FAO/IAEA Standard Operating Procedures for Mass-Rearing Tsetse Flies

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1. Introduction

1.1. Scope

This document is intended for use in laboratories and institutions that maintain colonies of tsetse flies. It outlines procedures for colony initiation and mass-rearing, and includes some aspects of fly production quality control. Due to a lack of facilities that maintain large colonies of tsetse flies, the procedures described herein are basically those developed at the Entomology Unit, FAO/IAEA Agriculture and Biotechnology Laboratory, Seibersdorf, Austria.

1.2. Background

Area-wide integrated pest management (AW-IPM) programmes using the sterile insect technique (SIT) depend, during a defined period, on a reliable supply of large numbers of high-quality sterile insects for release. The insects are reared in special large rearing facilities or factories under defined conditions.

Tsetse mass-rearing is simplified in the laboratory because only two developmental stages need to be considered, i.e. the adult and pupal stages, and the only dietary requirement is warm vertebrate blood for adult feeding. Reproduction in tsetse flies is by adenotrophic viviparity, where the female gives birth to live offspring. The larvae are nourished within the mother by secretion from highly modified accessory glands, and are larviposited at an advanced stage of development. Pupation takes place within a few hours. Larval production from fertilized females begins about 20 days after female emergence. The female produces a single larva at intervals of about 9–10 days, depending on the temperature. The duration of the pupal stage is about 30 days, with males requiring 1–2 days longer than females to emerge. The maximum adult female lifespan is about 100–120 days, equivalent to the time required to produce 9 larvae. However, in the laboratory in a typical production cage, the average is about 4 pupae per female. If the adult emergence rate is 90%, over a period of 3 months a female will produce an average of 1.8 emerged males and 1.8 females. The colony will not increase in size if the number of pupae per initial female is less than 2.2.

In traditional colony maintenance, males and females are separated at emergence until sexually mature, and then are introduced into cages for mating. After a period of 2 or 3 days the sexes are separated again, and the females are kept for larval production while males are discarded. Because of the low reproductive rate, females are kept for a considerable period of time to produce a sufficient number of insects both to maintain the colony and provide males for sterilization and release. The design of the rearing system is such that the cage holding the adults allows flies to feed on blood and the deposited third-instar larvae to crawl out. Fly performance is determined by monitoring survival and larval production.

This rearing manual is written as a series of standard operating procedures (SOP). Procedures for rearing blood-feeding insects such as tsetse flies are rather unique (Cohen 2003). Several papers describing various procedures for rearing tsetse flies have been published (Bauer and Wetzel 1976; Wetzel

and Luger 1978; Wetzel 1980; Oladunmade et al. 1990; Feldmann 1993, 1994a, b, c; Bandah 1994; Wanyonje 1994; Zdarek and Denlinger 1995; Gooding et al. 1997; Malele and Parker 1999; Opiyo et al. 1999, 2000; Olet et al. 2002; IAEA 2003; Parker 2005). However, only one of them (IAEA 2003) mentions the most recent rearing system in which flies (held in stationary cages) are fed by bringing blood to them (Tsetse Production Unit 3) (TPU 3) (sections 3.1.2. and 3.2.3.).

1.3. Rationale

After the successful eradication of *Glossina austeni* Newstead from Unguja Island, Zanzibar, other tsetse eradication programmes integrating the SIT are being planned in mainland Africa. The affected African countries through their Heads of State and Government adopted the decision AGH/Dec. 156 (XXXVI), July 2000, to use the SIT to eventually eradicate tsetse flies from the African continent. As a result, the Pan African Tsetse and Trypanosomosis Eradication Campaign (PATTEC) was established. However, eradication can only be achieved when an efficient and reliable male production system is in place that generates good quality flies for sterilization and release — flies that compete successfully with wild males to mate with wild females. Currently there are very few rearing centres in the affected countries, and more need to be established (FAO/IAEA 2004). A standardized rearing system will help ensure an adequate supply of quality flies, and allow for comparisons to be made over time and between rearing facilities.

2. Colonization

Tsetse colonies can be initiated with one or more of the following methods:

- Collecting pupae in the field
- Collecting female adults in the field
- Obtaining pupae from an established colony elsewhere

Field collection of pupae is difficult, and the yield of pupae may be very small, depending on the species. Collecting female adults in the field is much easier, but the wild females may be infected with pathogenic trypanosomes leading to the risk that animals used for *in vivo* blood feeding, or the insectary staff, could be infected. For this reason field-collected females are usually kept separately, and only the pupae they produce are used to form the new colony. Obtaining pupae from an established colony is the easiest method, but the strain will not be exactly the same as the local strain.

Adult tsetse flies are usually maintained at 24°C and 70–80% RH. Savannah species require less humid conditions, and riverine species may require conditions above 80% RH. Pupae can be maintained under the same conditions as adults, but experience has shown that species such as *Glossina tachinoides* Westwood survive better at 23°C and 82.5% RH, and *G. austeni* pupae, after they are 20 days old, survive better at 85–88% RH.

Lighting should be subdued and indirect, using tungsten lamps or high-frequency fluorescent-tube lights on a 12:12 photoperiod. Light distribution is important because it influences the distribution of flies within a cage. Uneven distribution of light in cages may encourage crowding towards the light source, leading to increased fly mortality.

The success of colony establishment depends on the survival and productivity of flies. After successful mating, female flies should live long enough to produce pupae. When using insects collected from the field, it is important to observe mating behaviour and any abnormalities that could interfere with colony establishment. It has been observed that with some species, although flies may mate successfully and females become inseminated as evidenced by the filled spermathecae, the sperm may be non-motile. Therefore, periodically, a sample of male flies should be dissected, and the testes and accessory glands examined for evidence of development and sperm maturation. In *Glossina pallidipes* Austen, salivary gland hyperplasia (SGH) is associated with sterility and aspermia among males. The occurrence of this virus-induced condition or other pathogens in a colony may interfere with the successful establishment of the colony.

2.1. Colonization from Field-Collected Material

Collection of Pupae

This is a tedious procedure; it requires pupae to be located in the ground over large areas, large numbers of people, and considerable time. If the expertise exists, and the particular tsetse species concentrates pupae at defined breeding sites, it is possible to carry out efficient pupal searches. However, the collected pupae will be of unknown age, complicating the initial set-up of adult cages. It should be pointed out that, apart from obtaining pupae of unknown age, some of the pupae might contain hymenopteran or dipteran parasitoids, while others may be dead. However, colony initiation using field-collected pupae eliminates the introduction of pathogenic trypanosomes into the colony. Field-collected pupae should be quarantined to avoid introducing any parasitoids into the main insectary.

Collection of Female Adults

For many species, efficient trapping systems are available to attract wild flies for collection (Bandah 1994, Kuzoe and Schofield 2004). The first requirement is to successfully transfer the live adult females to an insectary; if the insectary is located far from the trapping area, special transport protocols may be necessary. Careful handling of flies, from the time they are caught in traps to the time they reach the insectary, is essential. Once in the insectary, the females have to be maintained as long as possible to maximize larval production. The colony performance and rate of adaptation should be followed through several generations. This includes monitoring the readiness of flies to feed during the first days of life, which influences the subsequent survival, mating, and timely production of pupae.

Field-collected adult females may be infected with trypanosomes, and once in the insectary they could infect hosts on which they are maintained in an *in vivo* feeding system (Wanyonje 1994) (section 3.2.5.). It is important that any infected females do not transmit trypanosomes to hosts, other flies or insectary workers. Field-collected females must not be introduced into the colony.

Trauma experienced during trapping and transport may, during the first few days after capture, result in females aborting eggs or larvae at different developmental stages, and flies caught in traps may fail to feed in the insectary. The handling of flies, from the moment they are caught in traps to the time they reach the insectary, will determine how many will survive long enough to take the first meal in captivity and thus minimize mortality due to starvation.

The trapping site and the insectary may be separated by a considerable distance, requiring a travel time of several hours by road or 1–2 hours by air. If there is an airport in the vicinity, it is possible to arrange air transport of live adult flies from the field station, where flies are housed after trapping, to the insectary. An air shipment of adult flies requires that arrangements with the airline be made in advance, and that a schedule for dispatch and reception is

prepared. If feeding can be done at the field station, flies should be shipped the day following feeding. Male flies can be removed at the field station, permitting the shipment of only females to the insectary.

The exact number of wild female tsetse required to establish a good laboratory colony is unknown, but two factors must be considered: genetic diversity (heterozygosity) of the colony (Parker 2005), and the relatively low reproductive rate of tsetse flies. In addition, as is usually the case, the initial survival and productivity of flies in the new colony will be low. Therefore, to initiate a new colony, a rather large number of wild females are required. Experience with tsetse indicates that the number required will be at least several hundred, and it is advisable to plan on collecting several thousand wild females. Of course, the final desired size of the colony will be achieved more quickly if the starting size is large rather than small. Once the survival and productivity of the colony have stabilized and are satisfactory, the colony size should double every 3–4 months (section 3.6.).

Transporting Adults by Air

The best procedure is **not** to transport field-collected female adults to the main insectary or rearing facility which houses the fly colony, but instead to bring these adults to a field station or field insectary where they can be held and fed, and pupae collected from them. These produced pupae can then be transported more easily to the rearing facility. However, if no field station is available, then the collected female adults have to be transported to the main insectary. (**Note:** If pupae are transported from the field station to the insectary or rearing facility, sections 2.2.1. and 2.2.2. provide guidelines. If pupae are collected from wild female adults held at a field station, it is assumed that the pupae do not harbour any parasitoids and can be safely received in the insectary. However, if pupae are found in soil in natural conditions, this cannot be assumed, and such pupae brought to the insectary must be held in a quarantine area.)

After successfully collecting adult females in traps, it is essential that these females be transported to the insectary quickly and very gently. The work of field collection must not be lost by poor packaging and shipment of the flies. Also the details of the shipment must be communicated promptly to the insectary staff so that there is no delay in handling the flies on arrival. Transporting flies is more difficult than transporting pupae, and precautions to provide good care of the flies are essential.

Air transport is the best means of transport because it is quick and avoids rough treatment of the flies. However, if ground transport is the only option, then great care must be taken in handling the flies to prevent fly mortality due to shaking and crowding of the flies, and overheating, during a long road trip.

Delays in obtaining the shipment after the flight arrives at the airport must be avoided, and if possible arrangements with customs and other official agencies should be made in advance to expedite the clearance of the

package. Preparations in the quarantine area of the insectary must be ready to receive the shipment of wild female flies as soon as it arrives.

HEADING: 2.1. Colonization from Field-Collected Material	SUBHEADING: 2.1.1. Collection of Pupae
DATE:	AUTHORIZATION:
COMMENTS:	

Aim: To establish a colony of tsetse flies using pupae collected from the field

Equipment and Materials

- Transport vehicle, equipment and containers (which have not been in contact with insecticides)
- Quarantine room
- Holding room
- Feeding room (optional)
- Cleaning area
- Air conditioner
- Humidifier
- Sterilizing oven
- Trolleys or shelves (for keeping cages of flies)
- Emergence cages
- Holding cages
- Sieve (coarse, usually used to sieve flour)
- Vials or match boxes
- Cotton wool
- Sticks (about 45-cm long)
- Separating tube
- Feeding trays and silicone membranes
- Heating mats
- Feeding table/surface
- Refrigerator
- Deep-freeze
- Quality-tested blood (section 4.5.2.)
- Live hosts (if *in vitro* feeding system is unavailable)
- Temperature and RH monitor (with visual display)
- Maximum/minimum thermometer
- Thermocouple thermometer or Thermoscan IR thermometer
- Timer
- Compound microscope
- Stereoscopic microscope (for dissection)
- Dissecting instruments
- Microscope slides
- Cover slips
- Normal saline

HEADING: 2.1. Colonization from Field-Collected Material	SUBHEADING: 2.1.1. Collection of Pupae
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure:

1. Locate the breeding site.
2. Carefully inspect the site, loosening the sand or soil with a stick and sweeping it outwards.
3. Search the loosened soil for pupae.
4. The sand or soil may also be sieved using a coarse flour sieve that will allow fine sand and soil to pass through, reducing the amount of debris and thus permitting the pupae to be more easily recognized.
5. Collect the pupae and store them in a vial or matchbox lined with cotton wool.
6. On arrival at the insectary, transfer the pupae to a quarantine area and place them in an emergence cage in a holding room maintained under controlled conditions (Annex 4.). Retain the pupae for 35–40 days.
7. Prevent the escape of any emerging parasitoids by placing the emergence cage in a large cage with fine-mesh netting.
8. Remove emerged flies using the separating tube, identifying females and males.
9. Keep females and males in separate cages and feed (section 3.2.).
10. Initially, when flies of the appropriate age are not available, keep females and males in a 1:1 ratio in the same cage starting 3 days after emergence.
11. After 7–10 days, remove the males.
12. If available, cage 3-day-old females together with ≥10-day-old males at a female:male ratio of 1:1. After 3–4 days, remove the males.
13. Place the appropriate number of females in production

HEADING: 2.1. Colonization from Field-Collected Material	SUBHEADING: 2.1.1. Collection of Pupae
DATE:	AUTHORIZATION:
COMMENTS:	

cages (Annex 3.), and maintain the females as in a normal colony (section 3.).

14. If there is a shortage of males, after 1 or 2 days reuse males for mating, otherwise discard them.
15. Record survival and production data (section 3.6.).
16. At intervals, dissect a sample of males to examine testes for sperm motility and salivary glands for salivary gland hyperplasia (SGH).
17. If females do not produce pupae as expected, dissect a sample of females and examine their spermathecae. If the mean spermathecal value (section 5.2.) is low, modify the mating procedures to ensure that females are mated, e.g. leave males and females together longer, or add more males to the cage.

HEADING: 2.1. Colonization from Field-Collected Material	SUBHEADING: 2.1.2. Collection of Female Adults
DATE:	AUTHORIZATION:
COMMENTS:	

Aim: To establish a colony of tsetse flies using pupae produced by wild female adults

Equipment and Materials

- Transport vehicle, equipment, traps and containers (which have not been in contact with insecticides)
- Collecting cages
- Cool boxes with ice packs
- Holding room
- Feeding room
- Separating tube
- Chiller (optional)
- Appropriate traps
- Emergence cages
- Production cages
- Membrane-feeding equipment (section 3.2.)
- Quality-tested blood
- Live hosts (if membrane-feeding system is unavailable) (section 3.2.5.)
- Stereoscopic microscope (for dissection)
- Dissecting instruments
- Compound microscope
- Glass slides
- Cover slips
- Normal saline

HEADING: 2.1. Colonization from Field-Collected Material	SUBHEADING: 2.1.2. Collection of Female Adults
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure:

1. Select trapping sites that could yield a sufficient number of flies.
2. Trap flies using appropriate traps.
3. Empty the traps, and remove a sample of flies.
4. Dissect female flies, and examine the spermathecae (sections 2.1.1. and 5.2.) and uterus.
5. Establish the insemination rate and the reproductive status of wild-caught female flies prior to the main collection; this will influence the decision to include (or not include) males in female production cages.
6. Set up a sufficient number of traps during the peak activity time.
7. Service a trap every 2–3 hours, removing the collecting cage containing trapped flies.
8. Place the collecting cage in a moist and, if possible, dark container, e.g. a cool box.
9. Add ice packs to the box to keep it cool, but avoid chilling the flies.
10. Return to the field station.
11. Separate the sexes and species, and place 20–30 females in each production cage (diameter 20 cm) labelled by species and date of collection.
12. Do not include males in production cages when the insemination rate is 85% or higher.
13. If the insemination rate is low, add males to a production cage at a ratio of 4 females to 1 male.
14. Kill all excess males to avoid infestation of the field station.
15. Transfer production cages to the holding room maintained at 23–24°C and 75–80% RH.

HEADING: 2.1. Colonization from Field-Collected Material	SUBHEADING: 2.1.2. Collection of Female Adults
DATE:	AUTHORIZATION:
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16. Allow flies to “settle down” before feeding. (If trapping is done in the evening, feed flies the following day.)
17. Feed flies on membranes, if available (section 3.2.), or on live hosts (section 3.2.5.).
18. Field-collected females may be infected with trypanosomes, and precautions must be taken to prevent live hosts or other flies from also becoming infected (section 2.1.). Insectary staff must take care that they themselves do not become infected.
19. During feeding, cover cages with a damp dark cloth to increase relative humidity and prevent flies from being distracted by nearby movement.
20. Check the immediate feeding response of the flies, and determine and record the percentage of flies that feed.
21. If the feeding response is poor, check that the membrane being used is the correct one for the tsetse species (it may be too thick or too thin for the particular species) (sections 3.2. and 3.2.1.).
22. Check the temperature of the blood, light intensity and relative humidity, and adjust as required.
23. When the flies have fed, return the cages to the holding room.
24. Clean and sterilize all feeding equipment (sections 3.2. and 3.2.4.).
25. Collect pupae produced by wild females, and ship them to the insectary.
26. On day 25 after larviposition, transfer pupae to Petri dishes and place in an emergence cage.
27. When flies begin emerging, separate the sexes and treat them as in section 3.
28. Maintain the flies until they stop producing pupae.
29. Monitor the survival and pupal production for each

HEADING: 2.1. Colonization from Field-Collected Material	SUBHEADING: 2.1.2. Collection of Female Adults
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batch of flies (section 3.6.).

30. Establish the colony using flies that emerge from the pupae deposited by wild-caught flies. However, hold and monitor the F_1 and subsequent generations separately until the “pupae per initial female” (PPIF) is greater than 2, then pool the offspring to produce and maintain the colony (section 3.).
31. If required, continue collecting wild females and harvesting pupae until the colony is established.

HEADING: 2.1. Colonization from Field-Collected Material	SUBHEADING: 2.1.3. Transporting Adults by Air
DATE:	AUTHORIZATION:
COMMENTS:	

Aim: To transport flies by air from the trapping site to the insectary

Equipment and Materials

- Field station with adult-holding and feeding facilities (sections 3.1. and 3.2.)
- Live hosts (for feeding flies, if an *in vitro* feeding system is unavailable)
- Cages of adult flies
- Metal or cardboard boxes (insulated), or cool boxes
- Equipment for communicating between the field station and insectary
- Temperature recorder (electronic) e.g. Hobo (optional)
- Quarantine area in insectary or rearing facility

HEADING: 2.1. Colonization from Field-Collected Material	SUBHEADING: 2.1.3. Transporting Adults by Air
DATE:	AUTHORIZATION:
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Procedure

1. Confirm availability of flight the day before shipment, and inform receiving station of shipment plans.
2. Pack cages containing flies horizontally in cool boxes or insulated metal boxes lined with damp cloth or tissue paper to maintain a high relative humidity.
3. If necessary, wrap ice packs and include them in the box. Ice packs should not be in direct contact with the flies.
4. Ensure that cages are tightly packed to avoid movement during shipment. Cover the tops of the cages with damp cloth or tissue paper before closing the box.
5. Transport the box of flies to the airport, and weigh the box.
6. Pay, and obtain a receipt, for the shipment.
7. Include with the box a copy of the air waybill (AWB) number, date of shipment, and number of flies sent.
8. Seal or lock the box, and give the box of flies to the airline staff.
9. To be sure that the box is really sent, a project staff member should stay in the airport until the aircraft departs.
10. Communicate to the receiving station the AWB number and the time the aircraft departed.
11. At the airport of destination, the receiving officer, having the AWB number, collects the box of flies and quickly delivers it to the insectary.
12. On arrival at the insectary, place the box in the quarantine area and remove the cages of flies.
13. Feed the flies immediately in the quarantine area,

HEADING: 2.1. Colonization from Field-Collected Material	SUBHEADING: 2.1.3. Transporting Adults by Air
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either on live animals or using the membrane system.

14. The following day, remove any dead flies from the cage.
15. Note and record any abortions, and monitor the survival and pupal production of each batch of flies (section 3.6.).
16. Transfer the pupae from the quarantine area to the main insectary.
17. As described in Procedure 2.1.2., establish the colony using flies that emerge from the pupae deposited by wild-caught flies. Hold and monitor the F_1 and subsequent generations separately until the “pupae per initial female” (PPIF) is greater than 2, then pool the offspring to produce and maintain the colony (section 3).

2.2. Colonization from an Established Laboratory Colony

Pupae of known age from an existing tsetse fly colony may be used to establish a new colony. This is the easiest procedure for establishing a colony. The flies are adapted to the laboratory, their productivity is known, and additional pupae are readily available. One drawback is that the origin or history of the colony may not have been accurately documented. Information on origin, date, diet and procedures used in the original colony is invaluable. Colony data from the original colony will give insight into the performance of the new colony. These include percentage daily mortality, survival, pupal weight and sex ratio, among others. When using pupae from an established colony, initially mating procedures are adjusted to take into account that females emerge first and have to wait for males to emerge before mating can take place.

Transporting Pupae by Air

Pupae may be shipped from one rearing facility to another, but using special arrangements. Tsetse pupae are very sensitive to environmental stress, and are unable to withstand extremes in temperature and relative humidity during development. Therefore, it is important to apply special conditions during transport. The total travelling time will influence the packaging material chosen. Prior to shipment, explore import clearance procedures and requirements of the receiving country. Perfect timing is imperative because freshly formed pupae, and pupae that are almost fully developed, are very sensitive to disturbance. If pupae are shipped when they are fully developed, some flies could emerge during transit. Therefore, the best shipment period is between 2 and 3 weeks after larviposition.

The number of pupae to be shipped influences the type of packaging. Large consignments can be packed in Styrofoam® containers of appropriate size. To avoid pupae being left exposed to the hot sun during summer and to the cold during winter, the package should be clearly marked with the temperature range within which it could be stored.

Handling Pupae Received from an Established Laboratory Colony

Pupae from another colony are assumed to be free of parasitoids, but should be kept separate from, and not mixed together with, other pupae that may be in the laboratory. The pupae are held until adult emergence begins. Pay special attention to fly feeding and mating behaviour, survival and productivity.

HEADING: 2.2. Colonization from an Established Laboratory Colony	SUBHEADING: 2.2.1. Transporting Pupae by Air
DATE:	AUTHORIZATION:
COMMENTS:	

Scope: Shipment of Pupae

Equipment and Materials

- Balance (accuracy of ±1 mg or better)
- Forceps (soft)
- Pupal-counting machine (optional)
- Temperature and relative humidity recorder (e.g. Hobo) (optional)
- Tape (for securing containers and package)
- Cotton wool
- Styrofoam® boxes
- Petri dishes (plastic, perforated)

HEADING: 2.2. Colonization from an Established Laboratory Colony	SUBHEADING: 2.2.1. Transporting Pupae by Air
DATE:	AUTHORIZATION:
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Procedure

1. Arrange and obtain import authorization or permit from receiving country.
2. Count and weigh the 2-week-old pupae intended for shipment.
3. Put pupae in perforated Petri dish, box or tube, lined with cotton wool.
4. Cover pupae with a layer of cotton wool, close container, and seal with tape.
5. Pack no more than 1–2000 pupae together in a cardboard box or in perforated plastic Petri dishes.
6. Mark the container with species name, date of larviposition, number of pupae, weight, and method used in the laboratory to feed flies.
7. Include a temperature and relative humidity recording instrument in the package for later identification of problems that might have been encountered during transport (optional).
8. Note date when the package leaves the insectary.
9. If possible, use a courier service to ship the container by air.
10. At point of dispatch, place the container with pupae into a padded envelope, and include fully completed copies of all authorization documents or permits in English (and another language if appropriate) allowing the shipment of pupae into the receiving country.
11. Label packet with the following statement: **FRAGILE, STORE BETWEEN 20–25^{0}C**.
12. Clearly mark the consignor's and consignee's names and contact information in waterproof ink on the envelope or packet.

HEADING: 2.2. Colonization from an Established Laboratory Colony	SUBHEADING: 2.2.1. Transporting Pupae by Air
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13. Obtain receipt, and note the air waybill (AWB) number.
14. Inform the receiving country (by e-mail, facsimile or telephone) of the number of pupae sent, date of departure, expected date and time of arrival, and the AWB number for ease in tracking.
15. Avoid timing a shipment so that it would arrive on a weekend.
16. Advise the receiving laboratory, on receipt of the package, to inspect it carefully, and report back on any leakage, mechanical damage, wetness, mould, etc., and to record these observations for future reference.

HEADING: 2.2. Colonization from an Established Laboratory Colony	SUBHEADING: 2.2.2. Handling Pupae Received from an Established Laboratory Colony
DATE:	AUTHORIZATION:
COMMENTS:	

Aim: To establish a colony of tsetse flies using pupae originating from existing tsetse colonies

Equipment and Materials

- Holding room
- Feeding room
- Trolleys (for keeping cages of flies)
- Chiller (a converted deep-freeze)
- Separating tubes
- Trays (plastic)
- Petri dishes
- Emergence cages
- Production cages
- Air conditioner
- Humidifier
- Heating mats (polyvinyl chloride (PVC)) or aluminium plates, with an adjustable heat source
- Feeding trays (anodized aluminium, with diamond-shaped surface)
- Silicone membranes
- Sterilizing oven
- Washing machine
- Dishwasher
- Deep-freeze (for bulk storage of blood)
- Refrigerator (for keeping blood ready for use)
- Decontaminated quality-tested blood for feeding
- Hand-sterilizing solution (antiseptic)

HEADING: 2.2. Colonization from an Established Laboratory Colony	SUBHEADING: 2.2.2. Handling Pupae Received from an Established Laboratory Colony
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. When the pupae arrive at destination, take them to the fly-handling area.
2. Wash hands with unscented soap, and dry with tissue paper or an electric handdryer.
3. Wipe hands with sterilizing solution (antiseptic).
4. Open the package, and count and record the number and condition of pupae; remove any damaged pupae. Record the date of larviposition and date of arrival.
5. Put the pupae in Petri dishes under emergence cages. (Pupae from another colony are assumed to be free of parasitoids, but should be kept separate from, and not mixed together with, other pupae that may be in the laboratory.)
6. Transfer pupae to the appropriate incubation or holding conditions.
7. When flies start to emerge, separate the sexes (section 3.3.3.).
8. Count and record any abnormal-looking flies by sex, then discard abnormal flies.
9. Load production cages with normal-looking flies.
10. Label production cages with date, source of flies, and sex.
11. Transfer cages of normal-looking flies to the holding room.
12. Feed flies on membranes or host animals (section 3.2.).
13. Observe the immediate feeding response of the flies.
14. Mate 2–3-days-old females (6-days-old for *G. pallidipes*) with ≥10-days- old males by holding the sexes together for 2–3 days before separation (section

HEADING: 2.2. Colonization from an Established Laboratory Colony	SUBHEADING: 2.2.2. Handling Pupae Received from an Established Laboratory Colony
DATE:	AUTHORIZATION:
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3.3.4.).

