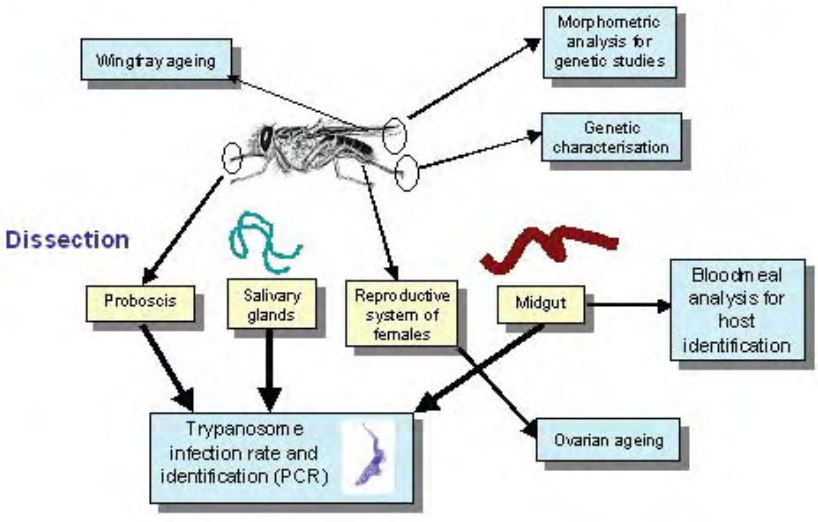
3.2. ENTOMOLOGICAL PROCEDURES

In addition to the basic required information such as the identity of the tsetse species present in an area, their sex and apparent density, there are additional parameters, knowledge of which will contribute to the development of control/eradication planning and monitoring. Among these additional parameters are:

- the trypanosome infection rates in tsetse and trypanosome species identity,
- the age structure of the tsetse population,
- their feeding habits (hosts),
- mortality rates,
- nutritional status,
- genetic structure of the tsetse population.

Unless many staff is available for the main, surveying activity of the project, it may not be possible for the survey teams, responsible for deploying traps and harvesting tsetse from them, to also carry out the additional entomological data collection. It will not be practical for tsetse brought back to the laboratory at the end of a day of collection to be dissected by the same people at the end of that day. Furthermore, many tsetse flies captured will be dead by the time they reach the laboratory and will be unsuitable for dissection. For those practical reasons, it is preferable to have an additional team who will be responsible for collection of the additional data. Traps for those data may be fewer in number than those used for the basic survey so that they can be visited, and the flies collected at more frequent intervals so that they are still alive and fresh for dissection. The distribution of those traps may be based on different criteria from those of survey traps according to what information is needed in the area.

FIGURE 3.6
Scheme illustrating possible processing procedures for captured tsetse flies



3.2.1. Identification of Tsetse Species and Sex

Tsetse species can be identified using conventional identification keys of the sort that are available in standard texts (e.g. Machado 1954, 1959 (for *palpalis* group tsetse), Buxton 1955, Mulligan 1970, FAO 1982a, section 1, these guidelines) or by using interactive computer-based identification programmes such as the computer identification package developed by L'Institut de Recherche pour le Développement (IRD)/Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD) and made available on a CD–ROM.

Generally, the species inhabiting a particular area are known and local information will be available. However, in areas with more than one species present, it will be necessary for survey staff to be able to distinguish between species and this ability is not always available without some training on identification and the use of identification keys. In other areas, whilst one species may predominate there may be other species that are detected rarely and may be overlooked if insufficient attention is paid to identification. There are also countries in which tsetse subspecies coexist, for example, *G. m. centralis* and *G. m. morsitans* in Zambia; subspecies of *G. fuscipes* in Congo or *G. palpalis palpalis* and *G. p. gambiensis* in West Africa. These subspecies are not readily distinguished without microscopic examination, yet there are important implications to the targeting of just one of these subspecies in a control/eradication programme.

Distinguishing the sexes is relatively easy and quickly learned (FAO 1982a, section 1, these guidelines). Data for male and female captures should be analysed separately. The total of males and females should be added up for each day, either over all trap positions, or in each of the vegetation types or areas. Arithmetic mean catches per day per trap are then calculated for the full sampling period. The data can be displayed as a graph of numbers, on the vertical axis, against time, on the horizontal axis.

There are two commonly used types of logarithms, logarithms to base 10 ($\log_{10}$) and natural logarithms ($\log_e$ — e is a constant equal to 2.71828). For the plot of numbers against time, natural logarithms should be used.

3.2.2. Trap and Tsetse Distribution Maps

The coordinates of all trap sites will be entered into a database and mapped on a base map of the survey area. This map can be updated weekly to display survey progress. This will be linked to data on trap catches so that weekly updated maps of tsetse distribution and apparent density can be produced on screen or as hard copies if required. There should be feedback of these maps to field survey teams to facilitate monitoring of progress and assess the need for any modifications of the survey protocol/work-plan.

3.2.3. Estimation of Apparent Density

It is not possible to precisely estimate the density of a tsetse fly population in a given area by trapping. All that can be done is to estimate the apparent density, changes in which are expected to roughly reflect changes in the real density of the population. The apparent density is relative to the type of sampling tool (trap) used, and is expressed as the average number of flies caught per trap per day (flies/trap/day or FTD). The apparent density is calculated by dividing the total number of tsetse flies captured (ΣF) by the product of the

number of functioning traps used to catch them (T) and the number of days for which the traps were operational (D).

$$FTD = \Sigma F / T \times D$$

If a trap is not operational for some reason, perhaps it was blown down or the cage was blown or knocked off, that trap day should be excluded from the sum of trap days.

