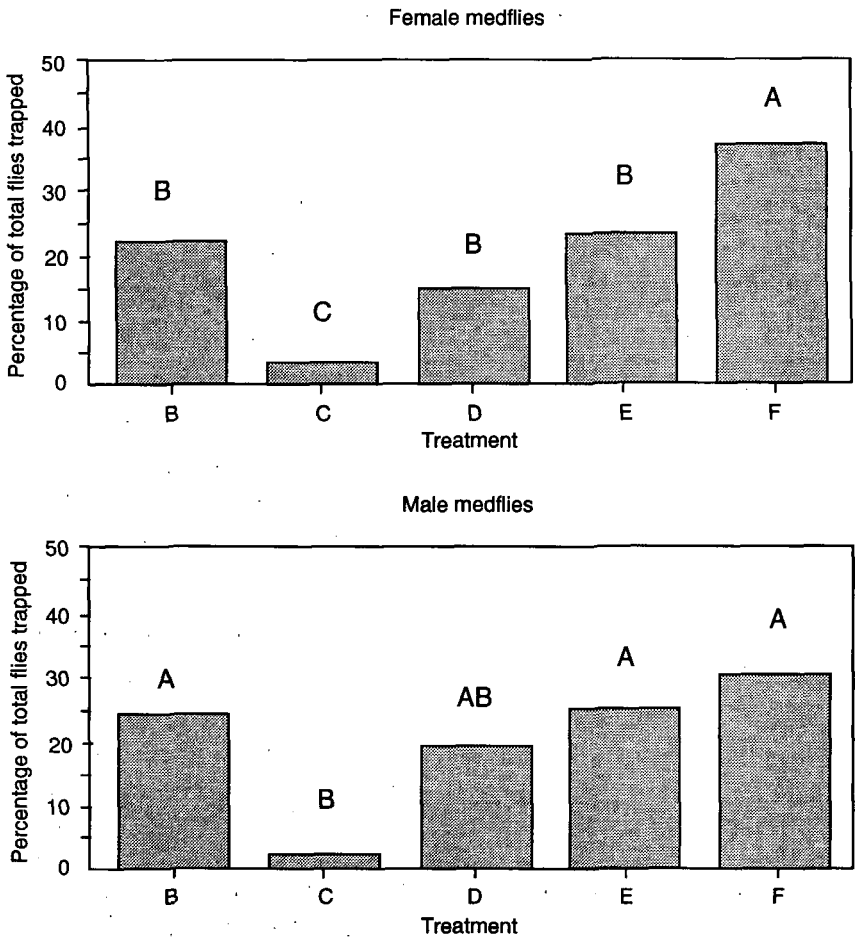


FIG. 4. The percentage of female and male medflies trapped with various protein baits. A: Water (not shown); treatment B: five torula yeast pellets; C: Nulure + 1% borax; D: Nulure + 3% borax; E: Nulure + 5% borax; F: Nulure + 10% borax; The effect of bait treatment was analysed by one way analysis of variance followed by Duncan's mean separation test (P = 0.05). n = 90 for each treatment.

provided. Subsequently, Jang et al. [6] determined the electroantennogram responses of male and female medflies to these nine compounds and to an additional 56 compounds collected from air passed over male medflies. In 1991, we reported on the qualitative and quantitative analyses of three principal components of the volatiles emitted by laboratory reared and wild male medflies during photophase [7]. The attractiveness of the pheromone blend at four release rates was investigated in field trials conducted with wild medfly populations in Guatemala [7]. In subsequent trials

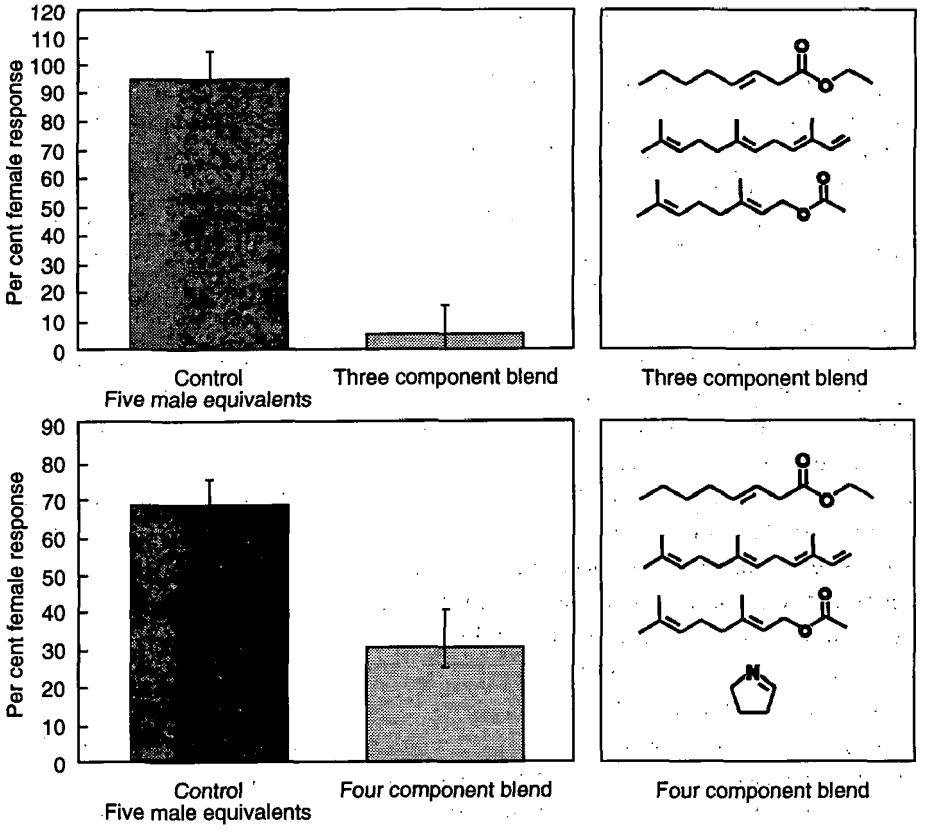


FIG. 5. Response of female medflies to a three component and four component synthetic pheromone blend (n = 16).

using the flight tunnel bioassay system described earlier, it appears that additional components may result in a significantly improved lure for female medflies. A comparison between the feral and factory reared female medfly response to a control equivalent to five caged males versus the previously field tested three-component blend containing ethyl-(*E*)-3-octenoate, geranyl acetate and *E*, *E*-α-farnesene is shown in Fig. 5. It is apparent that the three-component blend is considerably less attractive. We determined that the addition of delta 1 pyrolline results in a considerable increase in attractiveness (Fig. 5). Although we have improved the attractiveness of our previously field tested three-component blend, additional tests are being conducted to delineate a blend that will be equal and perhaps better than that emitted by male medflies.

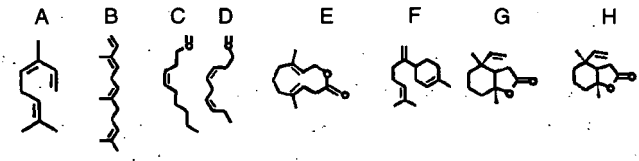
5. *Anastrepha suspensa* PHEROMONE

Research that began in 1972 and which has continued to date has not yet resulted in a pheromone based trap for the Caribbean fruit fly (caribfly), *Anastrepha suspensa* (Loew). The most current information on the pheromone components can be found in a recent article by Rocca et al. [8] and references therein. Initial studies on the chemical nature of the pheromone extracted from the abdomens of sexually mature male caribflies resulted in the identification of (*Z*)-3-nonenol and (*Z*, *Z*)-3,6-nonadienol. Subsequent investigations of the abdominal extracts of male flies resulted in the identification of two additional components, the lactones, anastrephin (*trans*-hexahydro-*trans*-4, 7α-dimethyl-4-vinyl-2-(3H)-benzofuranone) and epi-anastrephin (*trans*-hexahydro-*cis*-4, 7α-dimethyl-4-vinyl-2-(3H)-benzofuranone). Laboratory bioassays of these compounds showed that all were individually attractive to females, but a blend of all four components was the most attractive to females [9]. A synthetic mix of the four components, however, failed to attract flies in field trials [10]. A fifth component, a macrolide (*E*,*E*)-4, 8-dimethyl-3, 8-decadien-10-olide), was identified, synthesized and has been named 'suspensolide'. Additionally, β-bisabolene, ocimene and (*E*, *E*)-α-farnesene have been reported as volatiles emitted by the caribfly.

Admittedly, the chemistry of the male caribfly pheromone is complex. Analysis of some of the pheromonal components is further complicated by their thermal lability. For example, the identification of suspensolide was not completed until 1988 and the identification of (*E*, *E*)-α-farnesene not until 1992. Elucidation of these components in earlier research was hampered by the lack of appropriate analytical methodologies now available to researchers. Not all components of the volatile blend produced by calling males have been proved to have pheromonal activity. We have begun studies to test the female response to the putative male pheromone components. We were able to obtain high rates of attraction of females to caged males using the flight tunnel bioassay system. The testing of synthetics is awaiting the completion of the synthesis of some of the pheromonal components.

6. OTHER *Anastrepha* FRUIT FLY PHEROMONES

Considerable research on the pheromone chemistry of *Anastrepha ludens* (Loew), the Mexican fruit fly, has been conducted (Ref. [8] and references therein). Again, this research has not yet resulted in a pheromone based trap. It is interesting to note that there is considerable overlap between the pheromone components produced by *A. ludens* and *A. suspensa* (Fig. 6). Analysis of compounds from *A. fraterculus* suggests that this pheromonal system may be similar to that of the caribfly and the Mexican fruit fly. This commonality does not appear to extend to the other *Anastrepha* species, however. In some of our recent investigations of the



	A	B	C	D	E	F	G	H
A. suspensa	Y	Y	Y		Y	Y	Y	Y
A. ludens	N	Y	Y		Y	N	Y	Y
A. obliqua	N	Y	N		N	N	N	N
A. striata	N	N	N		N	N	N	N
A. fraterculus			?		?	?	?	?
A. serpentina	N	N	N		N	N	N	N

FIG. 6. Comparison of volatiles emitted from several species of male Anastrepha. *'Y' indicates the presence of the compound, 'N' indicates the absence of the compound and '?' indicates that the compounds are likely present (A: ocimene; B: (*E, E*)-α-farnesene; C: (Z)-3-nonenol; D: (*Z, Z*)-3, 6-nonadienol; E: suspensolide; F: β-bisabolene; G: anastrephin; H: epi-anastrephin).*

pheromonal compounds released by virgin male *A. obliqua*, *A. striata* and *A. serpentina*, we were unable to detect many of the compounds found to be released by *A. suspensa* and *A. ludens*.

7. CONCLUSIONS

The success in developing better attractants for fruit flies will require considerable efforts in understanding the biology of the insect, especially as it relates to the attractiveness of the semiochemical being investigated. Additionally, a great deal of effort will be required to isolate, identify, synthesize and formulate the attractive semiochemicals for use in the field. An integrated approach between the chemistry of attractive chemicals and an understanding of the insect response to the semiochemicals should provide a significant series of excellent lures for the detection, and perhaps even control, of many economically important fruit flies.

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EFFECT OF THE SEX RATIO ON EGG COLLECTION AND HATCH OF THE MEDITERRANEAN FRUIT FLY (DIPTERA: TEPHRITIDAE)

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Abstract

EFFECT OF THE SEX RATIO ON EGG COLLECTION AND HATCH OF THE MEDITERRANEAN FRUIT FLY (Diptera: Tephritidae).

When the female:male ratio in the adult cages of artificially reared Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), was increased from 50:50 to 80:20 (the initial number of total adults was always the same) a 50% overall increase in the fertilized eggs collected per cage occurred during the first 2 weeks of adult life. Smaller increases occurred when the sex ratios were between 50:50 and 80:20, and there was a decrease (compared with the 80:20 sex ratio) when the sex ratio was 90:10 because the males needed several days to fertilize all, or nearly all of the females. At high female:male ratios, there was no increase in the eggs sticking on the oviposition net, in spite of the increased egg laying per surface unit of oviposition net.

1. INTRODUCTION

In area wide sterile insect technique programmes, hundreds of millions of insects may be mass produced and released weekly. To reduce rearing and release costs, rearing technique innovations are made. For example, genetic sexing strains of the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), have been developed to separate the sexes so that only males are released [1]. When the sexes are easily separated, it is possible to make any desired sex ratio in the mass rearing adult cages. If the number of eggs collected per cage could be substantially increased, the rearing costs could be reduced significantly. In the present work, a study was made of the effect of different female:male ratios on egg collection and fertilization in two different adult density experiments.

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TABLE I. EGGS COLLECTED PER CAGE PER DAY FROM MEDITERRANEAN FRUIT FLIES KEPT AT SEVEN DIFFERENT SEX RATIOS

(Insect density at start, 0.3 flies/cm^3 cage volume, or 0.7 flies/cm^2 cage surface; oviposition net surface/cage, 66.5 cm^2)

Sex ratio (% females)	Eggs collected per cage per experiment day[a]	
	Days 4, 5, 6, 7, 8	Days 15, 22
90	18 289 ± 1254a	3450 ± 668a
80	17 119 ± 882a	4032 ± 574a
70	14 208 ± 886b	1866 ± 483b
60	11 489 ± 1323c	2405 ± 839b
50	10 609 ± 586c	2409 ± 316b
40	9 006 ± 579d	2441 ± 510b
30	7 035 ± 839e	2370 ± 268b

[a] Four cage replications per treatment. Eggs laid on days other than those indicated were removed but not counted. For each column, means with the same letter do not differ significantly ($P > 0.05$; Duncan's multiple range test [3]).

TABLE II. PER CENT HATCHABILITY OF EGGS OF MEDITERRANEAN FRUIT FLIES KEPT AT SEVEN DIFFERENT SEX RATIOS

(Same experiment as in Table I)

Sex ratio (% females)	Per cent egg hatch[a]	
	Days 4, 5, 6, 7, 8	Days 15, 22
90	59.7 ± 2.3d	64.4 ± 5.6b
80	75.4 ± 2.2b	80.7 ± 4.3a
70	68.7 ± 9.5c	66.5 ± 7.8b
60	80.6 ± 3.0ab	62.4 ± 8.6b
50	80.4 ± 1.8ab	62.1 ± 6.2b
40	78.5 ± 2.0ab	61.4 ± 5.1b
30	83.2 ± 0.8a	61.9 ± 3.5b

[a] Four cage replications per treatment. Eggs laid on days other than those indicated were removed but not counted. For each column, means with the same letter do not differ significantly ($P > 0.05$; Duncan's multiple range test [3]).

TABLE III. EGGS COLLECTED PER CAGE PER DAY FROM MEDITERRANEAN FRUIT FLIES KEPT AT FIVE DIFFERENT SEX RATIOS

(Insect density at start, 0.16 flies/cm^3 cage volume, or 0.33 flies/cm^2 cage surface; oviposition net surface/cage, 184 cm^2)

Sex ratio (% females)	Eggs collected per cage per experimental day[a]	
	Days 1, 2, 3, 4, 5, 6, 8, 10	Days 15, 17, 22, 24, 29, 31
90	13 223 ± 420a	2677 ± 639a
80	11 972 ± 686b	2231 ± 665ab
70	10 644 ± 183c	2352 ± 204ab
60	9 125 ± 171d	2059 ± 329ab
50	7 248 ± 190e	1679 ± 381b

[a] Four cage replications per treatment. Eggs laid on days other than those indicated were removed but not counted. For each column, means with the same letter do not differ significantly ($P > 0.05$; Duncan's multiple range test [3]).

TABLE IV. PER CENT HATCHABILITY OF EGGS OF MEDITERRANEAN FRUIT FLIES KEPT AT FIVE DIFFERENT SEX RATIOS

(Same experiment as in Table III)

Sex ratio (% females)	Per cent egg hatch[a]	
	Days 1, 2, 3, 4, 5, 6, 8, 10	Days 15, 17, 22, 24, 29, 31
90	65.6 ± 2.7d	32.4 ± 4.1c
80	74.4 ± 0.4c	46.7 ± 5.2b
70	75.2 ± 0.8c	54.2 ± 2.8a
60	79.2 ± 1.4b	55.2 ± 1.3a
50	81.2 ± 1.1a	53.8 ± 4.2a

[a] Four cage replications per treatment. Eggs laid on days other than those indicated were removed but not counted. For each column, means with the same letter do not differ significantly ($P > 0.05$; Duncan's multiple range test [3]).

2. MATERIALS AND METHODS

The insects used were from a colony established in the IAEA Laboratory at Seibersdorf in October 1983 from pupae that had been collected from guava in the Sohag Governorate in Egypt. The rearing procedures were as described by Hooper [2]. Small plastic cages (11 cm × 11 cm × 15.3 cm) were used, with four cages (replicates) per sex ratio treatment in both experiments.

Two successive experiments were run. In the first, the adult flies were kept for 3 1/2 weeks in cages at an initial density of 0.3 flies/cm^3 (600 flies per cage). A 9 cm diameter hole, covered by a 0.1 mm^2 hole synthetic oviposition net (the total oviposition surface per cage was 66.5 cm^2) was prepared for oviposition in one of the two small sides (11 cm × 11 cm) of the cage. Sex ratios of 30, 40, 50, 60, 70, 80 and 90% females were compared. Insect mortality, egg collection (eggs inserted through the net and dropped on moistened filter paper plus those that stuck on the oviposition net) and egg hatch (100 eggs from those dropped on the filter paper) were examined per cage on the experiment days (adult day minus 2) shown in Tables I and II. The cages were kept at 25 ± 2°C, 55–65% RH and a 12 h photoperiod. The light intensity was about 2800 lux inside the cages.

In the second experiment, the adult flies were kept for 4 1/2 weeks in cages at an initial density of 0.15 flies/cm^3 (300 flies per cage). An 8 cm × 11.5 cm rectangular hole, covered by an oviposition net, was prepared for oviposition on each of the two opposite large sides (11 cm × 15.3 cm) of the cage (the total oviposition surface per cage was 2 × 92 cm^2 = 184 cm^2). Sex ratios of 50, 60, 70, 80 and 90% females were compared. Again, insect mortality, egg collection and egg hatch (from 500 eggs that had dropped) were examined on the experiment days shown in Tables III and IV. The cages were again kept at 25 ± 2°C and 55–65% RH but with a 14 h photoperiod.

In both experiments, the first experiment day was usually day 3 of adult life. Adults were of variable size, having been collected as larvae on day 2 of their emigration from the diet to the pupation medium. The flies were provided with water and solid adult food containing three parts sucrose to one part hydrolized yeast.

Data were analysed by analysis of variance (ANOVA), and significant differences between the means ($P < 0.05$) were detected by Duncan's multiple range test [3].

3. RESULTS

In both experiments, there were no significant differences in the longevity of either sex when compared among the different sex ratios tested. When the longevity of the two sexes was compared for the different sex ratio treatments of

the second experiment, male longevity was always shorter than female longevity, by 12.5–18.5%.

Tables I and III show the egg collection rates per cage in the two experiments. The total egg collection per cage per day increased by 72 and 82% when the sex ratio was changed from 50 to 90% females in the first and second experiment, respectively. The collection of eggs per cage per day increased, usually significantly ($P < 0.05$), in all successive increases in the female:male ratio. In the second part of both experiments, a significant increase in egg collection again occurred when the lower female:male ratios were compared with the higher ones. Nevertheless, the differences were not as striking as in the first part of the experiments, and often no differences could be detected between successive increases in the female:male ratios.

Tables II and IV show the percentage eggs fertilized in the different sex ratios of the two experiments. In the first part of the experiments, egg hatch decreased by 26 and 19% when the sex ratio was changed from 50 to 90% females in the first and second experiment, respectively. In the first experiment, the decrease occurred only in the higher female:male sex ratios, while in the second experiment the decrease was small, but constant, in successive increases in the female:male ratio. In the second part of the first experiment, no significant decrease in egg hatch was observed with successive increases in the female:male ratio, while in the same period of the second experiment a striking decrease in egg hatch was observed in the two highest female:male ratios.

TABLE V. PER CENT INCREASE IN FERTILIZED EGGS COLLECTED PER CAGE WITH INCREASE IN FEMALE:MALE RATIO OF MEDITERRANEAN FRUIT FLIES KEPT AT TWO DIFFERENT DENSITIES AND OVIPOSITION SURFACE SIZES

Increase in females (%)	Per cent increase in fertilized eggs/cage[a]	
	Experiment 1 (Tables I, II)	Experiment 2 (Tables III, IV)
30 → 40	20.8	—
40 → 50	20.7	—
50 → 60	8.6	22.8
60 → 70	5.4	10.8
70 → 80	32.2	11.3
80 → 90	-15.4	-2.6

[a] Only for days 4, 5, 6, 7, 8 and 1, 2, 3, 4, 5, 6, 8, 10 of Experiments 1 and 2, respectively.

Table V combines egg collection and fertilization and shows the effect of the sex ratio on the overall collection of fertilized eggs during the first 2 weeks of the experiments. In both experiments, when the female:male ratio was changed from 50:50% to 80:20%, there was an overall increase in the fertilized eggs, by about 50%. A further increase in the female:male ratio, to 90:10%, caused a decrease in the fertilized eggs.

A detailed study of egg hatch in the first experiment showed that only 1–2 days were needed for the female:male ratios of 60:40, 70:30 and 80:20 to reach the percentage egg hatch of the 50:50 sex ratio. The corresponding time for the 90:10 female:male ratio was 4 days. Also, after the second week of adult life, the percentage eggs that hatched with the 90:10 female:male ratio decreased much faster than in the other sex ratios, corresponding to the increase in percentage males per cage. Similar changes also occurred in the second experiment.

The collection of eggs per cage was 26–46% higher in the first experiment for the corresponding sex ratio treatments than for the second experiment (Tables I, III). There were no significant differences in the percentage of eggs that stuck on the oviposition net among the different sex ratios in both periods of the two experiments.

4. DISCUSSION

The major economic species of tephritids (fruit flies) are in the subfamilies of Dacinae and Trypetinae. In Dacinae, the sexual behaviour has been studied mostly in the genus *Dacus*, where the males are polygamous but the females do not remate frequently. In Trypetinae, different mating frequencies have been found for females of the different species of *Anastrepha*, *Ceratitis* and *Rhagoletis* [4]. In the Mediterranean fruit fly, males are polygamous while females generally are not, depending on the amount of sperm stored in their spermathecae [5]. Among laboratory reared flies, of the females that remated (about 60% of the total), 54% remated once, 25% twice, 12% three times and the remaining between four and six times. The amount of sperm stored in the spermathecae declined considerably only after 20–25 days following initial mating, and remating increased with the decrease in stored sperm. This suggests that one successful mating can fertilize most of the eggs produced in an average female's life and certainly for the 2 weeks that the flies are kept in mass rearing facilities.

Following the recent successful efforts to develop genetic sexing strains in the Mediterranean fruit fly [1], the present results clearly show that high female:male ratios can be used in the mass rearing of the fly. Female:male ratios of around 80:20 are recommended, since they result in the highest possible collection of fertilized eggs per cage during the first 2 weeks of adult life. A further increase in the

proportion of females does not increase the collection of fertilized eggs because it takes several days for the low number of males to achieve fertilization of the females.

Although in Experiment 1 there were twice as many females as in Experiment 2, egg collection was only 26–46% higher. As has been reported [6], apparently this resulted from the longer duration of light in Experiment 2 (14 versus 12 hours, respectively), and the reduced oviposition surface and increased insect density in Experiment 1. The finding that there was no increase in eggs sticking on the oviposition net as a result of increased egg laying per unit of oviposition surface in the highest female:male ratios confirms that sticking depends on fly activity, which results in dirt on the net [7, 8], and not the number of eggs laid. Finally, the reduced survival rates of males compared with females for all the sex ratios tested indicate that reproduction is not a major survival stress for the female. Competition among the polygamous males to secure matings could be one of the factors, among others, which reduced the male lifespan. Nevertheless, it is noted that no significant difference was observed in the present study in male survival between treatments of low and high female:male ratio, i.e. high and low male mating competition, respectively.

In conclusion, to achieve a substantial reduction in the mass rearing costs of genetic sexing strains, a ratio of 80% females to 20% males in the cages is recommended. This results during the first two weeks of adult life in about a 50% increase in fertilized eggs collected per cage compared with cages containing a 50:50 ratio. The possible negative effects of high female:male ratios on the quality of flies, in particular their sexual behaviour, should be examined.

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POSTER PRESENTATION

IAEA-SM-327/1P

RECOVERY OF FERTILITY IN IRRADIATED *Epestia cautella* (WALKER) (LEPIDOPTERA: PHYCITIDAE)

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1. INTRODUCTION

The recovery of damaged sperm of *Ephestia cautella* irradiated with 10 krad[1] was determined when: (1) irradiated males were repeatedly mated with new unirradiated females; and (2) irradiated males were held after treatment for a period of time before allowing them to mate with unirradiated females.

2. MATERIAL AND METHODS

2.1. Effect of repeated mating on the mating ability and fertility of irradiated males

Twenty-nine 1-d-old males were irradiated with 10 krad at a dose rate of 4584 rad/min. After treatment, the males paired singly with 2-d-old females. The females were removed after 24 h and subsequent oviposition observed. Fresh 2-d-old females were introduced to the males for 24 h. The same procedure was followed for four successive days. Males in the control groups were mated in the same manner as the irradiated males. Every two days, all the eggs laid by each female in each group were counted and a sample taken to determine the percentage egg hatch. The females were dissected after death and examined for the presence of spermatophores.

2.2. Effect of delayed mating on the mating ability and fertility of irradiated males

Four different groups of irradiated males were mated at various intervals (0, 1, 2 and 3 d) after treatment. In each group, the males were paired, singly, with

[1] 1 rad = 1.00×10^{-2} Gy.

2-d-old females for 24 h, after which the males were removed and oviposition observed. Four groups of unirradiated males aged 1, 2, 3 and 4 d old were taken as controls. The percentage egg hatch and mating ability were determined as described in Section 2.1.

3. RESULTS AND DISCUSSION

3.1. Effect of repeated mating

The mating ability of irradiated and unirradiated males was very high in the first copulation; after that it started to decline. At any copulation, the mating ability of irradiated males did not differ significantly from that of the control (Table I).

It is possible that in late mating the males were unable to mate because they had already depleted their supply of sperm and accessory gland secretions during the early mating [1].

The results show that both irradiated and unirradiated males were most fertile in the first mating, but then their fertility declined during the following mating. There was no significant difference in the fertility of irradiated males in the second, third and fourth matings. The fertility of these males was markedly lower than the control males, regardless of the number of matings involved (Table I).

It has been known that mature sperm are the most radioresistant stage of spermatogenesis: spermatids are less resistant and would suffer greater damage. This could explain the higher fertility in early mating than in late mating.

TABLE I. EFFECT OF REPEATED MATING ON MATING ABILITY AND FERTILITY OF IRRADIATED *Ephestia cautella* MALES (10 krad)

Parameter	Male group	Time of mating after treatment (d)			
		1	2	3	4
% mating	Control	(20/20) 100a	(20/20) 80b	(20/20) 100a	(7/20) 35c
	Irradiated	(26/26) 100a	(23/26) 88a	(19/26) 73ab	(10/26) 38c
Mean % fertility	Control	44.8a	37.5a	20.8b	13.1b
	Irradiated	19.9c	7.8d	9.7d	3.1d

Note: Numbers followed by the same letter are not significantly different.
Numbers in parentheses denote (number of mated males)/(number of tested males).

TABLE II. EFFECT OF DELAYED MATING ON MATING ABILITY AND FERTILITY OF IRRADIATED *Ephestia cautella* MALES (10 krad)

Parameter	Male group	Time of mating after treatment (d)			
		0	1	2	3
% mating	Control	(25/26) 96a	(24/28) 86a	(22/27) 81a	(17/28) 61b
	Irradiated	(23/24) 96a	(18/25) 72a	(19/25) 76a	(13/26) 50b
Mean % fertility	Control	41.1a	40.3a	33.9a	36.7a
	Irradiated	12.6b	17.6b	17.7b	11.2b

Note: Numbers followed by the same letter are not significantly different.
Numbers in parentheses denote (numbers of mated males)/(numbers of tested males).

3.2. Effect of delayed mating

When the irradiated males were paired on the same day of treatment, their mating ability was higher than at any other time. The mating ability of the control males declined steadily with age. There was no significant difference in the mating ability of irradiated and unirradiated males (Table II). Irradiation appears, therefore, to have no effect on the ability of the male to mate and only age is important in the reduction in male mating ability [2].

When irradiated males were mated at various intervals after treatment, their fertility did not change significantly. A very similar result was recorded in the control males (Table II).

Thus, the age or storage of sperm in the reproduction system for various intervals before mating did not have any effect on male fertility.

These results are very important in applying the sterile male technique against *E. cautella.*

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BIOCONTROL

(Session 6)

Chairman

S. BARBOSA
FAO

PROTEIN HYDROLYSATE COMPONENTS ATTRACTIVE TO TEPHRITIDS

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Abstract

PROTEIN HYDROLYSATE COMPONENTS ATTRACTIVE TO TEPHRITIDS.

Volatiles from protein hydrolysates have for a long time been known to attract tephritids. Many volatiles from protein hydrolysates have previously been identified, but no highly attractive materials have been determined. There have been few studies on the very low boiling components, other than ammonia. Because protein hydrolysate is more attractive to tephritids at alkaline rather than at slightly acidic conditions, vapours from Nu-Lure insect bait (NLIB) at pH4.5 and pH8.5 were examined by gas chromatography/mass spectrometry (GC/MS). Compounds identified by GC/MS did not evoke very high responses, therefore attention was focused on ammonia. Because ammonia is a gas at ambient temperatures and at atmospheric pressure, a slow release system was devised. Ammonia was tested with walnut husk flies in California and was found to be primarily attractive to the female flies.

1. INTRODUCTION

Even though volatiles from protein hydrolysates have for a long time been known to attract tephritids [1–10], attractants accounting for most of the activity have not been chemically identified, although there are indications that some components have been found to be somewhat active. The standard methods of distillation and extraction have been applied, and many compounds have been identified [4, 5, 10]. Because such distillation and extraction methods did not yield very active isolates, it was decided to look at components with very low boiling points, specifically ammonia.

It is well known that when the pH of protein hydrolysate is adjusted to higher pH values from the pH of about 4.5 at which most protein hydrolysates are received, the attractancy to tephritids is increased considerably [4, 10]. Although ammonia has been mentioned [3, 4, 7, 9, 10], no definite explanation has been given as to what compound or compounds is/are responsible for the increased attractancy as the pH is increased. No strict correlation between increased rate of ammonia release to increased attractancy has been found [7]. This paper describes some of our investigations of ammonia.

2. MATERIALS AND METHODS

The protein hydrolysate used in this study was Nu-Lure insect bait (NLIB), provided by the California Department of Food and Agriculture, arranged by R.T. Cunningham, Tropical Fruit and Vegetable Research Laboratory, Agricultural Research Service (ARS), United States Department of Agriculture (USDA), Honolulu, Hawaii.

The ammonium carbonate used, certified ACS grade, was purchased from the Fisher Scientific Company.

Cotton wicks were purchased from the Richmond Dental Cotton Company, Charlotte, North Carolina.

The gas chromatographs used were Hewlett Packard models 5830A and 5840A fitted with flame ionization detectors. The mass spectrometer used was a Finnigan MAT 4500 quadrupole fitted with a gas chromatograph. Cross-linked bonded methyl silicone columns (DB-1, 60 m $\times$ 0.32 mm i.d., 0.25 μm film, J&W Scientific, Inc.) and DB-WAX columns (same dimensions and source as the silicone columns) were used.

The isolation and chemical identification methods used were those developed for flavour studies by the group at the Western Regional Research Center, ARS, USDA, Albany, California.

The bioassay methods used were those developed at the Department of Biological Sciences, California State University, Hayward, California.

3. RESULTS AND DISCUSSION

The efficacy of ammonia as an attractant to tephritids has been debated [3, 4, 7, 9]. One of the reasons for conflicting results is perhaps that, because ammonia has a boiling point of -33.4°C at atmospheric pressure, it is very difficult to sustain an even, controllable supply of this material over a period long enough to observe insect responses accurately for days or weeks. To overcome this problem of accurate release of low boiling compounds, a slow release system was designed.

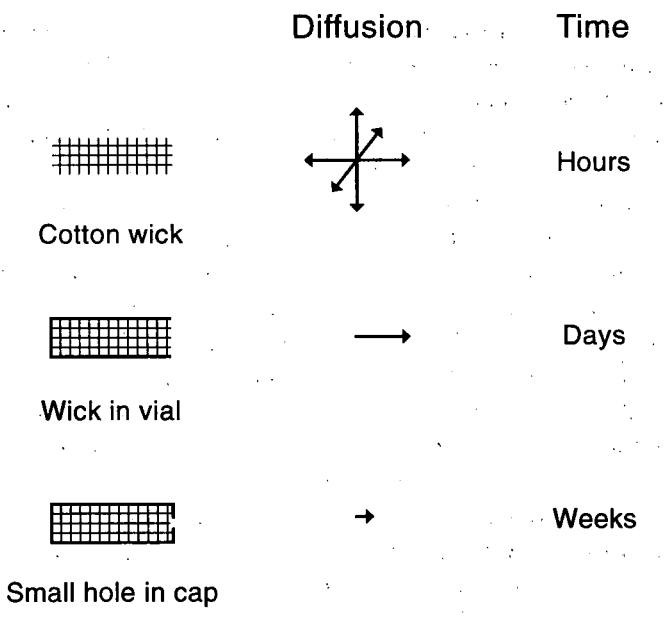

FIG. 1. Diagrams illustrating the reduction in diffusion by limiting the direction and reducing the orifice for the escape of ammonia.

In perfumery, slow release is achieved by dissolving a desired odorant in a high molecular weight substance, called a fixative. In this manner, vapour pressure is reduced, and slow evaporation is achieved. No simple fixative seems feasible for ammonia because it has such a low boiling point and is so polar.

The other principle which can be utilized involves reducing diffusion. Figure 1 illustrates diagrammatically how diffusion can be controlled. If an aqueous saturated ammonium carbonate solution is absorbed on a cotton wick, because diffusion can take place in many directions, ammonium carbonate is completely evaporated in a few hours. If the cotton wick is placed in a vial, diffusion is restricted to one direction. Vapours have only one escape path — out of the mouth of the vial. In this situation, ammonia may take a few days to dissipate. If the escape path is limited to a small hole in the cap of the vial, it may take weeks for the ammonia to evaporate completely.

Because ammonium carbonate is composed of a weak acid and a weak base, it dissociates to yield ammonia, carbon dioxide and water. One molecule of ammonium carbonate yields two molecules of ammonia.

Loss of weight due to the evaporation of ammonium carbonate is simply measured by weighing the bottle containing this material every day. If a bottle containing 30 g of ammonium carbonate, with a hole (6.25 mm) drilled in its cap, loses

0.3 g/d, this bottle will provide ammonia vapours for 100 d. To indicate the amount volatilized per second in terms of moles, the weight loss is divided by the formula weight of ammonium carbonate, and the result is the number of moles of ammonium carbonate dissociated per day. Because there are two moles of ammonia given off per mole of ammonium carbonate, the moles of ammonia emitted are double the moles of ammonium carbonate evaporated. To obtain the amount per second, one simply divides by the number of seconds in a day.

From simple experimentation and from simple calculations, the amount of ammonia given off can be accurately measured and easily calculated. With an inexpensive source available, with controlled and measurable amounts dispensed, optimum conditions for efficiency of trapping with ammonia can now be systematically approached. A report on bioassays with the walnut husk fly (*Rhagoletis completa* Cresson) is currently in preparation. However, a brief discussion of the effect of ammonia seems necessary to illustrate the importance of ammonia in attracting tephritids. The observations reported here are with the walnut husk fly.

Walnut husk flies were captured on Scentry Multigard traps baited with various lure substances during the summer of 1992 in an unsprayed English walnut orchard at the Ardenwood Historic Farm, East Bay Regional Parks, Newark, California. These were wild flies found under natural, field conditions. Four materials were used as lures: (1) bird faeces (fresh chicken droppings mixed with an equal volume of water); (2) NLIB at pH4.5; (3) NLIB at pH8.5; and (4) ammonia from powdered ammonium carbonate sprinkled on the sticky material on the traps or from ammonium carbonate dispensed with slow release systems. Water baited traps were used as controls. Each trap–lure combination was replicated five times for a total of 25 traps hung for continuous five-day periods during mid-season. Flies were removed and separated by gender from each trap daily.

Traps baited with bird faeces captured fewer flies than other materials tested and did not differ much from the water controls (Fig. 2). On the first day of observations, more than three times the number of walnut husk flies were captured on traps baited with NLIB at pH8.5 than on traps baited with NLIB at pH4.5. Moreover, twice the number of flies were captured on ammonium carbonate baited traps than on traps baited with NLIB at pH8.5. Both ammonium carbonate and NLIB at pH8.5 baited traps captured a far greater number of flies on the first day than they did on the remaining two days. This observation can be explained by the substantial decrease in ammonia concentration by the second day.

Ammonium carbonate baited traps captured significantly more female walnut husk flies than NLIB at pH4.5 or at pH8.5 (Fig. 3). Capture of females continued at a higher rate with use of systems capable of controlled slow release of ammonia versus the application of powdered ammonium carbonate (Fig. 4).

No significant differences in the capture of males were seen among the traps baited with the three substances from the first day to the end of the five trapping days. Equally low numbers of males and females were captured daily with

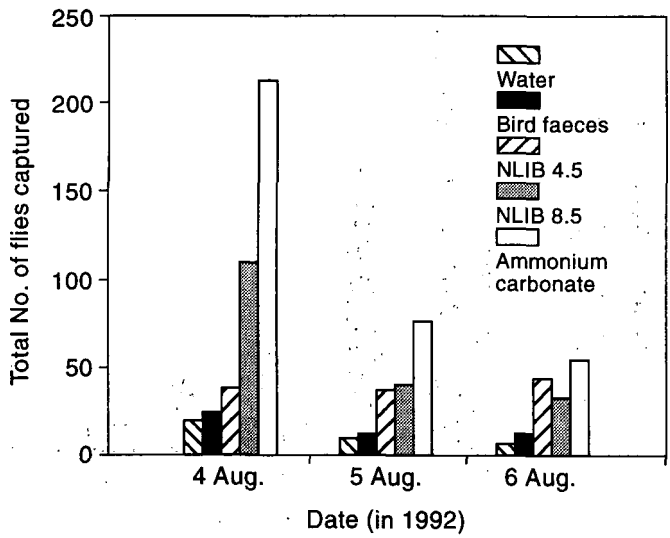

FIG. 2. Captures of walnut husk flies on yellow panel traps baited with various substances showing the rapid decline in attractiveness of ammonium carbonate and NLIB at pH8.5. Each bar represents the total number of flies captured on five replicates of each treatment.

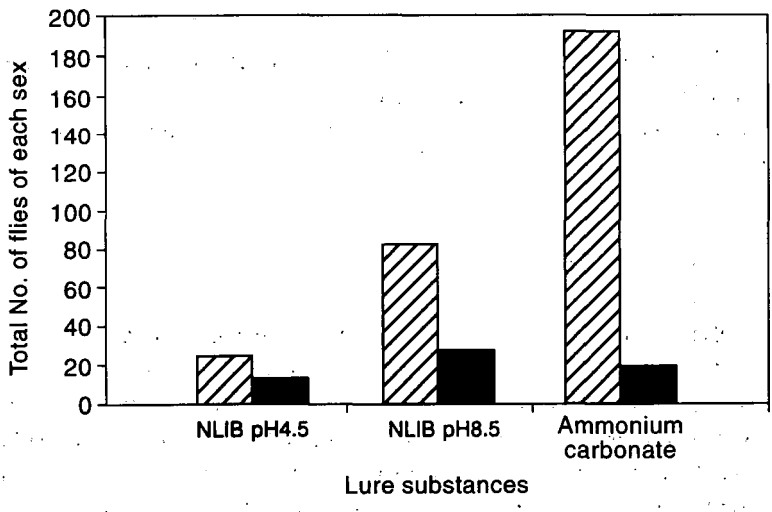

FIG. 3. First day captures of female versus male walnut husk flies on yellow panel traps baited with NLIB at pH4.5, NLIB at pH8.5 or ammonium carbonate. Each bar represents the total number of flies of each sex captured on five replicates of each treatment (▨: females; ■: males).