15. After sex separation, place the females into production cages.
16. Label production cages with a colony-unit identification number and the date, and add these cages to the colony.
17. Discard used males, but if males are in short supply, rest them for 1 or 2 days and then reuse them.
18. Continue collecting emerged flies until no more flies emerge from the batch of pupae.
19. Monitor survival and production (section 3.6.).
20. For each batch of pupae, record/calculate the emergence rate, the number of female and male flies that emerged and the condition in which they emerged (e.g. normal or crippled wings), the pupal period, and the percentage females (derived from pupae received) added to the colony.
21. Dissect a sample of non-emerged pupae to determine when development stopped and the likely cause of death (Annex 5.3.) (optional).
22. Discard non-emerged pupae 35–40 days after the larviposition date.

3. Routine Colony Maintenance

Tsetse flies are held in production cages kept on shelves on trolleys in a horizontal plane. The trolleys are kept in holding rooms, and are brought out during fly handling and feeding. At times, due to space limitations, both holding and feeding take place in the same room.

In the conventional tsetse rearing system, cages with flies are manually transferred for feeding, and then returned to the trolley for pupal collection. Adults must also be separated by sex for several purposes, and this has been done by hand on individual flies following immobilization at 4°C. The procedures described here are for conventional tsetse rearing, and for the improved systems of adult holding and feeding, and of sex separation.

The number of flies introduced into 20-cm-diameter cages depends on the species (or size) of the fly. See Annex 3 for examples of number of flies per cage for representative species (small, intermediate and large sizes of flies).

3.1. Adult Colony Holding

During mass-rearing, adult flies are kept in production cages placed on shelves on trolleys. In the conventional holding system, adults are held in cages kept on trolleys with wheels (Fig. 1) where larvae and pupae are collected (Fig. 2). To feed the flies, cages are manually transferred to a silicone membrane covering warm blood, and after feeding, the cages are returned to the trolleys. To reduce the possibility of predation by ants, water cups are welded to the trolley frame above the wheels and kept filled with water (Fig. 1). Larvae deposited by females drop onto a sloped sheet of aluminium below the expanded metal shelf holding the cages, and roll into the V-shaped larval collector where pupation takes place. Excretion from flies drops onto the same sheet of aluminium, preventing the fouling of cages and flies below. When the excretion dries, it may inhibit larvae from rolling into the pupal collector, and then larvae pupate on the sloped sheet. It is important to examine periodically the sheet for the presence of pupae, and if this occurs the sheet must be removed, cleaned and replaced.

When monitoring survival, trolleys with cages of flies are moved to the handling area, and dead flies are poured out from cages, sorted into categories and counted.

The improved colony-holding system is the tsetse production unit 3 (TPU 3) (Opiyo et al. 1999, Opiyo et al. 2000, IAEA 2003, Parker 2005). In the TPU 3, fly cages are held stationary, and blood in the feeding system (moved on rails) is taken to the cage-holding frame for fly feeding. Thus manual handling of cages with flies is greatly minimized using the TPU 3, and this reduces fly mortality.

The TPU 3 holding system (Fig. 3) consists of five stationary and five movable shelves; shelves in a set of five are stacked one above the other. On the stationary shelves are frames of cages, each frame holding nine round 20-cm-diameter cages (Fig. 4). The cage-holding frame has two support rods that cross the frame and hold the cages firmly in position (Fig. 5). The rods fit into grooves cut into the cages. To solve the problem of uneven light and potential uneven distribution of flies in a cage, the sloped sheet in the TPU 3 (onto which larvae drop before crawling into the pupal collector or trough) is made of transparent-plastic sheets (Fig. 6). The blood is heated by elements in individually controlled aluminium plates. The feeding trays are loaded with blood in the conventional way, as in the trolley membrane-feeding system. The mobile blood-feeding system (the five movable shelves) is moved on fixed rails (Fig. 3). During feeding, all five movable shelves (the feeding system consists of heating plates and trays of blood) are moved under the cages and raised to make contact with them. Two rows of cages on both sides (total of 180 cages) can be fed at one time. After feeding, the movable shelves are lowered and moved along the rail to the next set of cages.

As described in sections 2 and 3.1.3., and Annex 4, the environmental conditions for rearing tsetse flies are quite specific and very important. The adults of most tsetse species are maintained at 24ºC and 75–80% RH.

Savannah species require less humid conditions, while riverine species may require conditions above 80% RH. Subdued indirect lighting using tungsten lamps on a 12:12 photoperiod is recommended. Light should be evenly distributed to discourage overcrowding of flies towards the light source.

Fig. 1. Trolley holding cages of adults, showing water cups.

Fig. 2. Trolley showing sloped sheets and pupal-collecting trough.

Fig. 3. TPU 3 holding and feeding system on rails.

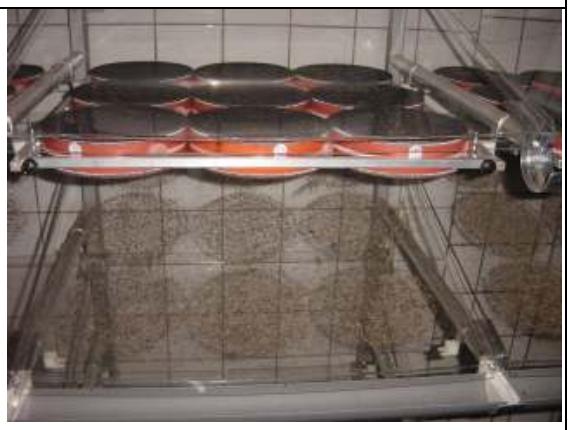

Fig. 4. TPU 3 cages (9) in a frame in position in the TPU 3 system.

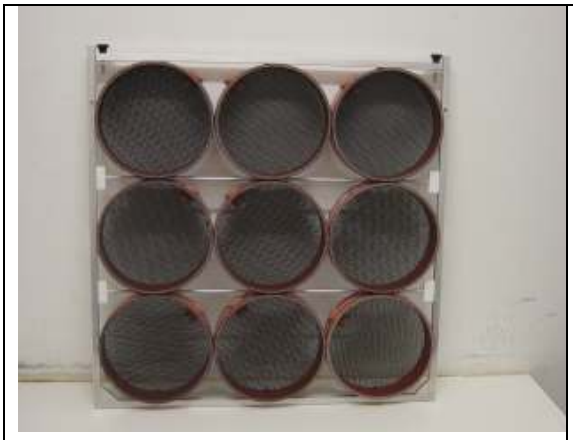

Fig. 5. TPU 3 cages (9) in a frame before being put into the TPU 3 system.

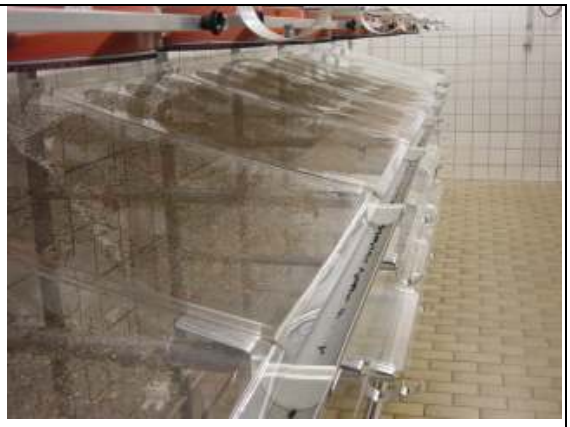

Fig. 6. TPU 3 sloped sheet below cages in frames, and pupal-collecting trough.

HEADING: 3.1. Adult Colony Holding	SUBHEADING: 3.1.1. Trolley Holding System
DATE:	AUTHORIZATION:
COMMENTS:	

Scope: A trolley system for holding cages of tsetse flies

Equipment and Materials

- Trolleys with shelves, sloped sheets and pupal collectors, and cups above the wheels
- Cages (with appropriate netting, Annex 2)
- Tungsten lamps with photoperiod controller
- Air conditioners
- Humidifiers
- Steam cleaner (to clean trolleys)
- Dishwasher (to clean cages)

HEADING: 3.1. Adult Colony Holding	SUBHEADING: 3.1.1. Trolley Holding System
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. Ensure that the trolley wheels are lubricated to facilitate movement.
2. Check the water level in cups, and fill when the level drops. (The water cups prevent ants from crawling up the trolley and feeding on the flies.)
3. Move trolley to the fly-handling area.
4. Transfer emerged separated flies into cages as required.
5. Label cages of flies with colony-unit identification marks, and date.
6. Place cages of flies onto the shelves of the trolley.
7. At the time of feeding, move the trolley to the feeding area.
8. Manually transfer cages of flies to the feeding membranes, inverting the cages and placing the normally upper side onto the membrane (permitting the flies to feed through clean netting) (section 3.2.2.).
9. After feeding, return cages to the correct shelf of the trolley, again inverting the cages so that the upper sides of the cages are now on top as before.
10. Return the trolley to the holding room.
11. Collect the larvae and pupae that have dropped onto the sloped sheets and then rolled into the pupal collectors (section 3.3.1.).
12. Clean the sloped sheets below the cages when they become dirty from fly faeces.
13. At the end of a unit lifespan, kill all remaining flies, and thoroughly clean (if possible steam-clean) the trolley frame, racks, shelves and sloped sheets, and store for future use. Also clean the cages, and if required repair

HEADING: 3.1. Adult Colony Holding	SUBHEADING: 3.1.1. Trolley Holding System
DATE:	AUTHORIZATION:
COMMENTS:	

or replace the netting.

HEADING: 3.1. Adult Colony Holding	SUBHEADING: 3.1.2. TPU 3 Holding System
DATE:	AUTHORIZATION:
COMMENTS:	

Scope: A TPU 3 system for holding a large number of cages and feeding the tsetse flies in these cages

Equipment and Materials

- TPU 3 system (cage-levelling and cage-holding frames, sloping plates, pupal collecting troughs, heating plates, rails, framework)
- Cages (with appropriate netting, Annex 2) designed for TPU 3
- Tungsten lamps with photoperiod controller
- Air conditioners
- Humidifiers
- Steam cleaner (for TPU 3)
- Dishwasher (to clean cages)

HEADING: 3.1. Adult Colony Holding	SUBHEADING: 3.1.2. TPU 3 Holding System
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. Transfer the frame for nine cages to the cage-levelling frame, and adjust so that all cages are at the same level.
2. Insert nine production cages holding flies into the cage-holding frame; secure tightly.
3. Transfer the frame with the nine cages to the stationary shelf, and hook into place.
4. The cages held in frames remain stationary during feeding.

HEADING: 3.1. Adult Colony Holding	SUBHEADING: 3.1.3. Environmental Conditions
DATE:	AUTHORIZATION:
COMMENTS:	

Aim: To maintain climatic conditions suitable for rearing tsetse flies

Equipment and Materials

- Lamps (tungsten) with photoperiod controller
- Light dimmer
- Light meter
- Air conditioners
- Maximum/minimum thermometer
- Temperature and relative humidity recorder (electronic) e.g. Hobo (optional)
- Humidifiers
- Hygrometer
- Electricity generator (optional)
- Well (for obtaining underground water) (optional)

HEADING: 3.1. Adult Colony Holding	SUBHEADING: 3.1.3. Environmental Conditions
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. The numerous equipment items that produce the appropriate physical environment for rearing tsetse flies require regular inspections and maintenance, periodic repairs and the replacement of parts.
2. A stock of replacement parts must always be kept on hand. It is important to order such items well in advance of the probable time when they may be needed. This is especially true for parts such as filters, fan belts and lamps, and for other parts that wear out quickly or regularly. In this context, having the same brand of a particular item, e.g. air conditioner, throughout the facility will make stocking of repair parts more justifiable.
3. Staff at a rearing facility should include an engineer trained in the maintenance and repair of environmental equipment. In addition, it is important to have access to commercial companies that can make major repairs to equipment.
4. If the facility has an emergency electricity generator, it is important that the staff engineer (and also a few other staff members including security guards) are trained in the basic operation of this generator. Supplies for the generator must always be on hand — fuel, oil, and essential spare parts such as fuses.
5. If the facility has a well for water, it is vital that water pumps and filters be inspected and maintained regularly.

3.2. Colony Feeding

If a reliable source of quality-tested blood is available, the membrane or *in vitro* feeding system is recommended (Feldmann 1994c, Gooding et al. 1997, Opiyo et al. 2000). The membrane for the *in vitro* feeding system is made of silicone, and was developed after several attempts with animal skins failed (Bauer and Wetzel 1976). A silicone membrane is reinforced with netting; the size of the netting determines the thickness of the membrane (section 3.2.1.). Small tsetse species are fed using a thin membrane, and larger species a thicker membrane. Bovine or porcine blood, or a mixture of both, has been used for colony maintenance, but the choice depends on the dietary requirements of a particular tsetse species. Tests using only bovine blood (at the FAO/IAEA Laboratories, Seibersdorf, Austria) demonstrated that most tsetse species can be maintained on bovine blood alone.

Colonies of tsetse flies can be maintained on sterile, fresh frozen, defibrinated blood, or blood to which anticoagulants have been added (section 4). The procedure for feeding flies using the membrane system aims to ensure that flies are given sterile blood in a suitable state. Before feeding, the blood is heated to the body temperature of mammals. A quantity of 100 ml of blood is sufficient to cover a surface area of 2000–2300 cm² and to feed 1500 flies. In the trolley membrane feeding system, cages of flies are removed from the trolley and placed on membranes lying flat on a tray containing warm blood (Fig. 7); after feeding the cages are returned to the trolley.

However, in the TPU 3 feeding system, the opposite occurs — blood is moved to the flies, and the cage-holding system is stationary. The mobile blood system is moved on rails that are fixed to the floor. Two rows of cages are fed at the same time. The blood is heated by elements in the heating plates; each plate is individually controlled. Temperature sensors are attached to individual heating plates which are connected to the temperature control box. The heating plates remain switched on, but can be programmed to heat during a specified period of time. It is vital that the temperature of the feeding membrane is correct, and therefore must be checked carefully, both before and after pouring the blood onto the tray (Fig. 8). Lower trays are filled with blood (Fig. 9), and the blood smoothed out under the membrane (Fig. 10), by a person standing on the floor, but upper trays are filled from a working platform. When the tray of blood is at the right temperature, it is pushed under the frame of nine cages (Fig. 11), and clips put into position to firmly press the tray onto the heating plate (Fig. 12).

All equipment (that comes into contact with the blood on which flies feed) must be thoroughly washed, sterilized, and stored in aseptic conditions before being used (section 3.2.4.). The equipment is rinsed with cold water before being washed by hand or in a dishwasher, and then it is sterilized. Silicone membranes are washed in a washing machine. Plastic wares are heat-sterilized at 80ºC, metal and glassware at 100ºC, and feeding trays with membranes at 120ºC overnight.

It is recommended that the *in vitro* feeding of flies takes place in a room that is not used for other daily routine colony work. Flies should be fed in environmental conditions that are as similar as possible to those under which they are held. Lighting should be subdued, indirect, and preferably from tungsten lamps, with illumination maintained at 12–14 lux. To reduce the distraction of flies during feeding, movement in the vicinity should be minimized, and cages covered with dark cloths. When dealing with riverine species, e.g. *Glossina palpalis palpalis* Robineau-Desvoidy and *Glossina fuscipes fuscipes* Newstead, it may be necessary to increase the relative humidity in the feeding areas; also the dark cloths covering the cages of flies during feeding can be dampened.

Both females and males should be fed at suitable intervals. To reduce the risk of bacterial contamination spreading throughout the colony, younger flies should be fed before older flies. If flies, after emergence, are separated using a chiller, flies should be given time to recover (2.5–3 hours) before feeding them. If flies are separated in the morning, they can be fed with a sterile feeding set-up in the afternoon or the following day, depending on the condition of the flies and their energy reserves (section 5.1.2.).

The feeding regimen used depends on the species and condition of the colony. However, a colony can be trained to adapt to a regimen convenient to the management of the rearing facility. At the Seibersdorf laboratory, flies are fed on Mondays, Wednesdays and Fridays. On Tuesdays and Thursdays only young flies (less than 1 week old) are fed. No feeding takes place on Saturdays and Sundays.

When the supply of blood is not assured, and where *in vitro* feeding cannot be performed, it is possible to maintain tsetse colonies on an *in vivo* system (Wanyonje 1994), using live hosts such as goats, rabbits, guinea pigs and cattle. However, it should be pointed out that the husbandry of host animals impacts on the colony performance. Rabbits must be fed a high-quality diet that is free from antibiotics and insecticides. The host animals can be trained to become accustomed to being restrained during the feeding period. Rabbits are trained to sit in restraining boxes and accept cages of flies that feed on their ears (the ears rest on padded platforms). Goats and cattle are restrained, and cages of flies are strapped to the body with elastic rubber bands.

The frequent use of host animals to feed tsetse flies raises the risk of over-challenging them. Some animals develop adverse skin reactions from tsetse bites. Antibiotics and coccidiostats in animal feed may affect the fecundity of tsetse flies, and animals undergoing treatment with antibiotics should not be used.

In endemic trypanosomosis areas, host animals may carry an infection at the time of purchase, escalating the probability of infecting the tsetse colony. Therefore, host animals should be checked regularly for parasites, and treated if found infected. The veterinary care of host animals is a major component of the *in vivo* tsetse feeding system.

Fig. 7. Feeding adults on membranes warmed by a heating mat.

Fig. 8. Checking the temperature of the membrane, TPU 3 system.

Fig. 9. Pouring blood onto a tray after lifting the membrane, TPU 3 system.

Fig. 10. Spreading blood with a paper roll, TPU 3 system.

Fig. 11. Pushing tray with membrane and blood under a frame of cages, TPU 3 system.

Fig. 12. TPU 3 feeding system with tray and heating plate, directly under cages. Two clips hold tray firmly onto the heating plate.

HEADING: 3.2. Colony Feeding	SUBHEADING: 3.2.1. Membrane Making
DATE:	AUTHORIZATION:
COMMENTS:	

Aim: To prepare silicone membranes for the *in vitro* feeding of tsetse flies

Equipment and Materials

- Mould (transparent-plastic, cut to the size of the required membrane with a groove at the edge)
- Rod (smooth, plastic, 4–7-cm thick and 70–80-cm long)
- Silicone (RTV 3500) and hardener (180 g silicone monomer and 9 ml hardener for thicker membrane, and 150 g silicone monomer and 7.5 ml hardener for thinner membrane)
- Netting (polyester) (thin netting for thin membrane, 130–150 µm thread diameter, 25 holes per cm^2; thick netting for thick membrane, 250–300 µm thread diameter, 16 holes per cm^2)
- Foil (polyethylene, 0.3-mm thickness, to cover the transparent-plastic mould)

HEADING: 3.2. Colony Feeding	SUBHEADING: 3.2.1. Membrane Making
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. Cut a piece of polyester netting to the size required to fit the feeding tray.
2. Boil the piece of polyester netting to remove chemical residues of impregnation and dyes, and dry in air.
3. Weigh the required amount of silicone monomer.
4. Measure the required volume of hardener.
5. Mix thoroughly the silicone monomer and hardener.
6. Place the dry netting on to a clean transparent-plastic mould.
7. Place the silicone and hardener mixture in the centre of the netting.
8. Cover the mixture with polyethylene foil.
9. Evenly distribute the silicone mixture on the mould by rolling the rod over the polyethylene foil.
10. Allow the silicone to harden at room temperature for 24 hours.
11. Gently pull off the polyethylene foil.
12. Remove the membrane from the transparent-plastic mould.
13. Cut off surplus silicone and netting around the thick edge of the membrane.
14. Wash the membrane in hot water without detergent, and then dry.
15. Until use, store the membrane between two layers of loosely rolled tissue paper.

HEADING: 3.2. Colony Feeding	SUBHEADING: 3.2.2. Trolley Membrane Feeding
DATE:	AUTHORIZATION:
COMMENTS:	

Scope: Membrane feeding of tsetse flies in cages held on trolleys

Equipment and Materials

- Trolleys (holding the cages of flies)
- Heating mats (polyvinyl chloride (PVC)) or heating plates (aluminium) with adjustable heat source
- Trays (anodized aluminium, diamond-shaped surface with a 1-cm edge)
- Membranes (silicone)
- Sterilizing oven
- Thermocouple thermometer
- Air conditioner
- Humidifier
- Washing machine
- Dishwasher
- Refrigerator (for holding blood at +4ºC)
- Blood (decontaminated, quality-tested, in glass bottles, at +4ºC)
- Feeding room (with adjustable light switch)
- Sterilizing or antiseptic solution

HEADING: 3.2. Colony Feeding	SUBHEADING: 3.2.2. Trolley Membrane Feeding
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. Switch on the heating mats or plates.
2. Wash hands with unscented soap. Dry hands with tissue paper or electric handdryer.
3. Wipe hands with sterilizing or antiseptic solution.
4. Wipe the feeding surfaces (benches and heating mats or plates) with sterilizing or antiseptic solution.
5. Transfer the sterile feeding trays and membranes from the oven on to the heating mats or plates.
6. Check the temperature of the heating mat or plate or the surface of the membrane using a thermocouple surface thermometer (aim for 35–37ºC).
7. Transfer quality-tested blood from the refrigerator (at +4ºC) (section 4.5.2.) to the feeding room, and wipe the surface of the bottle dry.
8. To reduce contamination of the feeding unit, close doors and switch off equipment that could cause air turbulence (fans, air conditioners and humidifiers).
9. When the temperature of the membrane surface is 35–37°C, lift two-thirds of the membrane at one corner of the tray, pour 100 ml of blood into the tray, and immediately replace the membrane. Avoid the formation of bubbles while pouring blood or when replacing the membrane on to the feeding tray.
10. Distribute the blood evenly on the feeding tray using a roll of tissue paper.
11. In the same way, pour blood into the other trays that are being used.
12. Switch on the fans, air conditioners and humidifiers.
13. Wait until the blood has warmed up.
14. Start the feeding procedure with young flies, and then

HEADING: 3.2. Colony Feeding	SUBHEADING: 3.2.2. Trolley Membrane Feeding
DATE:	AUTHORIZATION:
COMMENTS:	

follow with older flies to minimize the chances of introducing bacterial contamination.

15. When the membrane surface temperature has returned to 35–37°C (check temperature carefully), manually put cages of flies on to the membranes. (Invert the cages so that the normally upper sides face downwards, enabling the flies to feed through clean netting). Dim the lights in the feeding room.
16. As soon as cages are put onto the membrane, monitor the temperature of the feeding mats and membranes, and adjust as necessary. If the temperature is ≥39ºC, remove the cages and wait until the temperature has returned to 35–37ºC before replacing the cages.
17. If the flies are reluctant to feed, cover the cages with black and/or damp cloth.
18. Allow the flies to feed for 10–15 minutes.
19. Remove cages of fed flies and return them to the correct shelf on the trolley (again inverting the cages so that the upper sides are on top as before). The lights may be brightened during the period of changing cages, then dimmed again after the next group of cages has been placed onto the membranes.
20. If feeding takes place in a separate room, return the trolley with cages of fed flies to the holding room.
21. Continue the feeding procedure until all flies in the colony have been fed.
22. Aim to complete the feeding procedure within 3 hours. If more time is needed, before continuing the feeding procedure, completely replace the feeding set-up — trays, membranes and blood — with a new sterile set-up. (After 3 hours, the blood quality has deteriorated and is no longer usable.)

HEADING: 3.2. Colony Feeding	SUBHEADING: 3.2.2. Trolley Membrane Feeding
DATE:	AUTHORIZATION:
COMMENTS:	

23. Thoroughly rinse feeding trays and membranes in cold water to remove traces of blood.
24. Wash and sterilize all feeding equipment (section 3.2.4.).
25. Clean the room and feeding area.

HEADING: 3.2. Colony Feeding	SUBHEADING: 3.2.3. TPU 3 Membrane Feeding
DATE:	AUTHORIZATION:
COMMENTS:	

Scope: Membrane feeding of flies maintained in the TPU 3 holding system

Equipment and Materials

- TPU 3 blood-feeding system (mobile, on rails)
- Trays (anodized aluminium, diamond-shaped surface with a 1-cm edge)
- Membranes (silicone)
- Sterilizing oven
- Air conditioner
- Humidifier
- Dishwasher
- Washing machine
- Thermocouple thermometer
- Refrigerator (for holding blood at +4ºC)
- Blood (decontaminated, quality-tested, in glass bottles, at +4ºC)
- Feeding room (with adjustable light switch)
- Sterilizing or antiseptic solution

HEADING: 3.2. Colony Feeding	SUBHEADING: 3.2.3. TPU 3 Membrane Feeding
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. Switch on the blood heating plates, and wait 15–30 minutes for the plates to warm up.
2. Wash hands with unscented soap and dry with tissue paper or an electric handdryer.
3. Wipe hands with sterilizing or antiseptic solution.
4. Wipe the surface of the heating plates with sterilizing solution.
5. Place the sterilized trays with membranes on the heating plates. Ensure that each tray is flat on the plate, and is inserted under the fixed clips at the back of the plate. Add two additional portable clips at the front of the plate. Thus each tray is tightly pressed onto the heating plate in both the back and the front.
6. Check and confirm the temperature (35–37°C) of the membranes.
7. Transfer quality-tested blood from the refrigerator (at +4ºC) (section 4.5.2.) to the feeding room, and wipe the surface of the bottle dry.
8. Pour 250 ml of blood into each tray, and distribute the blood evenly using a roll of tissue paper.
9. Wait until the blood has warmed up.
10. Check the temperature of the membrane surface, and when the temperature has returned to 35–37°C, unlock the feeding system.
11. Move the feeding system to the cages with the youngest flies, and until each shelf with a tray of blood comes to rest directly below a frame with nine cages.
12. Using the hand crank, raise the heating plates with trays carrying warmed blood until the cages rest on the membrane surface.

