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The dissection of tsetse flies

Jérémy Bouyer, Wilfrid Yoni, Céné Bila, Marc Desquesnes and Laure Guerrini

Tsetse flies are the cyclic vectors of human and animal trypanosomoses in Africa. The trypanosome undergoes a development cycle in the tsetse fly before being transmitted to the host. Once infected, a tsetse fly remains infected throughout its life. The dissection of flies allows to investigate various organs for detection of trypanosomes. In females, it allows to determine their physiological age through the dissection of ovaries. In both sexes, the removal of certain organs such as the wings and the legs allows morphometric and genetic studies.

Dissection is carried out on anesthetized flies or freshly dead ones with the objective of searching for the possible presence of trypanosomes and the determination of the females' physiological age. Dissection requires suitable equipment and adapted procedures.

From capture to the dissection of tsetse flies

The capture of Tsetse flies requires an entomological survey protocol and the use of trapping equipment is described in the Geosaf technical guide No. 2. Tsetse traps can be deployed in different tsetse biotopes, and points of contact with their hosts (livestock, wildlife etc.) (Figure 1). In order for tsetse dissection to be successful, it is imperative that the latter not be dehydrated, a possibility if they stay for more than 2 or 3 hours in the traps' cage, fully exposed to the sun. To avoid this pitfall, cages should be harvested regularly, (every 2 to 4 hours if one wants to dissect all the captured flies) and placed in moist or cooled containers (figure 2). They have to be kept in these conditions for eventual transportation to the laboratory or for dissections in the field. In order to avoid damage to the flies during their removal from the cage, one should remove them using a test tube, or better still, anaesthetize them using the cold (20 minutes on ice or in a refrigerator at 4 °C, or 5 minutes in a deep freezer at –20 ° C).

Figure 1. Setting of an Epsilontrap. (Photo J. Bouyer)

Figure 2. Container humidification in order to preserve the insects. (Photo M. Desquesnes)

Equipment

- a binocular microscope (magnification of 6 x 10 = 60),
- a microscope (magnification of 40 x 10 = 400),
- Petri dishes,
- cover glasses and glass slides,
- fine forceps for watchmaker- no. 5,
- a test tube,
- Eppendorf tubes for collecting the infected organs,
- Wattman paper for blood meal collections,
- bleach and distilled water for cleaning,
- saline solution (Ringer's solution, refer to preparation formula outlined in table 1).

Table1. Ringer's solution preparation formula

Products	For 1000ml
Nacl	6,5g
Kcl	0,05g
$CaCl_2.2H_2O$	0,16g
$MgSO_4.7H_2O$	0,39g
$NaHCO_3$	0,20g

Prepare the solution to 900 ml using distilled water with a view to checking the pH (7.3), and then complete up to 1000 ml.

Figure 3. Dissection equipment.
(Photo J. Bouyer and C. Bila)

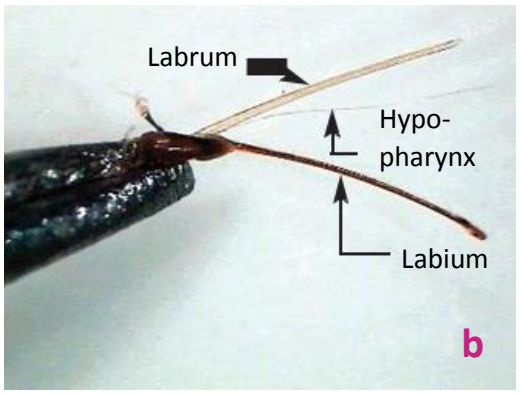
Detection of trypanosome infections

Removal of potentially infected organs

Dissection should always be carried out under a binocular stereoscopic microscope (magnification of X 60), the insect is placed in the petri dish, in a big drop of saline solution, which helps maintain the fly's organs in a hydrated and isotonic environment. If the fly is teneral, a slight pressure on the thorax releases the ptilinum (figure 4), an eversible pouch on the head. Dissection is unnecessary on a species that has not yet taken its first blood meal.

The dissection must be carried in the following order; start from the proboscis, then proceed to the salivary glands and finally end with the midgut so as to limit contamination of other organs by the large number of trypanosomes usually found in the latter. Thus, the proboscis is first firmly held on the thecal bulb (figure 5a) and removed; the labrum, hypopharynx and the labium are separated using a purse-string needle or directly using the tip of the fine forceps and placed between the cover glass and glass slide in one drop of saline solution (figures 5b and 7b).

Figure 4. Ptilinum.
(Photo J. Bouyer and C. Bila)