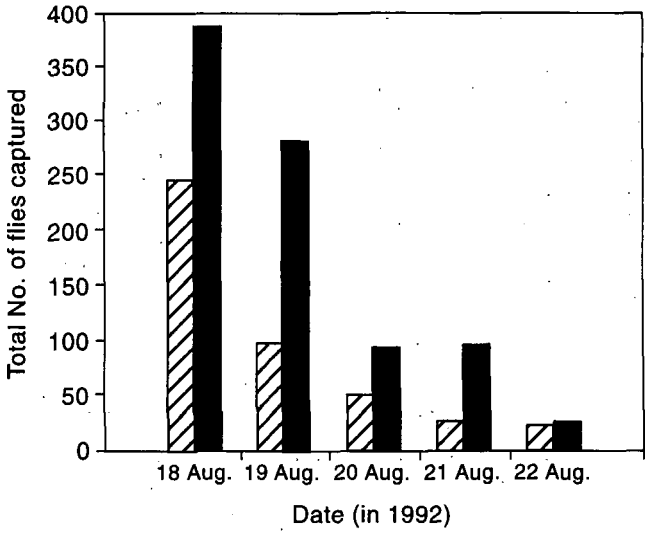

FIG. 4. Captures of walnut husk flies on yellow panel traps baited with 2 g of powdered ammonium carbonate versus a saturated solution of ammonium carbonate. Each bar represents the total number of flies captured on five replicates of each treatment (▨: ammonium carbonate powder; ■: ammonium carbonate tube).

NLIB at pH4.5. Perhaps an explanation for the low rate of capture of males with protein hydrolysate volatiles is that females need protein food to provide for the development of the eggs they carry, whereas male adults have no such urgent need for protein food.

4. SUMMARY

The efficacy of ammonia as an attractant to tephritids has been demonstrated. Ammonia from ammonium carbonate attracts more walnut husk flies than NLIB at pH8.5. The increased attractancy when NLIB is made basic may be explained by the increase in the ammonia concentration. The loss of attraction to NLIB at pH8.5 after one day may be explained by the decrease in the concentration of ammonia. Ammonia is a good attractant for females but not for males. A system for the slow release of ammonia has been devised by utilizing simple, inexpensive and commercially available chemicals and equipment to deliver controllable and easily measured amounts of ammonia for periods of weeks and months. The use of ammonia and the slow release systems can be easily applied for the survey and control of tephritids.

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ECOHORMONES FOR THE MANAGEMENT OF FRUIT FLY PESTS

Understanding plant–fruit fly–predator interrelationships

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Abstract

ECOHORMONES FOR THE MANAGEMENT OF FRUIT FLY PESTS: UNDERSTANDING PLANT–FRUIT FLY–PREDATOR INTERRELATIONSHIPS.

The flowers of several plant species of *Bulbophyllum* (Orchidaceae) and *Spathiphyllum* (Araceae) that release methyl eugenol (ME) or other phenylpropanoids, and several varieties of *Dendrobium anosmum* (Orchidaceae) that secrete raspberry ketone (RK) are attractive to fruit flies, which actively consume the ecohormone (fragrance) released. *Ocimum sanctum* stores large quantities of ME, which is released only when some part of the plant is damaged. When the male melon fly, *Bactrocera cucurbitae*, feeds on RK, the ecohormone is sequestered. Methyl eugenol, when consumed by male flies, is converted to other phenylpropanoid(s) for storage. *Bactrocera dorsalis* (taxon A) produced coniferylalcohol (CF); *B. dorsalis* (taxon B) produced three compounds: CF, 2-allyl-4,5-dimethoxyphenol (compound I) and Z-3,4-dimethoxycinnamyl alcohol (Z compound II); and *B. umbrosa* produced three compounds: compound I, E and Z compound II and 3,4-dimethoxy-hydroxyallyl benzene. Coniferyl alcohol and compound I are attractive to conspecific males, while CF attracts conspecific females and stimulates ovipositor extrusion at close range. The strong attractancy of fruit flies to certain lures can now be explained by demonstrating: (1) an anti-predation mechanism in several species of fruit fly through the endogenous production and secretion of allomone(s); and (2) chemical(s) responsible for male aggregation in a lek formation, after pharmacophagy. Compulsive feeding of ME by male flies produced a feeding deterrent effect on the house lizard, *Hemidactylus frenatus*. Lizards did not attempt to feed on fruit flies after initial exposure to flies that had fed on ME. On the other hand, sequestration of RK may not play an anti-predation role in the melon fly, but functions as a male aggregation pheromone. The anti-predation mechanism in the melon fly is the result of the endogenous synthesis of ethyl-hydroxybenzoate and 1,3-nonandiol in the male rectal gland at sexual maturity. Most mature male flies undergo reflex ejaculation of rectal gland content when they are under stress, e.g. when they are being anaesthetized with carbon dioxide or when they are being held by a feather forceps.

1. INTRODUCTION

Certain species of tephritid fruit flies are amongst the most important global pests infesting fruit and vegetable crops. In the tropics and subtropics, many species of *Bactrocera* (*Dacus*) are major, or potentially important, pests. Most *Bactrocera* species are strongly attracted to male lures, either methyl eugenol (ME) or cuelure (CL)/raspberry ketone (rheosmin/RK) [1–3]. Since the discovery in 1912 that *B. diversus* and *B. zonatus* were attracted to citronella oil [4], attempts have been made to explain the strong attractancy of fruit fly males to lures. The attractancy of Dacinae flies to, and their compulsive feeding on, certain male lures are unique characteristics of tephritid fruit flies; the reason for such a phenomenon is not fully understood [1, 3]. These lures play a central role in the chemical ecology of fruit flies. Methyl eugenol was first shown to be a strong attractant for fruit fly species in 1915 [5] and has been used successfully in monitoring and male annihilation programmes [6], and in estimating population and survival rates of native males in two species [7, 8] and fly movements between ecosystems [9]. In Malaysia, dacine fruit flies are serious pests of fruits and vegetables: two species of *B. dorsalis* complex (taxa A and B) and *B. umbrosa*, which are strongly attracted to ME, ME attracted species; *B. cucurbitae, B. caudatus* and *B. tau*, which are attracted to CL and RK, CL/RK attracted species; and *B. latifrons* (not attracted to latilure, alpha-ionol, under tropical conditions) and *B. arecae*, which are not attracted to the known lures. The biology, ecology, behaviour and control of economically important fruit fly species have been reviewed elsewhere [1–3]. In this overview, because of space limitation, emphasis is placed on the central role of fruit fly attractants, secreted as ecohormones, in understanding the complex interrelationships between plant, fruit fly (*Bactrocera* species) and predator.

2. CLASSIFICATION OF ECOHORMONES

Semiochemicals are chemicals (usually volatile) that affect the behaviour of a receptive animal at submicrogram or nanogram levels. They may be broadly divided into two main groups: ecohormones, which are released or secreted naturally into the environment, and para-ecohormones, which are animal or plant constituents not secreted naturally, or synthetic chemicals. An ecohormone with intraspecies activity is known as a pheromone. An ecohormone with interspecies activity which benefits the releaser is known as an allomone; one which benefits the receiver is a kairomone; and one which benefits both releaser and receiver species is a synomone. Therefore, an ecohormone may act as a pheromone as well as an allomone, a kairomone or a synomone. Many ecohormones involved in insect communication may be exploited as agents for the surveillance and monitoring of pests and/or pest control within an insect pest management programme.

3. PLANT–FRUIT FLY RELATIONSHIP

Plants form the focal point of the ecological activities of fruit flies, including host finding, oviposition, development, adult feeding and pharmacophagy (consumption of non-essential chemicals), courtship and mating behaviour, and protective behaviour against predators. Many of these behavioural patterns are mediated via chemical reception and some have been extensively reviewed [1–3].

3.1. Plant ecohormone and para-ecohormone

About 20 plant species from 16 families are known to contain either ME or RK; their role as kairomones in fruit fly ecology has been discussed elsewhere [3]. Here, we show that the chemicals do not function as kairomones, but actually as synomones or allomones. Several species and varieties of plants in Malaysia that contain either of the chemicals are reported on here.

Five species of *Bulbophyllum* (Orchidaceae), section Sestochilus (with rather large flowers, either solitary or in a subumbellate inflorescence), *Bu. cheiri, Bu. ecornotum, Bu. macranthum, Bu. patens* and *Bu. vinaceum,* attracted the Oriental fruit fly, *B. dorsalis,* complex species and the Atrocarpus fruit fly, *B. umbrosa,* which responds to ME [10]. The fruit fly orchid, *Bu. cheiri,* has a solitary flower containing eugenol, compound I and large amounts of ME [11] that are secreted as fragrance, acting as a synomone. The flies of ME-attracted species compulsively feed on the secreted ME and in return help in pollination [10]. In nature, a flower of a fruit fly orchid in bloom is usually covered with fruit flies. The flower stops producing the fragrance soon after pollination. It has a hinged lip, which moves in response to the fly's weight, thus helping the attracted fruit fly to remove the pollinarium and produce cross-pollination. The presence of compound I is interesting; it may function as an allomone against vertebrates (see Section 5) that can damage the flower. In the other *Bulbophyllum* species mentioned, the flowers also release a fragrance, which probably contains ME. These species are currently awaiting chemical analysis. Fruit flies may be the sole pollinator for these species of orchid.

The flowers of two species of *Spathiphyllum* (Araceae), *S. cannaefolium* and *S. commutatum*, attracted fruit flies. In Australia, floral spikes, bearing pollen, of *S. cannaefolium* have been shown to emit benzyl acetate and ME which attracted fruit flies [11]. However, the spike of the *S. cannaefolium* variety found in Malaysia contains eugenol, which is secreted [11]. Eugenol has a weak attraction for the ME attracted species. The flower fragrance attracts fruit flies and *Trigona* wasps, which help in pollination. Varietal differences in ecohormone also occur in the flowers of *Couroupita guianensis* (Lecythidaceae). A variety has been reported to contain ME [3]; however, the variety found in Penang contains eugenol (ME not detected)

that attracts a few fruit flies, which thus play a role along with other insects (*Trigona* spp.) as pollinators.

The orchid flower of *Dendrobium superbum* (synonymous with *D. Anosmum* (Orchidaceae)) has been reported to contain 4-phenyl-2-butanone [12]. Re-examination of the floral chemical constituents showed that all three varieties, one with white flowers, one with purplish flowers and one with white sepals and petals and purple lip flowers, contain 4-(4-hydroxyphenyl)-2-butanone (RK). Cuelure, when consumed through pharmacophagy by the male melon fly, is stored as RK in the rectum. If the fly feeds directly on RK, the ecohormone is sequestered [13]. Male melon flies, *B. cucurbitae*, have been observed to feed around the lip, petal and sepal of the *Dendrobium* flowers. It has not yet been observed that the melon fly acts as a pollinating agent for the orchid flower.

Healthy *Ocimum sanctum* plants, normally free from insect attack, do not attract the fruit fly, except when some part of the plant is damaged [14]. Two varieties of *O. sanctum* (Labiatae), one with a green calyx and white corolla, the other with a violet calyx and corolla, contain relatively large quantities of ME — more than 60% of the essential oil. Hence, ME, probably stored in numerous glands (well distributed on both the upper and lower leaf surfaces and having no pores, as seen under a scanning electron microscope), is released only upon tissue damage. This may be the plant's defence mechanism against foraging insects because ME is toxic to some phytophagous insects. Here, ME is a para-ecohormone that acts as an allomone-like deterrent upon release after plant damage. We believe that more varieties and species of *Ocimum* (e.g. *O. basilicum*, currently under study) with such a mechanism will be discovered.

Piper betel is normally free from insect damage. Healthy plants do not attract fruit flies. However, when a leaf is squashed in a cage containing ME attracted fruit fly species, many males are attracted. The leaf contains two phenyl propanoids — eugenol and hydroxychavicol [10]. It also contains numerous glands without pores. The chemicals act as a para-ecohormone in deterring phytophagous insects.

3.2. Plant kairomone

Female fruit flies probably locate host fruits through chemical as well as visual cues. The odour of fruit acting as a kairomone may attract female fruit flies to oviposit in the fruit, resulting in fruit damage caused by larval development. Infestation of 18 host fruit species by different fruit fly species has been reported [15]. A recent study using ripe host fruits (without ovipuncture) from 14 different species suspended under the canopy of a non-host tree in a village ecosystem showed that most female *B. dorsalis* (taxon B) flies (caught after landing) preferred to alight on banana (*Musa paradisiaca*), starfruit (*Averrhoa carambola*) and papaya (*Carica papaya*) (unpublished data from our laboratory). Female fruit flies are attracted to fruit odour, but the chemical nature of the kairomone has not been identified.

4. FRUIT FLY–FRUIT FLY RELATIONSHIP

4.1. Male response to lures

The number of *B. dorsalis* flies attracted to lure traps in the field showed two peaks in a daily cycle: a high peak between 0800 and 1000 h and a low peak between 1600 and 1800 h. Similar daily cycle peaks have been observed for *B. umbrosa*. Under subtropical conditions, different species have a different peak response in the daily cycle [3].

Conflicting data exist about the effect of age between 1 and 14 days on responsiveness to lures [3]. However, in Malaysia, all major pest species respond to lures at sexual maturity. In our wind tunnel studies, *B. dorsalis* (taxa A and B), *B. umbrosa* and *B. cucurbitae* began to respond to their respective lures after 5 d, with a high percentage of males responding at 12–14 d after adult eclosion. The response of the native male of *B. dorsalis* (taxon B) (marked after emergence) to ME traps set up in a village ecosystem showed that the first marked male trapped was 7 d old. The majority of marked males caught were between 10 and 16 d after eclosion [16].

4.2. Fruit fly pheromone

Analysis of the male fruit fly rectal gland after pharmacophagy showed that ME was converted to other phenylpropanoids for storage. *Bactrocera dorsalis* (taxon A) produced coniferyl alcohol (CF); *B. dorsalis* (taxon B) produced three compounds: CF, 2-allyl-4,5-dimethoxyphenol (compound I) and Z-3,4-dimethoxycinnamyl alcohol (Z compound II) [17]; and *B. umbrosa* produced three compounds: compound I, E and Z compound II and 3,4-dimethoxyhydroxyallyl benzene. Coniferyl alcohol and compound I are attractive to conspecific males while CF attracts conspecific females and stimulates ovipositor extrusion at close range [14]. The rectal compounds were released into the air during dusk, which coincides with the fruit fly courtship period [17]. The male rectal gland is known to be the source of the sex pheromone in fruit flies [18]. The three fruit fly species have been observed to form lek. Therefore, CF and compound I act as a male aggregation pheromone. Controversy currently exists over the possible interbreeding of taxa A and B (to be placed as separate species) flies in the wild. They were interbred in the laboratory, and intermediate morphological forms between the two were collected from the wild [19]. The ability to interbreed may be caused by the presence of CF, acting as sex pheromone, in the pheromone system of the two taxa, provided the sexes are in close proximity.

5. FRUIT FLY–PREDATOR RELATIONSHIP

It has now become possible to explain the strong attractancy of fruit flies to certain lures by demonstrating an anti-predation mechanism in several species of fruit fly through the endogenous production and secretion of an allomone after pharmacophagy. Compulsive feeding of ME by male flies produced a feeding deterrent effect on the house lizard, *Hemidactylus frenatus*. Lizards did not attempt to feed on fruit flies after initial exposure to flies that had fed on ME [20]. Some lizards would rather starve than feed on fruit flies (demonstrated in a no choice feeding experiment over a two to three week period). Lizards learn to avoid preying on fruit flies, probably through the characteristic yellow markings on the flies. It has been shown that compound I was a stronger feeding deterrent than ME against sparrows [21].

Besides functioning as a male aggregation pheromone, RK may not play an anti-predation role in *B. cucurbitae*. The anti-predation mechanism in the melon fly is achieved at sexual maturity [22], which coincides with the endogenous synthesis of ethyl-hydroxybenzoate and 1,3-nonandiol in the male rectal gland. The rectal compounds were not detected in the first week after eclosion, but they increased significantly with age, starting from two weeks old [23]. The mechanism probably involves endogenously synthesized chemicals. The chemical 1,3-nonandiol reduced the consumption of treated house flies but did not directly deter predation. It may play a role in conjunction with other components of the rectal gland [23]. Further investigation is necessary to determine the actual rectal component(s) involved as allomones in the anti-predation mechanism of the male melon fly.

Sexually mature male fruit flies congregate at dusk and produce a smoke-like substance originating from the rectum during courtship [24]. This substance, when sprayed onto the mate, may also protect females from lizards, especially during copulation, which normally lasts from dusk to dawn. The female flies may be protected through automimicry, because they resemble the males. Furthermore, both *B. latifrons* and *B. arecae* may be Batesian mimics to gain protection from predators. This is because the two species resemble *B. dorsalis* and they are much less abundant. However, their status as Batesian or Mullerian mimics is under review.

Sexually mature males have an escape mechanism. They have been observed to release droplets during escape when disturbed. Spontaneous ejaculation of rectal content has been observed in sexually mature males when under stress, e.g. when they are being anaesthetized with carbon dioxide or when they are being held by a feather forceps [20, 22]. Such behaviour may distract a potential predator and thus help the male flies to escape.

6. CONCLUSIONS

Phenylpropanoids in plants may act as ecohormones or para-ecohormones against insects. Owing to the special relationship that exists between fruit flies and

several species of *Bulbophyllum* orchids through synomonal fragrance, care should be taken in the implementation of fruit fly eradication programmes to avoid causing the extinction of the wild orchids. Plants make use of the para-hormones as a protective mechanism against phytophagous insects.

The abundance of plant species containing ME may affect the performance of the attractant in male annihilation, monitoring and surveillance techniques.

The pheromone systems of other fruit fly species should be investigated. Female attractants such as CF should be further investigated and developed for use in the management of fruit fly pests.

The male melon fly's ability to deter predation with attainment of sexual maturity shows a different anti-predation mechanism from that achieved by male *B. dorsalis* (taxon B). The latter needs to feed on ME and to convert the chemical to its analogues in the rectal gland in order to deter lizard predation. This indicates that ME attracted species may be considered more advanced than the CL/RK-attracted species, because the former spend less metabolic energy in producing allomones.

It is suggested that suppression of the anti-predation mechanism through the male annihilation technique may leave the female fruit flies unprotected against predators, which could further reduce the fruit fly population. Employing such a mechanism in an integrated fruit fly management programme warrants further investigation.

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CONTROL OF THE CHINESE CITRUS FLY, *Dacus citri* (CHEN), USING THE STERILE INSECT TECHNIQUE

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Abstract

CONTROL OF THE CHINESE CITRUS FLY, *Dacus citri* (CHEN), USING THE STERILE INSECT TECHNIQUE.

Artificial rearing of the Chinese citrus fly, *Dacus citri* (Chen), is described. It was found that the appropriate irradiation stage for sterilization treatment is at the last pupal phase, one or two days before emergence, and that 9 krad of irradiation is a suitable dose for sterilizing *D. citri*. A total of 56 000 and 95 000 irradiated sterile males of *D. citri* were released in the Zhonglian orchard (about 34 ha) in Huishui County, Guizhou Province, in 1987 and 1989, respectively. The release ratio of sterile to native fruit flies was 12.5:1 and 45:1. The percentage of oranges damaged by *D. citri* dropped from 7.5 to 0.005.

1. INTRODUCTION

It is known that *Dacus citri* (Chen) only occurs in China. This pest was first recorded in two provinces during the 1940s [1]; it was then found in four other provinces during the 1960s [2]. It occurs in all the citrus producing regions in China (except for Zhejiang and Guandong Provinces), ranging climatically from temperate to subtropical to tropical zones. *Dacus citri* is highly injurious to sweet orange and other citrus species, even though it produces only one generation per year. In some orchards, infestation of fruits by this pest has been estimated to amount to more than 50% [3]. During the 1950s, infestation in some counties in Sichen Province was higher than 80%. On the basis of a recent report from the Huaihua Plant Station in Hunan Province, infestation in some areas is still as high as 80%. In 1987, the infestation in Bangong township, Lodian County, Guizhou Province, was 22%; in one of these orchards it rose to 100%.

Over the last decade, China has made great economic progress. The living standards and purchasing power have increased and the demand for vegetables and fruits has become greater. To meet this increasing demand, large areas of orchard have been cultivated, especially for orange production. The area covered by citrus

orchards rose to 1 million hectares in 1989 and the annual output of citrus fruit increased to 4.56 million tonnes, from 0.9 million tonnes in 1980. Following the expansion of citrus orchards, great efforts have been made to protect citrus fruit and the damage caused by *D. citri* has been reduced. However, the losses caused by *D. citri* are still heavy. Therefore, it is necessary to find new ways of coping with *D. citri*.

2. MATERIALS AND METHODS

2.1. Artificial rearing

Wild pupae collected from Lodian County, Guizhou Province, southwest China, were placed in sand, soil or sawdust. The influence of temperature, the water content of the sand, etc., on the survival and emergence rates of the overwintered pupae were measured.

Paired adults were fed on a complementary nutrient and water in a glass jar; cellophane and an agar ball covered with gauze were also added for oviposition.

Incubation of the eggs was carried out in culture dishes. The eggs were placed on the filter paper, which was saturated with physiological saline.

The artificial larval diet put in the container (a diameter of 28 cm and a height of 15 cm) had a thickness of 2 cm for rearing.

2.2. Effect of radiation on the sterility of *D. citri*

One or two days before emergence the pupae were irradiated with 6, 9, 12 and 15 krad of gamma irradiation at a dose rate of 89.5 rad/min.[1]

The irradiated adults were placed in cages as mating crosses of S♀ × S♂, S♀ × N♂, S♂ × N♀ and N♀ × N♂. Each irradiation treatment was replicated four or five times and each cage contained five to ten pairs of adults covered with a citrus tree branch.

The mating time, lifespan and damage done to the citrus fruits, etc., were recorded.

2.3. Release test

The release of irradiated sterile Chinese citrus flies was conducted in the Zhonglian orange orchard in Huishui County, Guizhou Province, southwest China, which covers about 34 ha and has 4800 orange trees. The orange orchard is isolated

[1] 1 rad = 1.00×10^{-2} Gy.

by the surrounding hills, where there are neither citrus trees nor wild hosts of *D. citri* such as trifoliate orange.

The irradiated sterile pupae were mixed with fluorescent powder in containers and then placed in the cages. After the female adults were removed, the irradiated sterile male adults labelled with fluorescent powder were released. Six to ten sites evenly distributed in the orchard were chosen as the releasing locations. Traps, baited with a solution of brown sugar, hydrolytic protein and brewer's yeast, were used to capture the wild adults and recapture the released, labelled, sterile adults of *D. citri*.

3. RESULTS AND DISCUSSION

3.1. Artificial rearing

The survival and emergence rates of the overwintered pupae were 92.66 and 78.10% in the sand compared with 82.64 and 75.90% in the soil and 0 and 0% in the sawdust, respectively. Therefore, fine sand was considered to be a favourable substance for preserving the pupae. This also proved that the 10–15% water content in the fine sand was appropriate, and that the temperature should not drop below 0°C for several days.

The peak of emergence was at about 12–14 h. Mating occurred at least by day 7 after emergence, days 12–17 on average, and was most concentrated at 12–14 h under 100–1000 lux of illumination.

Dacus citri is a polygamous fly. Under single pair rearing of a total of 55 pairs, 60% mated once, 34.55% mated twice and 5.54% mated three times. The preoviposition period ranged between 23 and 69 d, most often 33–42 d. On average, the female laid 81.6 eggs three times within 10 d.

The best adult complementary nutrient was soybean powder + brown sugar + brewer's yeast.

Most of the females laid eggs on the surface of the glass jar; only a few eggs were laid on the cellophane and the gauze net covering the jar or the agar ball. Most of the eggs needed 40–60 d to complete development.

The artificial larval diet was selected from 31 formulas. Its ingredients were the powder of wheat germ, the powder of carrot, sucrose, wheat bran and brewer's yeast, juice of the green orange, agar, citric acid, methylparaben, etc. The percentage pupae obtained was 80.14. The larvae stage was about 66–79 d. Mature larvae of a lustrous light yellow colour pupated in the fine sand or soil.

3.2. Effect of radiation on sterility

A series of tests indicated that there was no significant influence of the radiation treatments (6–15 krad) on the emergence rate and lifespan of the adults (Tables I

TABLE I. INFLUENCES OF DIFFERENT RADIATION DOSES ON THE MATING RATE OF THE CHINESE CITRUS FLY, *D. citri*

Treatment (krad)	Crosses[a]	Mating rates (%)[b]		
		1985	1986	Average
6	S♂ × N♀	395	420	408**
	N♂ × S♀	310	300	305*
	S♂ × S♀	330	315	323*
9	S♂ × N♀	324	305	315*
	N♂ × S♀	310	315	313*
	S♂ × S♀	330	375	353**
12	S♂ × N♀	208	105	157
	N♂ × S♀	200	207	204
	S♂ × S♀	110	100	105*
15	S♂ × N♀	132	120	126
	N♂ × S♀	180	253	217
	S♂ × S♀	100	93	97*
Control	S♂ × N♀	216	198	207

[a] S: sterile; N: normal.

[b] ** and * denote significantly different at P = 0.01 and P = 0.05 (LSD), respectively, compared with the control.

TABLE II. INFLUENCE OF DIFFERENT RADIATION DOSES ON THE LIFESPANS OF CHINESE CITRUS FLY ADULTS[a]

Treatment (krad)	Lifespan of males (d)			Lifespan of females (d)		
	1985	1986	Average	1985	1986	Average
6	35.67	45.96	40.82 A	43.71	50.42	48.87 A
9	38.00	49.38	43.69 A	45.83	50.78	48.32 A
12	37.87	42.51	40.19 A	42.33	43.31	42.82 A
15	38.71	43.29	41.00 A	44.77	45.12	44.95 A
Control	35.42	44.91	40.17 A	32.95	45.73	39.34 A

[a] Means followed by the same letter are not significantly different at P = 0.01, LSD.

TABLE III. MATING TIMES OF IRRADIATED CHINESE CITRUS FLIES

Treatment and crosses	0800	0900	1000	1100	1200	1300	1400	1500	1600	1700	1800	1900	2000	*Total*
6 krad														
S♀ × N♂		2	6	10	18	11	17	15	22	8	7	4		120
N♀ × S♂	2		9	20	17	20	21	22	10	24	15	6	2	168
S♀ × S♂			6	5	18	16	17	14	16	18	9	6	1	126
9 krad														
S♀ × N♂		2	7	8	13	13	11	19	20	13	13	6	1	126
N♀ × S♂		1	6	8	12	14	10	18	17	15	13	7	1	122
S♀ × S♂		2	5	11	21	15	15	19	16	21	15	7	3	152
12 krad														
S♀ × N♂		2	4	8	16	8	8	8	3	3				60
N♀ × S♂			2	3	2	4	6	9	6	5	5			42
S♀ × S♂		2			7	5	3	5	3	3	2			30
15 krad														
S♀ × N♂		1	6	6	10	13	14	18	14	9	8	2		101
N♀ × S♂			3	5	3	11	7	12	3	2	2			48
S♀ × S♂				4	8	5	4	5	2	1	7		1	37
Control		1	2	7	7	13	9	12	7	10	7	3	1	79

TABLE IV. RELATION BETWEEN THE IRRADIATION DOSES AND THE CONTROL EFFECTS

Treatment and crosses	Total No. of fruits	Total No. of pierced fruits	No. of pierced fruits dissected	Dissected fruits		No. of damaged fruits dissected	No. of damaged fruits checked	Damage rate (%)
				No. of eggs	No. of larvae			
6 krad								
S♀ × N♂	31	17	12	13	6	7	9.92	32.00
N♀ × S♂	41	20	15	4		5	6.67	16.27
S♀ × S♂	29	23	15	2		1	1.53	5.28
9 krad								
S♀ × N♂	42	24	17			0	0	0
N♀ × S♂	28	9	6			0	0	0
S♀ × S♂	23	15	12			0	0	0
12 krad								
S♀ × N♂	13	8	5			0	0	0
N♀ × S♂	22	13	12			0	0	0
S♀ × S♂	16	3	2			0	0	0
15 krad								
S♀ × N♂	23	8	6			0	0	0
N♀ × S♂	27	15	9			0	0	0
S♀ × S♂	7	0	0			0	0	0
Control	34	17	12	1	70	9	12.75	37.50

Note: (1) The number of damaged fruits dissected is the number of fruits containing eggs and larvae from the dissected fruits.

(2) The number of damaged fruits checked is the number of damaged fruits dissected times the total number of pierced fruits divided by the number of pierced fruits dissected.

and II), and that the mating time of the irradiated *D. citri* was not changed (Table III). However, for both males and females treated with 6 and 9 krad, the mating rate increased significantly. Some control occurred at 6 krad, but complete control did not occur in the mating crosses until 9 krad was applied (Table IV).

Therefore, 9 krad of irradiation (for pupae irradiated one or two days before emergence) was determined as the appropriate sterile irradiation dose and irradiation phase [4].

3.3. Releasing test

Using the labelling and recapture method, the longest dispersal distance of *D. citri* was found to be about 1500 m in a favourable wind direction. It appears that movement and transportation of damaged citrus fruits containing the larvae of *D. citri* are the main ways of spreading infestation.

From the number of wild and treated adults of *D. citri* captured at different sites in the orange orchard and the times at which they were captured, it was deduced that *D. citri* prefers flying and is active in a breeze. Also, it was proved that irradiated sterile adult *D. citri* still retained the behaviour of native populations in the field.

A total of 56 000 and 95 000 sterile males irradiated with 9 krad were released at a ratio of 12.5:1 and 45:1 (sterile: native fruit flies) in 1987 and 1989, respectively. The percentage of oranges damaged by *D. citri* dropped from 7.5 to 0.005 during the same period [5].

In 1989, about 48 000 irradiated sterile female *D. citri* removed from the Zhonglian orange orchard were released in another small orange orchard, about 1 ha. The percentage of damaged oranges dropped from 0.55 to 0.003. Although the release ratio was not obtained, it appeared that release of irradiated sterile females was effective, although many of the orange fruits were scarred because the females still produced oviposition marks.

Because preliminary control of *D. citri* using the sterile insect technique has been successful, a larger release programme is to be carried out in all orange orchards with about 100 000 orange trees throughout Huishui County during the next five years.

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Bacillus thuringiensis ENDOTOXINS ACTIVE AGAINST *Chilo partellus* AND *Glossina morsitans morsitans*

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Abstract

Bacillus thuringiensis ENDOTOXINS ACTIVE AGAINST *Chilo partellus* AND *Glossina morsitans morsitans.*

Bacillus thuringiensis crystal endotoxins were isolated by centrifugation on linear sucrose gradients. Analysis of the crystals by gel electrophoresis revealed that the major component of the *Chilo partellus* active crystal endotoxin was a protein of M_r ~ 130 kilodalton. The *Glossina morsitans morsitans* active crystal endotoxin gave a major protein band of M_r ~ 120 kilodalton. Upon solubilization under alkaline pH and reducing conditions, the *C. partellus* and *G. m. morsitans* crystal endotoxin yielded protoxins of M_r ~ 63 and M_r ~ 64 kilodalton, respectively. Activation of the *C. partellus* protoxin with bovine trypsin resulted in no apparent change in the molecular weight. However, treatment with bovine chymotrypsin or *C. partellus* midgut homogenate resulted in a shift in the molecular weight of the protoxin to a toxin of M_r ~ 60 kilodalton. Similarly, treatment of *G. m. morsitans* protoxin with bovine trypsin gave a toxin of M_r ~ 62 kilodalton, but bovine chymotrypsin gave a toxin of M_r ~ 60 kilodalton. Staining with periodic acid Schiff reagent revealed that both the crystal endotoxins were glycosylated. The carbohydrate moieties were of the high mannose type, as shown by staining with fluorescein isothiocyanate conjugated-concanavalin A. Rabbit antibodies against *C. partellus* protoxin cross-reacted with the *G. m. morsitans* toxin.

1. INTRODUCTION

The use of synthetic chemical pesticides for the control of insect pests and disease vectors poses a great risk to human health and the environment [1]. Further, selected mortality of the more susceptible genotypes following repeated application of insecticides has resulted in the rapid development of resistance among several insect pests and disease vectors [2]. Consequently, the last half of this century has seen efforts to develop alternative pest management strategies that are non-polluting and environmentally benign. One such strategy that has attracted much interest involves use of the naturally occurring bacterium *Bacillus thuringiensis* (*Bt*), as a biopesticide. *Bacillus thuringiensis* (Berliner) is a gram positive soil bacterium that

synthesizes a parasporal, proteinaceous, crystalline inclusion during the sporulation cycle [3]. These inclusions consist of proteins known as delta-endotoxins or insecticidal crystal proteins (ICPs). Upon ingestion by a susceptible insect, the crystals are solubilized in the insect midgut to release the ICPs. The ICPs are then activated by midgut proteases to form the toxins. The toxins then interact with midgut epithelium, disrupting membrane integrity and leading ultimately to insect death [4].

Although much work has been reported on the use of *Bt* as a biopesticide, there is still little information on the molecular basis of the insecticidal properties of these toxins. Of particular interest also is the understanding of the molecular basis for selective toxicity of the toxins.

This paper reports on the physical–chemical properties of two *Bt* strains active against the economically important crop pest *Chilo partellus* and the disease vector *Glossina morsitans morsitans*. Understanding the properties of these endotoxins is considered an essential first step in elucidating their mode of action.

2. MATERIALS AND METHODS

2.1. Experimental insects and bacteria

Adult tsetse flies (*G. m. morsitans* Westwood) and third instar *C. partellus* larvae were supplied by the Insect and Animal Breeding Unit of the International Centre of Insect Physiology and Ecology (ICIPE).

Bacillus thuringiensis strain MF4B/2 was obtained from the ICIPE Microbial Bank. This strain was originally isolated in Kenya and found to be active against *C. partellus* [5]. The *Bt* (*Tikki*) strain active against adult *G. m. morsitans* was isolated from dead or dying flies at the Kenya Veterinary Research Laboratories, Kabete [6]. Strain MF4B/2 was grown in a modified nutrient broth medium [7]. The *Tikki* strain was grown in nutrient broth (Oxoid Ltd, Basingstoke, Hants, United Kingdom). After a starter culture (220 rev./min, 28°C, 24 h), the culture was aliquoted into media and incubated in a shaker incubator (220 rev./min, 28°C, 72 h).

2.2. Isolation of the endotoxin crystals

The MF4B/2 strain crystals were isolated as follows. The spores, crystals and cell debris were pelleted from a 72-h culture by centrifugation (10 000g, 10 min, 4°C). The resulting pellet was first washed with distilled water (× 3), layered on to a linear sucrose gradient (40–70%) and then centrifuged (8000g, 1 h, 15°C) in a Beckman Model L5-50 ultracentrifuge. The resulting interphases were washed free of sucrose by centrifugation (5000g, 20 min, 4°C) using distilled water. The interphase samples were checked for crystals microscopically after staining with Smirnoff

stain [8]. The interphase with the most crystals was layered on to a linear sucrose gradient (40–70%) and centrifuged (7000g, 1 h, 15°C) as described above. The resulting single interphase from this step was washed with water and stored at −20°C.

The relatively large *Tikki* crystals were isolated by single step, low speed centrifugation (1100g, 10 min, 4°C) of the 72-h culture. The crystals that settled at the bottom of the tube were then washed (× 3) with distilled water and stored at 4°C.

2.3. Solubilization and activation of the endotoxin crystals

The MF4B/2 crystals were dispersed in 50mM Na_2CO_3.HCl (pH10.5) containing 20mM dithithreitol (DTT) and incubated (3 h, 37°C). After incubation, the suspension was centrifuged (10 000g, 10 min, 4°C) in a Minifuge. The resulting supernatant and the pellet fractions were stored at −20°C. The *Tikki* crystals were solubilized in 50mM Na_2CO_3.HCl (pH9.5) containing 10mM DTT for 30 h at 37°C. After incubation, the suspension was centrifuged (10 000g, 10 min, 4°C). The supernatant fraction and the pellet fraction were stored at −20°C.

2.4. Enzymatic activation

The supernatant fraction resulting from solubilization of the MF4B/2 crystals was extensively dialysed against 0.1M Tris–HCl buffer, pH8.5, and incubated with commercial bovine trypsin (5:1, wt/wt) or bovine chymotrypsin (5:1, wt/wt) at 37°C for 1 h. After incubation, the mixture was centrifuged (10 000g, 10 min, 4°C) in a Microfuge. The same procedure was repeated using the solubilized *Tikki* protoxin.

2.5. Protein determination

Protein estimation was carried out using the Pierce Bicinchoninic acid (BCA) protein assay method. The absorbances were measured at 562 nm using a Model DU-50 Beckman spectrophotometer. Bovine serum albumin (BSA) was used as the protein standard.

2.6. Polyacrylamide gel electrophoresis

Electrophoresis on denaturing polyacrylamide gels was carried out according to Laemmli [9]. Gradients were cast using a BRL gradient maker. The low and high molecular weight standards were obtained from Bio-Rad. The gels were stained for protein with Coomassie Brilliant blue in 50% methanol/9% acetic acid and destained in 50% methanol/9% acetic acid.

2.7. Carbohydrate analysis

The presence of carbohydrate moieties on the MF4B/2 and *Tikki* endotoxins was determined using the periodate Schiff stain [10] and fluorescein isothiocyanate conjugated-concanavalin A (FITC-Con A) [11]. In the latter case, the protein samples were transferred electrophoretically on to nitrocellulose paper using the Nova-Blot system (Pharmacia). The nitrocellulose paper was then soaked in concanavalin A buffer (50mM NaCl, 50mM Tris-HCl, 1mM $CaCl_2$, 1mM $MgCl_2$, pH7.0) prior to staining with FITC-Con A.

2.8. Production of antibodies and immunological procedures

The toxin samples (~ 1.0 mg) were emulsified in complete Freund's adjuvant and injected subcutaneously at several sites into a New Zealand rabbit. A booster injection (~0.5 mg) in Freund's incomplete adjuvant was administered intramuscularly four weeks later. After a further two weeks, the rabbit was bled through the main ear artery and the serum prepared as previously described [12].

Double radial immunodiffusion [13] was performed on glass plates using 1% agarose in phosphate buffered saline (PBS: 0.2M NaCl, 0.02M sodium phosphate, 0.02% NaN_3, pH6.7). The samples were allowed to diffuse for 24 h at room temperature in a moist chamber. The plates were then extensively washed in PBS to remove the unreacted proteins and then dried using filter paper. Staining for protein was carried out using Coomasie Brilliant blue, as described in Section 2.6.

3. RESULTS

3.1. Isolation and properties of the endotoxin crystals

The *C. partellus* (MF4B/2) active endotoxin crystals were separated from the spores and cell debris by centrifugation in a linear sucrose gradient. The crystals formed a distinct band in the lower half of the centrifuge tube. The cell debris formed two bands in the upper half of the tube, while the spores formed a pellet at the bottom of the tube. The purity of the crystals obtained by this procedure was ascertained by Smirnoff staining. In the case of the *G. m. morsitans* (*Tikki*) active crystals, separation was achieved by a simple procedure involving centrifugation of the crystal, spore and cell debris mixture at a low speed. Under these conditions, the large *Tikki* crystals settled at the bottom of the tube, leaving the spores and cell debris in the supernatant fraction.

The MF4B/2 crystals were analysed by SDS-polyacrylamide gel electrophoresis (PAGE) and found to contain only one major polypeptide of M_r ~ 130 kilodalton (Fig. 1). The crystal endotoxin could be solubilized under

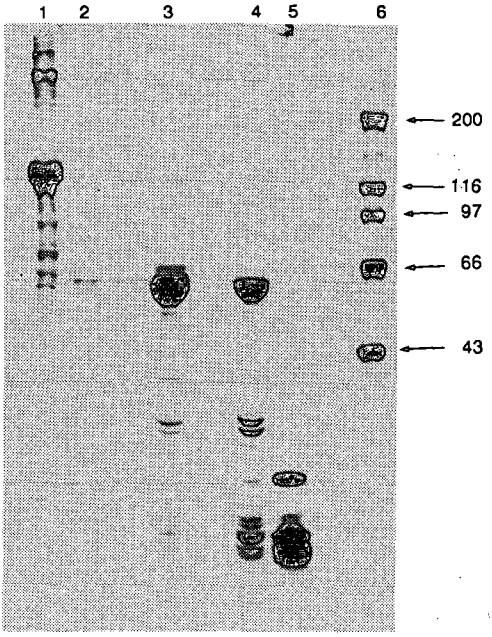

FIG. 1. Polyacrylamide gel electrophoresis of MF4B/2 crystal proteins. The protein samples were separated by dissociating gradient PAGE (4–15%), as described in Section 2. (1) Native crystal; (2) insoluble crystal (pellet); (3) solubilized crystal (protoxin); (4) toxin (trypsin treated protoxin); (5) bovine trypsin; and (6) molecular weight markers (Bio-Rad) (in kilodalton).