HEADING: 3.2. Colony Feeding	SUBHEADING: 3.2.3. TPU 3 Membrane Feeding
DATE:	AUTHORIZATION:
COMMENTS:	

13. Lift the transparent-plastic sloped sheet on which larvae drop before rolling into the larval collecting trough (in which they pupate).
14. Examine and ensure that the netting of cages is in contact with the membrane surface.
15. Return the transparent-plastic sloped sheet to its normal position.
16. Dim the lights, and allow the flies feed.
17. After 10 minutes, turn up the lights, and lower the heating plates with trays carrying blood.
18. Move the feeding system to a new set of cages, repeating the procedures.
19. Continue feeding until all the flies in cages along the row are fed.
20. After four feeding cycles, if necessary, top up blood in the trays. (Due to the risk of bacterial growth in the blood, do not feed flies for more than 3 hours with the same membranes and trays; change to sterile equipment.)
21. When all flies are fed, remove the feeding trays from the system.
22. Discard the residual blood from the feeding equipment, rinse, clean and sterilize (section 3.2.4.).

HEADING: 3.2. Colony Feeding	SUBHEADING: 3.2.4. Cleaning of Equipment Used in Membrane Feeding
DATE:	AUTHORIZATION:
COMMENTS:	

Scope: Cleaning of equipment used in membrane feeding

Equipment and Materials

- Sinks (large, for rinsing off blood from membranes and trays)
- Washing machine
- Dishwasher
- Sterilizing oven

HEADING: 3.2. Colony Feeding	SUBHEADING: 3.2.4. Cleaning of Equipment Used in Membrane Feeding
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. Rinse thoroughly in cold water all equipment that has been in contact with blood (to remove traces of blood).
2. Place all silicone membranes in a washing machine.
3. Put all trays in a dishwasher.
4. Put all glass bottles and plastic wares in a dishwasher.
5. Select the appropriate washer programmes and wash.
6. Equipment could also be washed by hand in cold water, and then rinsed with distilled water.
7. Put each washed silicone membrane on a washed tray, ensuring that the membrane is lying flat on the tray.
8. Overnight, sterilize the trays with membranes in an oven set at 120°C.
9. Leave the trays with membranes in the oven until required.
10. Sterilize glass bottles in an oven set at 100°C, and plastic wares at 80°C.
11. Store glass bottles and plastic wares in a clean place for future use.

HEADING: 3.2. Colony Feeding	SUBHEADING: 3.2.5. *In Vivo* Feeding
DATE:	AUTHORIZATION:
COMMENTS:	

Scope: *In vivo* feeding of tsetse flies

Equipment and Materials

- Rabbit-restraining boxes
- Crush (for restraining goats or cattle)
- Elastic bands (for holding cages onto host animals)
- Black cloth (for covering cages during feeding)
- “Insect-o-cutor”
- Polyvinyl chloride (PVC) cages
- Cages of flies
- Compound microscope
- Haematocrit centrifuge and reader
- Housing for host animals
- Shed (for feeding flies away from direct sunlight)
- Diminazine aceturate
- Veterinary drugs and supplies

HEADING: 3.2. Colony Feeding	SUBHEADING: 3.2.5. *In Vivo* Feeding
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. Select healthy animals for hosts.
2. Bleed and establish the packed cell volume (PCV) for each animal, and record.
3. If the hosts originate from a tsetse-infested area, examine the blood for the presence of trypanosomes.
4. Treat all infected animals with diminazine aceturate, and rest the animals for 2 weeks before using them.
5. When using goats, shave the surface on which flies are fed.
6. At the time of feeding, restrain the animal appropriately.
7. Wash with warm water the surface on which flies are to be fed, and dry with clean tissue paper or cotton wool.
8. Bring cages of flies to the feeding area.
9. Place cages of flies on the washed surface, and secure with elastic bands.
10. Cover cages of flies with a dark cloth.
11. Keep the cages on the host animal for 10–15 minutes, allowing the flies to feed.
12. Remove the dark cloth covering the cages, and observe the flies to see if they have fed well.
13. If the proportion of fed flies is low, replace the dark cloth onto the cages.
14. Allow more time for the flies to feed.
15. Return the cages of fed flies to the holding room.
16. Continue the feeding procedure until all flies in the colony have been fed.
17. Do not feed more than 400 flies on one rabbit per day.
18. More flies can be fed on goats and cattle than on rabbits.

HEADING: 3.2. Colony Feeding	SUBHEADING: 3.2.5. *In Vivo* Feeding
DATE:	AUTHORIZATION:
COMMENTS:	

19. Clean the ears of rabbits or flanks of goats and cattle on which flies have fed.
20. Return rabbits to the animal house, and allow goats and cattle to graze.
21. Wash the dark cloths, and then dry.
22. Clean the feeding area.
23. Once a week, examine all host animals for physical fitness and any signs of adverse skin conditions. Bleed host animals and examine for trypanosomes and estimate the PCV, and withdraw animals with low PCV.
24. Treat sick animals, and do not use them for feeding flies.
25. Rest weak and treated animals until they recover, or if possible replace them with new ones.

3.3. Using a Chiller to Sex Adults

Sections 3.3., 3.4. and 3.5. describe **three alternative methods** for handling pupae and adults, sexing adults, mating, and loading production cages. **Procedures in only ONE of the three sections should be followed — the three sections are mutually exclusive.**

1. Section 3.3. describes the procedures if a chiller is used to sex adults.
2. Section 3.4. describes procedures if self-stocking of production cages (SSPC) is done, specifically for *Glossina pallidipes*.
3. Section 3.5. describes procedures if pupae are separated with near-infrared (NIR) spectroscopy.

Pupal Handling and Adult Emergence

An adult female tsetse fly gives birth to a full-grown larva; it crawls around for a couple of hours, then pupates. Adverse conditions during this short period of crawling may affect subsequent development; ideally the larva should pupate in the holding room. Larvae and pupae are collected every morning (peak larviposition occurs in the afternoon) and put into open dishes marked with species, unit number and the date. Counting and quality assurance (QA) of pupae should be done on the following day to allow time for the puparium to fully harden.

Pupae are incubated in an incubation room or incubator for 25 days at 23–24ºC and 75–80% RH (in general, they may be kept under the same conditions as adult flies). *G. tachinoides* pupae perform better at 23ºC and 82.5% RH, and those of *G. austeni* do better at 85–88% RH after they are 20-days old. However, if self-stocking of production cages (SSPC) (section 3.4.) or near-infrared (NIR) spectroscopy (section 3.5.) is done, temperature control in the incubation room or incubator is critical. Pupae can be incubated under very stable environmental conditions if placed in an incubator with controlled temperature and relative humidity.

Handling of pupae from day 25 onwards depends on the sex separation technique being used. For chiller separation (section 3.3.), at about 25 days after larviposition, pupae are put into small Petri dishes which are then fitted under emergence cages. If desired, the relative humidity can be increased by placing the Petri dish containing 25-day-old pupae (covered by the emergence cage) on a damp sponge. As emergence proceeds, the emerging flies are trapped in the emergence cage where they expand their wings. The flies are now ready for sex separation, mating, and loading into production cages.

Sex Separation

In the standard method of mass-rearing, tsetse flies are separated according to sex, either for loading production cages or for processing for field release. Sex separation is usually only possible in the adult stage. Sexing is done manually on the basis of visual abdominal characters (Figs. 13 and 14), and is most easily done when flies have been immobilized by chilling to 4ºC. Males have a pointed abdomen, and the superior claspers or external genitalia are

heavily pigmented. In females, the abdomen is truncated, and has a pale appearance. For small colonies, the sexes can be separated by visually identifying the sex of a fly in a separating tube, and then removing the fly.

If manual sex separation is required, mature pupae are held in emergence cages which are made from polyvinyl chloride (PVC) tubes reshaped in water at 60°C to form rectangular cages approximately 13 cm high and 22.5 cm long. The height of the cages must permit air to be blown into the cage when they are placed in a chiller. A 6-cm-diameter hole is cut in the bottom of a cage to accommodate a Petri dish holding 400–500 pupae. Two pieces of plastic are attached to two sides of the hole to provide a groove along which another flat piece of plastic is inserted to close the hole at the time the emergence cage is taken to the chiller. In the standard method of handling flies, the difficulty in separating the sexes, except at the time of adult emergence, prevents the shipment of sterile male pupae to field projects. However, this could change if near-infrared spectroscopy is used to separate pupae (section 3.5.).

When flies begin to emerge, the emergence cage with the flies is transferred to the chiller, and the flies are immobilized by the cold air. Then, after dumping the chilled flies onto a counting plate in the chiller, males and females are visually separated (Fig. 15). Each day this procedure is repeated until no more flies emerge from the same batch of pupae.

The manual separation of flies through chilling is labour-intensive, and takes 23% of the total time invested in the standard method of rearing. Since chilling the flies may lead to latent damage to them, flies should be chilled for not longer than 5 minutes. The work of chilling flies may also lead to health problems for staff workers. Chilling for sex separation is done a number of times during the life of a fly — at emergence, after mating (for females), and several times for males.

Mating

At the time of emergence, tsetse flies are not sexually mature (Malele and Parker 1999). In the laboratory and for most species of tsetse, males become sexually active when about 1 week old, and females reach sexual receptivity when 2–3 days old. This is the time when maximum insemination rates are achieved. By this time, females have had the opportunity to feed twice and males several times. There are species differences; optimal fertility in *G. pallidipes* is achieved by mating 7–9-days-old females with 10-day-old or older males (Olet et al. 2002).

The traditional procedure for mating involves placing an equal number of 2–3-day-old females and 10+-day-old males into the same cage, leaving the sexes together for 2–3 days. Then the sexes are separated (with chilling), and females retained for production. Males are discarded or, if required, rested for 1 or 2 days and reused. If the sexes of adult flies are separated in a chiller, the mating of adult flies followed by separation would mean that females are chilled twice; this could affect their performance. The procedure is also labour-

intensive, and it has been estimated that the combined chilling activity takes up 46% of the labour time invested in rearing tsetse.

In a field sample of flies caught in a trap or from a bait-animal, virgin females are usually found very rarely. In addition, laboratory-reared *G. austeni* have been observed to mate in emergence cages even before the sexes have been separated. Further laboratory investigations showed that there was no need to age flies prior to mating. As a result, in the system known as day-0 mating with resident males, teneral flies of both sexes are placed in production cages at the time of emergence, at a ratio of 3 or 4 females to 1 male, and left together throughout the unit life. Mating takes place as the flies mature over the first 5–10 days. Eliminating the requirement to mature flies before mating, followed by chilling separation, has simplified the mating procedure, reduced the time invested in handling flies, and reduced the number to times that flies are chilled. However, for recently colonized species or strains, it is advisable to establish the appropriate mating regime prior to introducing this procedure into the colony routine.

Fig. 13. Male sexual characters.

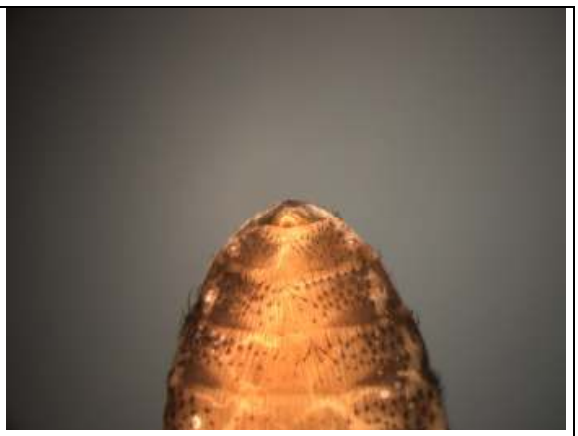

Fig. 14. Female sexual characters.

Fig. 15. Chiller, showing an emergence cage and adults immobilized by the cold.

HEADING: 3.3. Using a Chiller to Sex Adults	SUBHEADING: 3.3.1. Pupal Handling
DATE:	AUTHORIZATION:
COMMENTS:	

Scope: Handling of pupae when emerged adults will be sexed in a chiller

Equipment and Materials

- Forceps (soft, flexible)
- Trays (plastic)
- Dishes (polyvinyl chloride (PVC))
- Petri dishes (special, the two pieces are glued back-to-back)
- Sieve (coarse)
- Emergence cages
- Pupal incubation room
- Record books or sheets
- Incubator (with controlled temperature and relative humidity) (optional)

HEADING: 3.3. Using a Chiller to Sex Adults	SUBHEADING: 3.3.1. Pupal Handling
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. Mark dishes with colony unit identification number and date.
2. Each morning, collect larvae and pupae by tipping the pupal collector (trough) into PVC dishes.
3. Do not touch larvae or pupae with your hand.
4. Sieve out the debris from among the larvae and pupae.
5. Transfer pupae and larvae to medium-size PVC dishes marked with the colony unit number.
6. Place the PVC dishes on plastic trays.
7. Allow larvae to pupate and melanize, and pupae to harden, in the incubation room until the following morning.
8. Sort out malformed pupae and aborted larvae, count, record and discard.
9. Count and do a quality assurance (QA) on pupae on the following morning (sections 3.6. and 5.3.), and record.
10. Put pupae into open dishes, and hold in the incubation room or in an incubator with controlled temperature and relative humidity.

HEADING: 3.3. Using a Chiller to Sex Adults	SUBHEADING: 3.3.2. Adult Emergence
DATE:	AUTHORIZATION:
COMMENTS:	

Scope: Setting up emergence cages to collect emerged adults which will be sexed in a chiller

Equipment and Materials

- Emergence cages (plastic, screened)
- Pupae (in small Petri dishes, ready for adult emergence)
- Trays (to hold emergence cages)
- Sponges (thin sheet)
- Chiller

HEADING: 3.3. Using a Chiller to Sex Adults	SUBHEADING: 3.3.2. Adult Emergence
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. About 25 days after larviposition, transfer pupae to small Petri dishes (250–300 pupae per dish).
2. Cover the dishes of pupae with emergence cages (insert a dish with pupae into the hole cut in the bottom of an emergence cage). Label emergence cages with the colony unit identification number and the larviposition date.
3. Wet a sponge, and lay it on the bottom of a tray (to increase the relative humidity in the emergence cage).
4. Place emergence cage with dish onto the sponge in the tray.
5. Place emergence cages with trays into the incubation room, and wait for adult emergence.
6. Each day, check if any adults have emerged. If so, place emergence cages (but not the dishes with pupae) into the chiller, ready for separation of male and female adults. After chilled adults are removed from the emergence cages, recombine the cages and dishes with pupae and return them to the incubation room, waiting for additional adults to emerge in the following days.

HEADING: 3.3. Using a Chiller to Sex Adults	SUBHEADING: 3.3.3. Sex Separation of Adults
DATE:	AUTHORIZATION:
COMMENTS:	

Scope: Separating adult flies using a chiller

Equipment and Materials

- Chiller (converted chest deep-freeze with fly-counting surface)
- Separation tubes
- Brushes (small, e.g. camel-hair brushes, to manipulate flies)
- Emergence cages
- Petri dishes (with the sides glued back-to-back)
- Trays (plastic)
- Production cages
- Thermometer (range -10°C to +50°C)

HEADING: 3.3. Using a Chiller to Sex Adults	SUBHEADING: 3.3.3. Sex Separation of Adults
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. Transfer 25-days-old pupae to Petri dishes, and place under an emergence cage labelled with larviposition date and unit identification number.
2. When flies begin to emerge, close the hole where the Petri dish is fitted to the emergence cage.
3. Close the top of the Petri dish (holding the remaining pupae) to prevent any additional emerging flies from escaping.
4. Check and confirm the temperature (4°C) of the chiller.
5. Transfer the emergence cage with flies into the chiller.
6. When the flies are immobilized, pour the flies onto the cold fly-counting surface.
7. Using a brush to turn the flies, manually sex flies using visual characters.
8. Determine the physical appearance of emerged flies (normal or with crippled wings). Record, by sex, the number of normal flies and flies with crippled wings.
9. Every day, collect and separate the sexes of emerging flies (as described above).
10. In established colonies where day-0 mating with the resident male regime is practiced (section 3.3.5.), place flies in a production cage at the ratio of 4 females to 1 male at the time of sex separation (or at the ratio determined for the tsetse species or strain, Annex 3).
11. Otherwise, before mating, keep sexes separate until sexually mature.
12. The age of mating is also species-specific.
13. Calculate the adult emergence rate, sex ratio and pupal period for both males and females, and record (section 3.6.).

HEADING: 3.3. Using a Chiller to Sex Adults	SUBHEADING: 3.3.4. Matured Mating with Separation
DATE:	AUTHORIZATION:
COMMENTS:	

Scope: Mating of sexually mature tsetse adults

Equipment and Materials

- Production cages
- Brushes
- Chiller
- Separating tubes
- Thermometer (range -10°C to +50°C)

HEADING: 3.3. Using a Chiller to Sex Adults	SUBHEADING: 3.3.4. Matured Mating with Separation
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. Using the separating tube, put an equal number of 2-days-old females with 10+-days-old males in female cages, and leave for 2 days.
2. For *G. pallidipes*, the females should be 6–7 days old at the time of mating.
3. Do not feed flies before chilling separation.
4. Check and confirm the temperature of the chiller (4°C).
5. Place the cage holding mating flies in the chiller.
6. Wait until the flies are immobilized, but do not leave flies in the chiller for more than 5 minutes.
7. If flies take too long to become immobilized, remove the cage of flies and check and adjust the temperature of the chiller.
8. Pour immobilized flies onto the cold fly-counting surface.
9. Separate out females with a brush, and put females in production cages.
10. Label the cage with the colony unit identification number.
11. The number of females per cage depends on the species of tsetse fly (Annex 3).
12. Discard the males, but if there is a shortage of males, rest the males for 1 or 2 days and then reuse.
13. Let the flies recover for 2 or more hours before feeding.
14. Clean the chiller.

HEADING: 3.3. Using a Chiller to Sex Adults	SUBHEADING: 3.3.5. Day-0 Mating with Resident Males
DATE:	AUTHORIZATION:
COMMENTS:	

Scope: Day-0 mating with resident males

Equipment and Materials

- Production cages
- Separating tubes
- Brushes
- Chiller

HEADING: 3.3. Using a Chiller to Sex Adults	SUBHEADING: 3.3.5. Day-0 Mating with Resident Males
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. Separate emerged flies into males and females (section 3.3.3.) using a chiller.
2. Place newly emerged female adults in a production cage (Annex 3).
3. To the females add newly emerged males, so as to give a ratio of 3–4 females to 1 male (Annex 3).
4. Leave the males with the females until the end of the life of the production cage.

3.4. Self-Stocking of Production Cage (SSPC) for *Glossina pallidipes*

As mentioned earlier, sections 3.3., 3.4. and 3.5. describe **three alternative methods** for handling pupae and adults, sexing adults, mating, and loading production cages. **Procedures in only ONE of the three sections should be followed — the three sections are mutually exclusive.**

1. Section 3.3. describes the procedures if a chiller is used to sex adults.
2. Section 3.4. describes procedures if self-stocking of production cages (SSPC) is done, specifically for *Glossina pallidipes*.
3. Section 3.5. describes procedures if pupae are separated with near-infrared (NIR) spectroscopy.

The adult emergence pattern of tsetse depends on temperature (Zdarek and Denlinger 1995) and on the fly species, and can be manipulated to separate the sexes. Male and female pupae show differences in the rate of development — in general, females reach maturity before males. An example of the emergence pattern of *G. pallidipes* is shown in Fig. 16. The effect of temperature on the time of adult emergence is very species specific. For example, the pattern of adult emergence of females and males of *G. pallidipes* is different from, and less overlapping than, that of *G. austeni*.

Even if pupae are deposited on the same day, females subsequently emerge approximately 2 days earlier than males. When pupae, incubated at 23–24°C for 30–31 days after larviposition, are transferred to 26.5°C, the adult emergence spans a period of 4 days; during the first 2 days, only females emerge, and during the last 2 days, mostly males emerge (Fig. 16). The accuracy of sex separation, based on differences in the emergence time of females and males, can be enhanced by keeping individual pupae separated (one pupa not touching/stimulating another pupa) during emergence. Based on this behaviour, it is possible to separate the sexes at the time of emergence.

In the field, after the female adult has deposited a larva, the larva burrows into the ground and pupates; after a period of development the operculum opens, and the young adult, with unexpanded body and wings, wiggles its way to the soil surface. This behaviour, together with the ability of the newly emerged adult to penetrate the netting of a standard holding cage, led to the development of the self-stocking of a production cage (SSPC). In the SSPC, the physical set-up (differing mesh of the netting on the two sides of a cage) allows emerging adults to enter directly into the bottom of production cages but not to escape from the top. Thus, the SSPC eliminates the need for the manual sexing of flies (Opiyo et al. 2000), reduces labour costs, and yields higher-quality flies.

To use the SSPC, it is important to collect pupae at the same time each day. The pupal incubation room is maintained at 23–24°C and 75–80% RH, and the SSPC room maintained at an elevated temperature of 26.5°C. To have emerging flies enter production cages directly, the holes in the cage netting on one side (closest to the pupae) are large enough to allow a freshly emerged adult to crawl into the cage, but on the other side of the cage (away from the pupae) the holes are smaller, preventing the escape of the emerged adults.

The mechanism for holding pupae in a single layer under the production cage is milled cavities in boards or rubber rings that hold a predetermined number of pupae. The number of pupae required for loading the cages with the right number of flies, and in the right sex ratio, can be volumetrically estimated.

For colony production, the required number of flies to stock a cage in the right sex ratio is achieved by placing mixed-sex pupae together with male pupae (pupae from which females have already emerged in the preceding 48 hours) under each cage (Annex 3). After 48 hours, the cage contains all the emerged females together with a sufficient number of males required for insemination of females. The pupae that remain after the stocking of a production cage are male pupae that can be used to provide males for loading subsequent production cages or for production of sterile males. The established emergence dynamics indicates the time required for almost all of the female adults to emerge from the batch of pupae collected on the same day, and the total emergence duration of both male and female pupae.

NOTE: The number of additional male pupae, added to the mixed-sex pupae under a self-stocking production cage, is shown in Annex 3. For one cage of *Glossina pallidipes*, 17 male pupae are added to 133 mixed-sex pupae. (The expectation is that half of the 133 mixed-sex pupae will produce about 66 female adults for the cage, and the added male pupae will produce 17 male adults for the cage.) Due to the slower rate of emergence of male adults, very few if any of the male pupae from the mixed-sex group of pupae will produce male adults during the 48-hour period allowed for adult emergence into the SSPC cage. Thus, it is important that the 17 male pupae be ready to emerge when added to the mixed-sex pupae. Consequently, these 17 male pupae must be prepared in advance, and taken from a batch of pupae that were larviposited 2 days earlier that those in the mixed-sex group.

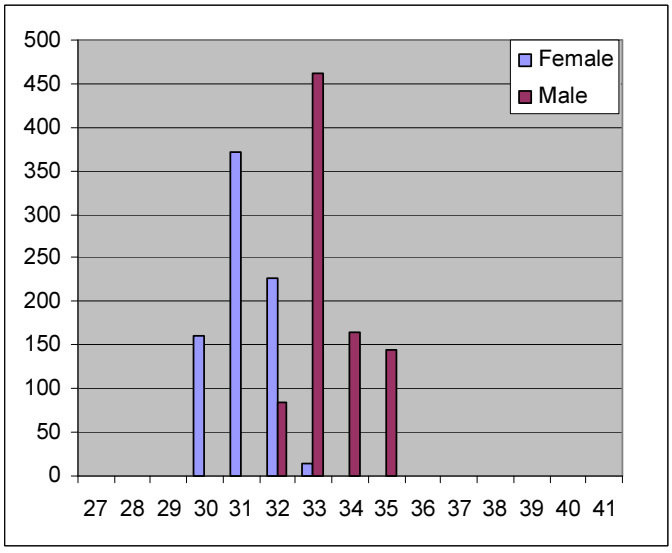

Fig. 16. Adult emergence pattern of *Glossina pallidipes* in a rearing facility. (Y axis = Number of adult females or males emerging per day) (X axis = Number of days since larviposition)

HEADING: 3.4. Self-Stocking of Production Cage (SSPC) for *Glossina pallidipes*	SUBHEADING: 3.4.1. Self-Stocking of Production Cage (SSPC) for *Glossina pallidipes*
DATE:	AUTHORIZATION:
COMMENTS:	

Scope: Self-stocking of production cage (SSPC) with the right number of adults, and in the right sex ratio, specifically described for *Glossina pallidipes*

Equipment and Materials

- Boards with milled cavities or rubber rings (that hold the required number of pupae)
- Production cages (holes in netting close to the pupae are large enough to allow newly emerged flies to crawl into the cage, and holes in netting away from pupae are small enough to retain newly emerged flies in the cage) (Annex 2)
- Incubation room (standard incubation conditions for pupae)
- Incubator (or SSPC room) with elevated temperature (26.5°C)
- Emergence cages (to retain adults that may emerge prior to transfer to the incubator or SSPC room)
- Record book or sheets

HEADING: 3.4. Self-Stocking of Production Cage (SSPC) for *Glossina pallidipes*	SUBHEADING: 3.4.1. Self-Stocking of Production Cage (SSPC) for *Glossina pallidipes*
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

Routine procedure — collecting and incubating pupae

1. Each day collect pupae at the same time of day (from pupal collector troughs).
2. Allow larvae to pupate, and pupae to harden, until the following day.
3. Sort pupae into abortions, soft pupae and normal pupae, and count and record.
4. Incubate well-formed pupae under standard pupal-holding conditions (in Seibersdorf — 23–24°C and 75–80% RH).

To establish emergence dynamics

1. A few days before adult emergence (5–7days), transfer pupae to a Petri dish, and keep it under an emergence cage.
2. Establish the normal length of incubation, i.e. until the first adults (pioneers) emerge.
3. When pioneers appear, count out the number of pupae required to load a production cage (Annex 3).
4. Arrange pupae in a single layer, and place a production cage with the larger-holed netting resting on the pupae.
5. Transfer pupae with production cages resting on them to the incubator or SSPC room with an elevated temperature (26.5°C).
6. Every day, at the same time of day, remove the cage and place a new production cage on the remaining pupae.
7. Count the number of females and males that emerge each day, and establish the emergence dynamics. Once the emergence dynamics has been established, the routine set-up of SSPC can begin, as follows.