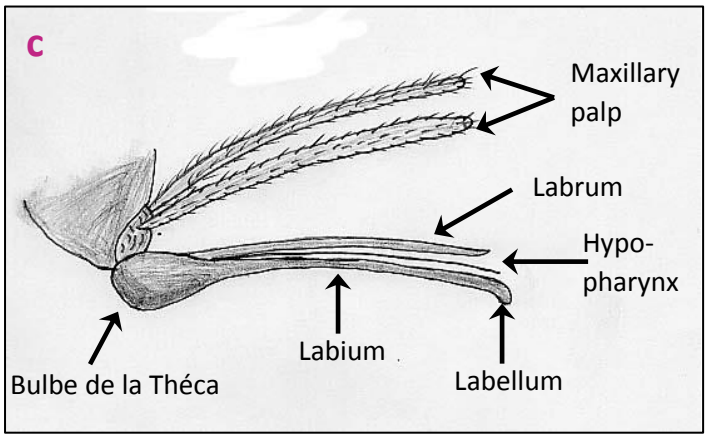


Figure 5. Dissecting proboscis:
a and b: removal
c: proboscis anatomy

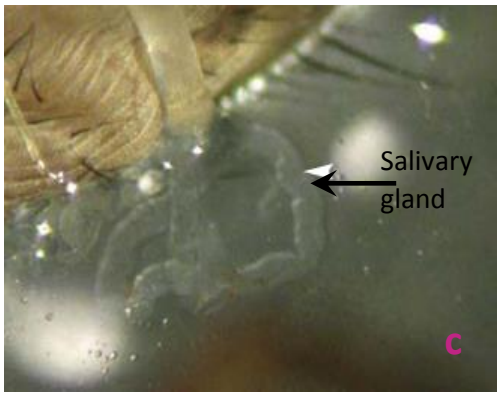


Figure 6. Opening-up the abdomen of a Tsetse fly:
a: the first abdominal segment is grasped.
(Photo W. Yoni and C. Bila)
b: it is then pulled back.
(Photo J. Bouyer and C. Bila)
c: the salivary glands now are visible in the anterolateral part of the abdomen.
(Photo W. Yoni and C. Bila)

After this procedure, the wings and the legs of the fly are removed from the body using the fine forceps (and can be collected in 90°c alcohol for population genetics studies), then the insect is placed on its dorsal side and held in place by the thorax in a big drop of saline solution for the extraction of the salivary glands and the midgut. The first abdominal segment of the ventral side (the first sternite) is grasped with the fine forceps (figure 6) and slowly and gradually pulled back to open the abdomen (figure 6b).

The salivary glands are retrieved from the lateral and anterior portions of the abdomen (figure 6 c) – these resemble two long and thin translucent tubes - and are then placed between the slide and coverslip in one drop of physiological saline. The midgut is then extracted from the abdomen and the attached Malpighian tubules and fat bodies are removed (figure 7a); the mid-gut is then arranged in a 'U' shape between slide and coverslip in Ringer's solution. All of the organs are then observed under the microscope (magnification X 400) for the detection of trypanosomes (figure 7b).

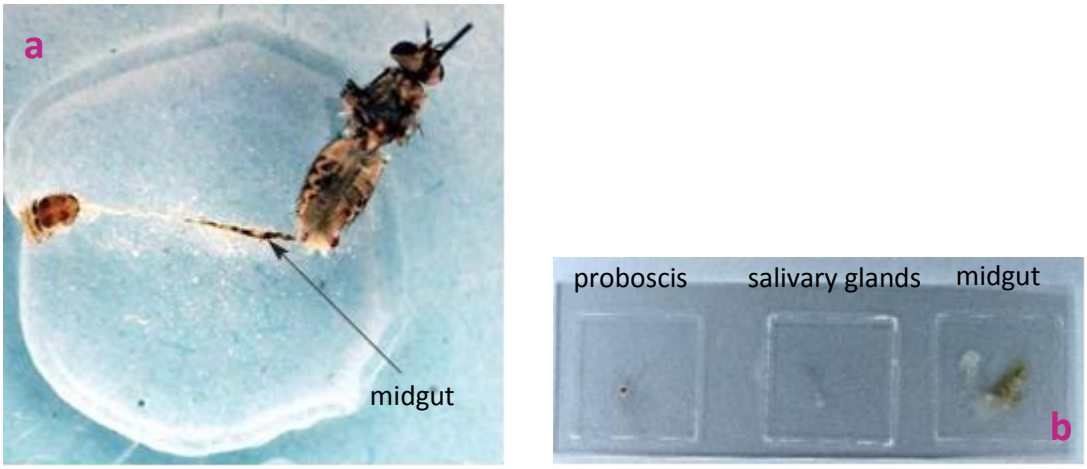


Figure 7. Harvesting of the midgut and placing organs on slide: a: dark midgut, b: placement of organs on a slide; From left to right: proboscis, salivary glands and midgut.
(Photos J. Bouyer and C. Bila)

An alternative abdominal dissection method can be used when one wishes to determine the physiological age of females.

Determining infection status

Trypanosomes' lifecycles differ depending on the species, thus, after a blood meal on an infected host, the spreading of the trypanosomes within the fly's organs allows us to determine the trypanosome species.

Trypanosoma vivax: Some trypanosomes ingested by the fly attach themselves to the walls of the food canal in the proboscis. They form colonies and multiply. The produced infective forms migrate to the hypopharynx where they settle and eventually form rosettes (figure 8a and 9b). Therefore these trypanosomes are only found in the mouthparts.

Trypanosoma congolense: after ingestion, this parasite first develops in the mid-gut and then migrates to the proboscis where it attaches itself to the wall where it multiplies. The infective forms migrate to the hypopharynx. Therefore these trypanosomes are only found in the intestines and in the mouthparts (Figure 9b).

Trypanosoma brucei: Their cycle is much more complex. After ingestion, these trypanosomes follow the route taken by the blood meal to eventually transform into procyclic forms in the midgut, and then into infective metacyclic forms in the salivary glands, and then migrate to the hypopharynx. Some Trypanosomes are therefore found in the midgut, the salivary glands (figure8 b) and the mouthparts (figure 9 c).

The presence of trypanosomes only in the proboscis consequently suggests a *T. vivax* infection, a midgut infection and proboscis suggests a *T. congolense* infection and an infection in all three organs (proboscis, salivaryglands and midgut) suggests a *T. brucei infection.*

However, microscopic examinations can give misleading results, because sometimes an organ's infection can go unnoticed or is temporary. As a result, this diagnosis is only indicative and must be confirmed by a laboratory PCR (Polymerase Chain Reaction) test using specific trypanosome primers.

