

***Quality Control for
Expanded Tsetse Production,
Sterilization and Field Application***



IAEA

International Atomic Energy Agency

Quality Control for Expanded Tsetse Production, Sterilization and Field Application

The following States are Members of the International Atomic Energy Agency:

AFGHANISTAN	GHANA	NIGERIA
ALBANIA	GREECE	NORWAY
ALGERIA	GUATEMALA	OMAN
ANGOLA	HAITI	PAKISTAN
ARGENTINA	HOLY SEE	PALAU
ARMENIA	HONDURAS	PANAMA
AUSTRALIA	HUNGARY	PAPUA NEW GUINEA
AUSTRIA	ICELAND	PARAGUAY
AZERBAIJAN	INDIA	PERU
BAHRAIN	INDONESIA	PHILIPPINES
BANGLADESH	IRAN, ISLAMIC REPUBLIC OF	POLAND
BELARUS	IRAQ	PORTUGAL
BELGIUM	IRELAND	QATAR
BELIZE	ISRAEL	REPUBLIC OF MOLDOVA
BENIN	ITALY	ROMANIA
BOLIVIA	JAMAICA	RUSSIAN FEDERATION
BOSNIA AND HERZEGOVINA	JAPAN	SAUDI ARABIA
BOTSWANA	JORDAN	SENEGAL
BRAZIL	KAZAKHSTAN	SERBIA
BULGARIA	KENYA	SEYCHELLES
BURKINA FASO	KOREA, REPUBLIC OF	SIERRA LEONE
BURUNDI	KUWAIT	SINGAPORE
CAMBODIA	KYRGYZSTAN	SLOVAKIA
CAMEROON	LAO PEOPLE'S DEMOCRATIC REPUBLIC	SLOVENIA
CANADA	LATVIA	SOUTH AFRICA
CENTRAL AFRICAN REPUBLIC	LEBANON	SPAIN
CHAD	LESOTHO	SRI LANKA
CHILE	LIBERIA	SUDAN
CHINA	LIBYA	SWEDEN
COLOMBIA	LIECHTENSTEIN	SWITZERLAND
CONGO	LITHUANIA	SYRIAN ARAB REPUBLIC
COSTA RICA	LUXEMBOURG	TAJIKISTAN
CÔTE D'IVOIRE	MADAGASCAR	THAILAND
CROATIA	MALAWI	THE FORMER YUGOSLAV REPUBLIC OF MACEDONIA
CUBA	MALAYSIA	TUNISIA
CYPRUS	MALI	TURKEY
CZECH REPUBLIC	MALTA	UGANDA
DEMOCRATIC REPUBLIC OF THE CONGO	MARSHALL ISLANDS	UKRAINE
DENMARK	MAURITANIA	UNITED ARAB EMIRATES
DOMINICA	MAURITIUS	UNITED KINGDOM OF GREAT BRITAIN AND NORTHERN IRELAND
DOMINICAN REPUBLIC	MEXICO	UNITED REPUBLIC OF TANZANIA
ECUADOR	MONACO	UNITED STATES OF AMERICA
EGYPT	MONGOLIA	URUGUAY
EL SALVADOR	MONTENEGRO	UZBEKISTAN
ERITREA	MOROCCO	VENEZUELA
ESTONIA	MOZAMBIQUE	VIETNAM
ETHIOPIA	MYANMAR	YEMEN
FINLAND	NAMIBIA	ZAMBIA
FRANCE	NEPAL	ZIMBABWE
GABON	NETHERLANDS	
GEORGIA	NEW ZEALAND	
GERMANY	NICARAGUA	
	NIGER	

The Agency's Statute was approved on 23 October 1956 by the Conference on the Statute of the IAEA held at United Nations Headquarters, New York; it entered into force on 29 July 1957. The Headquarters of the Agency are situated in Vienna. Its principal objective is "to accelerate and enlarge the contribution of atomic energy to peace, health and prosperity throughout the world".

QUALITY CONTROL FOR EXPANDED TSETSE PRODUCTION, STERILIZATION AND FIELD APPLICATION



COPYRIGHT NOTICE

All IAEA scientific and technical publications are protected by the terms of the Universal Copyright Convention as adopted in 1952 (Berne) and as revised in 1972 (Paris). The copyright has since been extended by the World Intellectual Property Organization (Geneva) to include electronic and virtual intellectual property. Permission to use whole or parts of texts contained in IAEA publications in printed or electronic form must be obtained and is usually subject to royalty agreements. Proposals for non-commercial reproductions and translations are welcomed and considered on a case-by-case basis. Enquiries should be addressed to the IAEA Publishing Section at:

Marketing and Sales Unit, Publishing Section
International Atomic Energy Agency
Vienna International Centre
PO Box 100
1400 Vienna, Austria
fax: +43 1 2600 29302
tel.: +43 1 2600 22417
email: sales.publications@iaea.org
<http://www.iaea.org/books>

For further information on this publication, please contact:

Insect Pest Control Section
International Atomic Energy Agency
Vienna International Centre
PO Box 100
1400 Vienna, Austria
email: Official.Mail@iaea.org

© IAEA, 2012
Printed by the IAEA in Austria
July 2012

IAEA Library Cataloguing in Publication Data

Quality control for expanded tsetse production, sterilization
and field application. – Vienna : International Atomic
Energy Agency, 2012.

p. ; 30 cm. – (IAEA-TECDOC series, ISSN
1011-4289 ; no. 1683)

ISBN 978-92-0-130110-9

Includes bibliographical references.

1. Quality control. 2. Insect sterilization. 3. Tsetse-
flies – Control. 4. Glossina. I. International Atomic
Energy Agency. II. Series.

FOREWORD

The use of the sterile insect technique (SIT) for the control of pest insects as part of an integrated, area-wide approach is widely accepted. Its application for the eradication of different tsetse flies, the vectors of human sleeping sickness and African animal trypanosomosis, is attracting increasing interest.

Following several initial demonstrations of the application of the SIT for tsetse control the technique was applied on the island of Unguja, Zanzibar, in the mid-1990s and, as the final component of an integrated control programme, led to the eradication of the only tsetse species on the island, *Glossina austeni*. This successful programme encouraged a number of countries to embark on projects with an SIT component for tsetse control, most Ethiopia.

In 2001 the Pan African Tsetse and Trypanosomosis Eradication Campaign (PATTEC) was launched by the Organization of African Unity (now African Union, AU) and, subsequently, six countries obtained funding from the African Development Bank (AfDB) in 2005 to support control programmes with an SIT component. A further six countries have subsequently requested AfDB funding for their programmes.

The FAO/IAEA coordinated research project (CRP) on Automation for Tsetse Mass Rearing For Use in Sterile Insect Technique Programmes, which was completed in 2001, led to the development of a semi-automated system for tsetse production. Using this new system, a large rearing facility was established in Addis Ababa, Ethiopia, to supply sterile males for an elimination programme in the southern Rift Valley.

The development of large-scale rearing highlighted the need for improved quality control procedures and, with this in mind, the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture established a CRP in 2003 entitled Improved and Harmonized Quality Control for Expanded Tsetse Production, Sterilization and Field Application with the Objective of Improving and Expanding the Quality Control Sections of the FAO/IAEA Standard Operating Procedures for Mass rearing Tsetse Flies.

Sixteen institutions from thirteen countries in Africa, Europe and Central America were awarded contracts or agreements under the CRP.

This publication is a report of the results and outputs of that CRP, including the new and revised quality control tests that resulted from it.

The IAEA officer responsible for the preparation of this publication was A. Parker of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture.

EDITORIAL NOTE

The papers in these proceedings are reproduced as submitted by the authors and have not undergone rigorous editorial review by the IAEA.

The views expressed do not necessarily reflect those of the IAEA, the governments of the nominating Member States or the nominating organizations.

The use of particular designations of countries or territories does not imply any judgement by the publisher, the IAEA, as to the legal status of such countries or territories, of their authorities and institutions or of the delimitation of their boundaries.

The mention of names of specific companies or products (whether or not indicated as registered) does not imply any intention to infringe proprietary rights, nor should it be construed as an endorsement or recommendation on the part of the IAEA.

The authors are responsible for having obtained the necessary permission for the IAEA to reproduce, translate or use material from sources already protected by copyrights.

CONTENTS

SUMMARY	1
Developing QUALITY control procedures to sustain a supply of high quality blood For mass rearing tsetse flies	5
<i>C.J. de Beer, G.J. Venter, F.T. Potgieter</i>	
Blood collection from local abattoirs for mass production of tsetse flies to be used in the sterile insect technique	25
<i>M.B. Byamungu, B. Kiimbisa, S. Matembo, G. Mashenga</i>	
Development of high temperature short time vertebrate-blood pasteurization equipment for tsetse fly diets	33
<i>I. Morávek, J. Lach, P. Takáč</i>	
Colony establishment of <i>glossina fuscipes fuscipes</i> and <i>glossina pallidipes</i> (origin tororo) at kaliti tsetse rearing and irradiation center, Ethiopia	41
<i>S. Mekonnen</i>	
Research results from the tsetse fly mass rearing laboratory at Cirdes and their application during 2003–2008	49
<i>A.Z. Bance, I. Kabore, I. Sidibe, J.B. Rayaisse, Z. Bengaly</i>	
Flight muscle development in the males of <i>glossina pallidipes</i> reared for the sterile insect technique	83
<i>F. Čiampor, Jr., Z. Pálošová, L. Mancošová, P. Takáč</i>	
Parturition in tsetse flies: Endocrine control	63
<i>J. Žd'árek, P. Takáč, D. Keszeliová, L. Šimo, L. Roller, J. Cvačka, M. Šanda</i>	
Recording and analysis of tsetse flight responses in three dimensions	97
<i>P.M. Guerin, A. Gurba, V. Harraca, J.-L. Perret, S. Casera, S. Donnet</i>	
Acoustic method for testing the quality of sterilized male tsetse flies (<i>Glossina pallidipes</i>)	101
<i>H. Kratochvil, A. Noll, J. Bolldorf, A.G. Parker</i>	
Effect of feeding blood treated with isometamidium chloride to <i>Glossina morsitans morsitans</i> on the flies' subsequent vectorial capacity, and evaluation of an <i>in vitro</i> feeding system for the maintenance of infected tsetse flies	109
<i>P. Van den Bossche, J. Van Den Abbeele, R. De Deken</i>	
Effects of Samorin® treatment and delayed infection on the establishment of trypanosomes in <i>glossina pallidipes</i>	113
<i>S. Okoth, P. Mukiria, G. Tinega, G. Murilla</i>	
BIBLIOGRAPHY	121
ANNEX: Proposed quality control progocols for mass rearing tsetse flies, behaviour and treatment to reduce vectorial capacity	123
LIST OF PARTICIPANTS	135

SUMMARY

1. BACKGROUND

When applying the sterile insect technique (SIT) to tsetse flies, one of the main constraints is the production of sufficient good-quality male flies for sterilization and release. The adenoviviparous reproductive system of tsetse results in the female flies producing at most one fully-formed larva each ten days which severely restricts the growth and productivity of tsetse colonies. Nevertheless, the current expansion of tsetse fly production in Africa is unprecedented. To ensure that this expansion does not impact on the quality of sterile flies it is essential that reliable, improved quality control (QC) methods be made available. Improvements in QC methodology will not only help to ensure the attainment of the production goals but also improve the quality of rearing and minimize production costs. It is a top priority that produced flies are monitored for their quality and suitability for release.

2. SCOPE

The research was divided into four main topics: blood diet, rearing, behaviour and vectorial capacity, with additional work in muscle development, endocrine control of larviposition and sound production.

3. OBJECTIVES

Research is required to improve existing methods, develop new tests and standards (especially in relation to reproductive behaviour, mating compatibility, field performance and irradiation), and harmonize quality control procedures among tsetse production facilities and area-wide SIT programmes. The overall objective was to improve and harmonize quality control for expanded tsetse production, sterilization and field application. Meeting this objective would improve and expand the quality control sections of the FAO/IAEA Standard Operating Procedures for Mass rearing Tsetse Flies.

4. RESULTS AND CONCLUSIONS

In the area of blood diet for tsetse colony maintenance, three groups looked at collection procedures, factors influencing diet quality and bacterial decontamination. With regards to preventing blood diet coagulation, defibrination was shown to yield the highest diet quality, and also the use of anticoagulants (heparin and acid citrate) gave acceptable quality and may be appropriate in some circumstances. The presence of therapeutic drugs in the blood is of concern, as both antibiotics and anthelmintics are toxic to tsetse. but it was found that bovine growth hormone has no effect. Blood from different species differs in its nutritive quality, the difference depending on the tsetse species, but in general some mixture of cow and pig blood is better than either alone. Conditions for collecting blood vary between locations, and some variation in blood quality was also seen with season. One of the main problems with using blood from the slaughterhouse is that it is not sterile and tsetse cannot tolerate high levels of bacteria in the diet. The blood is, therefore, normally irradiated to reduce the bacterial level, but large irradiators are not readily available in Africa and alternatives are needed. Earlier work had indicated that high temperature short time pasteurization may work and the Technical University, Bratislava, investigated this. Various conventional heat-exchange systems were investigated but they could not provide the necessary rapid temperature rise and fall to achieve pasteurization without coagulation. They, therefore, investigated microwave heating and were able to achieve very rapid heating rates but were not able to achieve the

necessary cooling to prevent coagulation. Further development of this idea may lead to a practical system for blood pasteurization, but this was beyond the finances available for this research contract.

Two groups worked on rearing issues, including emergence and holding conditions, handling of adults and feeding regime. Reducing feeding from 6 days per week to either 3 or 4 days per week was validated for *Glossina tachinoides* and *G. palpalis gambiensis*, and it was shown conclusively that the use of ATP as a phagostimulant was unnecessary for colony-adapted flies and could be eliminated, saving about EUR 27 per thousand sterile males produced. The use of day-zero mating (introducing males and females simultaneously to the production cages) had no impact on female fecundity so long as the correct ratio was observed. Evaluation of the tsetse production unit 3 (TPU-3) developed as a result of the previous CRP showed that it greatly reduces the time required for feeding whilst maintaining pupal mass and adult emergence.

Extensive and highly detailed studies of the courtship and copulation of tsetse revealed a wealth of information previously not seen. The courtship behaviour proved to be complex and lengthy and, contrary to prior reports, the female is far from a passive participant in mating, but is able to prevent intromission and thereby influence mate choice. This emphasises the importance of maintaining appropriate courtship and mating behaviour if the sterile males are to succeed in inseminating wild females. The interaction of vision and smell in initiating and orienting flight was investigated using 3D video analysis, coupled with electroantennograms to determine the attractive components in host odour. Odour is the principal trigger initiating flight, but in the absence of a visual cue the flight is poorly oriented with large swings from side to side. Despite many years of colony rearing, the tsetse flies were able to respond to odour and visual cues and to locate up-wind odour sources. As host location is an important component of mating strategy in some tsetse species, measurement and quality control of these behaviours is important and can be achieved with a wind tunnel and video system. Tsetse flies also produce characteristic sound, and although the function of these sounds is not yet identified, the sound production can be quantified with a simple system consisting of a microphone and laptop computer with software that can automatically measure the significant 'feeding sounds'.

Another aspect of the research was on vectorial capacity. It was shown that the likelihood of a fly becoming infected when it feeds on a parasitaemic host varies with age and feeding status. The probability is highest at the first feed, declining rapidly at subsequent feeds; the development stage of the peritrophic membrane appears to play an important role here. The risk of infection, however, rises again if the fly is starved for a significant period as the peritrophic membrane breaks down. As the released flies can be vectors of trypanosomes, it is important to reduce the risk of flies becoming infective, by reducing the risk of initial infection or by limiting the probability of the infection developing until the fly is infective. The simplest procedure is to feed all the flies before release. This both allows development of the peritrophic membrane and postpones the need for the fly to feed after release on a potentially infective host. A second procedure was investigated, involving the use of a standard trypanocidal drug isometamidium chloride. Feeding the flies once on blood containing 10 µg/mL isometamidium chloride effectively prevented infection by *T. congolense* and *T. b. brucei* for a period of 5 days. Prolonged starvation increases again the probability of infection by *T. congolense*, but not as high as in untreated flies and did not increase *T. b. brucei* infections.

An issue that is often raised regarding the quality of insects mass reared for the sterile insect technique is the development of the flight muscles. Earlier work suggested that the flight ability of mass reared insects is reduced, resulting in insects that are poorly competitive with wild insects. This work, however, demonstrated that although the first few days are critical for flight muscle development, rearing in standard cages does not significantly impair muscle development or flight ability. Electron microscope and immunochemical studies showed that the development of the mitochondria and myofibrils proceeded normally in the cages. In an attempt to allow more natural development to occur, a large cage was set up in a greenhouse with a free Living tsetse population. Problems with climate control and feeding, however, prevented any conclusive information being collected.

Other studies encompassed the rhythm of larviposition and oxygen consumption as a measure of fitness. Parturition in tsetse is controlled by a neuropeptide hormone that is conserved in other pupipara; extracts of calliphorid oviduct are active in inducing larviposition in tsetse. This parturition hormone was extracted and partially characterized by chromatography and mass spectrometry but a final sequence was not obtained and none of the putative sequences matched the (still incomplete) tsetse genome sequence. Oxygen consumption by pupae was measured with a Scholander respirometer, and it was proposed that this be used as a quality control parameter.

In summary, progress was made in all areas and resulted in modification or expansion of the existing quality control protocols in the FAO/IAEA Standard Operating Procedures for Mass rearing Tsetse Flies or proposals for new protocols.

Nine papers were published in peer-reviewed scientific journals as a result of this CRP, and 24 new or revised quality control protocols were proposed.

At the final Research Coordination Meeting (RCM) held in Addis Ababa, Ethiopia, 13–17 October 2008, some recommendations for future research were made by the participants. It was noted that, in general, some existing tsetse rearing protocols should be improved. It was recommended that collaborative research in the following specific subjects should be pursued:

4.1. Blood diet

- Tests should be done to determine if pre-mixtures of bovine/porcine blood give the same result as feeding flies X days on bovine blood and X days on porcine blood.
- More improvements in blood collection, processing and storage should be studied.

4.2. Mass rearing

- More improvements in the establishment of tsetse colonies from the wild should be studied.
- Develop a protocol for measuring, and set a standard for the proportion of flies feeding.
- Publish a protocol for mass marking flies for release. Work should include marking individual insects for mark release-recapture studies.

4.3. Rhythm of larviposition

- If the production of a colony decreases, environmental parameters of the rearing room including light intensity and duration of light/dark cycles should be checked and the circadian periodicity of larvae production evaluated.

- Larviposition and abortion – soft pupa issue. Why is there abortion? The measuring procedure should be improved.
- Include input from recent research on the existence of an oostatic hormone in tsetse flies.

4.4. Development of thoracic muscles

- Observations on the development of flight muscles in wild flies should be made to evaluate results obtained from laboratory flies.

4.5. Behaviour

- More information is needed on the competitiveness of sterile males under natural conditions in the different areas where the SIT could be applied, e.g. dispersal, mating compatibility, survival, and sensitivity to trap used.
- Based on scientific studies conducted during the CRP project, we concluded that it is important to document routinely the duration of mating in a mass reared strain as an indirect measure of the mechanical stimulation that mass reared males are providing to females. It is recommended that the duration of mating be related to the presence or absence of sperm in the spermathecae and to the percentage of filling. It is important to document these two parameters during a test of mating compatibility.
- The protocol for field cage mating behaviour is available as an eLearning course (<http://elearning.iaea.org/ATutor/users/browse.php>).
- Concerning quality control based on acoustic signals, it is necessary to set the control values for both good quality and poor quality flies to correctly evaluate recorded values. This should be done with all reared species.

4.6. Vectorial capacity

- The vector competence of all mass reared tsetse strains used for the SIT should be assessed.

4.7. Respirometry

- Respirometry is probably a prospective quality control test. However, to date only preliminary observations have been made. It would be useful to test this method further for the purposes of quality control procedures.
- Measurements of oxygen consumption and water vapour loss could help with fly handling procedures, i.e. reduce stress/mortality of flies produced for release and of flies being transported from the field for compatibility studies or other work in captivity.

4.8. Sterile male performance

- A new CRP on sterile male performance in the field is recommended. A single index of sterile male performance should be developed.

DEVELOPING QUALITY CONTROL PROCEDURES TO SUSTAIN A SUPPLY OF HIGH QUALITY BLOOD FOR MASS REARING TSETSE FLIES

C.J. DE BEER, G.J. VENTER, F.T. POTGIETER

ARC-Onderstepoort Veterinary Institute,
Old Soutpans Road, Private Bag X05,
0110 Onderstepoort,
South Africa

Abstract

Mass rearing tsetse flies *Glossina* spp. is dependent on the sustained availability of a high quality blood diet. In any mass rearing facility, the logistics for obtaining sterile, high quality fresh blood is challenging. An added complication is the influence of potential chemical, physical and microbiological elements present in the blood of donors, as well as contamination during collection, handling and storage. Research at the Agricultural Research Council – Onderstepoort Veterinary institute (ARC-OVI) is directed towards the development of quality control procedures for the supply of the in vitro diet used to maintain productive colonies of *Glossina brevipalpis* Newstead and *Glossina austeni* Newstead. Factors that may influence the blood diet, e.g. defibrination, feeding times, collection of blood in anticoagulants, treatment of blood with taste stimuli, repeated freezing and thawing of blood, effect of bovine growth hormones, and also a preference for bovine or porcine blood were tested. A 25 day bioassay was used to determine the effects of these factors on tsetse survival and reproduction. Defibrination of the blood for 10 to 15 minutes gave the best results for both species. It was found that *G. brevipalpis* should be fed three times per week for 5 minutes each time, and *G. austeni* three times per week for 10 minutes. Heparin, acid citrate dextrose (ACD), citric acid, citrate phosphate dextrose adenine (CPDA) and a combination of sodium citrate and citric acid were effective anticoagulants in the blood diets of *G. brevipalpis* and *G. austeni*. Blood treated with inosine triphosphate (ITP) gave the highest quality factor (QFC) values for both *G. austeni* and *G. brevipalpis*. Repeated freezing and thawing of blood definitely affects pupal production negatively; *G. brevipalpis* especially produced significantly smaller pupae. A premixed diet of equal amounts of bovine and porcine blood was found to be best suited for *G. brevipalpis*, and for *G. austeni* a mixture of 75% bovine and 25% porcine blood. In this study bovine growth hormones did not have any negative effect on tsetse fly survival and reproduction. Evidently certain blood dietary factors are important for the successful mass production of tsetse flies. Blood collection and feeding protocols can have a significant influence on colony maintenance and production.

1. INTRODUCTION

Tsetse flies are vectors of trypanosomosis, an economically important disease in Africa called nagana in domestic livestock and sleeping sickness in humans. Control of this debilitating disease includes vector control; the sterile insect technique (SIT) has proved to be a successful method of tsetse control. It entails the release of sterile male flies obtained by mass rearing, and this depends on the availability of a suitable blood diet for the flies [1]. *Glossina* species are obligatory haematophagous insects; both males and females feed on blood [2]. The female reproduces by adenotrophic viviparity, one larva at a time being nourished *in utero* by a secretion from the uterine gland [3]. Both the adult and larval stages are dependent on the same source of food. A high quality food source is essential for the growth and sustainability of the fly colonies [4]. In a mass rearing facility, the logistics of obtaining sterile, high quality blood remains problematic. An added complication is the variation in nutritional quality influenced by genetic, environmental, chemical and physical factors on blood composition. Other factors which play a role include chemicals and microbiological contaminants to which the blood is exposed during collection, handling and storage. Research at the ARC-Onderstepoort Veterinary Institute (ARC-OVI) has focused on the development and

improvement of blood collection procedures to ensure the supply of a quality product, comparable to fresh blood collected by a sterile method directly from donor animals, required to maintain viable healthy tsetse colonies of *G. brevipalpis* and *G. austeni*.

This study was part of an FAO/IAEA Coordinated Research Project, and the objectives were as follows:

- Maintain and expand existing colonies of *G. austeni* and *G. brevipalpis*; optimize tsetse rearing facilities.
- Investigate the effect of defibrination on the nutritional value of blood collected from a closed, quarantined cattle herd with known history.
- Resolve problems of high fly mortalities by:
 - Conducting experiments in which flies are fed at various feeding times and intervals.
 - Assessing the nutritional value of different blood diets by comparing the pupal size and fly emergence of locally bred and imported pupae.
- Evaluate the effect of anticoagulants on the nutritional value of blood diets.
- Investigate the effect on flies of feeding/taste stimuli, such as adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), inosine monophosphate (IMP), inosine triphosphate (ITP), guanosine monophosphate (GMP), guanosine triphosphate (GTP), cytosine monophosphate (CMP) and cytosine triphosphate (CTP) in tsetse blood diets.
- Investigate the effect of repeated freezing and thawing on the nutritional value of blood using the 25 day blood bioassay [5].
- Evaluate the use of porcine and bovine blood, as well as a mixture of the two, as a diet for *G. brevipalpis* and *G. austeni*.
- Determine the effect of growth hormone treatment to blood donors on the diet of tsetse flies.
- Determine the requirements that will improve the adaptation of wild flies to in vitro feeding and optimize their colonization.

2. MATERIALS AND METHODS

2.1. Blood bioassays

To evaluate blood diets, quality factors (QF) [6] were calculated using the 25 day bioassay [5] (subsequently referred to as the bioassay). No dissections were made, and QF values were adapted to exclude dissection results. EXCEL sheets with QF calculations (QFC) were provided by the FAO/IAEA (A.G. Parker, pers. comm.). Pupae were sorted by size into five distinct size classes using a pupal size-sorting machine [6]. This machine was calibrated according to the standards used by FAO/IAEA in Seibersdorf, Austria (A.G. Parker, pers. comm.). For *G. austeni* the measurements ranged between 2.3 and 3.0 mm, and for *G. brevipalpis* between 3.5 and 4.3 mm. The pupal size classes and weight (mg) range for class sizes for *G. austeni* were A (<18), B (18– <21), C (21– <24), D (24– <27), E (≥27) and for *G. brevipalpis* A (<56), B (56– <61), C (61– <66), D (66– <71), E (≥71) [5]. All tests using the bioassay were repeated at least twice. Only the tests with the best results were used in the analyses.

The following formula was used to calculate the values of the bioassays:

$$QFC = \frac{PA*11 + PB*17 + PC*19 + PD*20 + PE*22 + 0.616}{23.86*FS18}$$

where:

FS18 - Flies surviving to day 18
FS25 - Flies surviving to day 25
PA - No. of A (1) class pupae
PB - No. of B (2) class pupae
PC - No. of C (3) class pupae
PD - No. of D (4) class pupae
PE - No. of E (5) class pupae
PT - Total pupae

2.2. Existing colonies

Pupal shipments of *G. austeni* and *G. brevipalpis* were obtained on a 2–4-week basis from the Tsetse and Trypanosomiasis Research Institute (TTRI) in Tanga, United Republic of Tanzania, and FAO/IAEA laboratories in Seibersdorf, Austria. A total of 5618 *G. austeni* pupae were received from Seibersdorf during Oct.–Dec. 2002 and 25 000 from TTRI during Jul.–Oct. 2003. Since December 2003 a total of 21 450 *G. brevipalpis* pupae were received from FAO/IAEA.

Colonies were maintained on bovine blood collected at a commercial abattoir in Springs (Gauteng Province). All blood was irradiated at 2 kGy at Isotron, a commercial irradiation plant, and each batch of blood was quality tested prior to being used in the colonies. QFC values of >1 were obtained for all batches [6].

2.3. Blood collection

2.3.1. Blood collection from abattoir

2.3.1.1. Bovine blood

Equipment that came in contact with blood was washed and autoclaved. The apparatus used for blood collection [6] and defibrination consisted of:

- Three 40 L containers with tight-fitting lids; in the centre of each lid was a smaller screw-capped lid, through which the paddle/stirrer, attached to an electric drill, was fitted.
- The stirrer consisted of a 200-mm long stainless steel rod of 25-mm diameter to which the stirrer blade (380 × 170 mm) was attached. To agitate the full volume of blood, the blade extended to 20 mm from the bottom of the container. Each blade had thirty-eight holes (10 × 25 mm, 10 × 20 mm and 18 × 10 mm in diameter).
- The stirrer was driven by a SBE 1010 Plus Metabo electric drill (230V, 50–60Hz, 1010W and 4.6A). The speed was adjusted to maintain approximately 150 rpm.
- Aluminium sieve.
- 500 L container with lid.
- 5 L buckets.
- 5 L storage canisters.

Cattle were stunned and suspended from their hind legs. After the throat of the animal had been slit, the emerging blood was collected directly into buckets and transferred to the 40 L containers. Defibrination for 10–15 minutes followed, and the clotted fibrin was removed by hand. After defibrination the blood was pooled in the 500 L container from which 5 L storage canisters were filled before storage at –20°C.

2.3.1.2. Porcine blood

Pigs with a known veterinary history, kept at the ARC-Animal Improvement Institute for breeding and experimental purposes, were slaughtered at the BonAccord abattoir. Animals were stunned, the jugular vein exposed and severed, and the blood collected into a sterilized glass jar, containing glass beads, that was agitated for 10–15 minutes. The blood was then dispensed into 0.3 L containers and stored at –20°C.

2.3.2. Blood collection through bleeding of animal

Cattle from a closed quarantined herd with a known veterinary history were used. Animals were bled from the jugular vein by means of a trocar (stylet with a triangular point). Blood was drained directly (in a closed sterile system) into a sterilized glass jar containing glass beads, agitated for 10–15 minutes, dispensed into 0.3 L containers and stored at –20°C.

2.3.3. Bacteriology

Blood samples were taken throughout the collection processes to monitor for possible bacterial contamination. All bacteriological testing was done by the Division of Bacteriology at ARC-OVI. Blood found to be contaminated after irradiation was discarded.

2.4. Effect of defibrinating periods on blood quality

Eight cattle from the closed herd mentioned above were slaughtered (24/08/2004). Blood from each animal was collected into a bucket and distributed evenly between three 40 L containers. Agitation started when the first blood was poured into the container and continued for 5, 10 and 15 minutes, respectively, for container one, two and three, after the containers were filled. The clotted fibrin was removed and weighed after each cycle. The blood was then filtered through an aluminium sieve and the remaining fibrin removed and weighed. The volume of blood collected in each of the three containers was determined before and after the defibrination process and stored in 5 L containers at –20°C.

2.5. High fly mortality

2.5.1. Feeding times (duration) and frequency

To optimize the weekly feeding operation (duration of feeding and frequency of feeding) for both *G. brevipalpis* and *G. austeni*, four different combinations of feeding time (duration) and feeding frequency were tested (Table 1) using defibrinated gamma-irradiated bovine blood.

The experiment was conducted with colony flies of the same age. Two replicates of each feeding treatment were made. For *G. brevipalpis* both replicates initially each had 225 newly emerged female flies, and for *G. austeni* the two replicates had 1100 and 1200 newly emerged females, respectively. For both species and replicates, the four feeding treatments were continued for 17 weeks. Weekly mortality and pupal production were recorded. The daily percentage mortality, and pupae produced per female per 10 days, were calculated every week. The four feeding treatments were compared concurrently, using the 25 day test to obtain a QF value for each treatment.

TABLE 1. FEEDING TREATMENTS WITH FOUR COMBINATIONS OF FEEDING DURATION AND FREQUENCY

Treatment	Duration (min.)	Frequency
A	5	Daily (5 times/week – Monday–Friday)
B	10	Daily (5 times/week – Monday–Friday)
C	5	Alternate days (3 times/week – M, W, F)
D	10	Alternate days (3 times/week – M, W, F)

2.5.2. Pupal size

Locally produced and imported (from Seibersdorf) *G. brevipalpis* pupae were compared with respect to their size. Pupae were sorted into five size categories (sizes A–E, where A was the smallest and E the largest) (as described above). The size of *G. austeni* pupae was recorded only for locally produced pupae; the pupae from TTRI had already been received before the present research commenced.

2.6. Anticoagulants

In relation to the blood diets of both *G. brevipalpis* and *G. austeni*, anticoagulants (as opposed to defibrination) were tested in three experiments for their potential use in blood collection. The anticoagulants tested were acid citrate dextrose (ACD), heparin, sodium citrate, ethylene-diamine-tetra-acetic acid (EDTA), citric acid, citrate phosphate dextrose adenine (CPDA) and a combination of citric acid and sodium citrate. Blood was collected as described in Section 2.3.2. Blood defibrinated with glass beads was the control. The bioassay was used to evaluate the blood samples mixed with anticoagulants or defibrinated.

2.7. Feeding/taste stimuli

The nucleotides AMP (adenosine monophosphate), ADP (adenosine diphosphate), ATP (adenosine triphosphate), as well as mono- and tri-phosphates of inosine (IMP, ITP), guanosine (GMP, GTP) and cytosine (CMP, CTP), were tested for their efficacy as feeding/taste stimuli.

A small amount (0.055 g) of each compound was diluted in 10 mL of distilled water. The dilutions were kept in the freezer and used within three days, after which new solutions were prepared. Only 0.02 mL of each solution was used for 20 mL of blood. Blood collected from Springs Abattoir was used as the control. Each test was replicated twice.

2.8. Repeated freezing/thawing of stored blood

In 2004 it was noticed that locally produced *G. brevipalpis* pupae were significantly smaller than pupae obtained from the IAEA, Seibersdorf. It was speculated that the nutritional value

of blood used at ARC-OVI might have been of a lower quality than that used in Seibersdorf. It was found that, due to electrical power failures, the blood stored in the walk-in freezer at ARC-OVI (set at -30°C) was subjected a number of times to temperature fluctuations resulting in uncontrolled thawing and freezing. It was assumed that this would have negatively affected the blood quality, and therefore the nutritional value was suspect.

Bioassays were conducted to determine the effect of repeated freezing/thawing of five batches of blood collected in 2002 and stored in the -30°C walk-in freezer at the ARC-OVI. The QFC values obtained in 2002/2003 were compared with those obtained from bioassays conducted in 2005 with both tsetse species. Two replicates of each test were conducted.

2.9. Porcine, bovine, and mixture of porcine and bovine blood diets

Porcine blood, bovine blood and mixtures of porcine and bovine blood were evaluated in three experiments.

2.10. Bovine growth hormones

Animals from the same herd and age were divided into two groups — one group of 10 animals was treated with the bovine growth hormone (active ingredients trenbolone acetate and oestradiol) and the second group (untreated, also 10 animals) served as the control. Bioassays were conducted to study the effect of this growth hormone on the survival and development of tsetse flies.

2.11. Colonization of Zululand *Glossina brevipalpis*

Live *G. brevipalpis* flies were collected in northern KwaZulu Natal using H-traps baited with odour [7, 8]. The flies were fed on blood and kept at the northern KwaZulu Natal Tsetse Research Station (ZTRS). All pupae produced by these flies were sent to the ARC-OVI for rearing and to establish a colony. Flies that emerged from these pupae were held at $23\text{--}24^{\circ}\text{C}$, 75–80% RH and under subdued/indirect lighting, the same rearing conditions used for the existing colonies.

3. RESULTS AND DISCUSSION

3.1. Existing colonies

Figures 1–4 summarize the colonization of *G. austeni* and *G. brevipalpis* for 2002–2009. The colony sizes as of the end of week 31 of 2009 were 24 629 *G. austeni* female flies and 17 581 *G. brevipalpis* female flies (Fig. 1). Figure 2 shows the daily mortality. As of week 31 of 2009, the daily mortality was 1.79% for *G. austeni* and 1.13% for *G. brevipalpis*. High mortality was a problem in the rearing work; in week 24 of 2009 the daily mortality of *G. brevipalpis* was $>2\%$. The environmental parameters, feeding process and blood quality were assessed when high mortality occurred, and attempts made to resolve the problem as soon as possible. Pupal production as of the end of week 31 of 2009 was 14 663 for *G. austeni* and 8927 for *G. brevipalpis* (Fig. 3), and the fecundity (number of pupae/female/10 days) was 0.95 for *G. austeni* and 0.82 for *G. brevipalpis* (Fig. 4). The colonies were stable, and with the current staff capacity they will be maintained at the current levels, $\pm 25\,000$ *G. austeni* and $\pm 17\,000$ *G. brevipalpis*.

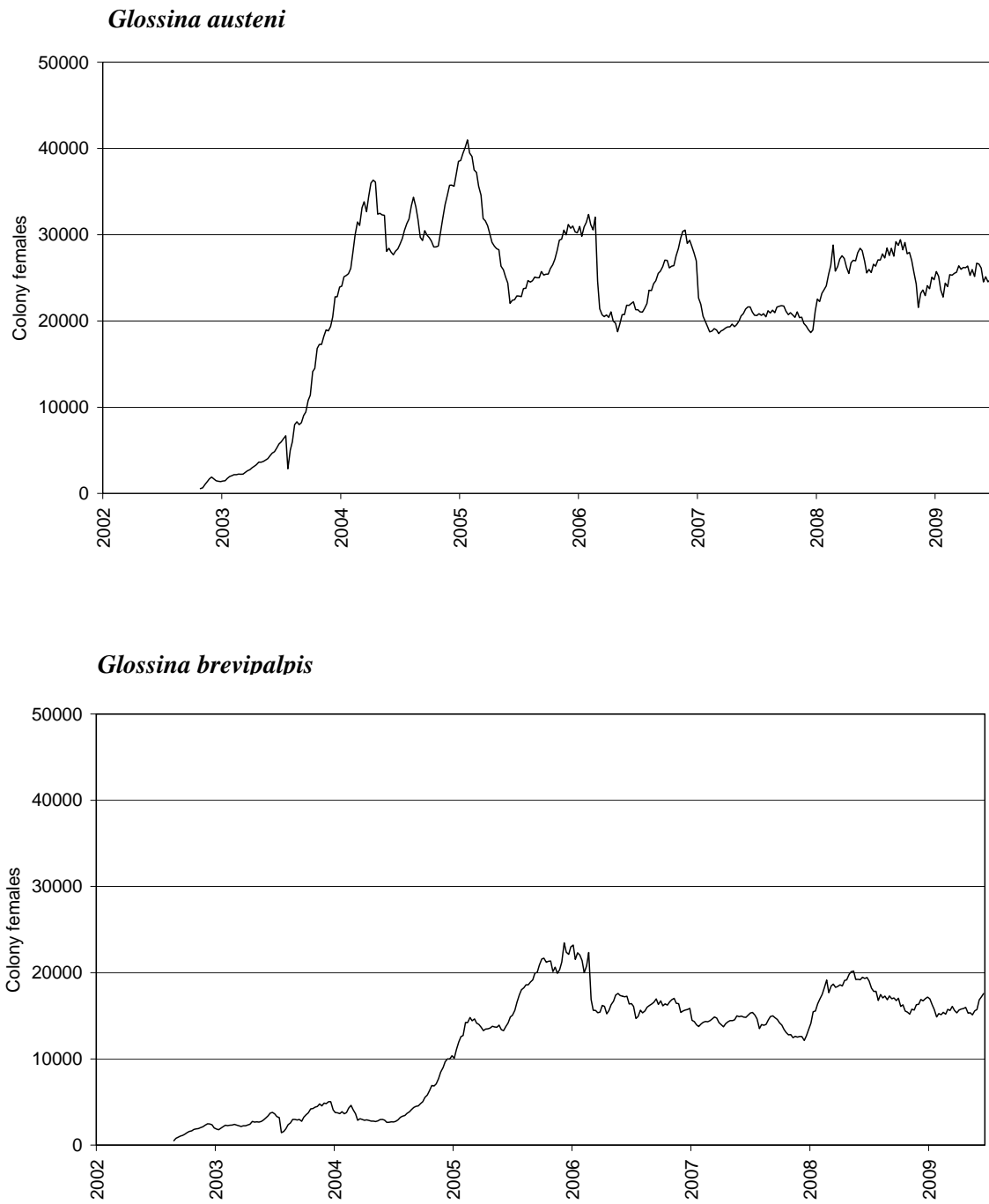
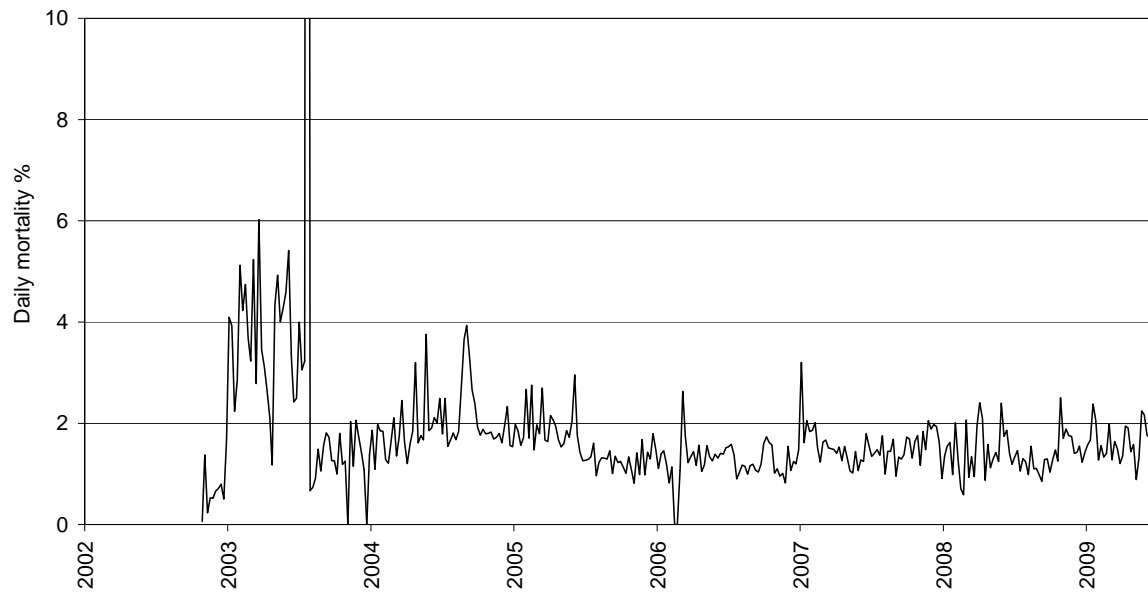


FIG. 1. Colony size (number of female flies) of two species of *Glossina*, 2002–2009.

Glossina austeni



Glossina brevipalpis

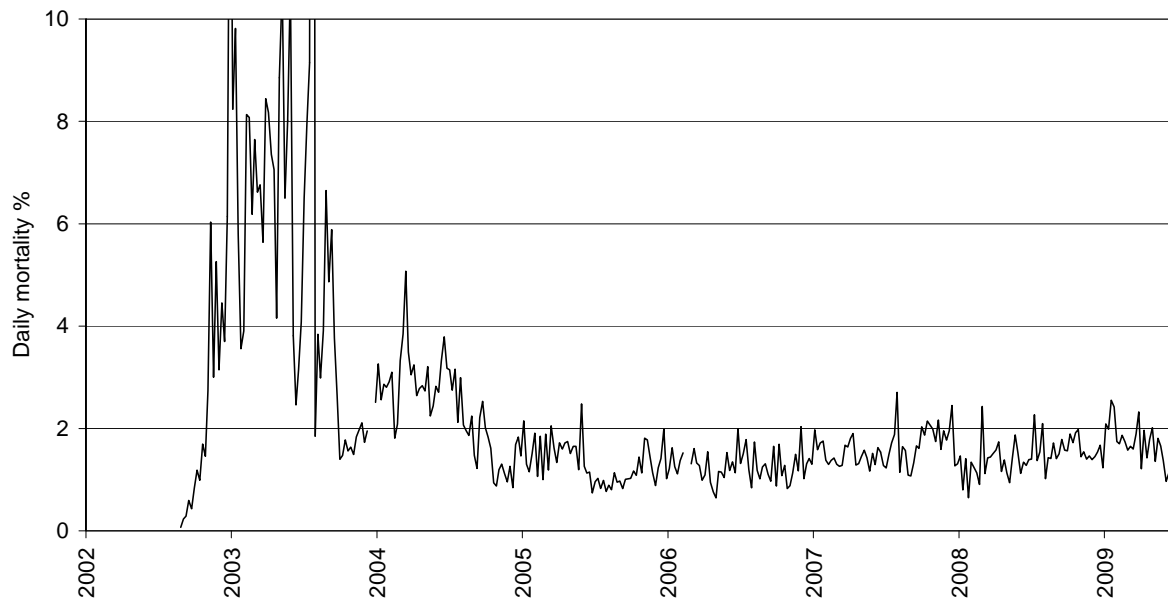


FIG. 2. Daily mortality (%) of two species of *Glossina*, 2002–2009.

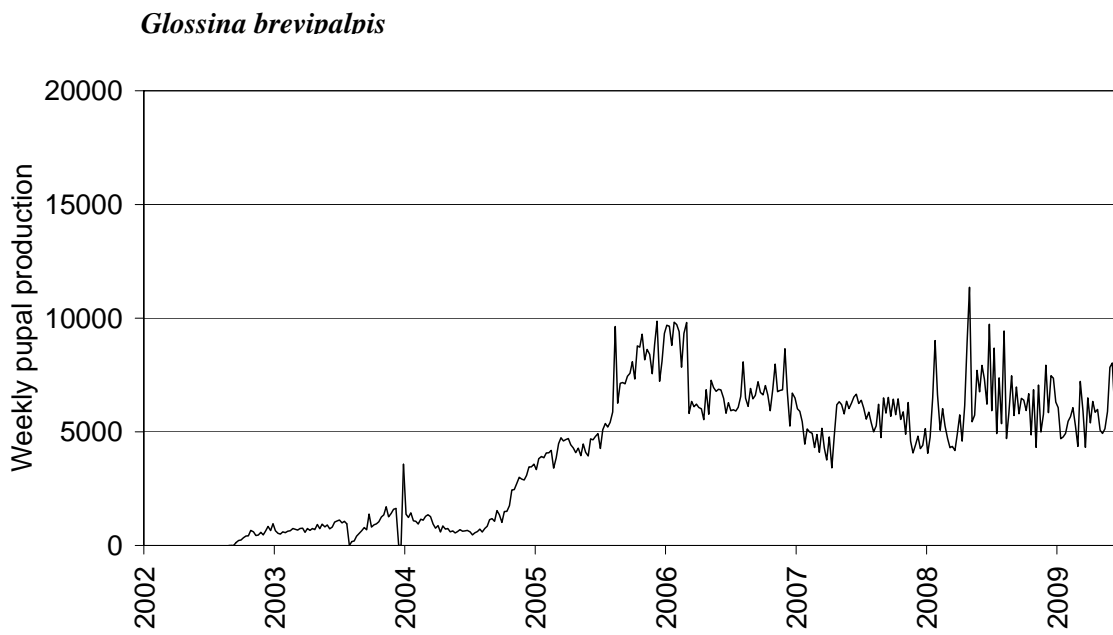
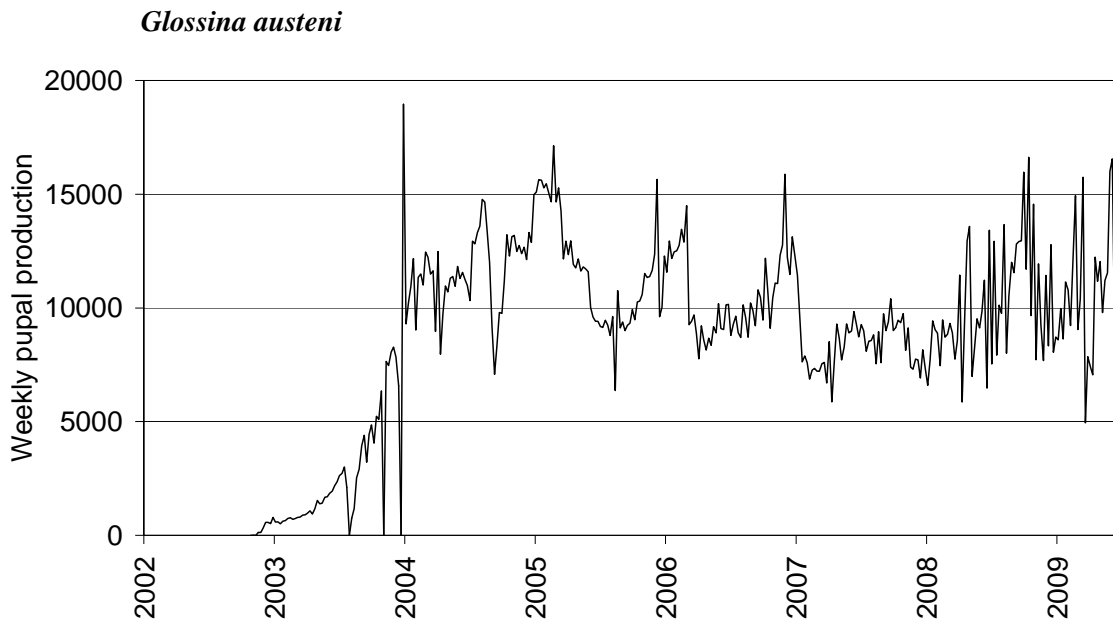


FIG. 3. Pupal production (number per week) of two species of *Glossina*, 2002–2009.