HEADING: 3.4. Self-Stocking of Production Cage (SSPC) for *Glossina pallidipes*	SUBHEADING: 3.4.1. Self-Stocking of Production Cage (SSPC) for *Glossina pallidipes*
DATE:	AUTHORIZATION:
COMMENTS:	

Routine procedure — SSPC production cages

1. On the day when it is expected (based on knowledge of the emergence dynamics) that females will begin to emerge, place the required number of mature mixed-sex pupae (133) and additional male pupae (17) in a single layer below each SSPC production cage (larger-holed netting resting on the pupae) (Annex 3). **(The 17 male pupae must be 2 days older than the mixed pupae.)**
2. Transfer pupae with SSPC production cages resting on them to the incubator or SSPC room with an elevated temperature (26.5ºC); adults will then emerge.
3. Remove the production cage with adults after the established duration for all females to emerge (usually 48 hours), and then transfer it to the holding room and maintain it in the usual way.
4. After cages have been loaded, only male pupae remain (providing males to mate with females from other mixed pupae in another cage).
5. Any male pupae remaining (after pupae have been used for production purposes) can be used for sterile male production.

3.5. Near-Infrared (NIR) Spectroscopy Separation of Pupae

As mentioned earlier, sections 3.3., 3.4. and 3.5. describe **three alternative methods** for handling pupae and adults, sexing adults, mating, and loading production cages. **Procedures in only ONE of the three sections should be followed — the three sections are mutually exclusive.**

1. Section 3.3. describes the procedures if a chiller is used to sex adults.
2. Section 3.4. describes procedures if self-stocking of production cages (SSPC) is done, specifically for *Glossina pallidipes*.
3. Section 3.5. describes procedures if pupae are separated with near-infrared (NIR) spectroscopy.

A new system using a single-kernel near-infrared (NIR) spectrometer (Fig. 17) is showing promise as a technique to separate the sexes in the late-pupal stage, providing the prospect of automated sexing (Dowell et al. 2005).

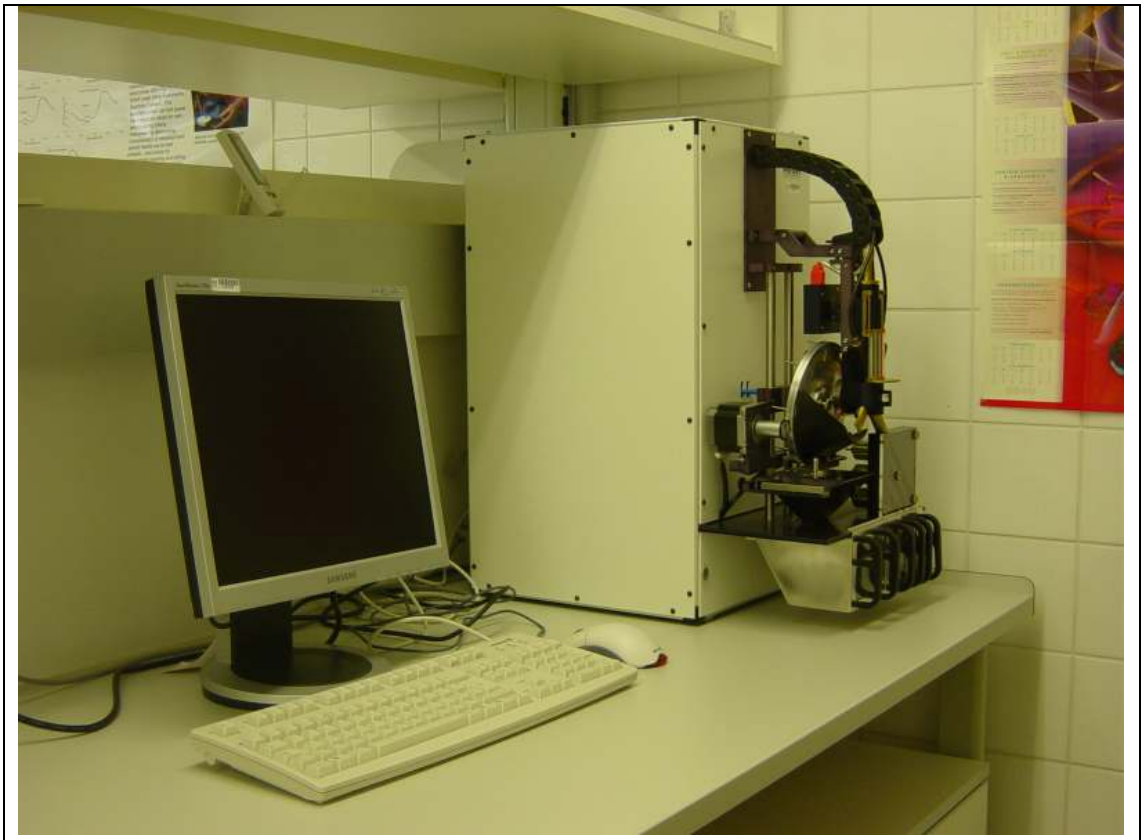

Fig. 17. Near-infrared (NIR) spectrometer and associated computer.

HEADING: 3.5. Near-Infrared (NIR) Spectroscopy Separation of Pupae	SUBHEADING: 3.5.1. Near-Infrared (NIR) Spectroscopy Separation of Pupae
DATE:	AUTHORIZATION:
COMMENTS:	

Scope: A single-kernel near-infrared (NIR) spectrometer can be used to separate pupae by sex

Equipment and Materials

- Near-infrared (NIR) spectrometer with computer support
- Dishes (plastic, for holding pupae)

HEADING: 3.5. Near-Infrared (NIR) Spectroscopy Separation of Pupae	SUBHEADING: 3.5.1. Near-Infrared (NIR) Spectroscopy Separation of Pupae
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. **TO BE COMPLETED**
2.

3.6. Data Collection and Analysis

The development and maintenance of a flourishing mass-reared colony of *Glossina* is dependent on steady quality-control parameters. Only the collection of proper data permit calculations to be made on these parameters, e.g. mortality, fecundity or pupal production, and prediction of possible excess flies or pupae in a given time period. After a period of accurate data recording at the beginning of the adaptation phase of a new strain, a more streamlined way of control can be adopted. Various forms have been developed to facilitate data collection, and an assessment of the data creates a clear picture on the status and dynamics of colony performance. The more accurately that data are collected, the quicker management can react to problems or changing needs.

A form to use is the weekly unit record sheet (Fig. 18) (section 3.6.1.). It follows the weekly performance of each unit, and contains weekly information on species, holding room, period of emergence, mating regimen, day of mating and separation, number of females per holding cage, diet, onset of production, and the number of pupae produced per day (section 5.). If necessary, pupal quality parameters such as weight or size are also added (section 5.3.). In addition, the information on mortality is displayed every second week or whenever a mortality check is done, indicating the reason for death. This regime is only applicable at rather low mortality rates — only then can small numbers of dead flies remain in the cage for 14 days. When the 'blood mortality' rate is high, it is important to identify the cause and correct the problem immediately. To reduce the chances of passing a bacterial contamination on to the live flies, all dead flies should be removed from the cage even if it is not yet time for the regular mortality check.

Every week a colony female unit is formed from emerged females collected from Monday to Saturday. The unit bears the respective week number 1–52 and the year (and 53 in leap years). The weekly unit record sheet contains the daily input of young females into the colony, and should contain all information on fly handling. The performance of each unit should be evaluated on a weekly basis, and expressed as daily mortality (% D.M.), fecundity (P/F/10d), mean pupal weight, and % A-class pupae (section 5.). Performance may be reviewed at weeks 4 and 8, and at the end of the unit life.

The performance of each unit is evaluated weekly, and is calculated as follows:

$$\%\ \text{D.M.} = \frac{\text{no. dead females} \times 100}{\text{no. females survived since last mortality check} \times \text{no. days elapsed since last check}}$$

$$\text{P/F/10d} = \frac{\text{no. pupae produced during previous week} \times 10}{\text{no. females recorded surviving previous week} \times 7}$$

Fecundity varies with the age of the females and among species.

The performance of the unit should also be assessed when the old unit is removed — by calculating the percentage surviving females and the number

of produced pupae per initial female (section 5.2.1.). The proportion of A-class pupae or mean pupal weight may also be calculated (sections 5.3. and 5.3.2.).

A summary weekly report will include the following:

- Mean colony female mortality (total mortality and blood mortality for both old and new units)
- Mean fecundity and proportion of A-class pupae
- Current emergence rate of the emergence control group
- Size of new colony unit formed
- Total number of colony females
- Number of pupae collected during the week
- Number of pupae or flies supplied for experiments or field projects

A data summary for a whole year is shown in Fig. 19.

Another major tool for data assessment is the blood quality-control sheet that gives a full picture of the blood, a vital component for *in vitro* rearing (section 4.3.3., Fig. 21).

A computer programme for a tsetse colony recording and reporting system (TCRRS) will soon be introduced for colony data collection and analysis (section 3.6.2.).

Entomology Unit

Joint FAO/IAEA Programme, Seibersdorf Laboratories

Record Sheet for Weekly Tsetse Colony Units

Species: *Glossina* _____ Loading SSPC / Chill

Females / cage _____ Mating Resident / Separate

Males / cage _____ Feeding, Treatment _____

/ Unit No. / year

DATE	No. of females dead	input	Σ
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11	No. of pupae		No. fem
12	daily tot.	daily A	died
13	weekly tot.	weekly A	surv.
14			
15			
16			
17			
Σ **wk2**			
18			
19			
20			
21			
22			
23			
24			
Σ **wk3**			
25			
26			
27			
28			
29			
30			
31			
Σ **wk4**			
32			
33			
34			
35			
36			
37			
38			
Σ **wk5**			
39			
40			
41			
42			

43				
44				
45				
Σ **wk6**				
46				
47				
48				
49				
50				
51				
52				
Σ **wk7**				
53				
54				
55				
56				
57				
58				
59				
Σ **wk8**				
60				
61				
62				
63				
64				
65				
66				
Σ **wk9**				
67				
68				
69				
70				
71				
72				
73				
Σ **wk10**				
74				
75				
76				
77				
78				
79				
80				
Σ **wk11**				
81				
82				
83				
84				

85			
86			
87			
Σ **wk12**			
88			
89			
90			
91			
92			
93			
94			
Σ **wk13**			
95			
96			
97			
98			
99			
100			
101			
Σ **wk14**			
102			
103			
104			
105			
106			
107			
108			
Σ **wk15**			
109			
110			
111			
112			
113			
114			
115			
Σ **wk16**			
116			
117			
118			
119			
120			
121			
122			
Σ **wk17**			
123			
124			Σ123 - 127
125			tot
126-7			A

Fig. 18a. Record sheet for weekly tsetse colony units.

WEEKLY SEIBERSDORF UNIT SUMMARY

Species *G. pallidipes* **Unit to** **08-Oct-06**
Code SEIB-pallidipes UGA **Check on** **11-Oct-06**
Week No **40** **2006**

Unit No.	Start	Died	Finish	Daily % Mort.	Pupae	P/f/10d	PPIF	%
40	840		840	-				
39	1680		1680	-				
38	830	7	823	0.06%	526	0.91	0.63	330%
37	800	13	787	0.23%	369	0.66	1.02	160%
36	1543	92	1451	0.87%	758	0.70	1.39	134%
35	673	50	623	1.10%	329	0.70	1.62	112%
34	340	38	302	1.68%	151	0.63	1.83	101%
33	803	98	705	1.84%	339	0.60	2.32	104%
32	459	118	341	4.16%	135	0.42	2.31	91%
31	407	78	329	2.99%	119	0.42	2.40	83%
30	249	102	147	7.25%	75	0.43	1.69	54%
29	144	70	74	9.07%	28	0.28	2.39	73%
28	140	62	78	8.02%	18	0.18	3.33	97%
27	95	73	22	18.86%	24	0.36	3.84	109%
26			0	-		-	2.41	67%
25			0	-		-	3.04	83%
24			0	-		-	2.48	67%
Out	0		0					
Preprod	2520	0	2520	-				
Produc.	6483	801	5682	1.86%				
Total	9003	801	8202	1.86%	2871	0.63		

Pupae	A	B	C	D	E	Total
38	3	8	260	233	22	526
37	1	8	86	199	75	369
36	1	19	231	365	142	758
35		5	95	168	61	329
34	1	6	45	80	19	151
33	4	15	97	168	55	339
32	1	11	31	65	27	135
31		2	32	59	26	119
30		8	33	28	6	75
29		2	10	14	2	28
28		3	11	4		18
27		1	15	6	2	24
26						
25						
24						
	11	88	946	1389	437	2871

Fig. 18b. Example of weekly unit summary.

Colony Summary

Code 2004

Year 0

Week	Start	Died	Colony Size	Daily Mortality %	Pupal production	P/F/10d	PPIF	Productivity
1	27581	704	26877	0.39%	9742	0.62	3.58	2.27
2	26410	624	25786	0.39%	10036	0.67	3.53	2.46
3	28306	1546	26760	0.76%	11446	0.70	3.75	2.09
4	29280	1471	27809	0.80%	10075	0.59	3.37	1.72
5	30329	1350	28979	0.78%	10758	0.61	3.24	1.78
6	28281	2060	26221	1.05%	11334	0.70	2.82	1.80
7	28741	1408	27333	0.74%	10596	0.64	2.61	1.91
8	27252	1485	25767	0.89%	10597	0.68	2.58	1.90
9	28287	1934	26353	0.95%	9652	0.59	2.25	1.60
10	26401	1215	25186	0.78%	9631	0.64	3.04	1.89
11	25739	1455	24284	0.82%	9166	0.63	2.07	1.82
12	26804	1735	25069	1.01%	10219	0.67	2.81	1.77
13	27589	1437	26152	1.03%	10101	0.64	3.27	1.67
14	27319	1919	25400	0.91%	9047	0.58	3.45	1.60
15	26704	1749	24955	1.02%	11477	0.76	3.84	1.98
16	26753	1751	25002	1.01%	10493	0.69	4.10	1.82
17	26455	1544	24911	0.88%	10419	0.70	3.87	1.94
18	26291	1649	24642	0.98%	11152	0.75	3.78	2.00
19	26363	1760	24603	0.99%	9535	0.64	3.98	1.69
20	26610	1722	24888	0.99%	10632	0.70	4.04	1.87
21	26174	1496	24678	0.87%	10460	0.71	3.99	1.98
22	26252	1637	24615	0.95%	10682	0.72	3.85	1.94
23	26386	1632	24754	0.93%	11265	0.75	3.38	2.06
24	26683	2064	24619	1.18%	10877	0.72	3.87	1.76
25	26271	1621	24650	0.92%	9275	0.62	3.83	1.71
26	26454	1548	24906	0.90%	9963	0.66	3.74	1.84
27	26547	2387	24160	1.57%	10328	0.69	4.25	1.47
28	26018	1471	24547	1.18%	10532	0.72	4.29	1.76
29	25890	1733	24157	1.03%	10367	0.71	4.54	1.86
30	26555	1344	25211	0.74%	11671	0.77	4.21	2.33
31	27099	1841	25258	1.08%	11249	0.73	4.27	1.86
32	26894	1683	25211	0.91%	10815	0.71	4.16	1.95
33	27537	1592	25945	0.89%	10816	0.69	4.08	1.91
34	27541	1437	26104	0.78%	11419	0.72	4.18	2.13
35	26409	1559	24850	0.88%	10924	0.73	4.13	2.04
36	26536	1579	24957	0.86%	12110	0.80	3.91	2.27
37	27477	2449	25028	1.96%	10605	0.68	4.17	1.32
38	27548	1038	26510	0.58%	10450	0.66	4.19	2.17
39	29030	1592	27438	0.85%	11749	0.70	4.02	1.98
40	28002	1760	26242	0.96%	8794	0.55	4.08	1.47
41	27667	1576	26091	0.86%	10536	0.67	4.74	1.88
42	27620	1731	25889	0.96%	10489	0.66	4.69	1.78
43	27118	1995	25123	1.12%	10966	0.71	4.52	1.78
44	26559	1554	25005	0.88%	9878	0.66	4.43	1.83
45	25821	959	24862	0.61%	10078	0.67	4.25	2.15
46	23174	691	22483	0.46%	8972	0.65	4.54	2.28
47	24163	1372	22791	0.82%	8866	0.61	4.81	1.75
48	24471	1716	22755	1.06%	8091	0.55	4.58	1.41
49	23487	1534	21953	0.97%	8242	0.58	4.22	1.56
50	22615	1205	21410	0.81%	8075	0.60	3.82	1.73
51	22284	1539	20745	1.04%	7297	0.55	3.58	1.43
52	20988	2225	18763	1.69%	6820	0.55	3.86	1.15
53	19126	1892	17234	1.45%	6465	0.59	3.91	1.31

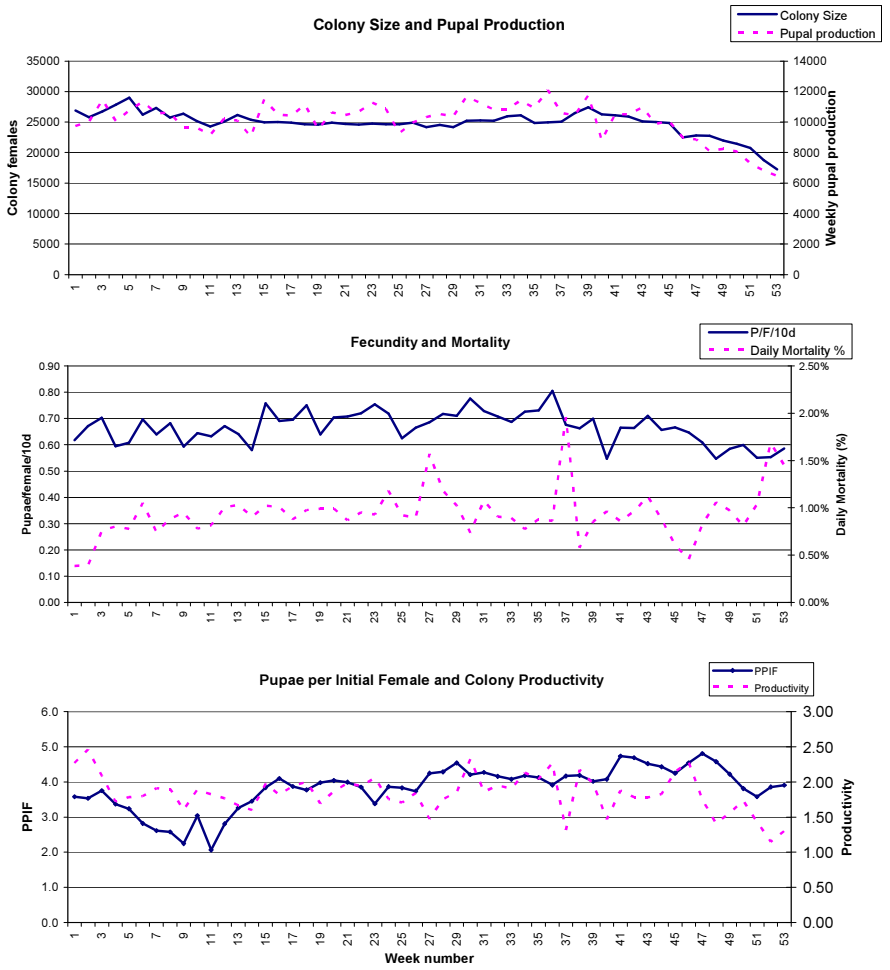

Fig. 19. Annual summary record sheet.

HEADING: 3.6. Data Collection and Analysis	SUBHEADING: 3.6.1. Weekly Unit Record Sheet and Annual Summary
DATE:	AUTHORIZATION:
COMMENTS:	

Scope: Weekly unit data and annual summary to monitor colony performance

Equipment and Materials

- Weekly unit record sheet (Fig. 18)
- Annual summary record sheet (Fig. 19)

(Note: These sheets are available as EXCEL spreadsheets from FAO/IAEA in Seibersdorf, Austria.)

HEADING: 3.6. Data Collection and Analysis	SUBHEADING: 3.6.1. Weekly Unit Record Sheet and Annual Summary
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. Familiarize yourself with terminologies
   - Mortality rate
   - % daily mortality
   - Types of mortality-starvation, blood pupae and other
   - Fecundity
   - Mean pupal weight
   - Pupal class size
   - A-size class pupae
2. Collect emerging flies on each working day.
3. Label cages with the unit number (the week number).
4. Enter on the record sheet the information on fly handling and unit formation (species, holding room conditions, period of emergence, day of mating, mating regimen, day of separation, number of females and males per holding cage, diet composition, and frequency of feeding).
5. Conduct a mortality check at 10–14 days after a unit is formed.
6. Remove any dead flies, and sort them according to category of mortality.
7. Calculate the percentage (%) daily mortality.
8. Collect and count the pupae.
9. Determine the fecundity (section 5.2.).
10. Sort pupae by weight and size (section 5.3.).
11. Determine the mean pupal weight and % A-class pupae.
12. Compute colony performance at 4 weeks, 8 weeks, and at the end of the unit life span (when cages from the old unit are removed).

HEADING: 3.6. Data Collection and Analysis	SUBHEADING: 3.6.2. Tsetse Colony Recording and Reporting System (TCRRS)
DATE:	AUTHORIZATION:
COMMENTS:	

Aim: **To be completed**

Equipment and Materials

- **To be completed**

HEADING: 3.6. Data Collection and Analysis	SUBHEADING: 3.6.2. Tsetse Colony Recording and Reporting System (TCRRS)
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. **To be completed**
2.

3.7. Control of Diseases, Parasitoids and Predators

Tsetse flies can pick up pathogenic trypanosomes when infected field-collected flies are fed on the same host or on the same membrane as colony flies. Procedures to avoid this include feeding flies from different sources on different live hosts, examination, removal and then treatment of infected hosts, feeding young flies before feeding older flies on the same membrane, or feeding on separate membranes.

Bacterial infection in colony flies is usually contracted from contaminated feeds. Infected individuals die and become the source of infection in other flies. When holding conditions, e.g. high relative humidity, favour the multiplication of bacteria, flies dying with blood in the abdomen provide a medium from which contamination can spread within the cage. Therefore, when blood mortality is observed, dead flies should immediately be removed from the cage.

Bacteria can also be introduced into the blood through non-sterile blood handling and feeding equipment, and during the filling of the feeding tray with blood while the membrane is lifted. If the blood used during feeding is not sterilized and maintained in the right conditions, the <1% residual bacteria could multiply and infect flies during feeding.

Salivary gland hyperplasia virus (SGHV) has been observed in *Glossina brevipalpis* Newstead, *Glossina morsitans* Westwood and *G. pallidipes*, but the highest incidence has been found in *G. pallidipes* where it has been associated with sterility among males. SGHV is associated with the enlargement of salivary glands and sterility in some tsetse species. *G. brevipalpis*, *G. morsitans* and *G. pallidipes* have been found infected in the wild. In the laboratory, an incidence of more than 50% has been found in male *G. pallidipes*. It is suspected that the inability to establish a colony of *G. pallidipes* arises from the occurrence of SGHV in colony flies. Research is ongoing to elucidate the mode of transmission so that a disease-control strategy can be developed.

There are hymenopteran parasitoids that lay eggs in tsetse pupae. After the eggs hatch, the larvae feed on the contents of the pupae, emerging later from the puparium as adults. When field-collected pupae are used to initiate a colony, some of these pupae may contain parasitoids, and therefore field-collected pupae should be quarantined and not kept together with colony pupae.

Ants have been known to invade colonies of tsetse to feed on the flies. An insectary can be isolated with a water moat that prevents ants from crawling into the insectary, but the moats must be inspected and cleaned regularly. Water cups fitted above the wheels of trolleys prevent ants from climbing up to feed on flies in cages on the trolley. (Depending on the location of the rearing facility, the need for water cups may apply to the TPU 3 system as well.) Branches from trees near the insectary building should be cut back so that they do not act as bridges for ants to get into the insectary.

HEADING: 3.7. Control of Diseases, Parasitoids and Predators	SUBHEADING: 3.7.1. Control of Bacterial Infections
DATE:	AUTHORIZATION:
COMMENTS:	

Aim: To control a bacterial infection in a tsetse colony

Equipment and Materials

- Blood-collection equipment (sections 4.1. and 4.1.1.)
- Blood-storage equipment
- Deep-freeze
- Membrane-feeding equipment (section 3.2.)
- Radiation source
- Bacterial-screening equipment (sections 4.3. and 4.4.)

HEADING: 3.7. Control of Diseases, Parasitoids and Predators	SUBHEADING: 3.7.1. Control of Bacterial Infections
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. Clean and sterilize all equipment used in blood collection, processing and storage (section 4.).
2. When not in use, store all sterilized equipment in sterile conditions.
3. Irradiate the blood at 1–1.5 kGy.
4. Store irradiated blood in the correct conditions (-20ºC).
5. Clean and sterilize the membrane-feeding equipment.
6. Before lifting a membrane to fill a tray with blood during preparations for feeding, switch off all equipment that would create air turbulence.
7. When blood mortality is detected in the colony, remove the dead flies in any affected cages.
8. Check the RH, and determine the likely cause of blood mortality; adjust according.
9. Take a sample of blood from the batch that was used to feed the flies, and plate it out to determine if there is any bacterial contamination (sections 4.3. and 4.4.)
10. Discard the blood if there are more than 10 bacterial colony-forming units (CFU). (If there are 10–15 CFU, and there is a severe shortage of blood, then the blood could be reirradiated and subsequently retested.)

HEADING: 3.7. Control of Diseases, Parasitoids and Predators	SUBHEADING: 3.7.2. Control of Salivary Gland Hyperplasia Virus (SGHV)
DATE:	AUTHORIZATION:
COMMENTS:	

Aim: To control the salivary gland hyperplasia virus (SGHV) in a tsetse colony

Equipment and Materials

- Dissecting instruments (for dissecting insects)
- Compound microscope
- Dissecting microscope
- Microscope slides
- Cover slips

HEADING: 3.7. Control of Diseases, Parasitoids and Predators	SUBHEADING: 3.7.2. Control of Salivary Gland Hyperplasia Virus (SGHV)
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. **To be completed**
2.