The apparent density (AD) data can be grouped for analysis at different levels. Firstly, at the trap level, giving a mean AD per trap, this is at the highest resolution, but will be subject to great variability and would therefore have to be interpreted cautiously, according to the number of days of trapping that were grouped to calculate the mean. If there are sufficient data (many flies being caught), this can be used to produce a detailed map of the tsetse distribution of an area, especially if a 1 km^2-grid survey was conducted. It can be useful in some circumstances to identify the vegetation types most associated with tsetse. The next level, to continue that association of tsetse with vegetation type would be to group traps deployed in the same vegetation type and determine the mean AD for those groups of traps. Thirdly, the traps in a particular grid can be grouped together and the mean apparent density for that grid can be estimated either overall, per season or per month (in the case of long-term monitoring rather than survey). The latter will give a general geographical distribution of tsetse by AD, not associated with vegetation. This can give an overview of a large area, but it must be borne in mind that with a 10 × 10 km grid square there could be a large amount of variation, depending on the uniformity of the habitat. Pooling the data in this way will reduce its variability.

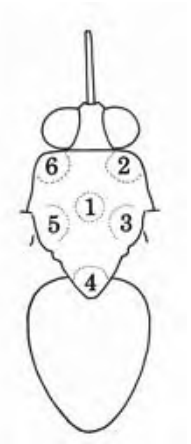
Analyses of variance (ANOVA) can be used to explore the effects of the main factors, e.g. habitat type, sampling method on the dependent variables such as apparent density or trypanosome infection rates. Multiple regression analysis is also useful to determine the importance of different variables (study area, ethnic group, habitat, distance from river) in determining tsetse apparent density or presence and absence. A common procedure is to transform variables to a normal distribution; tsetse catch numbers are usually transformed by taking the logarithm of the numbers of flies (n)+1, whilst an arcsin transformation can be used for disease prevalence data.

3.2.3.1. Mark-Release-Recapture Studies to Estimate Population Size

For some purposes it is useful, if not essential to know the approximate size of a tsetse fly population in a given area. For example, for AW–IPM with an SIT component, knowledge of the approximate number of flies in the area will enable calculations of the number of sterile males that will have to be produced and released.

The mark-release-recapture method to estimate population size is probably the most appropriate for this purpose, although it requires a high degree of technical expertise and supervision, if it is to be used to produce estimates with a sufficient degree of reliability to be of practical use. For that reason it will not be a routine procedure in most surveys but will have a place in surveys that are conducted prior to programmes that envisage the use of the SIT. For good estimates to be made a sufficient number of marked flies have to be recaptured and that requires both a reasonably efficient trapping device and a reasonably

FIGURE 3.7
Marking tsetse flies on the thorax for mark-release-recapture studies



Source: FAO 1982a

high natural population, as the proportion of marked flies that can be expected to be recaptured is relatively low.

The technique is as follows: tsetse flies are caught using a trapping device that will not damage the flies, leaving them with a probability of survival similar to that of uncaught wild flies after marking and release. The traps will be visited at short intervals (30 minutes) in order to process the flies before they have undergone stress in trap cages. Flies are marked, usually with oil paints, with a small spot on the thorax that will be readily seen if the fly is recaptured but which is not so big as to affect the flight and survival of the fly. Using combinations of different paint colours and different arrangements of marking the flies thorax it is possible to mark the flies in such a way as to enable the day of marking to be determined if the fly is recaptured (**Figure 3.7**). If, for example, one of the spots indicated in **Figure 3.7** is used to mark the month, a second site the week, and a third the day, using different colour codes on each spot, dates can be accurately marked and other spots can be used for other features, such as location.

The mathematics of estimating the population size, are relatively simple in theory — relating the proportion of marked flies recaptured and the proportion of marked flies in the total number captured during the occasion of that recapture to the total population size. However, there are various sources of error, such as the effects of immigration and emigration to and from the population and of birth and death rates that require more complicated mathematical procedures if accurate estimates are to be made. Within limits, a rough but reasonably accurate estimation will be sufficient for AW–IPM programmes that include an SIT component whilst more precise estimates will be necessary for research into tsetse population dynamics. Some of these procedures are reviewed by Leak 1998.

The Jolly method has been the most commonly used method to estimate the density of insect populations that are open, i.e. which are subject to emigration, immigration, birth and mortality, but the method requires that the release is made more than twice, which makes it logistically more challenging. Ito (1989) described alternative methods that could be based on a single-release and multiple recapture census for stable populations, i.e. the Hamada method (or modified Jackson positive method).

Flies can either be marked individually (e.g. oil paint spots) as described or group marked – marking large numbers of flies in the same way (e.g. with fluorescent paints or powders). Grass stems, fine brushes, or toothpicks can be used to mark the flies. It is important that the handling of the flies is minimal and does not affect their longevity or behaviour, that the marks do not fade, come off or in any other way become undetectable, and that the marked and unmarked flies can be captured with the same probability.