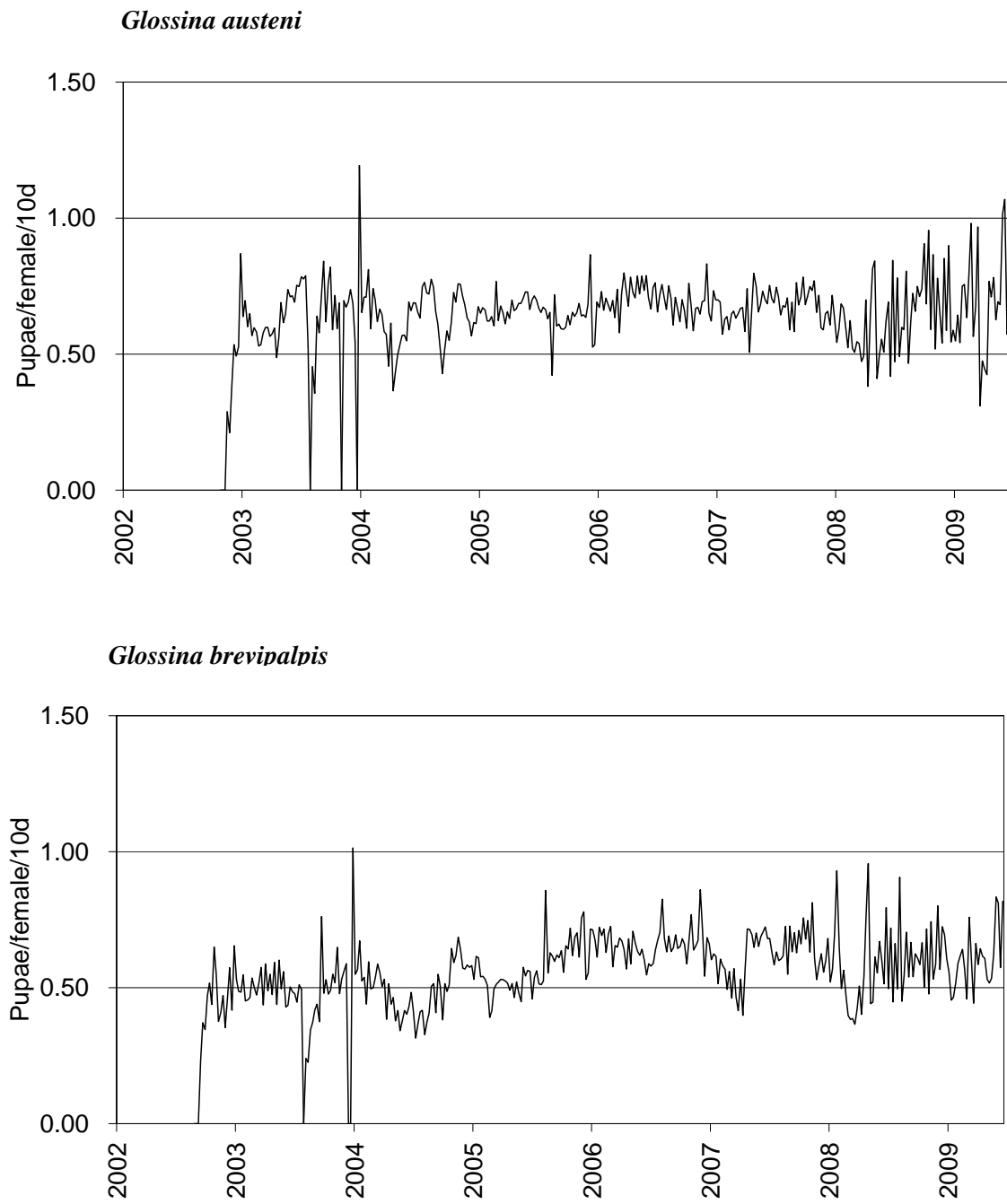


FIG. 4. Fecundity of two species of *Glossina*, 2002–2009.

3.2. Defibrinated blood

The survival of both *G. austeni* (Table 2) and *G. brevipalpis* (Table 3) females that fed on defibrinated blood for 5, 10 and 15 minutes was above 83% at day 25. In the case of *G. austeni*, a defibrination time of 10 min gave the best survival at day 25, 93% (Table 2). *G. brevipalpis* showed 100% survival at day 25 (Table 3) for both 10 and 15 min defibrination times. The blood defibrinated for 5 and 15 min gave the highest number of pupae for *G. austeni* (Table 2), however, the blood defibrinated for 5 and 10 min gave the best pupal weight distribution. A 10 min defibrination time gave the highest number of pupae, as well as the best pupal weight distribution, for *G. brevipalpis* (Table 3).

The QFC values indicated that blood defibrinated for 10 min gave the best results for *G. brevipalpis* (Table 3), and the blood defibrinated for 15 min the lowest value. However, for *G. austeni* (Table 2), blood defibrinated for 15 min gave the highest QFC value. This study showed that if the same blood is to be used for both *G. brevipalpis* and *G. austeni*, blood should be defibrinated for about 10 min; differences among the three treatments in their effects on the flies were small.

TABLE 2. EFFECT OF THREE DEFIBRINATING TREATMENTS ON *G. AUSTENI*

Treatment	Survival (%)		Pupal weight class					QFC value
	Day 18	Day 25	A	B	C	D	E	
5 min	96	90	1	3	8	8	4	1.28
10 min	93	93	4	1	5	7	5	1.22
15 min	90	86	2	9	1	8	4	1.3

TABLE 3. EFFECT OF THREE DEFIBRINATING TREATMENTS ON *G. BREVIPALPIS*

Treatment	Survival (%)		Pupal weight class					QFC value
	Day 18	Day 25	A	B	C	D	E	
5 min	93	93	4	4	2	5	2	1.06
10 min	100	100	1	4	7	8	4	1.26
15 min	100	100	4	0	4	2	4	0.96

3.3. High fly mortality

3.3.1. Feeding times (duration) and frequency

3.3.1.1. *G. brevipalpis*

No significant differences ($P=0.159$ (ANOVA)) were found among the four feeding treatments in the means of percentage daily mortality. However, there were significant differences ($\chi^2=34.618$; $df=3$; $P<0.001$) among the treatments in the number of flies still alive after 17 weeks (Table 4).

TABLE 4. SURVIVAL OF *G. BREVIPALPIS* FEMALES AFTER 17 WEEKS ON FOUR FEEDING TREATMENTS

Feeding treatment	Flies (no.)		
	Living	Dead	Total
A (5 min, 5×/wk)	71	379	450
B (10 min, 5×/wk)	33	417	450
C (5 min, 3×/wk)	56	394	450
D (10 min, 3×/wk)	23	427	450

A more detailed analysis (Chi-square test) was done to determine the differences among the proportions of surviving flies (Table 5). The probability that the proportions are different at the $P<0.05$ (*) or $P<0.0001$ (***) levels of probability, or not significantly different (n.s.), the degrees of freedom for error (df) and the Chi-square (χ^2) values are indicated.

TABLE 5. LEVELS OF SIGNIFICANT DIFFERENCES AMONG PROPORTIONS OF SURVIVING *G. BREVIPALPIS* FLIES

	A (5 min, 5×/wk)	B (10 min, 5×/wk)	C (5 min, 3×/wk)
B (10 min, 5×/wk)	*** ($\chi^2 = 14.883$; df=1)	-	
C (5 min, 3×/wk)	NS ($\chi^2 = 1.797$; df=1)	* ($\chi^2 = 6.035$; df=1)	-
D (10 min, 3×/wk)	*** ($\chi^2 = 26.241$; df=1)	NS ($\chi^2 = 1.542$; df=1)	*** ($\chi^2 = 14.209$; df=1)

Irrespective of feeding frequency, the number of flies surviving after 17 weeks with the two 5 min treatments (A and C) did not differ significantly, nor did the number of flies surviving after the two 10 min treatments (B and D) (Table 5). The two 5 min treatments (A and C) proved to be significantly better than the two 10 min treatments (B and D) (Table 5).

The four feeding treatments did not differ significantly ($P=0.140$) in the number of produced pupae/female/10 days (Table 4). Therefore, based on the survival of flies at the end of the 17 weeks, treatment A (feeding flies for 5 min daily (5 ×/wk)) proved to be the best feeding strategy. However, it did not differ significantly from treatment C (feeding flies for 5 min on alternate days (3 ×/wk)). Therefore, it is recommended that flies be fed for 5 min three times per week. This is the most practical and economical solution with respect to available labour and volume of blood used. This feeding duration and frequency is presently being followed to maintain the *G. brevipalpis* colony.

G. brevipalpis has a tendency to overfeed, and the results clearly indicated this when these flies were fed for 10 min.

3.3.1.2. *G. austeni*

For *G. austeni*, there were no significant differences ($P=0.919$ (ANOVA)) found among the four feeding treatments in the means of percentage daily mortality. However, there were

significant differences ($\chi^2=46.016$; $df=3$; $P<0.0001$) among the treatments in the number of flies still alive after 17 weeks (Table 6).

TABLE 6. SURVIVAL OF *G. AUSTENI* FEMALES SURVIVING AFTER 17 WEEKS ON FOUR FEEDING TREATMENTS

Feeding treatment	Flies (no.)		
	Living	Dead	Total
A (5 min, 5×/wk)	127	2173	2300
B (10 min, 5×/wk)	206	2094	2300
C (5 min, 3×/wk)	137	2163	2300
D (10 min, 3×/wk)	227	2073	2300

Similar to *G. brevipalpis*, a more detailed analysis (using Chi-square test) was done to determine the significance of differences among the proportions of surviving flies (Table 7).

TABLE 7. LEVELS OF SIGNIFICANT DIFFERENCES AMONG PROPORTIONS OF SURVIVING *G. AUSTENI* FLIES

	A (5 min, 5×/wk)	B (10 min, 5×/wk)	C (5 min, 3×/wk)
B (10 min, 5×/wk)	*** ($\chi^2 = 19.696$; $df=1$)	-	
C (5 min, 3×/wk)	NS ($\chi^2 = 0.326$; $df=1$)	*** ($\chi^2 = 14.567$; $df=1$)	-
D (10 min, 3×/wk)	*** ($\chi^2 = 29.995$; $df=1$)	NS ($\chi^2 = 1.020$; $df=1$)	*** ($\chi^2 = 23.631$; $df=1$)

Irrespective of feeding frequency, the number of flies surviving after 17 weeks with the two 5 min treatments (A and C) did not differ significantly from each other, nor did the number of flies surviving with the two 10 min treatments (B and D). Furthermore, regarding fly survival, the two 10 min treatments (B and D) were significantly better than the two 5 min treatments (A and C).

The four feeding treatments did not differ significantly ($P=0.520$) in the number of produced pupae/female/10 days. Therefore, based only on the survival of flies, the two 10 min treatments were clearly the best and significantly better than the 5 min treatments. Treatment D (feeding flies for 10 min on alternate days (3 ×/wk)) is considered the best, however, it did not differ significantly from treatment B (feeding flies for 10 min daily (5 ×/wk)). Currently, the *G. austeni* colony is fed daily (for 10 min 5 times per week), but it is recommended that they be fed on alternate days only (3 times a week) since this would be the most practical and economical solution with respect to labour and amount of blood used. This was also the feeding protocol used in the past, before a changeover to a daily feeding strategy, because of very high mortality due to starvation.

3.3.1.3. Bioassay

The experiment on feeding times (duration) and frequency was repeated using the bioassay to compare the various QFC values (no dissections were done). Table 8 gives the QFC values for both species on the four treatments. The results have not been analysed statistically, and will only be used to verify previous recommendations on the feeding strategies as given above.

Results obtained from the bioassay were consistent with the findings of the first experiment. For *G. brevipalpis*, the two 10 min treatments are not recommended (QFC<1 is unacceptable); the two 5 min treatments gave better QFC values. The former recommended feeding strategy (treatment C) showed a QFC value of 1.03, which is acceptable.

The QFC differences for *G. austeni* were small. The recommended feeding strategy (treatment D) gave the highest QFC value (1.36), which is very acceptable.

TABLE 8. QFC VALUES FOR *G. BREVIPALPIS* AND *G. AUSTENI* ON FOUR FEEDING TREATMENTS

Feeding treatment	<i>G. brevipalpis</i>	<i>G. austeni</i>
A (5 min, 5×/wk)	1.05	1.26
B (10 min, 5×/wk)	0.97	1.32
C (5 min, 3×/wk)	1.03	1.32
D (10 min, 3×/wk)	0.90	1.36

3.3.2. Pupal size

3.3.2.1. *G. brevipalpis*

The pupal size categories for *G. brevipalpis* pupae produced locally vs. imported pupae from IAEA, Seibersdorf, are given in Fig. 5. The results are shown as a percentage of the total pupae in size categories A–E.

Pupae produced locally fell mostly into the smaller size categories A–C compared with pupae from Seibersdorf which fell mostly into categories C–E. The QFC values obtained for blood used in the ARC-OVI colony were acceptable, i.e. QFC values of 1.42–1.51 for *G. brevipalpis* and 1.27–1.35 for *G. austeni*. However, it appeared that the nutritional value of blood used at the ARC-OVI might be of a lower quality than that of blood used in Seibersdorf. This aspect should be investigated to determine if there is any deficiency in the nutritional value of the bovine blood used at ARC-OVI. Also, it may be that pupae received from Seibersdorf were not randomly selected.

The pupal size categories for imported *G. austeni* pupae are unknown, since the pupae from TTRI were received in 2003 prior to the commencement of the present study. Figure 6 shows the size categories of pupae that were produced locally.

3.3.2.2. *G. austeni*

Although a relatively high percentage of pupae fell into size A category (smallest), the remaining pupae were normally distributed in the other size classes (B–E). The nutritional value of the local blood diet should be investigated for this species also.

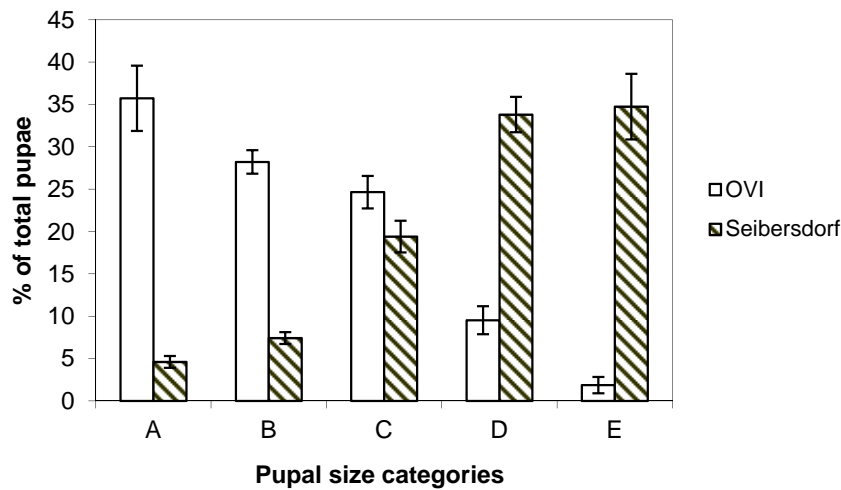


FIG. 5. Percentage of *G. brevipalpis* pupae in various size categories (A–E) obtained locally (ARC-OVI) vs. imported (Seibersdorf).

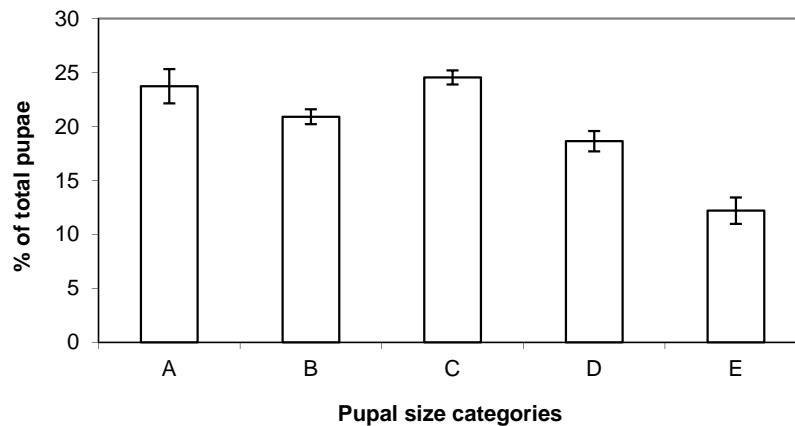


FIG. 6. Percentage of *G. austeni* pupae in various size categories (A–E) obtained locally.

3.4. Anticoagulants

Heparin, acid citrate dextrose (ACD), citric acid, citrate phosphate dextrose adenine (CPDA) and a combination of sodium citrate and citric acid were effective anticoagulants for use in preparing blood diets for *G. brevipalpis* and *G. austeni*. However, sodium citrate and ethylene-diamine-tetra-acetic acid (EDTA) were not suitable.

3.5. Feeding/taste stimuli

Survival of flies (both *G. austeni* and *G. brevipalpis*) that fed on blood treated with taste stimuli was above 80% in all treatments (Tables 9 and 10). When *G. austeni* was fed on blood treated with IMP or ITP (Table 9), there was 96% survival as well as high pupal production.

The lowest survival (80%) for *G. austeni* occurred when flies were fed on blood treated with GTP; however, these flies provided the most pupae in the highest weight class. *G. brevipalpis*, when fed on blood treated with five different stimuli, had 96% survival. The taste stimulus

CTP gave the lowest survival (83%) for *G. brevipalpis*. *G. brevipalpis* fed on IMP or ITP produced the most pupae as well as the highest number in the top weight class (Table 10). All the tested taste stimuli improved the survival and production of *G. brevipalpis* and *G. austeni*.

Blood treated with ITP gave the highest QFC values for both *G. austeni* and *G. brevipalpis* (Tables 9 and 10). All the taste stimuli resulted in QFC values >1, except for CTP in the case of *G. brevipalpis*. All the taste stimuli QFC values were above the QFC values for the controls. No significant differences among the QFC values for both *G. brevipalpis* ($P=0.07$) and *G. austeni* ($P=0.3$) were found.

Additional experiments were performed with ATP, ITP, CMP and GMP at a higher concentration. For *G. brevipalpis* blood treated with CMP gave a higher quality factor (QF) result than untreated blood, and for *G. austeni* blood treated with ATP, ITP and GTP gave better QF results than untreated blood.

TABLE 9. EFFECT OF A BLOOD DIET TREATED WITH VARIOUS FEEDING STIMULI ON *G. AUSTENI*

Feeding stimulus	Survival (%)		Pupal weight class					QFC value
	Day 18	Day 25	A	B	C	D	E	
IMP	96	96	2	7	4	6	4	1.21
ITP	96	96	2	7	6	4	5	1.26
GMP	93	93	2	3	8	3	4	1.17
CMP	93	90	0	7	4	4	3	1.13
ADP	96	86	4	0	9	5	0	1.07
AMP	90	86	2	2	5	5	6	1.21
CTP	93	93	3	3	2	7	3	1.11
GTP	86	80	2	3	3	3	7	1.17
ATP	86	83	7	6	5	1	2	1.16
Control	86	83	14	5	1	0	0	1.03

TABLE 10. EFFECT OF A BLOOD DIET TREATED WITH VARIOUS FEEDING STIMULI ON *G. BREVIPALPIS*

Feeding stimulus	Survival (%)		Pupal weight class					QFC value
	Day 18	Day 25	A	B	C	D	E	
IMP	96	96	1	5	7	6	4	1.06
ITP	96	96	1	5	7	6	4	1.25
GMP	96	96	4	8	3	2	3	1.11
CMP	93	90	3	2	4	5	2	1.05
ADP	96	96	8	8	2	2	0	1.05
AMP	90	90	7	7	2	1	0	1.22
CTP	86	83	9	4	3	0	0	0.98
GTP	96	96	4	4	4	5	0	1.03
ATP	93	93	3	5	8	1	0	1.05
Control	93	93	8	1	0	0	0	0.77

3.6. Repeated freezing/thawing of stored blood

All batches of blood were tested again in 2005. When compared with results obtained in 2003, the QFC values showed a decrease for both species, especially *G. brevipalpis* (Table 11). This could explain the decrease in pupal size obtained for *G. brevipalpis* reared at the ARC-OVI. Therefore, all blood collected in 2002 was discarded and fresh blood was collected from the Springs Abattoir. After bacteriological screening, 25 day bioassays were done. This blood is currently used to maintain both species.

TABLE 11. QFC VALUES OBTAINED FOR *G. AUSTENI* AND *G. BREVIPALPIS* IN 2005 (ON OLD BLOOD) AND 2003 (ON NEW BLOOD)

Batch (no.)	<i>G. austeni</i>		<i>G. brevipalpis</i>	
	2005	2003	2005	2003
3	1.16	1.10	0.73	1.15
4	1.0	1.29	0.98	1.24
5	1.09	1.35	0.76	1.17
6	1.19	1.34	0.81	1.26
7	1.03	1.27	0.76	1.21

In addition, the pupal sorter was calibrated again to standardize the size classes [6] (A.G. Parker, pers. comm.). To assist in the standardization process, pupae were obtained from the IAEA, Seibersdorf, pre-allocated in the respective size classes, and then resized in our sorter.

3.7. Comparison of porcine and bovine blood diets

A premixed diet of equal parts of bovine and porcine blood was found to be the best for *G. brevipalpis*, and a mixture of 75% bovine and 25% porcine blood best for *G. austeni*.

3.8. Bovine growth hormones

No significant differences (unpaired t-test) among QC values for *G. austeni* ($P=0.383$) or *G. brevipalpis* ($P=0.316$) were found when fed on blood from treated and untreated cattle (Table 12). The relatively high QF and QFC values indicated that the growth hormone did not have a significant effect on the development of the tsetse flies.

TABLE 12. QFC VALUES FROM *G. BREVIPALPIS* AND *G. AUSTENI* FED ON BLOOD FROM BOVINES TREATED AND UNTREATED WITH GROWTH HORMONES; DATA OBTAINED BY BIOASSAY

Treatment	<i>G. brevipalpis</i>	<i>G. austeni</i>
Hormones	1.36	1.19
Control	1.21	1.06

3.9. Colonization of Zululand *G. brevipalpis*

Over a period of one year 556 pupae were obtained from field-collected pregnant females. The progress of the colony is shown in Table 13.

In 2008 in the northern KwaZulu Natal *G. brevipalpis* strain in our tsetse colony, there were 18 first-generation (F₀) females, 11 second- (F₁), 7 third- (F₂), 12 fourth- (F₃) and 7 fifth-generation (F₄) females. At present there are no wild flies in our laboratory, but there are plans to start this colony again when funds become available.

TABLE 13. PROGRESS OF THE WILD FLY COLONY
FROM NORTHERN KWAZULU NATAL *G. BREVIPALPIS*
STRAIN (GENERATIONS F₀ TO F₃)

Generation	Females (no.)	Emergence (%)	Pupae (no.)	Pupae/female (no.)
F ₀	165	29.7	204	1.2
F ₁	61	29.9	94	1.5
F ₂	25	26.6	44	1.8
F ₃	14	31.8	23	1.6

Under the present laboratory conditions an estimated 60% of pupae emerged successfully, and production varied between 1.2 and 1.8 pupae/female. Although adult emergence was relatively high at 60%, the number of pupae produced per female was too low. This can be linked directly to poor blood diet and/or unsuitable holding conditions for the field-collected flies. Although we managed to breed a fifth generation of flies, this northern KwaZulu Natal strain's progress is extremely slow. Inputs of larger numbers of field pupae will be attempted.

4. CONCLUSIONS AND RECOMMENDATIONS

- The *G. brevipalpis* and *G. austeni* colonies are stable. From time to time there still are high mortalities (daily mortality >2%) for both species, but this is closely monitored.
- The same blood can be used for both *G. brevipalpis* and *G. austeni*, and should be defibrinated for 10 min but not longer than 15 min.
- *G. brevipalpis* should be fed for 5 min three times per week, and *G. austeni* for 10 min three times per week. This recommended feeding protocol appears to be optimal, practical and cost effective. Flies younger than one week old should be fed on a daily basis.
- The insectary should be maintained at 23.5°C and 70–75% RH.
- It is speculated that, due to possible deficiencies in the nutritional value of blood used at ARC-OVI, locally produced *G. brevipalpis* pupae are much smaller in size compared with pupae imported from Seibersdorf.
- Heparin, ACD, citric acid, CPDA and a combination of sodium citrate and citric acid can be used during blood collection. Citric acid gave the best results. The advantages of blood collection with anticoagulants versus defibrination should be analysed in more detail before recommendations can be made in this regard. Results showed that both procedures are suited for this purpose.

- Indications were that taste stimuli have a positive effect on fly survival and production. It is recommended that taste stimuli be used in colonies where flies are struggling to adapt to artificial feeding, i.e. starting up a colony from wild flies.
- Repeated freezing and thawing of blood had a definite negative effect on pupal production, especially for *G. brevipalpis* which produced significantly smaller pupae.
- A combination of bovine and porcine blood as the diet for *G. austeni* and *G. brevipalpis* improved pupal production and weight, as well as adult survival.
- From the data obtained it was evident that the tested bovine growth hormones did not have a negative effect on the development of colonized tsetse flies.

ACKNOWLEDGEMENTS

The authors thank FAO/IAEA for financial support, IAEA Seibersdorf Tsetse Unit for regular shipments of *G. brevipalpis* pupae, and co-workers in the Parasites Vectors and Vector-borne Diseases Programme of ARC-OVI for their assistance.

REFERENCES

- [1] KABAYO, J.P., TAHER, M., BARNOR, H., Use of oven-dried blood for *in vitro* feeding of tsetse flies, *Experientia* **44** (1988) 802–803.
- [2] BURSELL, E., BILLING, K.C., HARGROVE, J.W., MCCABE, C.T., SLACK, E., Metabolism of the blood meal in tsetse flies (a review), *Acta Trop.* **31** (1974) 297–320.
- [3] KABAYO, J.P., TAHER, M., Effect of dialysing diet components on the reproductive physiology of *Glossina p. palpalis*, *J. Insect Physiol.* **32** (1986) 543–548.
- [4] FELDMANN, U., “Guidelines for the rearing of tsetse flies using the membrane feeding technique”, *Proceedings: Techniques of Insect Rearing for the Development of Integrated Pest and Vector Management Strategies*, Vol. 2, (OCHIENG’-ODERO, J.P.R., Ed), ICIPE Science Press, Nairobi (1994) 449–471.
- [5] FELDMANN, U., “Some quality control parameters used in the rearing of tsetse flies”, *Proceedings: Techniques of Insect Rearing for the Development of Integrated Pest and Vector Management Strategies*, Vol. 1, (OCHIENG’-ODERO, J.P.R., Ed), ICIPE Science Press, Nairobi (1994) 13–29.
- [6] (FAO/IAEA) Food and Agriculture Organization of the United Nations/International Atomic Energy Agency, FAO/IAEA standard operating procedures for mass rearing tsetse flies, IAEA, Vienna, Austria (2006).
http://www-naweb.iaea.org/nafa/ipc/public/Tsetse_Rearing_SOP_web.pdf
- [7] KAPPMEIER, K., NEVILL, E.M., Evaluation of a proposed odour-baited target to control the tsetse flies *Glossina brevipalpis* and *Glossina austeni* (Diptera: Glossinidae) in South Africa, *Onderstepoort J. Vet. Res.* **66** (1999) 307–316.
- [8] KAPPMEIER, K., A newly developed odour-baited "H trap" for the live collection of *Glossina brevipalpis* and *Glossina austeni* (Diptera: Glossinidae) in South Africa, *Onderstepoort J. Vet. Res.* **67** (2000) 15–26.

BLOOD COLLECTION FROM LOCAL ABATTOIRS FOR MASS PRODUCTION OF TSETSE FLIES TO BE USED IN THE STERILE INSECT TECHNIQUE

M.B. BYAMUNGU, B. KIIMBISA, S. MATEMBO, G. MASHENGA

Tsetse and Trypanosomiasis Research Institute, P.O. Box 1026,
Tanga, United Republic of Tanzania

Abstract

The mass production of tsetse flies (*Glossina* spp.) for the sterile insect technique (SIT) requires a supply of quality blood. For some years already cattle blood has been used as food for laboratory reared flies. The blood is collected from an abattoir using standard procedures. The collection procedures, handling and storage require aseptic conditions to avoid contamination of the blood, which could be fatal to the flies. Fly mortality caused by low quality blood endangers the success of mass rearing. To rear healthy flies with good survival and production the blood should be of good quality — free of contamination and with a packed cell volume (PCV) above 25%. The present work involved the seasonal collection of blood from abattoirs in the United Republic of Tanzania (Tanga, Arusha, Dodoma, Dar-es-salaam). Dodoma was identified as having the best conditions for blood collection. To assess the quality of the blood as a diet for tsetse, blood was screened for the presence of bacteria, and the pathogens were identified. Protocols were developed for blood quality assurance.

1. INTRODUCTION

Feeding tsetse flies (*Glossina* spp.) in the laboratory has evolved from feeding on live hosts to a membrane feeding system [1]. Following successful colonization of tsetse flies using the membrane feeding technique, mass rearing has become a reality [2, 3]. Development of the technique has received added emphasis because of the need to mass rear flies for experimental work and eradication using the sterile insect technique. (SIT). As a result of the successful eradication of tsetse on the island of Zanzibar [4, 5], eradication programmes are being planned for certain areas in mainland Africa. The Tsetse and Trypanosomiasis Research Institute (TTRI) is preparing to mass rear flies for some of these areas.

For successful mass production, it is required that flies are kept alive and a healthy stock of insects is maintained which will produce a surplus of offspring. The offspring should be of comparable size, vigour and behaviour to insects in their natural habitat.

In mass rearing, flies are fed daily in an in vitro system on fresh bovine blood (FBB). The blood diet is of vital importance in producing flies with good performance. However, the maintenance of large fly colonies requires a considerable amount of blood of good quality. Various factors influence the quality of the blood diet, including the nutritional status of the animals to be slaughtered and contamination by micro-organisms such as bacteria. Therefore, it is important to assess the blood quality, and this is done by using measurable parameters including fly survival, fecundity and the number of offspring [2]. In view of the increasing demand for large numbers of healthy flies, there is a need to explore ways of ensuring that inexpensive and standard-quality blood is available. To reduce the cost incurred by importing blood, it is important to use locally available sources of blood. Using local blood will also help avoid the risks involved in importing blood such as disease in foreign livestock and blood shipments not received on schedule.

Blood was collected in an abattoir from cattle being slaughtered. The blood was defibrinated and pooled together before proportioning into two litre containers, which were then irradiated at 1 kGy. Bacterial screening was done, and packed cell volume (PCV) measured. The quality

control test was done by feeding female flies in the laboratory at TTRI. The tested blood was stored at -20°C until needed for feeding flies.

This work was done over a period of 5 years, starting in 2003, with the purpose of developing standard quality control procedures in blood collection, handling and storage for tsetse mass rearing under regional conditions.

2. MATERIALS AND METHODS

2.1. Animal selection for blood collection

The animal-holding ground was visited one day before blood collection, and there the manager, a veterinarian, made an assessment of the general condition of the animals. Selected animals were stamped and identified for slaughter. At the abattoir animals were checked again, and those fit for slaughter were passed. In one of the abattoirs visited (Dodoma), blood samples of sick animals (but diseases not yet identified) were sent to the Veterinary Investigation Centre (VIC) for further investigation, and animals found to be in critical condition were condemned.

2.2. Identification of local sources, seasonal collection and feeding flies

Local sources for blood sampling, with the exception of Tanga, were identified by contacting the Ministry of Livestock. Blood samples were collected on a seasonal basis, i.e. in the dry and wet seasons, from four abattoirs; Tanga, Arusha, Dar-es-salaam and Dodoma.

The slaughtering system differed among abattoirs:

- Tanga: Slaughtering was done on the floor, and blood collected from the jugular vein.
- Dar-es-salaam: Animals were slaughtered on the floor. The heart's vena cava was cut to allow quick draining of the animal's blood, and this permitted blood to be collected.
- Arusha: This abattoir is modern but slaughtering was done on the floor due to religious beliefs. Immediately after slaughtering animals were hung up, and blood was collected from the jugular vein.
- Dodoma: This is also a modern abattoir, and the construction meets the religious requirements. Animals were hung up and then slaughtered. Blood was collected from the jugular vein.

Bioassays were conducted by feeding the blood to tsetse flies (*Glossina austeni* Newstead) at TTRI. The quality control test was done for 25 or 30 days by checking feeding response, survival, abortions, number of pupae produced by day 18 and pupal class [6]. These parameters were used to calculate the quality factor (QF); the acceptable value was 1 or above. The QF is a bioassay that determines whether or not a blood diet is suitable for in vitro feeding [6].

The amount of blood to be collected depends on the size of the fly colony. It is important to ensure that there is enough blood in stock for at least four months, just in case there is a problem in the colony such as a disease outbreak.

2.3. Blood defibrination

At the abattoir, the blood was defibrinated manually using a defibrination kit [6]. Another trial was conducted using sodium citrate 0.015M as an anticoagulant [7] instead of the normal defibrination procedure. Bioassays were conducted to compare sodium citrate-treated bovine blood with defibrinated bovine blood on tsetse production and survival.

2.4. Bacteria screening, isolation and identification

Blood samples collected at different abattoirs in different seasons were screened for bacteria by mixing collected blood with nutrient agar in a laminar flow hood. The mixture was incubated at 37°C and checked for bacterial growth after 24 and 48 hours. Different colonies were cultured by transferring to different Petri dishes. Also, open Petri dishes with nutrient agar were placed in the fly handling, feeding and holding rooms. In addition, dead flies were crushed and cultured for bacterial growth.

Cultures were taken to the Central Veterinary Laboratory (CVL) (formerly Animal Disease Research Institute (ADRI)) for isolation, identification and amplification. The bacterial isolates (1 mL bacteria in 100 mL blood) were fed to test flies by conducting the bioassays for 25 days and calculating the quality factor (QF).

2.5. Equipment cleaning and sterilization

Equipment for blood collection was first washed in tap water, then rinsed in sodium hypochlorite solution (10%) followed by hot water, and finally rinsed in distilled water. Plastic equipment was oven-sterilized at 80°C, and heat-resistant items at 120°C, for not less than 6 hours.

2.6. Storage time and temperature monitoring

To establish the optimum storage time, bioassays were conducted by feeding flies on blood samples from collections in 2004, 2005, 2006 and 2007 and compared with feeding on blood collected in 2008. The blood was collected from four different abattoirs – Tanga (2004), Arusha (2005), Dar-es-salaam (2006) and Dodoma (2007 and 2008). Before frozen storage, the blood was tested and all samples had acceptable QF values. Blood was stored in freezers with temperature monitors that were observed every day. The optimum storage temperature (–20°C) was maintained.

3. RESULTS

3.1. Seasonal sampling

The overall bacterial count in dry seasons was significantly lower than in wet seasons, and blood samples from the Tanga abattoir showed the lowest bacterial count (Table 1).

Bacterial isolates of *Bacillus* spp. were commonly found, and in blood from the Tanga abattoir there were more bacterial species in the wet season than the dry season (Table 2).

In the insectaries at TTRI, there were six species of *Bacillus* and one of *Staphylococcus*, but other genera were not identified to the species level (Table 3). Bacteria were found more frequently in wet seasons than in dry seasons. *Bacillus pumilus* was isolated from dead flies.

3.2. Bioassay results

In each treatment for 25 days, 30 flies were fed daily on blood mixed with a bacterial isolate. *Bacillus cereus* var *mycoides* caused significantly lower survival and lower pupal production compared with other treatments, and *Bacillus firmus* caused significantly lower survival compared with some treatments (Table 4). However, pupal production was low in all treatments including the control when compared with the standard value [6].

There was a significant difference between the survival of flies fed sodium citrated blood and those fed defibrinated blood (Table 5). Pupal production was not significantly different between the two treatments, but production was below standard [6].

TABLE 1. BACTERIAL COUNT (NO. OF COLONIES) (MEAN±SD)

Abattoir	Dry season	Wet season	Overall ^a
Dar-es-Salaam	6.3±1.5	16.0±3.6	11.2±5.8 a
Tanga	4.3±5.2	4.6±5.0	4.5±3.6 b
Arusha	13.3±8.7	15.3±2.5	14.3±5.9 a
Dodoma	7.7±2.5	21.3±10.0	14.5±10.0 a
Overall ^b	7.9±5.4 b	14.3±8.1 a	

^a Means in a column followed by the same letter are not significantly different ($P<0.05$), Duncan's Multiple Range Test (DMRT).

^b Means in a row followed by the same letter are not significantly different ($P<0.05$), Duncan's Multiple Range Test (DMRT).

SD = Standard deviation.

TABLE 2. BACTERIA ISOLATED FROM COLLECTED BLOOD SAMPLES

Species of bacteria	Abattoir and season							
	Tanga		Dar-es-salaam		Arusha		Dodoma	
	Dry	Wet	Dry	Wet	Dry	Wet	Dry	Wet
<i>Bacillus</i>								
<i>licheniformis</i>		X		X				
<i>coagulans</i>		X		X				
<i>firmus</i>		X						X
<i>stearotherophilus</i>		X				X		
<i>cereus</i> var <i>mycoides</i>			X	X				
<i>pumilis</i>			X				X	
<i>lentus</i>					X			
<i>Staphylococcus</i>								
<i>saprophyticus</i>	X		X		X			
<i>intermedius</i>					X			
<i>Pseudomonas</i>								
<i>putida</i>					X			
<i>Streptococcus</i>								
<i>faecalis</i>					X			
<i>Acinetobacter</i>							spp.	
<i>junii</i>		X						
<i>lwoffii</i>		X		X				
<i>Enterobacter</i>								spp.

TABLE 3. BACTERIA ISOLATED FROM ROOMS IN THE INSECTARIES (TTRI)

Species of bacteria	Room and season						
	Working	Feeding	Holding		Handling		
	Wet	Dry	Wet	Dry	Wet	Dry	Wet
<i>Bacillus</i>							
<i>licheniformis</i>			X			X	
<i>coagulans</i>		X					
<i>subtilis</i>	X						
<i>stearothermophilus</i>			X				
<i>pumilis</i>	X				X		
<i>lentus</i>				X			
<i>Staphylococcus</i>							
<i>intermedius</i>	X		X		X		
<i>Pseudomonas</i> spp.	X		X		X		
<i>Corynebacterium</i> spp.			X				X
<i>Streptococcus</i> spp.			X		X		X

TABLE 4. SURVIVAL AND PUPAL PRODUCTION OF FLIES FED ON BLOOD INFECTED WITH PURE ISOLATES OF BACTERIA (MEAN \pm SD)^a

Species of bacteria	Survival (%)	Pupal production (no.)
<i>Bacillus coagulans</i>	85.4 \pm 2.0 a	9.0 \pm 1.4 a
<i>Bacillus firmus</i>	79.5 \pm 0.7 b	11.0 \pm 1.4 a
<i>Bacillus cereus</i> var <i>mycoides</i>	8.0 \pm 2.8 c	2.0 \pm 1.4 b
<i>Bacillus lentus</i>	88.5 \pm 2.1 a	9.5 \pm 0.7 a
Control	92.0 \pm 2.8 a	12.0 \pm 1.4 a

^a Means in a column followed by the same letter are not significantly different ($P<0.05$), Duncan's Multiple Range Test (DMRT).

TABLE 5. COMPARISON OF FEEDING SODIUM CITRATED AND DEFIBRINATED BOVINE BLOOD ON TSETSE SURVIVAL AND PUPAL PRODUCTION (MEAN \pm SD)

Treatment	Survival (%)	Pupal production (no.)
Sodium citrated blood	80.7 \pm 2.1	15.7 \pm 2.1
Control (defibrinated blood)	91.4 \pm 1.7	18.0 \pm 2.0
t-value	6.95**	1.4 ^{NS}

** Significant ($P<0.01$), NS = Non-significant

Comparing fly survival on blood collected in five different years, survival was rather similar, but in 2005 it was significantly higher than in 2004 and 2006; pupal production in 2007 was significantly higher than in other years (Table 6). Pupal weights were lower in 2004 and 2005 than in the following 3 years (data not shown).

TABLE 6. FLY SURVIVAL, PUPAL PRODUCTION AND PUPAL WEIGHT CLASS WHEN FED ON BLOOD COLLECTED IN DIFFERENT YEARS (MEAN \pm SD)^a

Year of collection	Survival (%)	Pupal production (no.)	Pupae in classes ^b A and B (%)	
			A	B
2004	82.0 \pm 7.2 b	19.3 \pm 3.1 b	11	31.6
2005	93.0 \pm 3.0 a	18.7 \pm 1.5 b	16	25.8
2006	82.7 \pm 5.8 b	16.7 \pm 3.5 b	25.3	28
2007	87.7 \pm 4.0 ab	25.7 \pm 1.5 a	4	6
2008	88.7 \pm 2.3 ab	20.3 \pm 2.1 b	10	13

^a Means in a column followed by the same letter are not significantly different ($P<0.05$), Duncan's Multiple Range Test (DMRT).

^b Weight class A is the smallest, class B the next smallest [6].

4. DISCUSSION

Animals originated mainly from individual livestock keepers in the northern, central and lake zone regions; some animals slaughtered in Dodoma were from ranches within the region. Seasonal sampling done in the four different abattoirs showed that there were differences in the set-up, slaughtering system and number of animals slaughtered according to season. More animals were brought for sale during the wet than the dry season, and they looked healthy due to the availability of pasture; few animals were available during the dry season. Nevertheless, blood collection in the wet season resulted in higher bacterial counts.

Even though the Tanga abattoir had the lowest bacterial counts in collected blood, the abattoir in Dodoma was selected as a collection centre for the following reasons: it is a modern abattoir, cold rooms for blood storage are available, and most of the animals slaughtered are well cared for and come from ranches.

There were no marked differences among the average values of packed cell volume (PCV); they ranged from 30 to 34 from one season to another, and were above the acceptable value of 25. Season could not be associated with PCV. Animals tend to be sold during times of favourable conditions. Also, animals are not exhausted because transportation to the marketing centres is either by truck or train.

Screening of bacteria in collected blood and in rearing rooms indicated that seven genera were present: *Bacillus* spp., *Staphylococcus* spp., *Pseudomonas* spp., *Streptococcus* spp., *Acinetobacter* spp., *Enterobacter* spp. and *Corynebacterium* spp. *Staphylococcus* spp. and *Streptococcus* spp. are normal flora, but can cause problems if ingested in abundance. Bacilli are free living bacteria, while *Corynebacterium* spp. and *Pseudomonas* spp. are obligate and

harmful bacteria. Experiments [8] done elsewhere revealed that some *Bacillus* spp. and *Pseudomonas* spp. caused mortality to laboratory reared flies. In the present work, mainly bacilli were isolated, suggesting that more hygienic standards are required in handling blood. Some of the bacterial isolates from dead flies, and from the holding, handling and feeding rooms, resembled those isolated from the blood diet. This implies that bacteria are being transferred from one room to the other, no doubt as a result of rearing activities. Control of bacteria in these rooms would probably minimize the contamination.

Of the bacterial isolates fed in blood to tsetse flies, *Bacillus firmus* reduced survival a little, but *B. cereus* var *mycoides* reduced it greatly, and this species also greatly reduced pupal production. *Bacillus coagulans*, *B. firmus* and *B. lentus* delayed larviposition, but no significant reduction in pupal production was observed. Pure isolates were used instead of different concentrations due to a lack of expertise, thus further tests are appropriate. Probably the effect of bacteria on the quality of the blood depends on the virulence of the bacteria, and the frequency of and time after ingestion [8]. Bacterial colonies could also affect the quality of the blood through their by-products.

Bioassays measuring pupal production from flies fed on defibrinated or citrated blood showed no significant differences between them. However, data on survival indicated that significantly fewer flies survived on citrated blood (sodium citrate 0.015M) than on defibrinated blood. Possibly better results would be obtained if the concentration was reduced. However, experiments [7] done in Vienna gave good results when using a similar concentration of sodium citrate.

Bioassays conducted on different batches of blood from 2004 to 2007 compared with the 2008 batch showed some significant differences in fly survival and pupal production, but this could not be related to storage time. However, flies fed on batches from 2004, 2005 and 2006 produced more pupae in classes A and B (thus low pupal weight) [6] compared with flies fed on batches from 2007 and 2008. For good-quality pupae, the fraction of A-class pupae should not exceed 10% [6]. This suggested that, even under stable storage conditions, blood could be stored for only one year without reducing the quality. Nevertheless, the tested blood samples were not all collected at the same location; this variable might have confounded the experiment, and therefore the potential for long-term (e.g. 5 years) blood storage cannot be ruled out.

5. RECOMMENDATIONS

- To successfully reduce bacterial infection in the membrane feeding technique, use prophylactic measures, e.g. sterilize membranes, underlying aluminium trays and blood.
- A high level of hygiene is required during blood handling, thus restrict movement in the blood handling room.
- Sterilize feeding rooms, probably using UV light.
- Conduct more research to identify pathogenic bacteria in the rearing system.

ACKNOWLEDGEMENTS

The IAEA provided funds for this work. The CVL (Bacteriology Department) assisted in isolating and identifying bacteria. The contribution of TTRI's Director, Dr. Msangi, in making this work a success is appreciated.

REFERENCES

- [1] FELDMANN, U., “Rearing tsetse flies for use in sterile insect technique vector control programmes”, Management of Insect Pests: Nuclear and Related Molecular and Genetic Techniques, (Proc. IAEA/FAO International Symposium, Vienna, Austria, 19–23 October 1992, STI/PUB/909), IAEA, Vienna (1993) 579–601.
- [2] MEWS, A.R., BAUMGARTNER, H., LUGER, D., OFFORI, E.D., Colonisation of *Glossina morsitans morsitans* Westw. (Diptera, Glossinidae) in the laboratory using *in vitro* feeding techniques, Bull. Entomol. Res. **65** (1976) 631–642.
- [3] MSANGI, A., et al., “Eradication of *Glossina austeni* Newstead on Unguja Island (Zanzibar) by the sterile insect technique. 2. Mass production and quality assessment of sterile flies”, Animal Trypanosomosis: Vector and Disease Control using Nuclear Techniques, (Proc. Second FAO/IAEA Seminar for Africa, Zanzibar, United Republic of Tanzania, 27 November–1 December 1995), Backhuys Publishers, Leiden, The Netherlands (1999) 219–229.
- [4] MSANGI, A.R., et al., “Success in Zanzibar: eradication of tsetse”, Area-Wide Control of Fruit Flies and Other Insect Pests, (Proc. International Conference on Area-Wide Control of Insect Pests, and the 5th International Symposium on Fruit Flies of Economic Importance, Penang, Malaysia, 28 May–5 June 1998), (TAN, K.H., Ed.), Penerbit Universiti Sains Malaysia, Pulau Pinang, Malaysia (2000) 57–66.
- [5] VREYSEN, M.J.B., et al., *Glossina austeni* (Diptera: Glossinidae) eradicated on the island of Unguja, Zanzibar, using the sterile insect technique, J. Econ. Entomol. **93** (2000) 123–135.
- [6] (FAO/IAEA) Food and Agriculture Organization of the United Nations/International Atomic Energy Agency, FAO/IAEA standard operating procedures for mass rearing tsetse flies, IAEA, Vienna, Austria (2006).
http://www-naweb.iaea.org/naifa/ipc/public/Tsetse_Rearing_SOP_web.pdf
- [7] YAMAGUCHI, A.Y., PARKER, A.G., GEMEINER, M., “Comparison of sodium citrate with defibrination for the processing of blood for tsetse mass rearing”, Integrating the Sterile Insect and Related Nuclear and Other Techniques, (FAO/IAEA International Conference on Area-wide Control of Insect Pests, Vienna International Centre, Vienna, Austria, 9–13 May 2005, Book of Extended Synopses, IAEA-CN-131/29P), IAEA, Vienna (2005) 272.
- [8] KAAYA, G.P., DARJI, N., Mortality in adult tsetse, *Glossina morsitans morsitans*, caused by entomopathogenic bacteria, J. Invertebr. Pathol. **54** (1989) 32–38.