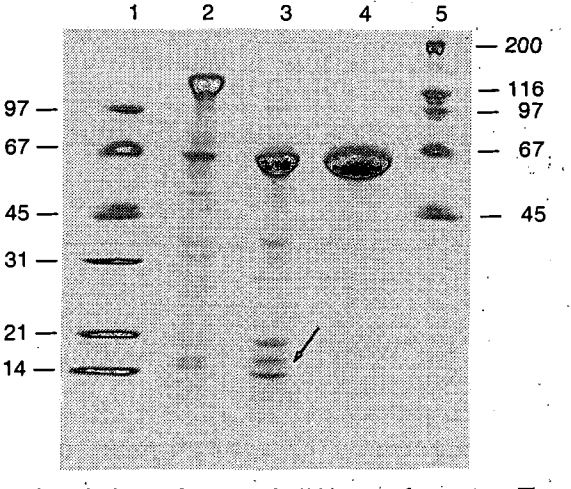

FIG. 2. Polyacrylamide gel electrophoresis of Tikki *crystal proteins. The protein samples were separated by gradient SDS–PAGE, as described in Section 2. (1) Low molecular weight markers; (2) native crystal; (3) insoluble crystal (pellet); (4) solubilized crystal (protoxin); and (5) high molecular weight markers (Bio-Rad) (in kilodalton).*

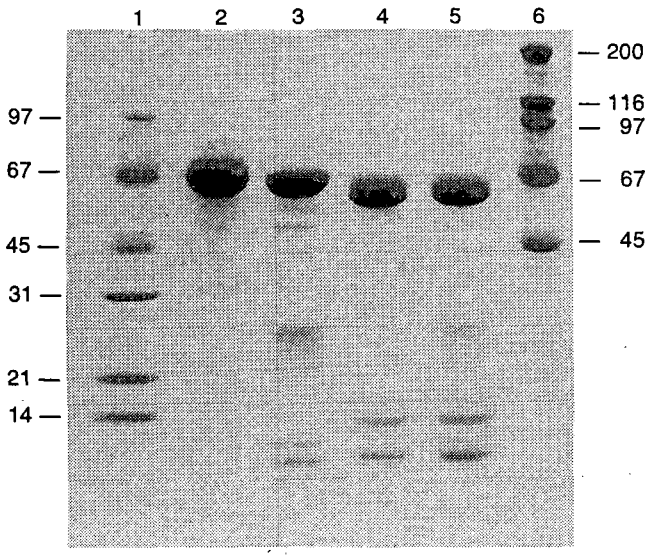

FIG. 3. Polyacrylamide gel electrophoresis showing activation of the Tikki *protoxin. The protein samples were separated by gradient SDS–PAGE, as described in Section 2. (1) Low molecular weight markers (Bio-Rad); (2) protoxin; (3) toxin (trypsin treated protoxin); (4) toxin (chymotrypsin treated protoxin); (5) toxin (chymotrypsin + trysin treated protoxin); and (6) high molecular weight markers (in kilodalton).*

high pH and reducing conditions to form the protoxin. Analysis by SDS–PAGE showed the MF4B/2 protoxin to be composed of a single major protein band (M_r ~ 63 kilodalton) (Fig. 1). Similarly, analysis of the *Tikki* crystals by SDS–PAGE gave only one major polypeptide band (M_r ~ 120 kilodalton) (Fig. 2). However, compared with the MF4B/2 crystals, the *Tikki* crystals required a longer solubilization time. The protoxin formed by this procedure had a molecular weight of M_r ~ 64 kilodalton (Fig. 2).

Enzymatic activation of the MF4B/2 and *Tikki* protoxins to the toxins gave interesting results. Treatment of the MF4B/2 protoxin with bovine trypsin resulted in no apparent change in the molecular weight of the protoxin (Fig. 1). Treatment with either *G. m. morsitans* or *Spodoptera exempta* midgut homogenates also gave similar results (data not shown). However, when the protoxin was treated with either commercial chymotrypsin or *C. partellus* midgut homogenate, a toxin of M_r ~ 60 kilodalton was formed (data not shown).

In the case of *Tikki* protoxin (M_r ~ 64 kilodalton), activation with commercial bovine trypsin gave a toxin of M_r ~ 62 kilodalton (Fig. 3). On the other hand, commercial bovine chymotrypsin or a mixture of trypsin and chymotrypsin gave a toxin of M_r ~ 60 kilodalton (Fig. 3). Similarly, *G. m. morsitans* midgut homogenate also gave a toxin with M_r ~ 60 kilodalton (data not shown).

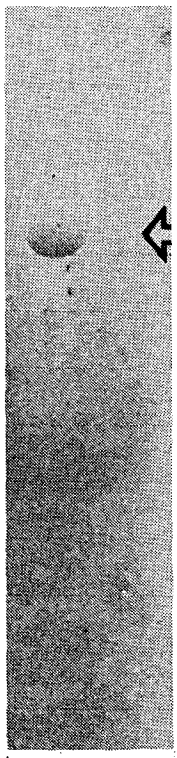

FIG. 4. Staining for carbohydrates. The protein samples were separated by gradient SDS–PAGE and the gel stained using the PAS stain, as described in Section 2. The arrow indicates the position of the protoxin band.

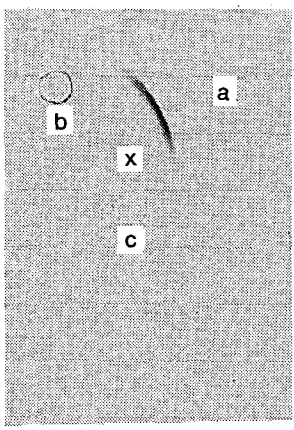

FIG. 5. Double radial immunodiffusion. The centre well had the antibodies against the MF4B/2 protoxin (x), while the peripheral wells had the following samples: (a) MF4B/2 protoxin; (b) Tikki *protoxin; and (c)* Ae. aegypti *active* Bt *protoxin.*

3.2. Carbohydrate moieties of the endotoxins

The presence of carbohydrate moieties on the MF4B/2 protoxin was tested by staining the SDS–PAGE gels with the PAS stain. As shown in Fig. 4, the protoxin reacted with the stain. The same result was obtained with the *Tikki* protoxin. Further, FITC–Con A also reacted with both the MF4B/2 and *Tikki* protoxins (data not shown).

3.3. Immunological studies

Antibodies to both the MF4B/2 and *Tikki* protoxins were prepared in rabbits. In double radial immunodiffusion studies, the antibodies against the MF4B/2 protoxin showed very slight cross-reactivity with the *Tikki* protoxin (Fig. 5). However, no cross-reactivity was detected with protoxin from another *Bt* strain active against *Aedes aegypti* (Fig. 5). In a related experiment, antibodies against the *Tikki* protoxin cross-reacted strongly with the protoxin of MF4B/2 (data not shown).

4. DISCUSSION

The lepidopteran stalk borer, *C. partellus*, is an important pest of maize and sorghum in many parts of the tropics. Infestations usually result in severe crop losses. Similarly, tsetse flies are the vectors of trypanosomes, the causative agents of African trypanosomiasis [14]. Current control measures for both *C. partellus* and *G. m. morsitans* rely largely on a number of chemical insecticides available in a variety of formulations. However, many of these compounds are also toxic to a wide range of insects and non-target organisms such as ants, spiders and parasitoids, as well as some predatory birds, which are of great value in regulating pest populations. An alternative to chemicals is use of naturally occurring pathogens for pest control. The most useful organisms to date are a group of toxin producing bacteria, *Bt* [15]. An attractive set of virtues of *Bt* is that it is readily degraded in the environment, has no harmful effects on non-target organisms and, so far, only one or two cases of resistance in the field have been reported [16]. Recent work at the ICIPE led to the isolation of several *Bt* strains, two of which are active against *C. partellus* and *G. m. morsitans* [5]. Development of these strains for field application is currently under way.

A major drawback in the use of *Bt* as a biopesticide is the rather narrow range of hosts susceptible to a particular isolate. To promote the widespread use of *Bt*, it would be important to increase the host range, possibly by genetic manipulations. However, before this can be achieved, it is essential that there should be a clear understanding of the molecular basis for selective toxicity and of the genetic basis for toxin production. This understanding could also provide a means of tailoring toxins of high potency for specific pests. The two strains mentioned above constitute

the basis for such studies in the project we are currently undertaking. This paper presents some initial findings on the biochemical characterization of the endotoxins derived from the two strains.

Although several procedures of isolating *Bt* crystals have been reported [17, 18], a linear sucrose gradient was found to be most suitable for isolating the MF4B/2 crystals. Over 90% of the crystals formed a discrete interphase in the lower half of the centrifuge tube. This was in contrast to the relatively large *Tikki* crystals, which had a higher density than that of the spores and consequently settled to the bottom of the tube during low speed centrifugation. These observations indicated that for each strain a specific procedure for isolating crystals has to be established. The freshly prepared MF4B/2 and *Tikki* crystals yielded a single major polypeptide band on SDS-PAGE, with other minor bands occasionally appearing. The intensity and number of these minor bands increased with storage of the crystals, probably due to endogenous proteases associated with the crystals. Solubilization converted the crystals into protoxins of $M_r \sim 63$ kilodalton in both cases. Compared with the *Tikki* crystals, the MF4B/2 crystals were more completely solubilized under high pH conditions and in the presence of a reducing agent. Very little solubilization was achieved in the absence of the reducing agent in both cases. Enzymatic conversion of the protoxins into the toxins gave interesting results. Compared with the *Tikki* protoxin, treatment with trypsin did not appear to alter appreciably the molecular weight of the MF4B/2 protoxin. Further, chymotrypsin caused a shift in the molecular weight of the MF4B/2 and *Tikki* protoxins. In the case of MF4B/2, chymotrypsin gave results similar to those of *C. partellus* midgut homogenate.

Many *Bt* toxins have been reported to be glycosylated [4]. In this study, both the MF4B/2 and *Tikki* protoxins were shown to be glycosylated by a general carbohydrate stain. Furthermore, both proteins reacted with the lectin concanavalin A, suggesting the presence of N linked high mannose carbohydrate chains. However, it is possible that the O linked glycosyl residues are also present on the protoxins. The exact role of the carbohydrate moiety on these toxins remains unknown. However, it has been proposed that they may play a role in insect toxicity [19]. Although MF4B/2 and *Tikki* had many similarities, they appeared to be immunologically distinct. However, it should be noted that the immunological relationship between the protoxins was tested using the relatively insensitive technique of immunodiffusion. It will be interesting to repeat the tests using the more sensitive technique of immunoblotting. Other studies aimed at characterizing these two *Bt* toxins more fully are under way.

ACKNOWLEDGEMENTS

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NATURAL FACTORS AS POTENTIAL INSECTICIDES

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Abstract

NATURAL FACTORS AS POTENTIAL INSECTICIDES.

In order to reduce environmental pollution, it is of great interest to find alternative methods for controlling insect pests. With the progress made in the isolation and identification of peptides and endogenous toxins from insects, the question can be raised whether or not these natural factors are potentially useful as insecticides. The aim of the present study was to test certain toxins and myotropic peptides isolated from insects for their usefulness as insecticides. Over twenty neuropeptides isolated from different insect species have been isolated and identified in the laboratory. To date, tests have been carried out on the influence of four neuropeptides on the food intake of L_1 larvae of *Mamestra brassicae*. Also, the crude haemolymph, as well as some of the purified fractions of the Colorado potato beetle, have been tested. At least one of the neuropeptides and some of the compounds present in the haemolymph and the purified fraction D have a negative influence on the development of L_1 larvae of *M. brassicae* after oral intake.

1. INTRODUCTION

To counteract the progressing resistance towards some commercial insecticides and to lower the environmental pollution caused by them, efficient and environmentally safe methods have to be developed continuously. The use of insect growth regulators designed from juvenile hormone was a first success in this respect. Especially in recent years, our knowledge of the endocrine system of insects has increased tremendously, particularly as the result of the identification of rather a large number of neuropeptides. Furthermore, progress has also been made in the isolation and identification of endogenous toxins from insects. The question can be raised whether or not peptides are potentially useful as insecticides. For the time being, large scale synthesis of peptides is far too expensive a process to be commercially viable for the production of insecticides. In addition, investigations on whether such peptides are active upon oral uptake still have to be carried out. However, the use of these peptides and protein toxins, provided they result in lethal effects following oral

uptake, might perhaps be realized by generating genetically transformed plants into which genes coding for peptides/proteins have been introduced.

Notable results have been attained with the insect toxins produced by *Bacillus thuringiensis*. The insecticidal activity resides in crystalline inclusion bodies produced during sporulation of the bacteria, which are composed of proteins specifically toxic against a variety of insects. Different strains of *B. thuringiensis* differ in their spectra of insecticidal activity. Most are active against Lepidoptera, but some strains specific to Diptera and Coleoptera have been identified.

The feasibility of engineering plants that defend themselves against lepidopteran insects that are sensitive to the *B. thuringiensis* Berliner insect toxin has been shown by Vaeck et al. [1]. However, some species belonging to the Noctuidae, such as *Heliothis* and *Spodoptera*, an important group of pest insects, are less sensitive to common strains of *B. thuringiensis*. In this regard, it might be essential to find other natural factors that cause lethal effects following oral intake. For transformation, however, the problem is to find out which proteins or peptide genes are also suitable for transfection into plants.

The aim of the first phase of our study was to identify peptides and proteins which, upon ingestion, cause lethal effects in a relatively short period.

2. TESTED PRODUCTS

2.1. Neuropeptides

In our laboratory, over twenty new insect neuropeptides were isolated and fully sequenced. Then they were synthesized. So far, we have tested four such synthetic peptides that were originally isolated from *Locusta migratoria* and *Sarcophaga bullata*. During isolation, the biological activity was monitored by observing the effect of fractions on changes in the frequency or amplitude of spontaneous contractions of the cockroach (*Leucophaea maderae*) proctodeum (hindgut). This bioassay is an excellent detection method for isolating peptides from different insect species.

Three of the four peptides tested showed myotropic effects. The one isolated from head extracts of *Sarcophaga* was a myoinhibiting peptide. Their amino acid sequences were:

(1) Thr-Asp-Val-Asp-His-Val-Phe-Leu-Arg-Phe-NH_2
(from head extracts of *S. bullata* [2]),

(2) Ala-Pro-Gln-Ala-Gly-Phe-Tyr-Gly-Val-Arg-NH_2
(from head extracts of *L. migratoria* [3]),

(3) Gly-Phe-Lys-Asn-Val-Ala-Leu-Ser-Thr-Ala-Arg-Gly-Phe-NH_2
(from accessory glands of the males of *L. migratoria* [4]),

(4) Ala-Ala-Leu-Gln-Glu-Lys-Ala-Gly-Ser-Ile-Val-Arg-Tyr-NH_2
(from the Malphigian tubules of *L. migratoria* [5]).

2.2. Insect toxins

We also tested toxic proteins present in the haemolymph of the Colorado potato beetle for possible lethal effects on *Mamestra* larvae. This haemolymph contains four different proteinaceous toxins [6]. Some are toxic to mammals and others to insects.

2.3. Simmondsin

Finally, we carried out some orienting experiments with Simmondsin, a glucoside isolated from the seeds of the jojoba plant, *Simmondsia californica*. This compound strongly reduces appetite and food intake in mammals. According to some endocrinologists, it may perhaps exert its action by affecting gastrin synthesis/release. Since insects also have gastrin like peptides, it was tempting to find out whether or not this compound could exert an effect on food intake in this insect species.

3. CHOICE OF TEST INSECT SPECIES

All the natural factors were tested on food intake and lethal effects in the phytophageous noctuid *Mamestra brassicae* L. This species occurs in large parts of Europe and Asia and can cause considerable damage to crops. Of extreme importance, however, for this species is that an artificial diet is available so that the insects can be reared all year round. As mentioned in Secton 1, some species of Noctuidae are less sensitive to common strains of *B. thuringiensis*, so we hoped that our tested natural factors may be effective.

4. EXPERIMENTAL SET-UP

As the larvae hatched from the egg they were divided into two groups of 20 larvae each. One group was fed a treated diet, the other group a normal diet. The compounds to be tested were dissolved in water and small cubes of artificial diet were covered with a given amount of the solution. The larvae were checked daily for repellent and lethal effects.

5. RESULTS

Preliminary results indicate that none of the tested natural factors has a repellent effect on the larvae. Only one of the neuropeptides (Ala-Pro-Gln-Ala-Gly-Phe-Tyr-Gly-Val-Arg-NH_2), when administered orally, has a lethal effect within five

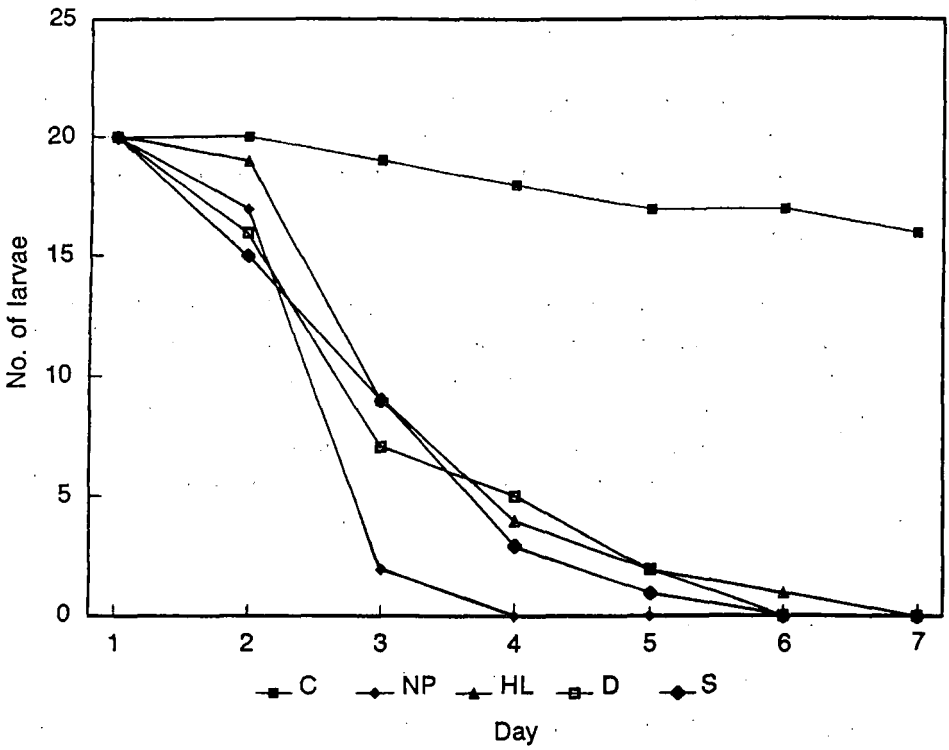

FIG. 1. Survival of the L_1 larvae of M. brassicae *following treatment with: crude haemolymph (HL) (500 µg/larva), purified fraction D (D) (1 mg/larva), neuropeptide (Ala-Pro-Gln-Ala-Gly-Phe-Tyr-Gly-Val-Arg-NH_2) (NP) (17.5 µg/larva), Simmondsin (S) (500 µg/larva) and control (C).*

days of emergence. The crude haemolymph, as well as one of the purified fractions, have a deleterious effect on the larvae of *Mamestra*. Simmondsin was also lethal for the larvae. Figure 1 shows the survival of the L_1 larvae of *M. brassicae* treated with the various natural factors.

6. DISCUSSION

At this point it is clear that at least some of the natural factors tested are lethal for the L_1 larvae of *M. brassicae*. However, none of the factors was totally pure, with the exception of Simmondsin. For the active neuropeptide, there was a possibility the impurities still present after synthesis are responsible for the lethal effects. However, the preliminary results of the feeding experiments with the purified neuropeptide gave the same results.

Further research is being carried out to find out if the alimentary canal is the primary target of the active compounds. Preliminary results with larvae treated with the active fraction isolated from the crude haemolymph of the Colorado potato beetle indicate that these compounds disturb the structure of the alimentary epithelium. Additional research is required before we can draw conclusions about the possibilities for practical use of all these compounds.

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QUANTITATIVE ANALYSIS OF THE FATE OF A PESTICIDE AFTER ITS APPLICATION TO INSECTS

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Abstract

QUANTITATIVE ANALYSIS OF THE FATE OF A PESTICIDE AFTER ITS APPLICATION TO INSECTS.

Utilization of biologically active compounds applied in insect pest management has been studied by a complex method using carbamate juvenoid radiolabelled in different positions with ^{14}C and/or ^{3}H. The technique enables the penetration, distribution and excretion of the applied compound and/or its metabolites to be followed quantitatively.

1. INTRODUCTION

Radiolabelling of insect growth regulators [1, 2] is a highly sensitive and effective technique for studying their fate, which is also closely related to environmental protection. From this point of view, the relation between the applied and utilized doses is of special importance. Knowledge of the sites of interaction of the investigated compound with the insect tissues and quantitative analysis of all radioactive compounds, including expired CO_2, make a comparison of the applied and utilized doses possible.

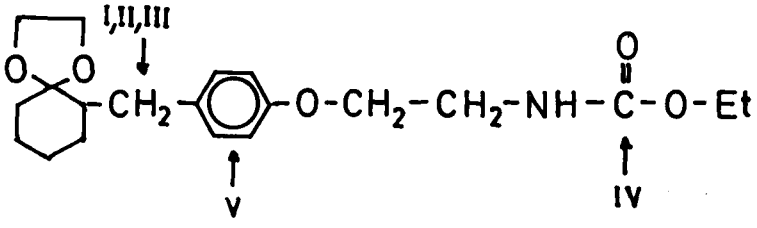
The results obtained with this technique prove that quantitative analysis of the fate of a pesticide after its application to insects is possible. At present, this technique is also being applied in the IAEA programme on tsetse fly control.

2. MATERIALS AND METHODS

The flesh fly, *Sarcophaga bullata* Parker, was used in the experiments. Larvae were reared on beef liver and pupariated in sawdust. One-day-old sugar fed females were employed. Since the strain of *Sarcophaga* used is partly autogenous, no liver was offered during the experiments.

Previously synthesized [3] carbamate juvenoid (signature W328) was labelled separately at the Institute of Nuclear Biology and Radiochemistry, Prague, in two different positions with ^{14}C using chemical synthesis, and independently with ^{3}H by the catalytic exchange solution–gas method. The radiochemical purity after preparation [4] was higher than 99%. The stability was examined during storage and chromatographic purification was carried out before application, if necessary. The labelled positions are summarized in Fig. 1.

An acetone solution (5 μL) of labelled W328 was topically applied to the upper part of the thorax. The flies were narcotized at given time intervals and then separated into three main parts (head, thorax and abdomen) to measure the macrodistribution of radioactivity. Washing was carried out three times in a 5 mL solution of chloroform–methanol (1:1). The body parts were dissolved in 1 mL of scintillation cocktail (Backman, BTS 450), then 15 mL of toluene scintillator were added. A liquid scintillation spectrometer, Backman LS 6000 SE, was used; the standard deviation was less than 1% in all the measurements.



Labelling	Specific activity (GBq/mmol)	No.
Benzyl-^{3}H	82.3	I
Benzyl-^{3}H	323	II
Benzyl-^{3}H	557	III
Carbonyl-^{14}C	0.185	IV
Benzene-U-^{14}C	2.294	V

FIG. 1. Labelling of the applied carbamate juvenoid W328 (molecular weight = 363.4).

To determine the microdistribution of radioactivity, the flies were sectioned at −20°C in a cryostat. The section thickness varied from 40 to 400 μm, which is an infinitely thick layer for ^{14}C; usually, 260 μm thick whole body sections are used. They were mounted on a microscopic slide lightly smeared with glycerol-albumin. For the radioactivity distribution, a computer controlled arrangement of special semiconductor detectors [5] having a measuring slit of 1 mm × 1 mm was used.

For the overall balance, ten flies were kept in a gas chamber lined with filter paper. Air (25–30 mL/s) first passed through the gas washing bottles filled with sodium hydroxide or water, then through the chamber, and finally through two bottles filled with 100 mL of 2M ethanolamine in methylcelosolve.

The radiochromatographic samples were prepared by extraction of sonified tissues using methanol–chloroform (1:1). The supernatant was cleaned up as described in Ref. [6]. After HPLC, standard detection using a UV/VIS detector at 230 nm and subsequent determination in a flow through detector fitted with a solid scintillator were carried out [4].

3. RESULTS AND DISCUSSION

The dependence of the barrier effect of the cuticle on the applied dose is demonstrated in Table I, expressed as a comparison of the radioactivity recovered by surface washing to the radioactivity applied [7]. It follows from the measured values that the percentage of applied radioactivity recovered increased with increasing dose. For example, the routes of surface decontamination after application of 0.081 μg of tritiated W328 differed considerably, depending on whether they were applied alone or simultaneously with ^{14}C. In the latter case, the values for both the radiotracers were comparable, although their positions in the molecule differed and the radioactivity applied differed considerably. Also, the time dependence of the radioactivity recovered differs according to the dose applied.

To appraise the influence of this barrier effect on the radioactivity levels in the body, we compared the results obtained with different doses of the same label (Table II). Although the relative radioactivity recovered by washing is strongly dependent on the applied dose, body radioactivity after washing is roughly proportional to the applied radioactivity. Within two orders of magnitude of the applied dose (0.206–13.7 μg per fly), the percentage of applied radioactivity measured within the whole body was only slightly different during a period of more than two weeks. Therefore, with increasing input of radioactivity in the range of a biologically active dose, the radioactivity of a certain tissue could be increased, if necessary. According to our measurements of the detailed radioactivity distribution (Table III), the fluctuations were partly due to excretion by the gut. The effects of washing were mostly evident on the thorax, because this was the site on which the label was applied.

TABLE I. RELATIVE RADIOACTIVITY (% OF THE APPLIED VALUE) RECOVERED FROM THE BODY SURFACE AFTER APPLICATION OF DIFFERENT DOSES OF DIFFERENTLY LABELLED W328 (THE MEANS OF FIVE FLIES)

Labelled W328	I		II + IV				III	
Dose (μg per female)	0.81		0.081 + 18.8				0.081	
Washed off per female	H-3		H-3		C-14		H-3	
Day	(%)	(ng)	(%)	(ng)	(%)	(μg)	(%)	(ng)
1	22.4	181	67.9	68	63.1	11.9	9.7	7.9
3	6.4	52	38.7	32	38.2	9.2	3.7	3.0
6	3.2	26	25.9	21	20.1	3.8	2.0	1.6
7	1.9	15	18.0	15	18.2	3.4	1.3	1.1

Note: The value of mass (g) was calculated from the appropriate specific activities of the labels (see Fig. 1), assuming their surface stability.

TABLE II. COMPARISON OF THE RADIOACTIVITY (% OF THE APPLIED VALUE) INSIDE THE BODY WITH THAT WASHED OFF FROM ITS SURFACE AFTER APPLICATION OF (^{14}C RING) W328 DOSES WITHIN THREE ORDERS OF MAGNITUDE (THE MEANS OF SIX FLIES)

Dose (μg per female)	0.206		1.72		13.7	
Day	Washed off	Body	Washed off	Body	Washed off	Body
1	6.5	6.1	23.8	10.4	48.2	8.5
3	4.1	4.3	7.1	7.8	33.6	8.0
6	3.2	3.3	6.4	8.4	18.5	7.9
10	1.0	3.7	1.4	4.8	5.9	5.6
17	0.6	3.0	0.8	4.2	5.0	4.2

TABLE III. INFLUENCE OF WASHING ON THE RELATIVE RADIOACTIVITY (% OF THE APPLIED VALUE) OF THE MAIN BODY PARTS AFTER APPLICATION OF 24.6 μg OF (^{14}C RING) W328 (THE MEANS OF THREE FLIES)

Day	Head		Thorax		Abdomen	
	+	−	+	−	+	−
1	0.3	4.0	2.1	65	2.3	9.0
2	0.3	1.3	2.1	32	2.7	8.2
3	0.25	1.2	2.0	32	4.4	8.5
6	0.18	0.6	1.9	14	2.0	0.8
10	0.10	0.3	1.3	12	1.2	3.8
14	0.08	0.2	1.0	12	0.3	2.5

Note: + denotes washed; − denotes unwashed.

TABLE IV. COUNTING RATES MEASURED IN THE WHOLE BODY BY THE SEMICONDUCTOR TOPOGRAPHIC TECHNIQUE ON DAY 4 AFTER APPLICATION OF 12.4 kBq OF (CARBONYL-^{14}C) W328 PER FLY

Tissue	Counts/min (SD ± 3%)
Thoracic cuticle	201
Head cuticle	63
Ovaries	47
Gut	46
Abdomen cuticle	24
Thoracic muscles	20
Brain	19

TABLE V. OVERALL BALANCE ON DAY 7 AFTER APPLICATION OF 198.3 kBq OF (CARBONYL-^{14}C) W328 PER FLY

Sample	Per cent applied activity
Ten unwashed flies	47.7
Expired CO_2	6.2
Excrements, cage surface contamination and water	31.0
Sugar	2.9
Total	87.8

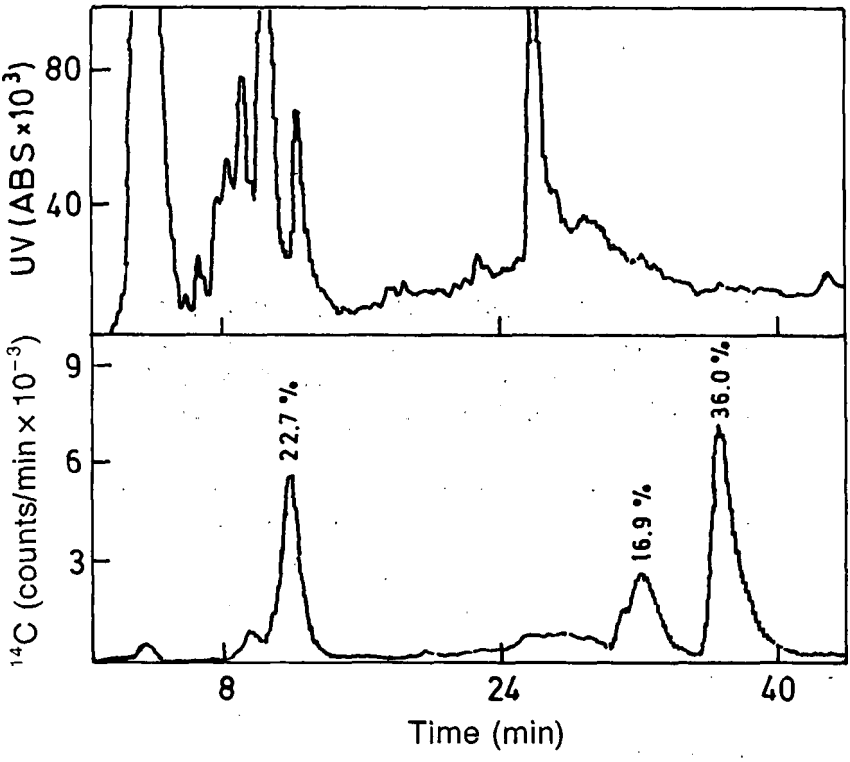


FIG. 2. Standard UV and radioactivity detection after high pressure liquid chromatography of an aliquot of the whole body extract from three flies on day 7 after application of 12.4 kBq of (carbonyl-^{14}C) W328 per fly.

Correspondingly, the microdistribution of the relative radioactivity (Table IV) shows relatively high values in the thoracic cuticle application site. The thoracic muscles contain much less radioactivity. An accumulation was found in the oocytes already four days after application.

From the measured values of radioactivity distribution (Tables II–IV) it follows that within the range of biologically active doses the lower values are preferable for effective utilization of the applied compound with low surface contamination. The applied compound is probably transferred through the cuticle into other distant body parts, with a certain amount penetrating to a haemolymph [8]. During penetration, there is a possibility of carbamate juvenoid metabolism in the cuticle, as has been demonstrated for organophosphorus insecticides [9]. The appropriate experiments are in progress.

Using (carbonyl-^{14}C) labelling, decarboxylation of the applied W328 could be analysed. In such a way, an overall balance was evaluated (Table V).

The radioactivity distribution within the insect body determines, in all cases, only the sites of interaction of the applied juvenoid with the appropriate insect tissues, but not the chemical character of the label. Also, the composition of the washings could be analysed.

High pressure liquid chromatography analysis is used to distinguish between the applied compound and its labelled metabolites or degradation products. Figure 2 demonstrates such a separation, from which it was calculated that 36% of the radioactivity of the original W328 was still present (retention time t_R = 36.6 min). Besides, two main radiopeaks were found, one of which probably corresponds to keton (16.9%) and the other lies in the region of the polar fractions (22.7%). Further radiochromatographic experiments are now in progress.

ACKNOWLEDGEMENTS

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USE OF JUVENOIDS FOR SUPPRESSION OF INSECT REPRODUCTION

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Abstract

USE OF JUVENOIDS FOR SUPPRESSION OF INSECT REPRODUCTION.

Juvenoids are bio-analogues of the juvenile hormones that are vital regulators of insect development and reproduction. Insects treated with juvenoids often perish owing to a block of embryogenesis or metamorphosis, but certain treatments cause sterility by disrupting gametogenesis and egg development in otherwise normal adults. Thousands of structurally diversified juvenoids are known, but only five compounds are currently on the market and a few others with promising properties are in the stage of laboratory testing. High species specificity and low environmental stability are the major obstacles in the commercialization of juvenoids. Juvenoids are used to suppress cockroaches, fleas and ants in households, to protect some stored commodities, to control mosquitoes and certain other dipterans, to defeat tortricids and homopterans in orchards, and to increase the silk yield in sericulture. Other applications, such as against various homopterans and against tsetse flies, are being developed. A combination of juvenoid treatments with biological control and their incorporation into sterile insect technique programmes deserve more attention.

1. INTRODUCTION

Insect development and reproduction are under hormonal control and are hampered when the hormonal regulation is disturbed. The practical potential of artificially induced hormonal imbalance was recognized by Williams [1], who discovered that topical applications of juvenile hormone (JH) extracts caused developmental defects with lethal consequences. Since then, the structure of five JHs has been elucidated. Juvenile hormones seem to be unique to insects, in which they control metamorphosis, reproduction, and several other processes and functions. There are differences in the role of JHs in various insect groups [2]. For example, in Lepidoptera, metamorphosis ensues after a drop in JH titre and egg formation often occurs in pupae when JH remains low. By contrast, in cyclorrhaphous Diptera, metamorphosis appears to begin in the last larval instar in the presence of JH, and egg production requires a high titre of this hormone.

The effects of JHs are mimicked by juvenoids, whose structure is often only vaguely related to the known hormones. A few juvenoids were isolated from plants and several thousand were synthesized. Examples of the structural variety of juvenoids can be found in Refs [3, 4] and in a number of other publications. Juvenoids have also been linked to inert moieties such as sugars and fatty acids to obtain juvenogens from which the hormonally active component is released upon enzymatic cleavage in the target insect. Research efforts were further directed to substances that interfere either with the production or with the action of JH [5]. These 'anti-JH agents', as well as the juvenogens, seem to be far from practical utilization.

Arguments for and against the use of juvenoids in insect pest management have been put forward for the last twenty years [6, 7]. It is agreed that juvenoids suffer two major disadvantages. First, the activities of juvenoids are invariably species specific and some insect groups are difficult to affect with any compound [8, 9]. Second, the desired effects in most pest control programmes are death or sterility, both of which occur days or weeks after the juvenoid treatments. Owing to these limitations, progress in juvenoid commercialization has been slow and to date only five compounds are available on the market. The flaws of juvenoids, however, are compensated for by their selectivity, which makes them safe to most non-target species, and their biodegradability, which prevents accumulation of residues in the food chain.

Available knowledge and experience indicate that juvenoids are not likely to become a general means of controlling insect pests. On the other hand, in certain cases their use appears to be the method of choice. The aim of the present paper, in which the effects and the current use of juvenoids are briefly reviewed, is to encourage considerations on combining juvenoid treatments with radionuclear, molecular and genetic techniques of pest control. Owing to lack of space the references listed are limited to reviews and selected reports published within the last few years.

2. VARIETY OF JUVENOID EFFECTS

Juvenoids have been reported to affect all the developmental stages of insects [10], but usually only applications to one or two stages are of practical significance in any given species. The most suitable stage must be identified experimentally. The following paragraphs illustrate the variety of effects that can be encountered after juvenoid treatments. The suppression of reproduction is emphasized.

2.1. Inhibition of embryogenesis

Juvenoids applied to the eggs either directly or via the maternal organism inhibit embryogenesis. The effects range from the blockage of cleavage to the hatch-

ing of slightly defective larvae that are bound to perish. Death usually occurs soon after hatching, but tobacco hornworm larvae eclosing from fenoxycarb treated eggs sometimes survive until the second instar [11].

2.2. Effects on polymorphism

Treatments of larvae in polymorphic species cause a shift towards a certain morph. Thus, juvenoids interfere with the development of casts in social insects, the seasonal polymorphism of aphids and phase dimorphism in locusts, lepidopterans and others. These effects have a potential for practical exploitation, but so far they have not been included in any application scheme.

2.3. Inhibition of moulting

Juvenoids inhibit the production of insect moulting hormones, the ecdysteroids, and this results in a temporal or permanent blockage in moulting. Moult is prevented in the larvae of most endopterygotes when they reach a body size incompatible with further larval development [12]. Such larvae often respond to juvenoids by continuous feeding without moulting, and may attain enormous body sizes. Eventually they perish, but pupation may take place when the juvenoid is disposed of. Precisely timed and dosed juvenoid application is used in sericulture to increase the size of silkworm larvae, which then produce more silk [13].

In the German cockroach and certain scale insects, juvenoids inhibit moulting and cause death in first instar larvae.

2.4. Interference with developmental diapause

The life cycle of many insects includes a facultative or obligatory diapause that occurs in various developmental periods and is controlled by differential hormonal mechanisms. Juvenoids have been reported to terminate early embryonic diapause, while our experience indicates that they have no effect on diapause at the very end of embryonic development when the fully formed first instar larvae are prevented from hatching. The effects of juvenoids on diapause in early larval instars have not been examined, except for a report on diapause termination in a planthopper [14]. Diapause in an early part of the last larval instar, i.e. before the programming for metamorphosis takes place (such diapausing larvae undergo in some species stationary larval moults), was induced and maintained with juvenoids in several endopterygotes. The effect of juvenoids is variable in the case of diapause occurring late in the last larval instar; diapause induction is facilitated but in some species juvenoids cannot revert stimulation of development by environmental cues. In contrast, juvenoids invariably terminate pupal diapause. Precisely dosed treatments induce continuation of normal development, whereas high doses bring about morphogenetic defects, as described in Section 2.5.

2.5. Derangement of metamorphosis

Inhibition of morphogenesis in metamorphosing insects is the most frequently described effect of juvenoids. In a few insect groups, juvenoid applications to the penultimate or newly ecdysed last instar larvae inhibit any development towards the adult form. Such insects ecdyse into an extra larval instar and may undergo metamorphosis one or more instars later. More frequently, however, the 'supernumerary' larvae are defective and perish. Juvenoid treatments performed in the course of the last instar cannot revert the morphological changes that have already taken place but inhibit their further progress: the insects moult to intermediate forms between larva and adult in exopterygotes or between larva and pupa in endopterygotes. Similarly, juvenoid applications to pupae cause development of pupal–adult intermediates. The 'intermediate' character of insects that ecdyse after a juvenoid treatment may be manifested only internally. The insects may appear as normal adults but their flight capacity, mating ability or fertility are impaired. As with all other juvenoid effects, the maximum extent of metamorphosis inhibition varied in different insects. Morphogenetic effects on larval–pupal transformation are absent in cyclorrhaphous Diptera and probably in some other groups as well.

2.6. Stimulation of reproduction

Stimulation of egg development is an important function of JH in many insect species and it is readily mimicked with juvenoids. Owing to this effect, the inhibition of imaginal differentiation described above is occasionally associated with precocious egg formation. Juvenoids also compensate for the lack of JH that causes imaginal diapause. In the bug *Eurygaster integriceps,* which has an obligatory imaginal diapause, the adults become sensitive to juvenoids only a couple of weeks after emergence. Except for this refractory phase, juvenoid treatments of diapausing adults induce reproduction and eliminate other diapause syndromes. For example, termination of diapause in certain water bugs and carabid beetles is associated with regeneration of the flight muscles, while in bark beetles and crickets these muscles are induced to degenerate.