An index y_i is calculated by the following equation:

$$y_i = \frac{10^4 m_i}{M_0 n_i}$$

Where M_0, n_i and m_i are, respectively, the number of individuals marked and released on day 0, that of individuals caught on day i, and that of marked individuals recaptured on day i. y_i is a standardized number of marked insects to be recaptured on day i, assuming that 100 marked insects are released on day 0 and 100 insects are randomly caught on day i. Provided that the wild fly density is constant, we can obtain a survivorship curve of marked individuals by plotting y_i against i. If the survival rate is constant it can be approximated by a linear regression equation:

$$\log y_i = \log y_0 + i \log S$$

Where S is the survival rate per unit time (as $< S < 1$, logs is always negative). Here y_0 is a theoretical value representing an expected number of recaptures on the assumption that 100 marked individuals released on day 0 are instantly intermingled into the wild population, and that 100 specimens are randomly caught before either disappearance or dilution occurs. It is now possible to estimate the number of individuals as:

$$\hat{N}_{J+} = 10^4 / y_0$$

3.2.4. Blood Meal Analysis

Analysis of undigested blood meals collected from the midguts of tsetse flies will allow the predominant hosts of the tsetse fly to be determined. This epidemiological data can be linked to significant habitats that might be utilized by tsetse flies (coinciding with the host habitat). The data can also be used to determine what sort of suppression technique might be suitable; notably the use of insecticide-treated cattle (livestock) will depend upon the proportion of feeds being taken from those cattle. In areas where tsetse fly density is low, it can be difficult to collect adequate numbers of recently fed flies to provide a suf-

ficient sample size for statistically valid conclusions to be drawn on the feeding habits of the tsetse fly population.

It is important to catch recently-fed flies and consequently it is preferable to collect captured tsetse flies at shorter intervals than are generally used for tsetse fly surveys, for example, hourly or two-hourly collection during the periods of tsetse fly activity. Most tsetse fly traps catch a higher proportion of hungry flies, as those are the segment of the population that are more active, looking for a host to feed on.

Alternative methods of capture that have been used in the past are collection from resting sites using hand nets and the use of artificial refuges. Both of those techniques aim to catch flies that have recently fed and that are looking for a place to rest whilst digestion starts and some of the large volume of water content taken in with the blood meal is excreted, allowing the flies' flight performance to improve. Capture of flies with hand nets is tedious and requires skills in identifying appropriate sites and use of the hand net, furthermore, in most situations a limited number of fed flies are likely to be captured. During a survey, ideally, blood meal samples should be collected from transect catches to get an indication of spatial variability in tsetse feeding preferences.

Another problem is the insufficient sensitivity of the techniques used (complement fixation) leading to a high percentage of unidentified blood meals. There have been recent improvements with the use of a polymerase chain reaction (PCR)-heteroduplex technique that amplifies cytochrome B and can distinguish between different host species (Njiokou et al. 2004). When tsetse are dissected (e.g. to detect trypanosome infections) and it is noticed that the fly has apparently fed recently, to collect the sample for blood meal analysis, take the midgut with forceps, and spread it on a piece of Whatman paper, carefully writing on the paper the number of the tsetse, date, and trap number. Allow the sample to dry then put it in aluminium foil to protect it from dust. When the survey has finished, samples can be sent to a specialized laboratory for analysis.

A further difficulty with blood meal analysis is the availability of specialized laboratories that can undertake the analysis. In the past, free blood meal identification services have been available at the Imperial College, UK, and the Free University of Berlin, Germany however neither of these services is still available. Whilst international institutes (the International Livestock Research Institute (ILRI), the International Centre of Insect Physiology and Ecology (ICIPE), and the Centre International de Recherche Développement sur l'Elevage en zone Subhumide (CIRDES)) have previously offered this service on an occasional and limited basis, such services are also not currently available. The Farming in Tsetse Controlled Areas (FITCA) Kenya project established a blood meal analysis laboratory through provision of equipment and training of personnel, at the Kenya Agricultural Research Institute-Trypanosomiasis Research Centre (KARI-TRC) hospital at Alupe in western Kenya and this laboratory may provide the service in future.

3.2.5. Dissection

In this section, a description of basic techniques for dissecting tsetse for ovarian ageing and determination of infection rates is given. In guidelines such as these, it is possible to describe the organs that are to be dissected, to illustrate them with diagrams and to give

general principles of the way to dissect the fly. The only way to really learn dissection techniques is to closely observe it actually being done and to practice dissection.

3.2.5.1. Handling of Caught Tsetse

If tsetse are to be dissected then they should be processed as soon as possible after returning to the field station so that as many as possible are still alive for dissection. Trypanosomes are less likely to be found in dead, dried flies and, therefore, dissection results from such flies are likely to be underestimated and misleading. Consequently, dissections should only be carried out on freshly killed flies.

- store all trap cages carefully after removal from the vehicle to await processing — storage in a fridge (not freezer) is desirable as this will immobilize the flies without killing them and reduce likelihood of escapes,
- take one cage at a time and immobilize flies by squeezing the thorax gently whilst removing them from the cage,
- record the number of the cage on the trapping data recording sheet,
- if more than one tsetse species may be present, check each tsetse individually and identify the species using the appropriate keys (or parts of a key as the possible tsetse species for the area will be known). If two subspecies may be present in the same area this may require dissection (e.g. of the inferior claspers for *palpalis* group male tsetse; the signum of female *fusca* group tsetse),
- separate the males and females of each species — count each and record on the data sheet,
- count and record on the data sheet biting fly numbers (Tabanidae including *Chrysops*, *Haematopota,* and *Stomoxys* spp.) identified at least to genus,
- if required, biting flies can be preserved in 70% ethanol for subsequent species identification, otherwise discard,
- hand tsetse flies to dissecting team (if separate), maintaining trap identification, and
- proceed with next cage.