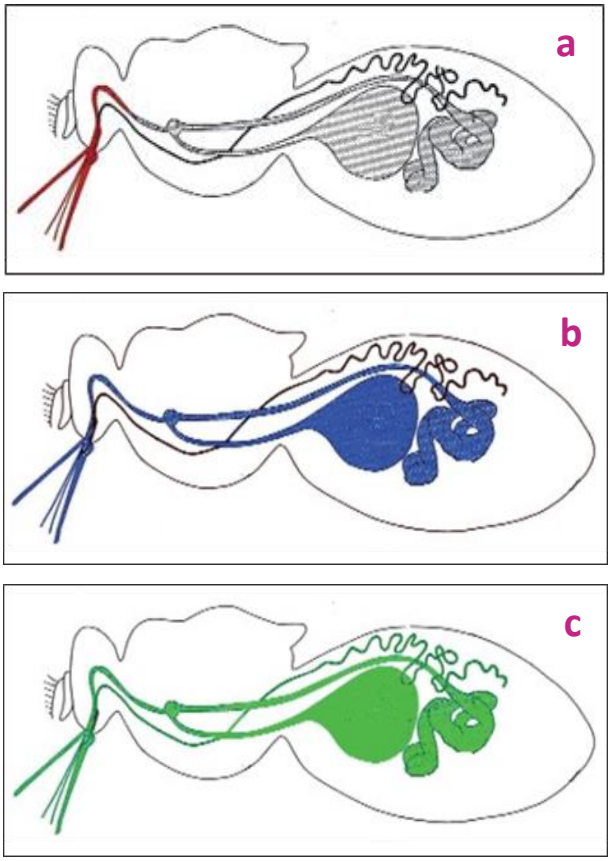


Figure 9. Anatomic location of trypanosomes in tsetse :
a: *T. vivax* (only in the proboscis);
b: T. *congolense* (intestine +/-proboscis);
c: *T. brucei* (intestine +/-salivary glands +/-
(Illustration D. Cuisance)

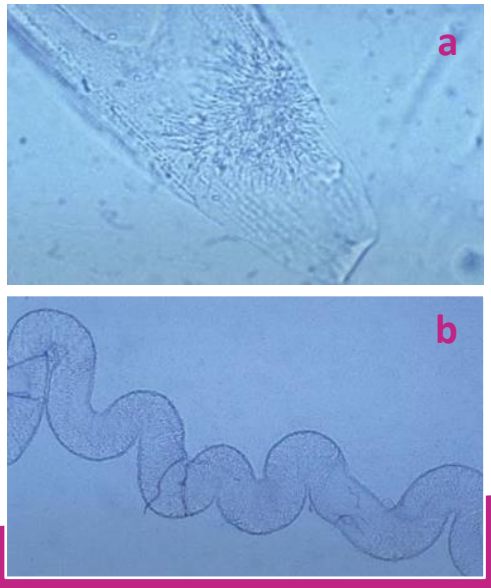


Figure 8a. Trypanosomes observed microscopically in tsetse organs: (a) A rosette of *T. vivax* in the hypopharynx; (b) *T. brucei* found in salivary glands.
(Photo by D. Cuisance)

Dissection of female tsetse for ovarian ageing

Dissection routine

This alternative dissection method of the abdomen is better suited for females because it involves the extraction of the reproductive tract and the determination of their physiological age.

Make an incision on either side of the fifth or sixth tergite (figure 10a) using the fine forceps. 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. Then pull back the cut part of the abdomen slowly, to reveal the female reproductive tract. This will help determine the physiological age of the female (figure 10b). The method described in the previous section is used to sample other organs suspected of harbouring trypanosome infections (proboscis, salivary glands, midgut, etc).

Figure10. Dissecting the female reproductive tract:
a: incision of the abdomen
b: extracting the reproductive tract
(Photo W. Yoni and C. Bila)

Determination of the females' physiological age

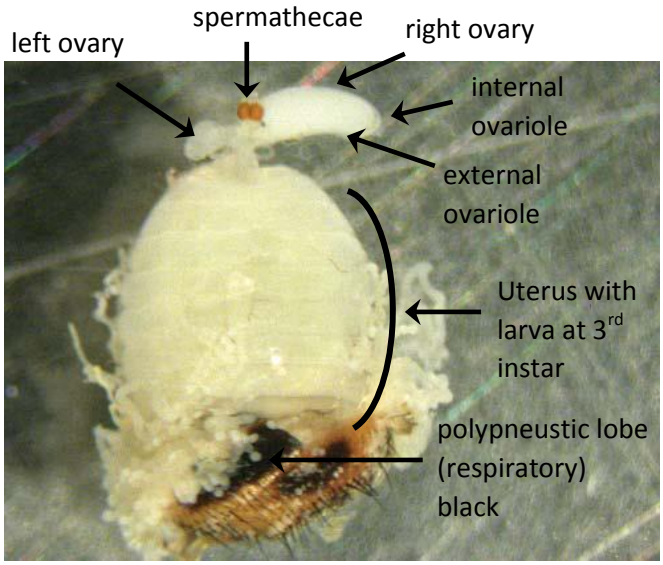
For estimating the age of female flies there is a much more accurate method than the wing fray method used for male flies. This is the ovarian analysis method. It involves dissection to examine the ovaries and uterus. Although it is more complicated because dissection is involved, the age of individual flies can be estimated.