HEADING: 3.7. Control of Diseases, Parasitoids and Predators	SUBHEADING: 3.7.3. Control of Parasitoids
DATE:	AUTHORIZATION:
COMMENTS:	

Aim: Control of parasitoids parasitizing tsetse in the colony

Equipment and Materials

- **To be completed**

HEADING: 3.7. Control of Diseases, Parasitoids and Predators	SUBHEADING: 3.7.3. Control of Parasitoids
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. If pupae are collected in the field, it is important to have a quarantine area in the rearing facility in which to hold these pupae.
2. If pupal parasitoids are found in the rearing facility, put 3-cm-deep fine sand into the pupal collecting troughs to prevent parasitization of the pupae produced in the holding room.
3. If feasible, it is useful to insect-proof the rearing facility, preventing the entry of unwanted parasitoids (as well as the escape of tsetse flies).
4. **To be completed**

4. Blood Collection, Processing and Quality Control

Tsetse flies are fed on quality-tested fresh defibrinated blood, but which has been stored in a frozen condition (Wetzel and Luger 1978).

To stop the blood from clotting, at the time of collection from animals being slaughtered, the fibrin is removed by mechanically stirring the blood or by adding anticoagulants. If slaughtering takes place on the floor, the blood collection equipment used is selected accordingly. Collection procedures are modified to suit the volume requirements. This procedure describes the collection and processing of animal blood at the abattoir for later use in feeding tsetse flies. However, a reasonable amount of aseptic blood can also be collected from live hosts using blood transfusion kits containing anticoagulants or sterile bottles containing glass beads.

After collection, a sample of blood from the batch is checked for microbial contamination, and bioassayed for its nutritive value using female adult flies. Procedures for identifying bacteria are provided.

All blood fed to flies must first be decontaminated, for example by treatment with radiation.

A procedure for shipping frozen blood to distant rearing facilities is described.

The reconstitution of freeze-dried blood is an alternative method of providing blood to feed flies, but is not used frequently.

4.1. Blood Collection

Before collection, make arrangements with the responsible authorities of the abattoir to ensure that the place, time and amount of blood to be collected are agreed upon. Alert the collection team (a minimum of three persons) to prepare for the work; note that some heavy lifting may be required. It is an advantage for the workers to have a good knowledge of basic biology and tsetse rearing procedures, and have the ability to handle mechanical equipment. In addition, the workers must be able to make sound judgments regarding the condition of animals (age and health) at the abattoir. The team must wear protective gear (white clothing, rubber boots and helmets).

Before arrival at the abattoir, all equipment that will be in contact with blood must be thoroughly cleaned and sterilized. The equipment used to collect blood (Fig. 20) depends on the amount of blood obtained on one occasion. The frequency of collection depends on the size of the tsetse colony and the amount of space available to store frozen blood at -20°C. In general, during one collection operation, it is economical to obtain as much blood as possible because transport, human labour and quality-control measures become cheaper with increasing quantity. If the ambient temperature is high or the storage facility is a long way from the collection point, it may be necessary to cool the tank of blood. Using two or three collection sets, blood can be collected from all slaughtered animals even if the rate of slaughter is high, e.g. 100 animals in 5–6 hours. Freezing slows down, but does not stop completely, the degradation of blood. It has been demonstrated that blood kept frozen at -20°C for many years retains its nutritive characteristic.

If animals are slaughtered on the floor, the blood collection equipment used is selected accordingly. Blood is collected from the cut neck of the animal in a bucket, and then immediately poured into a 25-l container for defibrination or into a container with anticoagulants.

Fig. 20. Blood collection equipment.

HEADING: 4.1. Blood Collection	SUBHEADING: 4.1.1. Blood Collection
DATE:	AUTHORIZATION:
COMMENTS:	

Scope: Collection of a large volume of blood in an abattoir where animals are hung by their hind legs and pulled up with a chain, with their heads hanging down

Equipment and Materials

- Vehicle (for transport to the abattoir, carrying equipment)
- Deep-freeze chests (or storage space maintained at –20°C)
- Containers (screw-capped, 20–25 l, polyethylene (PE), with a wide neck to allow a paddle/stirrer to be inserted) (The screw cap is equipped with a stand to hold the stirrer in an upright position. The container has a side inlet with a screw attachment for a plastic hose.)
- Hose (plastic, made of transparent silicone, with strong walls (4–5 mm thick, inside diameter 4 cm, outside diameter 5 cm), 1.5-m long to lead from the funnel to the inlet on the side of the container, should be autoclavable)
- Funnel (plastic or stainless steel, upper diameter 25–40 cm, with an outlet of 4 cm to fit the hose)
- Paddle (consists of a stainless steel rod of 1-cm diameter on which the oar-blade/stirrer is mounted)
- The size of the stirrer is determined by the dimensions of the mouth opening and the depth of the container. The stirrer should agitate the full volume of liquid, reaching close to the bottom of the container. The stirrer can be a mechanical handheld drill, an electric household drill or a battery-driven drill. If an electric drill is used, it should have a speed regulator and switch to control the driving speed. The drill should be strong enough to agitate a volume of 10–20 l of highly viscous liquid.
- When using electric stirring equipment, a ≥50-m length of waterproof extension cable is necessary to reach a power supply in the abattoir.
- Storage cans (5-l volume), preferably made of heat- and cold-resistant plastic, that are heat-sterilized before use and able to withstand prolonged storage at -20°C. It is advantageous if the can is rectangular in shape, making it easy to stack and economic in terms of storage space. The lid must have a screw cap with a rubber gasket that can be tightly closed.
- Collecting tank (made of sturdy low-density polyethylene (LDPE), volume up to 500 litres, with a wide neck and a tap to redistribute the blood)
- Sieves (metal, preferably aluminium ‘milk’ sieves, upper diameter 30–40 cm, lower opening to fit the neck of the collecting tank, equipped with a removable sieve to filter out residual particles from the defibrination)
- Trolley (to transport the filled collecting tank, with a platform high enough above the ground so that 5-l cans can be placed underneath the tap to redistribute the blood)

HEADING: 4.1. Blood Collection	SUBHEADING: 4.1.1. Blood Collection
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. Clean the blood-collection equipment.
2. Sterilize the equipment by exposing it to dry heat (at 80°C for polyethylene (PE) for at least 3 hours, and glass at 120°C) or autoclave for 15 minutes at 103.45 kPa (15 psi).
3. Sterilize the 500-l tank using steam.
4. When not in use, to minimize microbial contamination, store all sterilized equipment in autoclave bags or in an ultraviolet (UV) cabinet.
5. On arrival at the abattoir, assemble the collection equipment and check that it functions.
6. Negotiate with the butcher for permission to skin the neck of the animal before the jugular vein is cut.
7. When the butcher has cut the throat of the animal, direct the flow of blood into the funnel attached to a hose leading to the stirring container.
8. Collect as much blood as possible before terminating the procedure.
9. While one person controls the position of the funnel, the second controls the rotation of the stirrer, adjusts it to a moderate number of revolutions, and observes the filling of the container. Up to 15 l of blood can be expected from a bull, and up to 20 l can be collected in one stirring container.
10. After 10 minutes of stirring, the blood is defibrinated, and the stirring machine is stopped. (Meanwhile, if practical, use a second set of blood-collection equipment to collect blood from additional animals.)
11. Open the lid of the stirring container and remove the paddle with all the clotted fibres.

HEADING: 4.1. Blood Collection	SUBHEADING: 4.1.1. Blood Collection
DATE:	AUTHORIZATION:
COMMENTS:	

12. Pour the residual liquid through a ‘milk’ sieve into the collection tank so that fibres that were not caught by the paddle are removed.
13. Rinse the paddle of the stirrer with clean water to free it from fibrin.
14. As more animals are slaughtered, continue collecting blood. Using two or three sets of blood-collection equipment, blood can be collected from many animals, even if the slaughtering speed is high (100 animals in 5–6 hours).
15. Redistribute the blood from the tank into 5-l storage cans.
16. Mark the cans with an identification code (animal species, lot and year) using a water-resistant marker.
17. Close the cans tightly, and rinse all spilled blood from the outside of the storage cans.
18. Rinse all equipment with cold water before loading it into the vehicle.
19. Transport all cans to the storage facility and deep-freeze immediately, but keep one can from each batch separate and take it to the laboratory for quality assurance.
20. Thoroughly clean and sterilize all equipment that was in contact with blood, and keep until required again.
21. Record all information (date, quantity, code, number of cans, primary results of bacterial screening tests (section 4.3.1.), pH, osmotic pressure, consumption and output) electronically and in a hardcover record book.

4.2. Bulk Sterilization of Fresh Blood

This procedure describes the sterilization (decontamination), preservation and storage of animal blood, after collecting blood at an abattoir, for processing into diet for feeding tsetse flies. The procedure aims to provide nutrition at a constant quality to mass-reared fly colonies maintained over a long period for field programmes. The following is the standard procedure followed at the laboratory in Seibersdorf whenever blood is prepared for shipment to field programmes and after the quality assurance has been completed.

Access to an industrial gamma radiation source is a prerequisite for bulk sterilization at 1–1.5 kGy in which 5-l containers of blood in a frozen state are irradiated without further repackaging. The blood can be decontaminated at the time of shipment, or decontaminated and stored until shipment. A sample of decontaminated blood must be tested for microbial contamination to confirm sterilization.

To sterilize a small amount of blood, it is poured under sterile conditions into a container that fits into the chamber of a gamma cell.

To be completed:

Other techniques:
a. Pasteurization
b. Phenothiazine dyes
c. Ultraviolet (UV) radiation

HEADING: 4.2. Bulk Sterilization of Fresh Blood	SUBHEADING: 4.2.1. Irradiation
DATE:	AUTHORIZATION:
COMMENTS:	

Scope: Bulk sterilization of blood using gamma radiation

Equipment and Materials

- Gamma radiation source (industrial)
- Blood (frozen)
- Vehicle (for transporting frozen blood)

HEADING: 4.2. Bulk Sterilization of Fresh Blood	SUBHEADING: 4.2.1. Irradiation
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. Contact the responsible authorities of the gamma radiation source, and arrange the details of the irradiation work — date, time, and volume of blood to be sterilized.
2. Transport the frozen blood (in 5-l cans) from storage to the irradiation facility.
3. Expose the blood (in deep-frozen condition) to 1–1.5 kGy. (This treatment requires about 6 hours at room temperature, and during this time a thin layer of blood next to the plastic container thaws but does not warm up to more than 4ºC.)
4. Remove one 5-l can of blood and conduct bacterial screening to confirm sterilization (sections 4.3. and 4.4.).
5. Transport the blood in a frozen condition, and store.
6. Store the blood frozen until use or further processing and testing.

HEADING: 4.2. Bulk Sterilization of Fresh Blood	SUBHEADING: 4.2.2. Pasteurization
DATE:	AUTHORIZATION:
COMMENTS:	

Scope: Sterilization of blood using short-time high-temperature (STHT) pasteurization

Equipment and Materials

- **To be completed**

HEADING: 4.2. Bulk Sterilization of Fresh Blood	SUBHEADING: 4.2.2. Pasteurization
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. **To be completed**

HEADING: 4.2. Bulk Sterilization of Fresh Blood	SUBHEADING: 4.2.3. Ultraviolet (UV) Radiation
DATE:	AUTHORIZATION:
COMMENTS:	

Scope: Sterilization of blood using ultraviolet (UV) radiation

Equipment and Materials

- **To be completed**

HEADING: 4.2. Bulk Sterilization of Fresh Blood	SUBHEADING: 4.2.3. Ultraviolet (UV) Radiation
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. **To be completed**

4.3. Blood Quality Control

These procedures describe the quality assurance and data recording for blood used to feed large colonies of tsetse. The aim is to maintain high-quality mass-reared colonies of tsetse over long periods.

During collection and processing, blood can become contaminated with bacteria and chemicals. Under the influence of physiology, nutrition and disease, the composition of blood in animals varies among individuals of the same species. Due to these factors, a quality-control procedure was developed, and biological criteria for evaluating the suitability of blood used to feed flies were identified. Microbial screening of blood is done using blood from one 5-l can from each batch brought back to the laboratory after collection, and also on blood after decontamination. The second bacterial screening is carried out to confirm the efficacy of the sterilization procedure. On average, 96% of microbial contamination is eliminated by irradiation at 1.5 kGy.

A 25-day feeding test was developed with the aim of having a simple numerical system that adequately summarizes and combines the various data obtained from pupal production and dissections (Fig. 21). It is expressed as the quality factor (QF). For irradiated blood, a QF of >1 is acceptable. The assumption is that the larva/pupa will develop into a viable fly. Since both the female adult and the larva within the uterus are dependent on the same source of food, it follows that the availability of blood is one of the most important factors affecting reproductive physiology in tsetse.

Bacterial identification is discussed in section 4.4.

For the blood quality feeding test, a minimum quantity of 500 ml of blood is necessary, and is handled in the following way. A sample for bacterial testing is taken from the storage container, and blood foreseen to be used later for colony feeding is treated precisely in the same way as the test amount. The diet or a mixture of blood is portioned into 15-ml aliquots in 20 sterile vials. The vials are closed, and exposed to 1 kGy gamma radiation. After irradiation, another sample is taken for bacteriological screening to confirm decontamination. Once the result of the bacterial screening after irradiation is found to be acceptable, the blood is ready to be fed to the flies.

Entomology Unit, Joint FAO/IAEA Programme

IAEA Agriculture Laboratory Seibersdorf
Record Sheet for Tsetse Blood Diet Quality Control

BLOOD CODE: **BC06076** date collected 20.04.06 storage

date collected storage

Test-No.	6
Year	2006

date processed pH

additives osm. pr. mOsm

MICROBIAL SCREENING			(CFU/ml)
cont. A	cont. B	diet before γ	diet after γ
0 Sp/ml	Sp/ml	Sp/ml	Sp/ml

SURVIVAL & FECUNDITY

G. pallidipes
Test species

DISSECTIONS

day	DATE	dead st	dead bl	fem. surv.	No. of pupae prod.	No. of abortions in dish
1	5.5.06			30		
2	6.5.06			30		
3	7.5.06			30		
4	8.5.06			30		
5	9.5.06			30		
6	10.5.06			30		
7	11.5.06			30		
8	12.5.06			30		
9	13.5.06			30		
10	14.5.06			30		
11	15.5.06			30		
12	16.5.06			30		
13	17.5.06			30		
14	18.5.06			30		
15	19.5.06			30		
16	20.5.06			30		
17	21.5.06			30		
18	22.5.06	2		28	15	
19	23.5.06	1		27	3	
20	24.5.06			27	1	
21	25.5.06			27	1	
22	26.5.06			27		
23	27.5.06			27		
24	28.5.06			27		
25	29.5.06			27	2	
Σ		**3**		**27**	**22**	
26	30.5.06	1		26	1	
27	31.5.06			26	2	
28	1.6.06			26	7	
29	2.6.06			26	6	
30	3.6.06			26		
Σ		**4**			**38**	

females: date EM date MAT date SEP males: date EM

other remarks:

weight class distribution					
	A	B	C	D	E
Σ	**0**	**0**	**0**	**0**	**0**
Σ	**0**	**0**	**0**	**0**	**0**

N	Sp	UT
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		
11		
12		
13		
14		
15		
16		
17		
18		
19		
20		
21		
22		
23		
24		
25		
26		
27		
28		
29		
30		

Day 25

FS 18	28	PT	22	PB	0	PD	0	E + I	0	AB	0
FS 25	27	PA	0	PC	22	PE	0	II + III	0	BL	0

QF	
QFC	1.24

Day 30

FS 18	28	PT	38	PB	0	PD	0	E + I	0	AB	0
FS 30	26	PA	0	PC	38	PE	0	II + III	0	BL	0

QF	
QFC	1.70

Fig. 21. Record sheet for tsetse blood diet quality control.

HEADING: 4.3. Blood Quality Control	SUBHEADING: 4.3.1. Microbial Screening of Blood
DATE:	AUTHORIZATION:
COMMENTS:	

Scope: Microbial screening of blood

Equipment and Materials

- Laminar-flow bench (hood)
- Bunsen burner
- Balance
- Magnetic stirrer/hot plate
- Incubator
- Bacterial-colony counter/viewer
- Petri dishes (glass, autoclaved)
- Distilled water (autoclaved)
- Nutrient agar
- Syringe and needle (1 ml, disposable)
- Erlenmeyer flasks (various sizes)
- Blood (batch to be tested

HEADING: 4.3. Blood Quality Control	SUBHEADING: 4.3.1. Microbial Screening of Blood
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. Mix 2 g of agar with 100 ml distilled water in a 200-ml glass Erlenmeyer flask closed by cotton wool, stir gently and boil.
2. Autoclave the fluid agar at 125°C for 15 minutes.
3. Put the flask of agar in a water bath at 40°C.
4. Clean working bench and hands with antiseptic or sterilizing fluid.
5. Place sterile Petri dishes on the bench.
6. Wipe the outer surface of all containers before placing them on the bench.
7. Ignite the Bunsen burner.
8. Label the Petri dishes on the bottom of the smaller half with the blood code, and have one Petri dish for air and one Petri dish for nutrient agar.
9. Open the Petri dishes briefly, flame the open parts, and close them immediately.
10. Make sure the agar in the Erlenmeyer flask is between 40–45°C.
11. Lift the lids of the Petri dishes, and add 13 ml agar to the empty Petri dishes. Aim at an agar-layer thickness of ¼-⅓ of the internal depth of the dish.
12. Again lift the lids slightly from the Petri dishes and pour in 1-ml samples from the syringes.
13. Stir gently for about 15 seconds.
14. Wait until the mixture of agar and blood has solidified, and then turn the Petri dish upside down.
15. Incubate the inoculated Petri dishes in the upside-down position at 37°C for 72 hours.
16. Check the agar layers for bacterial colonies after 48 and 72 hours.

HEADING: 4.3. Blood Quality Control	SUBHEADING: 4.3.1. Microbial Screening of Blood
DATE:	AUTHORIZATION:
COMMENTS:	

17. Record the number of bacterial colonies found per sample.
18. If, after 72 hours, the irradiated blood contains NOT more than 10 colonies, feed the blood to tsetse for the 25- or 30-day quality-control test.
19. If the blood has more than 10 colonies, discard this can of blood, and take a new can and repeat the test.

HEADING: 4.3. Blood Quality Control	SUBHEADING: 4.3.2. Irradiation
DATE:	AUTHORIZATION:
COMMENTS:	

Scope: Sterilization of blood using a gamma cell

Equipment and Materials

- Gamma cell (gamma radiation)
- Blood (fresh)
- Vials (15 ml, sterile)
- Irradiation basket (cylindrical metal box)

HEADING: 4.3. Blood Quality Control	SUBHEADING: 4.3.2. Irradiation
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. Remove 500 ml of fresh blood from a 5-l can brought to the laboratory from each batch of blood collected.
2. Conduct bacterial screening on a sample of the blood (section 4.3.1.).
3. Portion the blood into 15-ml aliquots in 20 sterile vials.
4. Close the vials, and put them into the irradiation basket.
5. Open the gamma cell, put the basket into the irradiation chamber, and close the chamber.
6. Set the exposure time (according to the date) for 1 kGy.
7. Press the start button.
8. Retrieve the vials when irradiation is complete.
9. Take another 15-ml sample, and conduct bacterial screening to confirm decontamination.
10. Store the vials in a deep-freeze.

HEADING: 4.3. Blood Quality Control	SUBHEADING: 4.3.3. 25-Day Feeding Test
DATE:	AUTHORIZATION:
COMMENTS:	

Scope: 25-day feeding test

Equipment and Materials

- Scintillation vials (30, each 20 ml, polyethylene)
- Graduated cylinder (20 ml)
- Dispensing bottle (1 l, with 20-ml calibrated dispenser)
- Deep-freeze
- Laminar-flow bench (hood)
- Foil (aluminium, to cover autoclaved or sterilized glassware)
- Funnel (to fit 20-ml vials)
- Sieve (to collect floating fibres)
- Magnetic stirrer (to agitate blood — volumes between 100 and 1000 ml)
- Petri dishes (for nutrient agar plates)
- Syringes (1 ml, sterile, disposable)
- Incubator
- Bacterial colony-viewing/counting device
- Teneral female flies (30, mated in the appropriate way, and caged at 15 per standard 11-cm-diameter cage)
- Radiation source
- Stereomicroscope

HEADING: 4.3. Blood Quality Control	SUBHEADING: 4.3.3. 25-Day Feeding Test
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. Thoroughly clean and sterilize all parts of equipment that have come into contact with blood.
2. Sterilize in an oven by exposure to 80°C (polyethylene) or 120°C (glass and metal) for 3 hours, or in an autoclave at 120°C for 30 minutes, and then store properly to minimize the risk of microbial contamination.
3. At emergence, put 15 teneral females each in two standard 11-cm-diameter cages.
4. Mate flies in the appropriate way.
5. Feed flies daily.
6. For 25 days, feed from the same batch of blood.
7. Check for mortality, and dissect any dead female — examining for uterine content, insemination (section 5.2.), and mating scars where applicable, and record.
8. After 8 days, place the cages on a dish, and thereafter examine the dishes for abortions under the stereomicroscope on days 10, 15, 20 and 25 from the time of emergence.
9. Collect larvae and pupae.
10. Allow larvae to pupate, and record individual weight.
11. If a pupal size-sorting machine is available, at the termination of the test, sort by class all pupae, count, and note the various weight classes.
12. Dissect all females surviving 25 days, and examine for mating scars, insemination (section 5.2.), uterine content, follicle next in ovulation sequence (FNOS) (section 5.2.), and reproductive abnormalities such as blockage of the oviduct.
13. Calculate the quality factor (e.g. Fig. 21).

HEADING: 4.3. Blood Quality Control	SUBHEADING: 4.3.3. 25-Day Feeding Test
DATE:	AUTHORIZATION:
COMMENTS:	

Parameters used to calculate the blood quality factor (QF) (e.g. Fig. 21):

Parameter	Explanation

First reproductive cycle:

FS 18	number of females surviving on day 18
FS 25	number of females surviving on day 25
PT	total number of produced pupae
PA	number of A-class pupae
PB	number of B-class pupae
PC	number of C-class pupae
PD	number of D-class pupae
PE	number of E-class pupae

Second reproductive cycle:

E + I	number of inseminated females on day 25 with early pregnancy stage *in utero*
II + III	number of inseminated females on day 25 with late pregnancy stages *in utero*
BL	number of inseminated females on day 25 with oviduct blockage
AB	number of inseminated females on day 25 that aborted, empty uterus, follicle next in ovulation sequence (FNOS) is not mature

Calculation of the blood quality factor (QF):

QF = positive parameters from first reproductive cycle + positive parameters from second reproductive cycle – negative parameters from first reproductive cycle – parameters from second cycle / number of females

$$QF = \frac{[FS\ 25 + PT + (PB \times 0.3) + (PC \times 0.4) + (PD \times 0.5) + (PE \times 0.6) + (E + I \times 0.3) + (II + III \times 0.6) - (PA \times 0.2) - (AB \times 0.5) - (BL \times 1.0)]}{[FS\ 18 + FS\ 25]}$$

A QF value of 1.0 is the minimum acceptable value. For laboratories where tests are run regularly, experience shows that dissections can be omitted at day 25 without affecting the overall value, but the formula for calculating the QF value is modified (see below).

An EXCEL spreadsheet to calculate the QF is available at the Entomology Unit, FAO/IAEA Agriculture and Biotechnology Laboratories, Seibersdorf, Austria.

HEADING: 4.3. Blood Quality Control	SUBHEADING: 4.3.3. 25-Day Feeding Test
DATE:	AUTHORIZATION:
COMMENTS:	

To calculate the QFC (calculated quality factor), use the following formula:

$$QFC = \frac{[PA*11 + PB*17 + PC*19 + PD*20 + PE*22]}{[FS\ 18*23.86 + 0.616]}$$

An EXCEL spreadsheet to calculate the QFC is available at the Entomology Unit, FAO/IAEA Agriculture and Biotechnology Laboratories, Seibersdorf, Austria.

4.4. Bacterial Identification

The bacterial screening and 25-day feeding tests will indicate if there is a problem with the blood, but they do not specifically identify the cause. Occasionally it may be necessary to identify the bacteria present in the blood so as to determine if they are harmless or pathogenic to tsetse.

Individual pure bacterial colonies are first isolated by streaking or pouring. Then the colonies are tested with Gram stain, and further tested by the catalase or oxidase tests. Based on these results, the bacteria can then be identified using the appropriate Analytical Profile Index (API) strip, selected from the chart (Fig. 22).

The API strips are a standardized system for the identification of bacteria, using miniaturized biochemical tests and a database. The API strip consists of 20 microtubes containing dehydrated substrates. These microtubes are inoculated with a bacterial suspension, prepared in API Medium, to reconstitute the test. During incubation, the bacterial metabolism produces colour changes that are either spontaneous or revealed by the addition of reagents. The reactions are read according to the Reading Table, and the identification is made by referring to the Analytical Profile Index or using the identification software.