3.2.5.2. Equipment

A very useful tool for practical demonstration of dissection techniques is a teaching dissection microscope, which has two binocular eyepieces so that two people, the trainer and the trainee, can both look through the objective lens simultaneously. This allows the trainee to see exactly how the fly is being manipulated. A recently improved technological alternative is the use of a microscope connected to a monitor so that what is seen down the microscope is displayed on the monitor. One of these tools is recommended if a number of people are to be trained over a period of time.

Ideally, a dissecting microscope with fibre-optic illumination (cold light) should be used as this will prevent the saline buffer in which dissections are carried out from drying too rapidly. Dried saline will leave white crystals and dried tsetse organs will easily disintegrate and will be impossible to observe clearly.

3.2.5.3. Dissection for Wing Fray Analysis, Size Estimation and Determination of Ovarian Age

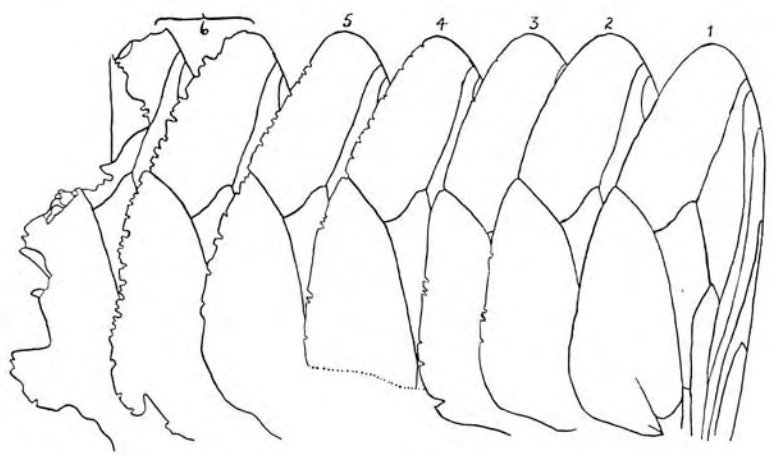
Determination of the physiological age structure of a tsetse population is of great importance in evaluating the progress of control/eradication programmes, especially those in which aerial spraying or SIT are used. During sequential aerial spraying of non-residual insecticides any tsetse captured within a spray block in the few days after spraying should be young flies that have just emerged from pupae in the ground. Any older flies captured must either have entered the spray block from the neighbouring area — indicating problems of re-invasion — or must result from some fault in the spraying operation.

Wing fray analysis

Relative age structure of a tsetse fly population can be determined for male and female flies by analysis of wing fray, although this is crude and subject to variability between species, sexes and seasons. Nonetheless, for pooled samples for each sex and species separately, it is a simple technique and can give a useful indication of changes in population age structure:

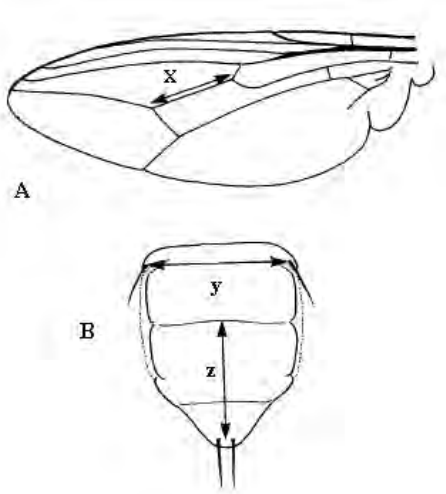
- gently but firmly squeeze one fly to immobilize it,
- with a pair of fine (watchmakers) forceps remove each wing from the fly, pulling from the base of the wing, taking care not to damage the trailing edge,
- place the wings on a slide with a drop of saline solution (or water) and place a cover slip on top,
- examine with a dissection microscope 25× magnification. Select the least damaged wing (to minimize overestimating wing fray (resulting from damage caused in the trap cage) and assess the degree of wing fray by making reference to **Figure 3.8**

FIGURE 3.8
Diagram showing the different wing fray categories



Source: Buxton 1955

FIGURE 3.9
Diagram to show measurements of (a) wing vein length and (b) thoracic size



Source: FAO 1982a

(with experience it is soon possible to distinguish between genuine fraying due to age and damage caused by handling/the trapping process), and

- record the wing fray category along with other details of the fly (species sex, trap number and date) on the recording sheet. Further details of the methodology for wing fray ageing techniques are available in the FAO training manual for tsetse control personnel, volume 1 (FAO 1982a) or Mulligan (1970).

Measurement of wing vein length

Whilst examining the wings for wing fray, a further procedure that can be carried out in order to obtain an estimate of the mean size of flies in the population is measurement of the wing vein of the hatchet cell (blade) as indicated in **Figure 3.9, upper.** Size-dependent mortality is known to occur in *G. morsitans* and *G. pallidipes*, and hence, changes in mean size of the population can therefore be used to estimate changing mortality rates. The measurement requires a graticule that is used in conjunction with the microscope and requires care in accurately measuring the vein length; otherwise the results will be meaningless. This is one of the reasons why dissections should all be carried out by a separate team from those conducting the survey trapping as if time is limited, insufficient care will be given to the dissections. The thorax size can also be measured across the dimension shown in **Figure 3.9, lower** as an additional measurement of size. Recently, more sophisticated morphometric methods of analysis have been developed (Patterson and Schofield 2005) as outlined in 3.3.3.