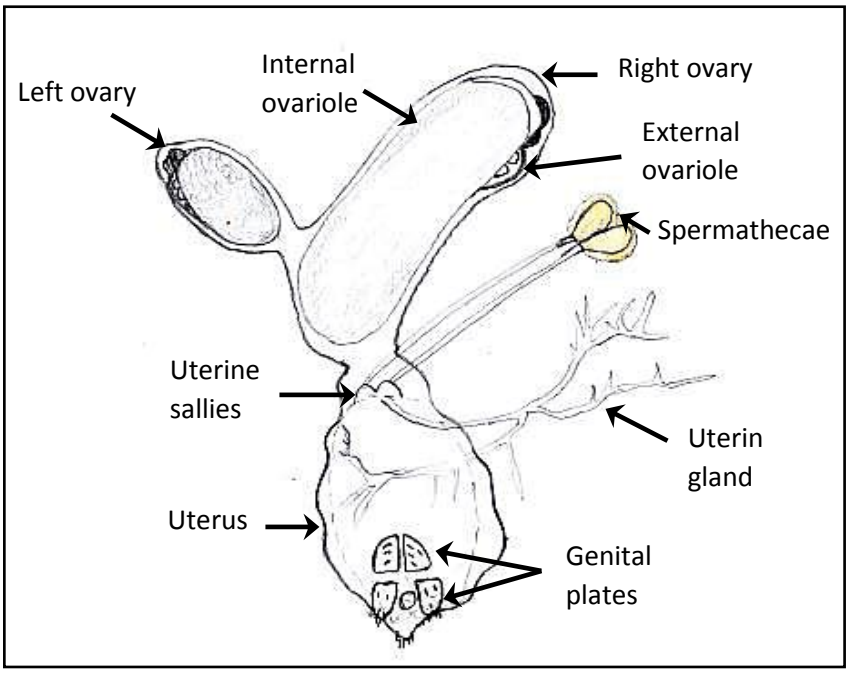
The reproductive tract of female tsetse flies consists of two dissymmetrical ovaries (left and right) due to a developmental difference; each of them has a translucent ovarian sheath containing two ovarioles of different sizes; an internal ovariole (IO) and an external ovariole (EO) (figure 11). Each ovary has two ovarioles, so the female has a total of four ovarioles. The oocytes mature separately and in a regular sequence, so that only one egg is passed into the uterus at a time. The eggs pass from the ovary to the uterus through a pair of oviducts;

these muscular tubes squeeze the mature egg down to where they join as a common oviduct and then into the uterus.

There are two spermathecae present, connected to the uterus by a duct, their role is to act as a sperm reservoir, which they preserve throughout the life of the female, which mainly mates once in general.

The tsetse fly's uterine gland, which is also connected to the uterus, synthesizes a secretion which feed the developing larva in the uterus, (figure 11). The tsetse fly's reproductive tract has a unique way of functioning, thus allowing us to easily determine the ovarian cycle of the fly, which can subsequently translated into the age of the fly (here presented for a temperature of 25°c, but the duration of each cycle varies with temperature). The young female fly has four ovarioles of different sizes when it leaves the puparium.