DEVELOPMENT OF HIGH TEMPERATURE SHORT TIME VERTEBRATE-BLOOD PASTEURIZATION EQUIPMENT FOR TSETSE FLY DIETS

I. MORÁVEK¹, J. LACH¹, P. TAKÁČ²

¹Department of Manufacturing Systems, Slovak Technical University

Namestie Slobody 17

812 31 Bratislava, Slovakia

²Institute of Zoology, SAV,

Bratislava, Slovakia

Abstract

Tsetse flies feed only on vertebrate blood, but the collection and processing of blood is expensive, it must be stored at -20°C requiring costly storage rooms and reliable electricity, and it must be irradiated to reduce bacterial contamination. This is tolerable for small colonies, but as colony size increases to service large-scale programmes, the supply and processing of blood becomes critical. Blood is normally collected from cattle at slaughter. This process is necessarily not aseptic, and large-scale collection is only possible where the animals are suspended for bleeding. One alternative to blood decontamination is using the High Temperature Short time Pasteurization (HTST) method. The food processing industry uses pasteurization to reduce bacterial load in a wide range of products. Our previous results indicated that for the control of the blood pasteurization process, to reach satisfactory bacteriological purity and at the same time to prevent the blood from coagulating, it is important to study temperature and time and also some other parameters that could predict blood coagulation. Crucial for blood coagulation is to study blood viscosity. Classical heat exchangers are not suitable for blood pasteurization. In such equipment the blood coagulation depends on temperature and time. Besides the relatively low temperatures, blood is coagulating with cumulative time until total shutdown of blood flow. After a series of experiments we found a solution using microwave systems. To verify the microwave heating concept, we built an experimental workstation. First we verified the accuracy of the applicator design from the aspect of output adaptation to the power source. Also we installed measuring equipment. This system complies with the requirements of quick heating with sufficiently high heat accumulation. By utilizing standard components for the base of the microwave generator, it is possible to markedly reduce the final price of the equipment.

1. INTRODUCTION

This paper reports on work to solve the difficulty of pasteurizing vertebrate blood. We used a new design of microwave generator equipment, obtained a maximum rate from the finished components and verified the work experimentally. In an earlier phase of the project, we utilized microwave components borrowed from S-TEAM company, and set up an experimental workplace. Nevertheless, even though the price was markedly reduced, the cost to the project was still great. It was convenient for a quick verification of the concept, but unacceptable for long-term experiments. Then we obtained the cooperation of RADAN company in the Czech Republic. It manufactured significant components of the microwave tract on easy terms. However, the hydraulic tract was used essentially as in an earlier phase of the project.

2. MICROWAVE BLOOD STERILIZATION SYSTEM

Below is a diagram (Fig. 1) of the microwave tract. A microwave generator with a frequency of 2.45 GHz and a power of 800 W was built from currently accessible components rationally put together as one unit, with compulsory air cooler and with controlling and safety components. Besides other aspects, using existing components is a big advantage because they are manufactured worldwide in large lots for microwave ovens. Thus, the price is low, and also spare parts are available.

A block diagram of a fully equipped microwave blood sterilization system is shown in Fig. 1.

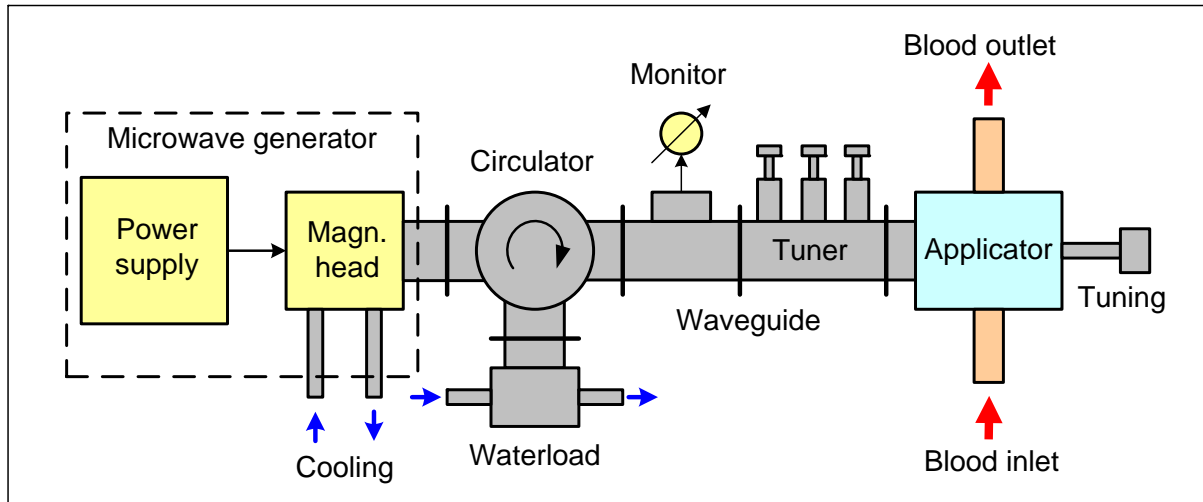


FIG. 1. Block diagram of a microwave sterilizer system (full equipment).

2.1. Microwave generator

The microwave generator consists of:

- magnetron head
- magnetron power supply

The magnetron head integrates a magnetron (microwave power-generating tube), a waveguide launcher, cooling equipment (air cooling) and overload protection circuitry.

The magnetron power supply provides high voltage for the magnetron (typically 4 kV) as well as magnetron filament heating voltage, and includes magnetron protection circuitry.

For development purposes, it is recommended that the power supply have low ripple and settable high voltage, providing for low ripple and settable microwave output power. The final version can use a less expensive high ripple power supply (the mean power averaged over the ripple period is then relevant). The power supply parameters must be matched to the magnetron used. Generally, manufacturers of microwave generators provide complete systems, i.e. a magnetron head with appropriate power supply.

2.2. Circulator and load

The circulator is a ferrite-based passive microwave component which, in combination with a load, serves for magnetron protection. Any reflected power that could potentially damage the magnetron is diverted to the load where it is absorbed and converted to heat. Both the circulator and the load must be rated for full generator power.

The circulator with the associated load is required for development purposes. While in principle if properly operated it is not necessary in the final version, to protect the magnetron against an accidental error the circulator/load is still recommended.

2.3. Waveguide

The recommended microwave transmission medium is the commonly used rectangular R26 (WR340) waveguide, having inner dimensions of 86.36×43.18 mm.

2.4. Power monitor

The monitor is a measuring device that serves to sense incident and/or reflected microwave power. It can be optionally used as an interlock, switching off the generator if the reflected power exceeds the magnetron-damaging level.

In principle, the monitoring device is not necessary in a developed and well-tested system.

2.5. Tuner

Due to high blood permittivity and its medium loss factor, the coupling will be inherently narrowband and sensitive to blood properties, which may vary from batch to batch and in dependence on temperature. Therefore, a method of tuning must be provided to adapt the applicator to these varying conditions.

The tuner can be external or internal to the applicator.

The external tuner is typically a three-stub tuner that can be manually operated or automatic.

Using a manual external tuner, the impedance match can be improved based on the monitored reflected power reading. The procedure requires some skill.

Using an automatic external tuner is a comparatively expensive solution but solves all coupling efficiency problems without an operator intervening when the processing plant is under full power.

As an internal tuner, the applicator internal matching stub (see Section 3) can be used for fine-tuning. However, the tuning range is limited and the tuning process cumbersome.

The system, stripped down to the absolute minimum, is shown in Fig. 2.

It is possible to use this reduced equipment only when blood parameters have already been measured and the applicator is set up to the attested condition.

3. APPLICATOR

The applicator is an essential system component, providing efficient coupling of microwave power to the processed blood. While other components of the system are commercially available, the applicator must be developed for this specific purpose. The coupling efficiency is expressed in terms of impedance match or return loss, which is a ratio in decibels of reflected power to incident power. The value of -10 dB or less is acceptable, assuring reflected, i.e. wasted, power below 10%.

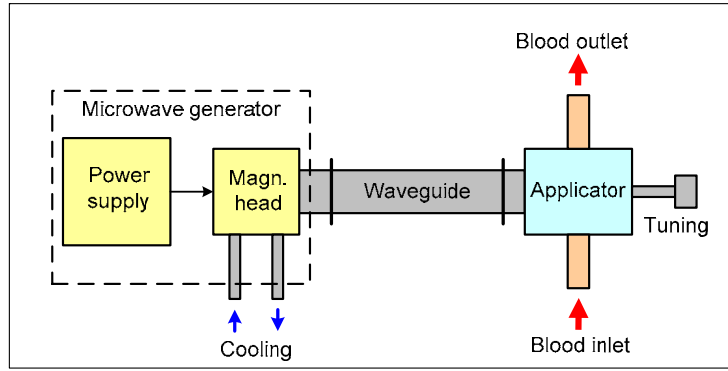


FIG. 2. Block diagram of a microwave sterilizer system (minimum equipment).

The applicator consists of a short-circuited waveguide section with an inserted PTFE tube (or more tubes) through which the processed blood flows with defined speed (example in Fig. 3). Impedance matching is achieved by:

- proper positioning of the tube in the waveguide, and
- inserting a matching metallic cylinder with defined dimensions at a defined distance from the tube.

To avoid the escape of microwaves through the PTFE tube, the tube must be guided through a metallic enclosure on the waveguide outside, where it must be ended and continue as a metallic tube for at least 30 mm. This can be part of the blood cooling device at the outlet. Then the conduit can again continue as a plastic unshielded tube.

4. BLOOD PROPERTIES

Based on published literature, a complex permittivity model of average blood can be expressed using the 1st-order Debye model as

$$\epsilon_r = \epsilon' - j\epsilon'' = \epsilon_\infty + \frac{\epsilon_s - \epsilon_\infty}{1 + j\omega\tau} = \left[\epsilon_\infty + \frac{\epsilon_s - \epsilon_\infty}{1 + (\omega\tau)^2} \right] - j \left[\omega\tau \frac{\epsilon_s - \epsilon_\infty}{1 + (\omega\tau)^2} \right]$$

with typical values $\epsilon_s = 55$, $\epsilon_\infty = 5$, $\tau = 8.5$ ps.

The broad topic of the experiments was the blood characteristics important from a behavioural aspect in the field of microwave radiation. We examined the temperature dependence and diffusion according to individual collections. The effect of frequency is very great, but our equipment works with constant frequency. Therefore, we oriented provisionally only according to sources in the literature.

To maximally eliminate disturbing factors, the measurements in the adapter were made directly in the applicator. Measurements were made depending on the temperature and on the samples from different collections. All measurements were done at a frequency of 2.45 GHz. The results of the measurements are shown in Figs 4 and 5. Based on our previous measurements and the literature, the massive diffusion of blood viscosity is known. That is also the parameter markedly affecting the process of microwave heating. The result of these effects is the necessity for precise knowledge of the mentioned parameters. It is possible to measure directly in the online process or to measure off line a small amount from a blended quantity. In the first case it is necessary to do quick automated trimming; the complex controlling system is too expensive. The second possibility of off-line sample measurement allows markedly simpler construction, but with higher demands on the qualifications of the workers.

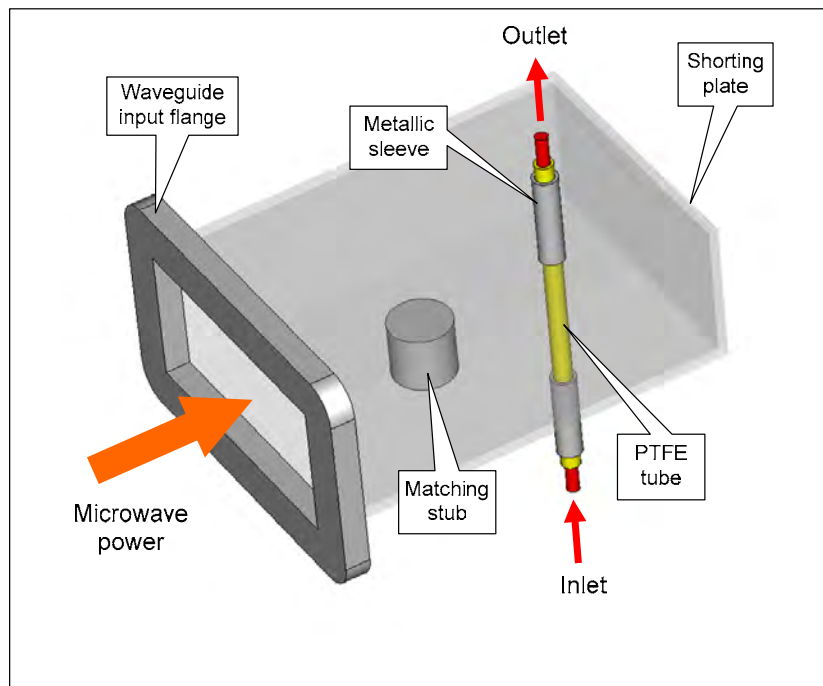


FIG. 3. Example of applicator.

Important conclusions are:

1. Temperature influence on the permittivity complex is great.
In the system it is important to stabilize the blood temperature before input to the applicator.
2. The influence of the individual samples of the measured blood is great.
It is important to measure the complex permittivity and viscosity, whether continually with the automated tuning or with the sample withdrawal from a blended quantity and hand-adjusted parameters.
3. Viscosity has great influence. Also, see previous point above.

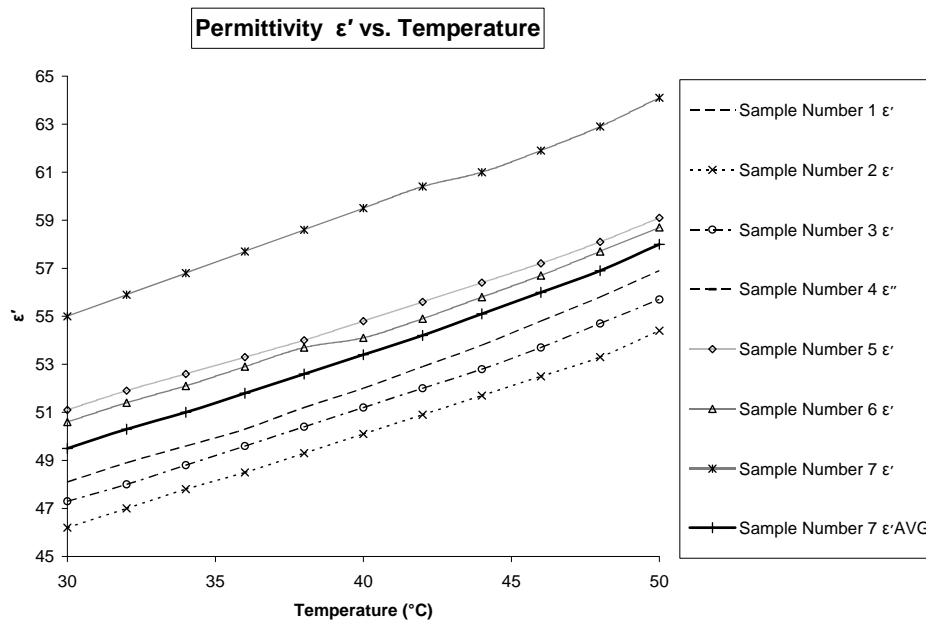


FIG. 4. Permittivity ϵ' vs. temperature.

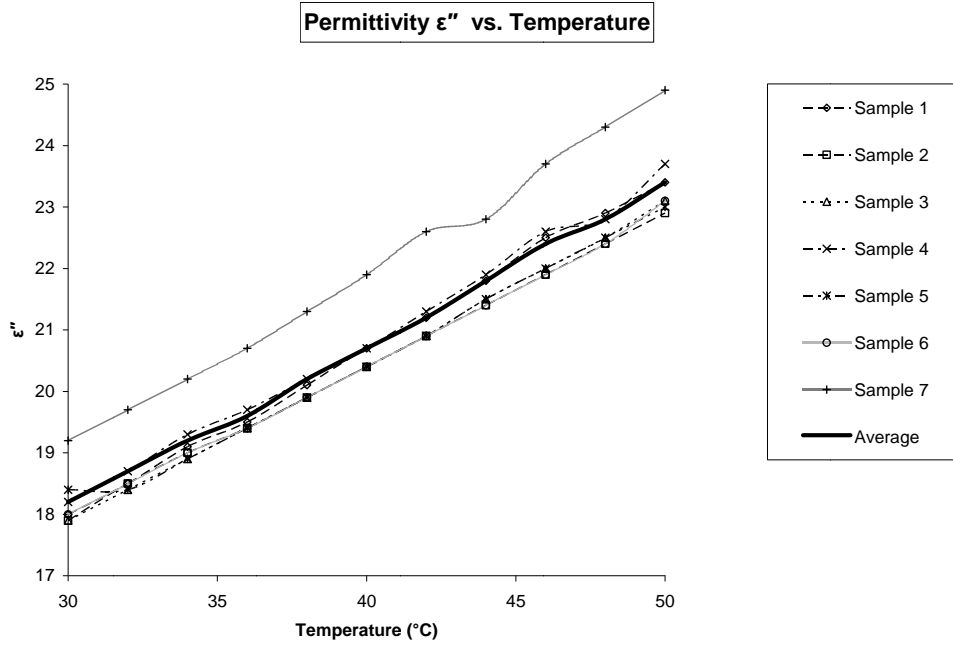


FIG. 5. Permittivity ϵ'' vs. temperature.

5. HYDRAULIC SYSTEM

The hydraulic system was developed from experience gained in an earlier stage of the project. The conclusions proved to be true, resulting in strict requirements for fluency and stability of flow. Also, there is the necessity of periodical system rinsing. In a module of the control system, the flow rate and pressure are observed as control parameters. The function of the hydraulic system is shown in the diagram below (Fig. 6).

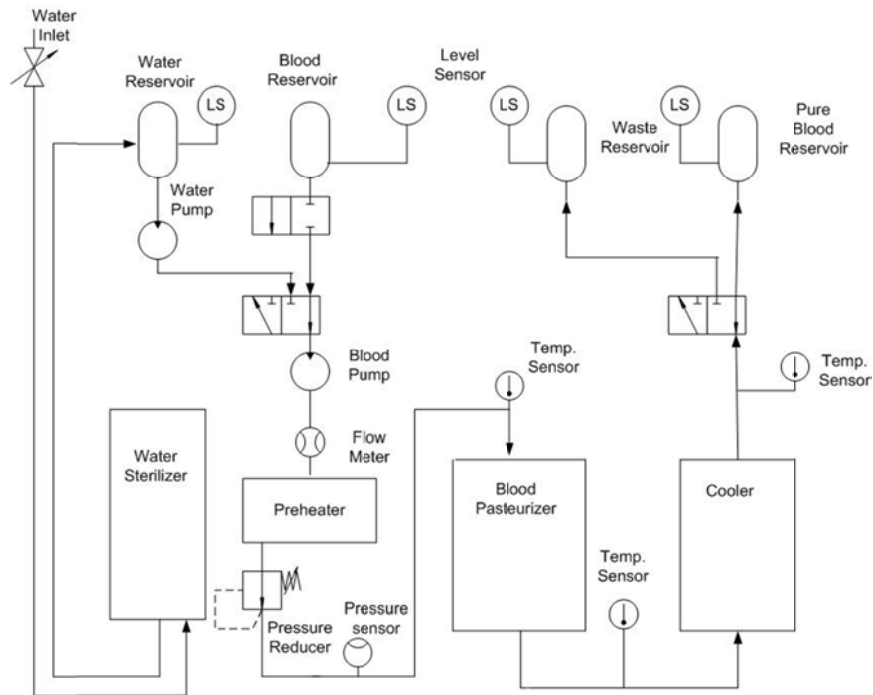


FIG. 6. Hydraulic scheme.

6. PROPOSAL TO SOLVE THE PROBLEM

We proposed and verified some ways to heat blood quickly and the complex of equipment related to this process. Constructional models were developed separately, some with organizations from research and industry.

The research results obtained in this project are important for the successful development of the prototype pasteurizing equipment heated with microwaves.

1. During blood treatment it is important to measure the blood parameters, which are crucial for the transformation of electromagnetic radiation to heat. The range of heat exposition, where 'microbiological inactivation' is reliable and the blood is not yet coagulating, is too narrow. The crucial step is to measure the viscosity and complex permittivity (Table 1). Whether these parameters will be measured continuously online or by dosage off-line can be decided after technical and economic issues are considered.
3. We recommend the development of a prototype of the pasteurizer with the dosage measurement parameters, and that after tests a final solution be found. The essential parts of the microwave tract and the hydraulic tract will be unaffected.
4. The expected cost to manufacture the prototype is approximately €30 000.

TABLE 1. PERMITTIVITY vs. TEMPERATURE

Complex permittivity ϵ (-)

Temperature (°C)	Sample Number														ϵ'_{AVG}	ϵ''_{AVG}
	1		2		3		4		5		6		7			
	ϵ'	ϵ''	ϵ'	ϵ''	ϵ'	ϵ''	ϵ'	ϵ''	ϵ'	ϵ''	ϵ'	ϵ''	ϵ'	ϵ''		
30	48.1	18	46.2	17.9	47.3	17.9	48.2	18.2	51.1	18.4	50.6	18	55	19.2	49.5	18.2
32	48.9	18.5	47	18.5	48	18.4	48.8	18.7	51.9	18.4	51.4	18.5	55.9	19.7	50.3	18.7
34	49.6	19.1	47.8	19	48.8	18.9	49.6	19.3	52.6	18.9	52.1	19	56.8	20.2	51	19.2
36	50.3	19.5	48.5	19.4	49.6	19.4	50.4	19.7	53.3	19.4	52.9	19.4	57.7	20.7	51.8	19.6
38	51.2	20.1	49.3	19.9	50.4	19.9	51.3	20.2	54	19.9	53.7	19.9	58.6	21.3	52.6	20.2
40	52	20.7	50.1	20.4	51.2	20.4	52.2	20.7	54.8	20.4	54.1	20.4	59.5	21.9	53.4	20.7
42	52.9	21.2	50.9	20.9	52	20.9	52.9	21.3	55.6	20.9	54.9	20.9	60.4	22.6	54.2	21.2
44	53.8	21.8	51.7	21.4	52.8	21.5	53.9	21.9	56.4	21.5	55.8	21.4	61	22.8	55.1	21.8
46	54.8	22.5	52.5	21.9	53.7	22	54.9	22.6	57.2	22	56.7	21.9	61.9	23.7	56	22.4
48	55.8	22.9	53.3	22.4	54.7	22.5	55.9	22.8	58.1	22.5	57.7	22.4	62.9	24.3	56.9	22.8
50	56.9	23.4	54.4	22.9	55.7	23.1	56.9	23.7	59.1	23	58.7	23.1	64.1	24.9	58	23.4

$f = 2.45255$ GHz

$v_A = 24^\circ\text{C}$

RH = 57%

BIBLIOGRAPHY

BILIK, V., Power Transmission in Microwave Systems, Internal Educational Document, Rev. B:07-May-2007, Slovak University of Technology, Bratislava.

GERLING, J.F., Waveguide Components and Configurations for Optimal Performance in Microwave Heating Systems, Gerling Applied Engineering, Inc., Modesto, CA 95358 USA (2000).

KAMARIAH, I., Microwave Characterization of Human Blood using Dielectric Waveguide Measurement System, Microwave Technology Center, University Teknologi MARA, Malaysia.

LOIDL, A., GULICH, R., WOLF, M., LUNKENHEIMER, P., Dielectric Spectroscopy on Blood, University of Augsburg (2008).

NAKAMURA, T., NIKAWA, Y., Precise measurement of complex permittivity of materials for telecommunication devices, J. Telecommun. Inf. Technol. **3** (2001).

POP, G.A.M., et al., Blood Electrical Impedance Closely Matches Whole Blood Viscosity as Parameter of Hemorheology and Inflammation, Department of Cardiology, Thoraxcenter, Hemodynamics Laboratory EE2322, Technical University, Delft, The Netherlands.

TANAKA, M., Molecular Dynamics Study of Microwave Heating of Water and Aqueous Solutions, National Institute for Fusion Science, Tokyo 509–529. mtanaka@nifs.ac.jp
<http://dphysique.nifs.ac.jp/indexE.html>

COLONY ESTABLISHMENT OF *GLOSSINA FUSCIPES FUSCIPES* AND *GLOSSINA PALLIDIPES* (ORIGIN TORORO) AT KALITI TSETSE REARING AND IRRADIATION CENTER, ETHIOPIA

S. MEKONNEN

Kaliti Tsetse Rearing and Irradiation Center, STEP,
P.O. Box 19917,
Addis Ababa, Ethiopia

Abstract

Colonies of *Glossina fuscipes fuscipes* Newstead and *Glossina pallidipes* Austen (origin Tororo) were established at Kaliti Center by acquiring pupae from Bratislava, Slovakia. The main objectives of the introduction of these species were to strengthen the release activities in the adjacent area where *G. f. fuscipes* exists, and to establish a back-up for the Arba Minch strain of *G. pallidipes*. Since April 2008, 20 300 *G. f. fuscipes* and 5300 *G. pallidipes* pupae were delivered to Kaliti. In week 38 the tsetse fly colony of *G. f. fuscipes* reached 14 800 females, and that of *G. pallidipes* reached 1500. The physical holding conditions for both species were $24\pm1^{\circ}\text{C}$ and 80–85% RH. The flies were kept in PVC cages at a density of 60 females/cage for *G. f. fuscipes* and 48 females/cage for *G. pallidipes*, with a male to female ratio of 1:4. They were fed 5 days per week on whole defibrinated bovine blood that was collected aseptically and irradiated at 0.5–1.5 kGy before storage at -20°C . The blood was presented to the flies through a silicone membrane at $35\text{--}37^{\circ}\text{C}$. Females of *G. f. fuscipes*, when 3 days old, were mated with 7 days old males, and females of *G. pallidipes*, when 7 days old, were mated with 10–12 days old males. The establishment of these two species was monitored by measuring regularly the following parameters: pupal production, pupal weight, emergence rate, PPIF, P/F/10 days and daily mortality.

1. INTRODUCTION

Tsetse flies (*Glossina* spp.) are viviparous blood-sucking African Glossinidae (Muscoidea) of immense economic importance as vectors of pathogenic Protozoa of the genus *Trypanosoma*, causing serious diseases of humans and domestic animals [1] and major economic loss by transmitting both human and animal trypanosomiasis [2]. The eradication of tsetse flies using the sterile insect technique (SIT) depends on mass rearing large numbers of females in order to produce sufficient male tsetse flies for the release operation.

The mass rearing of tsetse flies in the absence of living host animals has been successful in recent years [3, 4, 5, 6]. Tsetse rearing evolved from feeding on live hosts to an in vitro rearing system where blood is fed to flies through a silicone membrane [7]. *G. pallidipes* (Tororo (Uganda) strain) can be reared under laboratory conditions using rabbits as live hosts [8], or bovine blood held beneath a silicone membrane [9], as a food source.

Recently, the application of the SIT was demonstrated by the area-wide eradication of *Glossina austeni* Newstead in Unguja Island, Zanzibar [10, 11]. This case, plus earlier successful attempts in Burkina Faso [12] and Nigeria [13], inspired a continental strategy to progressively reduce isolated tsetse populations to unsustainable numbers. This undertaking will require the mass production of males for sustainable sequential release until major population reductions are achieved [14].

The present work focused on the colony establishment of two species, *G. f. fuscipes* and *G. pallidipes*, monitored using the following parameters: pupal production, pupal weight, pupal classes, emergence rate, PPIF, P/F/10days and daily mortality rate.

2. MATERIALS AND METHODS

Pupae of *G. f. fuscipes* and *G. pallidipes* were obtained from Bratislava (Institute of Zoology, Slovak Academy of Sciences), but originated in Tororo, Uganda and were adapted to laboratory conditions at the FAO/IAEA Agriculture and Biotechnology Laboratory, Seibersdorf, Austria. The flies were kept under standard colony conditions — temperature $24\pm1^{\circ}\text{C}$ and relative humidity 80–85% [7, 15]. Both colonies were fed in vitro on bovine blood obtained through a silicone membrane. Flies were fed 5 d/wk (except weekends) on defibrinated blood warmed to $35\text{--}37^{\circ}\text{C}$. The blood was subjected to 0.5–1.5 kGy gamma irradiation to ensure its sterility, and stored at -20°C before use. Soon after flies emerged they were chilled to 4°C to immobilize them, and then the sexes were separated by hand. Females of *G. f. fuscipes* and *G. pallidipes* were mated when 3 days old with 7 days old males, and when 7 days old with 10–12 days old males, respectively. Methods of data collection and recording were provided by the FAO/IAEA.

3. RESULTS

3.1. Colony size and pupal production

The colony size and pupal production of *Glossina f. fuscipes* are shown in Fig. 1; the colony size increased to 14 131 females and pupal production to 5574 pupae/week in week 52 of 2008. The colony size of *G. pallidipes* reached 1500.

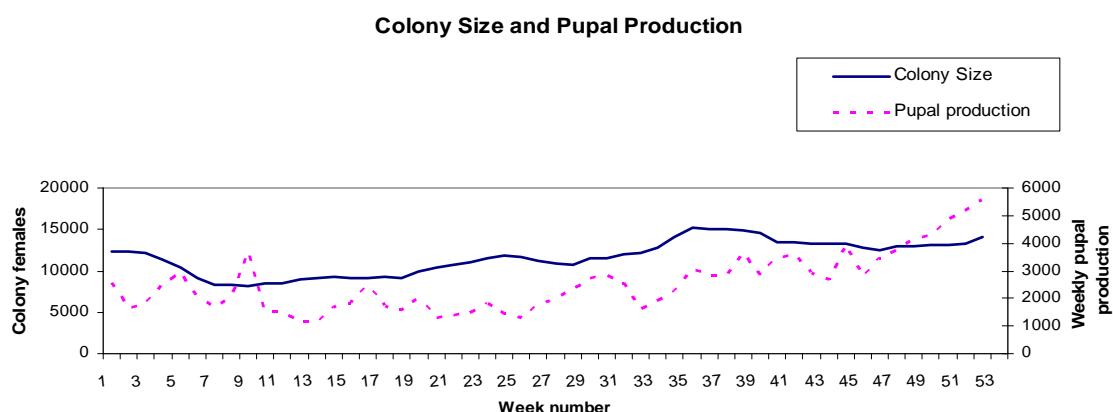


FIG. 1. *G. f. fuscipes* colony size and pupal production.

3.2. Fecundity and mortality

Fecundity of the flies was measured by the number of pupae produced by the initial females every 10 days (P/F/10d). It is influenced by mortality. The mortality rate was low (below 0.5%) for a few weeks, but was above 1% for many weeks in the one-year period shown in Fig. 2.

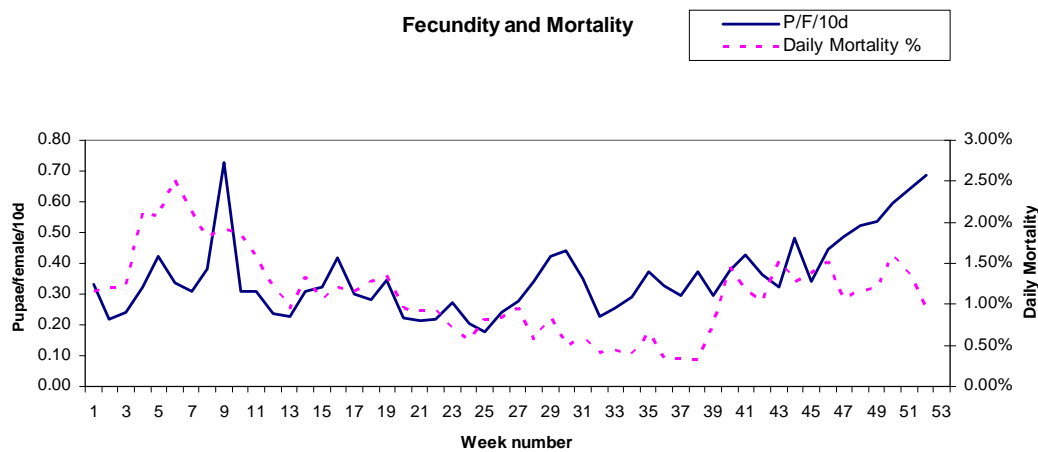


FIG. 2. *G. f. fuscipes* fecundity and mortality.

3.3. Pupae per initial female and productivity

Female productivity was measured as pupae per initial female (PPIF, total number of pupae produced in a given time divided by the number of initial females). The unit ‘PPIF’ is commonly used to assess the health of *Glossina* colonies. Productivity is a function of the current week’s mortality and fecundity (P/f/10d). The formula (written in two ways) for calculating productivity is:

$$\text{productivity} = (2(1-m)^{112} + (1-m^{20})) (1.6023) (f)$$

$$\text{productivity} = (2(1-m)^{112} + 1 - m^{20}) \times 1.6023 \times f$$

where m is daily mortality and f is p/f/10d. Roughly speaking, productivity is the ratio of colony size in three months to present colony size, so a value of 1 means that the colony would be static if this mortality and fecundity persisted indefinitely, greater than 1 the colony would grow and less than 1 the colony would shrink. The reason for introducing this calculation is because the overall measure of colony performance, PPIF, is only available after the unit is finished and so no use for taking corrective action. Productivity is intended to give equivalent information but based on current performance. Unfortunately, it is very sensitive to fluctuations in mortality, so it is not very stable.

The PPIF and productivity tended to increase slightly in the last half of the period shown in Fig. 3; at this time ‘mature mating’ was being practiced.

3.4. Pupal weight

The pupae produced in the colony were weighed individually within 3 days of larviposition. The mean pupal weights in the *G. f. fuscipes* and *G. pallidipes* colonies are shown in Figs 4 and 5, respectively.

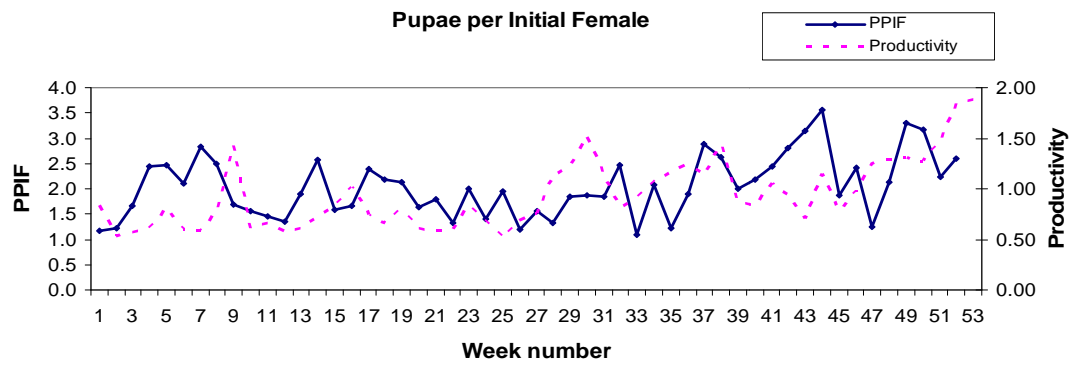


FIG. 3. *G. f. fuscipes* pupae per initial female and productivity.

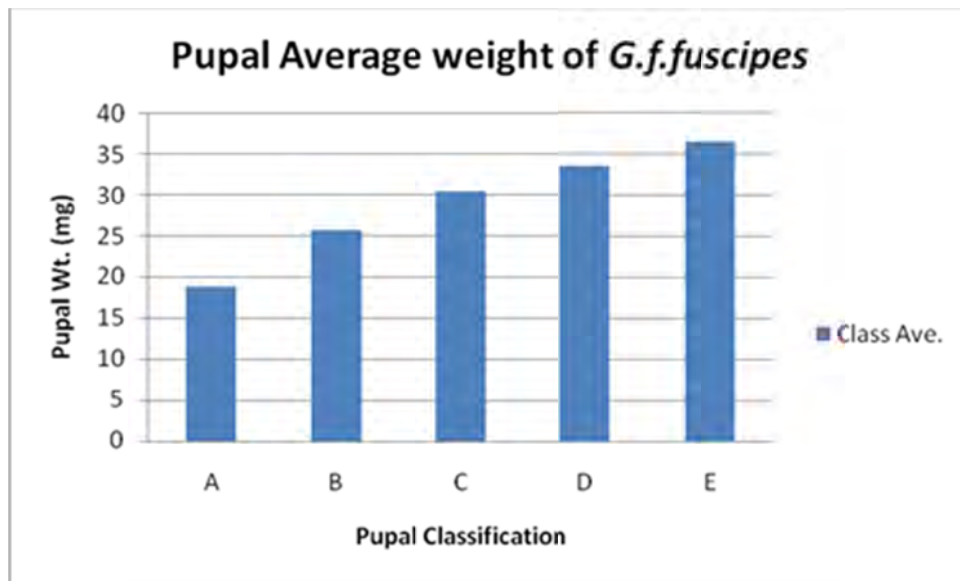


FIG. 4. *G. f. fuscipes* pupal weight.

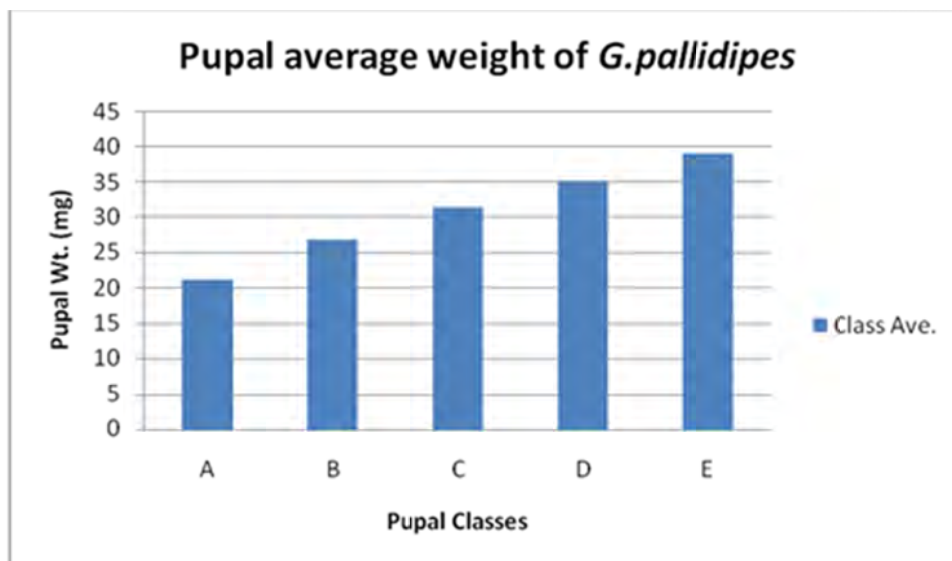


FIG. 5. *G. pallidipes* pupal weight.

3.5. Pupal classes

The pupal classes, A to E, are based on the individual weight of pupae; A is the lowest weight and E the highest. The pupal classes for *G. f. fuscipes* were defined by A = <22 mg, B = 22–28 mg, C = 28.1–32 mg, D = 32.1–36 mg and E = >36 mg. For *G. pallidipes* the pupal classes were defined by A = <23 mg, B = 23–29 mg, C = 29.1–33 mg, D = 33.1–37 mg and E = >37 mg. The pupal classes for *G. f. fuscipes* (Fig. 6) show that numbers in the classes A and E are low, and most of the pupae fall into classes B, C and D; however, the pupal classes for *G. pallidipes* (Fig. 7) show that numbers in classes A and B are low, and most of the pupae fall into classes C, D and E.

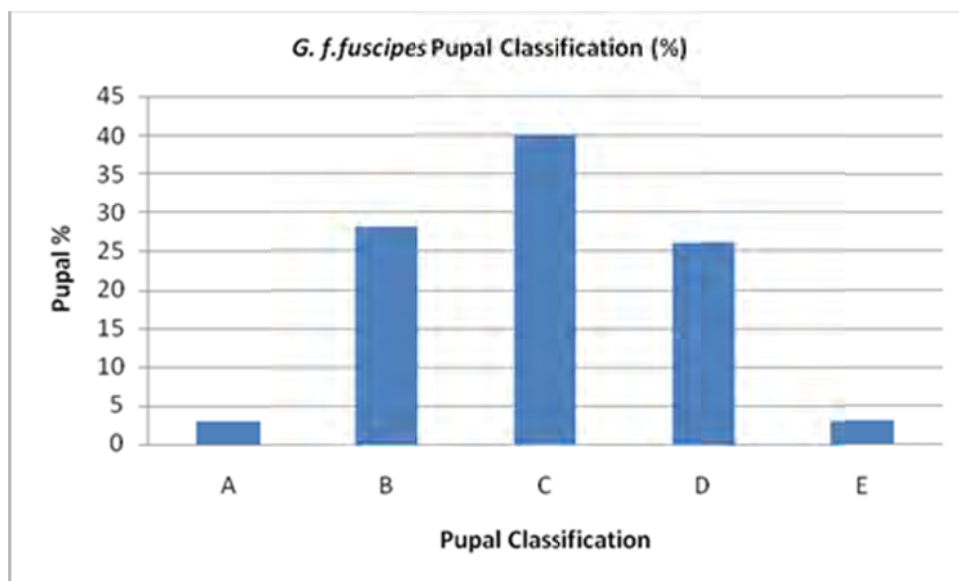


FIG. 6. *G. f. fuscipes* pupal classification.

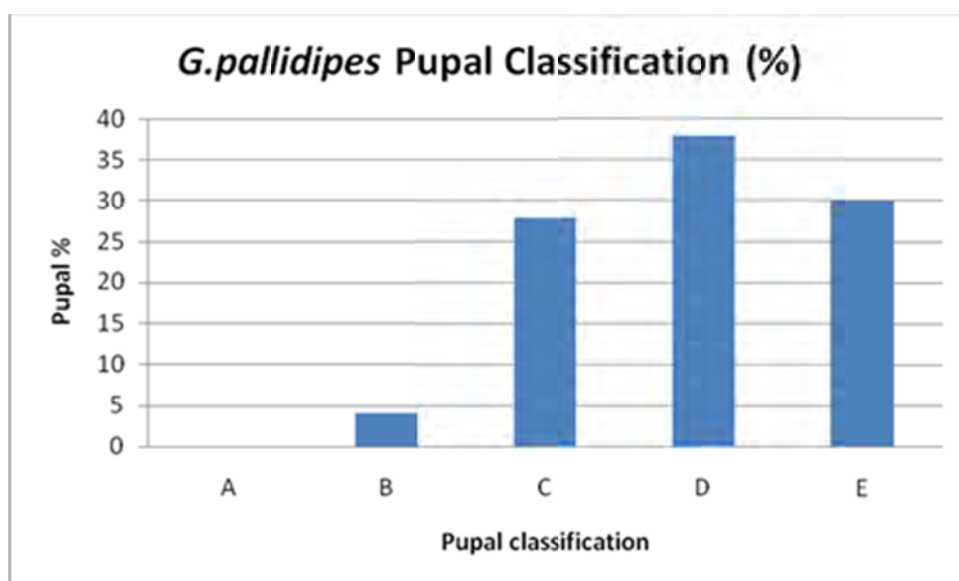


FIG. 7. *G. pallidipes* pupal classification.

4. DISCUSSION

4.1. Mortality rate

Gooding et al. [21] stated that, in a population of breeding females with a stable age distribution, the overall mortality rate should not exceed 1.2% per day. In our colonies the daily mortality was often above 1%, and therefore an assessment of the cause(s) of this mortality should be investigated. Langley and Pimley [16] found that, when female flies were mated on day 3 of adult life with 10–15 days old males from a goat-fed colony, their longevity was about 60 days. However, in the case of *G. f. fuscipes*, a high survival rate was obtained in our colony when 3 days old females were mated with 7–10 days old males.

4.2. PPIF

The data on PPIF for *G. f. fuscipes* and *G. pallidipes* range from 1.1 to 3.6, and 0.4 to 3.1, respectively. Langley and Pimley [16] obtained results ranging from 4–7 using different food sources. Mews [4] reported 7 pupae per initial female when flies were fed on cow blood, and 6.8–7.8 when fed on rabbits. According to Jordan [17], values can vary greatly, from 1.6 to 14. The PPIF required for the establishment of a colony should be ≥ 3 , and thus the values obtained for both colonies were usually rather low. Fecundity was usually also low. As a result, the growth of the colonies overall was less than satisfactory.

4.3. Pupal weight

Kettle [18] found that the weight of a pupa depends on the amount of blood taken by a female during pregnancy, with a highly significant correlation between puparial weight and quality of blood ingested. These results were confirmed by Jordan [17] who found that heavier pupae were produced by well-nourished females. Similar results were reported by Langley and Pimley [16] who found a weight of 26–28 mg from in vitro feeding, but when fed on rabbits the weight was 30–32 mg [19]. Other authors reported 25.2 mg on cow blood [20] and 25.4–30.5 mg on rabbits [4]. In an experiment in which flies were fed on goats, Jordan [17] found that the mean weight of puparia varied from 27.3 to 32 mg. The present values obtained from in vitro feeding of the colony (Figs 4 and 5) are in line with other in vitro feeding results [3].

4.4. Pupal classes

Puparial class is a good overall quality indicator of the effectiveness of colony maintenance; each weight class can be defined using a size-sorting machine [21]. The mean pupal weights should approximate the values developed by Zegler and Russ [22, 21], and no more than 10 % of the puparia should be in weight class A [23]. This was always the case in our colonies (Figs 6 and 7). In our work the pupal classes were determined by weight rather than by size, so there could be a slight variation in our comparisons. These are the normal distribution, as Malele and Parker [24] showed in the distribution of pupal classes of *G. austeni*.

ACKNOWLEDGEMENTS

Financial support from the IAEA through a Coordinated Research Project is gratefully acknowledged. We thank the ESTA and STEP for support and permission to do this work. We also thank all staff members of the Kaliti Center for their cooperation.