FIGURE 3.10
Ovarian age categories

Ovarian age category	Configuration	Category / uterine contents	Estimated age (days)	Later category	Age range
0	 Non-ovulated	-	0-8	8a 8b 8c	80-84 84-87 87-90
1	 1-ovulation	1a 1b 1c	8-12 13-16 16-19	9a 9b 9c	90-94 94-97 97-100
2	 2-ovulations	2a 2b 2c	20-24 24-27 27-30	10a 10b 10c	100-104 104-107 107-110
3	 3-ovulations	3a 3b 3c	30-34 34-37 37-40	11a 11b 11c	110-114 114-117 117-120
4	 4,8,12-ovulations	4a 4b 4c	40-44 44-47 47-50	12a 12b 12c	120-124 124-127 127-130
5	 5,9,13-ovulations	5a 5b 5c	50-54 54-57 57-60	13a 13b 13c	130-134 134-137 137-140
6	 6,10,14-ovulations	6a 6b 6c	60-64 64-67 67-70	14a 14b 14c	140-144 144-147 147-150
7	 7,11,15-ovulations	7a 7b 7c	70-74 74-77 77-80	15a 15b 15c	150-154 154-157 157-160

Source: FAO 1982a

Dissection for ovarian ageing

A more accurate method of ageing tsetse, applicable only to female flies, is the determination of their age based upon ovarian dissection (**Figure 3.10**) to determine the number of eggs produced by the female and the stage in the ovarian cycle at the time of dissection. This dissection technique, obviously for female flies only, requires some practice, ideally with tsetse of a known age before reliable results can be obtained so it is less appropriate for instructions of the sort provided here, it requires demonstration.

Guidelines for ovarian dissection

To dissect a female tsetse fly for ovarian ageing, first remove the wings and legs of the fly with fine forceps, simply because these may otherwise get in the way of the dissection.

Place the fly with its dorsal side uppermost on a glass slide with some buffered saline sufficient to prevent the dissected organs from drying out. Make an incision of about one mm on either side of the fifth or sixth tergite. Gently grip the tip of the abdomen below the incisions and pull backwards. A side-to-side movement will assist in tearing the abdomen across. When the abdomen tears, the internal organs will be exposed and should float in the phosphate buffered saline buffer so that the uterus, spermathecae and ovaries can be readily seen. Check the position of the spermathecal duct entering the uterus as this will indicate whether the reproductive organs are in the correct configuration or if they have become twisted or turned over. Observe the contents of the uterus, and if required, the percentage of sperm in the spermathecae. Make an initial observation of the configuration of the ovarioles, i.e., is the most developed on the left inside, left outside, right inside or right outside? Do the same for the next in size and so on. This will let you compare with the diagram in **Figure 3.10**, to determine the physiological age of that female fly. It will also enable you to determine which ovarioles you need to dissect to look for a follicular relic. Having determined that, remove fat bodies and other parts that might get in the way, then, using a higher magnification use fine mounted needles or good quality fine forceps to break the outer membrane of the ovarioles and release the developing egg.

This can then be examined for the presence or absence of a follicular relic. In some age configurations, there is only one possibility to look for a relic and if the dissection is messed up, for example because the egg is broken, there is no possibility to go further. In other configurations there can be a second chance if the first one is unsuccessful.

The key to success, is keeping the tissues moist, avoiding twisting or turning of the reproductive system and careful dissection avoiding puncturing of developing ovaries. If they are punctured, whitish contents come out and it becomes impractical to reliably observe the status. When correctly positioned the spermathecal ducts should be seen entering the uterus from above — provided the right and left ovaries have not twisted, they should then be in the correct position to determine which is the largest ovariole and the sequence. You can also verify that the order is in the correct configuration.

If the gut is full, contents of the gut, when broken can obscure the reproductive system, so when dissecting, don't pull the abdomen so far back that the gut is broken.

The gut may be carefully detached near the rectum to remove the fly's body from the reproductive organs afterwards.

Drawbacks to ovarian ageing are that it requires a skilled technician to perform the dissection accurately and that skill requires not only adequate training, with tsetse of known ages available for verification, but also a sufficient amount of practice. Training can be given but without sufficient practice and experience the validity of data obtained may be questionable. For that reason attempts have been made to develop automated methods for age determination, for example, based upon pteridine accumulation in the compound eyes, however, these attempts have met with difficulties and are not yet available for routine use. The methodology for ovarian dissection is described further in Mulligan (1970) and the FAO training manual for tsetse control personnel, volume 1 (FAO 1982a).

It is important to emphasize that ovarian ageing of female tsetse provides the dissector with the physiological age of the fly, not the calendar age (as the development of the ovaries is temperature dependant).