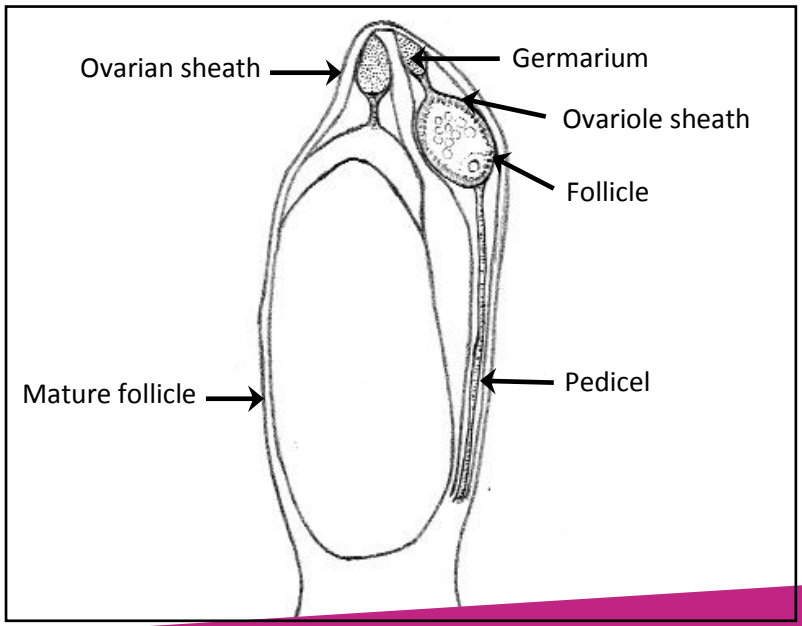
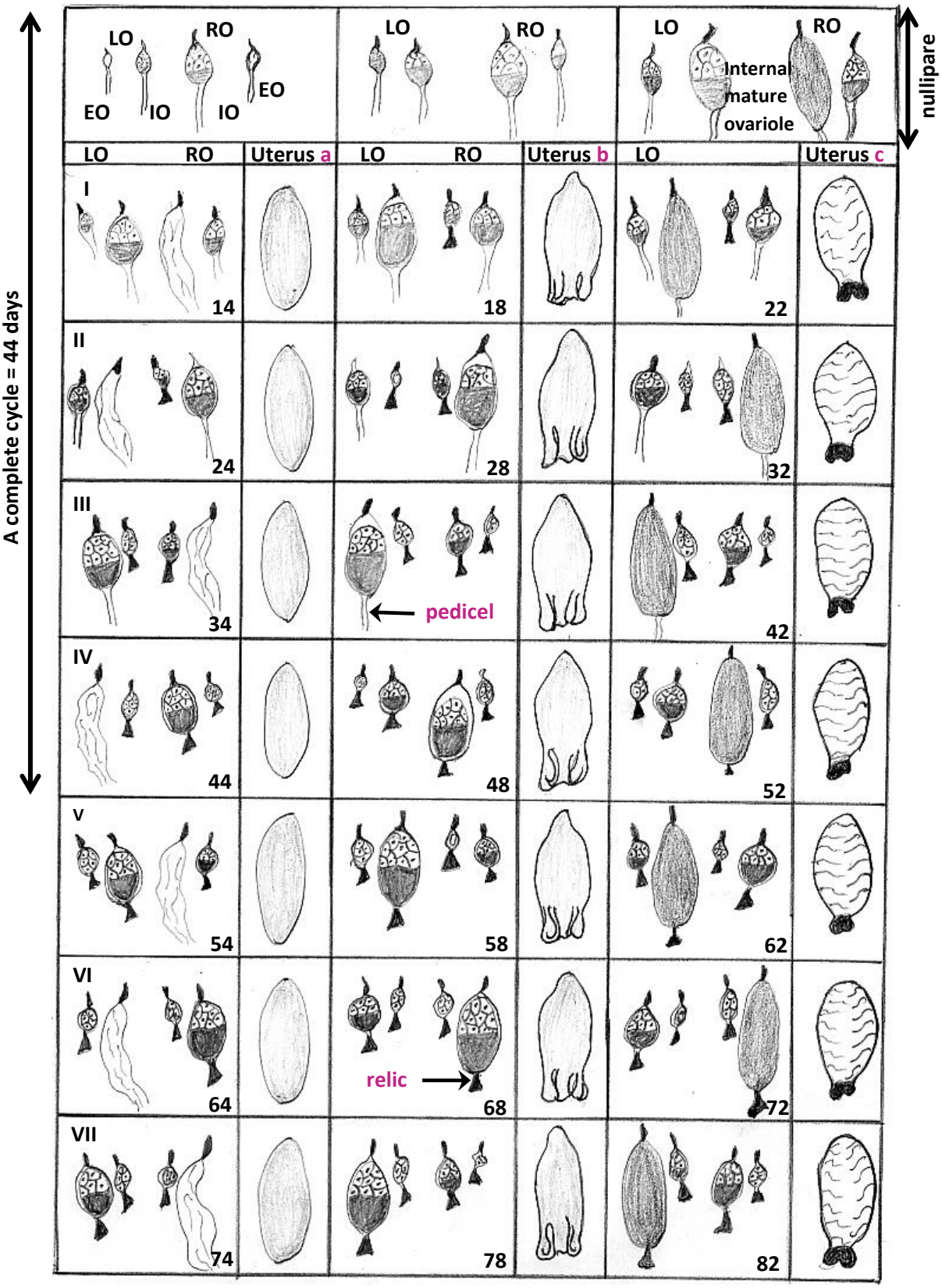


Figure 11. Tsetse fly reproductive tract.
(Photo and drawings W.Yoni and v. Bílá, according to J. Itard, tsetse flies, 1986)

The largest of the oocytes, which matures first, is always the internal ovariole of the right ovary, successively followed by the internal ovariole of the left, the external ovariole of the right and finally external ovariole of the left. The four ovarioles develop successively and reach maturity in the same order. The oocytes mature separately and in a regular sequence, so that only one egg is passed into the uterus at a time. A mature oocyte passes down to the oviduct by tearing itself from the distended follicular tube. It is the upper fragment of this tube that retracts into a wrinkled mass that makes up the follicular relic (or scare). In this way a single egg is produced in the female fly at intervals of about 9–10 days (more or less) at 25°c and the same ovariole produces a mature egg approximately every 40 days.

When determining age, the genital tract is extracted with the posterior abdomen (figure 10b) and stripped of fat tissue. Then turn the genitalia until its ventral side faces downwards (see figure 11), identify the biggest ovariole, and then use 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. You must also observe the uterine contents to see whether the uterus is empty or has 1st, 2nd or 3rd stage larva) and finally read the following table to be able to determine the fly's physiological age (See box 1).



Box 1. Ovarian age categories in female tsetse.

LO = left ovary;
RO = right ovary.
Each box describes the ovaries' evolutionary stages, from left to right:
- external ovariole to the left,
- internal ovariole to the left,
- internal ovariole to the right,
- external ovariole to the right.

The indicative age in days for a temperature of 25°c is found at the bottom right of the illustrations.
Upper-left in the first column, number of cycles in Roman numerals.
(drawings W. Yoni / source J. Itard, 1966).

0' Age group: In nulliparous females, ovulation is yet to take place (four pedicels can be seen):

-d5: the female has the biggest internal ovariole to the right;
-d8: the largest follicle (internal ovariole to the right);
-d12: mature egg, internal ovariole to the right ready to descend into the uterus.

'I to VII' age group: ovulation and the three larval development stages can be seen by observing the uterus:

-a: the uterus contains an egg (the ovary which had the last ovulation contains an open sack): simple ovoid shape;

-b: the uterus contains an immature larva (stage I or II), the last ovulation's ovariole contains a follicular relic (see day 48 box 1). Respiratory lobes visible, but they have same colour as the rest of the body;

-c: the uterus contains a mature 3rd stage larva (complete growth, black respiratory lobes and one of the ovarioles has reached maturity).