REFERENCES

- [1] WILLETT, K.C., Trypanosomiasis and the tsetse fly problem in Africa, *Annu. Rev. Entomol.* **8** (1963) 197–214.
- [2] RAWLINGS, P., CEESAY, M.L., WACHER, T.J., SNOW, W.F., The distribution of the tsetse flies *Glossina morsitans morsitans* and *G. palpalis gambiensis* (Diptera: Glossinidae) in The Gambia and the application of survey results and trypanosomiasis control, *Bull. Entomol. Res.* **83** (1993) 625–631.
- [3] BAUER, B., WETZEL, H., A new membrane for feeding *Glossina morsitans* Westw. (Diptera, Glossinidae), *Bull. Entomol. Res.* **65** (1976) 563–565.
- [4] MEWS, A.R., BAUMGARTNER, H., LUGER, D., OFFORI, E.D., Colonization of *Glossina morsitans morsitans* Westw. (Diptera, Glossinidae) in the laboratory using *in vitro* feeding techniques, *Bull. Entomol. Res.* **65** (1976) 631–642.
- [5] MEWS, A.R., LANGLEY, P.A., PIMLEY, R.W., FLOOD, M.E.T., Large scale rearing of tsetse flies (*Glossina* spp.) in the absence of a living host, *Bull. Entomol. Res.* **67** (1977) 119–128.
- [6] WETZEL, H., LUGER, D., *In vitro* feeding in the rearing of tsetse flies (*Glossina m. morsitans* and *Glossina p. palpalis* (Diptera: Glossinidae), *Tropenmed. Parasitol.* **29** (1978) 239–251.
- [7] FELDMANN, U., “Guidelines for the rearing of tsetse flies using the membrane feeding technique”, *Proceedings: Techniques of Insect Rearing for the Development of Integrated Pest and Vector Management Strategies*, Vol. 2, (OCHIENG’-ODERO, J.P.R., Ed), ICIPE Science Press, Nairobi (1994) 449–471.
- [8] LEEGWATER-VAN DER LINDEN, M.E., “Recent advances in the rearing of *Glossina pallidipes* Austen”, *Isotope and Radiation Research on Animal Diseases and their Vectors*, *Proceedings of the International Symposium on the Use of Isotopes for Research and Control of Vectors of Animal Diseases, Host Pathogen Relationships and the Environmental Impact of Control Procedures*, IAEA/FAO, Vienna, 7–11 May 1979, STI/PUB/525, IAEA, Vienna (1980) 413–422.
- [9] JAENSON, T.G.T., TAKKEN, W., Rearing of *Glossina pallidipes* using membrane-feeding technology, *Entomol. Exp. Appl.* **27** (1980) 102–104.
- [10] VREYSEN, M.J.B., et al., *Glossina austeni* (Diptera: Glossinidae) eradicated on the island of Unguja, Zanzibar, using the sterile insect technique, *J. Econ. Entomol.* **93** (2000) 123–135.
- [11] DYCK, V.A., et al., Monitoring the incidence of trypanosomosis in cattle during the release of sterilized tsetse flies on Unguja Island, Zanzibar, *Revue Élev. Méd. vét. Pays trop.* **53** (2000) 239–243.
- [12] POLITZAR, H., MEROT, P., BRANDL, F.E., Experimental aerial releases of sterile males of *Glossina palpalis gambiensis* and *Glossina tachinoides* in a biological control operation, *Revue Élev. Méd. vét. Pays trop.* **37** (1984) 198–202.
- [13] OLADUNMADE, M.A., et al., “Eradication of *Glossina palpalis palpalis* (Robineau-Desvoidy) (Diptera: Glossinidae) from agropastoral land in central Nigeria by means of the sterile insect technique”, *Sterile Insect Technique for Tsetse Control and Eradication*, *Proceedings of Final Research Co-ordination Meeting*, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Vom, Nigeria, 6–10 June 1988, STI/PUB/830, IAEA, Vienna (1990) 5–23.
- [14] FELDMANN, U., HENDRICH, J., Integrating the sterile insect technique as a key component of area-wide tsetse and trypanosomiasis intervention, PAAT Technical and Scientific Series 3, FAO, Rome (2001).
<http://www.fao.org/DOCREP/004/Y2022E/Y2022E00.HTM>

- [15] FELDMANN, U., “Some quality control parameters used in the rearing of tsetse flies”, Proceedings: Techniques of Insect Rearing for the Development of Integrated Pest and Vector Management Strategies, Vol. 1, (OCHIENG’-ODERO, J.P.R., Ed), ICIPE Science Press, Nairobi (1994) 13–29.
- [16] LANGLEY, P.A., PIMLEY, R.W., Quantitative aspects of reproduction and larval nutrition in *G. morsitans morsitans* Westw. (Diptera, Glossinidae) fed *in vitro*, Bull. Entomol. Res. **65** (1975) 129–142.
- [17] JORDAN, A.M., Forty generations of inbreeding of *G. morsitans morsitans* Westwood (Diptera: Glossinidae), Bull. Entomol. Res. **70** (1980) 557–562.
- [18] KETTLE, D.S., Medical and Veterinary Entomology, 2nd ed, CABI Publishing, Wallingford (1995).
- [19] JORDAN, A.M., CURTIS, C.F., Productivity of *G. austeni* Newstead maintained on lop-eared rabbits, Bull. Entomol. Res. **58** (1968) 399–410.
- [20] DEAN, G.J.W., PAGOT, J., WILLIAMSON, B.R., Survival and reproduction of *Glossina morsitans* Westw. in different types of cages exposed to variable and constant climatic conditions, Bull. Entomol. Res. **58** (1969) 773–785.
- [21] GOODING, R.H., FELDMANN, U., ROBINSON, A.S., “Care and maintenance of tsetse colonies”, Molecular Biology of Insect Disease Vectors: A Methods Manual, (CRAMPTON, J.M., BEARD, C.B., LOUIS, C., Eds), Chapman and Hall, London (1997) 41–55.
- [22] ZELGER, R., RUSS, K., Puppentrennmaschine mit zwei Walzenpaaren, Z. Angew. Zool. **63** (1976) 257.
- [23] (FAO/IAEA) Food and Agriculture Organization of the United Nations/International Atomic Energy Agency, FAO/IAEA standard operating procedures for mass-rearing tsetse flies, IAEA, Vienna, Austria (2006).
http://www-naweb.iaea.org/nafa/ipc/public/Tsetse_Rearing_SOP_web.pdf
- [24] MALELE, I.I., PARKER, A.G., Mating age of *Glossina austeni* Newstead, Acta Trop. **72** (1999) 319–324.

RESEARCH RESULTS FROM THE TSETSE FLY MASS REARING LABORATORY AT CIRDES AND THEIR APPLICATION DURING 2003–2008

A.Z. BANCE, I. KABORE, I. SIDIBE, J.B. RAYAISSE, Z. BENGALY

Centre International de Recherche-Développement sur l'Élevage en Zone Subhumide,
N°559, rue 5-31 angle avenue du Gouverneur Louveau 01, B.P. 454,
Bobo Dioulasso 01, Burkina Faso

Abstract

Some reduction in feeding frequency was possible. *Glossina morsitans submorsitans* Newstead easily tolerated a reduction from six to three days per week (Monday, Wednesday, Friday) without reducing female productivity. However, *Glossina palpalis gambiensis* Vanderplank and *Glossina tachinoides* Westwood tolerated a reduction from six to only four days per week. Even without ATP, blood that had been frozen enabled tsetse flies to achieve a high level of productivity. Using ATP unnecessarily increased the cost of producing *Glossina* by about 0.027 Euros per fly. Regarding handling tsetse flies, the procedure of day-zero-mating, which aims to minimize fly manipulation with one's hands, was applied to both *G. m. submorsitans* and *G. p. gambiensis*. The results showed that using emerging males (zero days old) for mating has no negative effect on female productivity. The optimal fly density in each new TPU-3 cage was 64 females and 16 males, i.e. 80 flies per box for all three species. Regarding applying the sterile insect technique (SIT), experiments with irradiated males of *G. p. gambiensis* showed that the percentage sterility induced to inseminated females was 89.8, 94.3 and 95.2% at doses of 102, 112 and 122 Gy, respectively. Biological parameters linked to sexual activity, e.g. insemination rate, spermathecal filling rate and survival rate, were not changed by the different irradiation doses. Finally, validation of the TPU-3 holding/feeding system showed that it reduced the feeding time considerably (by 80%) when compared with the old manual system. Productivity parameters of *G. p. gambiensis* using the TPU-3 system were: pupal weight 28.1 ± 0.6 mg and adult emergence $89.0 \pm 1.7\%$.

1. INTRODUCTION

Three species of tsetse flies are mass reared at CIRDES: *Glossina morsitans submorsitans* Newstead, *Glossina palpalis gambiensis* Vanderplank and *Glossina tachinoides* Westwood. Research on these species has the following objectives: to understand their biology, physiology and ecology, to improve methods of applying the sterile insect technique (SIT) and to provide inexpensive sterile males for the SIT. Since 2003 CIRDES has conducted tests to improve and harmonize the quality control of mass reared tsetse flies. These tests involved feeding tsetse flies (feeding frequency, use of frozen blood, evaluation of the importance of ATP in feeding behaviour), determining the optimum density of tsetse flies in TPU-3 cages, determining the irradiation dose of *Glossina* males, assessing the competitiveness of irradiated males, and validating new rearing techniques using the TPU-3 holding/feeding system [1].

2. MATERIALS AND METHODS

2.1. Feeding

2.1.1. Frequency of feeding *Glossina*

In the past, tsetse flies were fed six days out of seven [2]. Tests were conducted to reduce production costs but maintain the productivity and quality of the flies (Table 1).

TABLE 1. PROTOCOL FOR STUDYING FEEDING FREQUENCY

Feeding frequency	Species	Females/cage (no.)	Replicates (no.)	Total females (no.)	Parameters measured
3 days/7	<i>G. p. gambiensis</i>	150	4	600	• mortality
	<i>G. m. submorsitans</i>	150 and 100	3	400	• larviposition date
4 days/7	<i>G. p. gambiensis</i>	150	4	600	• weight of pupae
	<i>G. m. submorsitans</i>	150 and 100	3	400	• abortion rate
	<i>G. tachinoides</i>	150 and 100	2	250	• productivity
5 days/7	<i>G. p. gambiensis</i>	150	4	600	• hatching rate
	<i>G. tachinoides</i>	150 and 100	2	250	
Control (6 days/7)	<i>G. p. gambiensis</i>	150	1	150	
	<i>G. m. submorsitans</i>	150	1	150	
	<i>G. tachinoides</i>	150	1	150	

2.1.2. Using frozen blood to feed Glossina

Two treatments of 900 females of *G. p. gambiensis* with 6 replicates each were made. These two treatments were randomized with two types of blood:

Blood 1: frozen blood without ATP or glucose (ATP- and Glu-)

Blood 2: frozen blood with ATP and glucose (ATP+ and Glu+)

These females, at three days old, were mated with some six days old males prepared beforehand, and fed with these two types of blood.

A control, flies fed with fresh blood with ATP and glucose (Blood 0), was prepared.

2.1.3. Evaluating the importance of ATP in feeding Glossina

Five treatments with four replications of three females per replication of were carried out with individual cages. These treatments done randomly were assigned to this various types of blood. The five types of frozen blood constituted were:

- Blood 1 (bl 1) - bovine blood with added ATP and glucose before gamma irradiation
- Blood 2 (bl 2) - bovine blood with added glucose before, and with added ATP after, gamma irradiation
- Blood 3 (bl 3) - bovine blood with added glucose but without ATP
- Blood 4 (bl 4) - bovine blood without glucose or ATP
- Pig blood (pb) - with glucose and ATP

In the beginning, the weight of the females at adult emergence was determined by weighing the cages before and after inserting the flies. The weight of absorbed blood was determined by weighing a cage with flies before and after feeding. To determine average weight, it was calculated by the quotient of the difference in weight of the females at the end and at the beginning of the experiment and the number of days.

2.2. Optimum density in the new cages of TPU-3

Five replicated treatments of 60, 70, 80, 100 and 120 flies of the three species were made, of which 45, 53, 60, 75 and 90, respectively, were females. Flies in these treatments were fed regularly 6 days per week (6 days/7).

Five replicated treatments of 60, 70, 80, 100 and 120 flies of the three species were made, of which 45, 53, 60, 75 and 90, respectively, were females. Flies in these treatments were fed regularly 6 days per week (6 days/7).

2.3. Reducing the manual mating process by using zero days old males (*G. m. submorsitans* and *G. p. gambiensis*)

Within the framework of automating industrial production, the objective of this experiment was to reduce labour costs by using zero days old males for mating instead of males already a few days old. During the first two days of adult emergence, many more females than males emerge; the proportion of males increases only on the third and fourth day [3]. Traditionally, mating was a laborious process; emerged flies were sexed and females were mated with six- or seven days old males. The following experiment tested this objective (Table 2).

TABLE 2. SUMMARY OF MATING PROTOCOL AT ‘ZERO DAYS’

Species	Treatment	Control
	30 females/cage (camembert)	30 females/cage (camembert)
<i>G. m. submorsitans</i>	180 females (2 days old) X 60 males (0 days old)	90 females (2 days old) X 30 males (6 days old)
<i>G. p. gambiensis</i>	240 females (2 days old) X 80 males (0 days old)	120 females (2 days old) X 40 males (6 days old)

2.4. Tests within the framework of the SIT

2.4.1. Determining the effective irradiation dosage of *Glossina* males

By evaluating the effects of irradiation on the biological parameters of *G. p. gambiensis*, this experiment aims at ensuring the quality of the sterile males produced for the releases in Mali. Four batches of 90 males each, of which three were exposed to radiation at various doses, were mated with six consecutive batches of females at 48-hours interval at a ratio of 1:1. On the fourth day after flies emerged, they were irradiated with a Cesium 137 source at doses of 102, 112 and 122 Gy, respectively. Flies in the fourth batch, the control, were not irradiated.

On the sixth day, the 360 males were mated with 360 two days old females. After 48 hours, they were withdrawn using a tube and immediately mated again with new two days old females at a ratio of 1:1. This process continued until the 6th successive mating for each batch of males. The number of females to be mated was always identical to the number of males that survived the previous mating. At the end of the sixth consecutive coupling, the males were separated from the females and put in the male Roubaud cages to observe their survival rate. After 45 days of observation, the females were dissected to determine the degree of insemination and the rate of spermathecal filling [4, 5]. The conditions of maintenance and feeding of the flies were identical to those of the fly colonies.

2.4.2. Competitiveness of *G. p. gambiensis* irradiated males

A test of compatibility between the stock of *G. p. gambiensis* colonized at CIRDES for more than 20 years and that coming from the project zone in Mali has already been done in collaboration with the IAEA [4]. Now a test of competitiveness was made to ensure the quality of sterile males produced at CIRDES.

The wild stock of *G. p. gambiensis* originated in Tienfala-Baguinéda, Mali. The flies were captured at the edge of the Niger River and at two of its branches in the south of Bamako, Mali, in December 2001. For each of the 10 release sessions, 40 *Glossina* males from Mali and 40 from Burkina Faso were collected on the same day. Females of Mali stock were collected three or four days later. The release took place in the 'field cage'. The observations and parameters of follow-up were the same. However, different from the test of compatibility, the males and females of Mali stock were fed on goats from emergence to the day before release. Males from CIRDES were the only ones marked with the acrylic paint, fed on artificial membranes, and then irradiated with 112 Gy on the day before release.

2.5. Vectorial competence of *G. p. gambiensis* Tienfala-Baguinéda stock (Mali)

The vectorial competence, with respect to *Trypanosoma vivax* and *Trypanosoma congolense*, of the stock of *G. p. gambiensis* collected in Tienfala-Baguinéda was evaluated in comparison with *Glossina* reared at CIRDES.

The trypanosomes used were:

- *T. congolense* (savannah type), isolated in Dinderesso, Burkina Faso. For the experiment, the duration of the cycle of this parasite is 25 days.
- *T. vivax*, isolated in Folonzo, Burkina Faso; duration of the cycle of this parasite is 15 days.
- *T. vivax*, stock of Tienfala-Baguinéda, isolated in Mali and preserved at CIRDES. The duration of the cycle of this parasite is 15 days.

The infecting animals were two goats for *T. vivax* of Tienfala-Baguinéda and *T. vivax* of Folonzo and two rabbits for *T. congolense*. These animals were experimentally infected with the various stocks of trypanosomes.

Batches of 60 flies per sex and stock were infected by *T. vivax* stock of Tienfala-Baguinéda:

Batch V1: 60 males *G. p. gambiensis* in Tienfala-Baguinéda

Batch V2: 60 females *G. p. gambiensis* in Tienfala-Baguinéda

Batch V3: 60 males *G. p. gambiensis* in CIRDES

Batch V4: 60 females *G. p. gambiensis* in CIRDES

For feeding, cages of *Glossina* that had fasted for approximately 48 hours were put for 10 minutes on the backs of animals that had been shaved beforehand.

2.6. Validation of TPU-3 holding/feeding system

The new TPU-3 holding/rearing system and the former rearing system using mobile carts were compared (Table 3). Observations on the cost of labour and the productivity of flies in these two systems were made.

The two systems used the same blood diet as the colonies in the insectary. Mating of the females was done 3 days after emergence with 6 days old males at the ratio of one male to three females.

TABLE 3. VALIDATION PROTOCOL FOR REARING SYSTEMS

Rearing system	<i>G. p. gambiensis</i>		<i>G. m. submorsitans</i>	
	Replications (no.)	Females/rep. (no.)	Replications (no.)	Females/rep. (no.)
Former system	4	400	5	600
TPU-3 system	4	360	5	576

3. RESULTS

3.1. Feeding

3.1.1. Frequency of feeding *Glossina*

By 64 days, for *G. p. gambiensis*, the data analysis did not reveal any significant differences in the biological parameters due to changing the feeding frequency (Table 4). Evidently, it is sufficient to feed this species three times per week. However, given the trends in some of the parameters, if further tests were conducted some improvement in fly quality from feeding more than three times per week might be shown.

TABLE 4. EFFECT OF FEEDING FREQUENCY ON *G. P. GAMBIENSIS*

Feeding frequency (no./wk)	Parameter ^a				
	Mortality (%)	Mean pupal production/ female/day (no.)	Mean pupal weight (mg)	Abortions (%)	Adult emergence (%)
3	1.5±0.1	0.04±0.002 a	23.7±0.87 a	0.8±0.31 a	90.3±1.31 a
4	1.2±0.1	0.04±0.007 a	24.6±0.95 a	0.6±0.38 a	89.9±1.79 a
5	1.2±0.1	0.04±0.004 a	24.2±0.20 a	0.6±0.87 a	88.8±2.31 a
6 (control)	1.2	0.05 a	26.98 a	0.88 a	93.18 a

^a Means (±SD) in a column followed by the same letter are not significantly different ($P<0.05$), Duncan's Multiple Range Test (DMRT).

Also, for *G. m. submorsitans*, the data analysis did not reveal any significant differences in the biological parameters due to changing the feeding frequency (Table 5). Evidently this species is suited to a being fed three times per week.

As for *G. tachinoides*, the females had a daily mortality rate of 1.2±0.2, 1.09±0.5 and 0.6 % in 64 days, and the production of pupae per female per day was 0.04±0.004, 0.03±0.005 and 0.03 for females fed four, five and six times per week, respectively. Emergence was higher than 91%. It may be acceptable to feed *G. tachinoides* four times per week.

For males, at 30 days the death rate was 83.6 ± 6.6 , 65.5 ± 9.1 and 70.9% , respectively, for bloods 1, 2 and 0 (control). However, female daily mortality was low (Table 6). Even though blood 2 appeared to produce more pupae than the other two diets, there were no significant differences in pupal production on the three types of blood (Table 6). Freezing the blood before use appears to be favourable for mass rearing.

TABLE 5. EFFECT OF FEEDING FREQUENCY ON *G. M. SUBMORSITANS*

Feeding frequency (no./wk)	Parameter ^a				
	Mortality (%)	Mean pupal production/female/day (no.)	Mean pupal weight (mg)	Abortions (%)	Adult emergence (%)
3	0.8 ± 0.50	0.06 ± 0.01 a	30.1 ± 1.82 a	1.2 ± 0.08 a	91.7 ± 6.23 a
4	1.0 ± 0.47	0.06 ± 0.00 a	30.1 ± 0.95 a	2.2 ± 0.35 a	95.2 ± 2.80 a
6 (control)	0.5	0.1 a	30.3 ± 1.3 a	1.0 a	97.1 a

^a Means (\pm SD) in a column followed by the same letter are not significantly different ($P < 0.05$), Duncan's Multiple Range Test (DMRT).

TABLE 6. EFFECT OF FEEDING FROZEN BLOOD TO *GLOSSINA*

Blood ^a fed to flies	Parameter				
	Mortality (%)	Mean pupal production/female/day ^b (no.)	Mean pupal weight (mg)	Abortions (%)	Adult emergence (%)
Blood 1	1.1 ± 0.2	0.05 ± 0.008 a	24.3 ± 1.0	1.7 ± 0.89	91.6 ± 3.1
Blood 2	1.1 ± 0.1	0.06 ± 0.005 a	24.9 ± 1.3	1.0 ± 0.71	89.3 ± 5.2
Blood 0 (control)	1.2	0.05 a	27.0	0.9	93.2

^a Blood 1 = frozen blood without ATP or glucose; Blood 2 = frozen blood with ATP and glucose; Blood 0 = fresh blood with ATP and glucose (control).

^b Means (\pm SD) in a column followed by the same letter are not significantly different ($P < 0.05$), Duncan's Multiple Range Test (DMRT).

3.1.3. Evaluating the importance of ATP in feeding Glossina

The weight of blood absorbed by females of *G. p. gambiensis* was 12.2 ± 3.3 mg, 7.2 ± 4.0 mg, 5.9 ± 4.3 mg, 5.7 ± 5.1 mg and 15.8 ± 11.2 mg on the day following their emergence, and 19.2 ± 6.0 mg, 19.5 ± 4.9 mg, 16.7 ± 10.5 mg, 17.7 ± 6.1 mg and 26.1 ± 15.3 mg after a diet of 24 hours on bloods bl 1, bl 2, bl 3, bl 4 and pb, respectively. Evidently more blood was taken up if it contained ATP. The amount of blood absorbed increased with the number of days of feeding as well as with the presence of ATP in the blood.

As for average weight gained, it was higher on blood without ATP and glucose (bl 3 and bl 4). In addition, these are the same females that had the highest weights at emergence, 17.0 ± 1.7 mg and 17.3 ± 2.1 mg, compared with 15.5 ± 0.4 mg, 16.7 ± 1.8 mg and 14.7 ± 1.3 mg for the others (bl 1, bl 2 and pb).

3.2. Optimum density in the new cages of TPU-3

For *G. p. gambiensis*, the high density of 120 flies per cage (90 females) appeared to cause high mortality, low pupal production and low adult emergence (Table 7). Pupal weight was similar in all treatments.

TABLE 7. EFFECT OF FLY DENSITY IN TPU-3 CAGES ON *G. P. GAMBIENSIS*

Treatment	Flies (no./cage)			Parameter			
	Females	Males	Total	Daily mortality (%)	Mean pupal production/female/day (no.)	Mean pupal weight (mg)	Adult emergence (%)
1	45	15	60	0.9±0.3	0.08±0.01	27.8±1.2	91.7±2.9
2	53	17	70	0.9±0.3	0.08±0.02	26.7±1.6	93.5±2.1
3	64	16	80	0.7±0.2	0.07±0.00	25.3±1.2	94.3±3.0
4	75	25	100	1.0±0.2	0.05±0.02	25.4±3.7	95.7±1.6
5	90	30	120	1.4±0.3	0.02±0.03	26.6±1.6	79.0±20.1
Control	150	50	200	1.2	0.05	27.0	93.2

For *G. m. submorsitans*, the mortality rate was 1.2±0.2, 1.2±0.2, 1.3±0.3, 1.4±0.3, 1.3±0.3 and 0.5, and pupal production 0.1±0.02, 0.1±0.02, 0.02±0.02, 0.05±0.01, 0.03±0.04 and 0.07, in cages with 60, 70, 80, 100 and 120 flies, respectively. The mean weight of pupae was higher than 29 mg except in cages with 90 females where 28.11±1.30 mg was observed.

For *G. tachinoides*, the mortality rate was 0.7±0.1, 0.8±0.1, 1.3±0.3, 1.0, 1.2 and 0.6, and pupal production 0.05±0.01, 0.05±0.02, 0.03±0.01, 0.06, 0.03 and 0.03, in cages with 60, 70, 80, 100 and 120 flies, respectively. The mean weight of pupae was higher than 16 mg except in cages with 90 females where 15.98 mg was observed.

Based on these observations, it is concluded that the optimum density is 80 flies/cage (64 females and 16 males, i.e. 4 females for every male).

3.3. Reducing the manual mating process by using zero days old males (*G. m. submorsitans* and *G. p. gambiensis*)

Both tsetse species showed no significant difference between using zero days old and six days old males (Table 8).

3.4. Tests within the framework of the SIT

3.4.1. Determining the effective irradiation dosage of *G. p. gambiensis* males

In the presence of two days old virgin females, males mated immediately. Mating occurred 24 to 48 hours after putting males and females together throughout the six consecutive matings. Overall, 467 females were mated with males of batch 1 irradiated at 102 Gy, 474 females with males of batch 2 irradiated with 112 Gy, 493 females with males of batch 3 irradiated with 122 Gy, and 453 females with control males (Table 9).

At the end of the six consecutive matings, i.e. males were then 16 days old, survival of batch 1 was 71.1%, batch 2 was 76.7%, batch 3 was 82.2% and the control was 74.4%. These rates decreased to between 25.6 and 15.6% until males were 45 days old

The degree of sterility achieved during six consecutive matings was between 89.8 and 95.2% (Table 10).

The degree of sterility achieved from irradiation at 102–122 Gy was 90–95% (Table 11).

TABLE 8. FLY PRODUCTIVITY WHEN MATED WITH ZERO-DAYS-OLD MALES

Treatment	Parameter			
	Pupal production ^a (no.)	Abortions ^b (no.)	Mean pupal weight (mg)	Adult emergence (%)
<i>G. m. submorsitans</i> control	0.6	0.01	30.3	98.4
<i>G. m. submorsitans</i> males 0 days old	0.6	0.02	30.6	97.7
<i>G. p. gambiensis</i> control	0.8	0.02	25.1	97.6
<i>G. p. gambiensis</i> males 0 days old	0.8	0.01	24.5	98.8

^a Production of pupae by initial females.

^b Number of abortions by initial females.

TABLE 9. SURVIVAL (%) OF *G. P. GAMBIENSIS* MALES AFTER IRRADIATION

Treatment	Days after irradiation					
	7	14	21	28	35	45
1 – Irradiated at 102 Gy	97.8	78.9	64.4	55.6	22.2	15.6
2 – Irradiated at 112 Gy	98.9	82.2	71.1	54.4	25.6	25.6
3 – Irradiated at 122 Gy	98.9	87.8	62.2	55.6	23.3	21.1
Control (not irradiated)	98.9	77.8	70	62.2	32.2	20

TABLE 10. DEGREE OF STERILITY (%) OF *G. P. GAMBIENSIS* MALES AFTER IRRADIATION

Treatment	Mating number (consecutive, in sequence)						Mean
	1st	2nd	3rd	4th	5th	6th	
1 – Irradiated at 102 Gy	83.8	95.1	91.4	87.7	92.5	86.0	89.8
2 – Irradiated at 112 Gy	91.1	95.1	94.5	92.8	93.9	96.9	94.3
3 – Irradiated at 122 Gy	96.4	96.3	94.9	91.8	96.2	92.2	95.2
Control (not irradiated)	0	0	0	0	0	0	0

Irradiated and non-irradiated males had good insemination capacity and a good aptitude to fill the spermathecae of the females in the first three matings. From the first to third mating the level of insemination was equal to or higher than 94.2%, but at the fourth mating it decreased below 90%. Differences among irradiated and control flies became significant at the fifth mating when the insemination rate was 84.8% for the control but it ranged between 60.7 and 67.2% for the irradiated males. However, at the sixth mating, the insemination rate was 76% or higher, whatever the treatment (Table 12).

The mean level of filling of the female spermathecae was between 72.9% and 75.6% (Table 13).

In summary, there were no significant differences in survival and sexual performance among irradiated males and non- irradiated controls.

TABLE 11. EFFECT ON FEMALES IF MATED TO IRRADIATED MALES OF *G. P. GAMBIENSIS*

Treatment to males	Parameter				
	Pupal production (no.)	Mean weight of pupae (mg)	Degree of sterility (%)	Daily mortality (%)	Adult emergence from produced pupae (%) (no. emerged/ total number)
1 – Irradiated at 102 Gy	141	22.1	89.8	1.9	85.8 (121/141)
2 – Irradiated at 112 Gy	81	22.1	94.3	1.7	84.0 (68/81)
3 – Irradiated at 122 Gy	70	22.2	95.2	1.8	92.9 (65/70)
Control (not irradiated)	1346	24.4	0	1.8	97.3 (1309/1346)

TABLE 12. LEVEL OF FEMALE INSEMINATION (%) BY IRRADIATED MALES OF *G. P. GAMBIENSIS*

Treatment	Mating number (consecutive, in sequence)						Mean
	1st	2nd	3rd	4th	5th	6th	
1 – Irradiated at 102 Gy	98.4	98.4	98.6	86.8	60.7	76	86.5
2 – Irradiated at 112 Gy	100	98.6	94.7	86.2	67.2	85.5	88.7
3 – Irradiated at 122 Gy	100	96.4	94.2	89.7	63.2	78.4	87.0
Control (not irradiated)	100	100	96.4	85.7	84.8	76.4	90.5

TABLE 13. LEVEL OF FILLING OF FEMALE SPERMATHECAE (%) BY IRRADIATED MALES OF *G. P. GAMBIENSIS*

Treatment	Mating number (consecutive, in sequence)						Mean
	1st	2nd	3rd	4th	5th	6th	
1 – Irradiated at 102 Gy	92.7	92.7	94.6	68.2	39.8	47	73.7
2 – Irradiated at 112 Gy	95.8	95.6	86.5	71.8	35.4	49	74.0
3 – Irradiated at 122 Gy	89.0	92.9	86.8	81.5	48.7	56.2	75.6
Control (not irradiated)	97.8	92.1	86.4	64.1	57.4	47.1	72.9

3.4.2. Competitiveness of *G. p. gambiensis* irradiated males

The index related to mating was 0.51 for irradiated males and 0.50 for wild flies (Table 14), indicating that the competition is balanced [6].

The mean level of female insemination was 88.7%; the level of insemination achieved by the irradiated and non-irradiated males was 89.7% and 87.5%, respectively. The mean mating duration of irradiated and non-irradiated males was the same (Table 15).

Ultimately, in the test of competitiveness the irradiated males had a sexual behaviour similar to that of wild males in the field, and there was no sexual incompatibility between the stock of *G. p. gambiensis* produced industrially and the wild stock. Moreover, the time before mating the duration of mating for, and the filling of spermathecae by, irradiated males were comparable with those of wild males.

TABLE 14. MATING INDICES IN THE COMPETITIVENESS TEST

Replication (no.)	Index relating to mating		Mating propensity	Mating performance (Irradiated vs non-irradiated)
	Irradiated	Non- irradiated		
1	0.13	0.88	0.27	-0.094
2	0.64	0.36	0.47	+0.020
3	0.50	0.50	0.36	0.000
4	0.62	0.38	0.70	+0.011
5	0.52	0.48	0.90	+0.001
6	0.56	0.44	0.53	+0.008
7	0.56	0.44	0.30	+0.012
8	0.77	0.23	0.43	+0.041
9	0.47	0.53	0.50	-0.004
10	0.29	0.71	0.71	-0.024
Mean	0.51±0.06	0.50±0.06	0.52±0.06	

TABLE 15. MATING PARAMETERS IN THE MALE COMPETITIVENESS TEST

	Time before mating (minutes)		Mating duration (minutes)		Filling of spermathecae (%)	
	Irradiated	Non-irradiated	Irradiated	Non-irradiated	Irradiated	Non-irradiated
Max.	124.8	117.2	73.8	66.6	100.0	100.00
Min.	52.0	45.7	29.8	37.0	22.5	34.0
Mean	88.7±8.4	78.5±8.7	49.5±4.6	49.8±2.8	76.7±7.2	74.7±6.4

3.5. Vectorial competence of *G. p. gambiensis* Tienfala-Baguinéda stock (Mali)

Flies of both sexes from Tienfala-Baguinéda and CIRDES, i.e. those infected by *T. vivax* from Tienfala-Baguinéda, were dissected 15 days after the first infecting meal was taken by the flies. Only the proboscis was found to be contaminated. The proportion of non-infected males was higher than that of the non-infected females. Thus, with *G. p. gambiensis* of CIRDES, 42.2% of males and 30.4% of females did not have trypanosomes in their proboscis. However, with *G. p. gambiensis* of Tienfala-Baguinéda, 66.7% of males and 34.9% of females did not carry any trypanosomes in their proboscis. Regardless of the source of the flies, trypanosomes were more abundant in the biting organ of females than in that of males (Table 16). This suggests that transmission of trypanosomosis (*T. vivax*) to animals in the zone of Tienfala-Baguinéda was probably due to females of *G. p. gambiensis* [7].

There was no significant difference between the rates of the infected females of the two stocks, but a statistically significant difference between the rates of infected males was found. This significant difference between the rates of infected males is at the basis of the statistically significant difference observed at the level of the total rate of infection between the two stocks (Table 17). This experiment suggests a higher vectorial competence of *G. p. gambiensis* of CIRDES compared with that of *G. p. gambiensis* of Tienfala-Baguinéda [8]. This study will continue with a collection of wild stocks of *Glossina* (*G. p. gambiensis* and *G. m. submorsitans*) from locations where there is a strong prevalence of *Trypanosoma congolense*.

TABLE 16. PROPORTION (%) OF FLIES (*G. P. GAMBIENSIS*) FROM CIRDES AND TIENFALA-BAGUINÉDA THAT WERE INFECTED WITH TRYPANOSOMES

Sex	CIRDES	Tienfala	Difference
Male	57.8	33.3	Significant
Female	69.6	65.1	Non-significant
Total	63.7	49.4	Significant

TABLE 17. PROPORTION (%) OF FLIES (*G. P. GAMBIENSIS*) BY LOCATION THAT WERE INFECTED IN TERMS OF THE ABUNDANCE OF THE PARASITE (*TRYPANOSOMA*)

Parasite abundance (no. of trypanosomes)	CIRDES		Tienfala-Baguinéda	
	Males	Females	Males	Females
0	42.2	30.4	66.7	34.9
1–10	28.9	26.1	14.3	20.9
10–50	20	23.9	11.9	16.3
50–100	4.4	2.2	7.1	11.6
> 100	4.4	17.4	0	16.3

3.6. Validation of TPU-3 holding/feeding system

A comparison of the TPU-3 system with the former system showed that the TPU-3 needed only 20% of the time required for feeding flies in the old system. This is a major reduction in the cost of labour. Even though the version of TPU-3 that existed at CIRDES was an improvement over the old system, nevertheless some problems with the TPU-3 system were still found, as follows:

- Some hotplates malfunctioned
- The ground of the room required adjustment
- Some pupae were lost due to consecutive openings between two cones
- Netting of the cages lacked tightness.

4. CONCLUSIONS AND FUTURE RESEARCH

This research, a partnership between CIRDES and the IAEA, resulted in improvements in mass rearing *Glossina*. Reduced feeding times, using frozen blood and eliminating the addition of ATP to the blood made it possible to save time and lower the production cost. Mating females with males that are zero days old reduced manual labour. The tests on the doses of irradiation, and the competitiveness between *G. p. gambiensis* of CIRDES and *G. p. gambiensis* of Mali, permitted the SIT to be applied in the field.

In future, research should be conducted on the following topics:

- Competitiveness of males reared on frozen blood without ATP
- Self-stocking of production cages [1]
- Assessment of production costs regarding:
 - system of freezing blood
 - size of the fly colony
 - availability of cattle for slaughter at the abattoir
 - cost of electricity.

ACKNOWLEDGEMENTS

The IAEA provided some financial support for this research.

REFERENCES

- [1] (FAO/IAEA) Food and Agriculture Organization of the United Nations/International Atomic Energy Agency, FAO/IAEA standard operating procedures for mass rearing tsetse flies, IAEA, Vienna, Austria (2006).
http://www-naweb.iaea.org/nafa/ipc/public/Tsetse_Rearing_SOP_web.pdf
- [2] BAUER, B., FILLEDIER, J., KABORE, I., Large scale rearing of tsetse flies (Diptera-Glossinidae) in the C.R.T.A. Bobo-Dioulasso, Burkina based on *in vitro* feeding techniques, Revue Élev. Méd. vét. Pays trop. **37** (1984), numéro spécial, I.E.M.V.T., 9–17.
- [3] ŽD'ÁREK, J., DENLINGER, D.L., Changes in temperature, not photoperiod, control the pattern of adult eclosion in the tsetse, *Glossina morsitans*, Physiol. Entomol. **20** (1995) 362–366.
- [4] MUTIKA, G.N., OPIYO, E., ROBINSON, A.S., Assessing mating performance of male *Glossina pallidipes* (Diptera: Glossinidae) using a walk-in field cage, Bull. Entomol. Res. **91** (2001) 281–287.

- [5] MUTIKA, G.N., OPIYO, E., ROBINSON, A.S., Effect of low temperature treatment on the quality of male adult *Glossina pallidipes* (Diptera: Glossinidae) in relation to the sterile insect technique, *Entomol. Sci.* **5** (2002) 209–214.
- [6] CAYOL, J.P., VILARDI, J., RIAL, E., VERA, M.T., New indices and method to measure the sexual compatibility and mating performance of *Ceratitis capitata* (Diptera: Tephritidae) laboratory-reared strains under field cage conditions, *J. Econ. Entomol.* **92** (1999) 140–145.
- [7] CIRDES, Rapports scientifiques (2000) 19–24 and (2001) 24–30.
- [8] DJITEYE, A., BAUER, B., VAN DER VLOEDT, A., FELDMANN, U., VREYSEN, M.J.B., “Influence combinée de la basse température et de l’irradiation durant la dernière phase pupale sur la capacité vectorielle des mâles de *Glossina palpalis gambiensis*”, *Management of Insect Pests: Nuclear and Related Molecular and Genetic Techniques*, Proceedings of a symposium jointly organized by IAEA and FAO, Vienna, Austria, 19–23 October 1992, STI/PUB/909, IAEA, Vienna (1993) 567–578.

PARTURITION IN TSETSE FLIES: ENDOCRINE CONTROL

J. ŽD'ÁREK¹, P. TAKÁČ², D. KESZELIOVÁ², L. ŠIMO²,
L. ROLLER², J. CVAČKA¹, M. ŠANDA¹

¹Institute of Organic Chemistry and Biochemistry, AV CR,
Prague, Czech Republic

²Institute of Zoology, SAV,
Bratislava, Slovakia

Abstract

A problem in tsetse mass rearing facilities is the increased incidence of abortions of underdeveloped larvae or pupariation of larvae within the mother's uterus. We analysed the problem by investigating neural, hormonal and environmental factors controlling parturition. Input from the mother's brain is essential for normal parturition, since a female whose brain is disconnected by ligation fails to deposit her larva. The expulsion of a larva is stimulated by a putative parturition hormone present within the female's uterus. The hormone also elicits abortion when injected into neck ligated females at earlier stages of pregnancy. This report describes attempts to reveal the chemical nature of this hormone by purification of extracts of uteri of *Glossina* females and identification of behaviourally active fractions using a MALDI-MS instrument. Genomic (BLAST) analysis of the identified sequences did not reveal a significant match with any protein with bioactive properties in other species. However, similarity with various enzymes or structural proteins (and hypothetical proteins) was detected occasionally. In the *Glossina* genomic and cDNA databases no nucleotide sequence corresponding to the deduced AA sequences was found. Perhaps the deduced sequences are too short to obtain more significant hits both in protein and nucleotide databases. We also made investigations to elucidate environmental influences and physiological mechanisms associated with tsetse parturition. We found that the circadian rhythm of parturition of flies kept in Bratislava (*G. m. morsitans*, *G. f. fuscipes* and *G. pallidipes*) is less pronounced than under natural conditions. The loss of synchrony in the laboratory may have three possible causes: (i) genetic – absence of selective pressure, (ii) environmental – low intensity or absence of an entraining light or temperature stimulus, and (iii) physiological – impaired sensitivity to olfactory stimulation by a hypothetical 'oviposition' pheromone that is believed to synchronize larviposition. We also monitored and analysed the motor patterns of larviposition behaviour by measuring changes in haemocoel pressure before, during and after parturition. Using the same technique we monitored muscular contractions of the female engaged in copula with the male. Finally, we recorded the behaviour and survival of larvae prematurely deposited or artificially aborted.

1. INTRODUCTION

During our participation in the co-ordinated research project 'Improved and harmonized quality control for expanded tsetse production, sterilization and field application' we focused on some neglected aspects of physiological and behavioural processes underlying larval development and adult reproduction of this uniquely propagating fly. Understanding of basic physiology and behaviour is a prerequisite of successful and efficient mass rearing of the tsetse flies for the SIT programmes. Our research was based on the results of previous investigations made in our laboratories earlier and in the Tsetse Research Unit of the International Centre of Insect Physiology and Ecology, Nairobi, Kenya. We investigated neural, hormonal and environmental factors controlling parturition and analyzed behavioural patterns associated with this process using an original tensiometric technique. We also monitored and recorded hemocoelic pulsations associated with copulation and sperm transfer during mating. Finally, we investigated behaviour and viability of larvae prematurely deposited or artificially aborted at various times before expected time of parturition.

2. CHEMICAL IDENTITY OF THE PARTURITION HORMONE (PH)

The objective was to isolate and chemically identify a hormonal factor(s) that stimulates normal parturition or precocious abortion in pregnant tsetse females. The existence of such a factor was suspected long ago [1, 2], and its physiological action, chemical character and the tissue of origin have been investigated [3]. Nevertheless, the molecular identity of the factor(s) named parturition hormone (PH) remained elusive. Therefore, to fill this gap, state of the art analytical methods (HPLC, MALDI TOF, LTQ Orbitrap XL hybrid mass spectrometer) were employed to isolate and identify the active principle.

2.1. History and rationale for the search of the PH

A tsetse female gives birth to a single third-instar larva every 9–10 days. When, for example, *G. m. morsitans* females are reared at constant temperature in a controlled photoregime, larvae are typically deposited late in the photophase [4, and references therein], suggesting that parturition under natural conditions is an event of the late afternoon. Field observations confirmed that this is the case [5]. When reared through the pregnancy cycle in constant light or darkness, the fly exhibits no obvious rhythm of parturition [6], but the rhythm is completely restored after a single light:dark cycle following a constant light period [4]. It is assumed that the decision of a female to lay a larva is controlled by two mechanisms: neural and neurohormonal. When nervous connections from the mother's head are severed by neck ligation or nerve transaction, the female fails to deposit her larva [1, 2]. The fact that blood or brain homogenate from females that have recently given birth elicits a parturition response when injected into neck ligated females suggests the presence of a hormone that stimulates parturition. The richest source of a factor that prompts a neck ligated pregnant tsetse female to expel the contents of her uterus was found to be the uterus itself [3]. The active principle of uterine extract, called a parturition hormone (PH), will cause a neck ligated female carrying a full-term larva to give birth within minutes of injection. If the female is carrying a younger larva, her offspring will be aborted either immediately or with a delay. In any case, such a premature larva does not pupariate and dies. Furthermore, it was found that PH activity disappears after pepsin digestion, indicating its peptide/protein nature. Even though PH activity is present in the uteri of several tsetse species, neck ligated females of *G. m. morsitans* and *G. pallidipes* proved to be the most reliable bioassay system for testing the efficacy of uterine extracts and other agents in eliciting the parturition response [7]. The fact that extracts of the uterus of the grey flesh fly *Sarcophaga bullata* (Parker) and genital ducts of insects from diverse taxa (the silkworm *Bombyx mori* (L.) and the desert locust *Schistocerca gregaria* (Forskål)) also exhibit PH activity in the tsetse bioassay [3] suggests that this hormone or structural homologues may be present in insects in general and play a regulatory role during oviposition/larviposition.

2.2. Materials and methods

Work was performed in collaboration with the Zoological Institute of the Slovak Academy of Sciences, Bratislava, where mass production colonies of three *Glossina* species were kept. Since the bioassay had already been elaborated and routinely performed on *G. morsitans centralis* Machado [3], the first tests for parturition hormone (PH) activity were performed on this species. However, shortly after beginning the investigations in 2006, the Slovak colony of *G. m. centralis* was replaced with *G. m. morsitans*, and the work continued using this species. Unfortunately, it was found that the circadian rhythm of larviposition, so crucial to the PH bioassay, was greatly suppressed in the colonies of all tsetse species kept in Bratislava (see below). As a result, the bioassay used in the study was limited in sensitivity and accuracy.

Aqueous extracts of *G. f. fuscipes* and *G. m. morsitans* uteri were purified using Size Exclusion chromatography, Reverse Phase HPLC chromatography and HPLC-MS TIC chromatography. The separated peaks of the active fraction of the last purification step were subjected to the matrix assisted laser desorption/ionization technique for mass spectrometric analyses of biomolecules (MALDI-MS) that allow for denovo amino acid (AA) sequencing.

BLAST analysis of the deduced AA sequences was performed to search for homologous proteins (BLASTP) in related insects and identical AA (BLASTP) or corresponding nucleotide sequences (TBLASTN – protein vers. transl. DNA) in *G. m. morsitans*. We utilized the free accessible databases and search tools at the NCBI web page and GeneDB website of the Wellcome Trust Sanger Institute. Default parameters were used for searching *Glossina* genome and *Glossina* EST databases and relaxed search options (Expect value 100–1000) were applied for fishing in other insect databases. Promising matches were further analysed by free bioinformatics tools on the JustBio website (<http://www.justbio.com/>, Clustal W 1.8 Aligner, Translator) and by the signal peptide prediction software (SignalP 3.0).

2.3. Results of the first trial

2.3.1. Chromatography

A heated crude extract from uteri of *G. m. morsitans* induced deposition of larvae in 80% of neck ligated females injected at late stages of pregnancy. The material was subjected to a stepwise chromatographic procedure using an HPLC instrument. The most active fraction from the first purification step with retention time (RT) of 12–13 min induced 56% of abortions (Fig. 1A). This fraction was purified further. Among fractions from the second purification, the highest abortion (93%) was induced by the fraction with RT of 7.8–9.8 min (Fig. 1B). Subsequent purification of this fraction yielded an active fraction with RT of 6.7 min; this fraction induced 66.6% of abortions (Fig. 1C). The next RP-HPLC purification step revealed an active peak with RT of 6.4 min. Application of this fraction resulted in 86.6% of abortions (Fig. 1D). The last purification step on a Toshiba Biosep microcolumn (RT 2.3–4.6 min) induced also 86.6% of abortions of neck ligated females (Fig. 1E).

2.3.2. Mass spectrum analysis

The active fraction from the last purification on RP-HPLC was subjected to further separation on High Performance Liquid Chromatography-Mass Spectrometry Total Ion Current (HPLC-MS TIC). The analysis showed interesting tendencies in the profile of fractions and a better sensitivity. Five peaks were detected in the fraction (Fig. 2): Peak 1, RT=9.1 min; MS base peak = 1734.126; Peak 2, RT=11.0 min; MS base peak = 1778.938; Peak 3, RT=11.4 min; MS base peak = 2355.894; Peak 4, RT=12.0 min; MS base peak = 1448.708; Peak 5, RT=13.3 min; MS base peak = 1953.102.

2.3.3. Amino acid sequencing

Each peak was analysed by continual spectral analysis and AA sequencing, and in total 85 possible sequences were suggested, including 15 of Peak 1, 14 of Peak 2, 42 of Peak 3, 3 of Peak 4 and 11 of Peak 5.

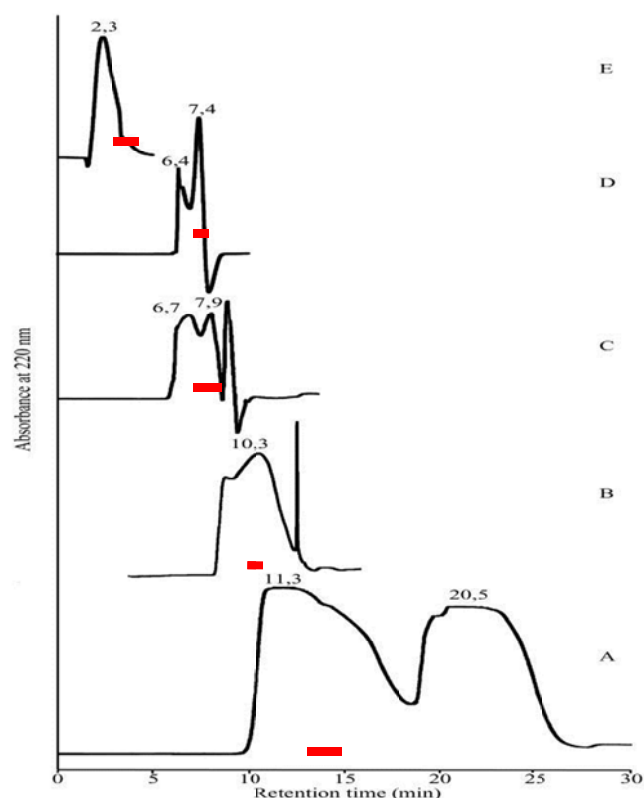


FIG. 1. Chromatographic (HPLC) analysis of the heated (80°C/5 min) crude uteri extract of tsetse fly *G. m. morsitans*. The peaks containing PH activity are indicated by short horizontal bars.

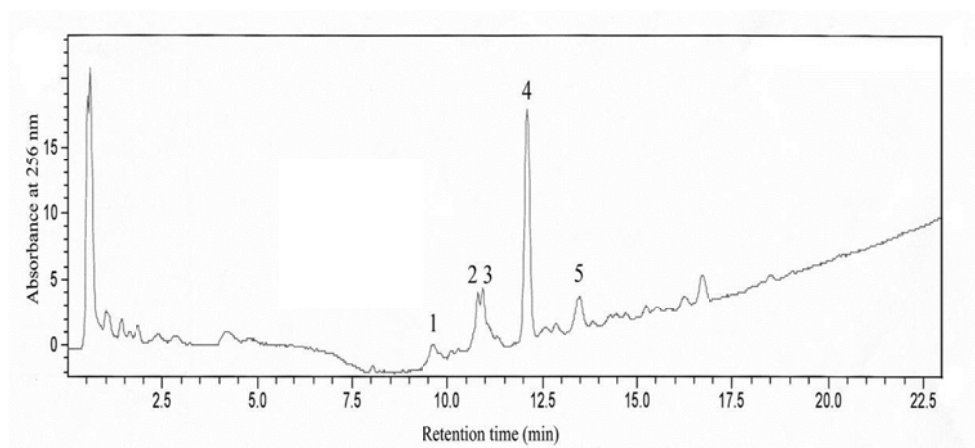


FIG. 2. HPLC-MS TIC chromatogram showing the main components of the active fraction from the last RP-HPLC purification step. (The numbers indicate the peaks used for AA sequence analyses.)