3.2.5.4. Dissection for Trypanosome Infection

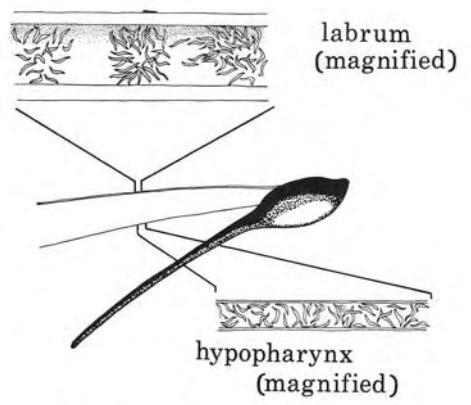
Trypanosome infection rates in tsetse can be determined either by dissection and observation of the parasites in organs of the fly (based on which, an imprecise identification of the trypanosome species subgenera can be made), or using molecular biological diagnostic procedures (DNA probes and polymerase chain reaction (PCR)). There are advantages and disadvantages to both methods. The basic dissection method is crude as it cannot precisely identify the trypanosome species. Inaccuracies can also result from mixed infections, immature infections and the inability to distinguish between some trypanosome species of the same group that are pathogenic to domestic livestock or humans or not, e.g. *Trypanosoma simiae*, which is indistinguishable from *T. congolense*, but which, although it can be pathogenic to domestic pigs, is primarily a parasite of warthogs and is not pathogenic to cattle. Diagnosis based on molecular biological techniques is not very different in sensitivity to the microscopical technique, but can accurately identify the trypanosome species. A drawback of PCR is that it requires sophisticated laboratories, specialist staff and reagents and is more expensive to perform. Problems have arisen in the past with trypanosome strains for which primers were not available. For most tsetse surveys associated with control/eradication programmes it is likely to be sufficient to determine trypanosome infection rates based on dissection alone, however, subsamples could be collected for diagnosis by molecular techniques if required and when possible PCR could be performed on mouthparts only, as this will give a rapid estimation of mature infections and will save the time and money used to identify trypanosomes in the midgut, which is less useful because it is known that a large proportion of trypanosome infections in the guts of tsetse do not mature.

Guidelines for trypanosome infection dissection

- Put the fly on its back in the middle of a glass slide with a drop of saline solution or phosphate-buffered saline (PBS) if available,
- looking at the fly through a dissecting microscope at low magnification, use a pair of fine, spring scissors to make an incision in each side of the abdomen close to the thorax,
- holding the thorax of the fly with one pair of fine forceps, use a second pair to grip the base of the abdomen and gently pull back the abdomen so that the "skin" slowly tears whilst the gut and other internal contents stretch out but do not break,
- in the drop of saline solution the salivary glands can usually be seen, one on either side of the gut, as clear, silvery transparent tubes; use one pair of fine forceps to carefully pick up each of these glands, pull from the fly and place on a separate part of the slide with a drop of saline and cover with a cover slip,
- remove the head and thorax of the fly to the other end of the thorax, cutting the gut close to the thorax,
- with the flat part of a mounted needle, squeeze the abdominal contents out from the "skin" into the middle of the slide,

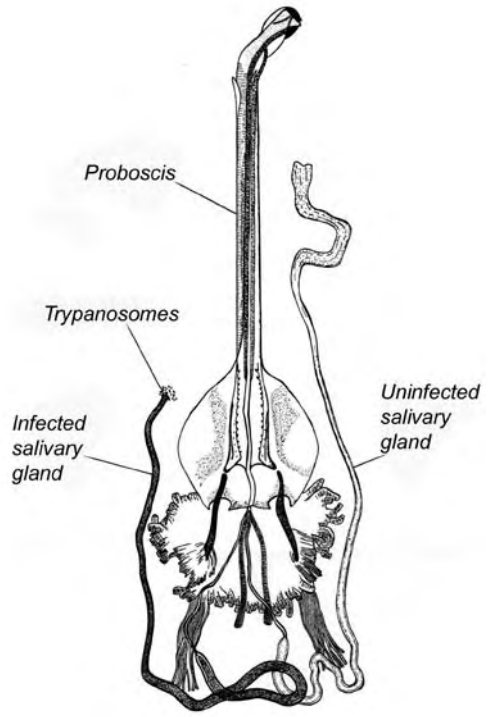
- discard the abdomen skin,
- place a cover slip over the gut contents and a drop of saline and press down gently,
- move the slide so that the end with the head is visible under the dissecting microscope; increase the magnification if necessary,
- cut the head of from the thorax and discard the thorax,
- with the head upside down on the slide hold the head with a pair of forceps and use the flat surface of a mounted needle (not the point) push down and forward so as to push the whole proboscis away from the head so that it detaches. The head can then be discarded or stored separately (for pteridine age analysis), whilst retaining the proboscis on the slide,
- increase the magnification as required so that the proboscis is clearly visible under the microscope. Use two mounted needles, one in each hand, to tease apart the labrum, hypopharynx and labium, starting from the end of the proboscis with the thecal bulb. Take care not to tear the structures rather than tease them apart, otherwise some tendon-like organs can sometimes be confused for the hypopharynx by an inexperienced dissector,
- discard the thick, brown labium as this will prevent the coverslip from sitting correctly over the mouthparts and will obscure vision,
- place a cover slip over the labrum and hypopharynx, with a small drop of saline,
- examine each of the organs, the labrum, hypopharynx, salivary gland and midgut for the presence of trypanosomes which, if present will be seen usually in motion, under a compound microscope using a 10× eyepiece and 25× objective, and
- record the presence of trypanosomes under the appropriate column of the recording sheet. If no trypanosomes are seen, record as negative.