Imaginal diapause is often terminated with juvenoid doses that cause derangements of reproduction when administered to the non-diapausing adults (see Section 2.7). No explanation has been offered for this interesting phenomenon. Induction of normal reproduction in an unsuitable season, however, would have detrimental consequences for the pest population. Such a premature termination of diapause with juvenoids was proposed as a control strategy for the pear psylla [15].

2.7. Inhibition of reproduction

2.7.1. Indirect effects on reproduction

Most of the juvenoid effects listed above have indirect consequences for reproduction. Obviously no progeny are produced when the treated insects die from morphogenetic defects. The supernumerary larvae and intermediate forms, which ecdyse after treatment at the metamorphosing stages, can survive for a long time, but incomplete development of the reproductive system prevents their reproduction. An exception is found in viviparous aphids that deliver their parthenogenic progeny via a rupture in the body wall. In most insects, however, reproduction is impaired when juvenoids cause even a minor morphological abnormality in the emerged adults, such as incomplete genitalia rotation in the males or distorted ovipositor in the females of cyclorrhaphous Diptera. The interference of juvenoids with polymorphism, moulting and diapause may also inhibit reproduction. For example, juvenoids suppress the reproductive potential of termite colonies by causing a shift from the development of reproductives to that of soldiers.

2.7.2. Direct reproductive failures

Administration of juvenoids to the last instar larvae, pupae or adults causes defects in the differentiation of gametes and follicular cells. The differentiation is inhibited in the same manner as any other morphogenetic process and, in cases when it is separated from the morphogenesis of other body tissues in time or by threshold sensitivity to juvenoids, one can obtain sterile, but in all other respects normal, adults. Inhibition of spermatogenesis by juvenoid treatment of larvae has been achieved in a number of species. Both under in vivo and in vitro conditions it has been demonstrated that spermiogenesis is more readily blocked than spermatogenesis. Differentiation of oogonia begins in larvae rather exceptionally and its blockage with juvenoids has been described only in the fall webworm [16]. In this species, meiosis in spermatogonia begins in the fourth larval instar and mature sperm are formed in the seventh (last) instar. The oogonia enter meiotic prophase in the sixth instar and diversify into oocytes and trophocytes in the second half of the last instar. Juvenoid applications to larvae do not affect the proliferation of stem cells and primary gametogonia, but at least in males they prevent the last round of mitoses by which the cysts of synchronously developing gametocytes are formed. In both sexes, juvenoids inhibit the onset of meiosis and block further progress of gametogenesis at various steps. Gametocytes whose development is blocked degenerate. No gametes are formed in either sex when webworm larvae receive the juvenoid hydroprene at a rate of 10 μg/specimen in each from the third to the seventh instar. In the case of the fall webworm, such specimens moult into larval-pupal intermedi-

ates and perish. In the German cockroach, however, it is possible to cause permanent sterility in externally normal adults by administering juvenoids to the larvae [17].

Juvenoids also inhibit the differentiation and function of follicular cells and this results in the formation of faulty egg chambers. The chambers either fuse to contain more than one oocyte or remain unusually small, and/or they appear normal but either vitellogenesis or chorion and micropyle formations are defective. Imperfect egg chambers are usually resorbed but occasionally they sustain and non-developing eggs are laid. Their sterility, however, may also be due to juvenoid deposition in the egg and consequent blockage of embryogenesis (see Section 2.1). The latter case occurs frequently after juvenoid applications to adults. Females are obviously more susceptible to this effect, but treated males may transfer juvenoids to the eggs during mating. High doses of juvenoids administered to the reproducing females of the firebrat, *Thermobia domestica,* cause irreversible degeneration of entire ovarioles. Such an effect has not been reported for any other insects and exemplifies great differences in the sterilizing potential of juvenoids for different species. In many cases, the cause of reproductive failure is not precisely known and may be unrelated to gonads. For example, reduction in fecundity in *Spodoptera litura* has been attributed to juvenoid interference with a humoral stimulation of oviposition [18].

3. PRACTICAL USE OF JUVENOIDS

3.1. Commercially available compounds

Zoecon Corporation (now Sandoz Crop Protection Ltd) of California commercialized three esters of 3,7,11-trimethyl-dodeca-2,4-dienoic acid more than a decade ago. The juvenoid kinoprene was introduced under the name Enstar for the control of homopterans on ornamental plants in greenhouses (applied at 0.1 g/m^2; these and all following data refer to the active ingredient). Two other juvenoids had a much broader spectrum of application. By 1985, the juvenoid methoprene was formulated as Altosid to control mosquitoes (280 g/ha) and horn flies (0.02% in mineral blocks for a feed through application); as Apex against sciarid flies in mushroom cultures (1 g/m^2); as Kabat for application in tobacco storehouses (10 ppm sprays), and as Dianex and Diacon to protect stored peanuts (0.0207 g/m^3 in aerosol formulation and 1.12 g/100 kg peanuts in spray). Bait formulations Pharorid (Zoecon) and Lafarex (Lachema, Czechoslovakia) proved very effective against the pharaoni ant. The third juvenoid of Zoecon Corporation, the hydroprene, was developed for use against fleas (Precor or, in combination with a pyrethroid, Raid Flea Killer II Plus) and cockroaches (Gencor, or Gencor Plus in a mixture with Permethrin).

Dr. R. Maag Ltd (Switzerland) developed for commercial use the juvenoid fenoxycarb, which is characterized by the phenoxyphenoxy moiety and a carbamate functional group. Fenoxycarb is used under the name Insegar (Ciba Geigy, Switzer-

land) to control tortricids and homopterans in orchards (the doses vary from 0.25 to 1 kg/ha with one to three sprays per season), fire ants in the households (5–10 mg/colony), mosquito larvae in water reservoirs (0.03–0.1 ppm), and some stored product pests (4–10 ppm). Fenoxycarb is now also manufactured in Japan, but its broad use in agriculture is being threatened by reports that fenoxycarb contamination has prevented cocoon spinning in commercially reared silkworms in certain parts of Italy and France [19].

The fifth commercially available juvenoid, named pyriproxyfen, is produced by the Sumitomo Chemical Company Ltd, Japan. As Sumilarv, it is particularly recommended for the control of fly development in waste products (0.2 g/m^2) and of mosquitoes in water reservoirs (0.01 ppm). Good results have also been obtained with the use of pyriproxyfen against aphids, psyllids, white flies and scales [20–22], and also against the triatomid bug, *Rhodnius prolixus* [23].

There are at least two juvenoids of interest in the testing stage. A compound resembling fenoxycarb, ethyl 2-{4-[(1,4-dioxaspiro[4,5]dec-6-yl)methyl]phenoxy} ethylcarbamate, was discovered at the Czechoslovak Academy of Sciences. It exhibits high activities on several insect groups, psyllids in particular [24]. A structurally unrelated compound, 4-chloro-5-(6-chloro-3-pyridylmethoxy)-2-(3,4-dichlorophenyl)-pyridazin-3(2H)-one, Nissan Chemical Industries, Japan, shows very promising effects on leafhoppers [15, 25].

3.2. Further perspectives of juvenoid exploitation

Juvenoids harbour a potential for use with other environmentally friendly techniques of sustained pest suppression. A combination of juvenoids with the use of bioagens, such as parasitoids and predators, is an emerging trend in some pest control schemes. Such an approach is possible because juvenoids are often destructive for the pests but are safe for their natural enemies [26, 27]. There are indications that juvenoid treatments may even enhance the population growth of some parasitoids; we observed that treatment of the eggs of the sun bug, *Eurygaster integriceps*, extends by several times the period in which they can be infested with the parasitoid *Azolcus grandis*.

It is possible to envisage the use of juvenoids as part of various sterile insect technique programmes. For instance, the high ovicidal activity of both fenoxycarb and pyriproxyfen on the codling moth could be exploited for suppressing the natural population before sterile insect release. Pyriproxyfen has also been identified as an effective tsetse sterilant that acts by inhibiting metamorphosis in the offspring of treated flies [28]. Luring adults to traps in which they become contaminated with pyriproxyfen was devised as a method for achieving a considerable population reduction in tsetse [29]. This approach should certainly be considered in tsetse eradication schemes.

Juvenoids have successfully been tested as a quarantine treatment of fruits [30] and this offers a possibility for using them as a supplement to commodity irradiation. In summary, exploitation of juvenoids for enhancing the efficacy of radio-nuclear, molecular and genetic techniques deserves attention.

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RESEARCH AND DEVELOPMENT ON THE TSETSE FLY

(Session 7)

Chairman

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AGE DEPENDENT SAMPLING BIASES IN TSETSE FLIES *(Glossina)*
*Problems associated with estimating mortality from sample age distributions**

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Abstract

AGE DEPENDENT SAMPLING BIASES IN TSETSE FLIES *(Glossina)*: PROBLEMS ASSOCIATED WITH ESTIMATING MORTALITY FROM SAMPLE AGE DISTRIBUTIONS.

For a closed (island) population of *G. morsitans morsitans* Westwood, the probability per week of capturing females on ox fly rounds was about 0.3 in the first week of life, less than 0.2 for 27 to 35-d-old flies and greater than 0.4 for flies more than 80 d old. For open populations, the relative changes in capture probability were measured from the ovarian age distributions of trap and ox fly round samples. They were used (with the island data) to show that the age dependent sampling bias of traps for female *G. m. morsitans* increased more than sixfold over the first 80 d of life. The age dependent bias for *G. pallidipes* Austen taken from odour baited traps is probably at least as serious as for *G. m. morsitans*. Estimates of daily mortality from the mark–recapture studies were always (up to 20 times) higher than estimates from ovarian age samples taken at the same times. The mortalities recalculated from samples adjusted for sampling biases were closer to, but still lower than, the mark–recapture estimates. Odour baited targets are successful in controlling tsetse populations, despite the relatively low probability of treating young females. If sterilants instead of insecticides were used on the targets, young females could be treated indirectly via treated males, which transfer the sterilant to virgin females during copulation.

1. INTRODUCTION

Attempts to control tsetse flies *(Glossina)* in much of Africa rely increasingly on the use of odour baited targets [1]. Because of their low natural birth rate, a population can be eradicated by superimposing, on the natural death rate, a mortality due to the targets such that the total mortality is greater than about 3.5% per day [2–4] by an amount that depends on the level of pre-adult mortality and the desired rate

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of population reduction. In deciding on the required target density, it is necessary to know the natural death rate of the adult population. Changes in mortality during the eradication campaign will also produce information on the performance of the targets under different circumstances, and may warn about operational problems or indicate points at which the overall mortality decreases as a consequence of the density dependent processes.

Mark–recapture methods [5, 6] can be used to measure mortality [7, 8] for closed populations but, under more natural open condition it is difficult to separate the effects of mortality and emigration, and the methods are generally complex and time consuming. Under certain conditions [9], mortality can be estimated more simply from sample age structures, one condition being that the sample should be random with respect to age or, if not, that the age bias be known and the sample corrected accordingly.

The ovarian age structure [10] of female tsetse flies *Glossina morsitans morsitans* Westwood and *G. pallidipes* Austen depends on the system used to capture the flies [11]. Two sampling methods are of importance in the current context: (1) stationary odour baited traps, since these are widely used to survey tsetse populations and to collect samples for analysis of the ovarian age structure [3, 12]; and (2) mobile ox fly rounds, since these were used in an experiment where tsetse mortalities could be estimated simultaneously by mark–recapture and by analysing sample ovarian age structures [7]. It is also the only technique for which estimates have been produced of the absolute probability of capture of tsetse flies of different ages [13]. These studies are used here to estimate the age dependent sampling bias of odour baited traps and its effect on estimates of mortality in adult tsetse flies.

2. METHODS

2.1. Estimating mortality from ovarian age data in tsetse flies

In the past, mortalities have been estimated from ovarian age data by non-linear regression [3, 12], but the method assumes equal variance in each of the n_i (the number of sampled flies in age class i). It can be shown that the maximum likelihood estimate for the mortality is obtained by solving, for φ, the equation

$$x_2(1 - \varphi^4) - x_1(1 + \varphi + \varphi^2 + \varphi^3) + 4\varphi^4 x_3 = 0 \qquad (1)$$

where

$$x_1 = \sum_{i=1}^{7} n_i; \quad x_2 = \sum_{i=1}^{7} i n_i; \quad \text{and } x_3 = \sum_{i=1}^{7} n_i$$

and where $\varphi = \exp(\mu + \lambda)$ for mortality μ and growth rate λ. (Flies in ovarian category zero have generally been excluded from such analyses, since they are judged to be severely underrepresented in field samples.) The mortality μ can then be estimated if the growth rate λ is known; the latter is obtained from changes in population levels estimated, for the present study, by multiple mark–recapture [5, 6].

The complex form of Eq. (1) is due to the pooling of older flies in ovarian categories 4–7. Thus, ovarian category 4 contains flies from age categories 4, 8, 12, etc., which cannot be separated by the dissection technique alone; analogous problems exist for ovarian categories 5, 6 and 7. The older age classes can be partitioned approximately on the basis of their wing fray [13]; this technique is used *in Section 3.*

2.2. Estimating age dependent sampling bias

For a population of N flies, suppose n_i are in age category i and that for a sample of M_j flies (using technique j) $m_{i,j}$ are in age category i. Then we define the bias ($b_{i,j}$) for age category i and capture system j by

$$b_{i,j} = (m_{i,j}/M_j)/(n_i/N) \tag{2}$$

Conversely, if the $b_{i,j}$ are known, the n_i are estimated by

$$n_i = (m_{ij}N)/(M_j \cdot b_{ij}) \tag{3}$$

Note that the population age *distribution* can be estimated even if N is unknown. It also follows that the biases of the two different sampling systems are related by

$$b_{i,2} = (m_{i,2}/M_2)(M_1/m_{i,1})b_{i,1} \tag{4}$$

3. RESULTS

In a study of a closed (island) population of *G. m. morsitans* [13], the absolute age dependent bias was estimated for female flies uniquely marked and released at birth and recaptured on ox fly rounds. The probability of capture per week (the approximate interlarval period on the island) can be described by a quadratic function of age (Fig. 1(a)). This type of experiment has so far not been possible to perform in open populations. However, the island results (Fig. 1(a)) can be used in conjunction with ovarian age samples taken from open populations to estimate the age dependent biases for the latter situation.

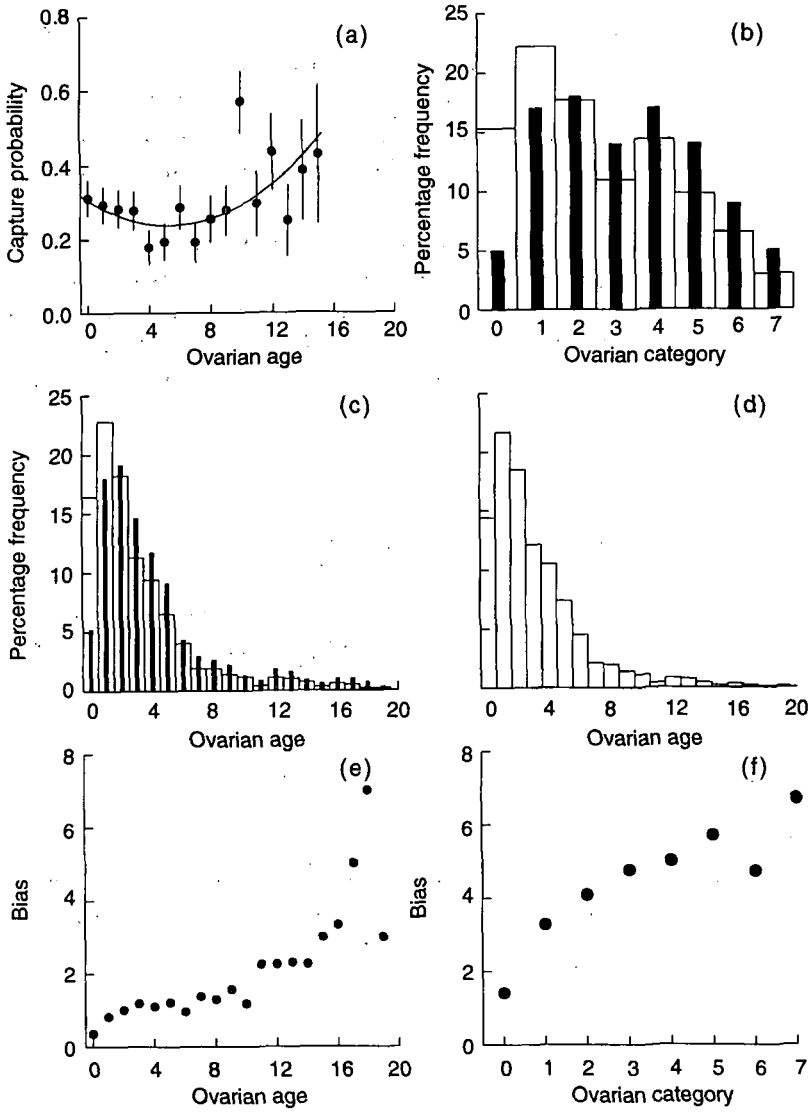

FIG. 1. Age dependent sampling biases in female G. m. morsitans. *(a) The relationship, for flies recaptured on an ox fly round [13], between the age and probability of capture (± standard deviation) in successive seven-day periods. (b) Frequency by ovarian category for flies caught in odour baited traps (solid bars) and on ox fly rounds (open bars) [11]. (c) As for (b), but flies in ovarian categories 4–7 distributed to older categories according to wing fray [13]. (d) Estimate of true age distribution using information from (a) and (c). (e) Estimated age dependent sampling bias for flies caught in odour baited traps calculated using the estimated true distribution (d) and the trap sample (c). (f) As for (e), but the distributions in (c) and (d) are pooled prior to calculation to give only ovarian categories 0–7, as in (b).*

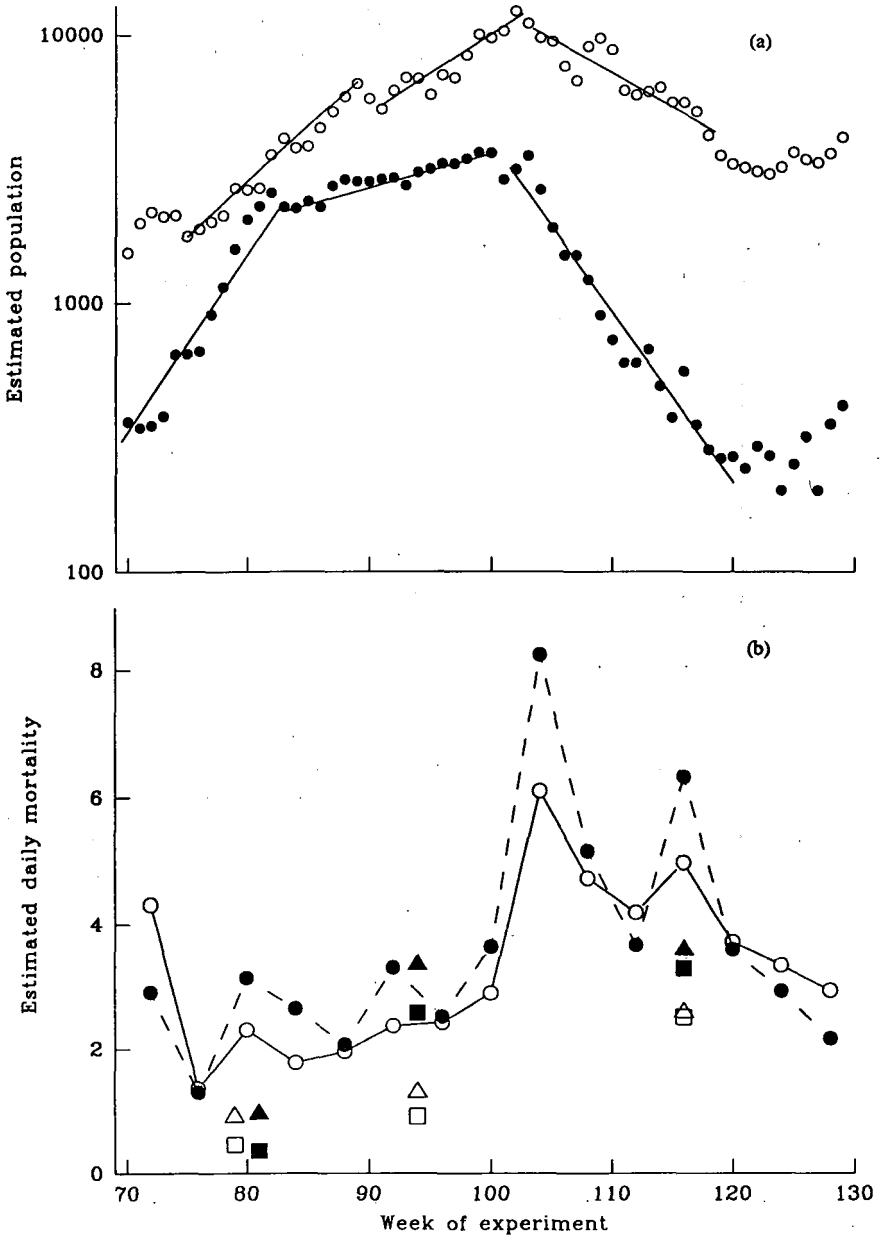

FIG. 2. Population levels and mortality rates for female G. m. morsitans *and* G. pallidipes *(all filled symbols) on Antelope Island, Lake Kariba, Zimbabwe [7]. (a) Weekly mark-recapture estimates of the total population numbers (logarithmic scale). Lines are fitted by regression to estimate growth rates over times shown. (b) Estimates of mortality resulting from: mark-recapture (circles), analysis of original ovarian age distribution (squares) and of 'corrected' distributions (triangles).*

Note that Fig. 1(a) shows the absolute probability of capture at various ages, rather than the bias. The latter is obtained by multiplying each of the probabilities (P_{ij}) by the constant factor

$$\sum_{i=1}^{k} n_i \Big/ \sum_{i=1}^{k} m_{ij}$$

where k is the number of age classes and the n_i and m_{ij} are defined in Section 2.2.

Figure 1(b) shows the percentage in each ovarian age class for samples of *G. m. morsitans* taken from odour baited traps and ox fly rounds in a study on an open population [11]. Flies in ovarian categories 4–7 can be partitioned, using the wing fray of individual flies, to produce an approximate sample age distribution (Fig. 1(c)). The true age distribution (Fig. 1(d)) can then be estimated (from Eq. (3)), using the ox fly round sample age distribution (Fig. 1(c)) and the capture probability function (Fig. 1(a)).

The age dependent sampling bias for the traps (Figs 1(e) and 1(f)) is now estimated (from Eq. (4)), using the estimated true age distribution (Fig. 1(d)) and the trap sample age distribution (Fig. 1(c)). The bias changes more radically with age for traps than for the ox fly rounds (see Figs 1(a) and 1(e), noting the tenfold difference in scale), increasing approximately linearly with age for most of the fly's life.

The vital parameters of island populations of *G. m. morsitans* and *G. pallidipes* [7] have been estimated by mark–recapture (Fig. 2). During this experiment, three ovarian age samples of trapped flies were also obtained. The daily mortalities estimated by mark–recapture were up to five times higher than those from age structure analysis for *G. m. morsitans* and up to 20 times higher for *G. pallidipes* (Fig. 2(b)). This could be explained by a bias in trap samples towards old flies. The mortalities estimated from age structures 'corrected' using the bias estimates (Fig. 1(d)) were indeed higher than the originals, but still substantially lower than the mark–recapture estimates in each case (Fig. 2(b)).

4. DISCUSSION

The differences between mortality estimates from mark–recapture and age distribution analysis are only partly explained by an age dependent trapping bias of the order estimated in Section 3. The remaining difference is not easy to explain. The bias function used has, perforce, been derived by a circuitous route and involves many assumptions regarding the equality of behaviour of the tsetse flies at three different localities and times in Zimbabwe. The trapping systems used on the island [7] and at Rekomitjie [11] were also somewhat different. It may be that the bias was even more pronounced on the island than assumed here.

Alternatively, mortality may be overestimated by the mark–recapture method. However, theoretical and computer simulations show that the method used results in mortality estimates with biases that are less than 5% of the mean [8, 14]. Since the changes in the capture probability of *G. m. morsitans* females on ox fly rounds were also small (Fig. 1(a)), there is no reason to suppose that the mark–recapture estimates of mortality are seriously in error as a result of the changing probabilities of recapture by this system during the fly's life.

Part of the discrepancy might be due to the fact that mark–recapture mortalities were averages for *all* adult flies, whereas the estimates derived from ovarian dissections ignored the first ovarian category, which has above average mortalities [13]. In addition, one cannot be certain that the island population had a stable age distribution at the time the samples for ovarian ageing were collected.

At the practical level, the results suggest that odour baited targets are successful in controlling tsetse populations, despite the fact that the (important) younger elements of the female population are treated with a relatively low probability. This difficulty could be overcome, and the technique thereby enhanced, by using sterilants instead of insecticides on the target [15]. The young elements of the female population could then be treated indirectly via the males, which pick up the sterilant from the target and transfer it to virgin females during copulation.

ACKNOWLEDGEMENT

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DETECTION AND IDENTIFICATION OF TISSUE SPECIFIC LECTINS OF THE TSETSE FLY, *Glossina tachinoides* *Midgut lectin activity with lipopolysaccharide binding specificity**

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Abstract

DETECTION AND IDENTIFICATION OF TISSUE SPECIFIC LECTINS OF THE TSETSE FLY, *Glossina tachinoides*: MIDGUT LECTIN ACTIVITY WITH LIPOPOLYSACCHARIDE BINDING SPECIFICITY.

Lectin that agglutinates human and animal red blood cells (RBCs) was demonstrated in midgut extracts of *Glossina tachinoides*. The highest haemagglutination titres were against pig and rabbit RBCs. Treatment of rabbit RBCs with pronase, trypsin, neuraminidase, bromelain, glutaraldehyde and periodate reduced the agglutination titres. The lectin is specific for amino, methyl and deoxy derivates of glucose, amino and methyl derivates of mannose, D-galactosamine, N-acetylneuraminic acid and trehalose. In addition, very high reactivity against the lipopolysaccharide of *E. coli* K 235 was found. Lectin is secreted to the midgut lumen. It consists of a 27 kilodalton protein component that is not glycosylated. Sandwich ELISA permits quantification of lectin in tissue samples.

1. INTRODUCTION

In insects, lectins take part in the humoral and cellular immune mechanisms. In vectors of infectious diseases, they may also react with the glycosylated components of transmitted pathogens [1]. Several species of *Glossina* possess haemolymph and gut lectins that are important for the life-cycle of transmitted trypanosomes, e.g.

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in *Glossina morsitans morsitans*, the lectins limit the susceptibility to trypanosomes and serve as signalling factors for maturation of *Trypanosoma congolense* [2]. Differences between species (strains) of flies in lectin secretion might be responsible for the species specific (or strain specific) barriers which exist between trypanosomes and vectors [3].

This paper is aimed mainly at identification of the binding specificity of the midgut lectin of *G. tachinoides* and its structural characterization.

2. MATERIALS AND METHODS

Adult tsetse flies, *G. tachinoides*, obtained as puparia from the Agency's Laboratory at Seibersdorf, were maintained on a mixture of cattle/pig blood.

The midgut was removed from teneral and non-teneral flies of both sexes, repeatedly homogenized, frozen and thawed three times at −70°C. The gut extracts were then centrifuged at 3000g for 10 min and the supernatant stored at −70°C.

In a haemagglutination activity (HA) test, four types of human (A1, A2, B and O) and seven types of animal red blood cells (RBCs) (rabbit, sheep, cattle, pig, goat, mice and chicken) were used. Rabbit RBCs were used native or treated with pronase, trypsin, bromelain, periodate and glutaraldehyde. An extract of *G. tachinoides* midgut was serially diluted in haemagglutination buffer TN (10 mM Tris-HCl, 0.15M NaCl, pH7.2) on microtitration plates. The same volume (50 μL) of erythrocyte suspension was added to each well. The reciprocal value of the highest dilution with a positive reaction was designated a titre.

In the haemagglutination inhibition test (HIT), the inhibitors (saccharides and glycoconjugates) were serially diluted in TN buffer. The midgut extract was adjusted to contain 1.5 units of haemagglutination activity (HAU) and the same volume (50 μL) of 1% suspension of rabbit or pig erythrocytes was added to each well. The lowest concentration of an inhibitor blocking 1.5 HAU was designated a 50% inhibition concentration.

Antibodies directed against the HA of *G. tachinoides* midgut were raised in rabbits and mice according to Ref. [4]. A 1% suspension of RBCs was agglutinated by midgut extract, washed three times in TN buffer and injected subcutaneously. The gammaglobulin fraction was isolated from the rabbit hyperimmune serum using caprylic acid according to Ref. [5].

Electrophoresis in polyacrylamine gel in the presence of sodium dodecyl sulphate (SDS-PAGE) was carried out in a discontinuous system according to Ref. [6] on a 10–15% linear gradient slab gel (thickness 1.5 mm) under reduced conditions. The gel, with markers of molecular masses (LMW kit, Pharmacia, Uppsala, Sweden), was stained with Coomassie Brilliant Blue R-250.

Transfer of proteins to the NC membrane was carried out according to Ref. [7]. The NC membrane was rinsed in T-PBS (PBS, pH7.4, 0.05% Tween 20).

Immunoblats were incubated for 2 h in 5% skim milk (Oxoid, London, United Kingdom), washed in T-PBS and incubated for 2 h with rabbit antiserum to *G. tachinoides* HA and then for 1 h with horseradish peroxidase-conjugated swine anti-rabbit immunoglobulins (SEVAC, Prague, Czechoslovakia).

In affinoblotting, NC strips blocked with 3% BSA (Serva, Heidelberg, Germany) were incubated with peroxidase labelled lectins (diluted 1:100) for 2 h. The lectins from the jack bean (Con A), garden pea (PSA), peanut (PNA), soybean (SBA) and wheat germ (WGA) obtained from Lectinola (Prague) were labelled with horseradish peroxidase (Serva, Heidelberg), as described in Ref. [8].

The peroxidase reaction product was developed by substrate solution with 0.6mM 3,3′-diaminobenzidine.

For indirect immunofluorescence assay, the midgut tissue was fixed in 3% paraformaldehyde at 4°C and embedded in JB-4 resin (Polysciences) in BEEM capsules. Sections (3 μm thick) were incubated with 3% BSA in PBS overnight at 4°C, then with rabbit antibodies directed against *G. tachinoides* HA diluted in 3% BSA for 30 min and with fluorescein-conjugated swine anti-rabbit immunoglobulins (SEVAC, Prague) for 30 min at 37°C. Sections post-stained with Evans blue were photographed using a Jenalumar (Zeiss, Jena, Germany) fluorescent microscope.

Sandwich ELISA was developed for the lectin measurement. Polystyrene microtitration plates (GAMA, České Budějovice, Czechoslovakia) were incubated overnight with 60 μL of rabbit gammaglobulins directed against *G. tachinoides* HA (20 μg/mL) in 0.1M carbonate buffer (pH9:6). Midgut extracts (50 μL) were serially diluted in PBS-Tw and incubated for 2 h at 37°C. Then the plates were incubated for 90 min with mice antiserum directed against *G. tachinoides* HA (diluted 1:1000) at 37°C and for 60 min with peroxidase-labelled swine anti-mice immunoglobulins (SEVAC, Prague). Orthophenylendiamine in phosphate–citrate buffer (pH5.5) served as a substrate and the plates were read at 492 nm.

3. RESULTS

Extract of teneral and non-teneral *G. tachinoides* midgut agglutinated rabbit, mice, pig and human A1 RBCs. The haemagglutination titres ranged from 1:10 to 1:10.240. The highest midgut extract titres were detected against rabbit and pig RBCs. Treatment of rabbit RBCs with pronase, neuraminidase, bromelain, glutaraldehyde and periodate reduced the agglutination titres.

The binding specificity of *G. tachinoides* HA was studied using the haemagglutination inhibition test with native rabbit and pig RBCs (Table I). Eight carbohydrates caused the inhibition of agglutination at concentrations of 250mM or below. These sugars comprised D-galactosamine, D-glucosamine, D-mannosamine, 2-deoxyglucose, methyl-a-D-mannoside, methyl-a-D-glucoside, N-acetylneuraminic acid and trehalose. Of the glycoconjugates used, the most effective inhibitors were

TABLE I. INHIBITION OF THE MIDGUT HAEMAGGLUTINATION ACTIVITY (HA) OF TENERAL FLIES (TF) AND NON-TENERAL FLIES (NTF), *G. tachinoides*, USING RABBIT AND PIG ERYTHROCYTES

Inhibitor	Concentration required for 50 rabbit erythrocytes		Inhibition of HA pig erythrocytes
	(TF)	(NTF)	(NTF)
Carbohydrates (mM)			
D-galactosamine	62	250	250
D-glucosamine	62	250	y
D-mannosamine	62	125	y
Trehalose	125	125	y
2-deoxy-D-glucose	y	y	125
Methyl-a-D-glucopyranoside	y	y	250
Methyl-a-D-mannopyranoside	y	y	125
N-acetylneuraminic acid	x	x	25
Glycoconjugates (μg/mL)			
Fetuin	39	156	10
Fetuin desialized	156	39	5
Bovine submaxillary mucin	156	78	39
Bovine submaxillary mucin desialized	z	z	20
Ovomucoid	z	z	312
Ovalbumin	z	z	156
Gelatine	z	30	10
Heparin	z	z	1
LPS *E. coli* K235	2	10	5
LPS *S. typhosa*	625	312	z

Note: LPS: lipopolysaccharide; x = $\geqslant$100mM; y = $\geqslant$250mM; z = $\geqslant$625 μg/mL.

Without inhibition effect in HIT using both rabbit and pig RBCs: D-glucose, D-galactose, D-mannose, L-fucose, D-arabinose, D-tagatose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, N-acetyl-D-mannosamine, lactose, maltose, fructose, cellobiose, mellibiose, stachyose, dulcit, inositol, hyaluronic acid, laminarin and peroxidase.

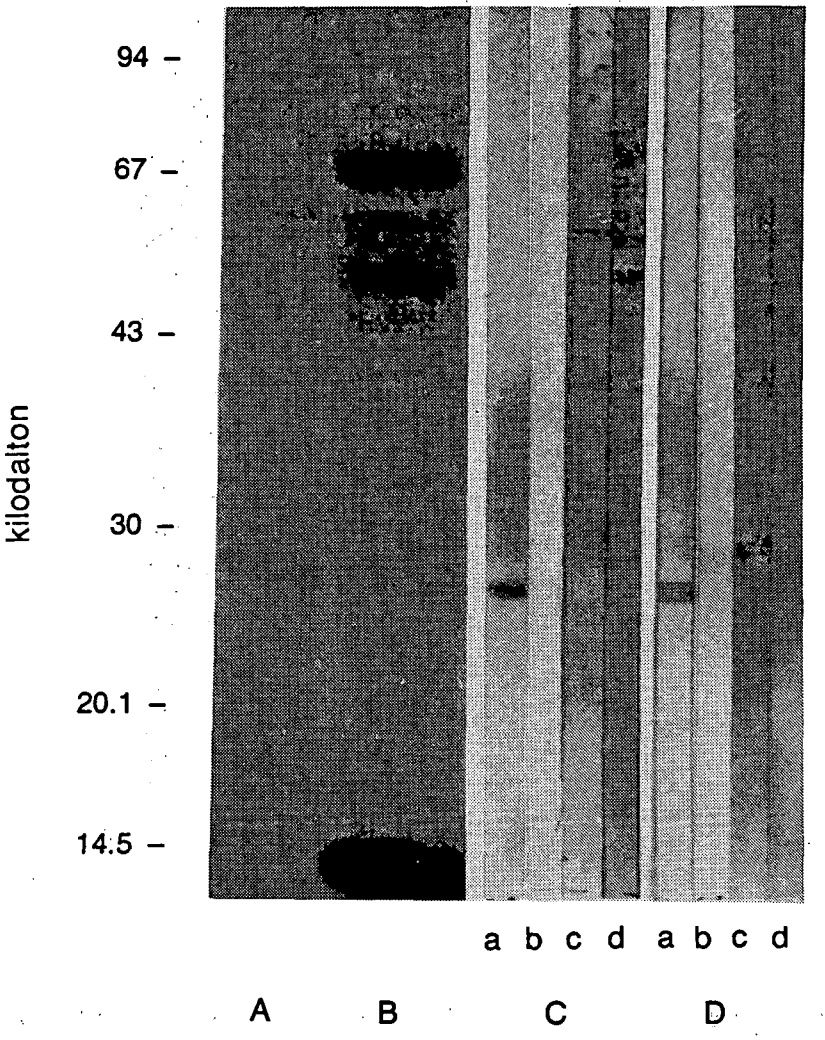

FIG. 1. SDS-PAGE analysis of G. tachinoides *midgut extracts from unfed flies (A) and from flies four days after feeding (B). Immuno- and affinoblotting analyses of the same extracts (C, D) with: (a) rabbit antiserum directed against the haemagglutination activity of* G. tachinoides *midgut; (b) control rabbit serum; (c) lectin Con A; and (d) lectin PSA.*

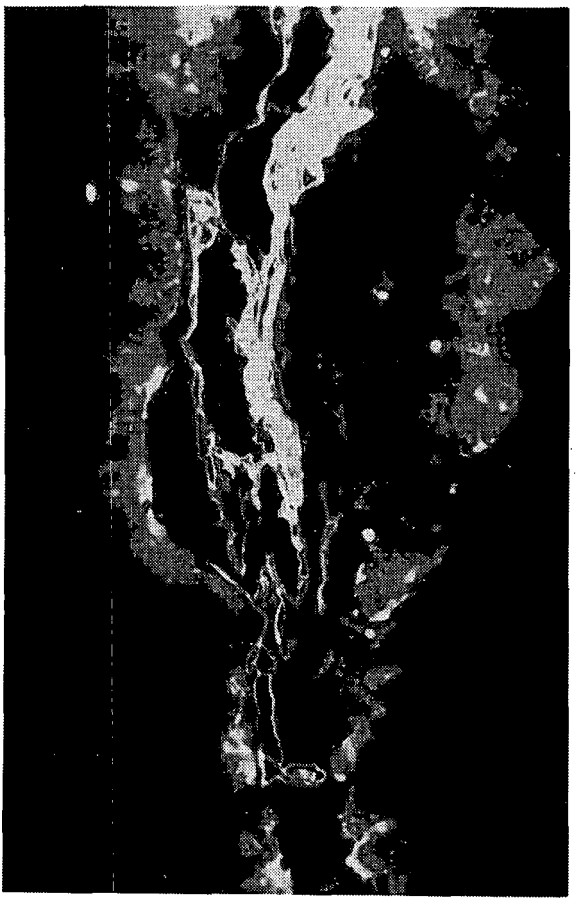

FIG. 2. Indirect immunofluorescence assay of G. tachinoides *midgut with rabbit antibodies directed against the* G. tachinoides *midgut lectin activity.*

lipopolysaccharide (LPS) from *E. coli* K 235 (with RBCs of both species) and glycoprotein heparin (with pig RBCs only). On the other hand, only a weak inhibition occurred with *Salmonella typhosa* LPS (Table I). Using rabbit RBCs, no qualitative differences were found between the binding specificity of midgut extracts from teneral and non-teneral flies of both sexes (Table I).

SDS–PAGE and blotting techniques were used for the identification and characterization of lectin components. Only one polypeptide subunit of 27 kilodalton was detected using immunoblotting with rabbit antibodies directed against the haemagglutination activity of *G. tachinoides* midgut (Fig. 1). The 27 kilodalton protein component did not react with the plant lectins in affinoblotting.

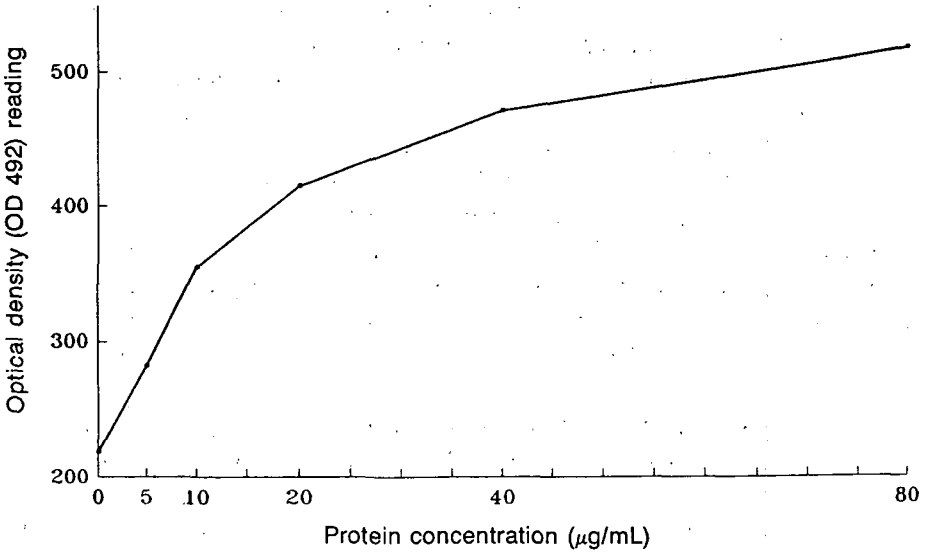

FIG. 3. Sandwich ELISA with G. tachinoides *midgut extract. Standard curve. The optical density (OD 492) reading was plotted against the protein concentration in the sample.*

In an indirect immunofluorescence assay, rabbit serum directed against HA of the *G. tachinoides* midgut extract reacted with the inner surface of the midgut epithelium and with the peritrophic membrane (Fig. 2). Control negative rabbit sera did not react with tsetse midgut tissue.