2.3.4. BLAST analysis

Despite the variability obtained we made a BLAST search for all identified sequences. The most frequently obtained sequences matched with parts of isocitrate dehydrogenase, vitellogenin, yolk protein, lectin, hemomucin, Cu/Zn superoxide and various ‘hypothetical protein’ sequences. Interestingly, partial conformity was observed with an antimicrobial peptide dipterin and a precursor of CCAP (crustacean cardiac-accelerating peptide).

However, the identity was observed in insignificantly short sequences of only five amino acids. Notably, synthetic CCAP has already been tested for PH activity [8] with negative results.

2.4. Results of the second trial

A uterus extract was purified by RP HPLC in the same way as in the first trial, and the PH active fraction of the last purification step was measured using a MALDI-MS instrument. The diagram showed a prominent peak at M/Z 655.615. This peak was fragmented. Based on the spectrum the following eight possible amino acid sequences were proposed: NPIVGR, PNIVGR, NPLVGR, PNLVGR, IPNVGR, LPNVGR, PINVGR and PLNVGR.

2.5. Results of the third trial

The uterus extract was purified by RP HPLC in the same way as in preceding trials, and the PH active fraction of the last purification step that proved to be most active in previous trials (Fig. 1E) was checked for biological activity (with a positive result) and then measured using a MALDI-MS instrument. The diagram showed a prominent peak at M/Z 655.574. This peak was fragmented and, based on the spectrum, the following seven possible amino acid sequences were proposed: GGPPATR, GNHATR, GPGPDGR, PNIGVR, NPLGVR, PNLGVR and IPNGVR.

2.5.1. BLAST analysis

BLAST analysis of the identified sequences did not reveal a match with any protein with bioactive properties in other species. As in the previous trial (experiment 1), similarity with various enzymes or structural proteins (and ‘hypothetical proteins’) was occasionally detected. In the *Glossina* genomic and cDNA databases no nucleotide sequence corresponding to the deduced amino acid sequences was found. Perhaps the deduced sequences are too short to obtain more significant hits both in protein and nucleotide databases. Moreover, the *Glossina* genome and EST projects are not yet finalized, hampering the making of relevant conclusions.

2.6. Conclusions

The fact that we did not lose biological activity even after five purification steps indicates that the purification procedure was properly designed. Nevertheless, the native PH molecule could possibly be larger than the short AA sequences identified. The integrity of the molecule may have been broken by the very first step of the procedure – removal of large proteins from the uterus homogenate by thermal denaturation. This could be circumvented by using another method of initial separation of peptides from large proteins such as molecular sieves, chemical denaturation by ammonium sulphate or methanol, dialysis, etc. Another bottleneck of the identification procedure was some unreliability of the bioassay performed on flies kept in Bratislava. Some of the experiments reported below were designed to elucidate possible causes of occasional failures of the test.

3. SCREENING OF SYNTHETIC PEPTIDES AND OTHER NEUROTROPIC COMPOUNDS FOR PH ACTIVITY

Since we have no definitive conclusion regarding the chemical identity of the active principle of PH, we attempted a short cut to identify at least its ‘generic’ class. Earlier, 35 already

identified synthetic insect neuropeptides and analogs were evaluated for PH activity to find out whether any of the known insect neuropeptides was capable of mimicking the effect of PH. When the classical bioassay employing neck ligated pregnant females was used, modest PH activity was observed for high doses of the myotropic peptide proctolin and a pyrokinin analog [9]. Recently, we have obtained several other synthetic pyrokinin peptides and analogues known to have ethotropic (myotropic) effects in *Drosophila* or *Neobellieria* [10] (and our unpublished observations). Modest activity of proctolin (threshold level 100 pmol) was confirmed, but we could not detect any activity in a synthetic pyrokinin Neb-PK-2 derived from the flesh fly neurosecretion [11]. Thus it is unlikely that PH is related to the peptides of the pyrokinin/PBAN family.

4. CIRCADIAN RHYTHMS OF LARVIPOSITION IN THE TSETSE COLONIES

Under natural conditions, parturition in the tsetse fly is a photoperiodically gated event, which reaches its peak in the late afternoon [5]. Good results of the bioassay for PH activity depend to a great extent on the synchrony of larviposition in the cohorts of experimental females. In the bioassay for PH activity the female to be injected with the tested sample should be neck ligated (or decapitated) several (4–6) hours before the expected time of parturition, i.e. at the time when the larva in the uterus is mature enough to be deposited but still before the endogenous hormone is released [3]. Only then is the test extremely sensitive (it detects doses of PH below 0.02 uterus equivalents) and reliable (less than 20% false positives). Since such parameters of the test could often not be obtained in the Bratislava laboratory, we searched for possible causes of the occasional failures. Our study began with investigating the *synchrony* and *circadian periodicity* of parturition in three tsetse species maintained in Bratislava (*G. m. morsitans*, *G. f. fuscipes* and *G. pallidipes*).

4.1. Methods

In each species two cohorts of females that emerged in a standard production colony within one week were observed for daily larviposition four times in their life, namely 8, 9, 11 and 13 weeks after emergence. Larvae deposited during each hour of the 24-h cycle were collected and counted. The observations were done on a day when colonies were not disturbed by feeding. Results were compared with data obtained earlier by one of us (J.Z.) in a similar experiment with a colony of *G. morsitans centralis* maintained and fed on rabbit ears at the International Centre of Insect Physiology and Ecology (ICIPE), Nairobi, Kenya, where most of the initial bioassays for PH were done.

4.2. Results

In all three species kept in Bratislava the larviposition gate was less pronounced (Fig. 3) than reported in previous studies (e.g. [4] and Fig. 4). When data from daily collections of larvae deposited (laid) by individual batches were subjected to the test for random distribution (Shapiro-Wilk test [12]), a significant non-random circadian distribution was most often recorded with *G. pallidipes* (Table 1). There were some differences in the number of larvae laid during photophase and scotophase (Fig. 5). The difference was most pronounced in *G. pallidipes* (LD, 68:32%), less in *G. f. fuscipes* (LD, 61:39%) and least in *G. m. morsitans* (LD, 55:45%). If the age of flies is a variable, no statistically significant differences among cohorts of different ages were found in any of the species (ANOVA, $p=0.05$).

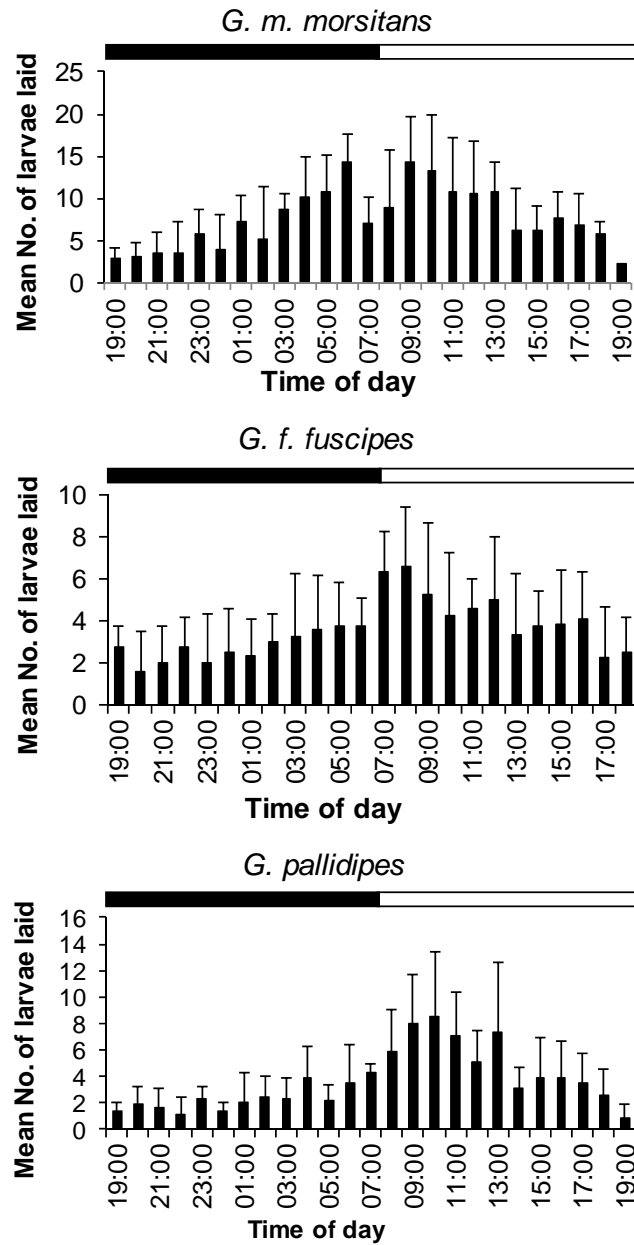


FIG. 3. Number of larvae deposited by females of three tsetse species (*G. m. morsitans*, *G. f. fuscipes*, *G. pallidipes*) kept in Bratislava under LD 12:12 photoregime. The numbers are means (\pm SD) of eight replicates with flies of various ages (8–13 weeks). The horizontal bars indicate scotophase (black) and photophase (white).

For comparison we reconstructed a diagram from data published by Žďárek et al. [4] using the ICIPE colony of *G. morsitans centralis*. Whereas very few larvae were deposited during the scotophase in the ICIPE colony and the parturition gate was well expressed covering only the second half of photophase (Fig. 5), our current experiments with *G. m. morsitans* showed only small insignificant differences between parturition at night or day, and a poorly expressed ‘parturition gate’ extended from the middle of scotophase to the middle of photophase.

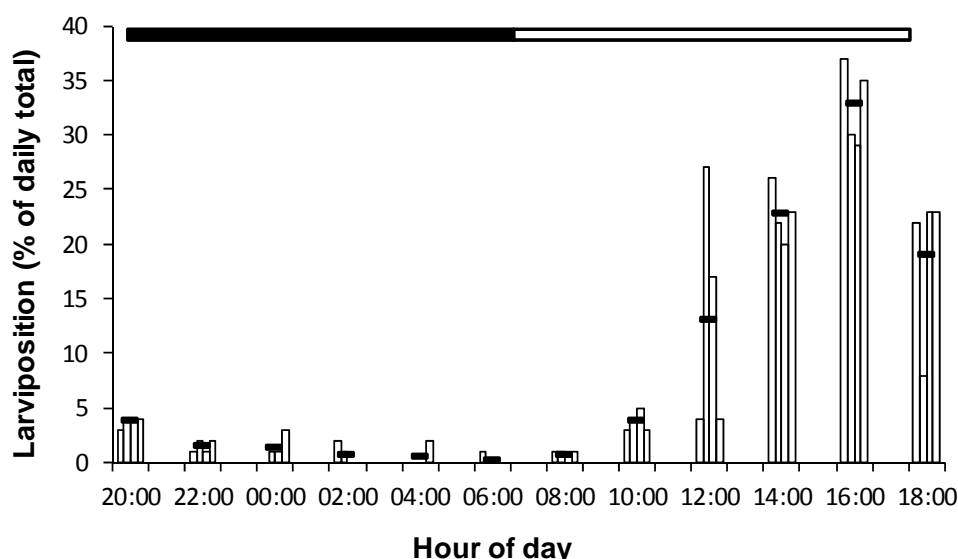


FIG. 4. Parturition on four consecutive days (columns) in *G. morsitans centralis* 4 to 10 weeks old reared at LD 12:12. Number of larvae laid during 2-h intervals is expressed as % of daily total. The bars indicate mean values for 2-h periods. The horizontal bar indicates the periods of scotophase (black) and photophase (white). (Adapted after Ždárek et al.[4])

TABLE 1. NON-RANDOM DISTRIBUTION OF PARTURITION DURING A CIRCADIAN PERIOD IN INDIVIDUAL BATCHES OF FEMALES OF VARIOUS AGES. VALUES ABOVE 95% SIGNIFICANCE ARE IN **BOLD ITALICS** (SHAPIRO-WILK TEST [12])

A e (weeks)	<i>G. m. morsitans</i>	<i>G. pallidipes</i>	<i>G. f. fuscipes</i>
8	<i>p=0.0008028</i>	<i>p=0.02365</i>	<i>p=0.1223</i>
8	<i>p=0.206</i>	<i>p=0.1104</i>	<i>p=0.01422</i>
9	<i>p=0.008779</i>	<i>p=0.00006935</i>	<i>p=0.01602</i>
9	<i>p=0.008341</i>	<i>p=0.02316</i>	<i>p=0.01061</i>
11	<i>p=0.001477</i>	<i>p=0.03737</i>	<i>p=0.08498</i>
11	<i>p=0.0578</i>	<i>p=0.0005951</i>	<i>p=0.1487</i>
13	<i>p=0.4357</i>	<i>p=0.02298</i>	<i>p=0.1558</i>
13	<i>p=0.0189</i>	<i>p=0.00002706</i>	<i>p=0.2718</i>
Total	<i>p=0.225</i>	<i>p=0.01141</i>	<i>p=0.1762</i>

4.3. Discussion

The distribution of larviposition in three tsetse species maintained in Bratislava (*G. m. morsitans*, *G. pallidipes* and *G. f. fuscipes*) has less expressed circadian periodicity than reported earlier for tsetse females from the outdoors [5] and laboratory [4]. This loss of synchrony may be due to various factors: (i) *genetic* – the absence of selective pressure in colonies kept in the laboratory for many generations, (ii) *environmental* – low intensity or absence of an entraining light (or temperature) stimulus (*Zeitgeber*) in the dimly illuminated constant environment of an insectary (light intensity of 0.1–3 lux depending on the position of a cage in the rack), and (iii) *physiological* – decrease in sensitivity to olfactory stimulation by hypothetical ‘oviposition’ pheromones [13] that are believed to synchronize larviposition in the field.

Although tsetse flies are viviparous, aggregation of the immature stages occurs [14]. The presence of an aggregation semiochemical in two species of tsetse has been confirmed [15]. Results of a recent preliminary experiment designed to test the validity of assumption (ii) above suggest that illumination intensity during photophase may indeed affect expression of circadian rhythms of larviposition (Fig. 6). These findings may have significance for proper management of tsetse mass rearing facilities, namely for more precise aging of puparia in the automated sexing system.

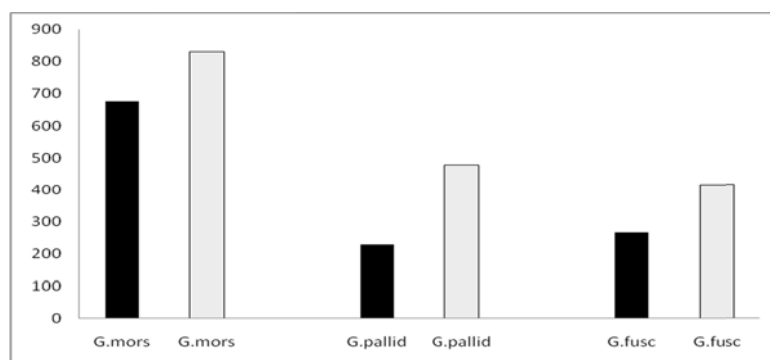


FIG. 5. Number of larvae deposited by females of *G. m. morsitans*, *G. pallidipes* and *G. f. fuscipes* during scotophase (black bars) and photophase (grey bars). Totals of eight 24-h observation periods.

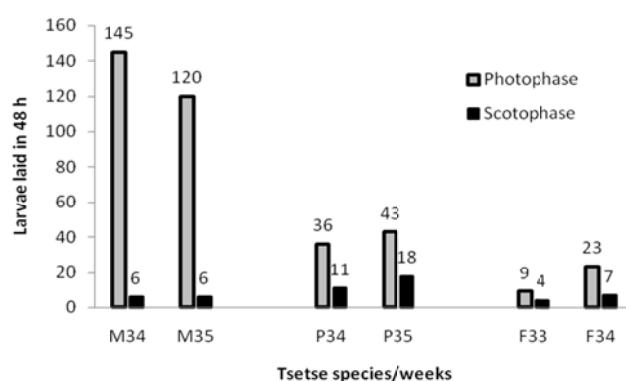


FIG. 6. Number of larvae deposited by females of *G. m. morsitans* (M), *G. pallidipes* (P) and *G. f. fuscipes* (F) during photophase (grey) and scotophase (black) after the intensity of illumination in the insectary during photophase was increased about five times. Totals of two 24-h observation periods.

5. HEMOCOELIC PULSATIONS BEFORE AND AFTER PARTURITION IN THE TSETSE FLY

The monitoring of hemocoelic pressure changes is a powerful technique to detect subtle muscular contractions within the insect body [16, 17]. They reflect both the autonomous movements of intersegmental muscles associated with ventilation of tracheae and air sacs (called extracardiac pulsations or coelopulses by Sláma [18]) and the movements of skeletal musculature underlying various stereotyped behavioural episodes such as ecdysis and adult emergence [21], pupariation and/or pupation [19, 20] as well as larviposition [22].

The activity of a pregnant female changes several hours before parturition. Deposition of the larva is characterized by a stereotypic sequence of behaviour [2]. About 5–6 h before parturition the larva descends slightly in the uterus, and the female becomes conspicuously restless. As parturition approaches the female again becomes quiet. The actual act of expulsion of the larva is initiated by relaxation of the sphincter of the vulva and usually lasts for only a few seconds. Both nervous and hormonal signals are involved in its coordination [2, 23, 24, 25]. The mother and larva together determine its precisely timed occurrence [6].

Following parturition some females produce a distinct sound, which is probably associated with rearrangement of the abdominal organs severely displaced by the growing larva. Within 1–2 h of parturition the next egg is ovulated and released into the uterus initiating the next gonotrophic cycle [26]. Ovulation is also controlled by hormonal factors [27] but probably not by the same regulators that control parturition [23, 28].

The neuromuscular mechanisms involved in tsetse parturition have already been studied using a barographic technique to monitor hemocoelic pressure changes [22]; the work provided an overall picture of events accompanying parturition. However, with the chart recorders then used it was almost impossible to analyse the process in more detail. Hence, we decided to repeat and extend this research with improved recording of the barographic data. The original tensiometric device was coupled with a modern PC-based programme for data acquisition and analysis [8].

5.1. Materials and methods

All experiments were performed with pregnant females of *G. m. morsitans* taken at the age of 6–12 wk from a standard stock colony in Bratislava. Details of the technique were described in Žďárek et al. [29]. Briefly, a hypodermic needle was inserted into the thorax of the female to be measured and her hemocoel connected through a hydraulic system filled with saline to the tensiometric device. The cannulated flies were provided with a styrofoam ball for tarsal contact to freely allow walking (Fig. 7). Sampling of changes was done at a frequency of 5 Hz by an A/D converter Drak 4 (Papouch, Prague, CZ), and digitized data were visualized and analysed using RainbowReader software (Jiří Semecký, Prague, CZ). On a pressure curve (barogram) distinctive patterns of muscular contractions that characterize particular phases of parturition behaviour and ventilation movements are reflected in pulsations of hemocoelic pressure. The barograms thus serve as an objective record of the muscular actions associated with these major reproductive events. Making measurements was time consuming because of uncertainties in predicting the actual time of parturition in the poorly synchronized fly populations (see above). Sometimes the recording was interrupted due to disruption of the hydraulic system between the fly and the tensiometric device, and in some cases records were deemed not physiologically relevant because the act of parturition was delayed or failed to occur when the fly was cannulated. From more than 30 attempts, only five complete records were obtained that were good enough to allow some generalizations.



FIG. 7. Copulating pair of G. m. morsitans with the female cannulated by a hypodermic needle that is hydraulically connected to a tensiometric (barometric) sensor (not in the picture).

5.2. Results and discussion

A complete representative barogram from a single female that documents the stereotypic pressure pulses occurring in association with parturition and ovulation is shown in Fig. 8. For several hours before parturition the female rested and usually did not show much muscular activity, only minute pressure pulsations that reflected regular ventilation movements (vm) associated with active breathing [18]. One or two hours before expulsion of the larva much larger pressure pulses occurred (mc). At first they were infrequent but then became more numerous and regular, announcing the impending parturition. These pulses had mostly positive but occasionally also negative values in relation to the baseline. Each pulse was associated with a distinct bobbing action of the female's proboscis. The rhythmic movements of the proboscis reflected these pressure changes and thus provided a reliable visible indicator of the internal pulsing. Throughout this period the fly occasionally walked (rotated the ball) for a few seconds, groomed the head, wings or abdomen but no pressure pulses were associated with these activities. Evidently not all muscular activity elicits a response detectable by the barograph. The large pressure peaks must be generated by a specific action of the overall somatic musculature, most probably by simultaneous and synchronous muscular contractions similar to that observed when pressure changes were monitored during puparium formation [30]. Since the larva actively participates in the birth process, we cannot exclude that its own peristaltic movements contribute to the recorded pressure changes preceding parturition. We speculate that the larval movements provoke the mother to intensify the labour, because preceding the moment of larval expulsion there was a dense train of high pressure peaks. The last peak, during which the larva was leaving the uterus, was distinctly extended in time (Fig. 8, upper trace). Following parturition, the large pressure peaks disappeared, the baseline first dropped and then slowly went up and down several times, and finally the small regular ventilation movements (vm) resumed. The sound produced by the female shortly after parturition was not reflected in the barogram.

Another vigorous muscular activity was recorded about 2 h after parturition, when both positive and to a greater extent negative deflections from the baseline occurred (Fig. 8, lower trace). It is noteworthy that this event coincides with the anticipated time of ovulation [26]. A

very similar pattern of late pulsations was also reported in a previous study [31]. Dissections performed then revealed that the egg was already in the uterus immediately after the presumptive ovulation pressure pulses. We assume that the described barographic activity occurring regularly some 2 h after parturition is likely associated with ovulation. Ovulation in tsetse females is a three-step process — rupture of the follicle, muscular contraction of the ovary and oviduct, and opening of the oviductal sphincter [23].

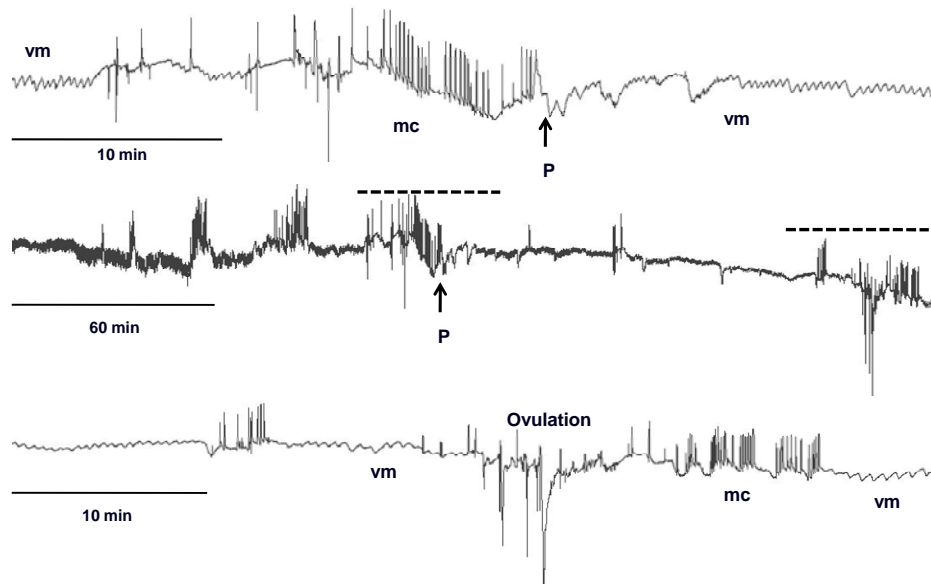


FIG. 8. A representative barogram of hemocoelic pressure changes in a *G. m. morsitans* female before and after parturition (P, arrow). Middle trace – the overall picture; sections indicated by dotted lines above the middle trace are 6 times extended in time in the upper and lower traces; vm – ventilation movements; mc – synchronous muscular contractions coinciding with bobbing of the proboscis.

5.3. Conclusion

Barographic records confirmed that parturition is the culmination of a long period of covert preparatory muscular activity in the stationary fly rather than the consequence of a few seconds of muscular labour.

6. COPULATION BEHAVIOUR IN *G. M. MORSITANS* AND *G. PALLIDIPES*

A paper read by R.D. Briceño at the 3rd RCM on ‘Improved and Harmonized Quality Control for Expanded Tsetse Production’ in Muguga, Kenya in May 2007 [32], and the personal discussions that followed, inspired us to develop a method of continuous recording of hemocoelic pressure changes in a tsetse female before, during and after copulation. The above authors described at length the functional morphology and anatomy of the genitalia of male *G. pallidipes*, and the involvement of various genitalic traits in courtship and copulation. The paper deals primarily with male mating behaviour, and the degree of active participation by a female is only postulated. Some of the male genitalic traits probably function to stimulate the female, while others function to restrain her. Our contribution to the elucidation of the mechanics of copulation was based on monitoring female behaviour before, during and after

copulation by recording hemocoelic pressure changes that reflect muscular contractions associated with this epigamic activity.

After the Final RCM in Addis Ababa in October 2008 a joint session with R.D. Briceño was arranged at the FAO/IAEA Laboratory in Seibersdorf in which simultaneous barographic and video recording of copulation in *G. pallidipes* was performed. The aim of the experiments was to: (i) compare the activities of *G. m. morsitans* and *G. pallidipes* females, and (ii) correlate visually observed mating activities of the male with continuously recorded changes of internal (haemocoelic) pressure in the female. The results of this joint session will be published elsewhere.

6.1. Materials and methods

Virgin females and males of *G. m. morsitans* 7–10 days old were used. The female to be monitored was connected hydraulically through a hypodermic needle inserted into her thorax with a tensiometric sensor, and internal pressure fluctuations were recorded by data acquisition software (Fig. 7). Sampling of pressure changes was done at a frequency of 5 Hz by an A/D converter Drak 4 (Papouch, Prague, CZ), and the data were visualized and analysed using RainbowReader software (Jiří Semecký, Prague, CZ). The female tethered on the needle was offered a small styrofoam ball for tarsal contact to freely allow walking. The fly was enclosed in a glass vial or other transparent container with a hole through which the needle was inserted. When the female calmed down a virgin male of equal age was introduced into the container. Usually the pair began to copulate within a few minutes (Fig. 7). If not, the male was replaced by a fresh one. Four good records of completed copulation were obtained.

Essentially the same technique was employed when the experiment involving simultaneous barographic and video recording of *G. pallidipes* copulation was performed. Four good records of complete copulation were obtained. Special attention was paid to the final phase of copulation to see whether or not the copulation was completed with sperm transfer (consummated).

6.2. Results

An example of the barographic record of hemocoelic pressure fluctuations of a copulating *G. m. morsitans* female is shown in Fig. 9. Before contact with the male was established, the female produced regular and slow muscular contractions indicating ventilation movements. The rhythm of contractions changed immediately after the male made the first copulatory attempts. The pulses became more frequent and less expressed. Several minutes after the onset of copulation pulsation slowed down and only before the very end of copulation were a few irregular prominent pulses recorded. We assume that this change is a response to the male behaviour, since the most vigorous activity of the male occurred immediately before the end of copulation, when sperm was transferred [33]. Ejaculation is assumed to occur when the male's body trembles, and the time of ejaculation can be identified on the female's barogram by a short interruption of the breathing pulses that are replaced by prominent pressure peaks. After separation of the pair, hemocoelic pulsation within the female's body decreased. Indications of any other male mating activities could not be detected by viewing barograms from the female.

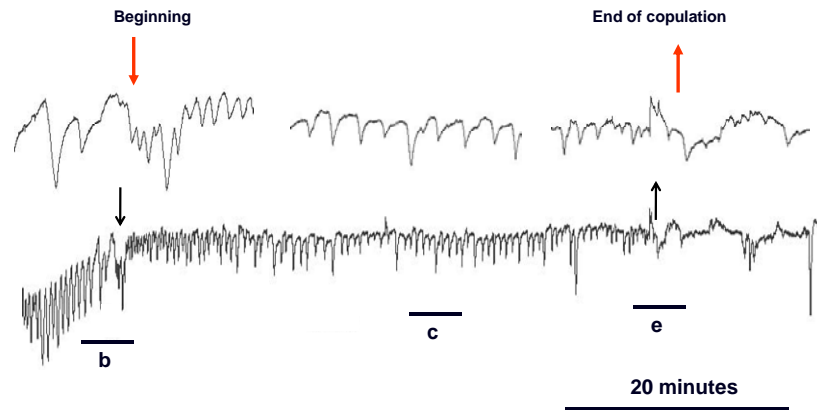


FIG. 9. A representative barogram of hemocoelic pressure changes in a *G. m. morsitans* female before, during and after copulation with a virgin male (lower trace with the time-scale bar). Fast chart speed records of short periods at the beginning (bar 'b') and end (bar 'e') of copulation, as well as in the middle of sexual interaction (bar 'c'), are shown above the main trace.

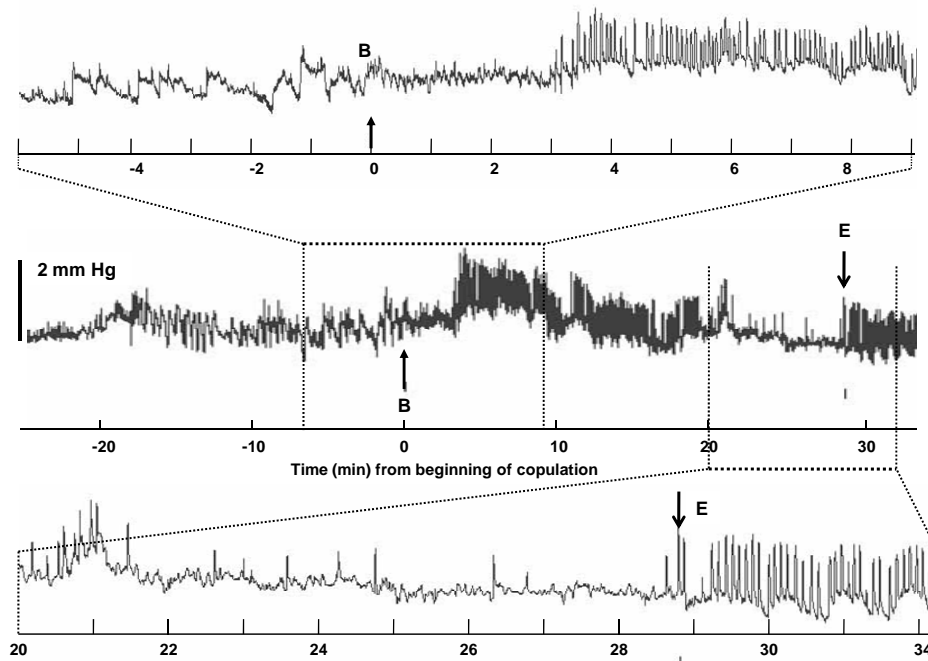


FIG. 10. A representative barogram of hemocoelic pressure changes in a *G. pallidipes* female before, during and after copulation with a virgin male (middle trace). Fast chart speed records of time periods at the beginning (B) and end (E) of copulation are shown in the top and bottom traces, respectively.

In a subsequent experiment with *G. pallidipes* females, a copulating pair was monitored simultaneously by barograph and video recorder [32], and the two records were compared. A representative barogram obtained from a copulating *G. pallidipes* female is presented in Fig. 10. Even though the final evaluation of the results has not been completed, some preliminary conclusions can be made.

The general pattern of hemocoelic pressure fluctuations in copulating *G. pallidipes* females is exemplified in Fig. 10. After the male's initial attack (B) the pressure patterns changed

indicating different motor patterns of muscular activity. The pressure fluctuations became more frequent, regular and increased markedly in amplitude. This enhanced activity lasted for several minutes and then ceased abruptly, remaining low until the end of copulation. Only a few bursts of pressure were observed shortly before separation of the male (E). When the female was free, the intense pressure fluctuations were resumed immediately. This increased pressure activity in the female was not observed after an incomplete (non-consummated) copulation (diagram not shown), suggesting that the enhanced postcopulatory activity may be stimulated by sperm in the female's genitalia. Evidently the pressure fluctuations do not directly reflect activities of the male. Rather they reflect the general metabolic state of the female; regular periodical pressure fluctuations are mostly reflections of ventilation movements caused by contractions of the abdominal intersegmental muscles [34]. These respiratory movements can be affected indirectly by the male's activities.

6.3. Discussion and conclusions

Following final analysis and evaluation, the results will be published. However, some tentative conclusions can be made:

- Pressure fluctuations shown on barograms do not directly reflect activities of the male. Rather they reflect the general metabolic state of the female; the regular pressure fluctuations are probably reflections of ventilation movements caused by contractions of the abdominal intersegmental muscles [34], and these can be affected indirectly by the male's activities.
- A male's initial actions such as mounting the female and attempting to copulate stimulate increased metabolic activity in the female, which gradually subsides during the second half of copulation. Male's drumming and shaking associated with the final (consummatory) phase of copulation (when sperm is transferred to the female) slightly stimulate pressure changes in the female; this is not observed when copulation is not completed (consummated). These general changes in pressure patterns during copulation were also observed in *G. m. morsitans* females.
- Ventilation in the female increases immediately after the male separates from the female, and this enhanced metabolic activity is terminated if the male makes a second attempt (not shown). Enhanced ventilation after copulation was not observed in *G. m. morsitans* females.
- Females of both species may mate a second time shortly after a first copulation, but the act is probably not consummated; they may not allow the male's aedeagus to penetrate fully.

7. BEHAVIOUR AND SURVIVAL OF LARVAE DEPOSITED BEFORE THE PARTURITION GATE

A frequent problem in tsetse mass rearing facilities for SIT programmes is an increased incidence of abortions of underdeveloped larvae or pupariation of larvae within the mother's uterus. Below we attempt to analyse the problem and search for its causes by investigating the behaviour of larvae forcibly aborted at various times before the normal term of larviposition.

7.1. Materials and methods

We compared the appearance and behaviour of larvae of *G. m. morsitans* deposited naturally with those that were expressed forcibly from the mother's uterus at various times before expected natural birth. The behaviour of the larvae during and after parturition, weight of freshly laid larvae and the later developmental fate of the individuals were recorded. Twenty to thirty larvae were observed in each group.

7.2. Results and discussion

Larvae deposited normally during the parturition gate participated in parturition by producing peristaltic movements that facilitated escape from the uterus, and when free they immediately began to crawl. Their mean weight (\pm SD) was 28.57 ± 4.34 mg. They pupariated normally within 2 h. Larvae expelled mechanically (aborted) before the parturition gate were divided into four categories:

1. Larvae that appeared similar to those deposited normally were active during expulsion, showed no abnormal coloration of the cuticular surface, and in due course formed normal puparia, but sclerotization of the puparial cuticle was preceded by melanization of the still-soft cuticle and tanning was delayed by several hours. Weight 26.13 ± 4.06 mg
2. Larvae that appeared similar to those deposited normally but were unable to crawl normally, i.e. to produce anterograde (forward moving) locomotory peristalsis. Instead, they repeatedly retracted the front segments of the body and produced weak retrograde peristalsis. The cuticle surface was a yellowish colour. These larvae eventually became melanized but remained soft and did not pupariate. Weight 19.16 ± 4.21 mg
3. Larvae were even smaller (mean weight 11.16 ± 3.36 mg), unable to crawl and their movements were reduced to feeble rhythmic pulsations of the front segments. The cuticle and polypneustic lobes became melanized but did not sclerotize. No puparium was formed.
4. Very young larvae that did not melanize nor sclerotize the cuticle and polypneustic lobes. Intrapuparial development was successfully completed only in larvae of category (1). In several cases, however, the adult emergence of flies in this category was postponed by several days when compared with normally deposited individuals. This interesting phenomenon deserves further investigation because it may have consequences for the timing of emergence in the automated sexing system.

In conclusion, the commitment of brain and other tissues to metamorphosis in tsetse larva is done only a few hours before parturition, when a critical amount of nutrients has been obtained. Unlike in other cyclorrhaphous larvae [35, 36], the critical amount of food necessary for initiating pupal and adult development in tsetse is rather high and more or less constant. This is a developmental adaptation to the peculiar reproductive strategy based on adenotrophic vivipary in the genus *Glossina*. It also explains why undernourishment of the mother causes abortion of a larva rather than prolongation of its intrauterine development.

8. EFFECTS OF A DIPTERAN OOSTATIC HORMONE ON REPRODUCTION IN THE TSETSE FLY (PRELIMINARY STUDIES)

Oostatic or antigonadotropic peptides have been described in a number of insect species. Borovsky et al. [37] isolated a member of this class of regulatory hormones from a mosquito and described it as a decapeptide containing six C-terminus proline residues (H-YDPAPPPPP-OH). In Diptera the compound was found to affect oogenesis through modulation of ovarian ecdysteroid synthesis, gut trypsin synthesis or the release of a neurohormone regulating egg development in the ovaries (signalling termination of vitellogenesis). The gonadotropic effects of the peptide and its C-terminus shortened analogues were also extensively tested on the flesh fly *Neobellieria bullata* (see Slaninova et al. [38]) at the IOCB, Prague. Peptides injected at a dose of 10 nmol/fly affected oogenesis and subsequent hatchability of eggs laid during the first and second gonotrophic cycles. Truncated analogues were even more effective than the original decapeptide. The most active were tetra- and penta-analogues.

Since the peptides were originally discovered and intensely studied in blood feeding insects – mosquitoes, and later they appeared to be active also in the flesh fly, a representative of cyclorrhaphous Diptera, we investigated any effects on oogenesis and associated gonadal functions in the tsetse female.

8.1. Materials and methods

Cohorts of 10 days old mated females of *G. m. morsitans* were injected with 3 nmol (N=26) and 0.3 nmol (N=22) of the oostatic pentapeptide (H-YDPAP-OH) (dissolved in 1.5 μ L of saline) into the thorax, and females injected with 1.5 μ L of Ringer saline (N=22) served as controls. Each group of injected flies was kept in a standard cage and checked daily (for a period of 99 days) for food intake, mortality and larviposition. The peptide was synthesized by J. Hlavacek (IOCB, Prague).

8.2. Results and discussion

No evident differences in feeding behaviour among the three experimental groups were observed. Mortality in the 3-nmol group was significantly higher than in the remaining groups during the first week after injection (Fig. 11). Cumulative data on larviposition showed a decrease in larval production per female in the hormone-injected females during the later gonotrophic stages (Fig. 12). Due to the rather limited numbers of treated insects these results can be considered as preliminary only (they helped to establish the threshold of toxicity) and do not allow proper statistical analysis. The experiment was repeated with larger sets of insects but the results are not yet available; a paper for publication is in preparation.

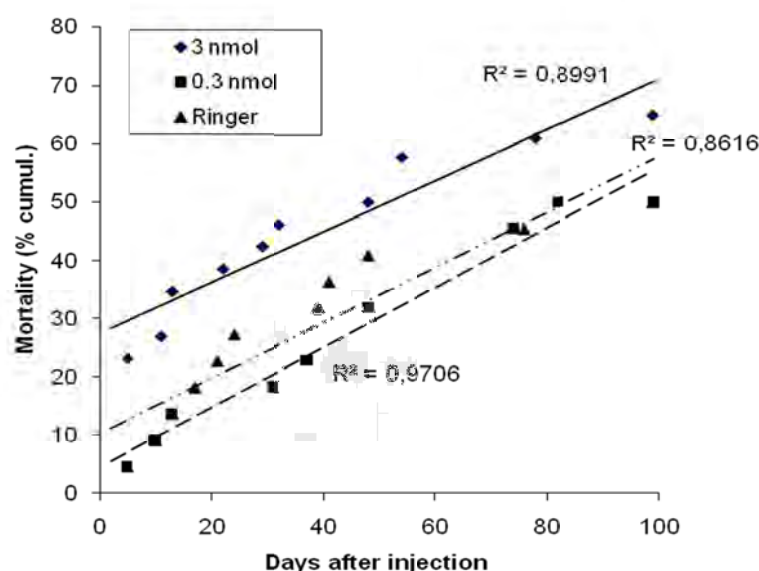


FIG. 11. Mortality of *G. m. morsitans* females injected with 3 nmol (solid line) or 0.3 nmol (dash line) of the oostatic pentapeptide H-YDPAP-OH, or with a solvent (PBS) (dash and dot line) as control.

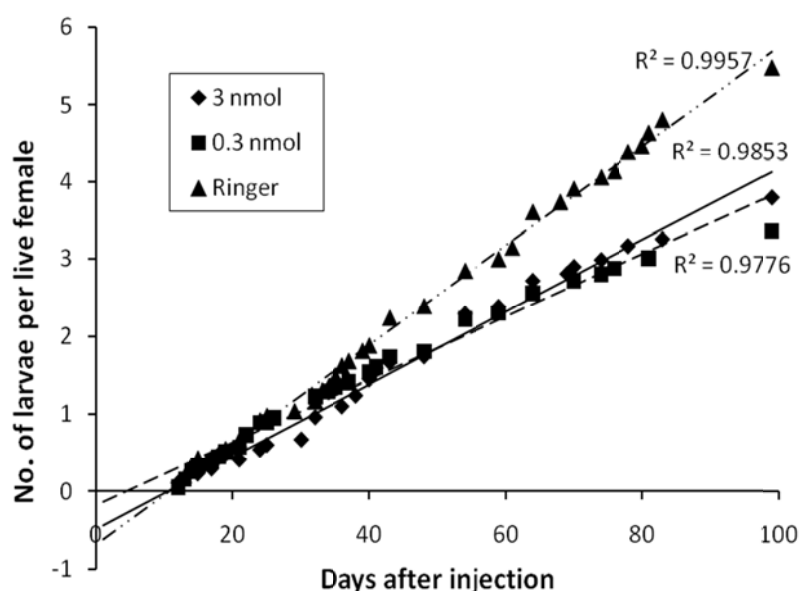


FIG. 12. Larviposition by females injected with 3 nmol (solid line) or 0.3 nmol (dash line) of the oostatic pentapeptide, or with a solvent (PBS) (dash and dot line) as control. The values represent the means per one survived female.

ACKNOWLEDGEMENTS

This work was supported by IAEA Research Contract No. 13405 and by financial support to the Zoological Institute, SAV. The technical assistance of Ms. J. Titzenthalerova (Prague) and Ms. J. Weisova is gratefully acknowledged. We are indebted for gifts of peptide samples from Dr. R.J. Nachnan (USDA, ARS, College Station, TX. USA) and Dr. J. Hlavacek (IOCB, CAS, Prague, CZ).

REFERENCES

- [1] NKOUKA, N., Controle neuro-endocrine de l'ovulation et de la parturition chez un insecte vivipare *Glossina fuscipes fuscipes* (Diptera, Muscidae). C. R. Acad. Sci. Paris Ser. D **285** (1977) 77–80.
- [2] DENLINGER, D.L., SAINI, R.K., CHAUDHURY, M.F.B., Parturition in the tsetse fly *Glossina morsitans*: pattern of activity, sound production and evidence for control by the mother's brain, J. Insect Physiol. **29** (1983) 715–721.
- [3] DENLINGER, D.L., ŽD'ÁREK, J., A hormone from the uterus of the tsetse fly, *Glossina morsitans*, stimulates parturition and abortion, J. Insect Physiol. **43** (1997) 135–142.
- [4] ŽD'ÁREK, J., DENLINGER, D.L., OTIENO, L.H., Does the tsetse parturition rhythm have a circadian basis? Physiol. Entomol. **17** (1992) 305–307.
- [5] PHELPS, R.J., JACKSON, P.J., Factors influencing the moment of larviposition and eclosion in *Glossina morsitans orientalis* Vanderplank, J. Entomol. Soc. S. Africa **34** (1971) 145–157.
- [6] DENLINGER, D.L., Who controls the rhythm of tsetse parturition: mother or larva? Physiol. Entomol. **8** (1983) 25–28.
- [7] ŽD'ÁREK, J., DENLINGER, D.L., Species variation in the response of tsetse flies (*Glossina* spp.: Diptera, Glossinidae) to parturition hormone, European J. Entomol. **94** (1997) 381–383.

- [8] ŽD'ÁREK, J., NACHMAN, R.J., DENLINGER, D.L., Parturition hormone in tsetse *Glossina morsitans*: activity in reproductive tissues from other species and response of tsetse to identified neuropeptides and other neuroactive compounds, *J. Insect Physiol.* **46** (2000) 213–219.
- [9] ŽD'ÁREK, J., MYŠKA, P., ZEMEK, R., NACHMAN, R.J., Mode of action of an insect neuropeptide leucopyrokinin (LPK) on pupariation in fleshfly (*Sarcophaga bullata*) larvae (Diptera: Sarcophagidae), *J. Insect Physiol.* **48** (2002) 951–959.
- [10] NACHMAN, R.J., STREY, A., ZUBRZAK, P., ŽD'ÁREK, J., A comparison of pupariation acceleration activity of pyrokinin-like peptides native to the flesh fly: Which peptide represents the primary pupariation factor? *Peptides* **27** (2006) 527–533.
- [11] VERLEYEN, P., et al., Fraenkel's pupariation factor identified at last, *Dev. Biol.* **273** (2004) 38–47.
- [12] SHAPIRO, S.S., WILK, M.B., An analysis of variance test for normality (complete samples), *Biometrika* **52** (3 and 4) (1965) 591–611.
- [13] MCCALL, P.J., "Chemoecology of oviposition in insects of medical and veterinary importance", *Chemoecology of Insect Eggs and Egg Deposition* (HILKER, M., MEINERS, T., Eds), Wiley InterScience (2002) 265–289.
- [14] NASH, T.A.M., TREWEM, M.A., MOLOO, S.K., Observations on the free larval stage of *Glossina morsitans morsitans* Westw. (Diptera, Glossinidae): the possibility of a larval pheromone, *Bull. Entomol. Res.* **66** (1976) 17–24.
- [15] LEONARD, D.E., SAINI, R.K., Semiochemicals from the anal exudate of larvae of tsetse flies *Glossina morsitans morsitans* Westwood and *Glossina morsitans centralis* Machado attract gravid females, *J. Chem. Ecol.* **19** (1993) 2039–2046.
- [16] SLÁMA, K., Insect hemolymph pressure and its determination, *Acta Entomol. Bohemoslov.* **73** (1976) 65–75.
- [17] ŽD'ÁREK, J., "Neurohormonal factors involved in the control of pupariation", *Neurohormonal Techniques in Insects* (MILLER, T.A., Ed.), Springer-Verlag, New York (1980) 154–178.
- [18] SLÁMA, K., Regulation of respiratory acidemia by the autonomous nervous system (coelopulse) in insects and ticks, *Physiol. Zool.* **67** (1994) 164–174.
- [19] ŽD'ÁREK, J., ZAVADILOVÁ, J., SU, J., FRAENKEL, G., Post-eclosion behaviour of flies after emergence from the puparium, *Acta Entomol. Bohemoslov.* **81** (1984) 161–170.
- [20] ŽD'ÁREK, J., DENLINGER, D.L., Metamorphosis behavior and its regulation in tsetse flies, *Bull. Entomol. Res.* **83** (1993) 447–461.
- [21] DENLINGER, D.L., ŽD'ÁREK, J., Metamorphosis behavior of flies, *Annu. Rev. Entomol.* **39** (1994) 239–262.
- [22] DENLINGER, D.L., ŽD'ÁREK, J., Rhythmic pulses of haemolymph pressure associated with parturition and ovulation in the tsetse fly, *Glossina morsitans*, *Physiol. Entomol.* **17** (1992) 127–130.
- [23] ROBERT, A., GRILLOT, J.P., GUILLEMINOT, J., RAABE, M., Experimental and ultrastructural study of the control of ovulation and parturition in the tsetse fly *Glossina fuscipes* (Diptera), *J. Insect Physiol.* **30** (1984) 671–684.
- [24] ROBERT, A., STRAMBI, A., STRAMBI, C., Haemolymph ecdysteroid levels in female tsetse fly *Glossina fuscipes* (Diptera) during the first reproductive cycle: a comparison between virgin and mated females, *J. Insect Physiol.* **32** (1986) 665–671.
- [25] ROBERT, A., STRAMBI, A., STRAMBI, C., GONELLA, J., Opposite effects of ecdysone and 20-hydroxyecdysone on *in vitro* uterus motility of a tsetse fly, *Life Sci.* **39** (1986) 2617–2622.
- [26] DENLINGER, D.L., MA, W.C., Dynamics of the pregnancy cycle in the tsetse *Glossina morsitans*, *J. Insect Physiol.* **20** (1974) 1015–1026.