Some features of the reproductive organs do not appear in the table above (Box-1):

-post larval stage: the uterus is empty due to a recent larviposition. A developed egg ready to ovulate can be seen in the ovaries. A follicular relic can be found at the base of each of the four ovarioles (depending on the fly's age and the preceding ovulation);

-abortion: the uterus is empty due to an interruption of the normal cycle, this scenario is shown by the presence of a relic(s), ovarian configuration (the relative size of the four follicles) and the presence of an ovarian follicle that did not reach its mature stage during ovulation. The abortion rate is very important to evaluate to infer the competitiveness of sterile males using Fried's index.
Since the ovulation cycle resumes from stage V, the presence of follicular relics allows us to make distinctions between the following age groups; I and V, II and VI and III and VII. Beyond stage VII, distinctions are no longer possible, in such cases, one will then need to take into account other features of the fly such as wing wear and tegument firmness. It should also be noted that in practice, it is extremely rare to find tsetse flies that are older than 82 days under natural conditions.

Wing fray analysis and age determination in males

Sampling and methodology

With a pair of fine (watchmakers) forceps remove each wing from the fly, pulling from the base of the wing and place the wings on a slide and place a coverslip on top for geometric morphometrics analyses. Photograph and analyse the wings using specialized software (http://www.mpl.ird.fr/morphometrics), which allows obtaining the coordinates of the landmarks on the wings and conduct their analysis (Fig. 12). It is therefore possible to determine the species, sex and morphometric features of a tsetse fly from a digital photograph of the wings, and even to calculate distances between populations. In order not to alter their transparency, the wings are placed dry between two brackets glued together with Canada balsam, be careful not to apply it on the wings. Using the software, nine distinctive points located at the intersections of the wing veins on the digital image can be digitized and are denominated “landmarks” (figure 12).

Statistical analyses are then performed on the coordinates of the landmarks (procrustes superimposition, Mancova, symmetry size and shape, discriminant or principal component analyses).

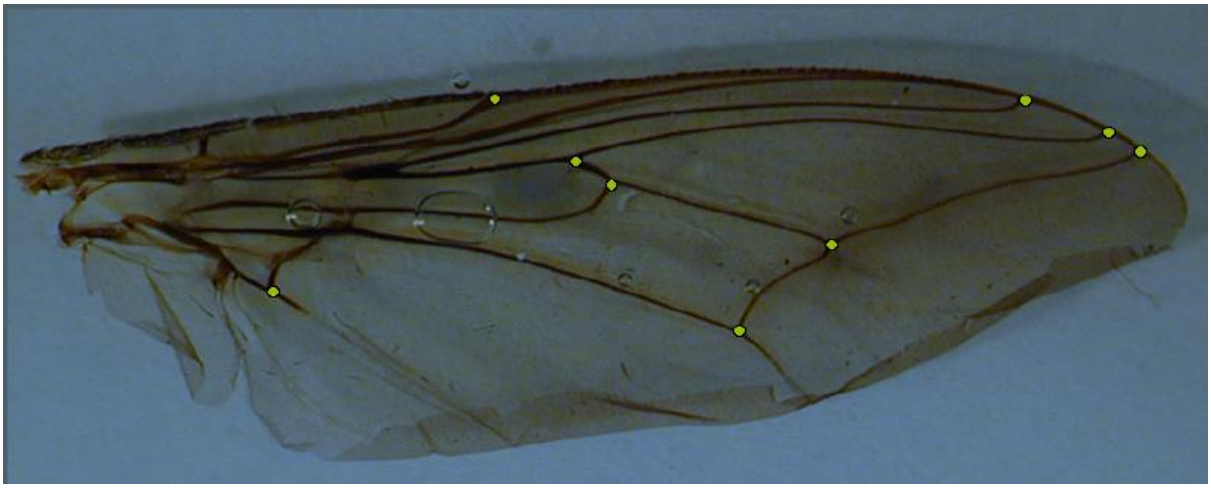


Figure 12. Photograph of a wing and location of 9 landmarks used for geometric morphometrics analyses.
(Photo P. Solano)

Determining age in males

Tsetse flies' average survival age is 90 days. In males, six categories of wing wear or Wing Fray (WF) have been calculated and defined, which now allow us to define a tsetse sample's average age (figure 13).

-1st category: Wings are perfect, with the whole hind margin of the wing intact;
-2nd category: hind margin showing very slight or suspiciously genuine damage such as might be caused during capture;
-3rd: definite wear, but confined to that part of the wing close to the notch where the vein joins the wing edge;
-4th category: shows some fraying both before and beyond the notch, but with long, undamaged sections;
-5th category: hind margin presenting saw-edged appearance, without long, undamaged sections;
6thcategory: hind margin showing heavy damage, including rounded indentations or large portions of the wing missing, giving a tattered appearance to the wing.