In sandwich ELISA, the optical density of the samples was dependent (non-linearly) on the protein concentration (Fig. 3).

4. DISCUSSION

In the present study, high haemagglutinin activity was found in *G. tachinoides* midgut. Contrary to results reported for midgut lectin of *G. m. morsitans* [9], lectin activity was detected also in teneral flies, although in the lower titres.

The specificity of the carbohydrate binding activity was very similar to that of *G. tachinoides* hindgut lectin reported by Ingram and Molyneux [10] against human RBCs. D-glucosamine, D-mannosamine, D-galactosamine and 2-deoxyglucose were effective inhibitors in both cases. In our study, inhibition occurred also with trehalose (using rabbit RBCs only), sialic acid, methyl mannoside and methyl glucoside (using pig RBCs only). In contrast, the agglutination activity of hindgut lectin against human RBCs was inhibited by low concentrations of N-acetyl-D-galactosamine and L-fucose [10].

The *G. m. morsitans* midgut lectin responsible for the stimulation of trypanosome maturation [2] was also inhibited with amino, N-acetyl and methyl derivates of glucose and mannose [10]. This suggests that *G. tachinoides* midgut lectin could play a similar role in the life-cycle of the transmitted trypanosomes.

The LPS of *E. coli* K 235 was the most effective inhibitor of *G. tachinoides* midgut lectin. As LPS is a typical surface component of the cell wall of gramnegative bacteria, the strong specificity of the *Glossina* lectin suggests its possible role in defence against microbial infections. The lectin is secreted to the midgut lumen, possibly in order to combat the microorganisms contaminating the ingested blood. Great differences in the inhibitory effects of LPS from two bacteria species may reflect the differences in LPS composition known to occur within gramnegative bacteria [11].

The LPS binding proteins have also been found in other insects, namely in the haemolymph of *Periplaneta americana* [12] and the midgut of *Triatoma infestans* [13].

The *G. tachinoides* lectin consists of a 27 kilodalton protein component(s) that is(are) not glycosylated. To the best of our knowledge, the structural characterizations of tsetse lectin have previously been reported only by Ingram and Molyneux [14] and Stiles et al. [15]. Lectin of *G. fuscipes fuscipes* haemolymph with a native molecule of 710 kilodalton comprised approximately ten non-covalently linked 70 kilodalton subunits; *G. palpalis* midgut agglutinin has a subunit of 67 kilodalton.

The preliminary results obtained with sandwich ELISA suggest that the method is a promising tool for quantifying tsetse lectins. We are now working on other assay modifications which should improve its sensibility and permit evaluation of antigenic similarities of lectins in different populations or species of tsetse flies.

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INFLUENCE COMBINEE DE LA BASSE TEMPERATURE ET DE L'IRRADIATION DURANT LA DERNIERE PHASE PUPALE SUR LA CAPACITE VECTORIELLE DES MALES DE *Glossina palpalis gambiensis*

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Abstract-Résumé

COMBINED INFLUENCE OF LOW TEMPERATURE AND IRRADIATION DURING THE LAST PUPAL PHASE ON THE VECTORIAL CAPACITY OF *Glossina palpalis gambiensis* MALES.

Glossina palpalis gambiensis pupae 25, 28 or 30 days old irradiated under ambient conditions with doses of 60 Gy, 80 Gy or 100 Gy (1 Gy = 100 rad) were exposed (or not exposed) for five days at 15°C (or incubated) before or after irradiation. The adult flies, whether in the teneral state or not, were fed for three successive days on animals infected with *Trypanosoma vivax, T. congolense* or *T. b. brucei* at the peak of parasitemia. As of the tenth day (post-infection), the rates of infection with *T. vivax* for flies in the teneral and the non-teneral state were, respectively: 98.9% and 97.9%, for the control group and 96.8% and 95.7% for those incubated and irradiated with 100 Gy on day 30. As of the twentieth day, the percentages of infection with *T. congolense* were, respectively, for the different states (teneral and non-teneral): 77.5% and 34.7% for the controls, 71.3% and 54.2% for the incubated group, 83.9% and 50.0% for those irradiated with 80 Gy on day 30, and 58.2% and 64.8% for those irradiated with 100 Gy on day 30. The rates of infection of the flies with *T. b. brucei* as of the twenty-fifth day were, respectively, for flies infected in the teneral state and in the non-teneral state: 91.1% and 54.7% for the controls, 77.0% and 44.9% for the incubated group, 51.3% and 35.0% for those irradiated with 80 Gy on day 30 and 73.1% and 69.5% for those irradiated with 80 Gy on day 28 and incubated. The percentages of sterility

† Décédé.

in males irradiated with various doses are: 71% (60 Gy on day 25), 75% (incubated and 60 Gy on day 25), 82% (incubated and 60 Gy on day 28), 100% (80 Gy on day 28) and 100% (100 Gy on day 30).

INFLUENCE COMBINEE DE LA BASSE TEMPERATURE ET DE L'IRRADIATION DURANT LA DERNIERE PHASE PUPALE SUR LA CAPACITE VECTORIELLE DES MALES DE *Glossina palpalis gambiensis*.

Des pupes, âgées de 25, 28 ou 30 jours, de *Glossina palpalis gambiensis* irradiées dans les conditions ambiantes à la dose de 60 Gy, 80 Gy ou 100 Gy (1 Gy = 100 rad), ont été exposées (ou non) durant 5 jours à 15°C (ou incubées) avant ou après irradiation. Les mouches adultes sont alimentées, à l'état ténéral ou à l'état non ténéral, pendant trois jours successifs sur des animaux infectés par *Trypanosoma vivax*, *T. congolense* ou *T. b. brucei* au pic de la parasitémie. A partir du 10^{e} jour (post-infection), les taux d'infection des mouches par *T. vivax* à l'état ténéral ou à l'état non ténéral sont respectivement: 98,9% et 97,9% pour le lot témoin, 96,8% et 95,7% pour les incubées et irradiées à 100 Gy au jour 30. A partir du 20^{e} jour, les pourcentages d'infection par *T. congolense* sont respectivement pour les différents états (ténéral et non ténéral): 77,5 et 34,7 pour les témoins; 71,3 et 54,2 pour les incubées; 83,9 et 50,0 pour les irradiées à 80 Gy au jour 30; 58,2 et 64,8 pour les irradiées à 100 Gy au jour 30. Les taux d'infection des glossines par *T. b. brucei* à partir du 25^{e} jour sont respectivement pour les mouches infectées à l'état ténéral et à l'état non ténéral: 91,1% et 54,7% pour les témoins; 77,0% et 44,9% pour les incubées; 51,3% et 35,0% pour les irradiées à 80 Gy au jour 30; 73,1% et 69,5% pour les irradiées à 80 Gy au jour 28 et incubées. Les pourcentages de stérilité des mâles irradiés aux différentes doses sont: 71 (60 Gy au jour 25), 75 (incubées et 60 Gy au jour 25), 82 (incubées et 60 Gy au jour 28), 100 (80 Gy au jour 28), 100 (100 Gy au jour 30).

1. INTRODUCTION

Le Laboratoire central vétérinaire de Bamako envisage d'utiliser la technique de l'insecte stérile, complétée par des traitements insecticides pour lutter contre la mouche tsé-tsé dans la zone agro-pastorale de Tienfala-Baguineda, et de déterminer à quelles conditions son éradication serait possible. L'entreprise est réalisable car il n'existe qu'une seule espèce de glossine (*Glossina palpalis gambiensis*) et la zone à traiter est isolée par des barrières naturelles. Il est programmé de lâcher des mâles stériles nourris et marqués sur place, issus de pupes irradiées en provenance du Centre de recherches sur les trypanosomoses animales (CRTA) de Bobo-Dioulasso.

Après des études préparatoires à l'Unité d'entomologie de la Division mixte FAO/AIEA (Laboratoire de Seibersdorf), des séries d'expériences ont été realisées au CRTA. L'objectif de ces expériences a été de déterminer une dose optimale d'irradiation des pupes et de connaître l'impact de la conservation à basse température ainsi que celui de l'état de réplétion des adultes avant l'infection sur le risque de la transmission des différentes espèces de trypanosomes pathogènes.

2. MATERIELS ET METHODES

Des pupes de *Glossina palpalis gambiensis* âgées de 25, 28 ou 30 jours ont été irradiées à l'aide d'un irradiateur gamma (^{137}Cs) dans les conditions ambiantes aux doses de 60 Gy, 80 Gy ou 100 Gy (1 Gy = 100 rad). Elles ont été également exposées (ou non) durant 5 jours dans un incubateur du type LKB Mini Coldlab réglé à 15 ± 1,5°C et 90% d'humidité relative, immédiatement avant ou après l'irradiation.

Après éclosion, les mouches mâles sont groupées dans des cages du type Roubeau à raison de 25–30 individus par cage et alimentées, durant 5 min, pendant trois jours successifs sur des animaux infectés: une chèvre pour *T. vivax* et des lapins pour *T. congolense* et *T. brucei*, tous au pic de la parasitémie. Dans chaque lot, deux catégories de mouches, dont le nombre varie de 100 à 120, sont infectées à différents états de réplétion: à l'état ténéral avec trois repas infectants aux jours 2, 3 et 4 post-émergence ou à l'état non ténéral avec deux repas ordinaires in vitro sur membrane de silicone aux jours 2 et 3 post-émergence, suivis de trois repas infectants aux jours 4, 5 et 6.

Dans le cas spécifique de *T. vivax*, deux lapins ont été infectés sans succès avec la souche Zaria 81/486/699. Le premier a éliminé l'infection (2 trypanosomes/champ) avant l'émergence des mouches, et les quelque 1000 mouches nourries sur le second, avec un pic de parasitémie de 8 trypanosomes/champ, n'ont pas été infectées. Une chèvre sahélienne a été directement inoculée par voie intraveineuse par une seconde souche (Banan 85/CRTA/78, précédant une administration de Dexamethason.

La parasitémie la plus élevée a été de 80 trypanosomes/champ, mais la chèvre est morte avant la fin de l'expérience (20^{e} jour post-infection, 40 trypanosomes/champ, hématocrite = 17%). C'est pour cette raison que certains lots ont reçu 1 ou 2 repas infectants et que d'autres n'en ont pas reçu du tout. Après les trois repas infectants, toutes les mouches sont alimentées tous les jours, durant 5 min, sur membrane à silicone avec du sang de bœuf hépariné, irradié et enrichi de glucose et d'adénosine triphosphate, et gardées dans une salle à la température de 24–26°C avec 75–85% d'humidité relative.

La mortalité est vérifiée avant les repas infectants et suivie tous les 5 jours. Les mouches infectées par *T. vivax* sont disséquées et examinées à l'aide d'une loupe binoculaire et d'un microscope, à partir du 10^{e} jour; celles qui sont infectées par *T. congolense* et *T. brucei* sont examinées respectivement à partir des jours 20 et 25 après le premier repas infectant.

L'étude de l'effet des différents traitements sur la fertilité a concerné 16 lots de mouches, constitué chacun de 30 femelles âgées de 2 jours, accouplées durant 3 jours avec 30 mâles âgés de 7 jours. Après séparation, la production des pupes et les avortements sont suivis chez les femelles jusqu'au 35^{e} jour.

TABLEAU I. DONNEES SUR L'EMERGENCE DE *Glossina palpalis gambiensis* APRES L'ACTION COMBINEE DE L'IRRADIATION ET DE L'EXPOSITION DES PUPES A BASSE TEMPERATURE (mars-mai 1990, nombre total = 36 285 pupes)

Séries	Traitement	Emergence				
		Pupes (Nb)	Total (%)	Mâles (%)	Durée pupaison (j)	
					Femelles	Mâles
A	Témoins	3444	95,6	53,4	28,8	31,2
B	15°C J20–25	3701	94,2	50,0	32,7 35,8	34,6 37,8
C	60 Gy J25	3189	91,2	47,5	28,2 31,2	30,1 33,0
D	60 Gy J28	2969	92,6	50,8	27,8 29,0	29,9 31,3
E	80 Gy J28	3146	91,9	48,6	27,8 31,1	29,9 33,2
F	80 Gy J30	3012	91,2	49,0	*	29,8 31,3
G	100 Gy J30	2216	92,6	49,6	*	29,1*
H	15°C J20–25 /60 Gy J25	2596	83,2	47,9	32,8 34,1	34,5 36,2
I	15°C J20–25 /60 Gy J28	2651	87,5	49,6	32,5 34,0	34,3 36,2
J	15°C J20–25 /80 Gy J28	2504	92,4	52,2	32,8 33,9	34,5 36,2
K	80 Gy J28 /15°C J28–33	2646	82,9	50,3	29,0 32,6	34,4 35,8
L	80 Gy J30 /15°C J30–35	2691	84,0	45,1	*	30,8 33,9
M	100 Gy J30 /15°C J30–35	1920	80,5	48,0	*	30,8

*: Une partie importante des mouches a émergé avant le second traitement.

J: Jours post-émergence. Basse température: 15°C.

Irradiation: 60, 80 ou 100 Gy (1 Gy = 100 rad).

Note: Les premiers nombres figurant dans les colonnes 6 et 7 indiquent le nombre de jours pour les pupes pondues en mars 1990, les seconds se réfèrent au mois de mai.

Pour les tests de compétitivité, des mâles traités, incubés ou irradiés ont été marqués et mis dans une cage d'éclosion avec des témoins (25:25, à 4 reprises) en présence du même nombre de femelles vierges. Les couples formés ont été immédiatement prélevés à l'aide d'un tube à essais, puis comptabilisés.

3. RESULTATS

3.1. Emergence des glossines (Tableau I)

Le taux moyen d'émergence des glossines est de 90,76%. Les pourcentages observés dans les différents lots sont: 95,6 pour les témoins, 94,2 pour les incubées, 91,8 pour les irradiées, 92,4 pour les incubées et irradiées et 82,6 pour les irradiées et incubées. L'incubation ou exposition à 15°C durant 5 jours augmente la durée de la vie pupale d'environ 4 jours; les moyennes enregistrées au mois d'avril 1990 sont respectivement pour les femelles et les mâles: 28,8 et 31,2 jours pour les témoins, contre 33,3 et 35,6 jours pour les incubées. Des variations ont été constatées suivant les mois de ponte, en effet chez les incubées, la vie pupale a une durée moyenne de 35,8 et 37,8 jours respectivement pour les femelles et les mâles issus de pupes pondues au mois de mars, contre 32,7 et 34,6 jours au mois de mai. D'une manière générale, les femelles émergent 2 jours avant les mâles, et l'irradiation provoque une émergence massive chez les pupes âgées.

3.2. Infections par les espèces de trypanosomes

3.2.1. *Trypanosoma vivax* (Banan 85/CRTA/78, Tableau II)

Les infections par cette espèce se limitent à la trompe des glossines: au labre et à l'hypopharynx. En général, les mâles nourris à l'état ténéral sur l'animal infecté par *T. vivax* n'ont pas un taux d'infection différent d'une manière significative de ceux qui ont pris leurs repas infectants à l'état non ténéral.

Les taux observés dans le lot témoin sont respectivement 98,8% et 97,9%, contre 96,7% et 95,6% dans le lot des pupes exposées à 15°C du jour 20 au jour 25 et irradiées à 80 Gy au 28^{e} jour. Cependant, les taux d'infection suivants 67,3% (ténéral) et 71,2% (non ténéral) observés dans le lot des pupes irradiées à 80 Gy au jour 28 et incubées sont inférieurs d'une manière significative aux taux des lots précédents. L'action de maintenir les pupes à basse température (à 15°C durant 5 jours) après irradiation semble diminuer le taux d'infection à *T. vivax* par rapport au témoin et au lot des pupes incubées et irradiées. Nous avons également constaté que le taux d'infection à *T. vivax* et la charge parasitaire augmentent avec le nombre de repas infectants, et que les mouches peuvent perdre l'infection avec le temps.

TABLEAU II. TAUX D'INFECTION DE *Glossina palpalis gambiensis* PAR *Trypanosoma vivax* (Banan 85/CRTA/78)

Traitement	Etat	Effectifs (Nb)	Repas infectants (Nb)	Survie au jour 10 (Nb)	Dissections (Nb)	Infection au labre et à l'hypopharynx (Nb)	(%)
Témoins	Ténéral	99	3	89	88	87	98,9
	Non ténéral	100	3	98	97	95	97,9
15°C J20-25	Ténéral	—	—	—	—	—	
	Non ténéral	100	1-2	91	90	79	87,8
60 Gy J25	Ténéral	100	1-2	94	93	80	86,0
	Non ténéral	95	1-2	91	90	82	91,1
60 Gy J28	Ténéral	—	—	—	—	—	
	Non ténéral	100	1	90	90	77	85,5
80 Gy J28	Ténéral	99	1-3	95	86	66	76,7
	Non ténéral	100	1-2	93	86	79	91,8
80 Gy J30	Ténéral	—	—	—	—	—	
	Non ténéral	60	1	51	51	39	76,5
100 Gy J30	Ténéral	—	—	—	—	—	
	Non ténéral	100	1	83	80	57	71,2
15°C J20-25 /60 Gy J25	Ténéral	99	3	87	82	71	86,6
	Non ténéral	100	3	87	86	59	68,6
15°C J20-25 /60 Gy J28	Ténéral	98	3	89	85	82	96,5
	Non ténéral	100	3	96	92	90	97,8
15°C J20-25 /80 Gy J28	Ténéral	100	3	94	93	90	96,8
	Non ténéral	104	3	94	93	89	95,7
80 Gy J28 /15°C J28-33	Ténéral	100	3	92	92	62	67,4
	Non ténéral	100	3	82	80	57	71,2

—: Les observations n'ont pas été faites, la chèvre étant morte avant la fin de l'expérience.
J: Jours post-émergence. Basse température: 15°C.
Irradiation: 60, 80 ou 100 Gy (1 Gy = 100 rad).

3.2.2. *Trypanosoma congolense* (Karankasso 83/CRTA/57, Tableau III)

Le développement de cette espèce commence au niveau de l'instestin moyen de la mouche avant d'envahir la trompe (le labre et l'hypopharynx). En général, les mâles qui ont pris leurs premiers repas infectants à l'état ténéral ont un taux d'infection supérieur à celui des mâles infectés à l'état non ténéral. Les pourcentages observés dans les différents lots sont respectivement: 77,5 et 34,6 pour les témoins, 71,2 et 54,2 pour les incubées, 59,7 et 23,5 (80 Gy au jour 28), 83,8 et 50,0 (80 Gy au jour 30). En revanche, les 58,2% et 64,8% trouvés chez les pupes irradiées à 100 Gy au jour 30 ne diffèrent pas d'une manière significative (écart réduit = 0,76).

La majeure partie (plus de 80%) des infections à *T. congolense* restent bloquées au niveau l'intestin moyen, n'atteignant donc pas la trompe. Une étude comparative a révélé que l'espèce de glossine riveraine (*G. p. gambiensis*: 89,74%, 70/78) s'infecte autant et même un peu plus que l'espèce de savane (*G. morsitans submorsitans*: 79,48%, 62/78); la différence n'est pas significative, écart réduit = 1,77. Cependant, *G. p. gambiensis* est un mauvais vecteur de *T. congolense*, car seules 27,77% et 7,14% des infections initiales de l'intestin atteignent respectivement le labre et l'hypopharynx, contre 91,93% et 82,25% chez *G. m. submorsitans* (écart réduit = 8,40 pour le labre).

3.2.3. *Trypanosoma brucei brucei* (Farakoba/80/CRTA/1, Tableau IV)

Les trypanosomes de cette espèce se multiplient d'abord dans l'intestin moyen de la glossine, avant de migrer vers les glandes salivaires et l'hypopharynx. A l'état ténéral les mâles de *G. p. gambiensis* contractent plus aisément les infections à *T. brucei* qu'à l'état non ténéral. Les taux d'infection observés dans les différents lots sont respectivement: 91,1% et 54,6% pour les témoins, 77,0% et 44,8% pour les incubées, 74,2% et 24,2% (60 Gy au jour 25), 63,7% et 50,0% (incubées et 60 Gy au jour 25). En revanche, chez les mouches irradiées à 100 Gy au jour 30, celles qui sont infectées à l'état ténéral ont un taux d'infection de 34,1% qui ne diffère pas d'une manière significative de celui des mouches infectées à l'état non ténéral (35,7%); écart réduit = 0,19.

Sur 799 infections de l'intestin moyen dues à *T. brucei*, 284 (35,5%) et 62 (7,7%) seulement ont évolué respectivement au niveau des glandes salivaires et de l'hypopharynx. Les taux de survie au 25^{e} jour des mâles infectés à l'état ténéral sont de 85,8% pour les incubées, de 59,0% pour les irradiées et de 77,5% pour les incubées et irradiées.

TABLEAU III. TAUX D'INFECTION DE *Glossina palpalis gambiensis* PAR *Trypanosoma congolense* (Karankasso 83/CRTA/57)

Traitement	Etat	Effectif (Nb)	Survie J20 (Nb)	(%)	Dissections (Nb)	Taux d'infection Intestin moyen (%)	Labre (%)
Témoins	Ténéral	100	97	97,0	80	77,5	1,2
	Non ténéral	100	83	83,0	75	34,7	1,3
15°C J20–25	Ténéral	99	91	91,9	87	71,3	2,3
	Non ténéral	99	91	91,9	83	54,2	3,6
60 Gy J25	Ténéral	125	79	63,2	72	66,7	2,8
	Non ténéral	121	90	74,4	73	37,0	4,1
60 Gy J28	Ténéral	120	62	51,7	61	75,4	3,3
	Non ténéral	120	77	64,1	62	66,1	16,1
80 Gy J28	Ténéral	100	92	92,0	72	59,7	1,4
	Non ténéral	163	116	71,2	85	23,5	3,5
80 Gy J30	Ténéral	110	90	81,8	62	83,9	14,5
	Non ténéral	100	52	52,0	52	50,0	7,7
100 Gy J30	Ténéral	120	101	84,2	79	58,2	10,1
	Non ténéral	110	72	65,4	54	64,8	18,5

Basse température: 15°C; J: jours post-émergence.
Irradiation: 60, 80 ou 100 Gy (1 Gy = 100 rad).

TABLEAU IV. TAUX D'INFECTION DE *Glossina palpalis gambiensis* PAR *Trypanosoma brucei brucei* (Farakoba 83/CRTA/57)

Traitement	Etat	Effectif (Nb)	Survie J25 (Nb)	(%)	Dissections (Nb)	Taux d'infection Intestin moyen (%)	Glandes salivaires (%)
Témoins	Ténéral	99	85	85,9	79	91,1	25,3
	Non ténéral	102	83	81,4	75	54,7	10,7
15°C J20–25	Ténéral	99	83	83,8	74	77,0	12,2
	Non ténéral	100	81	81,0	78	44,9	6,4
60 Gy J25	Ténéral	117	75	64,1	66	74,2	19,7
	Non ténéral	117	76	64,9	70	24,3	1,4
60 Gy J28	Ténéral	120	52	43,3	47	57,4	27,6
	Non ténéral	120	50	41,7	50	38,0	28,0
80 Gy J28	Ténéral	116	63	54,3	50	66,0	18,0
	Non ténéral	139	58	41,7	52	34,6	7,7
80 Gy J30	Ténéral	120	78	65,0	78	51,3	35,9
	Non ténéral	105	40	38,1	40	35,0	12,5
100 Gy J30	Ténéral	120	82	68,3	82	34,1	15,8
	Non ténéral	120	57	47,5	56	35,7	17,8
15°C J20–25 /60 Gy J25	Ténéral	120	84	70,0	69	63,8	30,4
	Non ténéral	120	61	50,8	60	50,0	16,7
15°C J20–25 /60 Gy J28	Ténéral	120	102	85,0	67	62,7	26,9
	Non ténéral	120	110	91,7	72	44,4	18,0
15°C J20–25 /80 Gy J28	Ténéral	120	93	77,5	65	67,7	29,2
	Non ténéral	120	88	73,3	75	41,3	22,7
80 Gy J28 /15°C J28–33	Ténéral	120	55	45,8	52	73,1	23,1
	Non ténéral	120	62	51,7	59	69,5	23,7
80 Gy J30 /15°C J30–35	Ténéral	—	—		—	—	—
	Non ténéral	75	36	48,0	36	50,0	16,7
100 Gy J30 /15°C J30–35	Ténéral	—	—		—	—	—
	Non ténéral	58	18	31,0	18	50,0	11,1

Basse température: 15°C. J: Jours post-émergence.
Irradiation: 60, 80 ou 100 Gy (1 Gy = 100 rad).

TABLEAU V. FERTILITE DES FEMELLES DE *Glossina palpalis gambiensis* INSEMINEES PAR DES MALES IRRADIES AU STADE PUPAL ET/OU EXPOSES A BASSE TEMPERATURE (Accouplement: 30 mâles/30 femelles)

Traitement		Survie femelles		Pupes		Avortements		Taux de stérilité
Femelles	Mâles	J25 (Nb)	J35 (Nb)	Total (Nb)	Par femelle	Total (Nb)	Par femelle	(%)
Témoin	Témoin	27	26	52	2,0	6	0,2	0,0
15°C J20–25	Témoin	29	29	66	2,3	5	0,2	-35,0
Témoin	15°C J20–25	24	23	42	1,8	7	0,3	9,0
15°C J20–25	15°C J20–25	29	29	65	2,2	4	0,1	-12,0
Témoin	60 Gy J25	29	29	17	0,6	47	1,6	71,0
Témoin	15°C J20–25 /60 Gy J25	29	28	14	0,5	64	2,3	75,0
Témoin	60 Gy J28	28	28	18	0,6	62	2,2	68,0
Témoin	15°C J20–25 /60 Gy J28	25	25	9	0,4	63	2,5	82,0
Témoin	80 Gy J28	22	21	0	0,0	66	3,1	100
Témoin	15°C J20–25 /80 Gy J28	30	30	7	0,2	67	2,2	88,5
Témoin	80 Gy J28 /15°C J28–33	24	24	1	0,4	65	2,7	98,0
Témoin	80 Gy J30 (N = 22)	26	26 /23	6	0,3	64	2,5	87,0
Témoin	80 Gy J30 15°C J30–35	19	18	2	0,1	49	2,7	94,5
Témoin	100 Gy J30	26	25	0	0,0	53	2,1	100
60 Gy J25	Témoin	23	23	0	0,0	29	2,3	100
80 Gy J28	Témoin	25	24	0	0,0	33	1,4	100

Basse température: 15°C. J: Jours post-émergence.
Irradiation: 60, 80 ou 100 Gy (1 Gy = 100 rad). % de stérilité (-) = plus fertile que le témoin.
26/23: 26 femelles dont 23 inséminées.
Note: Le signe - dans la neuvième colonne signifie une fertilité supérieure à celle du témoin.

3.3. Effets de l'incubation et/ou de l'irradiation sur la fertilité des mouches (Tableau V)

Les femelles de *G. p. gambiensis* non traitées (témoins) ou incubées, accouplées avec des mâles témoins, ont produit en moyenne 2 à 2,27 pupes par femelle. Les mâles incubés ont donné avec les femelles témoins ou incubées, respectivement, 1,82, et 2,24 pupes par femelle. Les femelles inséminées par des mâles irradiés au stade pupal ont donné un maximum de 0,64 pupes/femelle avec la dose de 60 Gy au 28^{e} jour. La production des pupes a été nulle chez les femelles accouplées avec les mâles irradiés à 80 Gy au jour 28 ou à 100 Gy au jour 30. Toutes les femelles irradiées (60 Gy au jour 25, ou 80 Gy au jour 28) sont devenues complètement improductives après 2,26 ou 1,37 avortements/femelle.

Les pourcentages de stérilité des mâles irradiés aux différentes doses sont: 71 (60 Gy J25), 75 (incubés et 60 Gy J25), 68 (60 Gy J28), 82 (incubés et 60 Gy J28) , 100 (80 Gy J28), 88,5 (incubés et 80 Gy J28), 100 (100 Gy J30).

Les tests de compétitivité réalisés entre les mâles traités et les témoins ont révélé que les premiers sont très combatifs pour la recherche des femelles. Les pourcentages d'élection enregistrés pour les mâles traités sont: 53 incubées, 57 (60 Gy J25), 51 (60 Gy J28), 47 (80 Gy J28), 50 (80 Gy J30) et 52 (100 Gy J30).

4. CONCLUSION

Les résultats obtenus montrent que les mâles de *G. p. gambiensis* irradiés au stade pupal ont une longévité leur permettant de transmettre toutes les espèces de trypanosomes pathogènes. L'action de nourrir les mâles avant de les lâcher réduit considérablement les risques d'infections par *T. congolense* et *T. b. brucei*, et les rend plus compétitifs, car ils vont directement à la recherche des femelles.

La technique de lâchers de mâles stériles est une méthode d'avenir qui est déjà compétitive du point de vue coût grâce à la maîtrise parfaite de l'élevage en masse des glossines. Il n'y a pas de doute que la lutte génétique demeure la seule méthode capable d'éradiquer une population de glossines sans porter préjudice à l'environnement.

Le lâcher et la recapture des femelles irradiées sont les moyens les plus fiables pour la détection d'une population résiduelle de glossines et la détermination très fine de ses limites de répartition.

La lutte génétique est envisageable d'ores et déjà dans les pays ou régions qui ne possédent ni insectarium, ni source gamma, mais disposent d'un modeste laboratoire nécessaire pour l'éclosion des pupes et la maintenance des adultes in vivo avant les lâchers.

Le maintien des pupes à basse température durant le transport est sans inconvénient. La voie est déjà bien ouverte pour les lâchers de mâles stériles sans

risque de transmission de la trypanosomiase. Des recherches sur le délai, la durée et l'intensité de l'exposition à basse température pourront probablement réduire l'infection par *T. vivax*, et l'augmentation du nombre de repas avant le lâcher des mâles stériles rendrait très peu probable les infections par *T. congolense* et *T. brucei*. En effet, trois repas pris sur membrane avant un seul repas infectant a réduit le taux d'infection de *G. p. gambiensis* par *T. b. brucei* à 26,66%, contre 59,61% (ténéral et un seul repas infectant) et 91,13% (ténéral et 3 repas infectants).

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REARING TSETSE FLIES FOR USE IN STERILE INSECT TECHNIQUE VECTOR CONTROL PROGRAMMES

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Abstract

REARING TSETSE FLIES FOR USE IN STERILE INSECT TECHNIQUE VECTOR CONTROL PROGRAMMES.

In past years, considerable progress has been achieved in the mass rearing of tsetse flies for use in vector control programmes. This was made possible through the introduction of the membrane feeding system and the development of strict quality control procedures for different developmental stages of tsetse and for various aspects that are relevant to insect production, such as blood diet quality screening. The maintenance of large tsetse fly colonies, e.g. more than 100 000 producing females that may provide more than 12 000 reproductively sterile males and equal numbers of surplus female material, is feasible without major efforts. Calculations of labour requirements and the costs involved for different mass rearing systems can now be conducted. Depending on the purpose for which the insects are reared, different types of mass rearing facilities may be erected (a small stationary insect factory, large breeding centre or mobile production plant). Sexually sterile tsetse from a mass rearing system may be used for different reasons in tsetse control or eradication campaigns, namely for eradication or control through the sterile insect technique, for ecological monitoring of a target tsetse population, particularly if it is under advanced control, and for transtaxon control of closely related species (or subspecies). Bottlenecks for a larger scale field use of mass reared tsetse remain for the time being: (a) the relatively long process of strain adaptation to mass rearing conditions, (b) laborious sexing procedures of mature insects upon emergence and after mating and (c) unavailability of simple genetic tools to collect baseline information on the suitability of a mass reared strain to combat an identified target population and to assess the degree of isolation of a target population.

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1. INTRODUCTION

Tsetse flies are larviparous insects with a very low reproductive capacity. During its three to four month lifespan, a female produces on average only five to seven offspring, hence the low natural population density of tsetse. In spite of this, many tsetse fly species are effective transmitters of pathogenic *Trypanosoma* species, and *Glossina* control in many regions of Africa is a prerequisite for sustainable agricultural production. The use of conventional area wide control methods, namely aerial spraying of insecticides, has been reduced in the past years because of (a) relatively high expense; (b) insufficient effectiveness against tsetse fly populations in densely vegetated habitats; and (c) environmental concerns resulting in legislative restrictions. Currently more favoured methods of tsetse control are field-by-field approaches that involve the participation of local communities for the application of insecticides in combination with attractive devices. Although involvement of the inhabitants of the region that is affected by trypanosomiasis is generally beneficial for the smooth conduct of vector control operations, any insufficiently controlled use of insecticides and trypanocides by individual farmers bears a considerable risk of inappropriate application and may promote the development of resistance among the trypanosome parasites and tsetse flies. The sterile insect technique (SIT) does not share the environmentally detrimental aspects of conventional area wide control programmes, because of its specificity against only the target pest insect. However, the success of SIT depends largely on the regular supply of sufficient and competitive sexually sterile insects from a mass rearing facility. This paper briefly describes the status and prospects for the mass rearing of tsetse flies and their use in vector control programmes.

2. REARING OF TSETSE FLIES

2.1. Methods of rearing tsetse flies

Both sexes of the tsetse fly are obligatory haematophagous. A variety of host animal preferences has been reported for different species [1] and there are distinct habitat requirements among various species [2]. Holding methods for tsetse flies in the laboratory have to reflect natural demands. These include the maintenance of an adequate breeding room climate, the provision of a suitable blood diet and the appropriate manipulation of different immature and adult stages for optimal colony female insemination and satisfactory fly performance. Until a few years ago, the maintenance of tsetse flies under laboratory conditions required the permanent availability of host animals for in vivo feeding. Nevertheless, fly colonies of considerable size were held using rabbits, goats or guinea pigs [3–5]. However, the maintenance of a host animal colony for in vivo feeding proved to be problematic and expen-

TABLE I. HOLDING CONDITIONS FOR TSETSE FLY SPECIES COLONIZED AT THE FAO/IAEA ENTOMOLOGY UNIT, SEIBERSDORF

Species	Pupae incubation and emergence of flies				Diet	Handling of young flies					Producing females	
	Incubation conditions	Mean female pupal period (d)	Rate of emergence (%)	% males	bovine: porcine (%)	Sexing method upon EM and SEP	Holding conditions	Feeding regimen	Age at 2 d mating (d)		Holding conditions	Feeding regimen
									Males	Females		
G. tachinoides	23°C, 82% r.h.[a]	32	93.7	48.4	75:25 (+0.1mM ATP)[b]	Immobilized at +4°C	24°C, 82% r.h.	M6	8–10	2–3	23.5°C, 80% r.h.	M4
G. p. palpalis	24°C, 88% r.h.	34	91.3	42.8	75:25 (+1mM ATP)	Immobilized at +4°C	24°C, 88% r.h.	M6	8–10	2–3	24°C, 88% r.h.	M5
G. f. fuscipes	24°C, 85% r.h.	36	91.4	50.5	75:25 (+1mM ATP)	Immobilized at +4°C	24°C, 85% r.h.	M5	8–10	2–3	24°C, 85% r.h.	M5
G. austeni	24°C, 80% r.h. (increased r.h. before EM)	34	96.0	51.1	100% bovine (+1mM ATP)	Immobilized at +4°C	24°C, 80% r.h.	M6	8–10	7–8	24°C, 80% r.h.	M5
G. pallidipes	24°C, 75% r.h.	33	77.8	50.5	100% bovine (+1mM ATP)	Immobilized at +4°C	24°C, 75% r.h.	M5	8–10	7–8	24°C, 75% r.h.	M5
G. m. submorsitans	24°C, 75% r.h.	33	89.6	32.0	75:25 (+1mM ATP)	Immobilized at +4°C	24°C, 75% r.h.	M5	8–10	2–3	24°C, 75% r.h.	M5
G. brevipalpis	24°C, 80% r.h.	36	94.7	47.7	75:25 (+1mM ATP)	Immobilized at +4°C	24°C, 80% r.h.	M5	8–10	2–3	24°C, 80% r.h.	M5

[a] r.h.: relative humidity.
[b] ATP: adenosine triphosphate.

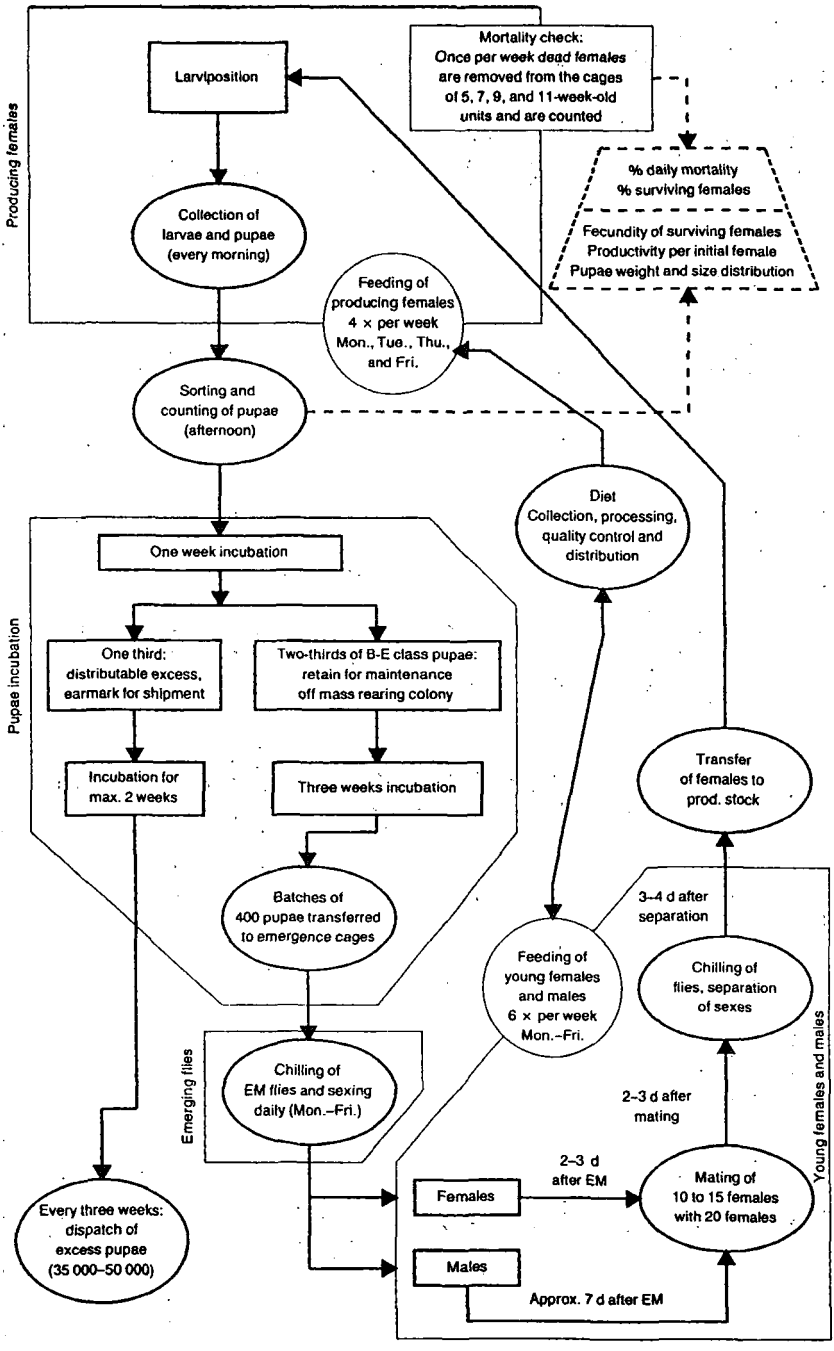

FIG. 1. Colonization of 100 000 female G. tachinoides *at the FAO/IAEA Entomology Unit, Seibersdorf. Flow chart of routine colony maintenance.*

sive [6]. The development of artificial membranes [7] and their successive modification for use with different tsetse fly species have greatly facilitated and reduced the cost for the laboratory maintenance of *Glossina*. To breed a colony of 30 000 *G. palpalis palpalis*, a host animal colony of 1000 guinea pigs (40–45% thereof are feeders, the rest being breeding stock, weaners or sick animals) needs to be maintained [6]. The same colony size, fed a quality tested diet using the in vitro feeding regimen, consumes 10–15 L of blood per week. The basic tsetse handling procedures and conditions of incubation of different developmental stages, as conducted at the FAO/IAEA Entomology Unit at the Agency's Laboratory at Seibersdorf, are summarized in Table I. The colonization procedures for mass rearing 100 000 *G. tachinoides* to produce surplus pupae for use in a SIT project in Africa are illustrated in Fig. 1.