FIGURE 3.11
Mouthparts (proboscis) of a tsetse fly, showing the labrum and hypopharynx (both magnified) with trypanosome clusters



Source: FAO 1982a

FIGURE 3.12
Diagram of the proboscis and salivary glands of an infected tsetse fly



Source: FAO 1982a

As the hypopharynx is a very fine transparent tube, avoid putting too much saline solution or the hypopharynx can float away and be difficult to relocate. It is helpful to place the hypopharynx across the labrum on the slide as they will usually stay together and avoid the problem of finding the hypopharynx after putting on the cover slip. If the proboscis is torn, rather than being teased apart, there are two transparent tendon-like structures that people may mistake for the hypopharynx; they should be easily distinguished, firstly because there are usually two of them seen rather than just one, secondly, because there is a tuft of (muscle) fibres at one end, and thirdly there are rings at intervals along their length, a bit like the rings on bamboo, whereas the hypopharynx is a plain, hollow transparent tube.

Trypanosome infections in the hypopharynx and labrum (proboscis) only, are classed as *Trypanosoma vivax*-type; in the proboscis and midgut only as *T. congolense*-type and in the proboscis, midgut and salivary glands as *T. brucei*-type. If an infection is detected in the midgut alone, that is classed as an immature infection (**Figure 3.11 and 3.12**)

Don't use needles that are too fine and sharp as they can tear the labium.

It is important to dissect out the hypopharynx as it is the site of maturation of *T. congolense* and *T. vivax*-type trypanosomes.

Good practice is to place the labrum and hypopharynx crossed over each other prior to dropping a cover slip on them as otherwise, when the cover slip is put on the hypopharynx may float away, and if it floats to the edge of the cover slip it is difficult or impossible to observe.

3.2.5.4.1. Salivary Glands

There are two main methods of dissecting out the salivary glands and either may be used according to personal preference. It is also usually possible to use the second method if the first fails.

Dissection method 1 — A simple and clean method, if it works, is to hold the fly by the thorax, on its back in phosphate buffered saline, after having removed the wings and legs and with a second pair of fine forceps held between the head and the thorax, but not closed or squeezing the neck gently and slowly move the head away from the thorax, moving the forceps from side to side to facilitate the gentle breaking of the external membrane of the "neck" without a sudden break which might jerk the head forward and break all the internal tissues including the salivary glands. If this is done carefully, the salivary glands will emerge cleanly, initially sticking together as one; the head can continue to be moved forward gently and a surprising length of salivary glands will emerge, separating into two distinct glands as they float in the phosphate buffered saline. After the first stage, in which the glands are thin, it becomes less likely that they will break and they can be pulled all the way out and covered with a cover slip. If however, they break they will retract into the thorax and abdomen and it will be difficult to retrieve them, although this is sometimes possible if they have not gone all the way inside the thorax — they are quite elastic. If the salivary gland do go back inside it is still possible to get them using the second method, as they have not disappeared.

Dissection method 2 — The second method is to make two small incisions on either side of the tergites close to the thorax (tergites 1–3), with the fly on its ventral surface. The "skin" of the abdomen is then pulled back with a side to side motion with forceps, whilst holding the thorax with a second pair. When the "skin" breaks, provided the dissection is done in a drop of phosphate buffered saline the internal organs should separate as they float, revealing the gut in the centre, with one salivary gland on either side. Sometimes, especially in an older, well-fed fly, they may be obscured by fat bodies and sometimes people mistake the malpighian tubules for the salivary glands. With a little experience however, the salivary glands are easily recognized by their translucent nature and structure of the outer membrane.

Don't let the salivary glands dry up as they are pulled out using the first method, as they will break very readily.

Don't pull them out to fast but be patient and slow.

When using the second method, again, make sure that the organs come out floating in saline.

The first method is preferable as there is less likelihood of trypanosomes from an infection of the gut being present in the phosphate buffered saline surrounding the salivary glands and being mistaken for a salivary gland infection.

3.2.5.4.2. Dissection of the Midgut and the Proventriculus

This dissection is the easiest to perform. After dissection of salivary glands using method 2, the residual contents of the abdomen can be put under a third cover slip after first removing the fat bodies and part of the hind gut.

Removal of the fat bodies and the hind gut, particularly in a recently fed fly will make examination easier as they will otherwise obscure the view, making detection of trypanosomes less easy.

Use light pressure on the cover slip to squeeze out the gut contents before examination.

Collection of samples for identification using PCR or other molecular diagnostic techniques — If facilities are available, or can be arranged for PCR identification, then the following methodology should be followed for collection of samples.

The standard procedure is to dissect out the organs of tsetse in which the trypanosomes are known to occur (mouthparts, salivary glands and midgut), and to grind these organs up before DNA extraction. In order to avoid contamination, the dissection instruments must be carefully cleaned between the dissection of each fly and also between the dissections of each organ of the fly. This cleaning is done first in a bath of sodium hypochlorite bleach, followed by rinsing in sterile distilled water. The proboscis is dissected from the fly first, followed by the salivary glands and lastly the midgut. After dissection, the organs (proboscis, salivary glands and midgut) will be stored separately; suspended in 50 µl of sterile distilled water in a sterile, sealed eppendorf tube, or in 70% alcohol before sample preparation. DNA extraction is now carried out using resins (Chelex®, Ready-Amp®) or commercial kits (Qiagen®, DNAzol-BD / polyacryl carrier®). The midguts and mouthparts need to be treated prior to the PCR but salivary glands can be processed directly after lysis in distilled water and/or by freezing/thawing (Lefrançois et al. 1998, Desquesnes and Davila 2002, Jamonneau et al. 2004).

