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A morphological study of phlebotomine sandflies and their role in the transmission of human leishmaniasis, with special reference to Namibia

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cover slip was carefully lowered into place on top of the gut it did not crush it. This was important as it is of interest to note the site of the infection in the gut (ie. foregut, stomodael valve, oesophagus, hindgut) or malpighian tubules, should it be infected. If there is too little saline the parasites are flushed from the gut. The gut was then examined for the presence of promastigotes, using a compound microscope with phase contrast and a 40x objective.

2.1.3 Isolation of *Leishmania* from Phlebotomines.

When the dissected gut was positive the circumference of the coverslip was flooded with sterile saline causing the coverslip to float so enabling it to be carefully removed. The coverslip was then flushed with sterile saline. The gut was teased apart with a sterile syringe needle (26 gauge), so as to release the promastigotes. The saline solution plus parasites was then drawn into a 1ml (tuberculin) syringe and inoculated into McCartney bottles containing blood slants of *ELON'S medium. Preferably 3-4 bottles per positive gut were inoculated so as to insure against loss through contamination. Providing care was taken to flame the slides, microneedles, syringe needles and McCartney bottle tops, relatively few cultures were contaminated when using the above method.

* The ELON'S medium, a modified NNN medium, was made in the SAIMR Media Department with defibrinated rabbit blood to which is added 20µg/ml of 5-fluoro-cytosine and 30 units of crystalline penicillin per ml to both the slant as well as the sterile saline overlay. The medium was incubated at room temperature for two to three days before use.

2.1.4 Age grading.

Age grading is the term used for the techniques developed to assess the age of an individual. Assessing the age of a pre-adult individual is usually not difficult but in order to understand the ecology of a species it is necessary to try to determine the age structure of the adults in a population. There are only two reliable age grading methods (Tyndale-Biscoe, 1984), one is based on cuticular growth and the other on the accumulation of follicular relics or dilatations in the ovarioles of individuals as a result of repeated ovipositions (Detinova, 1962). The latter method was the one used by myself having gained some training and experience from T J Wilkes (of the London School of Tropical Medicine and Hygiene, London, U.K.) when on a field course in southern France during 1980.

As previously described, the genital segments were detached from the gut and placed in a drop of saline on a separate slide. The ovaries were removed from the surrounding chitin which is discarded. Using natural light reflected from below, follicular relics in ovarioles are looked for [as first described by Polovodova (1949)] by stretching the ovarioles. Follicular dilatations are difficult to dissect out as the intima (inner wall surrounding each ovariole) is

very fragile and easily broken. Hence it is necessary to examine a number of ovarioles and a constant number of dilatations need to be recorded. An estimate of the number of ovarian cycles can be determined by counting the number of dilatations of a number of ovarioles.

2.2 LABORATORY TECHNIQUES.

The techniques used for mounting phlebotomines for taxonomic study follow:-

2.2.1 De-oiling.

Phlebotomines collected using the oiled card method need to be de-oiled before being cleared and mounted. I found the best method to be as follows:-

a) transfer specimens from the 70% ethanol (in which field sample is preserved) to lactic acid (conc.) for 15 minutes.

b) transfer to xylene for 15 minutes.

c) transfer to clean lactic acid for 15 minutes.

d) transfer to teepol (or equivalent detergent).

e) then wash twice in clean lactic acid and allow to clear as described in the following paragraph.

2.2.2 Clearing.

It is necessary to clear the chitin of the exoskeleton in order to see the morphology of certain internal structures ie. spermathecae and pharyngeal and cibarial morphology. The traditional solution for fleas and lice and some flies is 10% potassium hydroxide (KOH). However, although phlebotomines can be cleared with KOH it is very harsh and if the specimens are left in it for too long the spermathecae may disintegrate or become invisible. It was only used on flies that had been stored in 70% Ethanol for more than 1 year as these were difficult to clear. The following methods were preferred for fresh specimens:-

either

a) clear in Lactic Acid for 1 - 2 hours;

b) wash in distilled water before mounting in Faure's Gum Chloral.

or

a) clear flies in the solution *Marc André for a minimum of 2 hours but not longer than 24 hours (as rupturing of the thorax sometimes occurs) before mounting in Faure's Gum Chloral.

