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# On the Use of Membrane Feeding to Study the Development of *Trypanosoma brucei* in *Glossina*.

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*Abstract*

A method of infecting tsetse flies by feeding them through chicken skin membranes on stabilated populations of trypanosomes is described. The infectivity to flies of trypanosomes in mouse blood which had been preserved in liquid nitrogen for up to one year was maintained.

Three types of infection in flies were recognised, namely, initial, established and mature. Infections became established by the 5th day after flies had fed on suspensions of trypanosomes. The proportion of flies with initial infection varied with (1) the temperature at which the flies were maintained; (2) the number of trypanosomes in the infective feed; (3) the strain of trypanosome. The proportion of flies with mature infection varied with (1) the temperature at which the flies were maintained; (2) the temperature at which pupae were incubated. None of the treatments significantly altered the proportion of flies with established infections.

Of 39 *G. morsitans* which were proved to be infected, since mice on which they had fed subsequently became parasitaemic, trypanosomes were detected in the salivary glands of only 15 of the flies.

## Introduction

Since it was shown, about 60 years ago, that tsetse flies were biological vectors of trypanosomes, a large literature has accumulated concerning their relationships. Factors which appear to influence the development of trypanosomes in tsetse flies include temperature (DUKE, 1933; FAIRBAIRN & CULWICK, 1950; FORD & LEGGATE, 1961; KINGHORN & YORKE, 1912; KINGHORN, YORKE & LLOYD, 1913; TAYLOR, 1932), the species of mammalian host (CORSON, 1935 & 1936; DUKE, 1935b; VAN HOOF, 1947), the stage of infection in the mammal host (DUKE, 1935a; ROBERTSON, 1913), the composition of the population of trypanosomes in the blood (WIJERS & WILLET, 1960), the numbers of trypanosomes in the blood (VAN HOOF, 1947; PAGE, 1972), the age of the fly (HARLEY, 1971a; WIJERS, 1958) and the species of fly (HARLEY, 1971).

In this paper we describe a method for infecting laboratory reared tsetse flies by membrane feeding on stabilated populations of *Trypanosoma brucei*. We show (1) that when flies are fed on the same population of trypanosomes and are kept subsequently under the same conditions, reproducible results are obtained; (2) that the pattern of development of *T. brucei* in *Glossina morsitans* may be modified by changing the experimental conditions.

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## Materials and methods

*The organisms.* Most of the experiments were conducted with derivatives of *T. brucei* TREU 667; a second strain, TREU 1096, was also used. TREU 667 was isolated by the Edinburgh Veterinary Expedition (EVE) to East Africa in 1966 (REID et al., 1970). When the stabilates were received at the Centre for Tropical Veterinary Medicine (CTVM) of Edinburgh University, 2 rats were infected and the stabilates made from these rats were designated TREU 667. Mice were infected with 667 and at the 5th passage the infected blood was preserved and designated population 667-A. A single *G. morsitans* which had fed on 667-A infected a mouse. From this mouse 6 further mice were infected by syringe passage; their blood was preserved and formed population 667-B.

TREU 1096 was isolated from an ox at Ibadan, Nigeria; it was passaged 7 times in rats before being stabilated as TREU 1096. Two mice were infected with 1096 and, after 3 further mouse passages, the trypanosomes were preserved as 1096-A. One *G. morsitans* which fed on 1096-A infected a mouse. From this mouse 6 further mice were infected and the pooled blood was preserved as population 1096-B.

*G. morsitans* pupae were obtained from the Tsetse Laboratory, Bristol, or from the colony of the Zoology Department, a sub-colony of the Bristol stock.

Mice were provided from the breeding colony of the Zoology Department and were mostly Q strain; semi-lop eared rabbits were also bred and reared in the Zoology Department.

*Fly maintenance.* The flies of the Department colony are maintained at 26 °C and relative humidity of 65–70 %; they are fed on rabbits. The experimental flies, after sexing, were placed in individual polystyrene cages of 1/2 inch diameter (MEWS, 1969). Unless otherwise stated, the first feed of the flies was the infective feed given within 36 hrs of eclosion. The following day female flies were mated. Thereafter, flies were offered food on alternate days but flies that did not feed within 10 min were removed and offered food daily until a meal was taken.

The experimental flies were maintained on mice, one mouse being allocated to each fly. The apparatus for restraining the mice was constructed as described by COCKINGS et al. (1959). When testing for the effect of temperature, a relative humidity of 75 % was maintained by placing the fly cages in jars on perforated trays over a saturated solution of potassium tartrate (WINSTON & BATES, 1960).

The average mortality of flies in the experiments described was 3 %. Although the flies fed poorly on mice, examination of their feeding records showed no correlation between the number of feeds taken and the proportion of flies in which an infection became established.