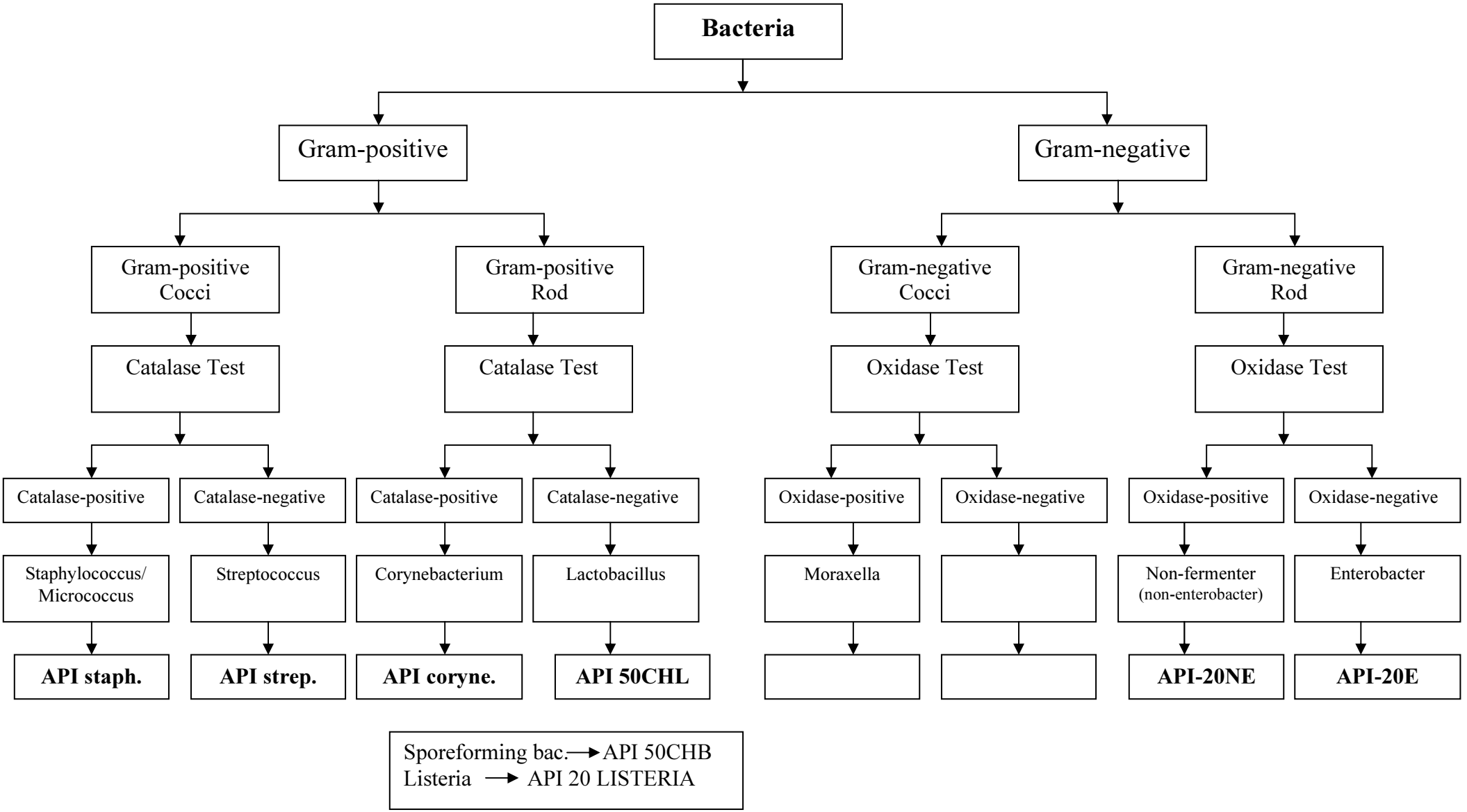

Fig. 22. Selection of API kit based on Gram staining, morphology and catalase or oxidase tests.

HEADING: 4.4. Bacterial Identification		SUBHEADING: 4.4.1. Bacterial Culture
DATE:		AUTHORIZATION:
COMMENTS:		

Scope: Mixed cultures for colony count

Equipment and Materials

- Sheep-blood agar plate (prepared)
- Blood (fresh)
- Spreader
- Bunsen burner
- Incubator
- Syringe and needle

HEADING: 4.4. Bacterial Identification		SUBHEADING: 4.4.1. Bacterial Culture
DATE:		AUTHORIZATION:
COMMENTS:		

Procedure

1. Using a syringe and needed, take up 0.5 ml of blood, and label it.
2. Open the sheep-blood agar plate, and pour in 0.2 ml of blood.
3. Flame the spreader to sterilize it, and when cool spread the blood over the whole plate.
4. Label the plate.
5. Incubate the agar plate for 48 hours.
6. Bacterial colonies can be recognized after incubation (Fig A).

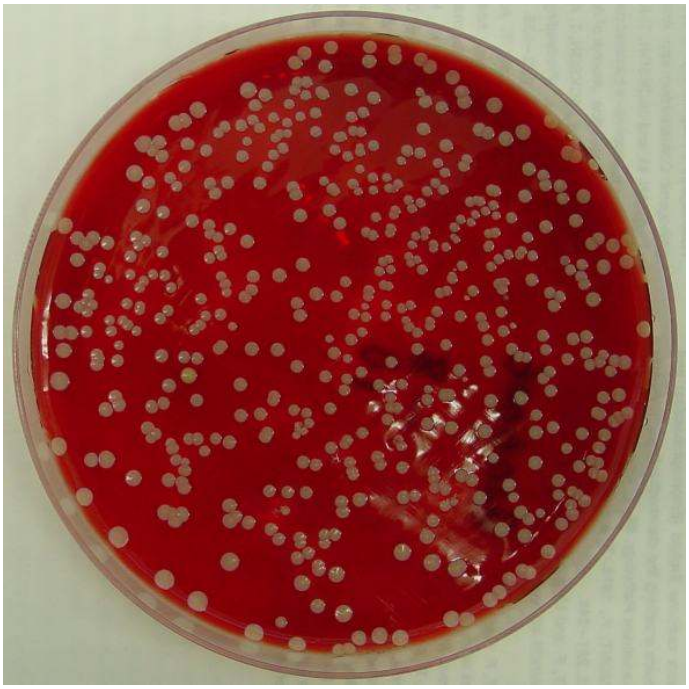

Fig. A

HEADING: 4.4. Bacterial Identification	SUBHEADING: 4.4.2. Bacterial Isolation by Streaking
DATE:	AUTHORIZATION:
COMMENTS:	

Scope: Isolation of pure bacterial cultures by the streak method

Equipment and Materials

- Inoculating loop (sterile)
- Bunsen burner or spirit lamp
- Sheep-blood agar plates or nutrient agar plates (prepared)
- Incubator
- Toothpicks (sterile)

HEADING: 4.4. Bacterial Identification	SUBHEADING: 4.4.2. Bacterial Isolation by Streaking
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. Using a sterile inoculating loop, spread a sample of the specimen over a small area at the edge of the plate of sheep-blood agar medium (Fig. B, ①).
2. Flame the loop to sterilize it again, and spread the inoculum from ① over the area ②.
3. Flame the loop to sterilize it again, and spread the inoculum from ② over the area ③.
4. Flame the loop to sterilize it again, and spread the inoculum from ③ over the area ④.
5. When done carefully, at each step this procedure results in a reduction in bacterial numbers.
6. Incubate the agar plate for 48 hours.
7. In area ④, discrete bacterial colonies can be recognized after incubation (Fig. B).
8. Pick up single colonies for identification with a sterile loop or sterile toothpick, and spread onto a new agar plate.
9. Label the plates.

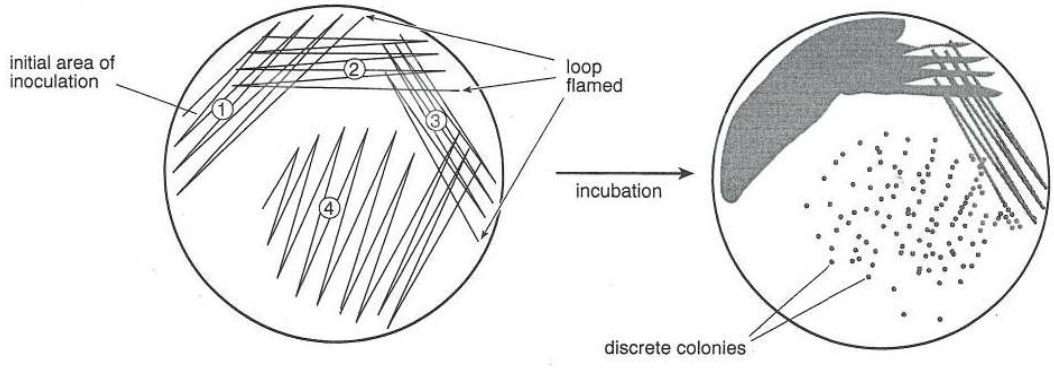

Fig. B

HEADING: 4.4. Bacterial Identification	SUBHEADING: 4.4.3. Bacterial Isolation by Pouring
DATE:	AUTHORIZATION:
COMMENTS:	

Scope: Isolation of pure bacterial cultures by the pour plate method

Equipment and Materials

- Nutrient agar (prepared, sterilized, and cooled to 45°C)
- Test tubes (sterile)
- Petri dishes (sterile)
- Inoculating loop (sterile) or toothpicks (sterile)
- Agar plates (prepared)

HEADING: 4.4. Bacterial Identification	SUBHEADING: 4.4.3. Bacterial Isolation by Pouring
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. Inoculate a dilution of the mixed culture into a test tube containing melted agar that has been cooled to 45°C.
2. Mix well to disperse the bacteria throughout the medium.
3. Pour the mixture into a sterile Petri dish, and allow it to solidify. Then go to 4 below.

Alternative Procedure

1. Place the inoculum in an empty sterile Petri dish.
2. Pour sterilized and cooled medium over the inoculum.
3. Swirl the dish to mix before the medium solidifies.

4. Incubate for 48 hours.
5. Colony growth takes place within the medium and also on the surface (Fig. C).
6. Pick up single colonies for identification with a sterile loop or toothpick, and spread onto a new agar plate.
7. Label the plates.

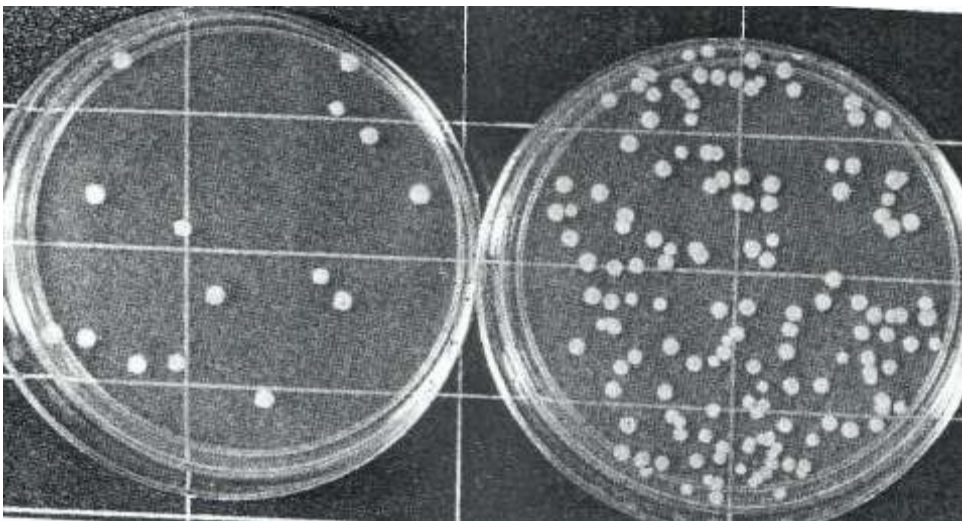

Fig. C

HEADING: 4.4. Bacterial Identification	SUBHEADING: 4.4.4. Gram Staining
DATE:	AUTHORIZATION:
COMMENTS:	

Scope: Identification of Gram-positive and Gram-negative bacteria

Equipment and Materials

- Bunsen burner
- Microscope slides (alcohol-cleaned)
- Compound microscope
- Crystal violet solution - solution 1
- Lugol's solution - solution 2
- Decolourizing solution - solution 3+4
- Safranine solution - solution 5
- Immersion oil
- Washbottle (with distilled water)

HEADING: 4.4. Bacterial Identification	SUBHEADING: 4.4.4. Gram Staining
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

Prepare a Slide Smear:

1. Record the reference number of the sample (a pure culture incubated for 24 hours in a Petri dish) on the side of the slide.
2. Place a drop of water on the slide.
3. Aseptically transfer a minute amount of pure colony from a Petri dish (sections 4.4.1., 4.4.2. or 4.4.3.) with a flamed loop, and then distribute the specimen.
4. The smear should be thin enough to dry completely within a few seconds.
5. After drying in air, heat-fix the smear by slowly drawing it two or three times through the upper portion of a Bunsen flame.
6. Let the slide cool.

Gram Staining:

7. Completely cover the smear on the slide with solution 1, and stain for 1 minute.
8. Gently rinse with water.
9. Completely cover the smear on the slide with solution 2, and stain for 1 minute.
10. Gently rinse with water.
11. Add decolourizing solution (either 3 or 4), one drop at a time, until the blue–violet colour of the specimen is no longer seen. **Using too much solution could result in a false Gram(-) result, and not enough solution may yield a false Gram(+) result.**
12. Gently rinse with water for 5 seconds.
13. Completely cover the smear on the slide with solution 5, and stain for 1 minute.

HEADING: 4.4. Bacterial Identification	SUBHEADING: 4.4.4. Gram Staining
DATE:	AUTHORIZATION:
COMMENTS:	

14. Gently rinse with water.
15. Let the slide dry.
16. Examine the slide with a compound microscope, using high power with immersion oil.
17. Record the appearance of the bacteria (rods – long thin bacteria, cocci – circular bacteria, or filamentous), and the results of the Gram staining:
    Blue-violet/dark blue - Gram-positive
    Pink to red - Gram-negative

HEADING: 4.4. Bacterial Identification		SUBHEADING: 4.4.5. Catalase Test
DATE:		AUTHORIZATION:
COMMENTS:		

Scope: Determination of the catalase reaction for Gram(+) bacteria

Equipment and Materials

- Slides (glass)
- Catalase reagent
- Inoculating loop (sterile)

HEADING: 4.4. Bacterial Identification		SUBHEADING: 4.4.5. Catalase Test
DATE:		AUTHORIZATION:
COMMENTS:		

Procedure

Slide Test:
1. Put one drop of Catalase reagent on a slide.
2. Pick up a colony with the sterile loop.
3. Smear the colony on the slide.

OR

Direct Test on Culture Medium:
1. Put one drop of Catalase on the colony.

Then:
Observe and record the immediate reaction:
- Immediate release of bubbles of oxygen - Catalase-positive
- No reaction - Catalase-negative

HEADING: 4.4. Bacterial Identification	SUBHEADING: 4.4.6. Oxidase Test
DATE:	AUTHORIZATION:
COMMENTS:	

Scope: Determination of the oxidase reaction for Gram(-) bacteria

Equipment and Materials

- Oxidase reagent
- Inoculating loop (sterile)
- Swab (sterile)
- Filter paper (sterile)

HEADING: 4.4. Bacterial Identification	SUBHEADING: 4.4.6. Oxidase Test
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. Touch a colony with a sterile swab.
2. Put a drop of oxidase reagent onto the swab.

OR

1. Place a colony onto sterile filter paper.
2. Put a drop of oxidase reagent onto the filter paper.

Then:

Observe and record the reaction:

- Development of a violet to purple colour
  - Oxidase-positive
- No reaction - Oxidase-negative

HEADING: 4.4. Bacterial Identification	SUBHEADING: 4.4.7. Use of API Kits
DATE:	AUTHORIZATION:
COMMENTS:	

Scope: Identification of pure bacterial cultures

Equipment and Materials

- Analytical Profile Index (API) strip
- Incubation box
- Ampoule of API medium
- Results sheet
- Reagents:
  - Different reagents are required for different API strips
- Pipettes
- Ampoule rack
- Ampoule protector
- Mineral oil
- McFarland Standard (according to kit)
- Analytical Profile Index (API) strip or identification software
- Reading Table
- General microbiology laboratory equipment

HEADING: 4.4. Bacterial Identification	SUBHEADING: 4.4.7. Use of API Kits
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

Preparation of the Strip:

1. Using an incubation box (tray and lid), distribute about 5 ml of distilled water in the tray to create a humid atmosphere.
2. Record the reference number of the sample on the elongated flap of the tray.
3. Remove the API strip from its individual packaging.
4. Put the strip in the incubation box.
5. Place the incubation box on top of the lid in such a way that it tilts the strip slightly forward.

Preparation of the Inoculum:

6. Obtain a pre-prepared pure culture on a blood-agar Petri dish of about 18 to 24 hours with the above mentioned reference number (sections 4.4.1., 4.4.2. and 4.4.3.)
7. Open an ampoule of the appropriate API medium (e.g. API Staph).
8. Pick up a few colonies with a swab and mix well into the medium to get a homogenous bacterial suspension.
9. Compare with the respective McFarland Standard (i.e. 0.5 McFarland for API Staph).

Inoculation of the Strip:

10. Using a pipette or PSIpette, fill the microtubes (only tube portion of the microtubes, not the upper part) with the inoculated API medium (i.e. API Staph medium).
11. To avoid the formation of bubbles inside the tubes, place the tip of the pipette or PSIpette against the side

HEADING: 4.4. Bacterial Identification	SUBHEADING: 4.4.7. Use of API Kits
DATE:	AUTHORIZATION:
COMMENTS:	

of the microtubes.

12. Fill the cupules of the Underlined tests (ADH and URE in API Staph) with mineral oil to ensure anaerobiosis.
13. Close the incubation box.
14. Incubate at 37°C for 24 hours.

Reading the Strip:

15. After the incubation period, develop the reactions by adding one drop of each required reagent, and then read all the reactions by referring to the Reading Table.
16. Record the results on the results sheet.
17. On the results sheet, the tests are separated into groups of three, and a value of 1, 2 or 4 is indicated for each. Add together the values corresponding to positive reactions within each group to obtain the 7-digit profile number.
18. In the Analytical Profile Index (API), find the numerical profile in the list of profiles.
    OR
19. Using the identification software, enter manually (via the keyboard) the 7-digit numerical profile.

4.5. Protocol for Tsetse Diet Preparation

As described in sections 4.1., 4.2. and 4.3., blood is collected from slaughtered cattle in an abattoir, stored in 5-l cans, frozen and bulk-sterilized. However, the blood in one can (taken directly from the abattoir) is tested for microbial contamination (based on a 1-ml sample, and incubating the culture at 37ºC for 24 hours), portioned into 15-ml aliquots in 20 sterile vials, irradiated (at 1 kGy in a gamma cell), and then tested again (procedures to identify bacteria are described in section 4.4.). If, evidently, the blood is satisfactory, blood from this batch is used in a 25-day feeding and quality factor (QF) test (section 4.3.3.) to confirm that the blood is of a good quality.

When blood will be needed within the next few weeks to feed flies, and provided that the batch of blood is of a good quality, an appropriate number of 5-l cans of frozen blood are brought into the cool room and stored at +4ºC for a few days. This blood is not thawed rapidly (as was once done under running cold water) because apparently rapid thawing reduces the quality of the blood. Instead, the blood is transferred from -20ºC frozen storage and kept at +4ºC in a refrigerator for several days, allowing the blood to thaw slowly.

When thawed, samples of the blood are checked again for microbial contamination. Most of the blood is poured into 1-l glass bottles (each filled only half full) for refreezing and storage at -20ºC until needed to feed flies. When needed, the blood is again thawed (slowly) at +4ºC, and the blood is then ready to be fed to flies (sections 3.2.2. and 3.2.3.).

HEADING: 4.5. Protocol for Tsetse Diet Preparation	SUBHEADING: 4.5.1. Thawing and Blood Portioning
DATE:	AUTHORIZATION:
COMMENTS:	

Scope: Blood preparations done between blood collection and readying blood to feed flies

Equipment and Materials

- Beaker (glass, 5-l volume)
- Funnels (metal, large and small)
- Sieve (fits into large funnel)
- Bottles (1-l, glass, sterile) and rubber stoppers
- Bleach solution (5 parts commercial bleach, e.g. Clorox, and 95 parts water, v/v)
- UV lamp (in a sterilization room)
- Agar (nutrient)
- Autoclave
- Boxes (plastic, for storage of bottles with blood)
- Syringe (1 ml) and needle (disposable)
- Petri dishes (sterile, for agar and blood)
- Bunsen burner
- Laminar-flow bench (hood)
- Water bath (held at 40ºC)
- Heat-sterilizing oven (dry heat)
- Distilled water

HEADING: 4.5. Protocol for Tsetse Diet Preparation	SUBHEADING: 4.5.1. Thawing and Blood Portioning
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. Estimate the amount of blood needed in the next few weeks.
2. Transfer an appropriate number of frozen 5-l cans of blood to a refrigerator to thaw SLOWLY at +4ºC. For example, do this on a Friday, planning to process some of the cans of blood on the following Tuesday, and the remaining cans on Thursday.
3. For cans stored at +4ºC until Tuesday, do not place the cans tightly together, permitting them to thaw. For cans stored at +4ºC until Thursday, place the cans together tightly (slows down the rate of thaw) until Monday, but on Monday separate them so that they thaw more quickly. The blood must be completely thawed when the next step in processing begins.
4. The 1-l glass bottles must be sterile. The day before they are to be half-filled with blood, clean and sterilize the bottles — wash, rinse with distilled water, and sterilize overnight in the heat-sterilizing oven (160ºC, hot dry air).
5. The rubber stoppers and sieve must also be sterile. Clean, rinse in distilled water, soak for 1 hour in bleach solution for disinfection, and place them overnight in a UV-room for sterilization.
6. Autoclave the nutrient agar solution for 15 minutes at 121ºC, and then put it in a water bath at 40ºC. Use the agar to check for microbial contamination in 1-ml samples of the blood taken during pouring (see below, and section 4.3.1. for details on the procedure). **The results of this check must be known before any of the blood is fed to flies.**

HEADING: 4.5. Protocol for Tsetse Diet Preparation	SUBHEADING: 4.5.1. Thawing and Blood Portioning
DATE:	AUTHORIZATION:
COMMENTS:	

7. Pour blood from a 5-l can into a large (5-l) glass beaker through a large metal funnel with a sieve. The sieve removes any fibrin or other solid particles in the blood.
8. During this pouring of the blood, with the help of another worker, take two 1-ml samples of blood with a sterile syringe and needle, one sample at the beginning of the pouring process, and the second sample in the middle. Using agar, conduct tests for microbial contamination (see above, and section 4.3.1.). **Each 5-l can of blood is tested.**
9. The actual partitioning of blood is done in a laminar-flow bench (hood) (which during the previous night had a UV lamp turned on to sterilize the interior); turn off the UV lamp but keep the clean air flowing. Working with your hands, and all blood and equipment, inside the bench, pour blood from the 5-l glass beaker into 10 1-l sterile glass bottles, filling each bottle only half full. Close each bottle with a sterile rubber stopper, and label each bottle with the batch number and date.
10. Place the half-filled bottles on their sides (horizontally) into plastic boxes, and transfer the boxes to a deep-freeze kept at -20ºC.
11. Provided that the blood is shown to be sufficiently free of microbial contaminants, these bottles of blood (about 0.5 l of blood in each bottle) are ready to use in feeding flies. The next section (section 4.5.2.) describes the procedure for the final preparation of blood used to feed flies.

HEADING: 4.5. Protocol for Tsetse Diet Preparation	SUBHEADING: 4.5.2. Preparing Bottles of Blood for Feeding
DATE:	AUTHORIZATION:
COMMENTS:	

Scope: Final preparation of blood before being used to feed flies

Equipment and Materials

- Blood (in 1-l glass bottles, half-filled, and frozen at -20ºC in a deep-freeze) (this blood has been checked twice for microbial contamination)
- Refrigerator (at +4ºC)

HEADING: 4.5. Protocol for Tsetse Diet Preparation	SUBHEADING: 4.5.2. Preparing Bottles of Blood for Feeding
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. Based on the blood requirements in 2 or 3 days, transfer the required number of glass bottles of frozen blood from the deep-freeze to a refrigerator at +4ºC, allowing the blood to thaw SLOWLY for 48–72 hours. (The blood in a bottle should be completely thawed after 48–72 hours at +4ºC.)
2. When the blood has thawed, it is ready to use in feeding flies. However, keep the bottles of blood at +4ºC until the blood is actually needed to pour onto feeding trays (and under feeding membranes) (sections 3.2.2. and 3.2.3.).
3. If thawed blood is not used, it can be stored for a maximum of 3 days at +4ºC. If, after 3 days, this blood has still not been used, discard it.
4. If some of the thawed blood in a bottle is used to feed flies but the remainder is not used, this unused blood can be stored at +4ºC **in a separate place** for up to 3 days. Discard the blood if it is not used within these 3 days.

4.6. Shipment of Blood

The increased demand for applying the SIT against tsetse flies may result in tsetse production facilities being located in regions that lack a suitable local source of blood, and therefore requiring that blood be shipped to them. Prior to shipment, the blood must be decontaminated and tested for suitability as a tsetse diet. A radiation source is necessary for decontamination, and a tsetse colony is required for the 25-day feeding test (section 4.3.3.) to determine the nutritional quality of the blood. The 25-day test involves feeding a sample of newly emerged flies on the same batch of blood for 25 days, and monitoring their survival and productivity. Once the blood has been found to be suitable for feeding colonies of flies, it can be supplied to rearing centres either by air (small quantity) or shipped in a refrigerated container. Prior to shipment, shipping arrangements are made between the consignor and the consignee. Once a date has been agreed upon, the authorization documents are prepared by the importing country and made available to the consignor, and a veterinary certificate is obtained by the consignor.

HEADING: 4.6. Shipment of Blood	SUBHEADING: 4.6.1. Shipment of Blood
DATE:	AUTHORIZATION:
COMMENTS:	

Scope: Shipment of quality-tested decontaminated blood to tsetse rearing facilities

Equipment and Materials

- Container and carrier (to transport blood in a frozen state)
- Pro forma invoice
- Packing list
- Declaration letter “TO WHOM IT MAY CONCERN” describing shipping intentions and the content of the package
- Veterinary certificate signed by the veterinarian in charge of the district in which the blood was collected (certificate includes statements on notifiable Office International des Épizooties (OIÉ) diseases)
- Air waybill number
- Acceptance letter by the carrier with a guarantee of storage in a frozen state at destination
- Confirmation of availability of container and timing of shipment
- Import licence or authority to import blood
- Blood (fresh, deep-frozen, decontaminated)
- Styrofoam®
- Crates (plastic)
- Boxes (cardboard)
- Dry ice (for air shipment)

HEADING: 4.6. Shipment of Blood	SUBHEADING: 4.6.1. Shipment of Blood
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. Collect the required volume of blood and store in 5-l cans (sections 4.1. and 4.2.).
2. Determine the date of shipment by consignee.
3. Arrange with the carrier for collection and delivery of consignment to destination.
4. From the consignor, arrange documents to accompany the consignment.
5. From other sources, collect information to guarantee transportation of blood in a frozen state.
6. From the consignee, obtain documents pertaining to authorization to import blood.
7. Irradiate blood in a frozen state immediately before packing.
8. Using blood from one of the 5-l cans, conduct bacterial screening to confirm decontamination (section 4.3.1.), and record the results.
9. For shipment by air, pack the frozen blood in cardboard boxes insulated with 3-cm-thick Styrofoam® sheets.
10. If using a box with a capacity of 5–6 5-l cans, add 3–4 kg of dry ice.
11. On special request, 1-l plastic bottles are packed in the same way, but each are filled with only 700 ml of blood.
12. To avoid leakage, waterproof plastic crates may be used, but this increases the weight and cost of the consignment.
13. If shipment is in a refrigerated container, the frozen 5-l cans are strapped to a palette, and each palette is wrapped with strong plastic foil to allow the unit to be

HEADING: 4.6. Shipment of Blood	SUBHEADING: 4.6.1. Shipment of Blood
DATE:	AUTHORIZATION:
COMMENTS:	

handled by a forklift.