* this solution is made as follows:-
a) dissolve 40g Chloral Hydrate in 30ml distilled water,
b) when dissolved add 30ml Acetic Acid.

2.2.3 Mounting.

The chitinous exoskeleton of sandflies is very fragile. When mounting specimens in other than aqueous solutions, care must be taken to dehydrate them very slowly, otherwise the specimen will collapse and distort. For this reason an aqueous mountant is prepared. Its disadvantage, however, is that it is necessary to ring the coverslip with a non-aqueous solution so as to prevent the mountant dessicating (see Ringing Agents). The aqueous mountant, used here, is useful for rapid mounting and remounting of specimens and provides a clearer image of both the internal as well as the external morphological features. The mountant is a modified Faure's Gum Chloral (also known by some authors as Hoyer's medium) and similar to Berlese's Gum Chloral. It is made up as follows:-

a) 30g of Gum Arabic (it is important to pick large lumps of Gum as the powdered form does not make a satisfactory medium) is dissolved in 50 ml of distilled water;

b) when completely dissolved 20ml of glycerine is added and mixed well;

c) to this is added 200g of Chloral Hydrate which is stirred in and may take two to three days to dissolve completely; it should be stirred occasionally.

d) the solution is then filtered through a scintered glass filter and stored in an airtight container.

I have never had much success with mounting of phlebotomines in Canada Balsam as the specimens nearly always collapse and were distorted. The internal structures were not as clear when mounted in Balsam as they were in Faure's. I did, however, have limited success using the following recipe:-

a) dehydrate the specimens slowly through increasing concentrations of Ethanol commencing at 20% and transferring every 15 minutes (minimum) to 40%, 50%, 70%, 80% (twice), 96% (twice), 100% (twice),

b) rinse in cedar wood oil, clove oil (both make specimens very brittle) or preferably Beechwood Creosote (if clove oil or creosote are used the 100% Ethanol dehydration step is unneccessary),

c) mount in Canada Balsam.

Phlebotomines are mounted laterally, their heads are severed from the body and orientated such that the ventral aspect faces the coverslip. They are dissected and positioned in a drop of mountant and allowed to dry overnight at 37°C in an incubator, before additional medium and the coverslip are added. The completed mount

is then dried at 37°C for 2 - 4 weeks before a ringing agent is applied.

2.2.4 Ringing Agents.

When using aqueous mountants like Faure's it is necessary to ring the coverslip in order to prevent the mountant drying out. Two commercially available agents were used to seal mounts, these were ENTELLAN (Merck product) and GLYCEEL (Gurr product). As I use 18mm diameter coverslips, mounts are readily ringed using a ringing stage (turntable) and a size two artist's brush.

2.2.5 Cryopreservation of *Leishmania*.

Cryopreservation has many advantages over continuous animal passage, or culture on artificial media, as it preserves unchanged the physiological characteristics of individuals as well as reducing the likelihood of the introduction of a contaminant. The cryopreservation of ***Leishmania***, isolated from phlebotomine sandflies in Namibia and grown on artificial medium, was carried out as follows:-

a) using a 1ml tuberculin syringe 0.1ml Glycerol was added to a 1.8ml Nunc plastic screw-top vial which was then autoclaved (at

121°C for 15 minutes). Using a disposable syringe, 1ml of the supernatant (of the ELON'S medium) containing the growing promastigotes was drawn off and introduced to an autoclaved Nunc vial containing glycerol. Care was taken to mix the supernatant and the glycerol well by agitating the vial by hand. Nunc vials were then suspended in the vapour phase in a liquid nitrogen container.

Reconstituting the stabilate (frozen leishmanias) was performed every 8-12 months as follows:-

a) the Nunc tubes were removed from the liquid nitrogen and placed in running warm water (32-35°C) until liquid,

b) The unfrozen stabilate was then inoculated into ELON'S blood culture medium.

In some cases, within as little as a few weeks the growth was such that it was possible to refreeze the promastigotes.

CHAPTER THREE

SYSTEMATICS OF THE *SYNPHLEBOTOMUS* COMPLEX.