2.2. Status of mass rearing

Self-sustaining colonies of most *Glossina* species that are of economic importance can be reared under defined laboratory conditions using the membrane feeding system [8–10]. This not only provides standard fly materials for toxicological, physiological or behavioural studies to develop more effective insecticide formulations, improve attractive devices for tsetse control or conduct basic research, but is also the basis for mass producing high quality insects for release in tsetse control or eradication programmes in Africa. Figure 2 shows the number of *Glossina* spp. pupae supplied by the FAO/IAEA Entomology Unit to technical co-operation projects in Africa and to collaborative researchers over past years. Table II [6, 8, 11–13] summarizes the achievements of membrane fed tsetse colonies that were mass reared to provide excess fly material for use in vector control programmes.

2.3. Aspects of quality control

Quality assurance needs to be maintained for various stages of tsetse fly production [14, 15].

2.3.1. Quality of the host animals and blood diet

The health of animals kept for feeding tsetse requires permanent care. For smaller scale in vivo feeding, suitable animals can be bought upon demand. Before use as hosts to feed tsetse, they require quarantine observation and treatment against diseases, particularly an effective trypanocidal treatment in case the animals originate from an area with endemic trypanosomiasis. The feed mixture and vitamin supplementation have to reflect the special holding purpose of the animals as blood donors. In order not to interfere with tsetse colony performance, the host animal feeds need to be free of insecticides, antibiotics [16] or other toxins for tsetse. Limits

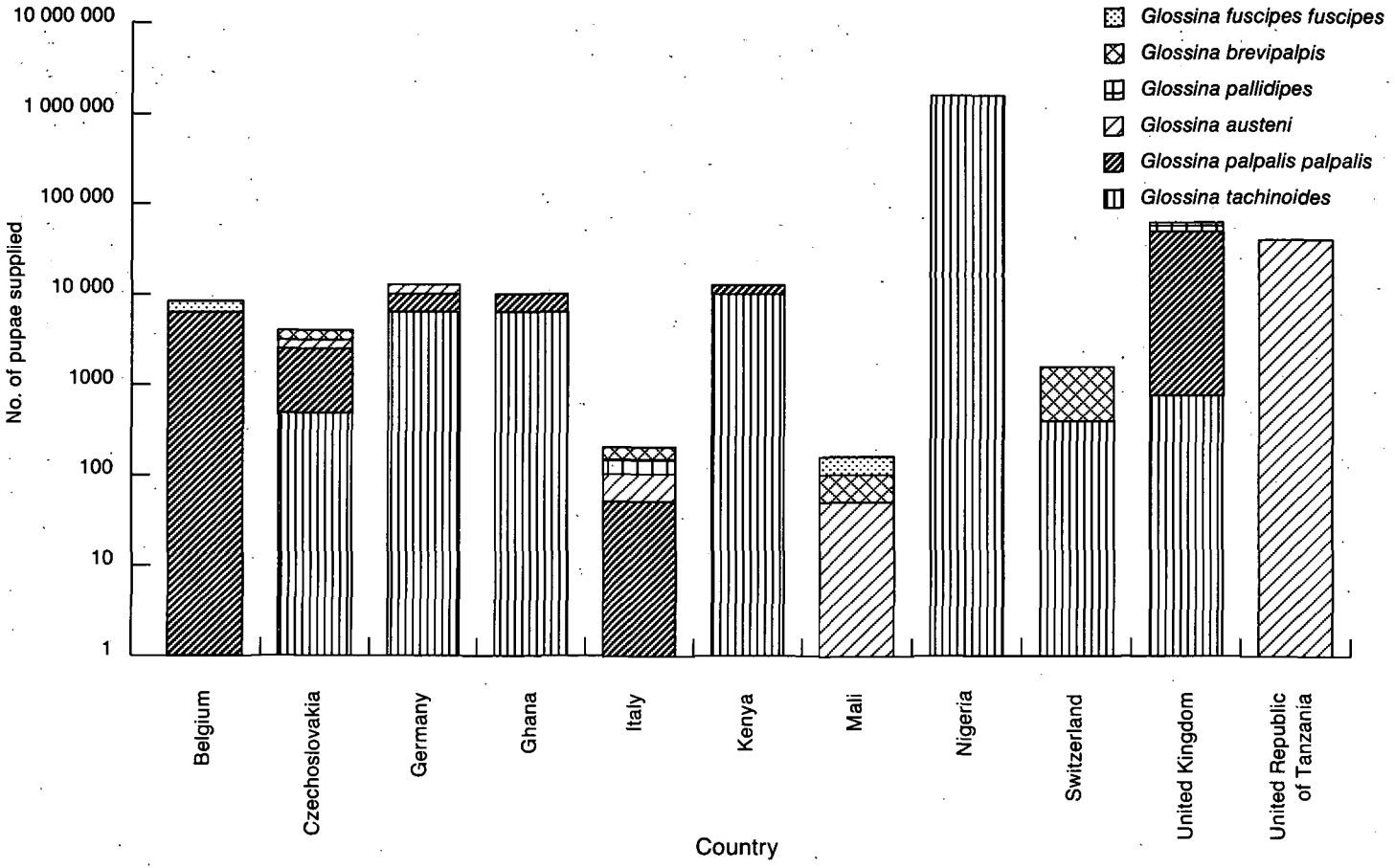

FIG. 2. Tsetse fly pupae supplied by the FAO/IAEA Entomology Unit to IAEA technical co-operation projects and to collaborative researchers.

TABLE II. IN VITRO FED *Glossina* spp. MASS REARED FOR THE PROVISION OF EXCESS FLIES FOR USE IN VECTOR CONTROL PROGRAMMES

Location	Institute	Species	Colony size	Source	Year
Burkina-Faso	CRTA,	*G. palpalis gambiensis*	159 415	'Rapport d'activité'	1983
	Bobo-Dioulasso	*G. tachinoides*	71 068	'Rapport d'activité'	1983
		G. submorsitans	15 822	'Rapport d'activité'	1983
		All of the above tsetse species	330 000	Ref. [8]	1984
Nigeria	BICOT, Vom	*G. p. palpalis*	139 427	Ref. [6]	1986
		G. tachinoides	87 409	Ref. [11]	1991
United Republic of Tanzania	TTRI, Tanga	*G. austeni*	89 418	Ref. [12]	Sep. 1992
Austria	FAO/IAEA Entomology Unit,	*G. p. palpalis*	80 000	Ref. [13]	1990
	Seibersdorf	*G. tachinoides*	130 740		1990
		G. austeni	43 650		Oct. 1992

for minimum weight and maximum fly challenge per host animal need to be established and strictly observed, e.g. for guinea pigs used as host animals, a 550 g minimum weight, a maximum challenge with 200 tsetse colony females at a time and their use for a maximum of twice per week as 'feeder'. The maintenance of large numbers of tsetse flies may require a self-sustaining host animal colony with established standards for colony performance (mortality and female fecundity) and for the condition of individual animals (offspring and feeder weight) [6].

Small amounts of blood for in vitro feeding of tsetse can be collected under sterile conditions from a known and healthy host that is not under any medication treatment. Once the microbial cleanliness of such blood has been confirmed, it can be offered to tsetse. Large tsetse colonies require a system of blood collection, microbial decontamination and diet testing with a quantifiable result, i.e. a diet quality control factor [14]. The system of diet quality control at the FAO/IAEA Entomology Unit includes a bioassay with a small group of test females [17] and microbial screenings at different stages of diet processing (Fig. 3). Thus, only an uncontaminated diet with sufficient nutritional quality is offered to the mass reared colony.

2.3.2. Immature stages of tsetse

The offspring quality is usually monitored routinely by weight one day after pupation or by size (width class, Ref. [18]). The appropriateness of incubation conditions for tsetse fly pupae can be determined by means of a weekly emergence control, i.e. the pupal period, per cent emergence rate and sex ratio are monitored from a group of 100 pupae. If the emergence rate is below 85%, all non-emergent pupae are dissected in order to determine the developmental stage as an indication of whether any interference with 'normal' intrapuparial development may have occurred. For many *Glossina* spp., an increased number of weak females is recorded if the peak of female emergence occurs before day 32 post-larviposition. Occasionally, at least once per month, some males from the weekly emergence control should be dissected in order to determine the development of their testes and sperm mobility. In particular, incubation temperatures above 27°C may affect spermatogenesis or sperm maturation. An adjustment of climatic conditions is usually sufficient to achieve an appropriate incubation of immature stages. Species with rather waxy puparia, such as *G. austeni*, require slightly increased humidity during the last third of the pupal period (Fig. 4). Without changing the climatic settings of the breeding room, a suitable microclimate can be created in the vicinity of the pupae by placing moist sponges below slightly modified pupal dishes in the standard emergence cages.

2.3.3. Adult flies

There is currently no automatic method to identify the sex of flies upon emergence or for separating males and females after routine colony female mating. The

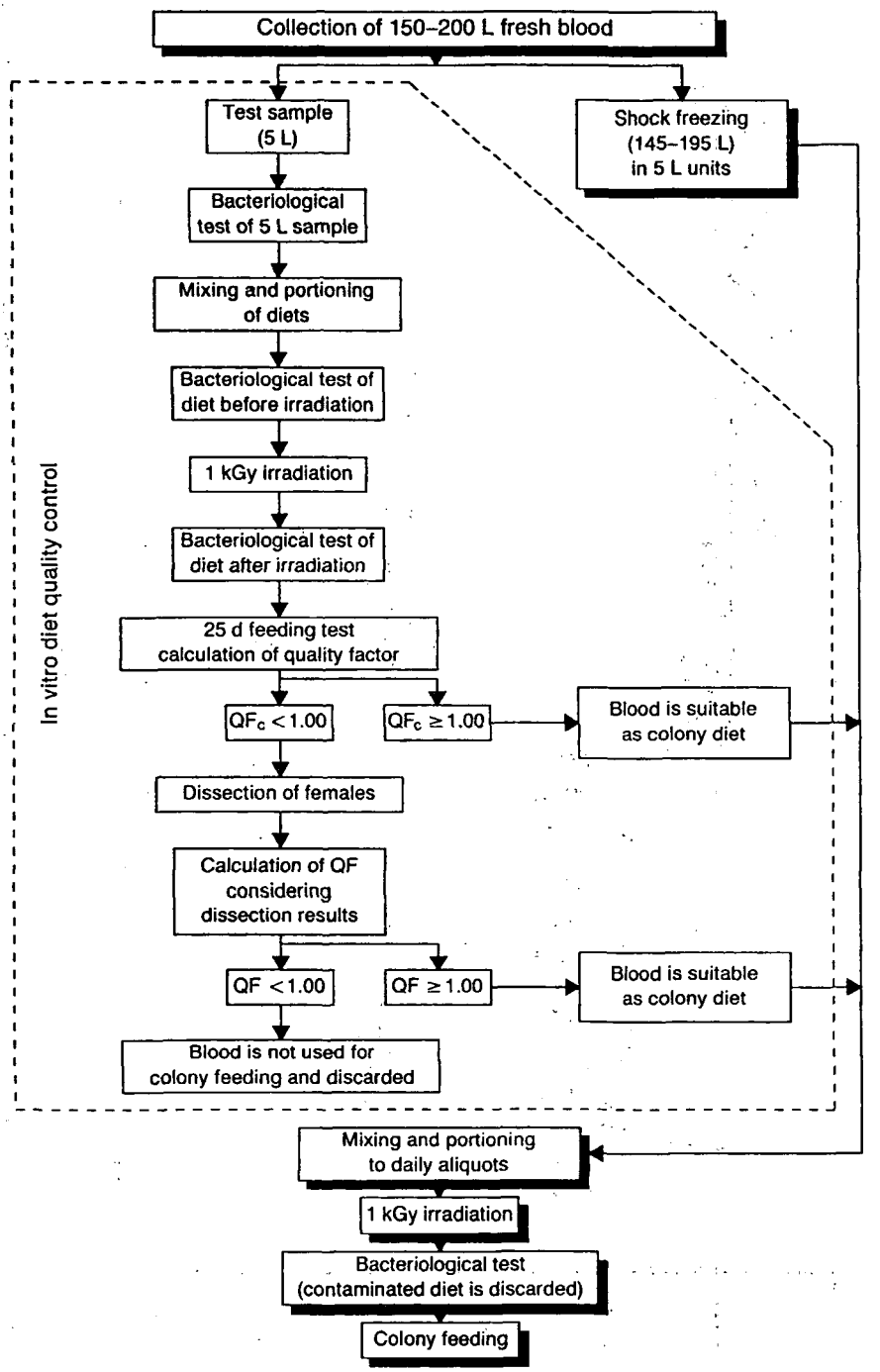

FIG. 3. Production of colony blood and diets.

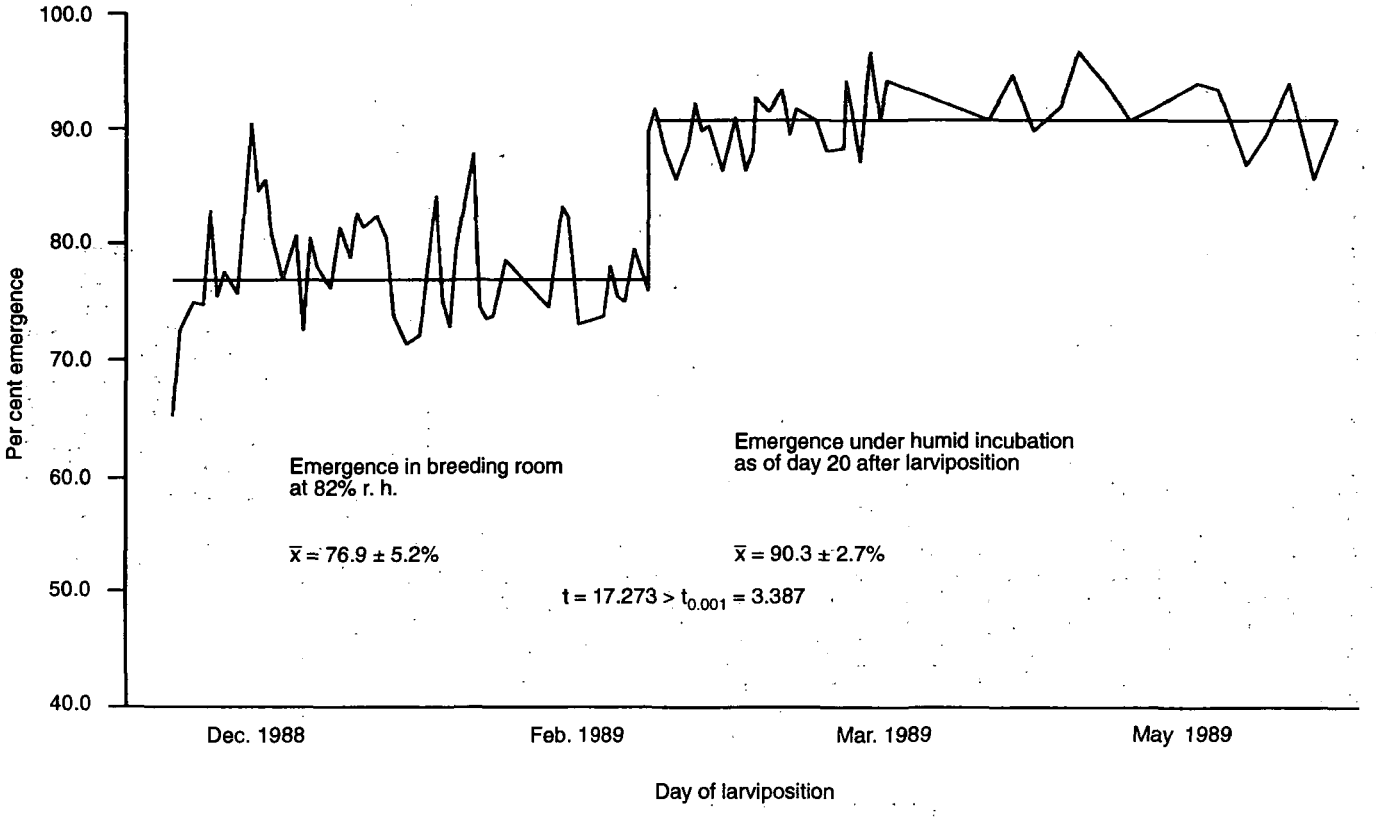

FIG. 4. Development of the adult emergence rate in the Glossina austeni *colony in relation to relative humidity during the late pupal stage (r.h.: relative humidity).*

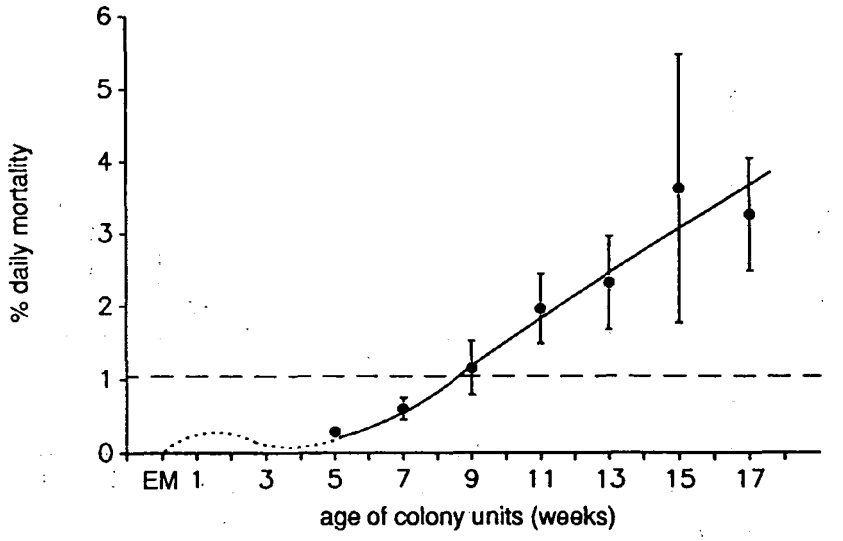

FIG. 5. Glossina tachinoides *under mass rearing conditions. The graph shows the percentage daily mortality in relation to the age of the female colony units (---- : colony mean; EM: emergence).*

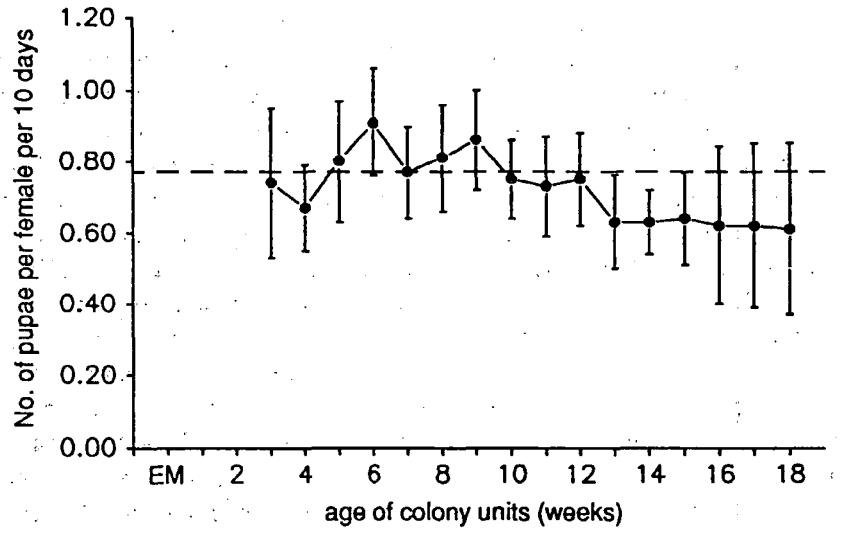

FIG. 6. Glossina tachinoides *under mass rearing conditions. The graph shows female fecundity in relation to the age of the female colony units (---- : colony mean; EM: emergence).*

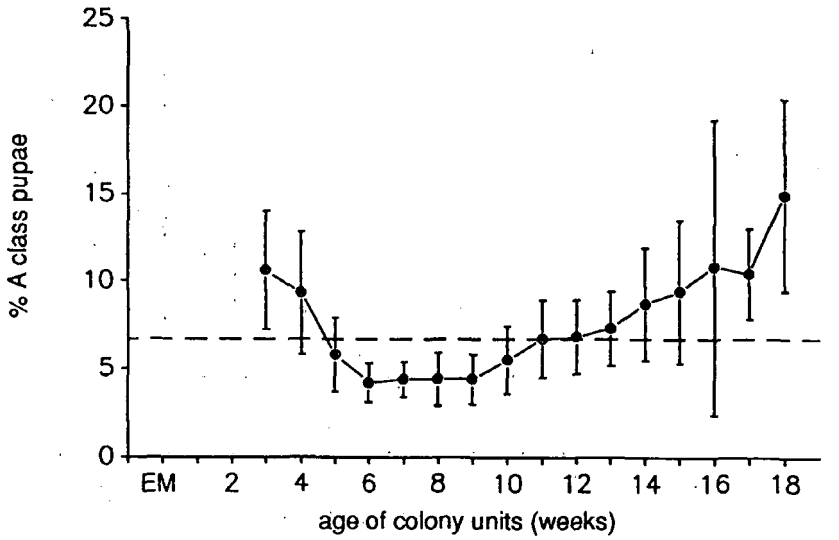

FIG. 7. Glossina tachinoides *under mass rearing conditions. The graph shows the percentage of produced small (i.e. A-class) pupae in relation to the age of the female colony units (---- : colony mean; EM: emergence).*

manipulation of young tsetse is very laborious but permits observations on the appearance, feeding response, activity and early mortality during the first days after emergence. Thereafter, the performance of tsetse colony females is quantified by the determination of (a) the survival or mortality, (b) the productivity or fecundity and (c) the offspring weight or size. The regular collection of data from weekly formed colony female units provides a means to establish quality standards on the above parameters for the entire colony and for different female ages. Figures 5–7 illustrate the age dependent pattern of female mortality, fecundity and quality of offspring production for a *G. tachinoides* colony under mass rearing conditions. In order to trace the sources for increased female *mortality* (if above 1.2% per day on average), it is necessary to (i) differentiate between the types of dead females (i.e. starved flies, flies with undigested blood in the abdomen and females with a larva that pupated in utero), and (ii) analyse the mortality in separate colony female units for patterns that may be age dependent, caused by inappropriate handling or related to the holding position in the breeding room (humidity and temperature gradients). Concerning the mean colony female *fecundity*, a value of 0.60 pupae per surviving female per ten days can be regarded as the minimum limit. Low female fecundity can be caused by different factors [14], such as bad insemination (use of premature males, inappropriate incubation of pupae, inadequate mating regimen), non-suitable climatic set-

tings of the holding room, stress (overcrowding, rough handling, inadequate illumination), intoxication or bacterial contamination. In many cases, when low female fecundity is recorded, an increased number of abortions (expelled at the early or late pregnancy stages) are also observed. Factors causing inferior performance of colony females need immediate correction in order to prevent detrimental effects on colony size or the supply of excess pupae to field projects.

2.3.4. Dispatched insect material

The quality of dispatched pupae and the suitability of methods used for packing and transport can be monitored as described above for immature stages. The quality of sexually sterile males can be assessed under laboratory conditions by means of mating competitiveness and survival tests in large cages or with flight activity simulations in specially designed flight mills.

2.4. Labour and cost for rearing tsetse

Investigations were conducted recently at the FAO/IAEA Entomology Unit on the *labour* requirements to maintain a 100 000 female *G. tachinoides* colony. Using the current colonization procedure, i.e. for young flies and producing flies, an M6 and an M4 feeding regimen, respectively (membrane feeding on Monday, Tuesday, Thursday and Friday), and maintenance of colony female units for 18 weeks, approximately 140 labour hours of experienced technicians are required. Of this time, 45% (or 63 h) is spent on the manipulation of young flies and 30% (or 42 h) is needed for blood collection, diet processing, quality control and feeding of colony flies. The remaining time is spent on pupae handling, removal of dead females from holding cages, data collection and evaluation, washing, cleaning and sterilizing of materials and other routine work. This is a reduction by 50 h when compared with the labour needed for colony maintenance in early 1991, when fly producing females were still fed five times per week and when old female colony units were disposed of at the age of 13 weeks instead of 17–18 weeks. Other *costs* for the colonization of tsetse at the FAO/IAEA Entomology Unit include buildings, equipment and materials and are summarized in Table III as they are actually incurred at Seibersdorf.

3. USE OF LABORATORY PRODUCED TSETSE IN VECTOR CONTROL PROGRAMMES

There may be either continuous demand for sexually sterile insects throughout the year until eradication of the target pest insect has been achieved, or flies are

TABLE III. COSTS FOR MASS REARING 100 000 FEMALE *Glossina tachinoides* AS INCURRED IN 1991-1992 AT THE FAO/IAEA ENTOMOLOGY UNIT, SEIBERSDORF (SALARIES EXCLUDED)

1. Facilities

No.	Item	Cost per unit (US $)		Amortization time (years)	Cost per year (US $)	Total cost per pupa (US $)
30 m^2	Fly holding/breeding room for producing females					
25 m^2	Feeding room					
45 m^2	Handling room					
10 m^2	For pupae incubation					
10 m^2	Fly holding/breeding room for young flies					
10 m^2	Washing area					
10 m^2	Blood processing					
10 m^2	Store					
150 m^2	Total	1 250	187 500	25	7 500	0.0029

TABLE III. (cont.)

No.	Item	2. Equipment Cost per one (US $)	Total cost (US $)	Amortization time (years)	Cost per year (US $)	Total cost per pupa (US $)
16	Fly holding trolleys	550	8 800	15	587	
5	Air conditioners	3 000	15 000	10	1 500	
3	Humidifying systems (incl. air ventilation systems)	3 500	10 500	10	1 050	
3	Climate control systems	1 500	4 500	10	450	
50	Pupae holding trays	4	200	5	40	
100	Emergence cages	20	2 000	5	400	
5000	SC-11 fly holding cages for young females	2.50	12 500	5	2 500	
1600	MC-20 fly holding cages for producing females	5	8 000	5	1 600	
2500	Male holding cages	2.50	6 250	5	1 250	
100	Silicone membranes	20	2 000	1	2 000	
100	Aluminium feeding trays	20	2 000	10	200	
3	Washing machines (1 each for cages and feeding trays)	1 000	3 000	5	600	
2	Heat sterilizing ovens	1 200	2 400	10	240	
1	Pupal sorting machine (quality control)	7 000	7 000	10	700	

TABLE III. (cont.)

No.	Item	Cost per one (US $)	Total cost (US $)	Amortization time (years)	Cost per year	Total cost per pupa (US $)
1	Pupae counting machine	3 500	3 500	10	350	
3	Fly chillers	1 850	5 550	5	1 110	
1	Laminar air flow bench	15 000	15 000	15	1 000	
3	Electric blood stirrers	800	2 400	15	160	
3	Blood collection sets	480	1 440	5	288	
4	Freezing cabinets (500 L)	1 000	4 000	10	400	
2	Refrigerators	600	1 200	10	120	
1	Set shelves, benches, tables, chairs	5 000	5 000	10	500	
			122 240		17 045	0.0066
1	Gamma radiation source	160 000	160 000	10	16 000	
					33 045 (including the gamma source)	0.0129

TABLE III. (cont.)

	Cost per year (US $)	Total cost per pupa (US $)
3. Supplies		
Blood, chemicals, glassware	20 000	0.0078
4. Overhead costs		
Running overhead costs at the Agency's Laboratory depend on the number of staff involved in each project	74 460	0.0290
Total costs	135 005	0.0526

released on a seasonal basis if other control methods cannot be used due to the inaccessibility of the control area, e.g. in the rainy season. Depending on the nature and location of the field programme, different types of rearing facilities may be erected, such as:

— A stationary insect factory for national campaigns with a relatively small treatment area,
— A large breeding centre for the provision of sterile insects to several neighbouring countries (regional programmes),
— Mobile insect factories (ship or land based container system) that can be shifted as the release area changes in accordance with the expansion of the eradication zone.

In addition to procedures for quality assurance of the released insects, the long distance transfer of tsetse from a rearing facility may require the co-operation of various relevant agencies, such as veterinary, quarantine and customs authorities, brokers and importation firms, airlines and other transport businesses.

3.1. Sterile males for SIT

'Conventional' means of tsetse control, e.g. the use of insecticides or trapping, are usually more efficient at the beginning of a treatment campaign than later, when the density of the target population is lower. The SIT has a converse efficiency pattern: it becomes more efficient with progressive reduction of the target insect population. Sexually sterile tsetse fly males can therefore be more efficiently utilized when the phase of release follows an initial period, e.g. three months, of 'conventional' population suppression. Sterile males need to disperse and behave like native fertile males. The release location, interval and the numbers of males released are chosen such that throughout the fly habitat the sterile males outnumber the fertile wild males by at least 10 to 1. This ratio must be maintained for at least three to four generation periods (one tsetse generation period is approximately 50 d). Thus, in the dense riverine fly habitat of the southern Guinea vegetation zone, the eradication of *Glossina p. palpalis* requires during the release phase some one hundred sterile males per linear kilometre of riverine forest per week. This number of males can be supplied by a tsetse colony of 1000 females.

3.2. Sterile females for sterile virgin female ecological monitoring

The collection of data on tsetse population density and dynamics is important for appropriate decision making in vector control operations. During the advanced stage of tsetse control, the density of the target population may be below the level detectable by trapping; furthermore, the collection of information requires extensive trapping and is laborious and relatively expensive. At this stage the release of labora-

tory reared, sexually sterile virgin females into the target habitat and their recapture and dissection (sterile virgin female ecological monitoring (SVFEM)) may have technical and economic advantages.

Preliminary results from tests with various tsetse fly species colonized at Seibersdorf (i.e. *G. p. palpalis, G. tachinoides, G. f. fuscipes, G. austeni, G. pallidipes* and *G. brevipalpis*) provide evidence that:

(a) Female *Glossina* spp. can be radiation sterilized sexually (ovary atrophy) by means of a 60 Gy gamma treatment one week before expected emergence.
(b) There is evidence that such radiation sterilized pupae may be shipped over a long distance without affecting their emergence (provided the packing and transport conditions and duration are adequate).
(c) Sexually sterile virgin females emerging from 60 Gy gamma ray treated pupae survive well and indicate no difference in mating behaviour and receptivity to insemination when compared with fertile virgin females.
(d) Sterile virgin females may be released into the fly habitat of the target population and be recaptured later. Inseminated recaptured females prove the presence of native males (i.e. the prevalence of a wild population).

For the detection of a relic tsetse target population, the time interval between release of the sterile females and their recapture may be extended, which increases the chances of sexual contact between released sterile females and native males, thus *amplifying* the monitoring results. In order to explore the feasibility and economics of SVFEM as a detector technique, releases and recaptures of virgin sterile tsetse females have to be conducted before, during and after vector control operations.

3.3. Tsetse for hybridization with closely related species or subspecies

The genetic implications of the hybridization of different species or subspecies have been reviewed [19]. In the *morsitans* group of tsetse, evidence for conspecific sperm selection by the female for egg fertilization, however, presents an obstacle for using the SIT for trans-specific eradication. For species of the *palpalis* group, similar evidence for conspecific selection of sperm has not been found.

3.3.1. Closely related subspecies in the palpalis *group*

Laboratory studies of *G. palpalis palpalis* and *G. palpalis gambiensis* indicated that there is no preference for selective inter- or intrasubspecific mating [20]. 'Fertile' intersubspecific crosses result in a reduced emergence rate among the F_1 offspring and a sex ratio distortion. Furthermore, most F_1 males are sterile (aspermia). Morphometrical studies on the male hypopygium in the field provide evidence for hybridization of *G. p. palpalis* and *G. p. gambiensis*. In natural habitats, where the possibility of contact between the two subspecies exists, only a very narrow corridor

of hybridization has been detected. This limited extent of the hybridization zone is another indication of the occurrence of hybridization in the field, as it is probably caused by the reduced emergence and the sex ratio distortion among hybrid offspring and the sterility of hybrid males.

3.3.2. *Closely related species in the* palpalis *group*

Preliminary results from laboratory investigations on the mating behaviour of *G. p. palpalis* and *G. f. fuscipes* [21] suggest the feasibility of using sterile, laboratory reared *G. p. palpalis* males and females for the control of *G. f. fuscipes*:

(a) *G. f. fuscipes* males, if given the choice of mating *G. f. fuscipes* or *G. p. palpalis* females, express a preference to mate with females of the other taxon.

(b) *G. p. palpalis* males mate with females of both taxa at random. However, the abdomen of *G. f. fuscipes* females that were mated by *G. p. palpalis* males show severe wounds and approximately 40% of such females die within few days after mating as a result of secondary microbial infections.

The above results suggest the use of laboratory produced sexually sterile flies from one species for the control of its own and two other species or subspecies, i.e. *G. p. palpalis*, *G. p. gambiensis* and *G. f. fuscipes*. The economic implications of the mass rearing of tsetse flies, i.e. producing only one species in the laboratory to combat at least three in the field, are obvious.

4. BOTTLENECKS AND R&D REQUIREMENTS FOR LARGER SCALE FIELD USE OF MASS REARED TSETSE

The establishment of a small laboratory colony from wild fly material using fresh sterile whole blood with the membrane feeding system is relatively simple for most tsetse fly species, provided there are adequate maintenance conditions. However, the transfer of such a strain to mass rearing procedures, such as the adaptation to (a) immobilization at +4°C for sex recognition, (b) mass processed blood diet, or (c) higher female densities in larger cages, usually lasts about two years and may cause undesired selective pressure. Information is required on the genetic diversity and the fate of symbionts during this process of adaptation. Efforts should aim at a reduction of the adaptation period to the mass rearing system.

Procedures for morphological sex recognition and separation, or the development of a genetic sexing technique without losses in fly performance would double the number of males that can currently be supplied by a tsetse colony and is the prerequisite for large scale mass production of sexually sterile tsetse flies for use in vector control projects. Once this has been achieved, the mass rearing of tsetse can be automated.

Basic information is needed on the genetic backgrounds of the target populations and mass reared strains in order to determine (1) the suitability of the colony fly material for release and (2) the existence of genetically isolated fly populations or the gene flow between neighbouring populations. The latter is very important to assess the risk of reinfestation of a determined eradication zone by fly populations in the border areas. If necessary, methods that permit the introduction of the genetic background of the wild target strain into the mass reared strain need to be elaborated.

Although it is known that the current procedures of fly handling prior to release reduce almost to zero the probability of a sterile male becoming infected, future improved, mass reared tsetse strains will have to be refractory to trypanosome infection. The interrelationship between trypanosomes, symbionts and RLOs and tsetse has to be further investigated in order to incorporate principles for refractoriness into mass reared strains.

The demand for sterile tsetse may fluctuate during the year and the insect production may be in excess of or below demand. Biological materials that are produced for pest insect control should be storable like conventional insecticides. Existing knowledge on the preservation of other insects [22] should be reviewed for their applicability to produce in advance and stockpile tsetse. Methods of shipping tsetse over a long distance from regional breeding centres to control projects are also expected to benefit from this research.

The efficiency of SVFEM would increase with enhanced attractivity of released virgin females to relic native males (colours, manipulation of the short range sex pheromone). First, however, detailed field studies are proposed to obtain further information on the feasibility and economic aspects of using sterile virgin females as detectors of tsetse populations before, during and after vector control operations.

Sexually sterile tsetse from a mass production facility can be used for eradication or control in combination with or in support of other regional or field-by-field applied methods of trypanosomiasis control. Provided the research topics identified above are sufficiently covered and the main bottlenecks eliminated, the use of tsetse flies from a mass production facility has considerable potential for the efficient and specific control of several economically important *Glossina* species. The proposed erection of regional mass breeding facilities close to international airports would permit the supply of sexually sterile tsetse to projects in neighbouring countries upon demand.

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HYBRIDIZATION OF *Glossina swynnertoni* WITH SUBSPECIES OF *Glossina morsitans* (DIPTERA: GLOSSINIDAE)

Implications for use of hybrid sterility and satyrs for genetic control of tsetse

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Abstract

HYBRIDIZATION OF *Glossina swynnertoni* WITH SUBSPECIES OF *Glossina morsitans* (DIPTERA: GLOSSINIDAE): IMPLICATIONS FOR USE OF HYBRID STERILITY AND SATYRS FOR GENETIC CONTROL OF TSETSE.

Adult *Glossina swynnertoni* Austen that emerged from puparia collected during 1989 and 1991 near Makuyuni, United Republic of Tanzania, and their progeny were hybridized with *G. morsitans centralis* Machado, *G. morsitans morsitans* Westwood, or *G. m. submorsitans* Newstead. All the *G. swynnertoni* females were receptive and mated readily, regardless of whether they were placed with conspecifics or members of a subspecies of *G. morsitans*. *Glossina swynnertoni* males attempted to mate with females of all three subspecies of *G. morsitans*; the order of increasing success in pair formation was *G.m. centralis*, *G. m. morsitans* and *G. m. submorsitans* . About 50% of the *G. swynnertoni* females were fertilized by conspecific males, about 25% by *G. m. centralis* and none were fertilized by *G. m. morsitans* or *G. m. submorsitans*. *Glossina swynnertoni* males fertilized from 35% (*G. m. submorsitans*) to 68% (*G. m. morsitans*) of their mates. All the F_1 hybrid males were sterile, but most of the F_1 hybrid females were fertile and could be backcrossed to either parental taxon. Recurrent backcrossing produced sterile and fertile males, and an X chromosome marker gene was used to demonstrate that a major cause of male hybrid sterility was an incompatibility of the sex chromosomes of *G. swynnertoni* with those of *G. m. centralis* and *G. m. morsitans*.

1. INTRODUCTION

The genus *Glossina* Wiedemann consists of 31 species and subspecies arranged in three subgenera [1, 2]. Adult tsetse flies are haematophagous and are the vectors of trypanosomes throughout most of sub-Saharan Africa. The subgenus *Glossina s. str.* includes seven species and subspecies and it is usually accepted that within this subgenus *Glossina swynnertoni* Austen is closely related to *G. morsitans* sensu

lato Westwood [1, 3, 4], and it has been suggested that *G. swynnertoni* recently evolved from *G. morsitans* as a species that is adapted to drier and more open savannah [5].

Many species or subspecies of tsetse will hybridize with members of closely related taxa. From a historical perspective, the most interesting species is *G. swynnertoni*. This species was one of the first to be studied in hybridization experiments in the laboratory [3, 6–8] and in the field [9], and it was the first species of insect against which genetic control methods were used [8, 10]. Vanderplank reduced the number of *G. swynnertoni* in an isolated population by releasing *G. m. centralis* Westwood at that site [8, 10]. He interpreted the reduction in the *G. swynnertoni* population as the result of hybrid sterility, but Ribeiro [11] interpreted this reduction as being the result of 'satyrist' behaviour, i.e. the tendency of males of one taxon to mate with females from another taxon. Ribeiro [11] proposed that satyrs may be useful in the genetic control of insects. This suggestion has been challenged, at least for use against subspecies of *G. morsitans*, by the demonstration that polyandrous females of this species generally use the sperm from their own taxon only [12].

The objectives of this study were to determine the hybridization relationships between *G. swynnertoni* and the three subspecies of *G. morsitans* and to use this hybridization data to determine the potential of hybrid sterility as a genetic control agent for use against populations of *G. swynnertoni* or subspecies of *G. morsitans*.

2. MATERIALS AND METHODS

Puparia of *G. swynnertoni* Austen were collected in the Naitolia Ranch, Rift Valley Seed Company Ltd, about 20 km south of Makuyuni (at the junction of highways A104 and B142) in the northern part of the United Republic of Tanzania during August 1989 (about 700 puparia) and during late February and early March 1991 (about 800 puparia). The puparia were transported by road (to Nairobi, Kenya, in 1989 and to Arusha, United Republic of Tanzania, in 1991) and then by air to Edmonton, Canada. In 1989, approximately 300 adults emerged and in 1991 approximately 250 adults emerged. In 1989, deformed adults were common and mortality was very high among the young flies, but in 1991 only a few adults were deformed. The flies were maintained in the laboratory at 25°C and 65–80% relative humidity by feeding six or seven days per week on rabbits.