- [27] CHAUDHURY, M.F.B., DHADIALLA, T.S., Evidence of hormonal control of ovulation in tsetse flies, *Nature* **260** (1976) 243–244.
- [28] SAMARANAYAKA-RAMASAMY, M., DENLINGER, D.L., Blockage of ovulation and insemination in the tsetse *Glossina morsitans morsitans* with reserpine, *Insect Sci. Appl.* **6** (1985) 159–161.
- [29] ŽD'ÁREK, J., SLÁMA, K., FRAENKEL, G., Changes in internal pressure during puparium formation in flies, *J. Exp. Zool.* **207** (1979) 187–196.
- [30] ŽD'ÁREK, J., DENLINGER, D.L., Wandering behaviour and pupariation in tsetse larvae, *Physiol. Entomol.* **16** (1991) 523–529.
- [31] DENLINGER, D.L., ŽD'ÁREK, J., Rhythmic pulses of hemolymph pressure associated with parturition and ovulation in the tsetse fly, *Glossina morsitans*, *Physiol. Entomol.* **17** (1991) 127–130.
- [32] BRICEÑO, R.D., EBERHARD, W.G., ROBINSON, A.S., Copulation behaviour of *Glossina pallidipes* (Diptera: Muscidae) outside and inside the female, with a discussion of genitalic evolution, *Bull. Entomol. Res.* **97** (2007) 471–488.
- [33] HUYTON, P.M., LANGLEY, P.A., Copulatory behaviour of tsetse flies *Glossina morsitans* and *G. austeni*, *Physiol. Entomol.* **7** (1982) 167–174.
- [34] SLÁMA, K., Extracardiac versus cardiac haemocoelic pulsations in pupae of the mealworm (*Tenebrio molitor* L.), *J. Insect Physiol.* **46** (2000) 977–992.
- [35] ŽD'ÁREK, J., SLÁMA, K., Supernumerary larval instars in cyclorrhaphous Diptera, *Biol. Bull. Woods Hole* **142** (1972) 350–357.
- [36] MYŠKA, P., ŽD'ÁREK, J., The role of 20-hydroxyecdysone in the CNS metamorphosis in flesh fly (*Neobellieria bullata*) larvae (Diptera: Sarcophagidae), *European J. Entomol.* **102** (2005) 21–26.
- [37] BOROVSKY, D., CARLSON, D.A., GRIFFIN, P.R., SHABANOWITZ, J., HUNT, D.F., Mosquito oostatic factor: a novel decapeptide modulating trypsin-like enzyme biosynthesis in midgut, *Fed. Am. Soc. Exp. Biol. J. (FASEB J.)* **4** (1990) 3015–3020.
- [38] SLANINOVA, J., et al., Activity and mechanism of action of insect oostatic peptides in flesh fly, *Bioorg. Chem.* **32** (2004) 263–273.

FLIGHT MUSCLE DEVELOPMENT IN THE MALES OF *GLOSSINA PALLIDIPE* REARED FOR THE STERILE INSECT TECHNIQUE

F. ČIAMPOR, JR., Z. PÁLOŠOVÁ, L. MANCOŠOVÁ, P. TAKÁČ

Institute of Zoology, Slovak Academy of Sciences,
Bratislava, SK-845 06, Slovakia

Abstract

The project's main goal was to study the influence of laboratory conditions on the development of flight muscles and the ability to fly in males of *Glossina pallidipes* Austen. Flight muscles can serve as an important criterion in the quality control of mass reared tsetse flies. All experiments were performed in the research and training facility in Bratislava which provided the flies. The experiments were generally performed by comparing different age groups and groups with different flight activity. To acquire data, several approaches were employed, i.e. classical measurements (residual dry weight, thoracic surface) as well as other alternatives – flight mill, electron microscopy and immunohistochemistry – to visualize and analyse muscle development. The results clearly identified differences in age groups. Slight changes in the development of flight muscles regarding different chances to fly were also detected, but these were not sufficiently significant to decrease the quality of males produced in mass rearing facilities. No distinct trends (rising or declining of amount of metabolites) in the groups studied were detected. The differences were in the amount of analysed metabolic components and the structure of the flight muscles. Our results suggest that, similar to other *Glossina* species, in *G. pallidipes* males the first days after emergence are crucial for successful muscle development. On the other hand, rearing in cages does not negatively influence the quality of males with respect to their ability to fly and actively search for females in the wild after release. We also compared the mating behaviour of irradiated and non-irradiated males. We initiated the development of a functional walk-in field cage in which to rear a small colony of *G. pallidipes* under semi-natural conditions. Our work suggested that outside climatic conditions and suitable cage components, e.g. food source, limit the successful realization of using such a cage for rearing tsetse flies.

1. INTRODUCTION

Interest in flight muscle growth in adult tsetse flies has increased in recent years because of the sterile insect technique (SIT). It has been suggested that flight muscles of tsetse flies reared in the laboratory develop poorly when compared with those of wild flies [1, 2, 3, 4, 5, 6]. If fully viable males are to be reared and then released, this potential problem must be assessed.

The development of thoracic musculature has been studied in several *Glossina* species. Bursell [1] and Langley [7] studied males of *Glossina swynnertoni* Austen. Their findings suggested a slight negative influence of laboratory rearing, and also behavioural differences associated with differences in the development of flight muscles between flies released in the field after confinement in the laboratory and their native counterparts. Dame et al. [2] found a similar retardation of development in laboratory reared *G. morsitans orientalis* Vanderplank. Langley [5] studied *G. morsitans* and *G. austeni* Newstead; he also demonstrated irregular development of the thoracic musculature. Not until 20 days post-emergence (after the ingestion of the tenth bloodmeal) were the thoraces comparable with those of wild flies.

Assessment of the dispersal ability of the target insect is one of the fundamental issues in SIT programmes. According to Bursell and Kuwengwa [6], the rate of development of flight muscles during the first hunger cycle of the adult tsetse can be increased to normal levels if the laboratory reared flies are induced to fly soon after they have taken their first bloodmeal.

Van Dam et al. [8] showed a correlation between flight and food source. Darlington [9] suggested that species with highly unstable habitats and food sources would have the greatest need for dispersal, and with stabilization of the environment and food resource a loss in dispersal power can occur. Concerning differences between sexes, studies of the flight behaviour found no differences in flight propensity between males and females [10, 11, 12, 13]. Van Dam et al. [8] suggested a difference in flight in relation to the time of day (morning versus evening). Evening flights occur more frequently because evening temperatures are usually higher than morning with the same light intensity.

Another important factor influencing the success of the SIT is mating ability. However, Buxton [14] reported that little is known about the mating behaviour of *Glossina pallidipes* Austen in nature. Limited mating studies have been carried out in the laboratory [15].

The behaviour of laboratory reared males in the field is of particular importance to the SIT for controlling *Glossina* species [3]. The development of the thoracic muscles in relation to the flight behaviour of *G. pallidipes* is, however, still unknown.

Another way to study development and activity of the flight muscles is to search for the ultrastructural changes during development of the fly or tracing changes in the utilization of energy within these processes [16]. Many physiological processes require muscular contraction, and we need to understand how muscles contract and release such that these movements are possible. Tsetse flies have asynchronous fibrillar muscles, which are characteristic for Diptera. Muscle fibres (myofibrils) are a multinucleate syncytium indicating that they arise by the fusion of many myoblast cells. Myofibrils are hexagonally arranged in the fibre, and thus there is no empty space.

Myosin and actin are fibrous proteins, and they form the most important components of muscle cells. Actin assembles into thin filaments which together with thick myosin filaments provide the framework for muscle contraction (filaments slide past each other). The energy for the actin-myosin interaction is derived from the hydrolysis of adenosine triphosphate (ATP) by an Mg^{2+} ATP-ase residing in the myosin molecule.

ATP analogs have played an important role in the study of crossbridge structure in insect flight muscle [17]. The hydrolysis of ATP is not directly coupled to the power stroke. Rather, the energy from ATP hydrolysis is stored in a strained protein conformation and subsequently released. Asynchronous muscles of Diptera are less sensitive to calcium flux, which is also correlated with reduced development of sarcoplasmic reticulum.

Mitochondria are very important for muscle growth and activity. Mitochondrial volume increases at a relatively uniform rate but myofibril volume increases until about the third and fourth bloodmeals. Sarcoplasmic volume correspondingly declines steeply at first, thereafter more slowly [18]. The ultrastructural changes in dipteran flight muscles were studied by Auber [19] in *Calliphora erythrocephala* Meigen and by Gregory et al. [20] in *Lucilia cuprina* (Wiedemann). Auber [19] reported that the number of thick filaments in a cross-section of a single myofibril increases from 669–1186 at emergence to about 2000 at 10 days, and does not rise significantly after that — up to 35 days after emergence. The myofibrils and mitochondria of tsetse flies are poorly developed structurally at the time of emergence. In the period from emergence to about the fourth bloodmeal both the mitochondria and the myofibrils increase in volume, whereas after the fourth feed only the mitochondria continue to increase in volume [21]. During growth there is also an increase in the overall diameter of the flight muscle fibres [22], which has been shown to be the combined result of an increase in

the diameter of the myofibrils by addition of new filaments and an increase in the size of the mitochondria [21, 23]. As the fly matures it develops more muscle and a greater density of mitochondria in that muscle, resulting in the production of enough energy to increase wingbeat frequency [24].

2. MATERIALS AND METHODS

For all experiments, males of *G. pallidipes* were provided by the rearing facilities in Bratislava (Slovakia) and Seibersdorf (Austria). Males were divided into groups of different age and with a different opportunity to fly (reared in cages of different size or induced to fly).

2.1. Morphology

Most studies noted that the residual (= not fatty) dry weight of the non-teneral thorax is much greater than of the teneral, and it is thought that the post-teneral development of thoracic musculature might account for the increased firmness of the thorax. Because muscle size is normally related to the overall size of the fly, thoracic measurements are used as an index of overall size [25, 26].

The relation between the size of the thoracic surface and the residual dry weight of the thorax was determined in teneral and non-teneral males of *G. pallidipes* of various ages held under laboratory conditions. All non-teneral flies were fed every day. Flies from tests were frozen for subsequent determination of the thoracic residual dry weight by the techniques of Loder [27]. The thoracic surface was measured using a stereomicroscope with an ocular grid, and correlation of the residual dry weight (RDW) and thoracic surface was analysed using ANOVA.

2.2. Flight mill

A flight mill was used to assess the flight performance of *G. pallidipes* males. For the experiment, three groups were set up according to the amount of space available for flight as well as the level of disturbance (increased number of flies in the cage, light stimuli). Within these groups, males of several ages fed every day were investigated. The following groups were tested: (1) one male reared in a small cage (diam. 4 cm, height 6 cm) under standard conditions (23–24°C, 70–74% RH), (2) 64 males reared in a standard cage (diam. 20 cm, height 5 cm) under standard conditions (23–24°C, 70–74% RH), and (3) 300 males reared in a big cage (45 × 45 × 45 cm) under standard conditions (23–24°C, 70–74% RH) disturbed by light for 10 min every hour from 0600 to 1800.

On the notum of each fly a thin wire was attached and mounted on a flight mill. Flies from each group were left to fly for 10 min.

2.3. Immunohistochemistry

For the histochemistry of thoracic muscles, males of various ages fed daily (1, 7, and 18 days old and 1-month old males of *G. pallidipes*) and kept in three types of cages (classic rounded rearing cage, large square cage with sides 60 cm, walk-in field cage) were investigated. In the square cage, flies were induced to fly by light. The muscles were frozen in liquid nitrogen and cryostat slices were prepared. Sections were placed on slides and immunohistochemically stained. Several different reactions were made to observe differences in muscle development.

2.4. Electron microscopy

Ultrastructural changes in the flight muscle growth in laboratory reared males of *G. pallidipes* were studied using transmission electron microscopy. Non-teneral flies of various ages were investigated to see the differences in muscle development due to the age of the flies. For transmission electron microscopy, muscle tissue was fixed in 2.5% buffered glutaraldehyde, postfixed in 1% osmium tetroxide and then conventionally prepared.

2.5. Mating behaviour [15, 28]

Mating competitiveness was tested with *G. pallidipes* flies that emerged from pupae stored at 23–24°C. On the day of the test all males were 10 days old and females 3 days old.

One group of males was chilled and irradiated at 110 Gy (on the day before test) in a commercial irradiator at Seibersdorf, Austria. Irradiated males were marked with orange colour using a dot of polymer paint on the notum. A second group of males was the control. Flies were not fed on the day of the test.

A walk-in field cage (3 × 3 × 2 m) was used for behavioural tests. It was placed inside a greenhouse with natural light conditions but with temperature and RH controlled. Two *Ficus* trees about 1.5-m high were placed in the cage. All tests began at 1000 and finished after 2 h. All environmental conditions were monitored every 30 min during the test. Temperature and RH were measured in the centre of the cage. Light intensity was averaged from three levels (top, foliage, bottom) using a light meter.

Irradiated and non-irradiated males were first introduced into the centre of the cage, followed immediately by virgin females. Mating pairs were collected in single tubes and the mating time was recorded. After mating, males were discarded, but females were kept overnight under standard colony conditions and dissected the next day to determine insemination. The test was replicated three times.

3. RESULTS AND DISCUSSION

3.1. Residual dry weight (RDW) and thoracic surface

Changes in the RDW of the thorax were investigated in *G. pallidipes* males divided into three groups: teneral, 3 days old and 5 days old flies. The lines were calculated from regression formulae. Statistical analyses revealed a statistically significant relationship between the thoracic surface and thoracic RDW at the 99% confidence level in all test groups (example in Fig. 1).

There were no statistical differences in the surface area among test groups, but differences in RDW were significant in relation to fly age, i.e. a gradual increase in RDW of the thorax with age.

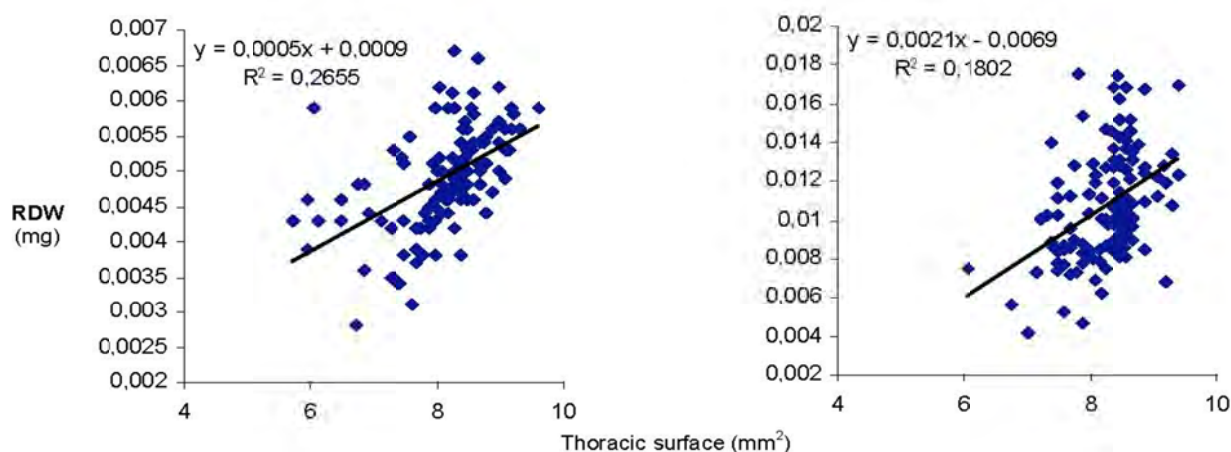


FIG. 1. Relation between thoracic surface and thoracic RDW in unfed tsetse flies ($N=128$; $F=45.19$; $P<0.001$) and in fed tsetse flies ($N=137$; $F=29.46$; $P<0.001$).

3.2. Ultrastructural changes in flight muscles

Since there were no ultrastructural data on the thoracic muscles of *G. pallidipes*, we described their anatomy in detail (Fig. 2). Observations with an electron microscope (EM) on the different fly groups clearly showed a progressive, age related increase in the volume of myofibrils in muscle fibres, but sarcoplasmic volume correspondingly declined. Development and differentiation of sarcomere units were clearly visible in connection with increasing age of flies (Fig. 3).

We also studied the ultrastructure of the thoracic muscles of flies reared in cages of different sizes (rearing cage, big square cage, walk-in field cage). Transmission electron microscopy (TEM) showed that age related changes in the size of sarcomeres and amount of myofibrils are not cage-size dependent. In the muscles of older flies, vacuoles, glycogen deposits and mitochondria of different size were observed. Flies (18 days old) kept in a walk-in field cage had slightly malformed mitochondria. Surprisingly, we observed a lack of M lines in flies taken from a rearing facility. Z lines were uneven, and myofibrils were separated from each other in regular segments by cisterns of sarcoplasmic reticulum (Fig. 4). These features differed in the test age groups.

Unfortunately, we lack comparable data from wild flies, so it is difficult to know if these changes in the anatomy of *G. pallidipes* flight muscles are related to rearing conditions.

3.3. Immunohistochemistry

Immunohistochemical experiments were designed to describe changes in the amount of flight muscles, metabolism of energy sources (ATP, glycogen) and enzyme activity as secondary parameters of flight muscle development and fitness according to different flight activities [29, 30]. The following tests and reactions were used:

- **Hematoxylin** – stains nuclei to blue and cytoplasm to red, to visualize amount of the flight muscles.
- **ATP-ases** – demonstrate activity of actomyosin ATP-ase. Two types of ATP-ases were used: myosin ATP-ase in CaCO_3 reaction and mitochondrial and cell membrane ATP-ases in Pb reaction. Marked myofibrillar actomyosin ATP-ase activity was observed in all age and cage groups, but significant trends within the groups were not recorded. It was shown that activity of ATP-ase localized in mitochondria and the cell membrane was higher in the

muscles of younger flies than the muscles of older ones, whereas the activity of ATP-ase localized in myofibrils increased with age. This is probably due to different activity and energy metabolism of mitochondria during the development of the fly, but present results do not sufficiently support this hypothesis.

- **PAS** – (periodic acid-Schiff reaction) was used to demonstrate the presence of glycogen. The amount of colour developed by the reaction is dependent primarily on the amount of reactive glycol structure present in the tissues. The results suggested that the amount of glycogen is not age or cage dependent.
- **SDH** – succinic dehydrogenase activity was used to monitor thoracic muscle energy metabolism. In general, enzyme activity increased with age, but there were no apparent differences among different age groups.

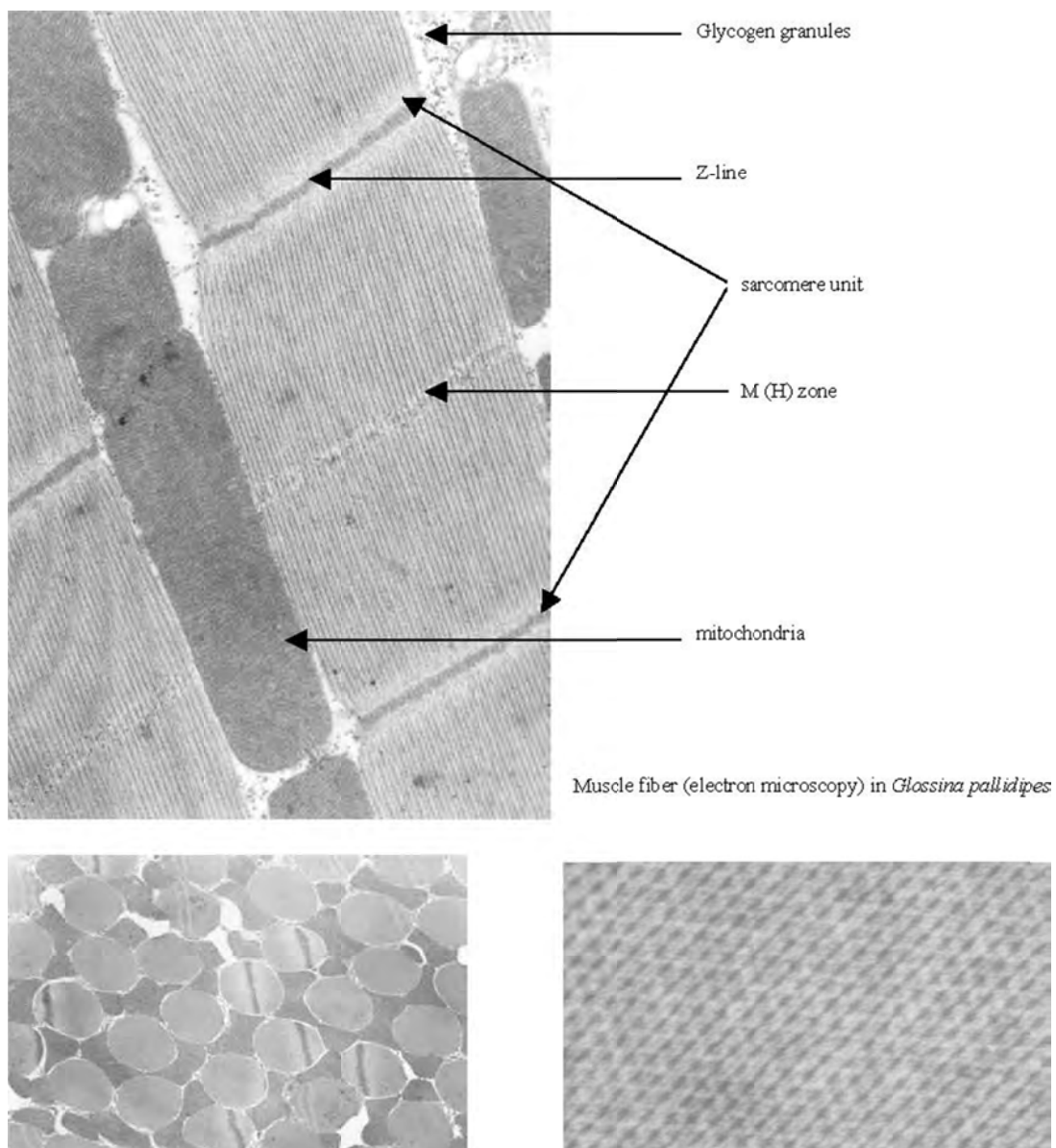


FIG. 2. Longitudinal and transverse sections of muscle fibres (EM) with hexagonally arranged myofibrils.

The results of the immunohistochemical experiments showed differences in muscle development in different ages and rearing conditions. The differences are in the amounts of analysed metabolic components, as well as in the structure of the flight muscles. The changes were clearly visible, however, there were no distinct trends in the rising or declining of the amounts of metabolites in all test groups.

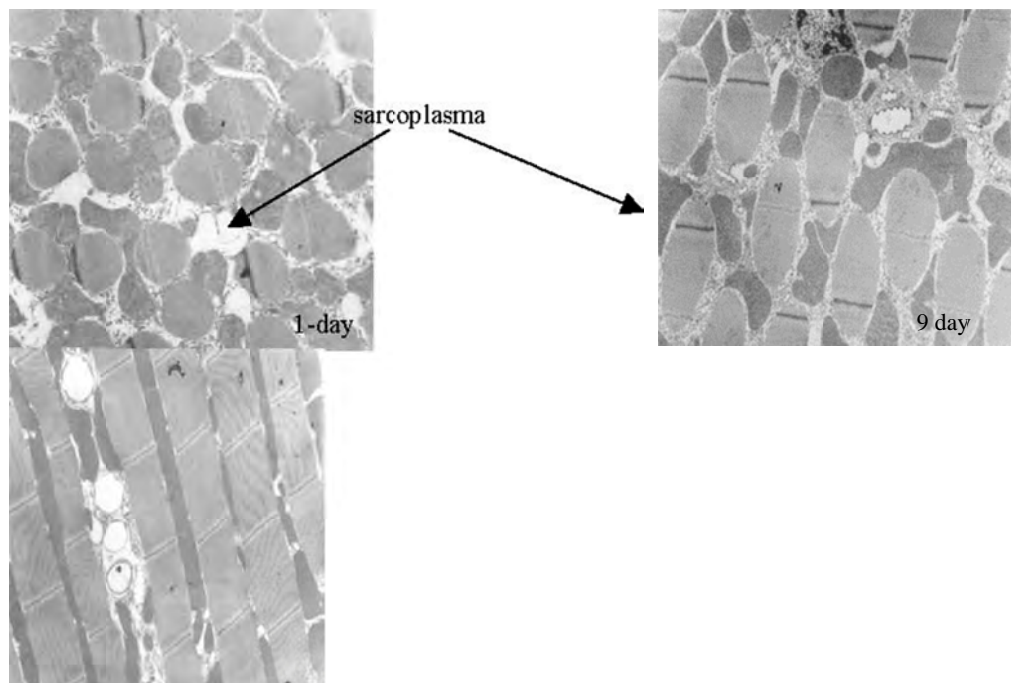


FIG. 3. Differentiation of sarcomere units, and decrease in sarcoplasmic volume with respect to the age of flies (1 day and 9 days old flies, electron microscopy).

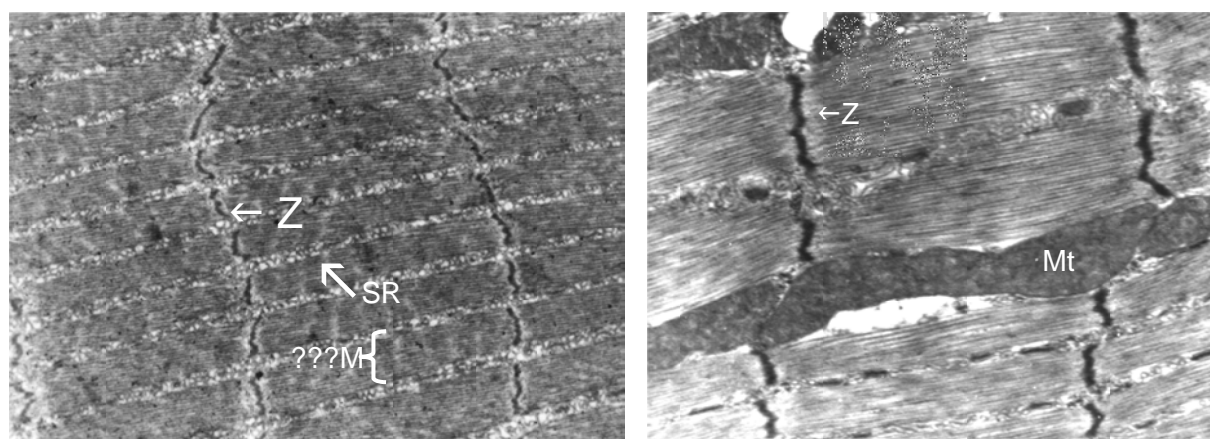


FIG. 4. Ultrastructure of dorsal flight muscles in 18 days old flies (Z – Z-line, M – M-line (missing), SR – sarcoplasmic reticulum, Mt - mitochondria).

3.4. Flight mill

Studies of the flight activity showed no significant differences in respect to different temperatures and light intensity during rearing. The data indicated a lower flight propensity after feeding. In spite of the fairly accurate measurement of distance flown, it cannot be correlated with distance flown under natural conditions.

In the first group, flight activity increased gradually from 1 to 16 days after emergence. The flight ability of males fed 20 and 25 times was distinctly lower. Within the second group, four significant divisions were observed: 1) unfed males, 2) males fed 1–4 times, 3) males fed 6–16 times, and 4) males fed 18–19 times with low flight activity. Within the third group, the flight activity increased almost continuously until the eleventh bloodmeal. Males fed 15 and 20 times showed decreased flight activity.

The results suggested that flight activity decreases distinctly after feeding; flies likely rest and digest the bloodmeal [31]. The activity also decreased after 20 days. This is likely correlated with the lifetime of males.

Finally, it should be noted that a flight mill, even though providing valuable data on tsetse flight activity, is not a fully reliable device for tsetse flies [32, 33]. It is well known that tsetse flies fly very economically, often resting and searching for a food source. This was observed when using a flight mill; often flies flew only halfway around and then stopped, even though the mill made the complete round.

3.5. Mating behaviour (Comparison of mating behaviour — irradiated and non-irradiated males) [15]

The propensity of mating (PM) is defined as the ratio of total number of pairs that mated and total number of the females released (Table 1). The relative mating index (RMI) is defined as the ratio of the number of pairs of one group (irradiated or non-irradiated males) and the total number of mating pairs; the value ranges from 0 to 1. The relative mating performance (RMP) is defined as the ratio of the difference between the number of matings of both groups and the total number of matings; the value ranges from –1 to +1. The spermathecal value (MSV) is obtained by assessing the content of the two spermathecae (S) in a mated female. Spermathecae are classed as empty (0), one quarter full (0.25), one half full (0.5), three quarters full (0.75) or full (1). Chi-square analysis and Z-test for comparison of proportions were carried out as appropriate (Table 2).

Flies tended to rest on the darker surface. Most of the successful mating pairs were formed in the first 50 min after release into the cage.

TABLE 1. AVERAGE NUMBER OF PAIRS, RELEASED FLIES, NON-FLIERS AND DEAD FLIES

Test	Released females	Irrad. males	Non-irrad. males	Total released	Non-fliers	Dead flies	Mating pairs	Pairs with irrad. males	Pairs with non-irrad. males
1	22	20	20	62	0	4	11	3	8
2	20	17	19	56	1	5	13	3	10
3	26	23	26	75	0	2	16	4	12
Total	68	60	65	193	1	11	40	10	30

TABLE 2. AVERAGE DATA OF RMI, PM, RPM, S, MSV AND MATING PERIOD (MIN)

Test	RMI irrad. ♂s	RMI non-irrad. ♂s	PM	RPM	S'theca 1 (irrad. ♂s)	S'theca 2 (irrad. ♂s)	MSV S 1	S'theca 1 (non-irrad. ♂s)	S'theca 2 (non-irrad. ♂s)	MSV S 2	Mating period with irrad. ♂s	Mating period with non-irrad. ♂s
1	0.27	0.72	0.5	0.45	0.25	0.25	0.5	0.5	0.5	1	17.3	14.1
2	0.23	0.77	0.65	0.54	0.25	0.25	0.5	1	0.5	1.5	19.4	12.45
3	0.25	0.75	0.62	0.5	0.25	0.5	0.75	1	1	2	13.2	17.5

The irradiated and non-irradiated males were allowed to mate with the non-irradiated virgin females at the ratio 2:1. The mating of treated (110 Gy) males with females produced 25% viable adults. Only one female from 68 mated with an irradiated male had empty spermathecae. RMI and MSV values were higher in females mated with non-irradiated males than in those mated with irradiated males, but an analysis of variance (Z-test, $P>0.05$) did not confirm a significant difference for both indices. No significant differences in the mating period were observed between irradiated and non-irradiated pairs.

3.6. Development of a walk-in field cage

To assess accurately the influence of laboratory rearing on flight muscle development, a comparison between naturally developed flies and laboratory reared flies should be made. However, in the experiments described above, we lacked naturally developed flies. Since we were unable to obtain flies caught in the wild, we developed a functional walk-in field cage [15], i.e. a cage with adequate space for free-flying flies, a food source and at least semi-natural environmental conditions. In this cage we could rear flies without the constraints of usual laboratory conditions.

The development of the walk-in field cage started in 2005. We used a small greenhouse (3 × 3 × 2 m), shaded by dense green fabric from the outside and painted on the inside. Two *Ficus benjamina* trees were placed in the greenhouse to provide places for the flies to rest and hide. We used a heater and a humidifier to create appropriate environmental conditions. For feeding, initially we used a small aluminium plate with a membrane heated by a water bath, but with no effect. A workable feeding unit is essential for a functional system, i.e. flies can emerge, feed and successfully survive. Therefore, in 2006, we improved the feeding unit by several steps. The first was the plate with blood heated by a water bath was put on the floor. Even if we used colour patterns and CO₂ as attractants known from the literature and experience, this device did not work, i.e. the flies ignored it.

The next step was a sphere hung from the top of the greenhouse. We also used a black and blue colour pattern, and CO₂ to attract flies (Figs 5–7). The blood was accessible from the bottom of the sphere, and it was heated either by an electric bulb (which did not work) or warm water pumped in a tube from a water bath (this worked) (Figs 8–10). As the flies are attracted also by movement, a tiny rope was attached to it to allow manual moving of the feeding unit. Even though we used several attractive features (colour pattern, movement, CO₂), the results were insufficient; only a few flies, about 1%, were able to locate the blood source and feed. Probably CO₂ is not a very useful attractant in such a small space, i.e. the greenhouse is very soon saturated with the gas making it impossible to locate the source, so we replaced it with alcohol extract or fresh leaves of the invasive plant *Lantana camara*. This improved attraction to 20%. The final step in the improvement of the feeding unit was attaching a blue stripe across the black feeding membrane (Fig. 11), which appeared to be very useful. This simple step increased attractiveness up to 82–93% if the environmental conditions were optimal. In 2007 we tested other chemical attractants, as suggested in the literature, and repeated experiments with *Lantana*. All attractants were compared with the feeding unit without an attractant. Each experiment was done in 5 days and, besides recording conditions inside (temperature, RH), outside weather conditions (temperature, RH, rainfall, wind) were also recorded.

***Lantana camara* extract and leaves** – We conducted experiments with *Lantana camara* leaves and alcohol extract in 2006 and 2007, but the results showed much less success in 2007. It is very likely that this poorer result was caused by outside weather conditions, which influenced conditions inside the greenhouse. Therefore, in future, more attention should be paid to decrease this influence (Figs 12 and 13).

Buffalo urine - Buffalo urine was applied using plastic odour-permeable bags, with 0.5 mL of attractant. The bags were attached close to the feeding membrane. The percentage of fed flies was 8–50% (Fig. 14), which suggested that outside conditions influenced the test.

Mix (p-cresol, octenol, 3-n-propylphenol in the proportion 8:4:1) - This mix of attractants was applied in the same way as buffalo urine. The percentage of fed flies during the experiment was 34–46% (Fig. 15).

Feeding unit without chemical attractant - This experiment was performed for comparison, testing if using attractants in the greenhouse was really necessary. The percentage of fed flies during the experiment was 20–34% (Fig. 16). Therefore, flies are attracted by colour pattern, shape and movement of the feeding unit without using chemical attractants, but with a lower effect.

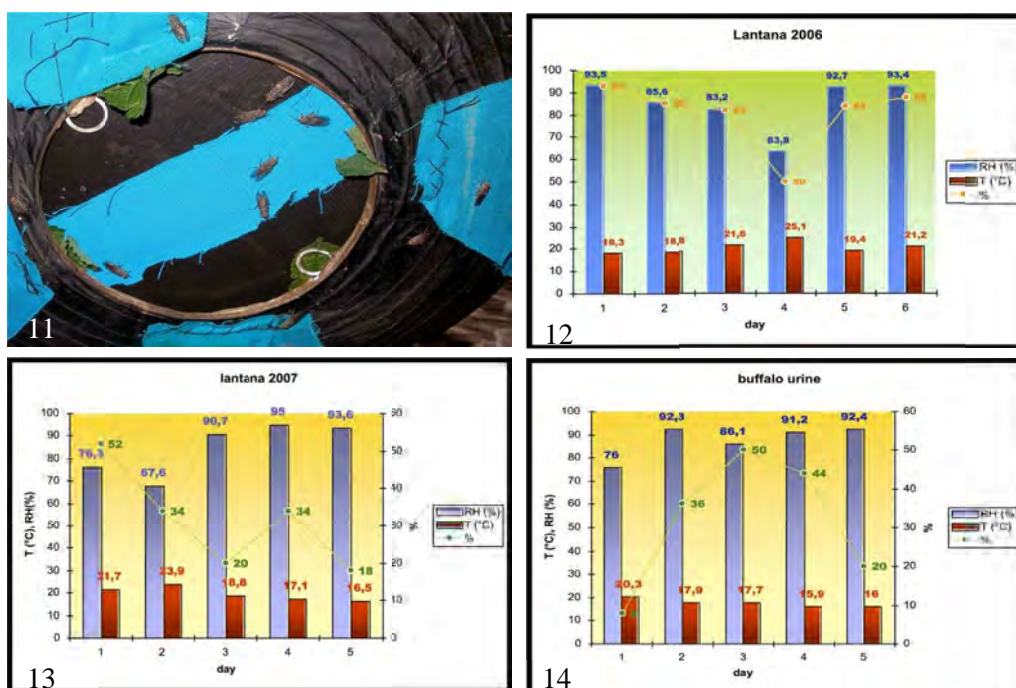
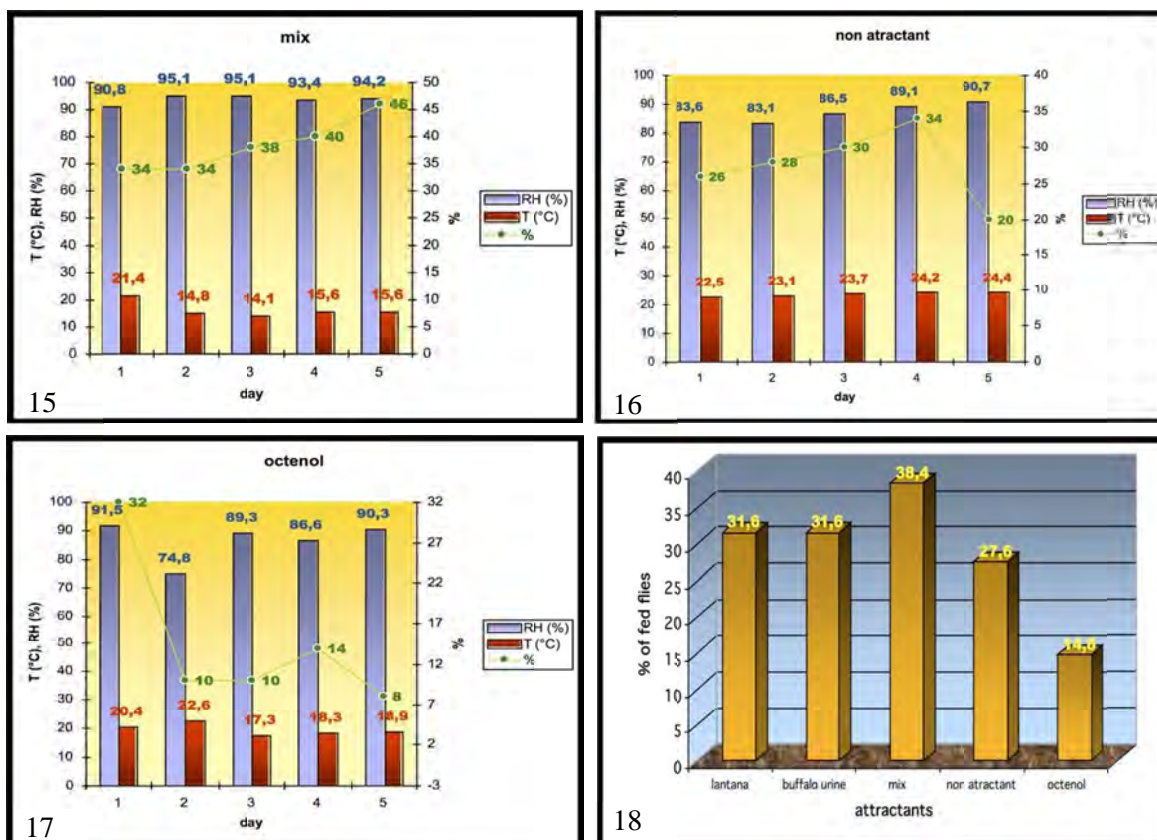


FIG. 11. Membrane with blue stripe and Lantana leaves attached close to the feeding membrane. FIGS 12–14. Results of tests with different attractants (blue columns – average RH during feeding period, red columns – average temperature during feeding period, line – feeding success (%)), X axis – five days of the experiment; 12 – Lantana year 2006, 13 – Lantana year 2007, 14 – buffalo urine.



FIGS 15–18. Results of tests with different attractants (blue columns – average RH during feeding period, red columns – average temperature during feeding period, line – feeding success (%)), X axis – five days of the experiment; 15 – attractant mix, 16 – test without attractant, 17 – octenol, 18 – average effectiveness of tested attractants (experiments done in 2007).

Octenol – Even though 1-octen-3-ol is described in the literature as a poor attractant, we tested it anyway. The percentage of fed flies during the experiment was 8–32% (Fig. 17), which resulted in the lowest attraction among chemicals tested.

Considering the results of the tests with attractants (Fig. 18), it is likely that their effectiveness was significantly influenced by overall in cage conditions rather than their direct attractiveness to flies. For example, we obtained different results when using *Lantana* in 2006 and 2007. Also, it is clear that the functioning of attractants was not temperature and humidity dependent. Unfortunately, despite our efforts, outside weather conditions influenced inside conditions, and thus the walk-in field cage did not work as a closed system. It is very likely that, in weather conditions such as in Slovakia, a much more isolated system should be developed to test and improve its components.

ACKNOWLEDGEMENTS

This study was supported by the IAEA Research Contract 12542. We thank A. G. Parker for invaluable help and comments during the survey, and A. Dyck for editorial help.

REFERENCES

- [1] BURSELL, E., Post-teneral development of the thoracic musculature in tsetse flies, *Proc. R. Entomol. Soc. Lond. (A)* **36** (1961) 69–74.
- [2] DAME, D.A., BIRKENMEYER, D.R., BURSELL, E., Development of the thoracic muscle and flight behaviour of *Glossina morsitans orientalis* Vanderplank, *Bull. Entomol. Res.* **59** (1969) 345–350.
- [3] DAME, D.A., BIRKENMEYER, D.R., NASH, T.A.M., JORDAN, A.M., The dispersal and survival of laboratory-bred and native *Glossina morsitans morsitans* Westw. (Diptera: Glossinidae) in the field, *Bull. Entomol. Res.* **65** (1975) 453–457.
- [4] LANGLEY, P.A., The control of digestion in the tsetse fly, *Glossina morsitans*: a comparison between field flies and flies reared in captivity, *J. Insect Physiol.* **13** (1967) 477–486.
- [5] LANGLEY, P.A., Post teneral development of thoracic flight musculature in the tsetse flies *Glossina austeni* and *Glossina morsitans*, *Entomol. Exp. Appl.* **13** (1970) 122–140.
- [6] BURSELL, E., KUWENGWA, T., The effect of flight on the development of flight musculature in the tsetse fly, *Glossina morsitans*, *Entomol. Exp. Appl.* **15** (1972) 229–237.
- [7] LANGLEY, P.A., The effect of environment and host type on the rate of digestion in the tsetse fly *Glossina morsitans* Westw., *Bull. Entomol. Res.* **57** (1966) 39–48.
- [8] VAN DAM, W.A., WILLIAMS, R.N., TAYLOR, R.A.J., Flight performance of some nitidulid beetles (Coleoptera) using a computer-monitored flight mill, *J. Agric. Urban Entomol.* **17** (2000) 143–151.
- [9] DARLINGTON, P.J., Carabidae on tropical islands, especially the West Indies, *Biotropica* **2** (1970) 7–15.
- [10] COOTER, R.J., Studies on the flight of black-flies (Diptera: Simuliidae). I. Flight performance of *Simulium ornatum* Meigen, *Bull. Entomol. Res.* **72** (1982) 303–317.
- [11] BLACKMER, J.L., PHELAN, P.L., Behaviour of *Carphophilus hemipterus* in a vertical flight chamber: transition from phototactic to vegetative orientation, *Entomol. Exp. Appl.* **75** (1991) 137–148.
- [12] MORIYA, S., A preliminary study on the flight ability of the sweetpotato weevil, *Cylas formicarius* (Fabricius) (Coleoptera: Apionidae) using a flight mill, *Appl. Entomol. Zool.* **30** (1995) 244–246.
- [13] SHIRAI, Y., Laboratory evaluation of flight ability of the Oriental corn borer, *Ostrinia furnacalis* (Lepidoptera: Pyralidae), *Bull. Entomol. Res.* **88** (1998) 327–333.
- [14] BUXTON, P.A., The natural history of tsetse flies, *Mem. Lond. Soc. Hyg. Trop. Med.* **10** (1955) 1–816.
- [15] MUTIKA, G.N., OPIYO, E., ROBINSON, A.S., Assessing mating performance of male *Glossina pallidipes* Austen (Diptera: Glossinidae) using a walk-in field cage, *Bull. Entomol. Res.* **91** (2001) 281–287.
- [16] BURSELL, E., TAYLOR, P., An energy budget for *Glossina* (Diptera: Glossinidae), *Bull. Entomol. Res.* **70** (1980) 187–196.
- [17] SCHMITZ, H., et al., Electron tomography of insect flight muscle in rigor and AMPPNP at 23°C, *J. Mol. Biol.* **264** (1996) 279–301.
- [18] BURSELL, E., Development of mitochondrial and contractile components of the flight muscle in adult tsetse flies, *Glossina morsitans*, *J. Insect Physiol.* **19** (1973) 1079–1086.
- [19] AUBER, J., La myofibrillogenèse de muscle strié - I. Insectes, *J. Microsc.* **8** (1969) 197–232.
- [20] GREGORY, D.W., LENNIE, R.W., BIRT, L.M., An electron-microscopic study of flight muscle development in the blowfly *Lucilia cuprina*, *J. R. Microsc. Soc.* **88** (1968) 151–175.

- [21] ANDERSON, M., FINLAYSON, L.H., Ultrastructural changes during growth of the flight muscles in the adult tsetse fly, *Glossina austeni*, J. Insect Physiol. **19** (1973) 1989–1997.
- [22] GLASGOW, J.P., GLASGOW, S., Histology of the growth of flight muscles in *Glossina* (Diptera: Muscidae), Proc. R. Entomol. Soc. Lond. (A) **37** (1962) 35–36.
- [23] ANDERSON, M., FINLAYSON, L.H., The effect of exercise on the growth of mitochondria and myofibrils in the flight muscles of the tsetse fly, *Glossina morsitans*, J. Morphol. **150** (1976) 321–326.
- [24] HARGROVE, J.W., Some changes in the flight apparatus of tsetse flies, *Glossina morsitans* and *Glossina pallidipes* during maturation, J. Insect Physiol. **21** (1975) 1485–1489.
- [25] BURSELL, E., The water balance of tsetse fly, Trans. R. Entomol. Soc. Lond. **111** (1959) 205–235.
- [26] BURSELL, E., The measurement of size in tsetse flies (*Glossina*), Bull. Entomol. Res. **51** (1960) 33–37.
- [27] LODER, P.M.J., Size of blood meals taken by tsetse flies (*Glossina* spp.) (Diptera: Glossinidae) correlates with fat reserves, Bull. Entomol. Res. **87** (1997) 547–549.
- [28] (FAO/IAEA) Food and Agriculture Organization of the United Nations/International Atomic Energy Agency, FAO/IAEA standard operating procedures for mass-rearing tsetse flies, IAEA, Vienna, Austria (2006).
http://www-naweb.iaea.org/nafa/ipc/public/Tsetse_Rearing_SOP_web.pdf
- [29] BURSELL, E., Substrates of oxidative metabolism in dipteran flight muscle, Comp. Biochem. Physiol. **52** (1975) 235–238.
- [30] D’COSTA, M.A., RICE, M.J., Localization and quantification of glycogen in the tsetse fly and the blowfly, Comp. Biochem. Physiol. **45 B** (1972) 483–485.
- [31] BURSELL, E., BILLING, K.C., HARGROVE, J.W., MCCABE, C.T., SLACK, E., Metabolism of the bloodmeal in tsetse flies, Acta Trop. **31** (1974) 297–320.
- [32] TAYLOR, R.A.J., NAULT, L.R., STYER, W.E., CHENG, Z.-B., Computer-monitored, 16-channel flight mill for recording the flight of leafhoppers (Homoptera: Auchenorrhyncha), Ann. Entomol. Soc. Am. **85** (1992) 627–632.
- [33] YAMANAKA, T., TATSUKI, S., SHIMADA, M., Flight characteristics and dispersal patterns of fall webworm (Lepidoptera: Arctiidae) males, Environ. Entomol. **30** (2001) 1150–1157.