3.0 OVERVIEW.

The subgenus ***Synphlebotomus*** of ***Phlebotomus*** comprises species which are either known or suspected vectors of visceral and dermal leishmaniases in the Afrotropical Region (except for two extralimital species). There are nine described species belonging to this subgenus. These are *P. **grovei***, *P. **katangensis***, *P. **rossi*** and *P. **taylori*** in southern Africa; *P. **celiae***, *P. **martini*** and *P. **vansomerenae*** in East Africa; and *P. **ansarii*** and *P. **eleanorae*** from Iran and India. This work concerns the discrimination of the southern African and the East African species and for this purpose this chapter is divided in two; Section 1 pertaining to Southern Africa and Section 2 to East Africa. ***Phlebotomus ansarii*** and *P. **eleanorae*** are extralimital and are excluded from this work.

SECTION ONE - Southern Africa.

3.1 INTRODUCTION.

In 1972 Abonnenc synonymised the species **Phlebotomus rossi** (De Meillon & Lavoipierre, 1944) with **P. katangensis** (Bequaert & Walravens, 1930). The former was described from a single male specimen collected from Mutare (Umtali), Zimbabwe, and the latter from two male specimens from Lubumbashi (Elizabethville), Zaïre. No further specimens of **P. rossi** were collected until entomological investigations on the dissemination of CL in SWA/Namibia, were undertaken. In 1974 a sample of **Synphlebotomus** was collected in the Karasberg mountains in the south of the territory. It was initially thought to be a new species. Lewis & Ledger (1976), in acquiring the original drawings of both species, decided that the SWA/Namibian specimens compared with the Umtali specimen of **P. rossi**. They considered both names (ie. **katangensis** and **rossi**) to be valid and so reversed Abonnenc's earlier decision.

Another species, **Phlebotomus (Synphlebotomus) grovei** Downes (1971), is distributed, discontinuously, from Owamboland in northern Namibia eastwards to the

Transvaal in the Republic of South Africa and northwards to Zimbabwe and Zambia. After its discovery in northern SWA/Namibia, there was some speculation that it might be the vector species as the first cases of CL appeared to originate from this area. To date, nothing is known of its vector capabilities but the documentation of VL in southern Zambia (Naik **et al.**, 1976), where the habitat is similar to that of northern SWA/Namibia where **P. grovei** occurs, suggests that its vector potential requires investigation. It is possible that **P. (Synphlebotomus) katangensis** described from Lubumbashi also occurs in NW Zambia, but no collecting has been undertaken here to verify this.

In 1981 another species of **Synphlebotomus**, **P. taylori** Davidson (1982), was described from male specimens collected north of Harare in Zimbabwe. Females of this species as well as **P. katangensis** are at present unknown and are thus not covered in this dissertation as it concerns the discrimination of those species to which females have been assigned.

Since the discovery of **P. rossi sensu lato** in SWA/Namibia further material has been collected from the original locality and from the northern Transvaal and Zimbabwe. Enough material has now been examined to

question whether P. rossi from the east (northern Transvaal/Zimbabwe) is the same as P. rossi from the west (SWA/Namibia). As can be seen from the map [Fig. 2] there appear to be two distinct populations separated from each other by hundreds of kilometres. In addition there are certain morphological differences and apparent differences in habitat preferences between the two populations.

The terminalia of the males of P. rossi (west) and P. rossi (east) are similar but distinct from those of P. grovei [Figs 7 & 8]. Identification of the females of P. rossi (east) and P. grovei present problems in areas of overlap. Overlap in the distributions of P. grovei and P. rossi (west) has thus far not been established.

3.2 HABITAT PREFERENCES OF P. ROSSI SENSU LATO.

P. rossi (west) has been found and collected exclusively from cliff or rock bound habitat in association with rock hyrax (Procavia capensis) mostly from their exit/entrance tunnels.

P. rossi (east) has been collected from termite hills with ventilation shafts and once from a cave together with P. rousettus Davidson (1981); it has

Fig. 7 Male terminalia of P. rossi sensu lato.

Fig. 8 Male terminalia of P. grovei.