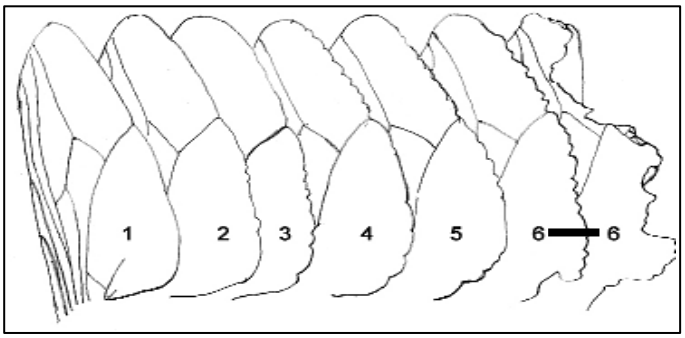


Figure 13. Tsetse wing fray categories.
(Drawing W. Yoni, according to Jackson, 1946)

The number of flies in each category is multiplied by a coefficient attributed to it (table 2), the product for all categories is then totalled, and the total divided by the number of flies in the sample to give the mean wing fray value (MWFV). The MWFV is then converted to a mean age of the sample by reference to the table below:

Table 2. MWFV calculation example for male tsetse flies

Wing fray category	Number of flies per category	Coefficient	Product
1	12	1,0	12
2	8	2,0	16
3	1	3,0	3
4	5	4,4	22
5	4	5,5	22
6	3	6,9	20,7
	33		95,7

MWFV = 95,7/33 = 2,9

Table 3. MWFV conversion table into the average male tsetse population age. (source: Tsetse fly control manual/volume 1/ FAO)

MWFV	Est.Age	MWFV	Est.Age	MWFV	Est.Age	MWFV	Est.Age
1,6	11d	2,8	21	3,9	31	5,1	41
1,8	12	**2,9**	**22**	4,0	32	5,2	42
1,9	13	3,0	23	4,2	33	5,3	43
2,0	14	3,1	24	4,3	34	5,4	44
2,1	15	3,3	25	4,4	35	5,5	45
2,2	16	3,4	26	4,5	36	5,6	46
2,3	17	3,5	27	4,6	37	5,8	47
2,4	18	3,6	28	4,7	38	5,9	48
2,6	19	3,7	29	4,8	39	6,0	49
2,7	20	3,8	30	5,0	40		

From the example, a MWFV of 2.9 converts to a mean male age of 22 days. (Table 3).

The method cannot be used to age individual flies as it is not sufficiently precise, but is used to obtain the mean age of a sample (/ILCA/ICIPE network training manual 1983).

It is possible to calibrate the method for a given environment by comparing the physiological age of females and their wing fray.

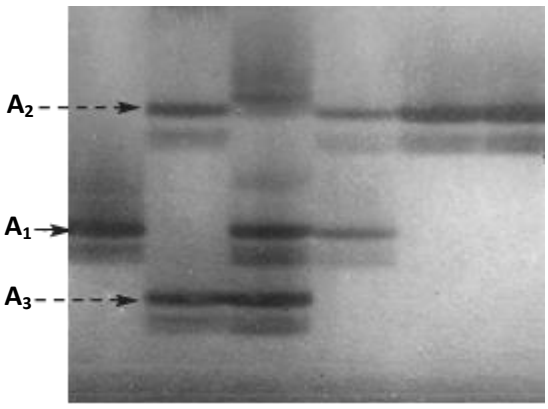
Other sampling techniques

Leg dissection for genetic analysis

A population genetics analysis of the tsetse fly and sequencing can be performed through the sampling of legs. The legs are removed using forceps and placed in Eppendorf tubes with 70° alcohol, the forceps must be thoroughly cleaned with bleach and distilled water after each harvest/ smearing.

The use of microsatellite DNA markers (figure 14) allows detection of gene flow between populations.

The results of such analyses will contribute to confirming or otherwise, the assumed degree of isolation of interaction of the target population. This allows us to estimate the percentage of 'foreign individuals' per generation in a given tsetse population. Such studies are very beneficial for the implementation of sequential eradication campaigns such as Area Wide Management (AWM) that make use of the Sterile Insect Technique (SIT).



Individuals' Genotypes	A_1A_1	A_2A_3	A_1A_3	A_2A_1	A_2A_2	A_2A_2

Figure 14. pGp 29 microsatellite loci migration in 6 individuals of *Glossina palpalis gambiensis*: interpretation of the individuals' genotype in denaturing polyacrylamide gels. (Photo S. Thévenon)

Sampling and analysis of blood meals

Both tsetse sexes are hematophagous and they feed on a variety of host animals, so the analysis of undigested blood meals collected during dissection will allow to determine what host animals are being fed on by tsetse flies in an area, and to obtain some idea of the preference in tsetse for certain food. This provides us with:

-a better understanding of tsetse ecology and tsetse behaviour in a given biotope;
-guidance on the tsetse's trophic preferences;
-guidance on the potential parasite hosts (trypanosomes carriers);

-relative epidemiological importance of the various species.

In order to identify what host animals are being fed on by tsetse, a portion of the intestine containing undigested blood meal is removed and smeared onto Whatman Grade 1 filter paper, with information on the fly as age, sex and species, location and date of capture (figure 15). A serological technique, using species specific antibodies (humans, domestic animals and wild fauna) or PCR using specific primers will then identify the host animal being fed on by tsetse.