In a preliminary experiment in 1989, *G. swynnertoni* males that had mated with *G. swynnertoni* females always attempted to mate with the females of all three *G. morsitans* subspecies, but were least likely to be accepted by *G. m. centralis* and most likely to be accepted by *G. m. submorsitans*. In a subsequent experiment, 19 virgin male *G. swynnertoni* that were one to two weeks old were individually placed with a female of one *G. morsitans* subspecies; if the male was rejected, it was placed with a female of another subspecies. Each female that rejected a *G. swynner-*

toni male was receptive when placed with a male of its own subspecies. Males were used up to five times, at 48–72 h intervals. Each day, *G. swynnertoni* males were placed with females in the order *G. m. centralis*, *G. m. morsitans* and finally *G. m. submorsitans*.

In the 1991 experiments, males were at least seven days old and females were three or four days old when tested. With the exception of 14 males that were mated twice with *G. swynnertoni*, all the males emerging from field collected *G. swynnertoni* were mated once only. However, the 15 laboratory reared *G. swynnertoni* males used to establish the hybrid pedigrees were mated four times, at 48–72 h intervals. The first mate was a *G. swynnertoni* female, after which one third of the males were placed with females in the order *G. m. centralis*, *G. m. morsitans* and *G. m. submorsitans*; one third in the order *G. m. morsitans*, *G. m. submorsitans* and *G. m. centralis*; and one third in the order *G. m. submorsitans*, *G. m. centralis* and *G. m. morsitans*.

Females that became pregnant were maintained, by the procedures mentioned above, to obtain offspring for further experiments. Hybrid and backcross females were mated with males from one of their parental taxa and maintained to obtain offspring for further fertility tests. Recurrent backcrosses (to *G. swynnertoni* or to the appropriate *G. morsitans* subspecies) were accomplished with the crosses *G. m. centralis* × *G. swynnertoni*, *G. m. morsitans* × *G. swynnertoni* and *G. m. submorsitans* × *G. swynnertoni*. (Throughout this paper the taxon of the female is specified first in an inter-taxon cross.)

Each hybrid or backcross male was mated with two to four females from his parental *G. morsitans* subspecies. Forty-eight hours after mating, each of the test females was dissected and the spermathecae examined (at 160×) for motile sperm. Those males that passed motile sperm were mated with several more *G. morsitans* subspecies females and each female was observed daily until she became pregnant, or for 28 d, at which time the spermathecae were examined for motile sperm.

Only one marker gene (*Pgm*, the locus for phosphoglucomutase) was available to distinguish the X chromosomes of *G. swynnertoni* from those of *G. m. centralis* and *G. m. morsitans*. The electrophoretic procedure used [13] gave phosphoglucomutase (PGM) bands with the following migrations, relative to the bromophenol blue marker: 0.58, 0.62 and 0.65 for *G. m. centralis*, 0.58 and 0.62 for *G. m. morsitans*, and 0.55 and 0.59 for *G. swynnertoni*. The origins of the Y chromosomes were established on the basis of the taxon of the sire.

3. RESULTS

3.1. Mating behaviour

Glossina swynnertoni males always attempted to mate with females of *G. swynnertoni*, *G. m. morsitans*, *G. m. centralis* and *G. m. submorsitans*. In the 1989

TABLE I. RESULTS OF PAIRING *G. swynnertoni* WITH *G. swynnertoni* OR WITH *G. morsitans*[a]

Taxon tested	Mated by *G. swynnertoni*[b]		Insemination and pregnancy[c]	
	Females	Males	*G. swynnertoni* females	Mates of *G. swynnertoni* males
G. swynnertoni[d]	100 (65)	100 (65)	25/53/61	25/53/61
G. m. centralis	100 (10)	43.5 (23)	2/3/8	4/4/10
G. m. morsitans	100 (10)	100 (11)	0/2/7	8/8/11
G. m. submorsitans	100 (10)	100 (16)	0/6/10	5/8/16

[a] The adult *G. swynnertoni* used in these experiments had emerged from puparia collected in the United Republic of Tanzania in 1991 and shipped to the University of Alberta, Edmonton, Canada.

[b] The numbers in these two columns are the percentage pairs that copulated (the number of pairs tested is in parentheses).

[c] The numerals in the last two columns are: No. of pregnant/No. of inseminated/No. of mated females alive 28 d after mating.

[d] Data for the same 65 pairs appear in all the columns.

experiments, of 19 *G. swynnertoni* males that were placed with females from one, two or three *G. morsitans* subspecies in 1 d, four mated with *G. m. centralis*, 15 mated with *G. m. morsitans* and 14 mated with *G. m. submorsitans*. Three males were rejected by *G. m. centralis*, then by *G. m. morsitans* and then accepted by *G. m. submorsitans*. On one occasion, a male was rejected by the females of all three *G. morsitans* subspecies; this male mated with two *G. m. submorsitans* and one *G. m. morsitans* on other days.

In the 1991 experiments, all the *G. swynnertoni* females accepted their mates, regardless of whether these were conspecifics or members of a *G. morsitans* subspecies (Table I). More than half of the *G. m. centralis* females rejected *G. swynnertoni* males that emerged from field collected puparia, but the rejected males were accepted by *G. m. morsitans* or *G. m. submorsitans* (Table I). Similar results were obtained using males from the first generation of a laboratory colony of *G. swynnertoni* (Table II).

TABLE II. RESULTS OF PAIRING LABORATORY REARED *G. swynnertoni* MALES[a] WITH FEMALES OF *G. m. centralis*, *G. m. morsitans* AND *G. m. submorsitans*

Taxon of females	Per cent mating (No. tested)	Inseminated and pregnant[b]	Offspring produced during three months
G. m. centralis	47 (15)	4/4/7	Four males: two females (6)[c]
G. m. morsitans	93 (15)	9/9/14	Four males: nine females (16)
G. m. submorsitans	100 (15)	7/7/15	Six males: one female (7)

[a] Males were from the first generation that was laboratory reared, and each had mated with a *G. swynnertoni* female before being tested with a *G. morsitans* female.

[b] The numerals are: No. of pregnant/No. of inseminated/No. of mated females alive 28 d after mating.

[c] The number of puparia is given in parentheses.

3.2. Insemination and fertilization of *G. swynnertoni* and *G. morsitans* females

In the 1989 experiments, none of the females that mated with *G. swynnertoni* became pregnant. Of 12 *G. swynnertoni* females examined, only three were inseminated, and none of the 65 females of the various *G. morsitans* subspecies were inseminated. At that time, the three *G. swynnertoni* males that were still alive were dissected; all had motile sperm in their testes. It is not known why the *G. swynnertoni* in the 1989 sample were unable to fertilize females.

In the 1991 experiments, only 41% of the *G. swynnertoni* females were fertilized by conspecifics, but 28 of the 36 females (78%) that did not become pregnant were inseminated (Table I). None of the *G. swynnertoni* females that mated with *G. m. morsitans* or *G. m. submorsitans* became pregnant, but two of those mating with *G. m. centralis* did (Table I).

Polling the data on males emerging from field collected puparia (Table I) with the data from males of the first generation of the laboratory colony (Table II) showed that *G. swynnertoni* males fertilized 47% of *G. m. centralis*, 60% of *G. m. morsitans* and 39% of *G. m. submorsitans*.

TABLE III. FERTILITY OF F_1 HYBRIDS PRODUCED BY CROSSING *G. swynnertoni (Gs)*[a] WITH *G. m. centralis (Gmc), G. m. morsitans (Gmm)* AND *G. m. submorsitans (Gms)*

Taxon		Males		Testing of hybrid females[b]	
Female	Male	Fertile	Sterile[c]	Maternal taxon male	Paternal taxon male
Gs	*Gmc*	0	2	1/1/2	1/1/1
Gmc	*Gs*	0	2[d]	5/5/5	1/1/1
Gmm	*Gs*	0	11	8/8/10	6/6/6
Gms	*Gs*	0	8[e]	1/2/2	2/2/2

[a] The data are from field collected and laboratory reared *G. swynnertoni* and include F_1 hybrids of the pedigrees *Gmc/Gs*, *Gmm/Gs* and *Gms/Gs*.

[b] The numerals are: No. of pregnant/No. of inseminated/No. of mated females alive 28 d after mating.

[c] The criterion for sterility was the inability to inseminate a female of the appropriate *G. morsitans* subspecies.

[d] Three other males died when less than one week old.

[e] One other male died when less than one week old.

3.3. Fertility of hybrid and backcross flies

All the F_1 hybrid males obtained by crossing *G. swynnertoni* with the *G. morsitans* subspecies were sterile, but most of the F_1 hybrid females could be fertilized by backcrossing to either parental taxon (Table III).

3.3.1. Pedigree Gmc/Gs

This pedigree began with four *G. m. centralis* females fertilized by *G. swynnertoni*. Three of the four F_1 males died when less than one week old; the fourth was sterile. One F_1 female (F_1 female A) was backcrossed to *G. swynnertoni* (Fig. 1) and the other F_1 female (F_1 female B) was backcrossed to *G. m. centralis* (Fig. 2).

Recurrent backcrossing to *G. swynnertoni* produced fertile females in backcross generations $Bx_1(s)$ to $Bx_3(s)$ (Fig. 1), but switching to recurrent backcrossing to *G. m. centralis* produced both fertile and non-fertilized females in backcross generations $Bx_1(s)$ to $Bx(s/c)$ (Fig. 1). Four of these non-fertilized females were not

inseminated; the fifth was not checked for sperm. Fertile males were not found in the first three backcrosses, but did occur among $Bx_4(s)$ and $Bx_4(s/c)$ (Fig. 1).

Most of the females produced by recurrent backcrossing to *G. m. centralis* were fertile (Fig. 2), with only two non-inseminated females found among the $Bx_3(c)$ females. Fertile backcross males occurred among the second to fourth backcross generations (Fig. 2).

All but two of the 39 backcross males that were placed with the females from all three *G. morsitans* subspecies were accepted by the females from all three subspecies. The exceptions ($Bx_3(s)$ and $Bx_4(s)$ males, Fig. 1) were rejected by the *G. m. centralis* females.

Of the 37 males that mated with the females from all three *G. morsitans* subspecies, two failed to fertilize *G. m. centralis*, four did not fertilize *G. m. morsitans*, two did not fertilize *G. m. submorsitans* and one fertilized *G. m. submorsitans* only. The remaining 28 males fertilized the females from all three *G. morsitans* subspecies.

All 15 backcross males that had an X from one taxon and a Y from another were sterile (Table IV). Fertile and sterile individuals occurred among the 76 males that had both sex chromosomes from the same taxon.

3.3.2. Pedigree Gmm/Gs

This pedigree began with nine *G. m. morsitans* females fertilized by *G. swynnertoni* that produced 16 puparia. All four F_1 males were sterile.

Four F_1 females were fertilized by backcrossing to *G. m. morsitans*, but only seven backcross (Bx_1) males and five Bx_1 females were obtained. Two Bx_1 males died when less than one week old and the others were sterile. All the Bx_1 females were mated with *G. m. morsitans* males, but none became pregnant and only two were inseminated.

The remainder of pedigree *Gmm/Gs* is shown in Fig. 3. In each backcross generation, there were both fertilized and non-fertilized females. Among the latter, some were inseminated (one each of $Bx_3(m)$, $Bx_2(s)$ and $Bx_3(s)$) and others were not (one each of $Bx_2(m)$, $Bx_3(m)$, $Bx_1(s)$ and $Bx_2(s)$). Only one of the 16 $Bx_1(s)$ males (Bx_1 male 13) was fertile and no further fertile males were found until Bx_3 (Fig. 3).

During these experiments, seven males (Bx_3 and Bx_4) were placed with females from all three subspecies of *G. morsitans*. The males were always accepted by *G. m. morsitans* and *G. m. submorsitans*, but three males were rejected by *G. m. centralis*. Another four Bx_3 males that were rejected by *G. m. centralis* mated with *G. m. submorsitans*, but were never offered *G. m. morsitans* as mates.

Almost all of the males were sterile, regardless of the taxonomic origin of their sex chromosomes (Table IV). Only two fertile males were tested with all three subspecies of *G. morsitans*; one male fertilized the females from all subspecies and the other male fertilized *G. m. morsitans* and *G. m. submorsitans* only.

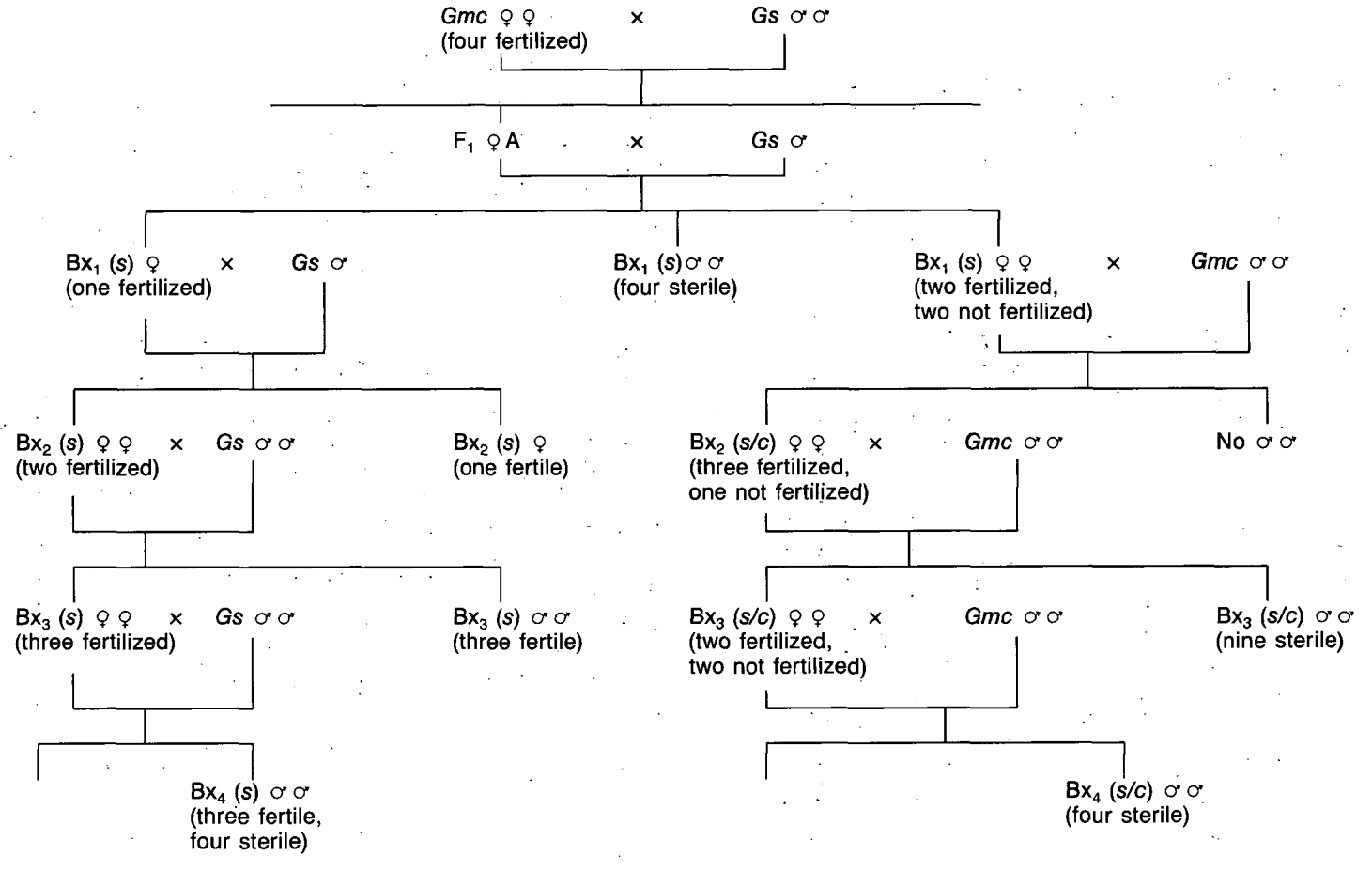

FIG. 1. Portion of pedigree Gmc/Gs *that involved recurrent backcrossing to* G. swynnertoni. (Gmc: G. m. centralis; Gs: G. swynnertoni.) *(See text for which of these females were inseminated.)*

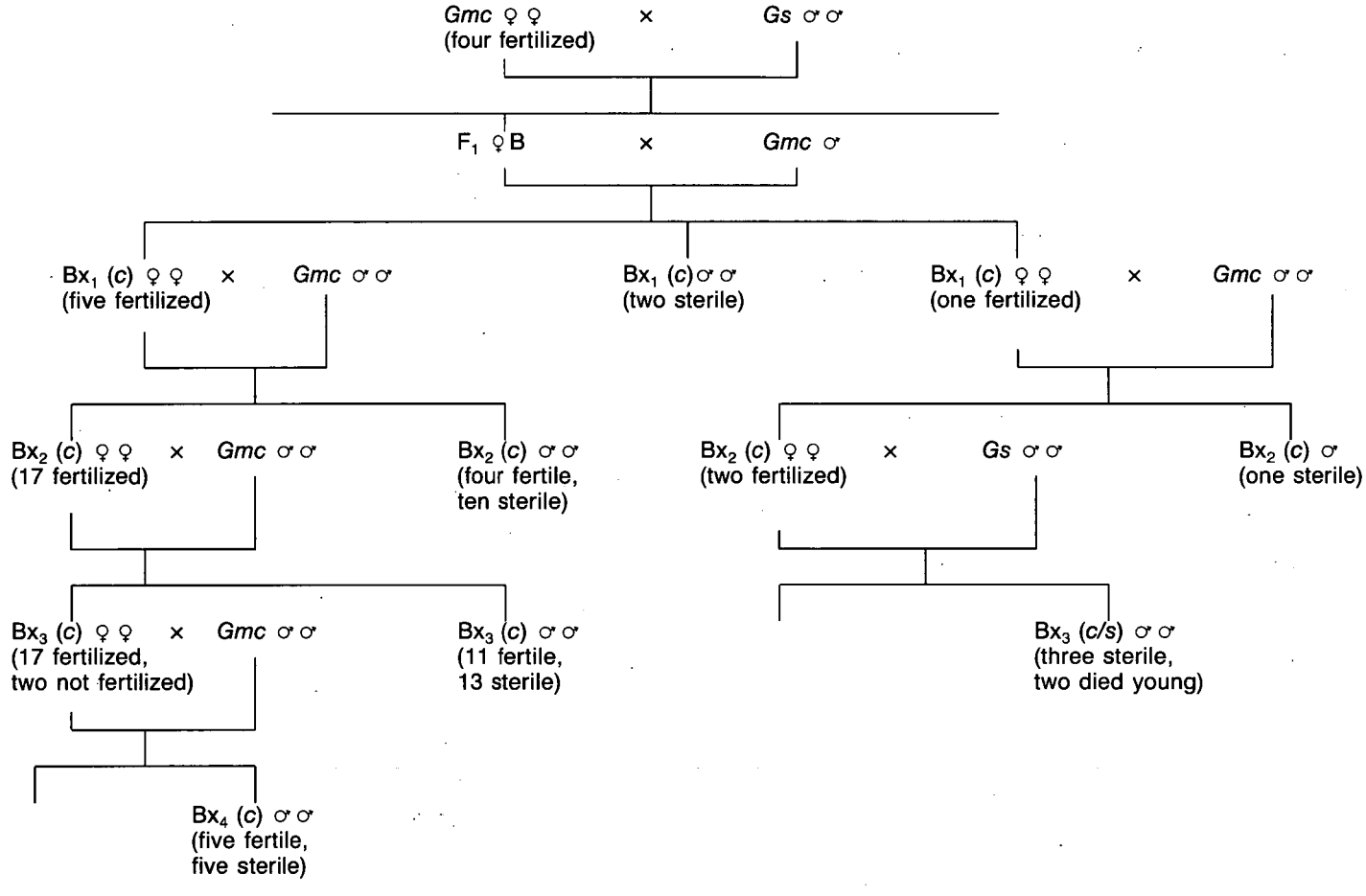

FIG. 2. Portion of pedigree Gmc/Gs *that involved recurrent backcrossing to* G. m. centralis. (Gmc: G. m. centralis; Gs: G. swynnertoni.) *(See text for which of these females were inseminated.)*

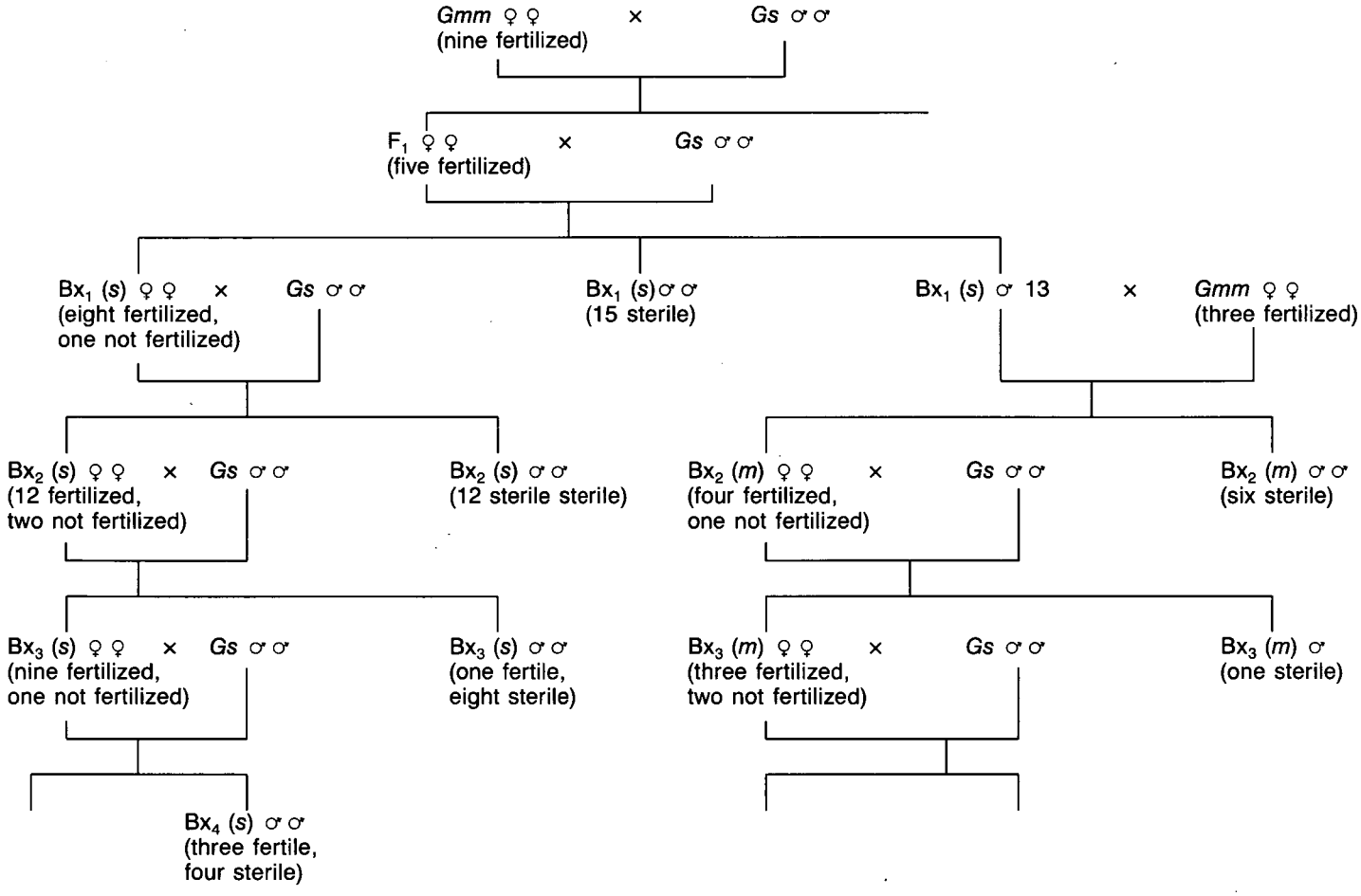

FIG. 3. Portion of pedigree Gmm/Gs *that involved recurrent backcrossing to* G. swynnertoni. (Gmm: G. m. morsitans; Gs: G. swynnertoni.) *(See text for which of these females were inseminated.)*

TABLE IV. INFLUENCE OF THE ORIGIN OF THE SEX CHROMOSOMES ON THE FERTILITY OF THE BACK-CROSS MALES[a]

Origin[b] of		No. of males	
X	Y	Fertile	Sterile
s	*s*	3	16
m	*s*	2	10
c	*c*	36	30
s	*s*	3	7
c	*s*	0	5
s	*c*	0	10

[a] Those in the upper part of the table are from the pedigree *Gmm/Gs* and those in the lower part are from the pedigree *Gmc/Gs*.

[b] The taxonomic origin of X was based on the electrophoresis of PGM; the origin of Y was based on the taxon of the father.

3.3.3. Pedigree Gms/Gs

This pedigree began with seven *G. m. submorsitans* females fertilized by *G. swynnertoni*, producing seven puparia. Five F_1 males were sterile; a sixth male died when less than one week old. The only F_1 female was fertilized by *G. swynnertoni* but produced only one sterile male.

4. DISCUSSION

The *G. swynnertoni* females used were those that emerged from field collected puparia. Under laboratory conditions, they mated readily with conspecifics and with the males of all three subspecies of *G. morsitans*. Similarly, *G. swynnertoni* males attempted to mate with conspecifics and with the females from laboratory colonies of *G. m. centralis*, *G. m. morsitans* and *G. m. submorsitans*, but were frequently rejected by *G. m. centralis*. The reluctance of *G. m. centralis* females to copulate

with *G. swynnertoni* was unexpected, since it had previously been reported that *G. m. centralis* and *G. swynnertoni* mated non-assortatively [9, 10]. The only reports suggesting any difficulties in mating *G. m. centralis* females with *G. swynnertoni* were the results of Corson [6], who was able to get only 12 of 18 *G. m. centralis* females to mate with *G. swynnertoni*, and the observation by Potts [7] that "... the inter-specific couplings were undertaken with greater reluctance than the intra-specific ones ...". The present results indicated that the tendency of females of all three subspecies of *G. morsitans* to mate with *G. swynnertoni* is inversely related to the geographical proximity of the *G. swynnertoni* and *G. morsitans* subspecies. This finding, although tentative, helps to clarify the ambiguous statement made by Vanderplank [3] that *G. swynnertoni* and the subspecies of *G. morsitans* "... will intermate, though the degree to which they do so varies with the geographical proximity of the species ...".

It has been proposed [14] that asymmetries in mating behaviour indicate the direction of evolution, with the derived species having females that accept males of both derived and ancestral taxa, while females of the ancestral taxon accept conspecifics only. If this hypothesis is applicable to tsetse, it would indicate that *G. swynnertoni* is derived from *G. m. centralis*, a proposal that is consistent with an earlier suggestion [5] that *G. swynnertoni* evolved from *G. morsitans* as a result of its ability to inhabit more open types of bush than are occupied by *G. morsitans*. In any case, the data suggested that premating barriers have begun to develop between *G. m. centralis* and *G. swynnertoni*, but that there is little to prevent mating of *G. swynnertoni* to either *G. m. morsitans* or *G. m. submorsitans*. If the mating barriers between *G. m. centralis* females and *G. swynnertoni* males exist under field conditions, they would impose limitations on the use of *G. swynnertoni* males for the control of *G. m. centralis*.

With respect to the fertility of interspecific crosses, the results of the present experiments were similar to most of the results of Vanderplank [3]. In the present study, *G. m. centralis*, *G. m. morsitans* and *G. m. submorsitans* males fertilized 25, 0 and 0% of *G. swynnertoni*; corresponding figures from the previous study were 6 and 0%, with the third cross not reported. Similarly, in the present study *G. swynnertoni* males fertilized 47% of *G. m. centralis*, 68% of *G. m. morsitans* and 39% of *G. m. submorsitans*; the corresponding figures from the previous study were 24, 58 and 0% (the latter being based on two pairs).

The most striking effect of the hybridization of females of *G. morsitans* was the suppression of their reproductive capacity. In our colonies of *G. morsitans*, females normally average six to eight puparia during the three months that they are maintained. However, females that were hybridized with *G. swynnertoni* produced an average of 1.0 (*G. m. submorsitans*), 1.5 (*G. m. centralis*) and 1.8 puparia/female (*G. m. morsitans*) during three months (Table II). All the females that were scored as pregnant had produced their first offspring within 28 d of mating, i.e. each had produced a mature larva as a result of the first or second ovulation. The low number

of offspring produced thereafter suggests that the females were sterilized as a result of their first pregnancy. Such an effect would have a profound effect on reducing the size of a population.

The ability of *G. m. centralis* males to mate with *G. swynnertoni* and to fertilize a small percentage of these females indicates that these males could serve as satyrs or as a means of introducing hybrid male sterility into a *G. swynnertoni* population. Either would have the effect of reducing the reproductive capacity of the *G. swynnertoni* population. This was, in fact, accomplished by Vanderplank with the marked reduction in or eradication of a *G. swynnertoni* population in the United Republic of Tanzania [8, 10]. The results of the present study do not shed light on whether the success of this control operation was solely the result of hybrid sterility (as claimed by Vanderplank [8, 10]) or the effect of satyrism (as suggested by Ribeiro [11]).

The ability of *G. m. morsitans* and *G. m. submorsitans* males to inseminate, but not fertilize, *G. swynnertoni* suggests that these males may be useful as satyrs for the control of *G. swynnertoni*. To explore this possibility, it will be necessary to determine whether polyandrous *G. swynnertoni* females use the sperm of conspecifics only (as is the case with *G. morsitans* subspecies [12]) and to establish whether interspecific mating can occur in nature.

All the F_1 hybrid males were sterile and failed to inseminate their mates. These results contrast with those of Vanderplank [10], who found that about 80% of the F_1 hybrid males from the *G. swynnertoni/G. m. centralis* crosses were able to inseminate, but not fertilize, females. The discrepancy in our results may be due to differences in the conditions under which we maintained the puparia and adults. However, three backcross males were found (one $Bx_4(c)$ male from pedigree *Gmc/Gs* and two $Bx_3(s)$ males from pedigree *Gmm/Gs*) that each inseminated the first female with which it mated; however, none of the females that were subsequently mated (six, six and four) became pregnant, and only one of these females was inseminated.

Most of the F_1 hybrid females could be fertilized by backcrossing to either parental taxon. The occcurrence of sterile females among the first three backcross generations was sporadic. Although the number of flies in each pedigreee was low, the occurrence of sterile backcross females was most common in the *Gmm/Gs* pedigree, and in that portion of the *Gmc/Gs* pedigree in which backcrossing was switched from *G. swynnertoni* to *G. m. centralis*. If genetic control of a *G. m. centralis* population were attempted by repetitive release of *G. swynnertoni* males, one would expect some of the backcrosses to be of this 'switch' type and this phenomenon, whatever its genetic basis, would contribute to a more rapid decline in the population.

Sterile males predominated among the first three backcross generations and made up approximately half the males of the fourth backcross generation. Among the backcross males, for which the origin of the sex chromosomes was determined

(Table IV), there were 42 fertile and 53 sterile males whose X and Y chromosomes came from the same taxon, and two fertile and 25 sterile males whose X and Y chromosomes came from two different taxa (χ^2 (with Yates correction) = 10 801, 1 degree of freedom, $P < 0.005$). Thus, a major cause of hybrid male sterility is an incompatibility of sex chromosomes from two different taxa. This is consistent with previous reports that X/Y incompatibility in tsetse is a major cause of hybrid male sterility [15]. However, it is clear that since 58% of the males that had X and Y chromosomes from one taxon were sterile, there must be other factors that influence hybrid sterility. Furthermore, the fertility of hybrid males was not tested with females from both parental taxa, and there may be maternally inherited sterility factors that will cause an asymmetry in the reproductive success of backcross males [15]. Such factors could further influence the effectiveness of backcross males in reducing the reproductive potential of a population.

The results reported here are preliminary and are based on two samples of *G. swynnertoni* taken from a population against which control operations had been carried out, and were also dependent on the use of inbred laboratory colonies of *G. morsitans* subspecies. Interpretation of the results must be tempered also by the fact that a relatively low percentage of *G. swynnertoni* females were fertilized by conspecific males. The above notwithstanding, the results suggest that in certain combinations there is a possibility for control of populations of *G. swynnertoni* and of the subspecies of *G. morsitans* by the effects of satyrism and by interspecific hybridizations that might occur by introducing members of one taxon into the habitat of another.

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THE VARIOUS VECTOR CONTROL ACTIVITIES OF THE CENTRE DE RECHERCHES SUR LES TRYPANOSOMES ANIMALES (CRTA)

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Abstract

THE VARIOUS VECTOR CONTROL ACTIVITIES OF THE CENTRE DE RECHERCHES SUR LES TRYPANOSOMES ANIMALES (CRTA).

The role of the Centre de recherches sur les trypanosomomes animales (CRTA) in anti-tsetse control will become more important because the centre will soon become an institution with a regional mandate. In the laboratory, studies are being carried out on olfactory attractants to riverine species, for instance from reptiles. The persistence and efficiency of pyrethroids are being tested, after impregnation on the tissues used for traps and targets, and after application on cattle. The rearing of ticks is also beginning and CRTA is continuing to rear different species of tsetse fly that can be used by other countries or organizations for their sterile insect technique programmes. In the field, many experiments are being carried out to improve traps and targets (shapes, colours, attractants, etc.) and entomological surveys are being carried out regularly in co-ordination with other programmes.

1. INTRODUCTION

The Centre de recherches sur les trypanosomes animales (CRTA) is in a transitional stage. Since it is financed by the European Economic Community, France and Italy, it is soon to become an institution with a regional mandate that covers, apart from vector control, various other aspects, such as the epidemiology and economics of parasitic diseases and their control, animal production and husbandry systems, including characterization of trypanotolerant cattle, resistance of crossbreeds, training of national staff at different levels, and extension activities in the field.

This transformation has come about at the request of the five Member States of the Communauté économique du bétail et de la viande (CEBV), namely Benin, Burkina Faso, Côte d'Ivoire, Niger and Togo. The name of the new centre will be the Centre international de recherche-développement sur l'élevage en zone subhumide (CIRDES).

2. RESEARCH ACTIVITIES OF THE VECTOR CONTROL UNIT IN THE LABORATORY

2.1. Isolation and identification of new odours

None of the substances attractive for savannah species such as *Glossina morsitans* or *G. pallidipes* (1-octen-3-ol, acetone or the phenolic fractions of urine) has proved to be effective against *G. p. gambiensis*. Therefore, part of our research is oriented towards other host animals of this tsetse species. Bearing in mind that more than 50% of the feed of *G. p. gambiensis* consists of bloodmeals taken from reptiles, it is worthwhile having a closer look at the mechanisms that are responsible for the attractiveness of these hosts to this tsetse. In fact, monitor lizards (*Varanus niloticus*), crocodiles (*Crocodylus niloticus* and *Osteolaemus tetraspis*) and snakes (*Python sebae*) are being evaluated for their attraction to *G. p. gambiensis* and *G. tachinoides*. Large scale bioassays with flies of known age — CRTA still disposes of about 150 000 producing females of *G. p. gambiensis, G. tachinoides* and *G. morsitans submorsitans* — allow rapid appraisal of the attraction of the various reptiles. The preferred feeding sites on the reptiles are also being recorded. Promising compounds or mixtures are then assayed in a field station to evaluate their ability to influence the effectiveness of a standard capture technique.

2.2. Effectiveness and persistence of pyrethroids

Servicing of the targets or traps accounts for approximately 30% of the total costs of a tsetse campaign based on the use of such devices. The incentive is to reduce the number of visits required per year for servicing. The possible ways of achieving this are: identification of pyrethroids with maximum persistence; increase in the amount of insecticide used for treatment and selection of the most suitable tissue — in general, synthesis tissues have proved to be more suitable than natural materials, e.g. synthetic cotton. After impregnation, the tissues are constantly exposed to sunlight and rain. Bioassays are conducted at regular intervals with defined numbers of flies of known age. So far, results have indicated that 'washing off', i.e. a decrease in the effectiveness of an insecticide due to rainfall, is less deleterious than the effects of sunlight itself. The maximum persistence of certain deltamethrin formulations was found to exceed nine months, approaching a situation where the effectiveness of a pyrethroid would last as long as the tissues themselves, hence making servicing unnecessary.

2.3. Treatment of cattle with acaricides/insecticides

Pyrethroids, originally conceived to combat ticks, have shown remarkable potential against tsetse, leading to a situation where the strategic deployment of

targets/traps and the treatment of cattle constitute the main pillars in rural areas for tsetse control. Currently, large scale bioassays are being conducted to evaluate all the commercially available pyrethroids. Flies of known age and reproductive state are released in the presence of a treated zebu and the following parameters are recorded: the persistence of a given product, the mortality rates, the knock down effects and their duration, the percentage of flies that failed to feed, as well as the effects on reproductive performance. Every second day, the treated animal is exposed to sun for about 3 h and thereafter rinsed with 50 L of water in order to provide a realistic view of the respective efficacies. Of all the products tested so far, none has shown any decrease in persistence when being rinsed with water even 2 h after treatment and thereafter every second day. On the other hand, sunlight has a deleterious effect on certain formulations. Some of the products show a distinct persistence, exceeding 90 d. A manual is in preparation that will summarize all the results and provide technical advice on the rational and strategic use of acaricides/insecticides for the simultaneous control of ticks and tsetse.

2.4. Rearing of tick species

Rearing of *Amblyomma variegatum* and *Boophilus geigyi* has started on live hosts. Priority is being given to monitoring and assessing the development of resistance to acaricides/insecticides. Indiscriminate and incoherent use of insecticides during the rainy season is likely to enhance the probability of a development towards resistance. Although chlorinated hydrocarbons (DDT, lindane, etc.) have basically been used up to now, resistance against pyrethroids may build up as well. The incidence of tickborne diseases and their economic impact will be evaluated in the field.

2.5. Improvements in the transport of sterile insects

The sterile insect technique has proved its efficacy in large scale eradication campaigns in the pastoral zones of Sideradougou, Burkina Faso, and Vom, Plateau State, Nigeria. CRTA/CIRDES, with its regional mandate, is prepared to deliver sterilized tsetse flies to any project in the region if required. Until recently, the fragility of the biological material has been a serious obstacle to the supply of tsetse over long distances. In general, either late stage puparia or young adult flies have been transported. There are now signs that storage of puparia at about 15°C does not adversely affect the emergence rate or the subsequent viability of the newly emerged flies. Therefore, supply of puparia over longer distances and taking several days may become feasible.

3. IMPROVEMENT IN THE EFFECTIVENESS OF TARGETS AND TRAPS

The shape and colour of the devices, apart from olfactory compounds, are requiring further work to define the optimum system. Further, it should be borne in mind that the system should be affordable and sustainable for the rural communities concerned. Comparisons between different trapping systems are being conducted in a field station in the presence of the tsetse species mentioned in Section 2.

4. LARGE SCALE TSETSE CONTROL

Large scale tsetse control is being carried out by integrating the periodic treatment of livestock with a pyrethroid in combination with the use of traps and targets in habitats inaccessible for treated cattle.

An immediate improvement in the overall health of the herd after a few acaricidal treatments is rapidly being perceived by the livestock owner, thus facilitating his further collaboration and his taking over of the costs of the treatments. The prerequisites of any intervention are thorough entomological and epidemiological surveys providing justification for interventions. Of increasing importance are socioeconomic analyses of the various situations. The main aspects should relate to a benefit/cost analysis of the control in areas of agropastoral land use and a comparative cost analysis of the different methods of control. The incidence of African animal trypanosomosis (AAT) is regularly monitored in sentinel herds. Consecutive entomological surveys, and dissection of captured flies to determine their physiological age and infection rate permit constant appraisal of the effects of the tsetse campaign. Ideally, this should allow assessment of trypanosomosis challenge.

However, in certain areas the results obtained so far indicate that incongruities exist between the incidence of AAT and tsetse challenge. More investigations are required to elucidate the dynamics of the tsetse–trypanosome–livestock relationship. The vectorial capacity of the different tsetse species must be better understood. Apparently, this capacity is low in *G. p. gambiensis* in an area (Samorogouan) where a tsetse campaign started about two years ago based on the treatment of the cattle population. Apparently, there exists a relic population of this species that does not seem to be affected by the tsetse campaign, presumably because it is partly feeding on reptiles. Can we afford to neglect this population because of its apparent low vectorial capacity and its present behaviour? Do we have to deal with other tsetse species (*G. m. submorsitans* were abundant before the campaign) that have a density below a detectable level and which could be responsible for an incidence of 20–25% AAT in the sentinel herds?