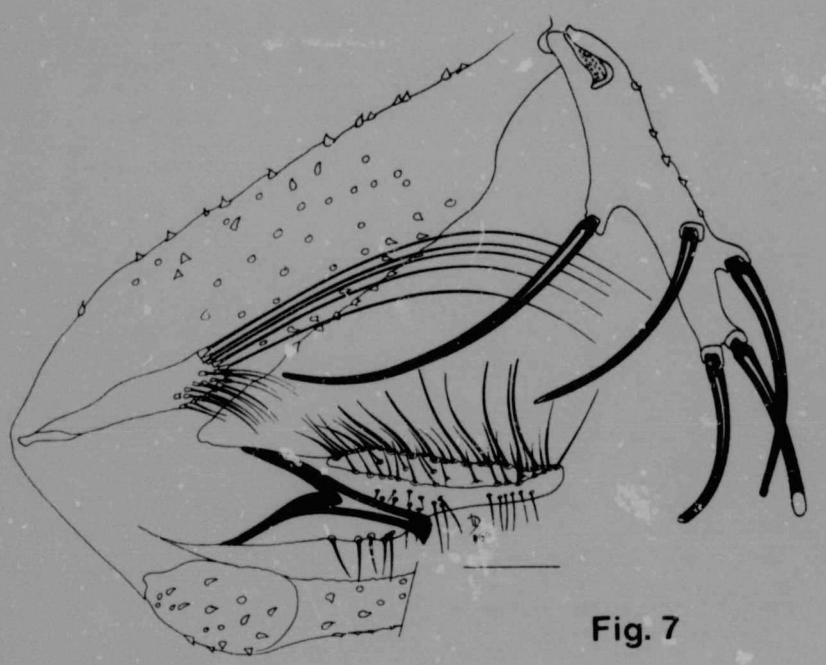


Fig. 7

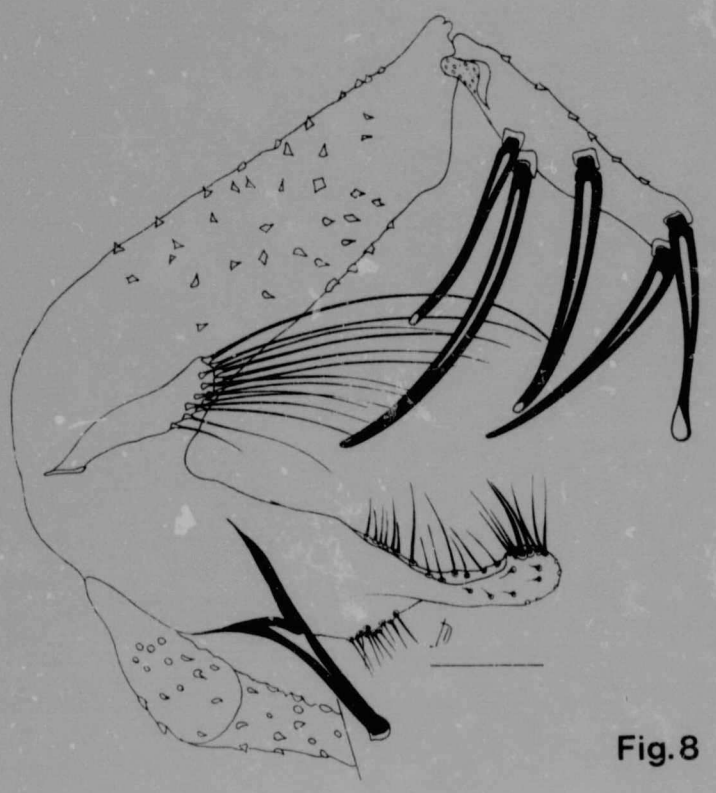


Fig. 8

never been collected from rock hyrax biotope in this part of its range. This may be because rock hyrax exist in a wetter biotope as opposed to semi-arid conditions in the west.

P. grovei has been collected in sympatry with **P. rossi** (east) and with further collecting may be found closely associated with **P. rossi** (west) in N SWA/ Namibia and W Botswana. **P. grovei** has been collected from termite hills with ventilation shafts as well as from ground burrows of mongooses (**Herpestes, Suricata**) and ground squirrels (**Xerus inauris**); it has not been collected from rocky biotope.

3.3 MORPHOLOGICAL DIFFERENCES.

3.3.1 Discrepancies in the ascoid formulae.

Ascoid formulae denote the number of ascoids (thin walled sensilla, perhaps functioning as chemical receptors) per antennal segment. Usually these are not known to vary within species. In the females of **P. rossi s.l.** and **P. grovei** the formulae are constant ie. 2/III-XV (meaning 2 ascoids on antennal segments 3-15; this feature is common to all known females of **Synphlebotomus**). The differences in the ascoid formulae of the males are shown in Table 2.

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