RECORDING AND ANALYSIS OF TSETSE FLIGHT RESPONSES IN THREE DIMENSIONS

P.M. GUERIN¹, A. GURBA¹, V. HARRACA¹, J.-L. PERRET¹, S. CASERA², S. DONNET²

¹Institute of Biology and ²Institute of Informatics,
University of Neuchâtel,
Rue Emile-Argand 11, Case Postale 158,
2009 Neuchâtel, Switzerland

Abstract

Recording and analysing three dimensional (3D) motions of tsetse flies in flight are technically challenging due to their speed of flight. However, video recording of tsetse fly flight responses has already been made in both wind tunnels and the field. The aim of our research was to study the way tsetse flies exploit host odours and visual targets during host searching. Such knowledge can help in the development of better trapping devices. We built a wind tunnel where it is possible to control environmental parameters, e.g. temperature, relative humidity and light. The flight of the flies was filmed from above with two high speed Linux-embedded cameras equipped with fish-eye objectives viewing at 60° from one another. The synchronized stereo images were used to reconstruct the trajectory of flies in 3D and in real time. Software permitted adjustment for parameters such as luminosity and size of the tsetse species being tracked. Interpolation permitted us to calculate flight coordinates and to measure modifications of flight parameters such as acceleration, velocity, rectitude, angular velocity and curvature according to the experimental conditions. Using this system we filmed the responses of *Glossina brevipalpis* Newstead obtained from a colony at the IAEA Entomology Unit, Seibersdorf, Austria to human breath presented with and without a visual target. Flights lasting up to 150 s duration and covering up to 153 m were recorded. *G. brevipalpis* flights to human breath were characterized by wide undulations along the course. When a visual target was placed in the plume of breath, flights of *G. brevipalpis* were more tightly controlled, i.e. slower and more directed. This showed that after multiple generations in a laboratory colony *G. brevipalpis* was still capable of complex behaviours during bloodmeal searching.

1. INTRODUCTION

Tsetse flies locate animals for a blood meal using visual cues and, outside the visual range, by using olfaction to detect host odours. A variety of traps and targets have been developed for controlling tsetse flies based on their responses to visual stimuli. The effectiveness of these devices can be improved when chemical stimuli identified from animals are dispensed in the vicinity. Recording and analysing three dimensional (3D) motions of tsetse flies are technically challenging due to their speed of flight. However, video recording of tsetse flies in flight has already been made in both wind tunnels and in the field in an effort to understand their responses to visual and olfactory cues. The aim of our research was to extend our knowledge of the way tsetse flies exploit these sensory inputs during host searching. Such knowledge could help in the optimization of traps and targets.

2. ELECTROANTENNOGRAM AND WIND TUNNEL FOR QUALITY CONTROL

The strength (in mV) of the electroantennogram (EAG) [1] responses in tsetse flies has been shown to correlate well with fly behaviour. For this reason the EAG is a very practical laboratory neurophysiological method to test the effects of candidate chemostimuli on tsetse species [2], and to examine the sensitivities of flies of different provenance, wild versus colony flies, for example, to known chemostimuli.

Wind tunnels provide a near physical mimic of the environment where factors such as temperature, relative humidity (RH), light and wind velocity can be manipulated to produce a particular experimental situation [3]. Test stimuli are delivered at the upwind end of the wind tunnel and carried by the laminar airflow downwind to where the insects are released. When the stimulus activates the insect it subsequently progresses upwind by anemotaxis to the source. Video recording permits quantification of the flight responses of tsetse to treatments. This provides a basis for comparing the flight responses of tsetse flies of different provenance.

3. RECORDINGS IN THREE DIMENSIONS OF *GLOSSINA BREVIPALPIS* NEWSTEAD IN THE WIND TUNNEL

A climatized wind tunnel was installed at the Laboratory of Animal Physiology, University of Neuchâtel, Switzerland to quantify the behavioural responses of tsetse flies to a range of chemical and visual stimuli from hosts and habitat. As tsetse flies are very fast flyers, it is difficult for the human eye to record quantitative behavioural differences of flies flying to different treatments. For this reason researchers have taken recourse to video analysis of tsetse flies in flight. We installed a 3D recording system for continuous recording of flight responses in tsetse. This provides data on quantifiable flight parameters such as speed, straightness, angular sum and angular velocity, i.e. sets of variables that are amenable to robust statistical analyses and permits comparison of quantitative aspects of the flight responses of tsetse flies to treatments.

The climatized wind tunnel (working area: 250 cm long 100×100 cm) was made of non-reflecting glass. A ventilator moved the humid- and temperature-controlled air ($85\pm1\%$ RH, $25\pm0.1^\circ\text{C}$) across the tunnel at 40 cm.s^{-1} through active charcoal cartridges and screens covered with mosquito netting placed at either end of the working area. Overhead illumination was provided by high frequency fluorescent lighting to provide ~ 700 lux on the floor. The floor of the wind tunnel was covered with a medium density fibreboard (4-mm thick, light brown), and the sides with white cotton sheets backed up by dark grey folded curtains forming random vertical bands along the sides of the tunnel. The light colours on the floor and the sides of the wind tunnel permitted contrast between insect and background, and the folded grey curtain provided lateral visual cues to the flying insects. Two video cameras equipped with objectives were placed 87 cm above the roof of the wind tunnel at 160 cm from each other. The two cameras were linked to a computer equipped with specially developed software *Crow* (University of Neuchâtel) that generates the 3D space. After 3D calibration, this programme registered the X, Y, and Z coordinates for successively detected points of a moving object permitting a determination of the position of a flying insect within the wind tunnel at any given time to an accuracy within 5 mm over 2.5 m. All analyses of tsetse fly trajectories were made using the software *CrowAnalyser* (University of Neuchâtel) which permits calculation of parameters characterizing each flight.

The data presented here are the responses of *G. brevipalpis* to human breath alone and to a 12 cm-diameter sphere covered with phtalogen blue cotton in a plume of human breath at the upwind end of the wind tunnel. The insects were from a *G. brevipalpis* colony maintained at the International Atomic Energy Agency, Entomology Unit, Seibersdorf Laboratories, Austria, for about 90 generations. *G. brevipalpis* (n=19) responding with at least one directed upwind flight to the source of human breath presented alone in the wind tunnel lasted between 4 and 141 s, covering distances between 6 and 153 m. Flights of another 10 *G. brevipalpis* that responded to human breath in the presence of a visual target lasted between 5 and 107 s, and covered distances between 6 and 40 m (Fig. 1). The longer flight times of *G. brevipalpis* exposed to breath alone occurred because the flies made successive upwind and downwind

flights in response to this treatment. In contrast, *G. brevipalpis* exposed simultaneously to breath and the visual target invariably made just one upwind flight followed by looping flights around and below the blue sphere.

The most notable aspects of the flights recorded for *G. brevipalpis* were the more directed and slower flights to the visual target placed in the plume of breath. From this we concluded that *G. brevipalpis* possesses the ability to hold flight parameters under stricter control in the presence of combined chemical and visual stimuli. The predominating role of the visual component of the responses was confirmed by the directed flights made by *G. brevipalpis* to the blue target even when it was placed outside the plume of breath. However, the flies first needed to be stimulated by breath to cause them to fly. Taken together, these findings showed that *G. brevipalpis*, issuing from multiple generations in a laboratory colony, were still capable of complex behaviours during bloodmeal searching. The flight tracks recorded provided evidence of the high degree of interaction between visual and olfactory sensory inputs that guide the behaviours of these tsetse flies.

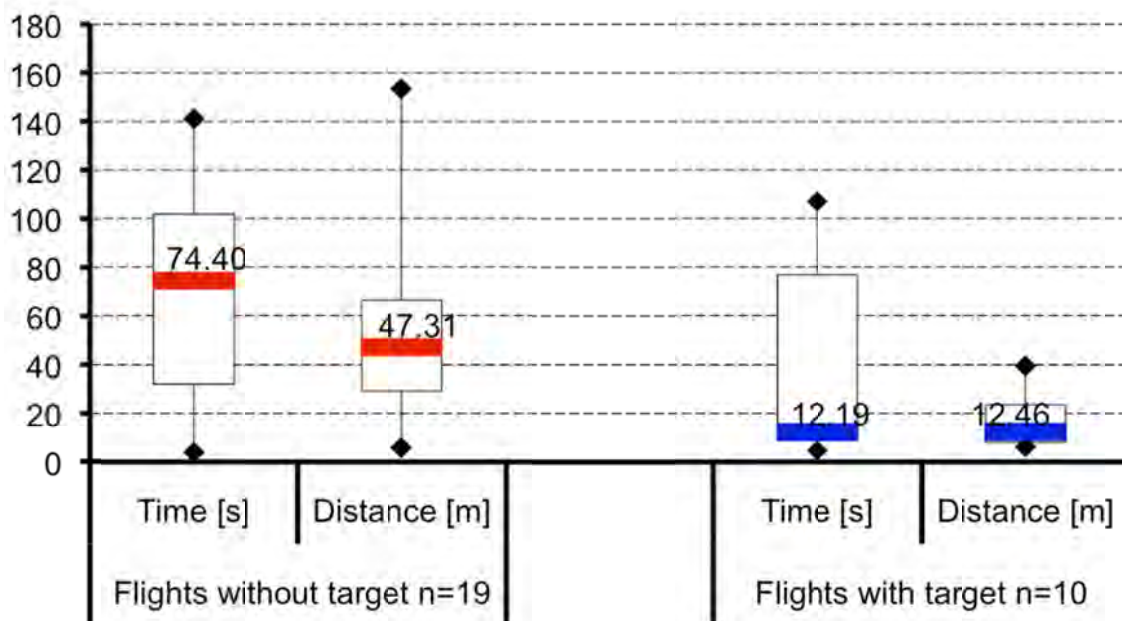


FIG. 1. Durations of flights and distances covered by *Glossina brevipalpis* in a wind tunnel in response to human breath, without and with a visual target upwind. Each box plot represents the position of the median, minimum, maximum and the 25th–75th quartiles of all tracks interpolated every 5 cm along the length of the wind tunnel.

REFERENCES

- [1] SCHNEIDER, D., Elektrophysiologische Untersuchungen von Chemo- und Mechanorezeptoren der Antenne des Seidenspinners *Bombyx mori* L., Z. Vgl. Physiol. **40** (1957) 8–41.
- [2] HARRACA, V., SYED, Z., GUERIN, P.M., Olfactory and behavioural responses of tsetse flies, *Glossina* spp., to rumen metabolites, J. Comp. Physiol. A **195** (2009) 815–824.
- [3] HUMMEL, H.E., MILLER, T.A., Techniques in Pheromone Research, Springer series in experimental entomology, Springer Verlag, New York (1984).

ACOUSTIC METHOD FOR TESTING THE QUALITY OF STERILIZED MALE TSETSE FLIES *GLOSSINA PALLIDIPE*S

H. KRATOCHVIL¹, A. NOLL², J. BOLLDORF³, A.G. PARKER⁴

¹Department of Evolutionary Biology, University of Vienna, Halsriegelstr. 34, Wien A-1090, Austria

²Institut für Schallforschung, Ö Ak d Wiss, Wohllebengasse 12–14, Wien A-1040, Austria

³Umweltbundesamt, Spittelauer Lände 5, Wien A-1090, Austria

⁴Joint FAO/IAEA Programme of Nuclear Techniques in Food and Agriculture, FAO/IAEA Agriculture and Biotechnology Laboratory, Seibersdorf A-2444, Austria

Abstract

Tsetse flies are able to emit different acoustic signals. An acoustic method to test the quality of sterilized male tsetse flies was developed. Differences in the sound characteristics between males and females, between sterilized and unsterilized males, and between males sterilized in air and nitrogen, were determined. Also, the acoustic parameters (frequency, time, sound pressure level) of the sounds that are useful as criteria for quality control were determined. It was demonstrated that only the so-called ‘feeding sounds’ can be used as a quality criterion. Both sexes emitted feeding sounds while feeding on a host. These sounds were also used to find sexual partners, and had an effect on male copulation success. An acoustic sound analysis programme was developed; it automatically measured sound activity (only feeding sounds) under standard conditions (random sample, relative humidity, temperature, light intensity).

1. INTRODUCTION

Releasing sterilized male tsetse flies is a well-proven and ecologically safe method of pest control. An acoustic method was developed for testing the fitness or quality of sterilized male tsetse flies *Glossina pallidipes* Austen. An acoustic method was selected because tsetse flies communicate acoustically. Both sexes can produce different types of songs. Many investigations have been conducted on the vocalization biology of tsetse flies [1–13].

Generally, the following sound types are distinguished:

1. Feeding sounds: These sounds are produced before, during and after feeding on hosts. Probably they are used to attract conspecifics and therefore also sexual partners.
2. Mating sounds: They are emitted during copulation. They serve to initiate and maintain copulation. Mating sounds can be distinguished from other sounds by differences in the temporal structure and temporal pattern.
3. Larviposition sounds: Immediately after larviposition, the female tsetse fly emits a characteristic sound that differs considerably from sounds produced during feeding and mating. It may attract other pregnant females to aggregate at appropriate sites with a suitable substrate for larval deposition.

Little is known about the function of the sound-producing organ of tsetse flies. Sounds are made by vertical wing vibrations [14]. According to Kolbe [1] and Popham et al. [4], flying muscles in the pterothorax are involved in sound production. Anderson [15] suspected that

specific muscle fibres in connection with tracheaeoles in the muscles are responsible for the high frequencies. At any rate, tsetse flies can only sing when they are not flying.

Finding acoustic characteristics in tsetse sounds that are correlated with physical conditions required extensive recordings and sound analyses. One focus was on analysing comparatively the female sounds. Another focus was on observing potential distinctions in the song quality of sterilized versus unsterilized males. An important aspect was to determine whether sterilization in nitrogen or air has an effect on male singing quality. Finally, the acoustic quality test method should be inexpensive and as practical as possible.

2. MATERIALS AND METHODS

The tests used individuals of *Glossina pallidipes* bred in the FAO/IAEA Agriculture and Biotechnology Laboratory in Seibersdorf, Austria. They originated from wild individuals collected as pupae in Uganda in 1986. They were transported quickly from Seibersdorf to the 'Biozentrum der Universität Wien' and kept in an anechoic chamber at about 25°C and a relative humidity (RH) of at least 90%. They were not fed because starvation was used to create different levels of fitness or activity. Recordings were initiated on the same day as the transport. The recordings were repeated every day under the same conditions and at the same time of day (0900–1200). Unfed tsetse flies retain a stable condition for 3 days. From the 4th day onwards, they become weaker; most die after 7–12 days.

Also, we initially attempted to record mating sounds, but it soon became clear that male *Glossina pallidipes* reliably emit only the feeding sounds.

The recordings were made in the anechoic chamber of the Biozentrum. The following equipment was available:

- Brüel u. Kjaer Condensermicrophone 4133 with a Preamplifier 2619 and a Measuring Amplifier Brüel u. Kjaer 2606 for measuring sound pressure level.
- Condenser Microphone AKG C461B. The recordings were made with a DAT-Recorder TASCAM DA-P1 and a Digital Recorder Marantz Professional.
- Acoustic analyses were made with the sound analysis program STX of the 'Institut für Schallforschung der Österreichischen Akademie der Wissenschaften', the programmes Raven and Adobe Audition.

The following acoustic parameters were measured: frequency spectrum, time parameters, sound pressure level, time pattern of frequencies and sound pressure levels, and singing activity (% of recording time). These parameters were measured daily and evaluated comparatively. Both males and females were analysed. We also examined potential differences between sterilized and unsterilized males, and those sterilized in air or nitrogen. Finally, we investigated under which conditions (RH, temperature, light, flies alone or in groups during the experiment, chemical stimuli) the males can be initiated to sing. Almost 1000 individuals were tested.

In cooperation with the 'Institut für Schallforschung der Österreichischen Akademie der Wissenschaften', software was developed to measure automatically the singing activity of the flies. For this purpose more than 100 recordings of feeding sounds were sectioned, i.e. typical sequences isolated, and evaluated statistically.

3. RESULTS

3.1. Characteristic criteria of the acoustic signals

Flight sounds of the tsetse flies were broadband, low frequency (mostly 180–800 Hz), predominantly noisy, with relatively indistinct harmonics (Fig. 1). Flight sounds were not emitted exclusively during flight but also during ground contact when standing and running.

Mating sounds were high frequency (390 Hz–20 kHz, main energy between 1.1 and 8 kHz) and strongly harmonic. These sounds were predominantly monotonic with marginal changes in frequency — especially at the beginning and end of the sequences. The duration of the sequences varied extremely (300 ms–1 min).

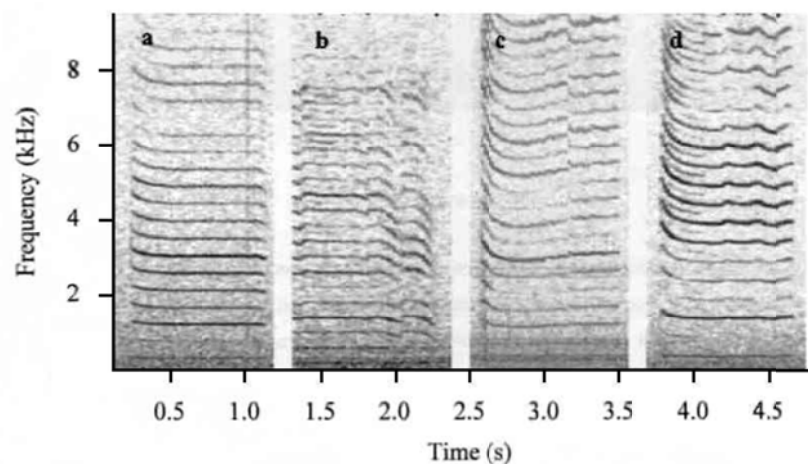


FIG. 1. Four different patterns of sounds produced by tsetse flies *Glossina pallidipes* were characterized by (a) a linear frequency pattern, (b) a decreasing frequency at the end of the sound, (c) slight frequency variations, and (d) a zigzag structure at the end of the sound.

Songs can be emitted only when tsetse are not flying. Sometimes songs pass directly into flight motion (wing movement during ground contact) (Fig. 2).

3.2. Comparative sound analysis

Fundamental frequencies of the feeding sounds did not differ between males and females.

Fundamental frequencies escalated with increasing temperature (17°C – 270 Hz, 25°C – 425 Hz).

The mean fundamental frequencies of females (170 Hz) were considerably lower than those of males (205 Hz).

The frequency of flight sounds was temperature independent.

There were no male/female differences in the temporal frequency characteristics.

There were no differences in temporal pattern of sounds between the sexes.

There were no measurable differences in sound pressure levels between males and females.

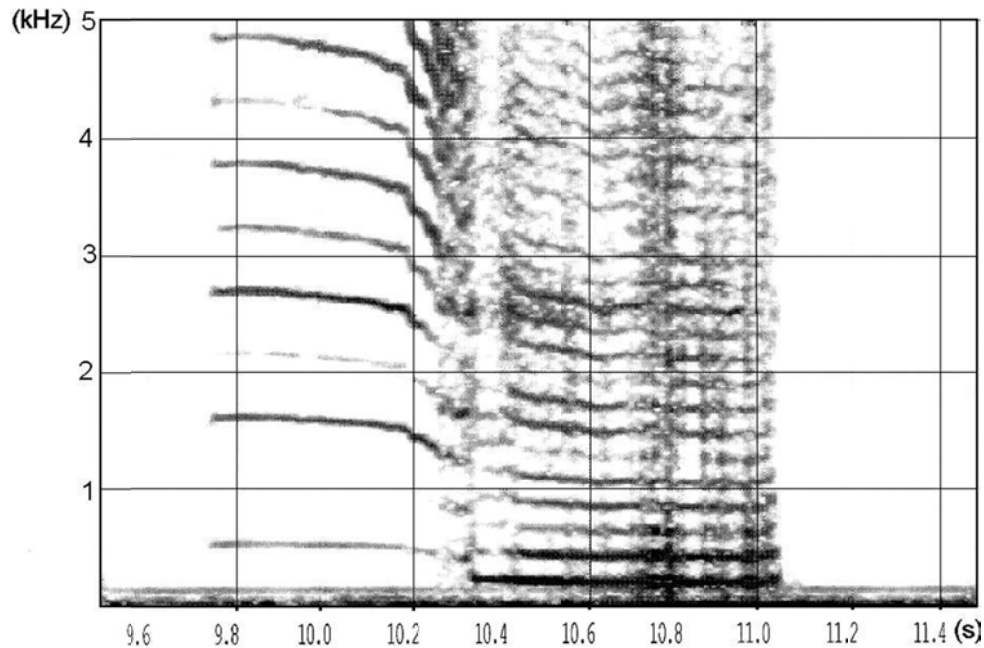


FIG. 2. *Glossina pallidipes*. Sonagram of a single song continuously changing into flying sound (buzzing sound).

3.3. Influence of sterilization

Sterilization lowered the frequencies of the songs; the fundamental frequencies in unsterilized individuals (both sexes) were higher. Individuals (both sexes) sterilized in nitrogen showed lower fundamental frequencies than air-sterilized ones.

3.4. Changes related to condition

A weakened condition caused no changes in the frequency or temporal patterns in sterilized or unsterilized male *Glossina pallidipes*. Also, a weakened condition had no influence on the sound pressure level of the songs. However, weakening had a conspicuous effect on the impetus to sing. Starving male tsetse clearly became weaker and their song activity decreased from the 4th day onwards. From the 8th day onwards, only a few songs were registered.

3.5. Measuring conditions

Male individuals, kept singly in the observation cage, could not be stimulated to sing. A minimum group of three individuals was required. The best combination was a group of five individuals.

The measurements should take place under the following conditions (Fig. 3):

Observation group: five sterilized male individuals

Temperature: 24°C

Relative humidity: minimum 80%

Light: 800 lux full spectrum on surface of the cage

Chemical stimulation: cup (3 cm diameter) with warm (30°C) cattle blood placed 1 cm under the floor of the cage

Measuring interval: 2 min

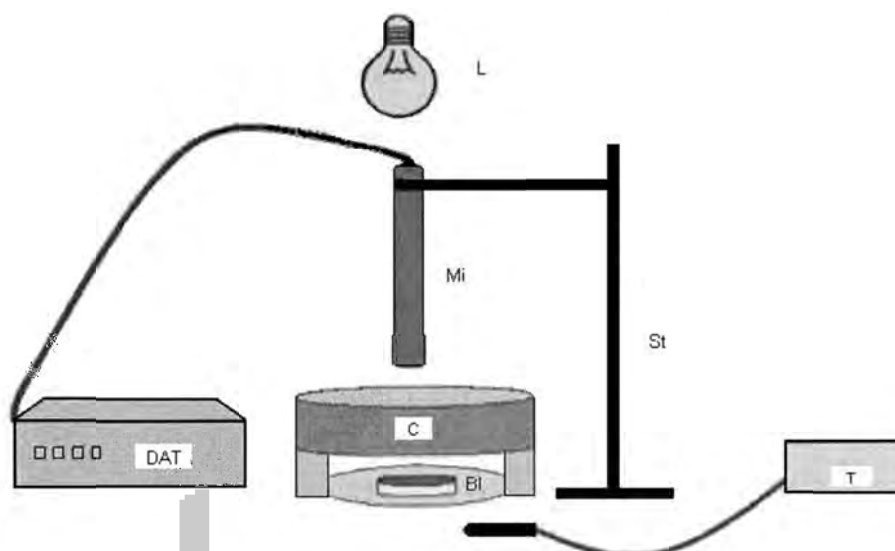


FIG. 3. Recording equipment for feeding sounds of sterilized male *Glossina pallidipes*.
Bl – blood, *C* – cage, *DAT* – digital sound recorder (computer), *L* – light, *Mi* – microphone, *St* – tripod, *T* – thermometer.

The results showed that singing activity was not uniform over the 5 min period but gradually decreased over this period (Fig. 4).

3.6. Analysis software

The sound analysis program STXtsetse is a supplement to the major sound analysis programme STX developed by ‘Institut für Schallforschung der Österreichischen Akademie der Wissenschaften’. As STXtsetse only operates in connection with STX, it will be available in the future in combination with a reduced version of STX.

An STXtsetse manual will be available. In brief:

After opening STX, STXtsetse can be called up. Two options are offered:

1. Calling up a stored file
2. A new record

The called file is displayed as a sonogram (Fig. 5).

Then a sector (task) must be selected, which covers only the background noise.

Arithmetic instruction displays a statistic in which the sound activity (% of recording time) is shown (Fig. 5).

3.7. Required equipment

- Temperature-controlled room adjusted to 24°C with high RH (minimum 80%)
- Computer with STXtsetse analysis programme [contact the FAO/IAEA Agriculture and Biotechnology Laboratory in Seibersdorf, Austria, regarding access to this programme]
- Professional condenser microphone
- Lux meter
- Hygrometer
- Light equipment for full-spectrum illumination
- Material for the experimental set-up
- Alternative: Digital sound recorder

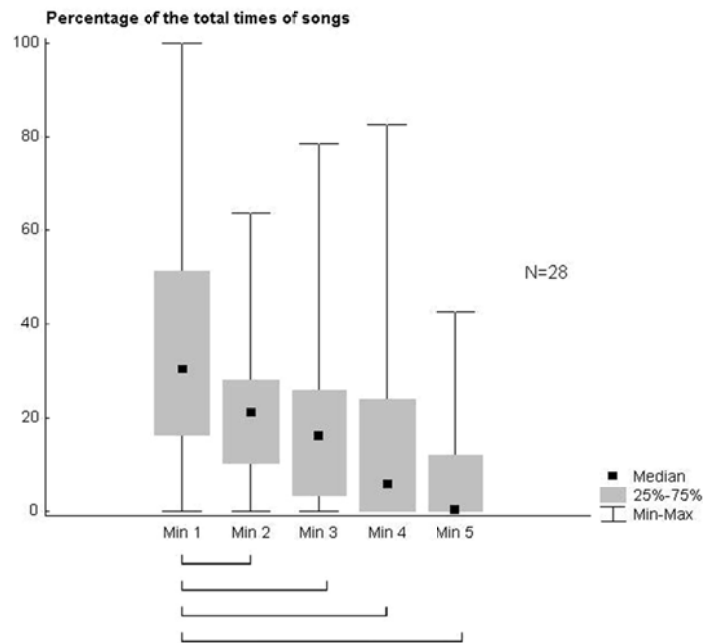


FIG. 4. *Glossina pallidipes*. Sound activity recorded over 5 minutes (%). The first minute was compared with minutes 2–5. It demonstrated the decrease in song activity.

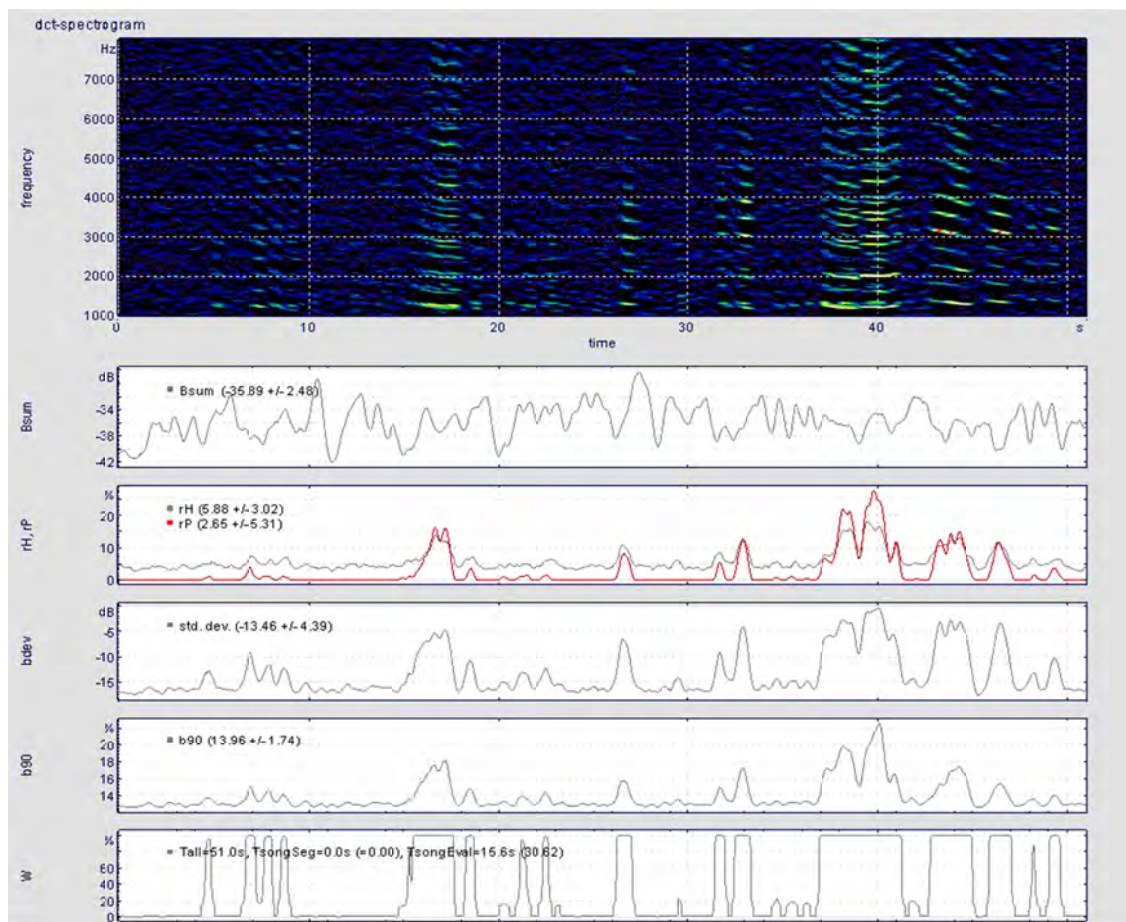


FIG. 5. Display of the software STXtsetse: Upper portion: Sonagram of the recorded sound. Lower portion: Time statistic of recorded songs. Tall – total time of record, Tsong Eval – time of song activity (15.6 s) and percentage of activity (30.62%).

4. DISCUSSION

It is an advantage that only the feeding sound is useful as a parameter for quality tests because this acoustic signal has a direct connection with mating success. Field application requires sterilized males that are competitive with wild males.

The goal of developing a simple test could not be entirely fulfilled; it turned out to be very complicated to trigger singing in male tsetse flies. One prerequisite was a minimum group of five individuals. Also, the RH, temperature and light conditions must be 'right'. Furthermore, the flies need a chemical stimulus in the form of warm cattle blood. The new software works as envisioned and can be learned quickly using the manual, although some practice is necessary for routine use. It uses filters to successfully separate the flight sounds from songs. The songs of the five males could not be distinguished individually because the differences in the frequencies were marginal. Therefore, the unavoidable incidental overlaps could be treated only statistically, i.e. with a sufficiently large random sample. Considerable practical experience is needed because we currently know very little about differences in the song activity of males from different breeding stocks.

ACKNOWLEDGEMENTS

This work was done within the framework of Cooperation Agreement No. 12537/RO between the IAEA and the University of Vienna. We thank the following contributors: Silvia Birnbaumer, Franz Maurer, Jana Bolldorf, Isabella Nagy, Leon Ploszczanski, Andreas Benkö, Florian Meyer, Melanie Pilat, Anton Lamboj, Werner Deutsch and Jonathan White.

REFERENCES

- [1] KOLBE, F.F., The modulated sounds made by the tsetse fly *Glossina brevipalpis* Newstead, Zool. Afr. **8** (1974) 241–258.
- [2] ERICKSON, I.E.C., MÖLLER, A.R., Sound production in tsetse fly *Glossina morsitans morsitans*, J. Ac. Soc. Am. **57** (1975) 984–986.
- [3] ERICKSON, I.E.C., MÖLLER, A.R., Tsetse fly *Glossina morsitans morsitans* produces ultrasound related to behaviour, Experientia **31** (1975) 788–790.
- [4] POPHAM, E.L., PARR, M.J., CHOWDHURY, V., Specific differences in tsetse fly sounds and their behavioral significance, Experientia **34** (1978) 1152–1154.
- [5] RUDRAUF, J.M., Comportement acoustico sexuel d'une glossine, *Glossina fuscipes fuscipes* Newstead, 1910, Ann. Zool. Ecol. Anim. **9** (1977) 389–406.
- [6] SAINI, R.K., Sound production associated with feeding behaviour of the tsetse *Glossina morsitans morsitans*, Entomol. Exp. Appl. **34** (1983) 1–8.
- [7] SAINI, R.K., Acoustic emission of tsetse, Insect Sci. Appl. **5** (1984) 341–344.
- [8] SAINI, R.K., Sound production associated with sexual behaviour of the tsetse fly *Glossina morsitans morsitans*, Insect Sci. Appl. **6** (1985) 637–644.
- [9] HUYTON, P.M., LANGLEY, P.A., Copulatory behaviour of the tsetse flies *Glossina morsitans* and *Glossina austeni*, Physiol. Entomol. **7** (1982) 167–174.
- [10] DENLINGER, D.L., SAINI, R.K., CHAUDHURY, F.B., Parturition on the tsetse fly *Glossina morsitans*, pattern of activity, sound production and evidence for control by the mother's brain, J. Insect Physiol. **9** (1983) 715–721.
- [11] ABDEL KARIM, E.I., BRADY, J., Changing visual responsiveness in pregnant and larviposition tsetse flies, *Glossina morsitans*, Physiol. Entomol. **9** (1984) 125–131.
- [12] HOWE, M.A., LEHANE, M.J., Post-feeding buzzing in the tsetse, *Glossina morsitans morsitans*, is an endothermic mechanism, Physiol. Entomol. **11** (1986) 279–286.

- [13] BAILEY, W.J., *Acoustic Behaviour of Insects: An Evolutionary Perspective*, Chapman and Hall, London (1991).
- [14] CHOWDHURY, V., PARR, J., The “switch mechanism” and sound production in tsetse flies (Diptera, Glossinidae), *J. Nat. Hist.* **15** (1981) 87–95.
- [15] ANDERSON, M., Spirally arranged muscles associated with tracheoles in tsetse flight muscles: their possible involvement in sound production, *Experientia* **34** (1978) 587–589.

EFFECT OF FEEDING BLOOD TREATED WITH ISOMETAMIDIUM CHLORIDE TO *GLOSSINA MORSITANS MORSITANS* ON THE FLIES' SUBSEQUENT VECTORIAL CAPACITY, AND EVALUATION OF AN IN VITRO FEEDING SYSTEM FOR THE MAINTENANCE OF INFECTED TSETSE FLIES

P. VAN DEN BOSSCHE, J. VAN DEN ABBEELE, R. DE DEKEN

Veterinary and Parasitology Departments,
Prince Leopold Institute of Tropical Medicine,
Nationalestraat 155,
2000 Antwerp, Belgium

Abstract

A repercussion of the release of large numbers of sterile male tsetse flies is the sudden increase in the number of disease vectors. To avoid the potential creation of a trypanosomosis epidemic, studies were conducted aimed at reducing the vectorial capacity of male tsetse flies by offering them a single bloodmeal containing the prophylactic trypanocidal drug isometamidium chloride. Experiments using the pair *G. m. morsitans* and *T. congolense* or *T. b. brucei* showed that a single treatment with isometamidium chloride protected flies from infection with susceptible and resistant trypanosomes for up to 5 days post-treatment. Starvation of isometamidium-treated flies increased their susceptibility to infection with *T. congolense*. Studies evaluating in vitro feeding of infected flies on citrated bovine blood showed that this feeding regime adversely affected the capacity of trypanosomes to develop in the fly.

1. INTRODUCTION

Tsetse-transmitted trypanosomosis is one of the major constraints to sustainable rural development in large parts of sub-Saharan Africa. Recent work on the island of Zanzibar has proven that the release of sterile male tsetse flies can be an effective method to eradicate tsetse in isolated pockets and thus result in a permanent solution to the trypanosomosis problem. However, a possible unwanted repercussion of the release of large amounts of sterile flies, in the initial phase of a sterile male control campaign, is an increase in the number of potential vectors of trypanosomes. To prevent sterile males from picking up trypanosome infections and acting as vectors, a first bloodmeal containing a trypanocide is usually given before release. The effectiveness of this treatment in preventing male and female tsetse flies from becoming infected was investigated. Moreover, the effectiveness of the isometamidium treatment in preventing (i) the establishment of trypanocidal drug-resistant trypanosome strains, and (ii) highly susceptible starved flies from becoming infected, was assessed.

Experimental infections of tsetse flies are important tools in the search for factors affecting the infection rate of the flies. An important drawback of such infection experiments is the transmission of the disease to experimental animals used to feed infected tsetse flies.

2. MATERIALS AND METHODS

2.1. Isometamidium chloride treatment and infection

2.1.1. Effectiveness of isometamidium chloride treatment on subsequent vectorial capacity of treated flies

Batches of teneral male and female tsetse flies (*Glossina morsitans morsitans* Westwood) were administered gamma-irradiated sterile defibrinated bovine blood containing either 10 or

100 µg isometamidium chloride/mL (for male flies) and 10 µg isometamidium chloride/mL (for female flies) as a first bloodmeal (day 0). Individual batches were infected with *Trypanosoma congolense* or *Trypanosoma brucei brucei* on day 3, 5, 10 or 20. Control flies were treated in a similar way but were not treated with isometamidium chloride. After the first bloodmeal all experimental flies were maintained on rabbits. Thirty days after infection, all flies were dissected and their infection status (immature infections and mature infections) was determined. The infection rates of the control groups and the groups treated with isometamidium chloride at 10 or 100 µg/mL blood were compared using a Poisson regression analysis.

2.1.2. Effectiveness of isometamidium chloride treatment on susceptibility of treated flies to infections with trypanocidal drug-resistant trypanosome strains

Batches of teneral tsetse (*G. m. morsitans*) were administered gamma-irradiated sterile defibrinated bovine blood containing 10 µg isometamidium chloride/mL as a first bloodmeal (day 0). Individual batches were infected on day 3 with one of three strains of *T. congolense* IL1180. The strains were isogenic apart from the mutation(s) underlying the isometamidium chloride resistance phenotype. The susceptible clone had a CD₅₀ (the curative dose that gives complete cure in 50% of the animals) in mice of 0.018 mg/kg. The resistant clones had a CD₅₀ of 1.8 mg/kg and 3.6 mg/kg for the low and high resistant clones, respectively. Control flies were treated in a similar way but did not receive isometamidium chloride. After the first bloodmeal all experimental flies were maintained on rabbits. Thirty days after infection, all flies were dissected and their infection status (immature or midgut infections and mature or proboscis infections) was determined. The infection rates of the control groups and the groups treated with isometamidium chloride were compared using the Poisson regression analysis.

2.1.3. Effect of starvation on susceptibility of isometamidium-treated tsetse flies to trypanosomal infections

Batches of teneral tsetse (*G. m. morsitans*) were administered gamma-irradiated sterile defibrinated bovine blood containing 10 µg isometamidium chloride/mL as a first bloodmeal (day 0). They were subsequently starved for 3, 5 or 6 days and then infected with *T. congolense* IL1180 or *T. b. brucei* EATRO 1125. Twenty one and thirty days after infection (for infections with *T. congolense* and *T. b. brucei*, respectively), all flies were dissected and their infection status (immature or midgut infections and mature or proboscis infections) was determined. The infection rates of the different experimental groups were compared using a logistic regression analysis.

2.2. In vitro feeding on citrated bovine blood and development of trypanosomal infections

Batches of teneral (less than 32-h old) male flies *G. m. morsitans* were infected with either *T. congolense* IL 1180 or *T. b. brucei* AntAR1. After the infected meal, the batches of infected flies were divided into two groups. The first group was maintained in vitro by feeding flies through membranes and citrated bovine blood obtained from the IAEA. The second group was maintained in vivo on rabbits.

3. RESULTS

The results of the experiments showed that:

- The administration of isometamidium chloride at 10 µg/mL in the first bloodmeal suffices to reduce significantly the *T. congolense* and *T. b. brucei* infection rates. This effect persists for at least 5 days after isometamidium chloride administration.
- The resistant trypanosome genotype does not result in an increased transmission by tsetse that received a single treatment with isometamidium chloride at 10 µg/mL blood.
- Starvation significantly increases the susceptibility of isometamidium-treated tsetse flies to infection with *T. congolense*. However, the infection rate of the treated flies was lower than would be expected of untreated controls. The infection rate with *T. b. brucei*, on the other hand, was low and was not affected by starvation.
- Maintaining infected tsetse flies in vitro on citrated bovine blood reduces the infection rate of *T. congolense* or *T. b. brucei* compared with flies maintained in vivo on rabbits.

4. CONCLUSIONS

Depending on the intrinsic vectorial capacity of the line of tsetse flies released in the field, it is advised that, before release, tsetse flies be given a bloodmeal that contains isometamidium chloride at, at least, 10 µg/mL. However, starvation increases the susceptibility of isometamidium-treated tsetse flies to infection with *T. congolense*. This observation needs to be taken into account when releasing sterile males by releasing the flies shortly after the bloodmeal containing isometamidium chloride and/or releasing flies during seasons when nutritional stress is low.

In vitro feeding of infected tsetse flies on citrated bovine blood may not offer an alternative to in vivo feeding.

ACKNOWLEDGEMENT

This research was conducted as part of Research Contract No. 12621 in collaboration with the FAO/IAEA in Vienna, Austria.

EFFECTS OF SAMORIN® TREATMENT AND DELAYED INFECTION ON THE ESTABLISHMENT OF TRYPANOSOMES IN *GLOSSINA PALLIDIPES*

S. OKOTH, P. MUKIRIA, G. TINEGA, G. MURILLA

Trypanosomiasis Research Centre,
Kenya Agricultural Research Institute (TRC-KARI),
Box 362, Kikuyu, Kenya

Abstract

The protective role of Samorin® (isometamidium chloride) when given with the bloodmeal, and the effect of the age of flies at infective feed, on the establishment of *Trypanosoma congolense* and *Trypanosoma vivax* in *Glossina pallidipes* Austen was evaluated. *G. pallidipes* was fed on fresh bovine blood containing 0, 6, 8, 10, 12 and 14 µg of Samorin/mL of blood on day 0. A control group was fed in vivo on an infected goat as first feed on the same day (day 0). On day 3 post-Samorin treatment, the flies were infected in vivo by feeding them on a goat infected with either *T. congolense* or *T. vivax*. Similar procedures were followed for the experimental groups infected 5, 10 and 20 days post-Samorin treatment. Thereafter flies were maintained on rabbits for 15 and 20 days for the *T. vivax* and *T. congolense* groups, respectively. Fly survival was assessed, and the flies were dissected to determine infection rates. Results showed that the age of a fly at infection significantly affected the refractoriness of *G. pallidipes* to *T. congolense* ($F_{4,20} = 5.241$; $P=0.0047$) and *T. vivax* ($F_{4,20} = 5.410$; $P=0.0040$) infections. However, the effect of Samorin treatment on infection per se was not shown to be significant ($F_{5,19} = 2.383$; $P=0.0733$ and $F_{4,20} = 1.106$; $P=0.3900$) for *T. congolense* and *T. vivax*, respectively. The age of flies at infection significantly reduced tsetse survival ($F_{4,20} = 7.584$; $P<0.001$ and $F_{4,20} = 10.755$; $P=0.0001$) for *T. congolense* and *T. vivax* groups, respectively. However, Samorin treatment did not significantly reduce survival in both *Trypanosoma* groups ($F_{5,19} = 0.642$; $P>0.67$ and $F_{5,19} = 0.485$; $P=0.783$). These results indicated that, for *T. congolense* and *T. vivax*, combining Samorin treatment at a concentration of at least 8 µg/mL, and delaying exposure of tsetse to infective feed for at least 3 days post emergence, induces sufficient refractoriness that may counter the increased challenge posed by the mass release of flies in sterile insect technique (SIT) programmes.

1. INTRODUCTION

African trypanosomosis is a debilitating parasitic disease transmitted by tsetse flies. Tsetse flies infest a total area of 10 million km² in 36 African countries. Annual losses incurred directly and in control programmes are estimated to be between US\$600 million and 1.2 billion [1, 2]. Control and/or eradication of tsetse flies may therefore considerably reduce the impact of the disease. Eradication of *Glossina austeni* Newstead from Zanzibar using the sterile insect technique (SIT) [3, 4] demonstrated the potential of the technique in an integrated intervention campaign against tsetse and trypanosomosis. Consequently, the Government of Kenya (GOK), in collaboration with the International Atomic Energy Agency (IAEA), launched such a programme targeting the eradication of *Glossina pallidipes* Austen from the Lambwe Valley.

Since the sustained release of sterile males increases the trypanosomosis challenge in the field, using the SIT in an eradication campaign requires that the vectorial capacity of the flies produced in the laboratory for release in the field be drastically reduced. To avoid an upsurge in this challenge following large-scale releases, released flies need to be rendered refractory to trypanosome infection. Leak [5] reported that the establishment of trypanosome infection in tsetse was limited in subsequent feeds if the first bloodmeal was uninfected. Moloo et al. [6] also showed that maturation of pathogenic trypanosomes in *Glossina morsitans morsitans* Westwood was completely suppressed when their first bloodmeal was spiked with isometamidium chloride (Samorin®) at between 8 and 12 µg Samorin/mL of blood. The

current study investigated the effect of Samorin-treated bloodmeal and age of tsetse at infection on the establishment of trypanosome infection in the *G. pallidipes* colony flies destined for mass release in area-wide eradication projects in Africa.

2. MATERIALS AND METHODS

2.1. Blood spiking with Samorin®

Freshly collected bovine blood was defibrinated by gentle swirling in a 250 mL conical Pyrex beaker containing glass beads for 10 min. It was then divided into 20 mL aliquots. A stock of Samorin-spiked blood containing 10 mg Samorin/mL of blood (wt/vol.) was prepared. The 20 mL blood aliquots were then spiked with Samorin by adding 0, 12, 16, 20, 24 and 28 µg of the stock to each 20 mL bottle resulting in concentrations of 0, 6, 8, 10, 12 and 14 µg Samorin/mL of blood. These were then used to feed the flies in the various groups.

2.2. Infection and monitoring of host

Four 8-months old Maasai black-head goats were used. The goats were quarantined for 2 weeks in insect-proof holding pens. The goats were monitored for possible prior infection with trypanosomes, and their packed-cell volume (PCV) was checked every other day for an additional 2 weeks. Two goats were infected intraperitoneally with *T. congolense* isolate EATRO 993, and the other two were similarly infected with *T. vivax* KETRI 2501. The infected goats were maintained on Napier grass, hay and water *ad libitum*. Parasitaemia and PCV were monitored daily.

2.3. Feeding of tsetse flies with Samorin-spiked blood

Five experimental groups of 50 teneral *G. pallidipes* each were fed on fresh bovine blood containing 6, 8, 10, 12 or 14 µg of Samorin/mL of blood on day 0. Two control groups (C_a and C_b) were not offered Samorin-treated blood.

2.4. In vivo infection of tsetse flies with trypanosomes

Two experimental groups designated control 'a' (C_a) were fed on either a *T. congolense*- or *T. vivax*-infected goat on day 0. The C_b group and the other five groups that had been fed on Samorin were fed on infected goats on day 3. Similar procedures were followed in the days 5, 10 and 20 experimental groups with flies that had been fed on Samorin-treated blood at the doses stated above. Flies that were allowed to but failed to feed on the infective feeds were excluded from the experiment.

2.5. Maintenance of infected flies

Infected and control flies for the day-3 group were maintained in vivo on rabbits on a 2 day-interval feeding regime. Mortality was checked daily, and the daily survival computed from survival data per treatment group. The flies infected with *T. congolense* were maintained for 20 days, while those infected with *T. vivax* were maintained for 15 days. Similar procedures were repeated in groups that obtained their first infective feeds 5, 10 or 20 days post first bloodmeal. To avoid reinfection of the flies with *T. congolense* or *T. vivax* during the in vivo maintenance, rabbits were replaced at weekly intervals.

2.6. Tsetse dissection

For the *T. congolense* infected group the mouthparts and midguts of flies were dissected and for the *T. vivax* infected group the proboscis was dissected. The infection status was determined by microscopy. The organs of uninfected flies were pooled into a 1.5 mL ampoule and preserved at -20°C for later molecular analysis.

2.7. Data management

Data were analysed in SPSS 12.0 and Stata 7 (StataCorp 2001). Survival and infection rates were compared among the groups using an analysis of variance (ANOVA). Where ANOVA was significant, the differences among means were separated using Fisher's PLSD pairwise comparison. The combined effect of delayed infective feed and Samorin treatment was assessed using probit regression.

3. RESULTS

3.1. Refractoriness to trypanosome infection

The effects of Samorin treatment and the age of tsetse flies at infection on refractoriness of the flies to trypanosomes were similar for both trypanosome species. In this experiment Samorin treatment did not significantly reduce infection of the tsetse flies by *T. congolense* ($F_{5,19} = 2.383$; $P=0.0733$). However, the infection rate in the Samorin control group (C_b) was higher than in the treatment groups. Similarly, the infection rate in flies infected by *T. vivax* was not significantly reduced by Samorin treatment ($F_{4,20} = 1.106$; $P=0.3900$) although the control group showed a higher rate of infection (Table 1). The age of tsetse at infection significantly reduced both *T. congolense* and *T. vivax* infection in flies ($F_{3,20} = 5.241$; $P=0.0047$ and $F_{3,20} = 5.410$; $P=0.0040$, respectively) (Table 2). For *T. vivax* there was a drastic reduction in infection rates from day 3 onwards, and day 0 infection rates were significantly higher than all the other days. For *T. congolense*, though, the day 0 infections were the highest, and the effects of the day of infection on subsequent infection rates were not as clear cut as in *T. vivax*.