It is evident that the actual methodology needs to be adjusted to fit the existing situation. Additional research is required, for instance, to identify bloodmeals or DNA probes for detecting infections in tsetse flies. Tracking of low density tsetse flies demands an improvement in the trapping devices; identification of new olfactory compounds could further enhance the response of the flies, thereby improving the trapping systems.

QUARANTINE

(Session 8)

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IRRADIATION AS A QUARANTINE TREATMENT OF AGRICULTURAL COMMODITIES AGAINST ARTHROPOD PESTS

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Abstract

IRRADIATION AS A QUARANTINE TREATMENT OF AGRICULTURAL COMMODITIES AGAINST ARTHROPOD PESTS.

The purpose of quarantine treatments is to eliminate, as far as possible, the risks of introduction or establishment of exotic pests in countries or regions where they do not already occur. Treatments may be applied to host commodities traded commercially or carried by travellers. Ionizing radiation is very effective when used as a quarantine treatment to disinfest fresh, dried or processed fruits, grains and other plant materials. It is highly effective in killing or inactivating arthropod pests, leaves no residues, and at the low doses required it can be used on most commodities without affecting the quality. The most important single pest group of quarantine importance internationally is arguably fruit flies in fresh fruits and vegetables. More than thirty species of fruit flies are recognized as serious quarantine pests. Dose-mortality studies with irradiation have shown that doses of 75–150 Gy prevent adult emergence. At two Task Force Meetings on Irradiation as a Quarantine Treatment convened by the International Consultative Group on Food Irradiation, a generic dose of 150 Gy was recommended against any fruit, based on extensive research data for these pests. Most fruits are relatively unaffected by quarantine disinfestation treatments of 100–300 Gy but some, for example, avocado, appear to be intolerant. Other pests of quarantine importance for which irradiation is an appropriate disinfestation treatment include certain moths (Lepidoptera), beetles (Coleoptera), bugs (Homoptera), flies (Diptera), thrips (Thysanoptera) and mites (Acarina). The Task Force Group also recommended that a generic treatment of 300 Gy, based on the inability to perpetuate the species, would be appropriate for any pest other than fruit fly. This was derived from extensive research on the codling moth, *Cydia pomonella* (L.), and the mango seed weevil, *Sternochaetus mangiferae* (Fabricius), with supporting results on eleven other pests from six orders.

1. INTRODUCTION

The purpose of quarantine treatments against arthropod pests is to reduce, as far as is practicable, the risk of introduction of species into areas where they do not already occur. Treatments are conventionally applied to bulk produce traded

commercially, but they are also applicable to items carried by travellers. Used as a quarantine disinfestation treatment, irradiation is highly efficacious and has other advantages for fruits, grains and agricultural produce which might be infested with unwanted pests.

Fruit flies, family Tephritidae, in fresh fruits or vegetables are arguably the most important quarantine pest of agricultural commodities [1]. The orders Acarina, Thysanoptera, Coleoptera, Lepidoptera and Diptera contain further pests of quarantine importance [2] against which irradiation can constitute a useful, and sometimes unique, method of disinfestation.

The potential of irradiation to disinfest agricultural and horticultural produce has been recognized since early in this century, although most of the research has been done in the latter half. The availability of gamma radiation sources, particularly ^{60}Co, has given rise to many dose–mortality studies on insect pests and on the effects of irradiation on the organoleptic qualities of fruit and other commodities.

A major conference on the radiation disinfestation of food and agricultural products [3], held in Hawaii in 1983, focused attention on irradiation as a quarantine disinfestation treatment. This was followed in 1986 and 1991 by Task Force meetings at Chiang Mai, Thailand, and Bethesda, Maryland, United States of America, respectively, convened by the FAO/IAEA sponsored International Consultative Group on Food Irradiation (ICGFI) to review irradiation as a quarantine treatment. Reports of these Task Force meetings [4, 5] identified the pests and host commodities for which irradiation was an effective method of disinfestation. They recommended generic and specific dosages of irradiation which would ensure quarantine security based upon the appropriate criteria for effectiveness.

A major factor motivating interest in irradiation for quarantine purposes was the cancellation of registration of the fumigant ethylene dibromide (EDB) in the USA [6]. This fumigant was very effective against fruit flies, economical to purchase and apply, and logistically very flexible, requiring only a gas tight room or tent. While it remained available, alternative methods, including irradiation, were greatly disadvantaged. The loss of EDB highlighted the risk to any treatment giving rise to residues and led to heightened interest in the physical residue free treatments, cold, heat and irradiation.

A prerequisite to the use of irradiation as a quarantine treatment for agricultural commodities was approval of its use on foodstuffs. The joint FAO/WHO Codex Alimentarius Commission, General Standard for Irradiated Foods [7], recommended approval of doses up to 10 kGy as safe for use on any food. Subsequently, the United States Food and Drug Administration [8] approved doses of up to 1 kGy for disinfestation of fresh fruits and vegetables. Many other countries followed suit and it is estimated that the process has been approved in a majority of the world based on population. Finally, in 1989 the United States Department of Agriculture's Animal and Plant Health Inspection Service amended its quarantine regulations to permit use of irradiation as a quarantine treatment [9].

In this paper, a review is made of the background regulatory requirements which all quarantine disinfestation treatments must meet, together with research demonstrating efficacy against quarantine pests, specific considerations which apply to irradiation, and monitoring of the dosage required to kill arthropod pests while avoiding any damage to the commodity.

2. FRUIT FLIES

The ultimate requirement of a quarantine treatment is to prevent the establishment of pests in new areas. This can be accomplished by killing the insect at the time of treatment or shortly afterwards, preventing its development to a reproductive stage and preventing reproduction through physical deformity or infertility.

For fruit flies, the Chiang Mai Task Force Group [4] recommended that the criteria for acceptance of a proposed quarantine treatment could be based on the dosage to prevent larval survival, pupation, emergence of adults capable of flight or of adults capable of reproduction. It was recognized that this could result in the presence of properly treated, but still living, insects in fruit at a post-treatment inspection [10]. Although not compromising quarantine security, they would present a problem for inspection authorities who would be uncertain of whether the treatment had been applied properly.

The problem has almost certainly been overestimated for fruit flies. The levels of infestation present in export quality commercial fruit need to be very low, regardless of quarantine requirements. Most markets have a nil tolerance for this type of pest based on quality, and consignments found to contain infested units could be expected to be rejected on this basis. This raises the principle that quarantine disinfestation treatments should not be used as a part of pest management needed to produce a quality product. For insect pests which do not affect the fruit quality, the problem is more valid. In a subsequent section, the means of identifying insects which have received a lethal reproduction inhibiting dose of irradiation, but are still alive at the time of inspection, are discussed.

The efficacy required of a quarantine treatment is usually defined in terms of the mortality induced in the pest. Other effects, including prevention of reproduction through physical defects or sterility, are more difficult to measure but can be equivalent. Mortality is usually quoted as a percentage or a probit value [10], e.g. 99.9968% is probit 9, and should have a statistical confidence level, usually 95% [11]. These give rise to the levels of testing required by quarantine authorities to demonstrate treatments experimentally, e.g. to demonstrate probit 9 at the 95% confidence level requires tests against 93 616 pests without survivors [12].

Although some of the variation could be attributed to differences among species, differences in age and stage as well as differences in experimental technique could account for most of the variation exhibited. Because of the nature of gamma

TABLE I. EFFECTS OF EXPOSURE OF FRUIT FLY LARVAE TO GAMMA RADIATION ON PREVENTION OF SUBSEQUENT ADULT EMERGENCE [5, 17–30]

Species	Host	Dose for no emergence (Gy)	Refs
Anastrepha ludens (Loew)	Grapefruit	50	[17]
(Mexican fruit fly)	Mango	100	[18]
A. obliqua (Macquart) (West Indian fruit fly)	Mango	100	[18]
A. serpentina (Wiedemann) (Sapodilla fruit fly)	Mango	100	[18]
A. suspensa (Loew)	Grapefruit	25	[19]
(Caribbean fruit fly)	Grapefruit	154	[20]
	Mango (Florida, USA)	17.5	[21]
	Mango (Dominican Republic)	25	[21]
	Mango (Haiti)	80	[21]
	Carambola	50	[22]
	Grapefruit	40	[23]
Ceratitis capitata (Wiedemann)	Papaya	25–75	[24]
(Mediterranean fruit fly)	Mango	150	[18]
Bactrocera cucurbitae (Coquillett)	Pumpkin	150	[23]
(Melon fly)	Mixed	100	[24]
B. dorsalis (Hendel)	Avocado	100	[24]
(Oriental fruit fly)	Mixed	100	[24]
	Mango	150	[25]
	Mango	100	[26]
	Mango	100	[26]
	Mango	150	[27]
B. jarvisi (Tryon) (Jarvis' fruit fly)	Mango	75–101	[15]
B. tryoni (Froggatt)	Mango	75–101	[15]
(Queensland fruit fly)	Apple	75	[28]
	Tomato	50	[28]
	Cherry	75	[28]
	Avocado	75	[28]
	Orange	75	[28]
B. zonatas (Saunders)	Guava	50	[29]
(Guava fruit fly)		50	[29]
		55	[29]
Rhagoletis indifferens (Curran) (Western Cherry fruit fly)	Cherry	97	[30]

radiation, it is virtually impossible to administer a uniform dose to any experimental sample of significant volume. Also, use of statistical regression analyses to predict the dose required for high efficacies such as probit 9 can lead to overestimation. The efficacy targeted and the criteria for effectiveness have a bearing on the results.

In general, exposure of eggs or larvae in fruit to a dose of 150 Gy or less prevented emergence of adults at an efficacy of probit 9 [1]. This was adopted as the generic dose applicable to all fruit flies of the family Tephritidae. Much greater doses would be required to cause probit 9 mortality of the stage treated, increasing the likelihood of damage to the fruit [13]. Where a lower dose has been shown to achieve probit 9 efficacy, it would be used; for example, for the Queensland fruit fly, *Bactrocera tryoni* (Froggatt), a dose of less than 100 Gy, possibly 75 Gy, has been shown to be effective [14, 15].

There are sound statistical data for 11 fruit fly species of major quarantine importance showing the dose to prevent adult emergence [16]. These are listed in Table I [5, 17–30], together with the fruit on which the efficacy was demonstrated. The fly species are from five genera with geographical distribution in Australia, Asia, North America, South America and Europe. These results provide strong assurance of the efficacy of the generic dose against any fruit fly of the family Tephritidae.

3. OTHER INSECTS

The 1986 Chiang Mai ICGFI Task Force Meeting on Irradiation as a Quarantine Treatment [4] recommended a generic dose of 300 Gy for insects other than fruit fly. On available evidence, this dose was judged to provide quarantine security against any stage of any insect pest using the criteria of prevention of "emergence of normal adults from treated eggs, larvae or pupae or to sterilise any adults emerging from treated larvae or pupae". A review presented at the 1991 Bethesda Task Force Meeting [31] identified insects from five orders and mites from two groups against which a disinfestation treatment with irradiation had been shown to provide quarantine security at doses of 300 Gy or less. These included three species of Lepidoptera, two of Coleoptera and two of Acarina. Effective quarantine doses ranged from 80 to 300 Gy (Table II) [5, 15, 32–42].

With the exception of the codling moth, *Cydia pomonella* (Linnaeus), and the mango seed weevil, *Sternochaetus mangiferae* (Fabricius), for which comprehensive mortality data were available [33, 38], these pests were not shown to have been killed to very high levels of treatment efficacy. This was due to the scope of the experiments and does not reflect on the treatment. It is a measure either of the lesser quarantine importance of most of these pests or of the early stage of research. However, all of the results supported the adequacy of 300 Gy as a generic treatment.

TABLE II. EFFECTS OF EXPOSURE OF ADULTS AND JUVENILES OF INSECTS AND ALLIED FORMS (OTHER THAN FRUIT FLIES) TO GAMMA IRRADIATION ON PREVENTION OF SUBSEQUENT ADULT EMERGENCE OR REPRODUCTION [5, 15, 32–42]

Pest species	Host	Effective dose (Gy)	Refs
	Lepidoptera		
Clepsis spectrana (Treitschke) (rose leaf roller)	Rose	200	[32]
Cydia pomonella (Linnaeus) (codling moth)	Apple, walnuts	139–177	[33, 34]
Epiphyas postvittana (Walker) (lightbrown apple moth)	Apple	199	[35, 36]
	Coleoptera		
Asynonchus cervinus (Boheman)	Citrus	150	[37]
Sternochaetus mangiferae (Fabricius) (mango seed weevil)	Mango	300	[15, 38]
	Hemiptera–Homoptera		
Brachycorynella asparagi (Mordvilko)	Asparagus	100	[39]
Quadraspidiotus perniciosus (Comstock) (San José scale)	Apple	300	[40]
	Diptera		
Liriomyza trifolii (Burgess) (serpentine leaf miner)	Chrysanthemum, tomato	80	[32]
	Thysanoptera		
Frankliniella pallidus (Steele)	Flowers	100	[32]
	Acarina		
Brevipalpis destructor (Baker)	Grapes	300	[41]
Tetranychus urticae (Koch)	Fruits, flowers	300	[42]

Mites responded in a similar fashion to insects. From the responses of mites as a group to irradiation it can be expected that 300 Gy will prove adequate. In addition to the mites given in Table II, a comprehensive study on the mould mite, *Tyrophagus putrescentiae* (Schrank), and the bulb mite, *Rhyizoglyphus echinopus* (Fumouze and Robin) [42], showed that mortality or adult sterility resulted from doses of 260 Gy. The only exceptional response of mites came from a preliminary study on cut flowers by Wit and de Vrie [32]. It can therefore be expected that the physiological response of mites to irradiation will be similar to that of insects.

Whereas quarantine restrictions on the entry of fruit fly host material almost invariably invoke treatments of the order of probit 8.7 (99.99%) or probit 9 (99.9968%), the efficacy required for other pests varies. An example of a pest for which a lower efficacy would be satisfactory is against the eggs of the Fuller rose beetle, *Asynonchus cervinus* (Boheman), on citrus fruit, where the requirement is that there are no live eggs on fruit at inspection. The condition can be met by a treatment of considerably lower efficacy than probit 9. A similar situation can apply to thrips and mites on cut flowers.

Irradiation is also highly appropriate for the disinfestation of stored products, although the justification for quarantine treatments against such cosmopolitan insects is questionable. Legume weevils (bruchids), *Callosobruchus* spp. and others, can be disinfested at doses as low as 70 Gy [43]. Cereal grains can be disinfested of the common grain beetles *Sitophilus, Rhyzopertha, Tribolium* and other species at doses below 200 Gy, but information on the moths *Sitotroga, Ephestia, Plodia* and other species is less precise. The dosages reported often appear to have been chosen arbitrarily and have possibly been influenced by operational factors related to the irradiation source. An important aspect of stored grain pests is that they can be irradiated with an electron beam source directed at a grain flow [44].

4. MODE OF ACTION OF IRRADIATION

All insect pests are susceptible to control by irradiation. The tissues most sensitive to irradiation are those containing a high proportion of dividing cells. During the immature development of insects, there are periods within each stage when cell division is highly active. This could explain the difference in response of insect developmental stages with age [45]. In adults, the two most important sites of cell division appear to be the gonads and gut cells. Disruption of the former can cause temporary or permanent sterility, and of the latter, death by starvation. At higher doses, other tissues may also be damaged, leading to more rapid mortality [46]. Particular sites of damage include the enzyme secretion systems, epidermal lipid secretion systems and the nervous system, especially the supraoesophageal ganglion, which exercises hormonal control over development [47, 48].

5. EFFECTS ON COMMODITIES

Because quarantine disinfestation treatments against arthropod pests involve very low doses of irradiation, most agricultural commodities are essentially unaffected. The quality attributes of fruit, food and other agricultural products were reviewed in depth at the 1983 conference on radiation disinfestation of food and agricultural products held in Hawaii [3]. Subsequent trials in Queensland [13, 49, 50] showed that lychee, mangoes (Kensington), capsicum, broccoli, chinese cabbage, melons, peaches, nectarines, passion fruit and tomatoes withstood a (generic) 300 Gy treatment without ill effects. Banana, carambola, navel orange, rambutan, persimmon, papaw, zucchini and rockmelons were relatively unaffected at a lower (fruit fly) dose of 75 Gy. Fruit which did not tolerate disinfestation were custard apple and avocado (except, possibly, cv Hass). Strawberries are well known for their high tolerance to irradiation (2 kGy), although this far exceeds any requirement for quarantine disinfestation. These trials assessed fruit on the basis of external colour, firmness, injury, internal colour, soluble solids, pH, acidity, vitamin C, sugars, acids, eating quality and disease.

Cereal grains are another major agricultural commodity which can be irradiated for quarantine purposes. Adverse effects on baking properties have been reported, but at insect or mite disinfestation doses of 200 Gy it is doubtful if irradiation of grains would have significant effects [51]. Spices are frequently irradiated at high doses to reduce their bacterial count, but this is done for health, not quarantine, purposes.

6. OPERATIONAL CONSIDERATIONS

6.1. Doses

Although the minimum doses for quarantine security may be very low, in practice a much higher dose may have to be given to ensure that all of the product being irradiated receives at least the minimum dose. Factors of 2:1 are common where boxes of fruit are to be irradiated; 3:1 can be needed. Careful attention to irradiation geometry is therefore required where the product to be irradiated is susceptible to damage. Fruits range in tolerance but almost all can be damaged at or above twice the generic dose of 300 Gy [13, 49, 50]. Where this is a problem, consideration can be given to combination treatments such as cold storage and irradiation [52].

6.2. Markers

Physical irradiation markers are a topic outside the scope of this paper. However, it would be of advantage to quarantine authorities if it could be determined

for insects found alive at an inspection following irradiation whether they had been irradiated. Studies on the Mediterranean fruit fly [48] and the Queensland fruit fly [52] have shown that the supraoesophageal ganglion fails to develop normally in irradiated larvae. Development can be expressed as a ratio of the size of the ganglion compared with a structure such as the crop. Another difference reported was the failure of ovaries and testes to develop and the loss of yellow colouration, e.g. of humeral calli, in flies emerging from the pupae of Queensland fruit flies irradiated at quarantine doses [53].

Biochemical diagnosis is also possible. A study using the technique of polyacrylamide gel electrophoresis on irradiated larvae of the Oriental fruit fly, *Bactrocera dorsalis* (Hendel), has shown reliable detectable differences in the protein band profiles [54]. This technique holds considerable promise as a relatively rapid diagnostic method.

6.3. Security after treatment

Irradiation does not confer any residual protection to disinfested produce, so measures must be implemented to ensure quarantine security after treatment. Where the product can be irradiated in insect secure packaging, e.g. fruit cartons, the only security measure necessary is separation of the unirradiated and irradiated stock. If the product is irradiated as individual pieces, e.g. fruit, then the whole facility must be made insect secure after treatment. Regulatory authorities will probably be involved in certification of the product.

6.4. Source options

Ionizing energy can be applied as gamma radiation, X radiation or as electron beams. Commercial facilities using all of these methods are now in operation. As far as the effects on arthropods are concerned, there should be no difference in effect between gamma and X rays. The choice between gamma rays (^{60}Co) and X rays (electric machines) becomes a commercial decision. However, for electron beam radiation, penetration is much less than either gamma or X rays and there is no certainty that the doses needed would be exactly the same. Modern linear accelerators possess the capability of operating in either the electron beam or X ray mode. The choice of the type of irradiation can then depend on the product to be irradiated.

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A BIOCHEMICAL MARKER FOR DETECTION OF IRRADIATED PUPAE OF THE ORIENTAL FRUIT FLY (*Dacus dorsalis*)

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Abstract

A BIOCHEMICAL MARKER FOR DETECTION OF IRRADIATED PUPAE OF THE ORIENTAL FRUIT FLY (*Dacus dorsalis*).

A biochemical method for detecting a radiation sensitive protein marker in pupae of gamma irradiated larvae of the Oriental fruit fly (*Dacus dorsalis*) using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is presented. Third instar larvae were irradiated at 100 Gy using a ^{60}Co dry gamma cell. SDS-PAGE protein profile analysis of pupae of the irradiated larvae revealed the absence of a specific protein band (ascribed to a gene product, Gs, of a putative radiation sensitive marker gene) in comparison to the protein profile of unirradiated control larvae. Absence of the Gs protein band from the electrophoretic gel profile was observed only in two-day-old pupae (day 3 post-irradiation) and even at a dose as low as 40 Gy. Thus, irradiation of immature (two-day-old) larvae at 100 Gy resulted in the subsequent absence of the Gs protein only after day 8 post-irradiation (three-day-old pupae), by which stage the pupae of the unirradiated larvae had already started to synthesize the Gs protein, as seen by the SDS-PAGE gel profile. The absence of this Gs protein band in the SDS-PAGE profile of pupae of the irradiated larvae could be used as a biochemical marker for establishing the efficacy of irradiation of the Oriental fruit fly.

1. INTRODUCTION

The quarantine security level for fresh agricultural products imposed by importing countries usually involves the minimum radiation dose that will prevent insects from emerging into viable adults. This is the criterion for establishing the efficacy of irradiation as a quarantine treatment [1]. A 300 Gy dose is the minimum dose required to treat any insect stage for such quarantine security [2]; for member species of the Tephritidae family, 100 Gy is the minimum dose [2].

Experimental data have shown that as a quarantine treatment, a dose of 100 Gy prevented the Oriental fruit fly, *Dacus dorsalis*, from emerging into an adult capable of flight [3]. The tolerance of fruit flies to radiation increases with age; this sensitivity to radiation depends on the species, the age and the stage of development as well

as the dose rate applied. Mature, third instar larvae are more resistant to a low dose of 25 Gy than larvae at the first and second instar stages [4].

Detection methods for irradiated foods are required to determine whether food has been irradiated effectively, i.e. whether the larvae or pupae found in a food package will emerge as viable adult flies. Several detection methods have been presented as part of the Programme on Analytical Detection Methods for Irradiation Treatment of Foods (ADMIT). A few are acceptable but, to date, no detection method has been adopted as an internationally agreed upon protocol for establishing the efficacy of irradiation treatment of foods [1].

Separation and identification of macromolecules using polyacrylamide gel electrophoresis (PAGE) show some potential for adaptation as an analytical technique for detecting radiation induced changes in protein biosynthesis. Our preliminary studies [5] on the electrophoretic profile of proteins extracted from *D. dorsalis* at different stages of development showed changes in certain proteins as the insect evolved into the adult stage. Distinct banding patterns of the proteins were observed in the electrophoretic profile at each stage of development. Thus, each profile could be used as an electrophoretic signature for a particular stage of insect development [5].

One of the recommendations made at the International Consultative Group on Food Irradiation (ICGFI) Task Force Meeting held in 1991 in Bethesda, Maryland, USA, was to develop a rapid, practical technique for determining whether insects have been irradiated adequately [2]. To address this problem, this paper presents a biochemical detection method for gamma irradiated larvae of the Oriental fruit fly using SDS-PAGE.

2. MATERIALS AND METHODS

Acrylamide, bis-acrylamide, ammonium persulphate, TRIZMA base, glycine, TEMED (tetramethyl ethylenediamine), bromophenol blue, Coomassie Brilliant blue, molecular weight markers (bovine serum albumin, chicken albumin, urease) were obtained from Sigma. Glycerine CP was purchased from a local chemical company. 2-mercaptoethanol was obtained from Fluka. $NaHPO_4$, EDTA, KCl, methanol and acetic acid were purchased from Baker Chemicals. Sodium dodecyl sulphate was obtained from Aldrich Chemicals. The homogenizer used was Dounce homogenizer purchased from VWR (USA). A Bio-Rad Mini-Protean II electrophoresis apparatus was used employing a Buchler power supply.

2.1. Irradiation

Dacus dorsalis larvae (5 to 6-day-old (third instar)) were irradiated at the multipurpose gamma irradiation facility of the Philippine Nuclear Research Institute

using a ^{60}Co dry gamma cell. Immature *D. dorsalis* larvae (two-day-old) were likewise irradiated in the same facility. After irradiation, the larvae were transferred to vials containing coconut coir dust and allowed to continue their life-cycle at room temperature.

2.2. Homogenization

Five pieces of post-irradiated larvae/pupae were homogenized in 0.4 mL buffer (10mM $NaHPO_4$, 400mM KCl, 1mM EDTA), pH7.6, in the cold using a Dounce homogenizer. The homogenate was centrifuged at 1500g for 5 min at 8°C and the soluble fraction was transferred to a clean test tube.

2.3. SDS-PAGE

A 100 μL sample buffer (3.3% SDS, 10% glycerine, 0.1M tris) was added to 100 μL of soluble fraction. 2-mercaptoethanol (10 μL) and 0.1% bromophenol blue (10 μL) were pipetted into the sample in buffer solution and heated at 80–90°C for 1 min or longer. The Laemmli method for slab gel electrophoresis was followed, with some modifications [6]. The 10–15 μL sample was applied to each electrophoresis well. A 7.5% separating gel was used, with a 3.5% stacking gel. The running buffer was 0.025M tris, 0.19M glycine. The proteins were electrophoresed at 25 mA for about 1 h or until the blue dye was approximately 2 cm above the lower end of the gel. Gel was stained in Coomassie Brilliant blue in 7% HAc for 1–2 h and destained in 7% HAc, 5% MeOH overnight, or until the blue dye background was removed.

3. RESULTS AND DISCUSSION

Mature (six-day-old) larvae irradiated at 100 Gy and 'electrophoresed' at day 3 post-irradiation (two-day-old pupae) showed the absence of a protein band when compared with the unirradiated pupae of the same age. This band, which we designated the Gs protein, only appeared at the start of day 3 post-irradiation (two-day-old pupae); it had evidently not been synthesized at an earlier stage (Fig. 1).

It has been shown that although larvae irradiated at a dose of 100 Gy go into pupation, they fail to emerge into adult flies capable of flight [7]. At this dose, a probit 9 (99.9968% mortality) quarantine security is reached for *D. dorsalis* [2]. In our studies, we observed delayed pupation of irradiated third instar larvae; day 3 post-irradiated larvae (two-day-old pupae) exhibited electrophoretic profiles showing the absence of the Gs protein band (Fig. 1).

To determine when the putative gene coding for the Gs protein is turned on for synthesis and how this is affected by gamma irradiation, immature, two-day-old

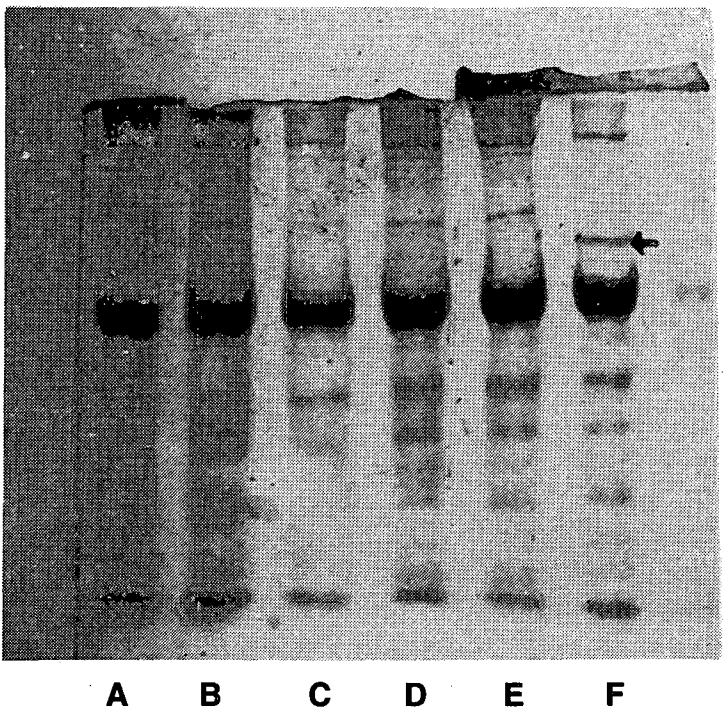

FIG. 1. The SDS-PAGE protein profile of seven-day-old larvae (A, B) one-day-old pupae (C, D) and two-day-old pupae (E, F) at day 1, day 2 and day 3 post-irradiated third instar larvae of D. dorsalis, *respectively. Dose is 100 Gy. The irradiated larvae are A, C and E, and the unirradiated control larvae are B, D and F. The arrow in F indicates the Gs protein.*

larvae were irradiated at 10, 25, 50 and 75 Gy. At day 4 post-irradiation, the electrophoretic profile showed identical protein patterns for both the irradiated and the unirradiated larvae at all the radiation doses tested. When irradiation of the immature larvae reached 100 Gy, the SDS-PAGE profile of six- and seven-day-old larvae (days 4 and 5 post-irradiation) did not differ from the protein profiles of larvae irradiated at lower doses. The GS protein band was definitely absent in the electrophoretic profiles derived from both the irradiated and unirradiated larvae; at days 4 and 5 post-irradiation, the insect had not yet turned into a pupa and so the protein profiles were still those of a larva. This suggests that at this early stage, when the insect was still in its larval stage, the putative gene coding for the Gs protein had not yet been transcribed. However, in one-day-old pupae (day 6 post-irradiation) the electrophoretic profile showed a faintly stained band of Gs protein, which became a

darkly stained band in three-day-old pupae (day 8 post-irradiation). In contrast, this Gs band was definitely missing in the pupae of irradiated larvae of the same age (Fig. 2). Experimental results suggest that at least one mutational event had taken place at the level of the genome and that the putative gene susceptible to such damage (and coding for the Gs protein) was responsible for the synthesis of a primary or secondary gene product necessary to transform pupae into adult flies. It has been demonstrated [4] that in the *D. dorsalis* life-cycle, immature larvae are the insect stage most sensitive to irradiation at a dose of 100 Gy or higher.

The electrophoretic protein profile of three-day-old pupae from irradiated (25–50 Gy) larvae at the third instar stage and tested for Gs protein already showed the absence of the Gs protein band at a low dose of 40 Gy (Fig. 3). The Gs protein bands from pupae of larvae irradiated at doses of 25, 30 and 35 Gy were still faintly

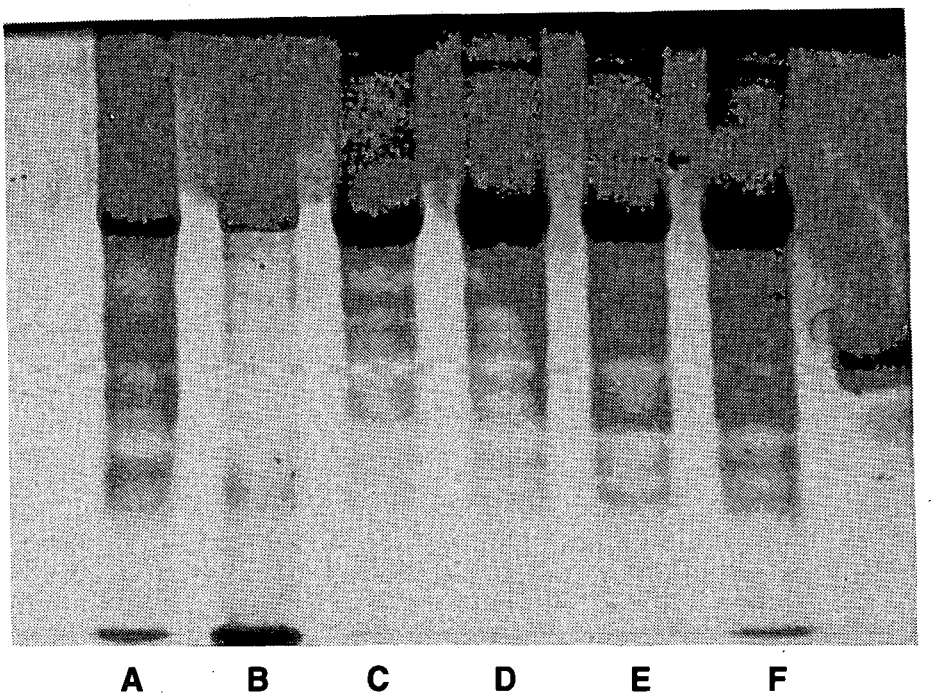

FIG. 2. The SDS-PAGE protein profile of seven-day-old larvae (A, B), one-day-old pupae (C, D) and three-day-old pupae (E, F) at day 5, day 6 and day 8 post-irradiated immature (two-day-old) larvae of D. dorsalis, *respectively. Dose is 100 Gy. The irradiated larvae are B, D and F, and the unirradiated control larvae are A, C and E. The arrow in E indicates the Gs protein.*

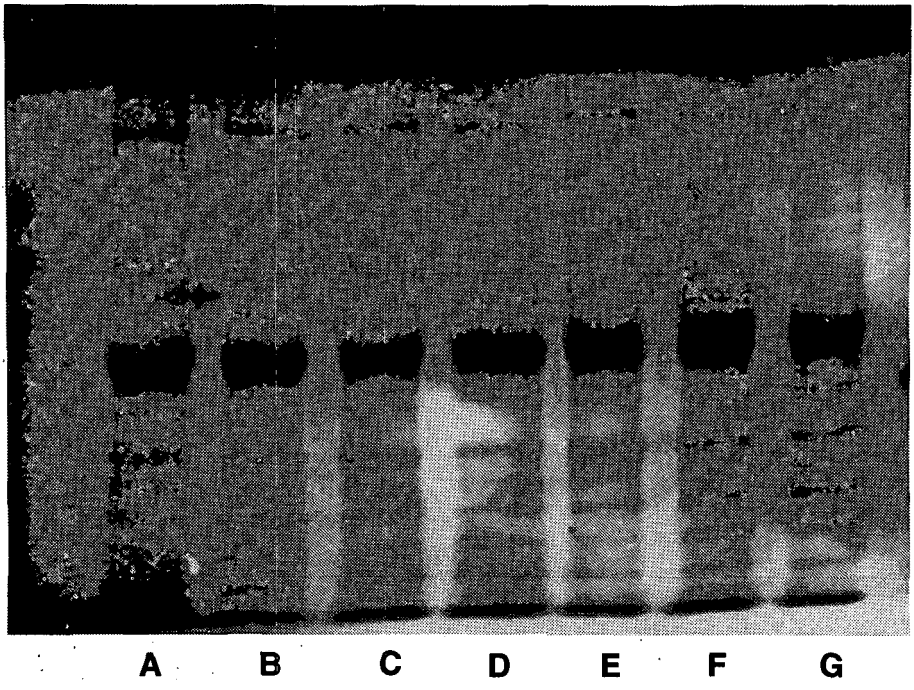

FIG. 3. The SDS–PAGE protein profile of three-day-old pupae (day 4 post-irradiation) of the third instar larvae of D. dorsalis *irradiated at various doses. The irradiated larvae are B (25 Gy), C (30 Gy), D (35 Gy), E (40 Gy), F (45 Gy) and G (50 Gy) and the unirradiated control larvae are A. The arrow in A indicates the Gs protein.*

present (compared with the electrophoretic profiles of the pupae from unirradiated larvae). This signifies that some form of damage (DNA lesion) had already been sustained by the Gs gene at a low dose of 25 Gy, although this dose may not be sufficient to disable (transcriptionally) the putative Gs gene completely.

The apparent molecular weight of the Gs protein, by linear regression, ranges between 105 and 115 kilodalton. This protein is detectable only at the pupal and adult stages of the *D. dorsalis* life-cycle and may be an obligatory protein in the maturation process towards the pupal stage and, beyond, into adult flies capable of flight.

Manoto and Resilva [7] have shown that in larvae exposed to 100 Gy, pupation takes place but not emergence into adults In our studies, the SDS–PAGE profile of pupae from the irradiated larvae showed clearly that these pupae do not emerge into adult flies because of the 'missing' protein component (Fig. 1). It was apparent that

a certain gene-enzyme system had been disrupted by gamma radiation and thus cellular production of certain key proteins had become inoperative, which explains why a certain protein band found in two-day-old control pupae was missing in two-day-old pupae (day 3 post-irradiation) of irradiated larvae. It is speculated that this particular Gs protein is necessary for the emergence of pupae into adults capable of flight.

4. CONCLUSIONS

Studies on the SDS-PAGE profile of proteins from pupae of irradiated larvae showed distinctly that a specific protein, designated as Gs, which is not present in the early stages of development (egg and larva), is needed in the transformation process of pupae into adult flies capable of flight. Absence of this putative Gs protein from the pupal stage was observed when the larvae were irradiated at a dose as low as 40 Gy. Our studies showed that the gene coding for this protein may have sustained a mutational lesion upon exposure of the larvae to a gamma dose as low as 40 Gy. Thus, as a result of radiation at this dose, this protein was found to be absent in the SDS-PAGE profile of two-day-old pupae of the irradiated larvae. This can be taken as proof that the irradiation of the pest, even at the immature larval stage, will prevent its emergence into an adult fly capable of flight. The absence of the Gs protein band in the SDS-PAGE protein profile of pupae therefore shows good potential as a biochemical marker for establishing the efficacy of irradiation of fruit flies. It is also an indicator that can be used by the quarantine services of a fruit importing country. The test, as implemented here, need not use SDS-PAGE; for example, some type of colour reaction could be used that is more specifically designed to detect directly the absence of the Gs protein in the soluble protein fraction of the pupae. This type of direct test would require further developmental studies, which are currently under way at our laboratory.

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POSTER PRESENTATION

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IRRADIATION AS A QUARANTINE TREATMENT FOR EUROPEAN RED MITE EGGS ON APPLES*

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Viable eggs of the European red mite, *Panonychus ulmi* Koch (Acari: Tetranychidae), on apples have been the concern of several importing countries, and exports require pre-shipment (i.e. quarantine) treatment to eliminate the live eggs [1]. The following treatments are suggested: fumigation with chemicals, modified atmosphere, cold treatment and ionizing radiation.

The objectives of this study were to determine the density of European red mite eggs on apples, and to determine the dose of gamma radiation required to prevent development of diapausing eggs of *P. ulmi*.

Apples with eggs of European red mite were treated with ^{60}Co gamma radiation at a dose rate of 140 Gy/min. After the treatment, the calyx end and stem cavity of each apple were ringed with white glycerine prior to incubation. Apples with eggs were incubated in darkness at 24°C for a period of 14 d. After this period, the apples were examined and the eggs and larvae counted.

Usually, 70–90% of the apples were infested with eggs of *P. ulmi*. The more infested apples in the sample, the more eggs were laid per apple. Distribution of mite eggs on apples was very characteristic. Aggregations of eggs were located in the calyx ends more often than in the stem cavities.

Preliminary experiments indicate that gamma radiation applied at 0.4–0.6 kGy inhibited completely the hatchability of eggs. About 60% of control eggs hatched.

The effects of lower doses of gamma radiation on the viability of diapausing eggs are presented in Table I. It is seen that wintering eggs of *P. ulmi* are very susceptible to irradiation. A dose of 100–125 Gy caused complete inhibition of egg hatchability. This dose is suggested for quarantine treatment of apples infested with diapausing eggs of the European red mite. A few larvae hatched at doses higher than 100 Gy. However, these larvae died soon after treatment without moulting into the next developmental stage.

* This research was carried out with the support of the IAEA under Research Contract No. 6942/RB.

TABLE I. HATCHABILITY OF WINTERING EGGS OF THE EUROPEAN RED MITE TREATED WITH GAMMA RADIATION

Apple variety	Dose (kGy)	No. of eggs treated	% hatched eggs	% dead eggs
Bancroft	0.0	428	66.4	33.6
	0.125	395	0.0	100.0
	0.25	430	0.5	99.5
Boiken	0.0	672	55.1	44.9
	0.1	682	0.0	100.0
	0.2	384	0.3	99.7
	0.3	308	0.0	100.0
	0.4	558	0.0	100.0

Apples are among those fresh fruits and vegetables which are relatively tolerant to irradiation stress at doses below 1 kGy. At these doses, only minimum detrimental effects of radiation on apples have been observed.

The embryonic stage of an animal is a time of extreme radiosensitivity [2, 3] and mites are no exception. However, the specific stage of embryonic development involved determines the observed radiosensitivity [4]. Usually, as the embryo achieves greater development, its resistance increases dramatically. Taking this into consideration, as well as the results which indicate the high susceptibility of the wintering eggs of *P. ulmi* to irradiation, one may conclude that these diapausing eggs are at the early stages of embryogenesis, before termination of diapause.

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