14. Fill the free space between the stacks with loose 5-l cans.
15. In both types of shipment, preparation for shipment and documentation are the same.

4.7. Reconstitution of Freeze-Dried Blood

The lack of a reliable and adequate supply of blood has led to the development of a procedure to extend the shelf-life of blood and make it easy to transport and store — by freeze-drying or lyophilization (Wetzel 1980). The blood is processed by the basic freeze-drying technique. Since it is highly hygroscopic, after freeze-drying the blood is immediately packed, sealed and stored in a dry place. Freeze-dried blood can be reconstituted and processed in the same way as fresh frozen blood for feeding tsetse flies. At the time of reconstitution, sterile glassware and sterile conditions must be maintained.

HEADING: 4.7. Reconstitution of Freeze-Dried Blood	SUBHEADING: 4.7.1. Reconstitution of Freeze-Dried Blood
DATE:	AUTHORIZATION:
COMMENTS:	

Scope: Reconstitution of freeze-dried blood

Equipment and Materials

- Laminar-flow bench (hood)
- Bunsen burner
- Flat-bottom flask (sterile)
- Magnetic stirring rod (sterile)
- Magnetic stirrer
- Balance (top-loading)
- Foil (aluminium)
- Distilled water (sterile)
- Blood (freeze-dried)
- Osmometer (semi-micro)
- pH meter
- Bottles (sterile, for storage of reconstituted blood)

HEADING: 4.7. Reconstitution of Freeze-Dried Blood	SUBHEADING: 4.7.1. Reconstitution of Freeze-Dried Blood
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. Weigh a sterile flat-bottom flask into which a sterile magnetic stirring rod has been inserted.
2. Add the freeze-dried blood.
3. For practical reasons, the volume to be mixed should not exceed 5 l.
4. Record the weight of the added freeze-dried blood.
5. Add (only a small amount at a time) the equivalent of 4.3 w/w of sterile distilled water to the freeze-dried blood in the flask.
6. Cover the flask with aluminium foil, and shake gently to wash down all particles adhering to the wall of the flask.
7. Transfer the flask to the magnetic stirrer, and switch it on.
8. Adjust the speed so that foam is not produced.
9. Continue mixing until a homogenous liquid is formed (a volume of 1 l of blood takes approximately 20 minutes to mix).
10. Check and adjust the pH and osmotic pressure to that of fresh blood (range pH 7.4–7.9 and osmotic pressure 230–290 mOsmol). Discard if a reading falls outside the range.
11. Dispense in required quantities, preferably inside a laminar-flow bench.
12. Label with batch number, date of collection of batch, and date of reconstitution.
13. Irradiate and treat as fresh frozen blood.
14. Wash and sterilize all equipment used (section 4.1.1.).

5. Fly Production Quality Control (QC)

Tsetse fly production is a labour-intensive operation, and therefore, to assure the product (excess pupae), the need to control aspects of production cannot be underestimated. Data must be collected on the vital parameters — mortality, fecundity, and the number and quality of pupae. Colony parameters are influenced by environmental factors, e.g. holding and incubation conditions, methods of fly manipulation, nutrition, and sources of toxicity and microbial contamination. Quality-control procedures include measures to achieve optimal rearing conditions at all times.

High fecundity and low mortality is correlated with pupal size and weight. Very small and light pupae result in a low emergence rate, and a low number of strong and viable flies. The size and weight of pupae are a reflection of the maintenance and feeding of the female fly, and of her ability to transfer the nutrients to her offspring.

Quality-control parameters and procedures have been discussed by Feldmann (1994b), FAO/IAEA/USDA (2003) and Calkins and Parker (2005).

5.1. Survival/Mortality and Stress Tests

The survival of flies in a colony varies according to species, and may also reflect the degree of adaptation of the species to the rearing conditions. Mortality is expressed as “percentage per day”, reflects an overall picture of the colony, and encompasses all age structures. This is necessary because mortality is age dependent, and should not exceed 1.2%/day. The distribution of mortality is recorded in different categories. Mortality could be due to starvation, blood in the abdomen, pupa *in utero*, etc. Classifying the mortality into different categories, and evaluating the mortality pattern, should facilitate tracing and eliminating the source of the problem. Each category indicates specific problems, and if the numbers are exceptionally high, the origin of the problem should be investigated. Dead flies should be removed from cages and counted about 10–14 days after a unit is formed. These data reveal mortality resulting from early fly handling. Mortality should be determined every 1 or 2 weeks. However, if mortality is high, the interval between mortality checks should be decreased to reduce contamination arising from flies that die with blood in the abdomen.

The longevity of adults under stress without blood is an indicator of fly quality. Tests are routinely conducted to determine the nutrient reserves available to the fly at the time of emergence. The result of the test is an indicator of the nutritional quality of the diet, and of the environment that affects the ability of the fly to store fat reserves during development and sustain the longevity of the adult fly.

HEADING: 5.1. Survival/Mortality and Stress Tests	SUBHEADING: 5.1.1. Survival/Mortality Test
DATE:	AUTHORIZATION:
COMMENTS:	

Aim: To determine the survival of flies in a tsetse colony

Equipment and Materials

- Trays (plastic)
- Forceps
- Cages of flies

HEADING: 5.1. Survival/Mortality and Stress Tests	SUBHEADING: 5.1.1. Survival/Mortality Test
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. Familiarize yourself with the following terminologies:
   a. **Starvation**: Dead females, with no undigested blood in the abdomen, could be due to: holding conditions that are too warm or too dry, unprotected direct exposure to fans, too low relative humidity in the feeding room, feeding regimen inadequate, diet not attractive, or the feeding unit not functioning. Low relative humidity is associated with increased starvation.
   b. **Blood mortality**: Dead females, with a large amount of undigested blood in the abdomen, could be due to: relative humidity too high or insufficient aeration in the breeding room, excessive diet ingestion, *in vitro* feeding membranes too hot, toxicity or microbial contamination.
   c. **Pupal mortality**: Dead females with larva that pupated *in utero* due to either toxicity to the female in the very late stage of pregnancy or too low relative humidity in the holding room.
   d. **Other mortality**: Due to other causes not included in the categories above.
2. Remove dead flies from cages at 10–14 days after the formation of a unit.
3. Sort dead flies according to the type of mortality.
4. Count dead flies and calculate the mortality rate.
5. At regular intervals, remove/sort dead flies and calculate the mortality rate. Increase or decrease the interval between mortality checks according to the mortality rate in the colony.
6. Calculate the mortality rate for all units in the colony

HEADING: 5.1. Survival/Mortality and Stress Tests	SUBHEADING: 5.1.1. Survival/Mortality Test
DATE:	AUTHORIZATION:
COMMENTS:	

(section 3.6.) and record.

HEADING: 5.1. Survival/Mortality and Stress Tests	SUBHEADING: 5.1.2. Stress Test
DATE:	AUTHORIZATION:
COMMENTS:	

Scope: A relative measure of nutrient reserves available to the adult fly at the time of emergence

Equipment and Materials

- Holding room
- Holding cages (polyvinyl chloride (PVC))
- Newly emerged unfed flies
- Separating tubes

HEADING: 5.1. Survival/Mortality and Stress Tests	SUBHEADING: 5.1.2. Stress Test
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. Put 30 female and 30 male recently emerged flies into a holding cage. Repeat five times, making five replicates.
2. Do not feed the flies.
3. Keep cages in the holding room at 23–24°C and 75–80% RH.
4. Each day remove and count the number of flies that have died, and record.
5. Based on the number of dead flies, calculate the average length of life (compare with the female's normal maximum lifespan of 100–120 days; section 1.2.).
6. When 50% of the flies are dead, the test is terminated.
7. The result is expressed in "number of days to 50% mortality".

5.2. Fecundity

Tsetse flies reproduce by adenotrophic viviparity (section 1.2.); each female produces a fully grown larva every 9–10 days, starting 19–20 days after emergence. Tsetse flies as a whole have a very low reproductive capacity. To maintain a colony at a constant size, each female added to the colony should produce at least three pupae during her lifetime (section 1.2.), provided the quality control (QC) parameters, e.g. portion of A-class pupae and emergence rate, are at acceptable levels. The minimum average fecundity of a colony should be 0.6 pupae per female per 10 days (0.6 P/F/10d) (sections 1.2., 3.6. and 5.2.1.). The ovulation and larviposition cycle of female tsetse flies varies under the usual holding conditions, and is between 8 and 10 days. The "follicle next in ovulation sequence'' (FNOS) is defined in the glossary (Annex 6) (Saunders (1961), Challier (1965), Van der Vloedt et al. (1978), and Vreysen et al. (1996).

At holding conditions of 23–24°C, the onset of production is at 18–19 days. If the onset of production is delayed and/or abortions are detected, the causes should be identified. A few females (fresh, not preserved) should be dissected and their spermathecae examined (Fig. 23). A drawing of the female reproductive system is shown in Fig. 24 (Pollock 1982). On pages 133–134 of Pollock (1982), instructions are given on how to dissect the female reproductive system.

The spermathecal value (SV) of females (measured after sperm transfer) is the proportion of the spermathecae that is filled with sperm after mating. After dissection of females in insect saline to estimate the volume of sperm plus spermathecal fluid in the spermathecae, each spermatheca is ranked as empty (0), one-quarter full (0.25), one-half full (0.5), three-quarters full (0.75), or full (1). The value for one spermatheca is added to the value for the other, giving a final SV. The maximum SV of one female adult is 2.0 (Nash 1955). The minimum acceptable SV is 0.5.

If the mean spermathecal value is low, the mating regimen should be reviewed, and the quality of males confirmed by dissection and examination of the testes for sperm mobility and the size of the accessory glands. The quality and quantity of offspring are other indicators. Pupae are collected daily and quality-controlled by either weight or a calibrated pupal size-sorting machine (section 5.3.). Also the emergence rate (%) should be determined (section 5.4.1.).

Fig. 23. Dissecting a tsetse adult using a stereoscropic microscope and dissection tools.

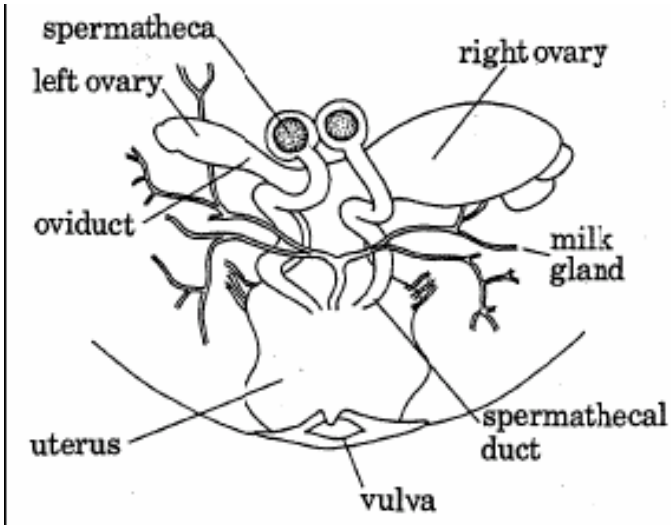

Fig. 24. Drawing of the tsetse female reproductive system, showing the two spermathecae. (Drawing from Pollock (1982), reproduced with permission.)

HEADING: 5.2. Fecundity	SUBHEADING: 5.2.1. Fecundity
DATE:	AUTHORIZATION:
COMMENTS:	

Aim : To determine the fecundity of a tsetse fly colony

Equipment and Materials

- Compound microscope
- Stereomicroscope
- Dissecting instruments
- Cover slips
- Microscope slides (glass)

HEADING: 5.2. Fecundity	SUBHEADING: 5.2.1. Fecundity
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. Collect pupae from the colony units, and record (section 3.3.1.)
2. Sort and quality-control the pupae (section 5.3.).
3. Using the number of pupae collected during the week, calculate the number of pupae produced per female per 10 days (P/F/10d) (see formula below).
4. If the onset of production is later than 19 days post-emergence, check the holding conditions.
5. Check for abortions of eggs and larvae on the sloped sheets below the fly cages.
6. If there is no indication of abortions, review the mating regime.
7. A review of the mating regime involves checking the ratio of males to females, the timing of mating, and the age at which females are most receptive to mating.
8. Confirm that males used for mating are mature.
9. Dissect a few males, and examine for sperm mobility in the testes.
10. Assess the developmental size of male accessory glands.
11. If results indicate poor male quality, check and revise the male-handling procedure and pupal-incubation conditions.

The fecundity of each female unit is determined as follows:

$$P/F/10d = \frac{\text{No. pupae produced during the previous week} \times 10}{\text{No. females surviving the previous week} \times 7}$$

5.3. Pupal Weight and Size

The optimal performance of the female tsetse, high fecundity and low mortality are correlated with pupal size and weight. The emergence rate and the number of strong viable flies are lower from very small and light-weight pupae than from normally developed pupae. Pupal size is measured by weighing pupae and measuring the diameter of pupae. Unequal rates of water loss at different facilities make sorting by diameter a preferred means of estimating the size distribution of a sample of pupae. However, both methods should be used for quality control. The size/weight of pupae is therefore an important quality-control parameter of offspring. It also reflects the maintenance and feeding of the female adult, and the transfer of nutrients from the female to a developing larva in the uterus.

The handling of pupae after collection depends on the colony size and the number of pupae produced. For a colony producing up to 50 pupae per day, pupae are weighed individually and the weights recorded. For larger colonies producing up to 1500 pupae per day, the pupae are either weighed together or are sorted by size and the size-groups are weighed separately. A downward trend in the mean size of pupae produced by a facility can arise from poor nutrition. There is a high probability that a small pupal size will result in poor performance of the offspring.

Pupal weight is influenced by the quality of diet, holding conditions and general rearing operations. Pupae lose weight throughout their development, with most of the weight being lost during the first few days after pupation. The rate of water loss by pupae at different facilities depends on the temperature and relative humidity of the holding conditions. Therefore, it would not be possible to compare pupal weight data between rearing facilities without stating the maintenance conditions. The age at weighing is critical, so if the data are to be meaningful, weighing should be done at the same time and under the same conditions. It is important that the lot of pupae is counted accurately. The use of electronic counters is not recommended for the determination of mean pupal weight.

Sorting by size or diameter gives better information on the size distribution of pupae. Tsetse pupae are sorted into five distinct size classes. The pupal size-sorting machine (Zelger and Russ 1976) is calibrated for each species of tsetse (Table 1), and separates pupae into five classes, with A-class the smallest and E-class the largest (Fig. 25). Special steel calibration sets, to calibrate the machine, are available from the FAO/IAEA Laboratory in Seibersdorf, Austria.

It is important that the fraction of A-class pupae, the smallest pupae, does not exceed 10%.

Table 2 shows the weight classes for different species of *Glossina*.

An example of the distribution of pupae by class is shown in Fig. 26.

Fig. 25. Pupal size-sorting machine.

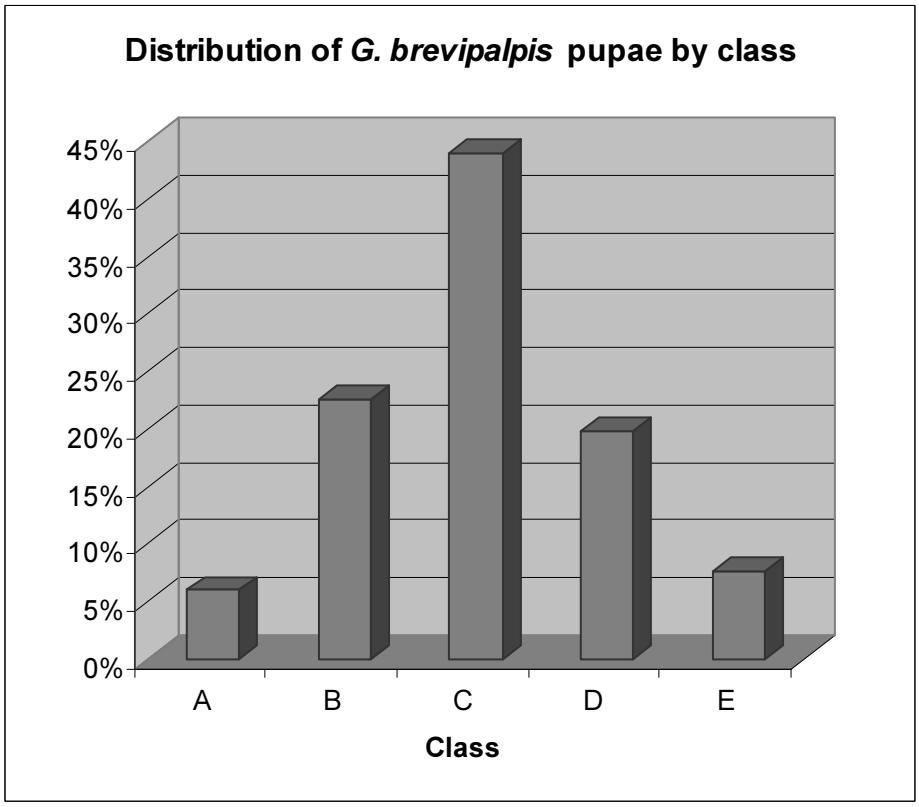

Fig. 26. Distribution of pupae by class.

Table 1. Calibration of pupal size-sorting machine — distance of sorting roll bars adjusted by means of a feeler-gauge (mm)

Species	At the beginning of Class A	At the end of Class E
Glossina pallidipes	2.50	3.60
Glossina austeni	2.30	3.00
Glossina morsitans	2.20	3.20
Glossina fuscipes	2.60	3.25
Glossina brevipalpis	3.50	4.25

Table 2. Definition of pupal weight classes for different species of *Glossina* spp.[1]

	Weight Class (mg)				
Species	A	B	C	D	E
G. austeni	<16	16– <19	19– <21	21– <23	>23
G. tachinoides	<14	14– <17	17– <19	19– <21	>21
G. palpalis	<22	22– <28	28– <32	32– <36	>36
G. pallidipes	<23	23– <29	29– <33	33– <37	>37
G. fuscipes	<22	22– <28	28– <32	32– <36	>36
G. brevipalpis	<56	56– <68	68– <76	76– <84	>84
G. morsitans	<18	18– <22	22– <26	26– <30	>30

[1] Based on weight-frequency distribution by means of the pupal size-sorting machine developed by Zelger and Russ (1976).

HEADING: 5.3. Pupal Weight and Size	SUBHEADING: 5.3.1. Weighing Pupae
DATE:	AUTHORIZATION:
COMMENTS:	

Aim: To obtain an accurate estimation of the mean weight of pupae by weighing a sample of pupae

Equipment and Materials

- Balance (with accuracy of ±1 mg or better)
- Forceps (soft, for handling pupae)
- Pupal-counting machine (optional)
- Dishes (polyvinyl chloride (PVC))
- Pupae (24 hours after collection)

HEADING: 5.3. Pupal Weight and Size	SUBHEADING: 5.3.1. Weighing Pupae
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. In the morning, collect larvae and pupae.
2. Transfer pupae to medium-size dishes marked according to the experimental or colony unit.
3. Allow larvae to pupate and melanize, and pupae to harden, in the incubation room.
4. Tare the balance.
5. From a sample of 1-day-old pupae, remove all trash, aborted larvae and any malformed pupae.
6. Count the number of pupae, and record.
7. Weigh the pupae together, and calculate the average weight per pupa.
8. For small colonies, individually weigh the pupae, and record.
9. Return pupae to the incubation room.
10. Calculate the mean weight, and standard error.

HEADING: 5.3. Pupal Weight and Size	SUBHEADING: 5.3.2. Sorting Pupae by Diameter
DATE:	AUTHORIZATION:
COMMENTS:	

Aim: To obtain an accurate estimation of the mean size of pupae by measuring the diameter of a sample of pupae

Equipment and Materials

- Pupal size-sorting machine (Fig. 25)
- Dishes (marked for collecting pupae from each class)
- Petri dishes (for holding pupae)
- Trays (plastic)

HEADING: 5.3. Pupal Weight and Size	SUBHEADING: 5.3.2. Sorting Pupae by Diameter
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. Calibrate the pupal size-sorting machine using a steel calibration set (23 pieces ranging in size from 2.2 to 4.4. mm). (The size settings are shown in Table 1.)
2. Run a sample of pupae through the machine.
3. When all the pupae have passed through, empty each batch into the marked dish corresponding to the mark on the collector.
4. Count the number of pupae in each class, and record.
5. Weigh the pupae in each class.
6. Calculate the percentage pupae in each class.
7. Draw a graph showing the distribution (e.g. Fig. 26).
8. The % A-class pupae should not exceed 10% of total pupae produced.
9. Return the pupae to the incubation room.
10. For each class of pupae, calculate the mean weight, and standard error, in mg.

5.4. Adult Emergence

The pupal period is temperature dependent. The mean pupal period of many tsetse species (incubation at 23–24ºC) is about 34 days, with the peak in female emergence occurring at 32 days after pupation. If amongst non-eclosed pupae a high percentage of late-stage males and females failed to emerge, it could have been caused by a low relative humidity during the late phase of incubation.

A significant deviation in the 1:1 sex ratio in the fly population may provide an early indication that there is a problem in the rearing system. The problem could be of a genetic nature or possibly a procedural effect. When the sex ratio deviates from an established standard, a review of the production process should be made. Each month the test for sex ratio is done on a sample of pupae.

HEADING: 5.4. Adult Emergence	SUBHEADING: 5.4.1. Adult-Emergence Rate
DATE:	AUTHORIZATION:
COMMENTS:	

Scope: To obtain accurate estimates of the percentage of pupae that will produce adults

Equipment and Materials

- Emergence cages
- Petri dishes
- Chiller (adapted for chilling tsetse adults)
- Forceps (soft)

HEADING: 5.4. Adult Emergence	SUBHEADING: 5.4.1. Adult-Emergence Rate
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. While in the incubation room (under the conditions appropriate to the tsetse species), once each week put 100 25-day-old pupae into a Petri dish under an emergence cage.
2. When adults start to emerge, remove the emergence cage and place it in a chiller.
3. Cover the Petri dish containing the remaining non-eclosed pupae.
4. When the adults are immobilized, pour them onto the cold surface.
5. Sort adults by sex, and count the number of adults.
6. Record the number of normal and deformed (crippled wings) adults.
7. Each day sort and count emerged adults until no more adults emerge from the batch of pupae.
8. Examine the remaining pupae, and make a note if there are any with partially emerged adults.
9. Calculate the emergence rate.
10. Calculate the mean pupal period of males and females.
11. If the emergence rate is below 85%, dissect all non-eclosed pupae, and record according to the following categories:
    a) Lysed pupal stage
    b) Early stage when there is no melanization
    c) Red-eye stage when only eyes are melanized
    d) Fully pigmented females or males but with cuticle still wet
    e) Males or females ready to emerge with dry cuticle
12. The results will show when development stopped, and indicate the likely causes of death.