TABLE 1. EFFECT OF TREATMENT OF TSETSE FLIES WITH SAMORIN ON REFRACTORINESS TO TRYPANOSOME INFECTION

Samorin concentration in blood at day 0 ($\mu\text{g/mL}$)	Mean infection rate \pm SE	
	<i>Trypanosoma congolense</i>	<i>Trypanosoma vivax</i>
$C_b(0)$	9.67 \pm 6.622	7.85 \pm 10.843
6	1.04 \pm 2.080	3.13 \pm 6.2
8	3.75 \pm 7.5	2.88 \pm 5.7
10	2.87 \pm 5.7	0
12	0.89 \pm 1.78	1 \pm 2
14	0	0

TABLE 2. EFFECT OF AGE OF TSETSE AT INFECTION ON REFRACTORINESS TO TRYPANOSOME INFECTION

Age at infection (days)	Mean infection rate \pm SE	
	<i>Trypanosoma congolense</i> ^a	<i>Trypanosoma vivax</i> ^a
C _a (0)	17 a	21.62 a
3	0 b	0 b
5	2.42 \pm 1.82 bc	0 b
10	7.41 \pm 2.8 ac	2.94 \pm 2.9 b
20	1.1 \pm 1.1 b	4.67 \pm 2.41 b

^a Means within a column followed by the same letter are not significantly different at $P=0.05$ LSD.

3.2. Survival of tsetse flies

Samorin treatment did not significantly affect tsetse survival among the *T. congolense* group ($F_{5,19} = 0.643$; $P>0.67$), and similarly the effect was insignificant on survival of the *T. vivax* infected group ($F_{4,20} = 0.485$; $P>0.783$) (Table 3). The age of tsetse flies at infection significantly reduced tsetse survival among the *T. congolense* infected group ($F_{4,20} = 7.584$; $P<0.001$). There was no significant difference in the mean survival among the control, 3- and 5 day groups. The 5 day group lived significantly longer than the day 10, but not any longer than the day 20 group. The effect of age of flies at infection among the *T. vivax* was also significant ($F_{4,20} = 10.755$; $P<0.001$) with respect to tsetse survival (Table 4).

TABLE 3. EFFECT OF SAMORIN TREATMENT ON TSETSE FLY SURVIVAL

Samorin concentration in blood at day 0 ($\mu\text{g/mL}$)	Mean survival \pm SE (%)	
	<i>Trypanosoma congolense</i>	<i>Trypanosoma vivax</i> group
C _b (0)	54.38 \pm 24.38	82.92 \pm 11.39
6	76.70 \pm 20.23	85.96 \pm 4.98
8	65.14 \pm 15.48	79.08 \pm 25.40
10	77.93 \pm 7.83	84.61 \pm 7.50
12	76.70 \pm 10.93	75.15 \pm 26.11
14	74.31 \pm 10.11	67.61 \pm 29.49
	NS	NS

TABLE 4. EFFECT OF AGE OF TSETSE FLIES AT INFECTION ON FLY SURVIVAL

Age at infection (days)	Mean survival \pm SE (%)	
	<i>Trypanosoma congolense</i> ^a	<i>Trypanosoma vivax</i> group ^a
C _a (0)	81.0 a	74.00 a
3	75.3 \pm 3.63 a	80.1 \pm 5.28 a
5	68.0 \pm 4.0 ab	79.8 \pm 3.88 a
10	53.3 \pm 3.41 c	72.0 \pm 2.87 ab
20	58.6 \pm 1.76 bc	53.3 \pm 2.71 c
	$p<0.001^{**}$	$p<0.001^{**}$

^a Means within a column followed by the same letter are not significantly different at $P=0.05$ LSD.

3.3. Combined effect of Samorin treatment and age of flies at infection

The combined effect of Samorin treatment and age of flies at infection significantly reduced both *T. congolense* ($F_{3,24} = 4.336$; $P < 0.002$) and *T. vivax* infections ($F_{3,24} = 1.423$; $P < 0.05$) in the tsetse flies. For *T. congolense*, 69.34% (SD = 4.482) of the variation in infection rate was contributed by Samorin treatment, and only 30.66% (SD = 1.982) was due to age of flies at infection. Similarly for *T. vivax* Samorin treatment was not significant and contributed only 47% of the variation in susceptibility of flies to *T. vivax* infection.

4. DISCUSSION

Strategies that interfere with the ability of tsetse to establish and transmit trypanosomes are of paramount interest in SIT-based area-wide pest eradication programmes. The released flies are known to transmit trypanosomes to both man and his livestock unless their vectorial capacity is sufficiently depressed. The findings in this study indicated that the trypanosome infection rate in tsetse flies treated with Samorin was lower than that for controls in both *T. congolense* and *T. vivax* groups. Infection rates correlated inversely, significantly for the *T. congolense* but not significantly for the *T. vivax* group, with the concentration of Samorin. There was progressive suppression of infection with increasing concentration of Samorin, and these observations agree with those of Moloo et al. [6] who showed that, when *Glossina morsitans* flies were offered Samorin-treated blood as a first bloodmeal, their ability to be infected with *T. brucei* and *T. congolense* was significantly depressed compared with those fed in the control. Kibugu et al. [7] reported similar findings in which treatment of flies with ethidium resulted in increased longevity. It is believed that trypanocidals have antimicrobial activity and thus protect the flies from entomophagous microbes, increasing their survival. Higher survival of flies improves their competitiveness and benefits the control operation.

In combination with Samorin treatment the age of tsetse at the time of infection significantly reduced both *T. congolense* and *T. vivax* infection in flies. The effect of age on *T. vivax* infection is more clear-cut than on *T. congolense* with the infection rate on day 0 being significantly higher than all the other days. For *T. congolense*, the infection rate for day 0 was significantly higher than for days 3, 5 and 20 but not so for day 10. Maudlin [8], Moloo et al. [9] and Welburn and Maudlin [10] also reported that, for *T. brucei* and *T. congolense* infections, the age of the tsetse fly at the time of the infective feed is an important determinant of infection rates. Similar results were reported for *T. brucei rhodesiense* where concentrations above 12 µg/mL were equally effective [11]. In our experiment, delayed infection past the age of 3 days significantly increased refractoriness of flies to *T. congolense* infection. The refractoriness increased with age although insignificantly after day 3. Therefore, for effective impartation of refractoriness and cost effectiveness, flies should be released only when they are at least 3 days old. A surprising observation was that despite the simple and localized life cycle of *T. vivax* and the non-involvement of the peritrophic membrane in the developmental cycle [12], delay in the day of infection had an effect on the infection rates and the infection rates were comparable with those of the *T. congolense* group.

In general, the combined effect of Samorin treatment and age of flies at infection significantly reduced *T. congolense* infection in *G. pallidipes*. Although the combined effect of Samorin and age of flies reduced *T. vivax* in *G. pallidipes*, age at infection had a bigger contribution to the suppression of infection. Therefore, it is evident that Samorin treatment alone at concentrations of 6–14 µg/mL did not show any definite advantage in conferring refractoriness to *G. pallidipes* with *T. vivax* infections. Using mark release-recapture methods, Bouyer [13] concluded that a Samorin dose of 5 µg/mL offered no protection to *Glossina*

palpalis gambiensis Vanderplank against infection by *T. vivax* and *T. congolense*, and it is evident that the role of delayed infection would not have worked in this assessment. Age at infection was important in conferring refractoriness to the flies for both trypanosome species. Van den Bossche et al. [14] found that, in addition to the Samorin-treated bloodmeal, a delay of 5 days before infection confers significant refractoriness on *G. pallidipes* to infection with *T. congolense* and *T. brucei*. Therefore, a combination of age above 3 days and a Samorin concentration of at least 8 µg/mL may be recommended to protect *G. pallidipes* against *T. congolense* and *T. vivax* in tsetse control campaigns involving the release of sterile males.

ACKNOWLEDGEMENTS

We gratefully acknowledge FAO/IAEA and KARI-TRC for funding this work. We thank K. Ndungu, P. Gitonga, P. Obore, S. Ekilaini and J. Murage for their invaluable efforts in making the laboratory work successful.

REFERENCES

- [1] CHENEAU, Y., A systematic approach to tsetse and trypanosomiasis control. In: Proceedings of the FAO Panel of Experts, Rome, 1–3 December 1993. FAO Animal and Production Health Paper No. 121 (1994), Rome, Italy.
- [2] FELDMANN, U., HENDRICH, J., Integrating the Sterile Insect Technique as a Key Component of Area-wide Tsetse and Trypanosomiasis Intervention, PAAT Technical and Scientific Series No. 3 (2001), FAO, Rome, Italy.
<http://www.fao.org/DOCREP/004/Y2022E/Y2022E00.HTM>
- [3] VREYSEN, M.J.B., et al., *Glossina austeni* (Diptera: Glossinidae) eradicated on the island of Unguja, Zanzibar, using the sterile insect technique, J. Econ. Entomol. **93** (2000) 123–135.
- [4] SALEH, K.M., MUSSA, W.A., JUMA, K.G., VREYSEN, M.J.B., Eradication of *Glossina austeni* from the island of Unguja confirmed: results of 2 years of post-eradication monitoring activities. In: Proceedings 25th Meeting of the International Scientific Council for Trypanosomiasis Research and Control, 27 September–1 October 1999, Mombasa, Kenya. OAU/IBAR, Nairobi, Kenya, 231–238 (2001).
- [5] LEAK, S.G.A., Tsetse Biology and Ecology: Their Role in the Epidemiology and Control of Trypanosomiasis, CABI International, Wallingford (1999).
- [6] MOLOO, S.K., KUTUZA, S.B., DESAI, J., Comparative study of the infection rates of different *Glossina* species for East and West African *Trypanosoma vivax* stocks, J. Parasitol. **95** (1987) 537–542.
- [7] KIBUGU, J.K., et al., The effect of ethidium (homidium bromide) on performance of laboratory tsetse, KETRI project report, Vol. 3 (2003).
- [8] MAUDLIN, I., Transmission of African trypanosomiasis: interactions among tsetse immune system, symbionts, and parasites. In: Advances in Disease Vector Research **7** (1991) 117–148, Springer-Verlag, New York.
- [9] MOLOO, S.K., KABATA, J.M., WAWERU, F., GOODING, R.H., Selection of susceptible and refractory lines of *Glossina morsitans centralis* for *Trypanosoma congolense* infection and their susceptibility to different pathogenic *Trypanosoma* species, Med. Vet. Entomol. **12** (1998) 391–398.
- [10] WELBURN, S.C., MAUDLIN, I., Tsetse-trypanosome interactions: rites of passage, Parasitology Today **15** (1999) 399–403.

- [11] INTERNATIONAL ATOMIC ENERGY AGENCY, Working material. Improved and harmonized quality control for expanded tsetse production, sterilization and field application, Second research co-ordination meeting within the FAO/IAEA Co-ordinated Research Programme, Vienna, Austria, 5–9 May 2005, IAEA-355-G3-RC-919.2 (2006).
- [12] OKOTH, S.O., KOKWARO, E.D., KIRAGU, J.M., MURILA, G.A., Susceptibility and transmission capacity of subpopulations of *Glossina pallidipes* to human infective *Trypanosoma brucei rhodesiense*, Trends in Medical Research **1** (2006) 75–85.
- [13] BOUYER, J., Does isometamidium chloride treatment protect tsetse flies from trypanosome infections during SIT campaigns?, Med. Vet. Entomol. **22** (2008) 140–143.
- [14] VAN DEN BOSSCHE, P., et al., Effect of isometamidium chloride treatment on susceptibility of tsetse flies (Diptera: Glossinidae) to trypanosome infections, J. Med. Entomol. **43** (2006) 564-567.

BIBLIOGRAPHY

- AKODA, K., HAROUNA, S., MARCOTTY, T., DE DEKEN, R., VAN DEN BOSSCHE, P., Investigations on the transmissibility of *Trypanosoma congolense* by the tsetse fly *Glossina morsitans morsitans* during its development in a mammalian host, *Acta Trop.* **107** (2008) 17–19.
- BRICEÑO, R.D., EBERHARD, W.G., Experimental demonstration of possible cryptic female choice on male tsetse fly genitalia, *J. Insect Physiol.* **55** (2009) 989–996.
- BRICEÑO, R.D., EBERHARD, W.G., Experimental modifications imply a stimulatory function for male tsetse fly genitalia, supporting cryptic female choice theory, *J. Evol. Biol.* **22** (2009) 1516–1525.
- BRICEÑO, R.D., EBERHARD, W.G., ROBINSON, A.S., Copulation behaviour of *Glossina pallidipes* (Diptera: Muscidae) outside and inside the female, with a discussion of genitalic evolution, *Bull. Entomol. Res.* **97** (2007) 471–488.
- BRICEÑO, R.D., WĘGRZYNEK, D., CHINEA-CANO, E., EBERHARD, W.G., DOS SANTOS ROLO, T., Movements and morphology under sexual selection: tsetse fly genitalia, *Ethol. Ecol. Evol.* **22** (2010) 385–391.
- KUBI, C., VAN DEN ABEELE, J., DE DEKEN, R., MARCOTTY, T., DORNY, P., VAN DEN BOSSCHE, P., The effect of starvation on the susceptibility of teneral and non-teneral tsetse flies to trypanosome infection, *Med. Vet. Entomol.* **20** (2006) 388–392.
- VAN DEN BOSSCHE, P., AKODA, K., KUBI, C., MARCOTTY, T., The transmissibility of *Trypanosoma congolense* seems to be associated with its level of resistance to isometamidium chloride, *Vet. Parasitol.* **135** (2006) 365–367.
- VAN DEN BOSSCHE, P., AKODA, K., DJAGMAH, B., MARCOTTY, T., DE DEKEN, R., KUBI, C., PARKER, A., VAN DEN ABEELE, J., Effect of isometamidium chloride treatment on susceptibility of tsetse flies (Diptera: Glossinidae) to trypanosome infections, *J. Med. Entomol.* **43** (2006) 564–567.
- ŽD'ÁREK, J., HLAVÁČEK, J., TAKÁČ, P., Delayed effects of a dipteran oostatic hormone on reproduction in the tsetse fly. In: *Biologically Active Peptides XI* (SLANINOVÁ, J., Ed.), Collection Symposium Series **11** (2009) 139–142.

PROPOSED QUALITY CONTROL PROTOCOLS FOR MASS REARING TSETSE FLIES, BEHAVIOUR AND TREATMENT TO REDUCE VECTORIAL CAPACITY

1. INTRODUCTION

Several quality control (QC) protocols related to mass rearing tsetse flies have been published [1]. At the final Research Coordination Meeting (RCM) held in Addis Ababa, Ethiopia, on 13–17 October 2008, some additional protocols were proposed by the participants. These draft protocols are described below.

To ensure that protocols are actually used, if possible they should be simple and use simple equipment. There is a need to harmonize available methods of quality control, consolidate all protocols on tsetse flies in one document and then circulate this document to all interested workers.

2. BLOOD DIET

2.1. Blood collection

2.1.1. Selection of an abattoir

- Location of abattoir — preferably near the rearing facility
- Slaughter system:
 - Modern systems
 - Hanging slaughter
 - Good water system
 - Reliable source of electricity
 - Cooling system (for blood storage)
 - Large number of animals slaughtered regularly

2.1.2. Selection of animals (antemortem)

- Communicate with abattoir authority about the collection date.
- Visit the holding ground a day before collection.
- At the holding ground the manager, who is also a professional veterinarian, makes an assessment of the animals based on their general condition before they are sold for slaughter. Selected animals are stamped and destined for slaughter.
- At the abattoir animals are checked again, and those fit for slaughter are passed.
- Selection of animals for blood collection, i.e. good physical condition:
 - Animal is not thin
 - Size is in relation to breed of animal
 - Hide of animal is in a good condition, no lesions, no ectoparasites
 - Animal should be active, should not look stressed
 - If possible, the medical history of the animal should be available
 - Age of animal, under 36 months

2.1.3. Blood collection and processing

2.1.3.1. Equipment and materials

- Electric drill to defibrinate the blood [appropriate speed of drill needs to be specified]
- Four litre containers – for the collection of blood from the animals (where funnel system cannot be used)
- Cool box and ice packs

2.1.3.2. Procedures

- Personnel collecting the blood should wear protective clothing (special clothes, gloves, masks and rubber boots).
- Animals are hung, then slaughtered; blood is collected from the jugular vein.
- The collection containers are four little buckets, and are changed after two to three collections. The collected blood is poured into 20 L containers and defibrinated by stirring either manually or with an electric drill for 10–15 minutes.
- An alternative to mechanical defibrination is the addition of anticoagulants to the blood. To use anticoagulants, make a stock solution of acid citrate and add to the blood collection container sufficient to ensure a final citrate ion concentration of 0.010–0.015 M.
- Consecutive collections could be done with a different set of collection equipment.
- The blood is sieved into a 20 L bucket and portioned into sterilized 2 L bottles. These are washed with clean water and kept in freezers for 48 h before transporting them to the rearing institution.
- The blood is kept in cool boxes with ice packs or ice blocks during transportation so as to remain in a frozen state.
- A sample of each batch of blood (1 bottle) is irradiated at 1000 Gy in a solid state, assuming that any microbes in the blood will be killed and quality factor (QF) tests will be conducted.
- The QF is calculated based on the survival, abortions and mortality of flies for 25 days. If the value is 1 and above the blood is assumed to be good for consumption by tsetse flies.
- Each bottle of the rest of the blood from that batch is irradiated at 1000 Gy and screened for microbes, assuming that individual bottles could be contaminated.
- A bottle with 0–4 colonies is categorized as excellent, 5–8 as good, and 9 or more colonies as bad and the blood discarded.

2.1.4. Microbial screening

- 1 mL of blood sample is mixed with nutrient agar in a sterilized Petri dish in a laminar flow hood.
- The mixture is stirred and incubated at 37°C for microbial growth for 24 and 48 h.
- To get discrete colonies initial cultures are sub cultured onto new plates before sending them to a diagnostic laboratory for further isolation and identification.

2.1.5. Cleaning of equipment and storage rooms

- Equipment is first washed in tap water and then rinsed in water with sodium hypochlorite (10%), and finally rinsed in distilled water.
- Plastic equipment is sterilized in an oven at 80°C for 24 h, and for heat-resistant materials the oven temperature is set at 120°C for 24 h.
- Storage freezers and laboratory rooms should be cleaned regularly.

2.2. Blood storage

2.2.1. Equipment

Chest freezers or walk-in freezers (storage space maintained at -20°C)

2.2.2. Procedures

- Blood should be placed in a storage space maintained at -20°C immediately after collection at an abattoir. The storage temperature of -20°C is continuously monitored.
- If blood is removed from storage it should be used immediately.
- Repeated thawing and freezing can have a negative effect on blood quality. If this occurs, then the blood quality needs to be assessed again.

2.2.3. Storage time limit for blood under tropical conditions

Under maintained storage conditions at -20°C, blood storage for at least 1 year is possible (Byamungu et al., this volume). Repeated thawing and freezing of blood is detrimental to blood quality (De Beer et al., this volume).

2.3. Combining bovine and porcine blood

Amendments can be made to include diets with a combination of bovine and porcine blood. A possibility of 50% bovine and 50% porcine blood can be used for *G. brevipalpis*, and 75% bovine and 25% porcine for *G. austeni* and *G. pallidipes*.

2.4. Blood pasteurization (alternative to irradiation)

See paper by Morávek et al., this volume.

3. MASS rearING TSETSE FLIES (PROCESS QUALITY CONTROL)

3.1. Feeding flies – general

- Thawing process for blood: overnight 4°C at the mass rearing facility in Kaliti (Ethiopia) and at the CIRDES (Centre International de Recherche-Développement Sur l'Élevage en Zone Subhumide), Bobo Dioulasso (Burkina Faso), in a container of water at room temperature at OVI (Onderstepoort Veterinary Institute), Onderstepoort, South Africa
- Feeding stimulants: ATP not necessary for colony flies
- Tray/membrane sterilization: as in manual [1]
- Pouring blood: as in manual [1]
- Feeding duration: as in the manual for mass rearing facility in Kaliti and CIRDES. Feeding for 5 min is sufficient for *G. brevipalpis* at OVI.
- Feeding frequency:
 - CIRDES: Frequency reduced to 3 days per week for *G. morsitans submorsitans*, and to 4 days per week for *G. palpalis gambiensis* and *G. tachinoides* at 1- or 2 day intervals.
 - OVI: Teneral *G. brevipalpis* and *G. austeni* are fed daily for the first 2 weeks, and thereafter are fed 3 days per week.
 - At the mass rearing facility in Kaliti *G. pallidipes* and *G. fuscipes* are fed 5 days per week.

- At the Tsetse and Trypanosomiasis Research Institute (TTRI) in the United Republic of Tanzania, *G. austeni* and *G. brevipalpis* preproducing flies (<18 days old) are fed 6 days per week and thereafter 3 days per week; *G. pallidipes* (from the wild) and *G. morsitans centralis* (from Seibersdorf, IAEA) are currently being fed daily during colonization of the flies.

3.2. Use of feeding/taste stimuli (ATP) to facilitate feeding of wild-collected flies

3.2.1. Equipment and materials

- Laminar flow hood
- Feeding/taste stimuli (ATP)
- Quality tested blood
- Balance
- Distilled water (autoclaved)
- 20 mL polyethylene vials
- Syringe and needle (5 mL, disposable)
- Spatula
- Automatic pipette (200–1000 µL)

3.2.2. Procedures

- Mix 0.055 g of feeding/taste stimuli with 1 mL of distilled water in a sterilized 20 mL polyethylene vial.
- Keep dilution for not more at 3 days at 3°C; a new solution needs to be prepared every 3 days.
- Transfer quality-tested blood from the refrigerator.
- Add 0.02 mL of feeding/taste stimuli solution to each millilitre of quality-tested blood.
- Proceed to feed flies.

3.3. TPU3 feeding operation

Specific points related to using the production line:

- At the installation at CIRDES, electrical discharge from the holding lines is regularly observed.
- In the opinion of operators at CIRDES, the control unit on the feeding unit is not user-friendly.

3.3.1. Problems

Unless the tray with the blood is clipped to the heating mat, the surfaces meeting the mat and the cages will not be flat. Consequently, some flies are not fed and mortality increases. Clipping the tray to the mat ensures even distribution of the blood, otherwise the blood flows to one side. Even distribution of the blood ensures equality of the temperature across the membrane surface.

In addition, repeated washing of the trays results in their being deformed.

3.3.2. Solutions

When a tray is uneven, 2–4 clips are used to pin the tray to the mat. This requires time.

To avoid deformation of trays when washing, put a flat support beneath them. Also, maybe a heavier tray would solve the problem.

3.4. SSPC (self stocking production cage)

- Day-zero mating: No problem with *G. pallidipes* at the mass rearing facility in Kaliti. No protocol for *G. fuscipes* available at the mass rearing facility in Kaliti, but it will be established shortly.
- No SSPC protocol at CIRDES at present, but it will be adopted for *G. palpalis gambiensis*. (CIRDES will adopt the SSPC in 2009.)

1.5. 3.5. Feeding in a field cage

See paper by Čiampor et al., this volume.

3.6. Larviposition and abortion

Take account of the circadian rhythm and the lighting *Zeitgeber* to synchronize larviposition in the second half of the afternoon.

3.7. Mortality

Under the TPU3 system at the mass rearing facility in Kaliti, mortality is controlled in cages with even and odd week numbers every other week.

Under the TPU3 system at CIRDES, mortality is controlled at weekly intervals for a colony size under 2000 flies. At higher colony sizes mortality will be controlled every 2 weeks.

At both locations all dead flies are removed from the cages.

3.8. Disease management and disease minimizing procedures

Pathogen control with pharmaceuticals – none being used at the moment but experiments are being conducted at the IAEA laboratories in Seibersdorf. Factors affecting expression levels of hyperplasia in virus-infected flies have to be established.

No protocol is described in the manual [1] for the removal of infected live flies.

No protocol is available for strain purification/selection of healthy insects.

3.9. Adapting flies from the wild to colony conditions

- Currently no experience at CIRDES, but earlier experience exists.
- mass rearing facility in Kaliti has current experience with adapting *G. pallidipes* from Arba Minch - the method described in the manual [1].
- Use feeding stimuli for field-captured flies to improve feeding (Section 3.2.).

- Combinations of bovine and porcine blood will improve survival and productivity of newly colonized flies - this combination has to be optimized for each species (Section 2.3.).

3.10. Strain management

At the mass rearing facility in Kaliti two strains of *G. pallidipes* exist – Arba Minch and Tororo strains. The following has been learned from maintaining these two strains:

- These two strains are compatible for mating and the strains of the salivary hypertrophy virus they carry are different by less than 2%.
- The hyperplasia virus has different effects on the productivity of the two strains; although the two strains show up to 10% hypertrophy, the level is higher in the Arba Minch strain.
- By maintaining the two strains it is hoped that colony levels can be boosted with the less-affected strain.
- Keep the two strains isolated for the reasons listed above.

3.11. Marking procedures

Mass marking for manual release at the mass rearing facility in Kaliti:

After irradiating the male flies they are marked with a fluorescent dye by applying the powder to 100 chilled flies per release box. Fluorescent powder is used to distinguish each release batch: red, orange or green for flies of different weeks.

Marking individual flies for manual release at CIRDES:

Male flies are chilled before irradiation and white, red or orange acrylic paint is applied to the top of the thorax to distinguish between the various release cohorts.

The mass marking protocol as it was used for the eradication programme on Unguja Island should be added to the manual.

4. RHYTHM OF LARVIPOSITION

Aim: To determine the regularity of larviposition in a particular colony

4.1. Equipment and materials

- Personal computer with MS Excel or similar software
- Luxmeter

4.2. Procedures

- Remove all puparia at the end of photophase (in the evening).
- Next morning collect all puparia produced during the scotophase (dark phase of daily cycle).
- In the evening collect all puparia produced during the photophase.
- Repeat steps 2 and 3 several times (optional).
- Evaluation:
 - Compare the number of puparia produced during photophase(s) with that produced during scotophase(s).

Notes: In a colony that behaves normally, the production of puparia during the scotophase (night time) should exceed production of puparia during photophase (day time) by 90%. If the results do not meet this criterion, the following parameters of external factors in the rearing room should be checked:

- a. The light intensity should be in the range of 10–50 lux during photophase (day time).
- b. The length of the light and dark periods should be 1:1 (LD 12:12).

This QC procedure should be performed as a routine check-up in case there is a decrease in production.

5. DEVELOPMENT OF THORACIC MUSCLES

Aim: To monitor fitness of males produced in mass rearing facilities by means of the development of the thorax

5.1. Equipment and materials

- Tsetse flies, 1, 7 and 18 days old males, taken from rearing cages and held under the same conditions as the whole colony
- Stereomicroscope
- Light microscope

5.2. Procedures

See paper by Čiampor et al., this volume.

Note: This protocol should be used occasionally to detect possible changes in normal fly development.

6. BEHAVIOUR

6.1. Mating behaviour

Aim: To monitor fitness of males produced in mass rearing facilities

6.1.1. Equipment and materials

- Virgin tsetse flies 5–10 days old (males and females), in the case of *G. pallidipes* flies 10–12 days old
- Stop watch
- Plexiglass chamber (50 × 50 × 50 cm)
- Video camera with microphone and close-up lenses (optional)

6.1.2. Procedures

- Set up recording conditions in a laboratory:
 - Temperature conditions 23–25°C
 - Relative humidity 80%
 - Room light
 - Recordings from 0800 to 1600

- Introduce a male 5 min before the female into the Plexiglas chamber.
- Wait for 15 min; if mating does not occur introduce a new pair.
- Record mating time for each pair (from the moment the male grabs the female until separation). If available, record each mating with a video camera.
- Record 30 matings.
- Record the number of rejections of the male by the female.
- Record the number of pairs that did not mate.
- After a mating, label each female and isolate it in a small cage.
- Feed the females.
- Dissect females 9–10 days after copulation.
- Record the presence or absence of a larva or egg in the uterus.
- Remove the spermathecae and under a light microscope estimate the degree to which the spermathecae are filled with sperm (use the following categories: 0–25%, 25–50%, 50–75%, 75–100%).
- Calculate the average for both spermathecae.
- Correlate the mating time with the presence or absence of larva and percentage filling of the spermathecae for each generation.
- Record the differences among generations.
- If possible, use mass reared males and wild females (optional).

Note: Females with sperm in their spermathecae but without a larva in the uterus should be judged as not ovulated; those with a larva in the uterus are assumed to have ovulated. Observations should be recorded every 3 months.

6.2. Flight

6.2.1. *Flight mill*

Aim: To monitor fitness of males produced in mass rearing facilities by means of flight activity

6.2.1.1. *Equipment and materials*

- Male flies held under colony conditions
- Flight mill device (See paper by Čiampor et al., this volume)
- Stop watch

6.2.1.2. *Procedures*

- Set up recording conditions in a laboratory:
 - Temperature 23–25°C
 - Relative humidity 80%
 - Room light
 - Recordings from 0800 to 1000
- Mount the male on to the flight mill by attaching it by instant-glue to the thorax.
- Start the watch and let the fly move for 10 min.
- Record the number of circles.
- Repeat measurements with 10 males.
- Repeat steps 2–5 for 15 days.

- Count the average flight activity (circles flown) for each day.
- Plot the average values on the chart, e.g. in MS Excel.

Note: If normally developed males are produced, the values of the flight activity should rise at least to day 11–12 (recorded in *G. pallidipes*).

6.2.2. *Wind tunnel*

See paper by Guerin et al., this volume.

6.3. Acoustic signals

See paper by Kratochvil et al., this volume.

Aim: To determine fitness of males produced in a mass rearing facility

6.3.1. *Equipment and materials*

- Five 3 day old males
- Personal computer with installed STX software
- Microphone
- Thermometer
- Hygrometer
- Lux meter

6.3.2. *Procedures*

- Set up recording conditions as follows:
 - Temperature 24°C
 - Relative humidity 80%
 - Light 800 lux
- Set up the recording device.
- Set up the software as follows:
 - Use the **I/O Setup** dialog to select the device for signal recording
 - Select the sampling frequency (**sampling freq.**)
 - Select the duration of background and signal analysis (**background time** and **analysis time**)
 - Adjust the analysis parameters in the **Settings** dialog (optional)
- Perform background analysis — select the **background time** and press the button **Background Analysis**.
- Place five 3 day old males in a rearing cage.
- Put the cage under the microphone.
- Place a Petri dish with warm bovine blood 1 cm under the cage.
- Perform signal analysis — set the **analysis time** to 2 min and press the button **Signal Analysis – Record**.
- Repeat recording with 10 groups of males.
- Compare recorded values with those obtained with irradiated males.

Note: Only directly recorded files can be analysed.

The Tsetse STX programme implements a method to extract tsetse fly ‘songs’ from a recording and measure the relative duration of the songs. This duration is an indicator of fly activity.

Hardware/Software Requirements: Desktop personal computer or laptop with Windows 2000/XP/Vista operating system, system memory at least 500 MB, free hard disk space 5 GB, graphic adapter, audio adapter supporting standard Windows MME drivers.

For software installation, put the CD in the computer, press Setup and follow instructions.

The background analysis must be performed: 1) before starting the first measurement, 2) any time the background noise has changed, or 3) if the I/O setup or the analysis parameters have been changed. Threshold values adjustment is necessary for the song detection. During background analysis, no flies should be present.

During signal analysis, a weighting function is computed, which is used to decide if tsetse flies are singing or not. The signal is classified as song if the value of the weighting function is above the selected threshold **Wth** (see **Settings** dialog). After finishing the analysis, a graphic is displayed which contains the following functions:

- The modified (filtered and smoothed) amplitude spectrogram (top)
- The parameters used to build the weighting function (middle)
- The weighing function (bottom)

In the title of the weighting function, the three values **Tall** (analysis duration), **Tsong** (summed duration of songs) and **RSD** (relative song duration = $Tsong/Tall*100$) are displayed.

All signals recorded and processed by this programme are stored in sound files located in the installation directory. The files are named **INL.yyyy.mm.dd.hh.mm.ss.WAV** for the background analysis and **ANA.yyyy.mm.dd.hh.mm.ss.WAV** for the signal analysis (yyyy.mm.dd and hh.mm.ss are the recording date and time).

The threshold values for the weighting parameters are computed during the background analysis and should not be changed by the user. It may be necessary to activate or deactivate a weighing parameter (using the checkboxes on the right side of the threshold values) or to adjust the value of **Wth**.

7. VECTORIAL CAPACITY

7.1. Assessment of prophylactic drug treatment on the tsetse fly’s vectorial capacity

Laboratory and field data show that non-teneral tsetse flies can act as vectors of trypanosomes. Hence, the release of large numbers of sterile male flies could result in an increase in the prevalence of trypanosomosis. To reduce the probability of a sterile male fly becoming infected and then a vector, treatment with a prophylactic trypanocidal drug may be necessary. A protocol for testing the efficacy of such a drug is presented below.

7.1.1. Protocol

- Tsetse flies
 - Teneral male tsetse flies (48–60 h old)

- Batches of 4×80 flies per treatment (i.e. dose rate of drug tested and control)
- Label cages in accordance with experimental groups
- Preparation of drug/blood mixture
 - Dilute prophylactic drug in 20 mL thawed defibrinated bovine blood to obtain required concentration (weight of active ingredient/mL of blood).
 - Mix drug/blood mixture slowly to ensure even distribution of drug in blood.
 - Store drug/blood mixture at 2–4°C for use within 1 h.
- Application of drug/blood mixture
 - Apply drug/blood mixture using normal membrane-feeding procedures.
 - Feed control group on defibrinated blood without drug.
 - Manually remove flies that are less than half-engorged from cages containing experimental flies using a tube.
- Maintenance of drug-treated flies and controls
 - Use the in vivo feeding system to maintain drug-treated flies and controls (e.g. rabbit ears, flank of goats).
 - Feed on alternate days.
 - Replace feeding host at weekly intervals.
- Preparation of infected host animals
 - Use documented strains of *Trypanosoma congolense*, *T. brucei brucei* or *T. vivax* that result in mature infections in at least 10% of the flies.
 - Infect host animal using standard infection procedures.
 - Measure parasitaemia daily.
- Infection of drug-treated tsetse flies and controls
 - Starve drug-treated flies and controls for 48 h before exposure to an infective feed.
 - Feed tsetse on an infected host with a parasitaemia of at least 20 trypanosomes/field of whole blood (x 400 magnification).
 - Manually remove flies that are less than half-engorged from cages containing experimental flies using a tube.
- Maintenance of drug-treated infected flies and controls
 - Use the in vivo feeding system to maintain drug-treated infected flies and controls (e.g. rabbits).
 - Feed on alternate days.
 - Replace feeding host at weekly intervals.
- Determination of infection rate
 - Starve drug-treated and control flies for 48 h before dissection.
 - Remove dead flies before dissection.
 - Twenty days (for *T. congolense*), 25 days (for *T. b. brucei*) and 15 days (for *T. vivax*) after the initial infective meal, dissect all surviving flies using the method described by Lloyd and Johnson [2] (i.e. dissect the midgut, mouthparts and/or salivary glands).
 - Examine mouthparts (for infections with *T. vivax*), mouthparts and midgut (for infections with *T. congolense*) and mouthparts, midgut and salivary glands (for infections with *T. b. brucei*) using a microscope (x 400 magnification) for presence of trypanosomes.
 - Summarize dissection results per batch of flies as follows:
 - Number of flies dissected
 - Number of flies infected in midgut, mouthparts, salivary glands
 - Number of dead flies
- Analyses
 - Compare mature and immature infection rates and survival between treatments statistically.

7.1.2. Equipment and materials

- Dissecting microscope
- Compound microscope
- Drug (check expiry date and active ingredient)
- Dissecting kits
- Silicon feeding membrane
- Sterile 25 mL tubes with screw caps (for blood storage)
- Defibrinated bovine blood
- Slides and cover slips
- Normal saline
- Clean host animals (e.g. no insecticide treatment, no antibiotic treatment, dewormed)
- Documented trypanosome strains

7.2. Treatment of sterile male tsetse flies with isometamidium chloride

Isometamidium chloride has proven to have a prophylactic action in tsetse flies. Feeding sterile male flies a bloodmeal containing isometamidium chloride reduces the flies' vectorial capacity, thereby reducing the probability of becoming infected with trypanosomes.

7.2.1. Protocol

- Preparation of isometamidium chloride/blood mixture
 - Dilute isometamidium chloride in the required volume of defibrinated bovine blood to obtain a concentration weight of 10 µg active ingredient/mL of defibrinated blood.
 - Mix isometamidium chloride/blood mixture slowly to ensure an even distribution of drug in the blood.
 - Store the isometamidium chloride/blood mixture at 2–4°C for use within 1 h.
- Application of drug/blood mixture
 - Apply isometamidium chloride/blood mixture twice, using normal membrane-feeding procedures, and on successive days.

REFERENCES TO THE ANNEX

- [1] (FAO/IAEA) Food and Agriculture Organization of the United Nations/International Atomic Energy Agency, FAO/IAEA standard operating procedures for mass rearing tsetse flies, IAEA, Vienna, Austria (2006).
http://www-naweb.iaea.org/nafa/ipc/public/Tsetse_Rearing_SOP_web.pdf
- [2] LLOYD, L.L., JOHNSON, W.B., The trypanosome infections of tsetse flies in Northern Nigeria and a method of estimation, Bull. Entomol. Res. **14** (1924) 225–227.

LIST OF PARTICIPANTS

- Bance, A. Z. Centre International de Recherche-Développement sur l'Élevage en Zone Subhumide,
N°559, rue 5-31 angle avenue du Gouverneur Louveau 01, B.P. 454,
Bobo Dioulasso 01, Burkina Faso
- Briceño, R. D. Escuela de Biología, Universidad de Costa Rica,
Ciudad Universitaria, Costa Rica
- Byamungu, M. B. Tsetse and Trypanosomiasis Research Institute, P.O. Box 1026,
Tanga, United Republic of Tanzania
- Čiampor, Jr., F. Institute of Zoology, Slovak Academy of Sciences,
Bratislava, SK-845 06, Slovakia
- De Beer, C. J. ARC-Onderstepoort Veterinary Institute,
Old Soutpans Road, Private Bag X05,
0110 Onderstepoort, South Africa
- Eberhard, W. Escuela de Biología, Universidad de Costa Rica,
Costa Rica
- Gemeiner, M. Institut für Medizinische Chemie,
Veterinärmedizinische Universität Wien,
Wien, Austria
- Guerin, P. M. Institute of Biology, University of Neuchâtel,
Rue Emile-Argand 11, Case Postale 158,
2009 Neuchâtel, Switzerland
- Kabore, I. Centre International de Recherche-Développement sur l'Élevage en Zone Subhumide,
N°559, rue 5-31 angle avenue du Gouverneur Louveau 01, B.P. 454,
Bobo Dioulasso 01, Burkina Faso
- Kratochvil, H. Department of Evolutionary Biology,
University of Vienna, Halsriegelstr. 34,
Wien A-1090, Austria
- Mekonnen, S. Kaliti Tsetse Rearing and Irradiation Center, STEP, P.O. Box 19917,
Addis Ababa, Ethiopia
- Morávek, I. Department of Manufacturing Systems,
Slovak Technical University, Namestie Slobody 17,
812 31 Bratislava, Slovakia
- Okoth, S. Trypanosomiasis Research Centre,
Kenya Agricultural Research Institute (TRC-KARI), Box 362,
Kikuyu, Kenya

- Parker, A. G. (Scientific Secretary) Joint FAO/IAEA Programme of Nuclear Techniques in Food and Agriculture, FAO/IAEA Agriculture and Biotechnology Laboratory, Seibersdorf A-2444, Austria
- Potgieter, F. T. ARC-Onderstepoort Veterinary Institute, Onderstepoort, South Africa
- Takáč, P. Institute of Zoology, Slovak Academy of Sciences, Bratislava, SK-845 06, Slovakia
- Van den Bossche, P. Veterinary Department, Prince Leopold Institute of Tropical Medicine, Nationalestraat 155, 2000 Antwerp, Belgium
- Žd'árek, J. Institute of Organic Chemistry and Biochemistry, AV CR, Prague, Czech Republic



IAEA

International Atomic Energy Agency

No. 22

Where to order IAEA publications

In the following countries IAEA publications may be purchased from the sources listed below, or from major local booksellers. Payment may be made in local currency or with UNESCO coupons.

AUSTRALIA

DA Information Services, 648 Whitehorse Road, MITCHAM 3132
Telephone: +61 3 9210 7777 • Fax: +61 3 9210 7788
Email: service@dadirect.com.au • Web site: <http://www.dadirect.com.au>

BELGIUM

Jean de Lannoy, avenue du Roi 202, B-1190 Brussels
Telephone: +32 2 538 43 08 • Fax: +32 2 538 08 41
Email: jean.de.lannoy@infoboard.be • Web site: <http://www.jean-de-lannoy.be>

CANADA

Bernan Associates, 4501 Forbes Blvd, Suite 200, Lanham, MD 20706-4346, USA
Telephone: 1-800-865-3457 • Fax: 1-800-865-3450
Email: customercare@bernan.com • Web site: <http://www.bernan.com>

Renouf Publishing Company Ltd., 1-5369 Canotek Rd., Ottawa, Ontario, K1J 9J3
Telephone: +613 745 2665 • Fax: +613 745 7660
Email: order.dept@renoufbooks.com • Web site: <http://www.renoufbooks.com>

CHINA

IAEA Publications in Chinese: China Nuclear Energy Industry Corporation, Translation Section, P.O. Box 2103, Beijing

CZECH REPUBLIC

Suweco CZ, S.R.O., Klecakova 347, 180 21 Praha 9
Telephone: +420 26603 5364 • Fax: +420 28482 1646
Email: nakup@suweco.cz • Web site: <http://www.suweco.cz>

FINLAND

Akateeminen Kirjakauppa, PO BOX 128 (Keskuskatu 1), FIN-00101 Helsinki
Telephone: +358 9 121 41 • Fax: +358 9 121 4450
Email: akatilauk@akateeminen.com • Web site: <http://www.akateeminen.com>

FRANCE

Form-Edit, 5, rue Janssen, P.O. Box 25, F-75921 Paris Cedex 19
Telephone: +33 1 42 01 49 49 • Fax: +33 1 42 01 90 90
Email: formedit@formedit.fr • Web site: <http://www.formedit.fr>
Lavoisier SAS, 145 rue de Provigny, 94236 Cachan Cedex
Telephone: + 33 1 47 40 67 02 • Fax +33 1 47 40 67 02
Email: romuald.verrier@lavoisier.fr • Web site: <http://www.lavoisier.fr>

GERMANY

UNO-Verlag, Vertriebs- und Verlags GmbH, Am Hofgarten 10, D-53113 Bonn
Telephone: + 49 228 94 90 20 • Fax: +49 228 94 90 20 or +49 228 94 90 222
Email: bestellung@uno-verlag.de • Web site: <http://www.uno-verlag.de>

HUNGARY

Librotrade Ltd., Book Import, P.O. Box 126, H-1656 Budapest
Telephone: +36 1 257 7777 • Fax: +36 1 257 7472 • Email: books@librotrade.hu

INDIA

Allied Publishers Group, 1st Floor, Dubash House, 15, J. N. Heredia Marg, Ballard Estate, Mumbai 400 001,
Telephone: +91 22 22617926/27 • Fax: +91 22 22617928
Email: alliedpl@vsnl.com • Web site: <http://www.alliedpublishers.com>

Bookwell, 2/72, Nirankari Colony, Delhi 110009
Telephone: +91 11 23268786, +91 11 23257264 • Fax: +91 11 23281315
Email: bookwell@vsnl.net

ITALY

Libreria Scientifica Dott. Lucio di Biasio "AEIOU", Via Coronelli 6, I-20146 Milan
Telephone: +39 02 48 95 45 52 or 48 95 45 62 • Fax: +39 02 48 95 45 48
Email: info@libreriaaeiou.eu • Website: www.libreriaaeiou.eu

JAPAN

Maruzen Company, Ltd., 13-6 Nihonbashi, 3 chome, Chuo-ku, Tokyo 103-0027
Telephone: +81 3 3275 8582 • Fax: +81 3 3275 9072
Email: journal@maruzen.co.jp • Web site: <http://www.maruzen.co.jp>

REPUBLIC OF KOREA

KINS Inc., Information Business Dept. Samho Bldg. 2nd Floor, 275-1 Yang Jae-dong SeoCho-G, Seoul 137-130
Telephone: +02 589 1740 • Fax: +02 589 1746 • Web site: <http://www.kins.re.kr>

NETHERLANDS

De Lindeboom Internationale Publicaties B.V., M.A. de Ruyterstraat 20A, NL-7482 BZ Haaksbergen
Telephone: +31 (0) 53 5740004 • Fax: +31 (0) 53 5729296
Email: books@delindeboom.com • Web site: <http://www.delindeboom.com>

Martinus Nijhoff International, Koraalrood 50, P.O. Box 1853, 2700 CZ Zoetermeer
Telephone: +31 793 684 400 • Fax: +31 793 615 698
Email: info@nijhoff.nl • Web site: <http://www.nijhoff.nl>

Swets and Zeitlinger b.v., P.O. Box 830, 2160 SZ Lisse
Telephone: +31 252 435 111 • Fax: +31 252 415 888
Email: info@swets.nl • Web site: <http://www.swets.nl>

NEW ZEALAND

DA Information Services, 648 Whitehorse Road, MITCHAM 3132, Australia
Telephone: +61 3 9210 7777 • Fax: +61 3 9210 7788
Email: service@dadirect.com.au • Web site: <http://www.dadirect.com.au>

SLOVENIA

Cankarjeva Založba d.d., Kopitarjeva 2, SI-1512 Ljubljana
Telephone: +386 1 432 31 44 • Fax: +386 1 230 14 35
Email: import.books@cankarjeva-z.si • Web site: <http://www.cankarjeva-z.si/uvvoz>

SPAIN

Díaz de Santos, S.A., c/ Juan Bravo, 3A, E-28006 Madrid
Telephone: +34 91 781 94 80 • Fax: +34 91 575 55 63
Email: compras@diazdesantos.es, carmela@diazdesantos.es, barcelona@diazdesantos.es, julio@diazdesantos.es
Web site: <http://www.diazdesantos.es>

UNITED KINGDOM

The Stationery Office Ltd, International Sales Agency, PO Box 29, Norwich, NR3 1 GN
Telephone (orders): +44 870 600 5552 • (enquiries): +44 207 873 8372 • Fax: +44 207 873 8203
Email (orders): book.orders@tso.co.uk • (enquiries): book.enquiries@tso.co.uk • Web site: <http://www.tso.co.uk>

On-line orders

DELTA Int. Book Wholesalers Ltd., 39 Alexandra Road, Addlestone, Surrey, KT15 2PQ
Email: info@profbooks.com • Web site: <http://www.profbooks.com>

Books on the Environment

Earthprint Ltd., P.O. Box 119, Stevenage SG1 4TP
Telephone: +44 1438748111 • Fax: +44 1438748844
Email: orders@earthprint.com • Web site: <http://www.earthprint.com>

UNITED NATIONS

Dept. I004, Room DC2-0853, First Avenue at 46th Street, New York, N.Y. 10017, USA
(UN) Telephone: +800 253-9646 or +212 963-8302 • Fax: +212 963-3489
Email: publications@un.org • Web site: <http://www.un.org>

UNITED STATES OF AMERICA

Bernan Associates, 4501 Forbes Blvd., Suite 200, Lanham, MD 20706-4346
Telephone: 1-800-865-3457 • Fax: 1-800-865-3450
Email: customercare@bernan.com • Web site: <http://www.bernan.com>

Renouf Publishing Company Ltd., 812 Proctor Ave., Ogdensburg, NY, 13669
Telephone: +888 551 7470 (toll-free) • Fax: +888 568 8546 (toll-free)
Email: order.dept@renoufbooks.com • Web site: <http://www.renoufbooks.com>

Orders and requests for information may also be addressed directly to:

Marketing and Sales Unit, International Atomic Energy Agency

Vienna International Centre, PO Box 100, 1400 Vienna, Austria
Telephone: +43 1 2600 22529 (or 22530) • Fax: +43 1 2600 29302
Email: sales.publications@iaea.org • Web site: <http://www.iaea.org/books>