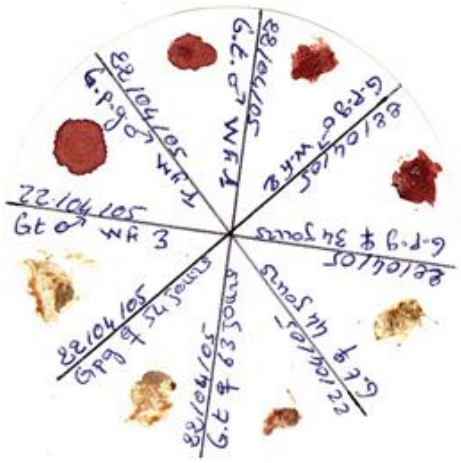
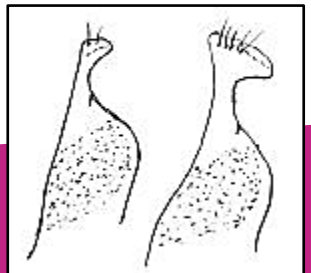


Figure 15. Preparing blood meal smears on filter paper.
(Photo W. Yoni)



Dissecting the male reproductive tract for taxonomy

The male tsetse presents at the ventral side of the posterior abdomen a tumefaction: the hypopygium that is in fact the folded male terminalia, just below abdominal sternite 5, which has hairy plates called the hectors (he).

The epandrium (ep) is a remnant of the tenth segment, enclosing the phallic apparatus and also containing the anus (an), forms a protrusion of the abdomen, allowing a quick distinction between males and females (figure 16) in the same way that the genital plates (gp), which are visible under a binocular microscope, allow differentiation between species in females of the same group.

Male reproductive organs' removal can be carried out for taxonomic purposes, the type of superior claspers in male (in the form of claws, the gap between the superior claspers is filled by the median lobes or the gap between the superior claspers is filled with membrane) help to distinguish the three species groups (subgenera and species) (figures 17 and 18).

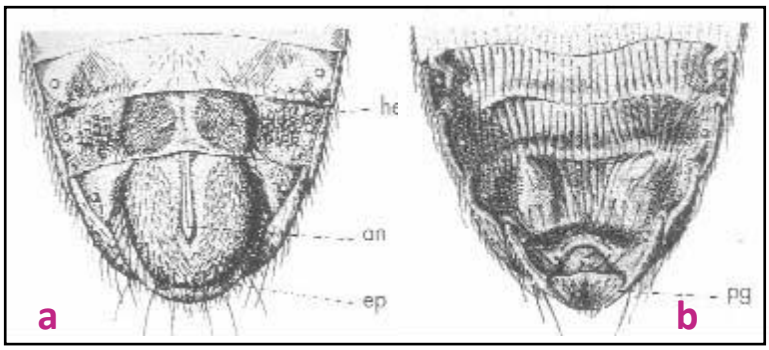


Figure 16. Ventral aspect of the terminal portion of the abdomen of both sexes abdomen of Tsetse flies:
a. male - female b.
(Image source: tsetse flies, HAT vectors: Biology and OCEAC-control-IRD, 2000)

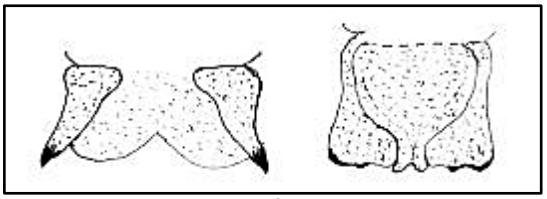


Figure 17. The types of superior claspers as shown in Nemorhina (a) and Glossina (b) subgenera
(Drawing W. Yoni; tsetse flies, HAT vectors: Biology and OCEAC-control-IRD, 2000)

Figure 18. The different shapes of the inferior claspers of *G. palpalis palpalis*, and *G. p. gambiensis* (b).
(Drawings by W. Yoni; tsetse flies, HAT vectors: Biology and OCEAC-control-IRD, 2000)

Conclusion

The dissection of tsetse flies is relatively easy, but it requires a good knowledge of tsetse anatomy and regular practice so as to be conversant with different scenarios and be prepared for any eventuality in both age determination in females and Wing Fray analysis in males. Entomological studies and dissections carried out in the field are a quick way of obtaining information relative to the health and disease situation of a given area, but these must all be connected to epidemiological surveys to estimate herd health and identify risk factors for selected diseases such as trypanosomosis. They are also very usefull to measure the impact of various control methods on the dynamics of tsetse populations.

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This manual is for policy makers, researchers and vector control specialists/ field workers.

This document was been produced with the assistance of the European Union, ACP Group of states in the framework of the project Geomatic technology transferred to animal health in southern Africa (GeosAf). Its contents only reflect the views of the authors and cannot be taken to reflect the position of the European Union

Jérémy Bouyer,[a,b,c,d] Wilfrid Yoni[e], Céné Bila[e], Marc Desquesnes[d,f] and Laure Guerrini[g,h]

a Institut Sénégalais de Recherches Agricoles (ISRA), Laboratoire National d'Elevage et de Recherches Vétérinaires, BP 2057, Dakar – Hann, Sénégal. b Unité Mixte de Recherche Contrôle des Maladies Animales Exotiques et Emergentes (CMAEE), Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD), 34398, Montpellier, France. c Unité Mixte de Recherche 1309 Contrôle des Maladies Animales Exotiques et Emergentes, Institut national de la recherche agronomique (INRA), 34398, Montpellier, France. d Unité de Recherche INTERTRYP, Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD), 34398, Montpellier, France. e Centre International de Recherche Développement sur l'élevage en zone subhumide (CIRDES), 01 BP 454, Bobo Dioulasso, Burkina Faso. f Faculty of Veterinary Medicine - Kasetsart University - Department Parasitology - Paholyothin road - Chatuchak - 10900 Bangkok Thaïlande. *g* Unité de Recherche AGIRs, Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD), 34398, Montpellier, France. h Department Environment and Societies, University of Zimbabwe, P.O. Box 1378, Harare, Zimbabwe.

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