How many flies should be dissected? — The answer to this question will depend on how many tsetse flies are expected to be caught. If the area is one of low density or if the procedures are being carried out after suppression, then a larger proportion (all live or fresh flies) will be dissected. The need is to dissect a sufficient number to provide a statistically acceptable estimation of the trypanosome infection rate or of the age structure of the population for each tsetse species. A target of 100 flies per sampling occasion period (or per month in the case of monitoring rather than survey) per species will enable seasonal patterns of infection or of changes in population age structure to be observed. There is generally small variation in trypanosome infection rates in a population over time in the absence of any external intervention. For age structure the dissections should be made over as short a period as possible. Dissection of 100 flies should be quite manageable in terms of the time required to dissect them, but even this relatively low number may be

hard to attain in some areas, in which case all live tsetse caught should be dissected. If there are distinct areas within the survey block, from which different infection rates might be expected, and if such a degree of epidemiological knowledge is required, then samples as above will be dissected from each such identified area. For most circumstances this will not be necessary.

3.2.6. Population Dynamics and other Entomological Assessment

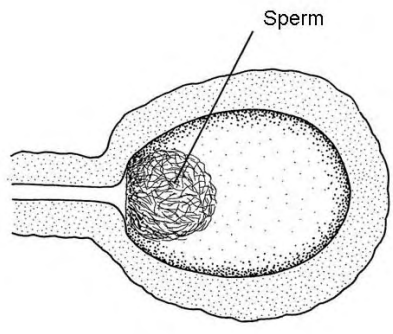
There are a number of techniques used for specific purposes that may or may not be included in a baseline survey, but are more likely to be employed during monitoring. However, it may be necessary to obtain such data during a survey for future comparison with post-intervention data.

Determination of insemination rates — by dissection of females and noting the proportion of the spermathecae that are filled with sperm (usually expressed in quarters). This is a simple dissection (**Figure 3.13)** that provides useful information for SIT programmes and does not require a great deal of skill.

Mortality rates — can be determined indirectly by determining changes in the population and the population age structure over time. Size frequency distributions, estimated from wing-vein length (hatchet cell length; **Figure 3.9**) can also be used to give an indication of mortality rates (see Leak (1998) for a review of procedures for determining mortality). The nutritional status of a population can be de termined by estimating the amount of fat and haematin (a product of blood digestion) in a sample of flies.

Temporal and spatial distributions and variations of the structure of a fly population — The spatial distribution of a tsetse population in grid squares/vegetation zones is important for the development of suppression strategies and for the selection of trapping

FIGURE 3.13
Appearance of sperm within the spermathecae for determining insemination rates



Source: FAO 1982a

sites for the monitoring programme. In practical terms, populations with a uniform distribution may be less complicated to suppress than a population aggregated into discrete foci (e.g. the Niayes infestation (orchards) in Senegal).

Calculating apparent density of *G. fuscipes* or other riverine flies based on a grid square value can be misleading as they are assumed to be relatively restricted to riverine vegetation cover.

Basic data on spatial distribution of tsetse in the survey area and their apparent densities determined at different seasons will enable the seasonal movements of tsetse to be assessed with respect to altitude and vegetation/climatic characteristics. These data will enable rates of dispersal throughout the area to be assessed. Analysis of the structure of the population in terms of its age and sex ratios will also contribute to planning of control/eradication.

3.3. GENETIC ANALYSIS

3.3.1. Objectives of Genetic Analysis in the Context of AW-IPM Programmes

One purpose of genetic analysis of tsetse flies may be to determine the degree of genetic isolation of neighbouring populations. This will contribute to determining whether or not a population is really isolated from another or whether there is gene flow, indicating movement of individuals between those tsetse populations. This is obviously important when considering AW–IPM, as re-invasion of cleared areas has to be prevented.

Despite the relatively low ability of tsetse flies to disperse, in comparison with other dipteran pests such as screwworm flies, there is a high potential for fly re-invasion into areas where control operations have been undertaken. Especially with *palpalis* group tsetse flies, which are considered to be quite restricted to riverine vegetation, it is often difficult to determine the extent to which they can disperse from one area of suitable habitat (river system) to another. An indirect method of determining the likelihood of this is through genetic characterization. Subpopulations, or demes, that exchange flies will be genetically much more homogenous (same gene frequencies) than those between which there is little or no genetic exchange (different gene frequencies). PCR techniques can now be used to rapidly characterize tsetse populations genetically, using either mitochondrial or microsatellite DNA markers (Solano et al. 1999, 2000, Krafsur 2003, Marquez et al. 2004). It is therefore desirable to collect samples for this purpose from the target area being surveyed and from neighbouring areas of tsetse infestation. Results of such analyses will contribute to confirming or otherwise, the assumed degree of isolation of the target population. Obviously this should be carried out early in the planning stage.

Recent studies using remote-sensing technologies have shown that in areas subject to human encroachment (in East and West Africa) tsetse fly populations become fragmented and in some cases isolated. Finding these "biological islands" by assessing their genetic isolation will undoubtedly help to target these populations for sustained vector control, possibly even eradication. However, such populations need to be identified and characterized prior to control operations. Molecular and morphometric techniques seem to have the potential to rapidly identify the levels of epidemiologically important population substructuring in tsetse vectors.