HEADING: 5.4. Adult Emergence	SUBHEADING: 5.4.1. Adult-Emergence Rate
DATE:	AUTHORIZATION:
COMMENTS:	

Calculations:

$$\text{Emergence rate (\%)} = \frac{\text{Total no. of adults emerged}}{\text{Total no. of pupae}} \times 100$$

$$\textbf{Pupal period (days)} = \textbf{(date of first emergence – larviposition date)} + \frac{\textbf{(date of last emergence – date of first emergence)}}{\textbf{2}}$$

[Note: The dates can be entered into an EXCEL spreadsheet (then use the formula as it stands), or the dates must be shown in terms of the number of days elapsed since a starting point, e.g. the first day of a calendar year.]

$$\textbf{Percentage females added to the colony} = \frac{\textbf{No. of females added}}{\textbf{Total no. of pupae}} \times \textbf{100}$$

HEADING: 5.4. Adult Emergence	SUBHEADING: 5.4.2. Sex Ratio
DATE:	AUTHORIZATION:
COMMENTS:	

Aim: To determine the ratio of females to males in a batch of reared adults

Equipment and Materials

- Emergence cages
- Mature pupae
- Petri dishes
- Chiller

HEADING: 5.4. Adult Emergence	SUBHEADING: 5.4.2. Sex Ratio
DATE:	AUTHORIZATION:
COMMENTS:	

Procedure

1. Place five samples of pupae, each sample with 100 25-day-old pupae, in Petri dishes under emergence cages.
2. Incubate the pupae under standard emergence conditions.
3. When adults begin to emerge, remove and sex the adults, and count the number of adults (sections 3.3.2. and 3.3.3.).
4. Continue until no more adults emerge from the batch of pupae.
5. Calculate the female:male ratio = $\frac{\text{No. of females}}{\text{No. of males}}$

Example: Female:male ratio = $\frac{52 \text{ females}}{48 \text{ males}}$ = 1.08:1

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7. Annexes

Annex 1. List of Equipment and Materials with Specifications

Annex 1.1. Standard Equipment and Materials

Agar (nutrient)
Agar plate (nutrient)
Air conditioner
Alcohol (for cleaning slides)
Animal house (for live hosts)
Autoclave
Balance (accuracy of ±1 mg or better)
Balance (top-loading)
Bottle (1 l, glass, for blood storage)
Bottle (with 20-ml calibrated dispenser)
Box (cardboard, metal, or Styrofoam®)
Box (metal, insulated)
Bunsen burner
Can (5 l, plastic, for frozen blood storage)
Cattle (for *in vivo* feeding)
Cloth (black)
Communication equipment
Container (for shipping frozen blood)
Container (polyethylene (PE))
Cool box
Cotton wool
Cover slip
Crate (plastic)
Crush (for restraining animals during *in vivo* feeding)
Cylinder (graduated)
Deep-freeze
Dishwasher
Dry ice
Elastic band (for *in vivo* feeding)
Equipment (laboratory, for microbiological techniques)
Filter paper (sterile)
Flask (flat-bottom)
Flasks (Erlenmeyer, various sizes)
Foil (aluminium)
Foil (polyethylene (PE))
Freezer (for bulk storage of blood)
Funnel
Goat (for *in vivo* feeding)
Hose (plastic)
Hose (silicone)
Hygrometer
Ice pack
Immersion oil (for compound microscope)
Laminar-flow bench (hood)
Lamp (tungsten, with photoperiod controller

Light dimmer
Lux meter
Microscope (compound)
Microscope slide (glass)
Mineral oil
Osmometer
Paper roll (used to smooth out blood poured under a membrane)
Petri dish (glass)
Petri dish (plastic)
Petri dish (sterile)
pH meter
Pipette
Rabbit (for *in vivo* feeding)
Rabbit-restraining box
Record book
Refrigerator
Saline solution (normal)
Scale (see 'balance')
Shed (protects animals from direct sunlight during *in vivo* feeding)
Sieve (coarse mesh, to separate pupae from debris during rearing process)
Sieve (flour sieve, coarse mesh, to sift sand/soil when searching for pupae)
Sieve (metal)
Sink (large, for rinsing blood from membrane and tray)
Sponge (thin sheet)
Steam cleaner
Sterilizing solution (antiseptic)
Stirrer/hot plate (magnetic)
Stirrer retriever (magnetic)
Stirring rod (magnetic)
Styrofoam®
Swab (sterile)
Syringe (1 ml, sterile, disposable) and needle
Tank (to hold blood collected in an abattoir)
Tape
Temperature and RH monitor (with visual display)
Test tube (sterile)
Thermometer (maximum/minimum)
Thermometer (range -10ºC to +50ºC)
Thermometer (thermocouple)
Timer
Toothpick (sterile)
Trap with collecting cage (to trap live flies in the field)
Tray (plastic)
Trolley (for transporting tank of blood)
Vehicle (for transporting equipment and personnel)
Vial
Vial (scintillation) (20-ml polyethylene)
Washbottle (for distilled water)
Washing machine
Water (distilled)

Annex 1.2. Special Equipment and Materials with Specifications		
Item	Specifications	Example of Supplier and/or Distributor
Analytical Profile Index (API) equipment and materials	Ampoule protector, ampoule rack, incubation box, McFarland Standard, medium, reading table, reagents for API strips, strips or identification software	Biomerieux S.A. LABSCO - Laboratory Supply Company GmbH & Co. KG
Bacterial colony-viewing/counting device	e.g. Colonicont, Schütt	LABSCO - Laboratory Supply Company GmbH & Co. KG
Blood-collection equipment	Blood-collection defibrination set, consisting of screw-capped 25-l container (polyethylene), funnel (stainless steel or plastic, upper diameter 25–40 cm) and hose (transparent silicone, autoclavable, with strong walls), paddle (stainless steel rod of 1-cm diameter on which oar-blade is mounted), and a separate electric drill (stirrer) approx 800 W, double insulated (insulation protected "schutzisoliert"), electronic adjustable and self-maintainable speed, and with (to be fitted) IP44 rubber plug (IP44 2p+E), aluminium sieve (milk sieve) with upper diameter 30–40 cm and removable sieve	Lindeberg GmbH, Prototypenfertigung & mech. Zentralwerkst.
Board with milled cavities (to hold pupae, used with SSPC) or rubber ring	Cavity or ring to fit hole in emergence cage	Local manufacture
Brush (small – used in sexing insects in chiller)	Similar to camel's hair brush	Local supply

Cage (PVC, 11-cm diameter, with netting and rubber stopper)	Round type: type SC-11, 110 x 3 x 45 mm. From PVC pipe. Hole: 26 mm. Oval type: 110 x 3 x 45 mm. From PVC pipe. Hole: 26 mm.	Schranz Walter – Tischlerei
Cage (emergence, with netting, hole and plastic cover)	200 x 4 x 100 mm, oval. From PVC pipe. Hole: 60 mm with slide closure. Netting of black polyester, fine mesh.	Schranz Walter – Tischlerei
Cage (production, self-stocking production and holding, PVC, 20-cm diameter, with netting and rubber stopper)	200 x 50 x 4 mm, with 26-mm hole in side for rubber stopper. Cage material foam core PVC water pipe. 100% polyester netting (mesh size depends on tsetse species and type of cage). Glue for cage netting, SABA S 3, (not bigger than 1 lit./tin)	Schranz Walter – Tischlerei American Scientific & Industrial Supplies
Catalase reagent		LABSCO - Laboratory Supply Company GmbH & Co. KG
Centrifuge (Micro-Haematocrit) and reader	Micro-Haematocrit Centrifuge, Hawksley model MHC, with rotor for 24 capillary tubes, cat. no. CE800-10	Labquip International Limited LABSCO - Laboratory Supply Company GmbH & Co. KG VWR International
Chiller (modified chest deep-freeze)	Tsetse fly chiller, working temperature +3°C, +/- 3 °C, modified chill-food display unit, modified to include a work platform in clear plastic, two air circulation fans and an electromechanical thermostat to replace the original digital thermostat. External dimensions length 1250 mm, width 790 mm, height 1200 mm.	Moro

Diminazene aceturate (trypanocide)	Trypanocidal drug for veterinary application, e.g. Berenil	Local supplier of veterinary drugs Intervet International Bv
Dish (for pupae)	Small, shallow, PVC	
Dissecting instruments (for dissecting insects)	Dissecting set in case, satinized, stainless steel, with incision scissors, fine pointed scissors, splinter forceps, dissecting forceps, 2 rounded scalpels, pointed scalpel, cartilage knife with scraper, double-button probes 1+2 mm, pointed dissecting needle, lancet	LABSCO - Laboratory Supply Company GmbH & Co. KG
Feeding tray	Anodized aluminium blood feeding trays, 480 x 480 x 10 mm, textured aluminium (14/16 G diamant)	Lindeberg GmbH
Forceps (for handling pupae)	Soft pliable forceps	
Gram stain materials (crystal violet solution, decolourizing solution, Lugol's solution, safranine solution)	Gram stain kit	Sigma-Aldrich Company Ltd. LABSCO - Laboratory Supply Company GmbH & Co. KG
Hardener (for membranes)	T35 @5 kg or 270-g bottle	Wacker Chemie GmbH, München Georg Schicht Handels Ges.m.b.H., Vienna
Heating mat	Rubberized heating mat, 35–45°C, regulation ±1°C, 220 V, heated surface 50 x 150 cm, overall size: 160 x 57 cm, with temperature regulator	Sohnnstrahl
Heating plate (aluminium)	Heating plate for tsetse feeding, consisting of a sealed unit with metal (aluminium) upper and lower face, with temperature isolation, complete with temperature sensing	PAKATECH Paral & Kalchhauser GmbH Moro

	element mounted underneath the plate. The centre (around the heating element) is filled with silicone rubber. Control unit for electrical heating plate, with timer 24 hour / 7 day, including RS232 interface. Temperature range: 35–45ºC, thermostat with digital display.	
Humidifier	Air humidifier, e.g. Defensor 3001, complete atomizer for direct room injection, for automatic water connection Mod. 1114232, incl. 1 basin with floater and drain Z69 reservoir capacity 4.5 lit., Humidistat H4 CH, Deflector hood Z66, Dust filter Z67	Axair Ltd.
Incubator (controlled temperature and RH, for pupae)	Climatic chamber with internal lights, controlled temperature, humidity and ventilation, humidifier and dehumidifier, temperature range: 10–50ºC, humidity range: 10–90%, volume: 404 ltr., multifunctional microprocessor controller, with exposition light in the door. Thermostat with day-night cycles with light, temperature and humidity control, time-pattern control.	LABSCO - Laboratory Supply Company GmbH & Co. KG Amex Export-Import GmbH
Incubator	Incubator for bacterial culture, chamber volume 73 litres, electronic temp. control, 5–70°C.	American Scientific & Industrial Supplies LABSCO - Laboratory Supply Company GmbH & Co. KG

Inoculation loop	Platinum-iridium wire 90/10, 0.5-mm thick, 60-mm long. Loop holder 160-mm long	LABSCO - Laboratory Supply Company GmbH & Co. KG
Insect electrocutor	UV lamp and high-voltage electrocutor, e.g. “Insect-o-cutor”	Egon Kulms, Ing.
Microscope, stereoscopic (for insect dissection and identification of marked individuals)	Stereo-zoom microscope, binocular, 7-45 X, upper variable quartz halogen lamp, lower fluorescent lamp, complete. Eyepiece micrometer.	SFSI (Service for Science and Industry, Inc.) Labsco (Laboratory Supply Company)
Mould (plastic, for making membranes) (also called ‘matrix’)	472 x 472 mm external membrane size, groove 6-mm wide x 4-mm deep	Augmeuller Kunststoff Verarbeiting
Netting (for cages)	100% polyester, mesh size depends on tsetse species and type of cage	Walther Berufskleidung
Netting (for membranes)	White 100%-polyester netting for membrane making (16 holes/cm^2, thickness 250–300 micrometer)	Walther Berufskleidung
Oven (sterilizing)	Heat sterilizing oven, Mod. UNE700, temp. range 5–250°C, with electronic microprocessor PID controller with digital timer, RS 232, capacity 416 lit.	LABSCO - Laboratory Supply Company GmbH & Co. KG
Oxidase reagent		Sigma-Aldrich Chemie GmbH
Petri dish (perforated with holes for ventilation, for pupae)	Plastic sterile disposable Petri dish (60 x 15 mm) with perforated holes made by hand with a hot wire	LABSCO - Laboratory Supply Company GmbH & Co. KG
Petri dish (glued back-to-back, used with emergence cage, for pupae)	Glued back-to-back plastic sterile disposable Petri dish (60 x 15 mm) to raise the level of the upper dish	LABSCO - Laboratory Supply Company GmbH & Co. KG
Petri dish (testing for bacteria in blood)	Glass Pyrex dish (100 x 15 mm)	LABSCO - Laboratory Supply Company GmbH & Co. KG

Pupal-counting machine	Pupal counter Elmor 600/A05, with discharge chute and foot control	Moll (Dr.) Rudolf - Elmor
Pupal size-sorting machine	Pupal size and separating machine with 5 chutes. Main frame in aluminium construction, vibration bolt with control unit, 2 polished sorting wheels (distance adjustable: 0,5 - 10,0 mm), 1 gear motor, 5 channels, 5 collecting hoppers.	PAKATECH Paral & Kalchhauser GmbH
Radiation source (gamma ray, industrial)	SVST Co-60 Industrial Scale Gamma Irradiator with 100 KCi Co-60 sources.	Transelektro
Rod (for making membranes)	Smooth, plastic, 4–7-cm thick and 70–80-cm long	Augmeuller Kunststoff Verarbeiting
Separating tube (for handling adult flies)	Glass tubing, bent to desired shape over a Bunsen burner	Local manufacture
Silicone (for membranes)	Silicone monomer, ELASTOSIL M3500 @ 1-kg or 5-kg can	Wacker Chemie GmbH, München, Germany Georg Schicht Handels Ges.m.b.H. Vienna
Spectrometer (single-kernel near-infrared, for sexing pupae)	(see model at the Seibersdorf laboratory)	
Sterilizing oven	Heat sterilizing oven, Mod. UNE700, temp. range 5–250°C, with electronic microprocessor PID controller with digital timer, RS 232, capacity 416 lit.	LABSCO - Laboratory Supply Company GmbH & Co. KG
Temperature recorder (digital)	32K StowAway TidbiT (-20–+50°C) temperature recorder, TBI32-20+50	Onset Computer Corporation
Temperature and relative humidity recorder (e.g. Hobo)	Digital temperature / humidity data logger, weatherproof, -30–+50°C, ±0.5°C, 0–100% RH, ±3%. E.g. HOBO LCD Temp/RH Logger H14-001	Onset Computer Corp

Thermometer (infrared)	Digital instant ear thermometer, e.g. “ThermoScan” (Braun)	LABSCO - Laboratory Supply Company GmbH & Co. KG
TPU 3 cage/feeding system (cage-holding frames, cage-levelling frames, cages, pupal-collecting troughs, heating plates, mobile trolley, guide rails, etc.)	TPU 3 system, with numerous components: Guide rails and mobile trolley. Heating plates with temperature sensor embedded in the plate for the control unit; control unit for heating plate, with timer 24 hour / 7 day, including RS232 interface. Feeding trays, 650 x 650 x 15 mm (internal), textured aluminium sheet 2 mm thick. Membrane mould, plastic sheet with milled groove 640 x 640 mm outside dimension, groove width 6 mm x 4 mm deep. Silicone membranes, 640 x 640 mm. Cage frames, 600 x 600 mm. Tsetse holding cage, 200 x 50 x 4 mm with 26-mm hole in side, complete with netting attached. Netting to be fixed with 3.5 mm galvanized steel spring (not glue). Cage material foam core PVC water pipe.	Moro Lindeberg GmbH, Prototypenfertigung & mech. Zentralwerkst
Trolley (for holding cages of flies and collecting pupae)	Older style of metal trolley, with shelves for cages of flies, sloping sheets, troughs to collect pupae, and water cups above wheels	Local manufacture

Annex 2. Cage-Netting Sizes

Species	Netting Size	
	Top	Bottom
G. austeni	130–150 µm thread, 13 holes per cm²	130–150 µm thread, 13 holes per cm²
G. morsitans	130–150 µm thread, 13 holes per cm²	130–150 µm thread, 13 holes per cm²
G. f. fuscipes	130–150 µm thread, 13 holes per cm²	250-300 µm thread, 8 holes per cm²
G. pallidipes	130–150 µm thread, 13 holes per cm²	250-300 µm thread, 8 holes per cm²
G. brevipalpis	130–150 µm thread, 8 holes per cm²	250-300 µm thread, 8 holes per cm²

Annex 3. Cage Densities (number of adults per cage)

Species	Adults			Pupae for Self-Stocking of Production Cage (SSPC) (Emergence rate = 95%)		
	Females	Males	Total	Mixed	Males	Total
G. austeni	100	25	125	222	28	250
G. tachinoides	100	25	125	222	28	250
G. morsitans	64	16	80	142	16	158
G. fuscipes fuscipes	64	16	80	142	16	158
G. palpalis palpalis	60	20	80	133	22	155
G. pallidipes	60	15	75	133	17	150
G. palpalis gambiensis Vanderplank	60	20	80	133	22	155
G. longipalpis Wiedemann						
G. brevipalpis	45	15	60	100	17	117

The number of pupae required to load one cage with females is based on the emergence rate, the sex ratio at emergence, and the cage density, e.g. for a 1:1 sex ratio, it is calculated as follows:

$$\text{No. of pupae to load one cage} = \text{Cage density} \times \frac{100}{\text{Emergence rate}} \times 2$$

Annex 4. Environmental Conditions

Condition	Species	% RH	°C	Light (Lux)	Special
Holding	*G. austeni*	75–80	23–24	Subdued/indirect	
	G. tachinoides	80–85	24–25	Subdued/indirect	
	G. morsitans	75–80	23–24	Subdued/indirect	
	G. f. fuscipes	75–80	23–24	Subdued/indirect	
	G. p. palpalis	80–85	24–25	Subdued/indirect	
	G. pallidipes	75–80	23–24	Subdued/indirect	
	G. longipalpis				
	G. brevipalpis	75–80	23–24	Subdued/indirect	
Feeding	*G. austeni*	65–70	21–22	12–14	The feeding response may be improved by covering the cages with a damp/dark cloth
	G. tachinoides	65–70	21–22	12–14	
	G. morsitans	65–70	21–22	12–14	
	G. f. fuscipes	65–70	21–22	12–14	
	G. p. palpalis	65–70	21–22	12–14	
	G. pallidipes	65–70	21–22	12–14	
	G. longipalpis				
	G. brevipalpis	65–70	21–22	12–14	
Pupal Incubation	*G. austeni*	75–80	23–24	Subdued/indirect	Increase to 80–85% RH at 2 days before emergence
	G. tachinoides	75–80	23–24	Subdued/indirect	
	G. morsitans	75–80	23–24	Subdued/indirect	
	G. f. fuscipes	75–80	23–24	Subdued/indirect	
	G. p. palpalis	75–80	23–24	Subdued/indirect	
	G. pallidipes	75–80	23–24	Subdued/indirect	
	G. longipalpis	75–80	23–24	Subdued/indirect	
	G. brevipalpis	75–80	23–24	Subdued/indirect	
SSPC	*G. austeni*	85	26.5	Subdued/indirect	Transfer from standard incubation conditions to SSPC conditions on the day of first emergence
	G. pallidipes	85	26.5	Subdued/indirect	
	G. brevipalpis	85	26.5	Subdued/indirect	

Annex 5. Problem Solving

Annex 5.1. High Mortality

When the mortality rate in the colony rises, examine the holding conditions to ensure that the equipment is functioning properly. Examine the dead flies, and categorize them according to the likely causes of death, e.g. starvation, blood in abdomen, pupal mortality, or other causes.

1. Flies with no undigested blood in the abdomen would have died of starvation due to:
   - The heating plate (and feeding membrane) was too cold
   - Relative humidity was too low
   - Pesticide toxicity
   - Disturbance during feeding
2. Females with a large amount of blood in the abdomen would have died because of:
   - Bacterial contamination (from contaminated blood, or inadequate sterilization of equipment)
   - Drug residue (toxicity)
   - The heating plate (and feeding membrane) was too hot
   - Relative humidity was too high
3. Pupal mortality occurs when females die with a larva that pupated in the uterus. The likely causes are pesticide toxicity in the very late stage of pregnancy, or the relative humidity in the holding room is too low.
4. There could be other causes of mortality, e.g. due to bad handling. This can be evaluated by determining the mortality pattern, e.g. when there is increased mortality of adults handled in the chiller on the same day or by one particular person, or of adults located in a localized place within the holding room.

Classifying mortality and evaluating the pattern facilitate tracing and eliminating the source of the problem.

Annex 5.2. Low Fecundity

The average fecundity of females should be 0.6 pupae per female per 10 days (0.6 P/F/10d); this yields an average of at least 3 pupae per female invested. The first larva should be deposited not later than 18–19 days after adult emergence. When these conditions are not met, do the following:

1. Determine the fecundity of each female unit.
2. Check the environmental conditions in the holding room, and adjust as required.
3. Check for aborted eggs and larvae on trays and sloped sheets, and in pupal-collecting troughs.
4. Abortions may be caused by improper holding conditions, stress, overcrowding, insufficient nutrition, toxicity and bacterial contamination.
5. Reproductive abnormalities may result in lowered fecundity. Dissect a sample of females, and examine the spermathecae (section 5.2.) to determine if they were inseminated.

6. Examine the content of the spermathecae for the presence of sperm and their motility. The spermathecae might be filled, but not contain motile sperm.
7. If there is insufficient insemination, revise the mating regime (sex ratio, age at mating of females and males, timing of mating).
8. Dissect a sample of males, and examine for the amount and motility of sperm in the testes, and the size of the accessory glands.
9. If the results show that male quality is poor, then revise the procedures for male handling and pupal incubation.

Annex 5.3. Low Adult-Emergence Rate

If the emergence rate is low, dissect a few of the pupae that apparently died, and determine when development stopped. If death occurred late in pupal development (when the adults were fully formed and ready to emerge), but the adults failed to open the operculum, increase the relative humidity just prior to emergence. Also examine the incubation conditions, and adjust as appropriate for the species.

Annex 6. Glossary

25-day test: A 25-day test was developed with the aim of having a simple numerical system that adequately summarizes and combines the various data obtained from pupal production and dissections. It is expressed as the quality factor (QF), and determines if the quality of blood is suitable for maintaining an adult to live and produce a larva/pupa during the first 25 days of adult life.

30-day test: A modification of the 25-day test for tsetse species with a slower first reproductive cycle. Pupal recording should be continued only until the first cycle is complete, even if this is less than 30 days.

ABL: Agriculture and Biotechnology Laboratory, NAAL.

Blood mortality: Female adults die with a large amount of undigested blood in the abdomen.

Chiller: A converted chest deep-freeze used to manually separate the sexes of tsetse adults.

Chilling separation: A procedure to separate the sexes of adult tsetse flies, involving the exposure of adults to 4ºC to immobilize them, and then visually separating them as either male or female.

Day-0 mating: Day-0 mating regime with resident males involves putting teneral adults of both sexes together in production cages right after emergence, and leaving them together permanently. Mating takes place as the adults mature over the first 5–10 days.

Dry pupal stage: A pupa with a fully developed and melanized fly once the adult cuticle is dry; the final stage before adult emergence.

Early-stage pupal stage: A pupa during development when the adult shape is fully formed but there is no melanization; the stage before the red-eyed pupa; also called “obtected pupal stage”.

Emergence cage: The emergence cage is made from a polyvinyl chloride (PVC) tube reshaped in water at 60°C to form a rectangular cage (about 13 cm high and 22.5 cm long). The cage must be high enough so that cold air is blown into it when the cage is placed in a chiller. A 6-cm-diameter hole is cut in the bottom of the cage to accommodate the Petri dish holding 400–500 pupae. To be able to close the hole when the emergence cage is placed in the chiller, two pieces of plastic are attached to two sides of the hole to provide a groove along which another piece of plastic is inserted.

Emergence rate:

$$Emergence\ rate\ (\%) = \frac{Total\ no.\ of\ adults\ emerged}{Total\ no.\ of\ pupae} \times 100$$

Entomology Unit: A unit of the Agriculture and Biotechnology Laboratory, NAAL.

FNOS: Follicle next in ovulation sequence. (References: Saunders (1961), Challier (1965), Van der Vloedt et al. (1978), and Vreysen et al. (1996). The term “FNOS” was coined by A. Van der Vloedt.)

Fully pigmented pupal stage: A fully developed and pigmented fly, but with the cuticle still wet; the stage before the dry pupa.

HDPE: High-density polyethylene.

Input: The number of newly emerged females that are added to a unit.

LDPE: Low-density polyethylene.

Lysed pupal stage: A pupa before adult development begins, with content lysed; the stage before early-stage pupal stage (obtected pupal stage).

Male pupae: Female adults emerge approximately 2 days earlier than males from pupae deposited on the same day. When pupae, incubated at 23–24°C for 30–31 days after larviposition, are transferred to 26.5°C, the adult emergence spans a period of 4 days; during the first 2 days, only females emerge, and during the last 2 days, mostly males. After the females have emerged, the remainder is mostly male pupae.

Mixed-sex pupae: Pupae before female adults have emerged.

NAAL: Agency's Laboratories, Seibersdorf (IAEA, Department of Nuclear Sciences and Applications).

Notifiable OIE disease: If a disease (that is on the "notifiable disease list" at the OIÉ) occurs in an area, it must be reported to the OIÉ (Office International des Épizooties) (but now referred to as the World Organisation for Animal Health).

Obtected pupal stage: See "early-stage pupal stage".

Other mortality: The mortality due to other causes that could arise from bad handling, e.g. prolonged chilling, insect age, and the trolley position in the holding room where there might have been overexposure to fans, etc.

PE: Polyethylene.

Percentage A-class pupae: The pupal size-sorting machine is calibrated for each species of tsetse, and separates pupae into five distinct classes, with A-class the smallest and E-class the largest. It is important that the percentage of A-class pupae (the smallest pupae) does not exceed 10%.

Percentage daily mortality (% D.M.):

$$Percentage\ daily\ mortality\ (\%\ DM) = \left(1 - \left(\frac{End\ females}{Start\ females}\right)^{\frac{1}{d}}\right) \times 100$$

where *d* is the number of days since the last mortality check, *Start Females* is the number of females at the start of the period, and *End Females* is the number remaining at the end of the period.

Production cage: A cage holding producing females (see "standard holding cage").

Pupa per female per 10 days (P/F/10d):

$$P/F/10d = \frac{\text{No. of pupae produced during the previous week} \times 10}{\text{No. of females surviving the previous week} \times 7}$$

Pupae per initial female (PPIF): The total number of pupae produced throughout the life of the unit divided by the original number of females (input).

Pupal mortality: Females with a larva that pupated *in utero*.

PVC cage: Polyvinyl chloride cage.

Quality factor (QF): A measure of the relative quality of the blood diet, measured by the 25-day feeding test (see "25-day test").

QFC: A calculated approximation of the QF, without any dissection results. This should be used only for routine quality control after the baseline QF values have been established.

Red-eye pupal stage: The stage during pupal development when the eyes are starting to melanize and look red; the stage before "fully pigmented".

Salivary gland hyperplasia (SGH): A pathological condition associated with sterility and aspermia among tsetse, principally *G. pallidipes* males. The salivary glands are enlarged to about four times the normal diameter, and become milk-white in appearance.

Salivary gland hyperplasia virus (SGHV): A virus that is present in wild tsetse populations at a low level, but can become widespread and affect colony production; causes SGH in some infected individuals.

Self-stocking of production cage (SSPC): In this procedure, newly emerged adults penetrate by themselves the netting of a "standard holding cage", and thus enter directly into a "production cage".

Standard holding cage: A cage cut from 200-mm-diameter polyvinyl chloride (PVC) drain pipe, 50 mm tall, with netting on both sides, and a 21-mm hole on the side plugged with a rubber stopper (permitting flies to be inserted or taken out).

Starvation mortality: Female adults die with little or no undigested blood in the abdomen.

TCRRS: Tsetse Colony Recording and Reporting System; a standard database system for the recording and reporting of tsetse colony data.

Tsetse production unit 3 (TPU 3): An improved colony-holding and colony-feeding system in which cages with adults are held stationary, and blood in the feeding system (moving on rails) is taken to the cage-holding frame; thus the adults are fed automatically without disturbing them.

Unit: Adults that emerged during a 7-day period, having been grouped together for handling and recording – the basic division of a tsetse colony for recording purposes.

UV: Ultraviolet radiation.

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