*Preservation of trypanosomes.* At the first peak of parasitaemia, when the trypanosomes had reached a concentration of $10^8$/ml in a group of mice, the mice were anaesthetized and bled from the heart. The pooled blood was thoroughly mixed and stabilates were prepared in lymph tubes as described by CUNNINGHAM et al. (1963). The stabilates were stored in a liquid nitrogen refrigerator. The trypanosomes were pleomorphic in all the populations that were used to infect flies.

*Infecting the flies.* Membranes were prepared from chicken skin. Fatty material was scraped off the dermis and the skin cut into pieces of the required size. These were spread out on top of self seal polythene bags and exposed to a Philips 60 Watt germicidal lamp for 15 min at a distance of 400 mm. After irradiation, the skins were placed in the polythene bags and the packets were stored at –20 °C.

Perspex feeding capsules were made in the workshop of the Zoology Department. Before use, they were boiled in water for 15 min; the feeding chamber was then exposed to UV irradiation as described above.

Ox blood was obtained once a week from the abattoir. Jugular blood was collected in a sterile flask containing glass beads, defibrinated and stored at 4 °C. One or more tubes of stabilate were removed from the cold store and, after weighing, the content was ejected into a sterile Bijou bottle fitted with a perforated cap. The emptied tubes were weighed to obtain an estimate of the volume of stabilate blood. Defibrinated ox blood was then added to the stabilates in the proportion of 100:1, and the bottle was placed on ice.

A chicken skin membrane was fitted over the feeding chamber of each capsule and the chambers were filled by injecting aliquots of the suspension of trypanosomes through the membrane. The fly cage was placed over the membrane; a maximum of 10 min was allowed for feeding. Flies that had not engorged within this time were discarded. Subsequently, a record was kept of the days when each experimental fly took a blood meal.

Mice were screened for infection by examination of a wet preparation of tail blood. If no trypanosomes were seen after scrutiny of 50 microscopic fields at $\times 400$ magnification, the animals were recorded as not infected. Mice were kept under observation for at least 6 weeks after the last feed taken by a fly. All manipulations requiring sterility were carried out in a Lamina Flow Work Station (Microflow Limited, Fleet, Hants.).

*Dissection of flies.* Flies were killed by exposing them to chloroform vapour. Dissection was carried out in 2 stages; the head was detached from the thorax and gently pulled away so that the entire salivary glands separated with the head. The alimentary canal was then dissected out and the parts were scanned for trypanosomes under a $\times 20$ objective of a phase contrast microscope.

## Results

### A. Development of a standard procedure

#### 1. Homeogeneity of the stabilates

The number of flies available on any one day was variable and seldom sufficed for one complete experiment. Hence groups of 10 flies were used in series until the required number had been accumulated. From the 3rd to the 12th day after the infective feed, one fly from each group was killed each day and examined for trypanosomes. The morphology of the trypanosomes and their position in the gut were recorded. The results of a typical experiment are given in table 1. Homogeneity of the stabilates was tested by calculating an index of dispersion for a number of samples using the formula

$$\chi^2 = \frac{S\,(x - \bar{x})^2}{\bar{x}\,q}$$

To test whether the variance of $\chi^2$ accorded with the binomial distribution the formula $\sqrt{2\chi^2} - \sqrt{2n-1}$ was applied to values obtained for $\chi^2$ (FISHER, 1941). We concluded on the basis of these calculations that the trypanosomes were randomly distributed between stabilates, that ox blood was a satisfactory medium for the infective feed, and that

*Table 1.* Infections in 200 *G. morsitans* which had fed on *T. brucei*, 667-A

| Group number of flies | Stabilate number | Day of dissection | | | | | | | | | | Number of flies infected |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | |
| 1 | 8 and 9 | | | | | | + | | | | + | 2 |
| 2 | 8 and 9 | + | | | + | + | | | | | | 3 |
| 3 | 8 and 9 | | | | + | + | | | | | | 2 |
| 4 | 8 and 9 | | | | + | + | + | + | | + | + | 6 |
| 5 | 8 and 9 | | | | | | | | | | | 0 |
| 6 | 8 and 9 | | | | | | | | | | | 0 |
| 7 | 11 and 12 | | | + | + | | + | | | | | 3 |
| 8 | 11 and 12 | + | | | | | | | | | | 1 |
| 9 | 11 and 12 | | | | | | | | | | | 0 |
| 10 | 16 and 17 | | | + | | | | | | | | 1 |
| 11 | 16 and 17 | | + | + | | | + | | | | | 3 |
| 12 | 16 and 17 | | | | | + | | | | | | 1 |
| 13 | 16 and 17 | | | | + | | | + | | + | | 3 |
| 14 | 16 and 17 | | + | | | + | | | + | | + | 4 |
| 15 | 18 | | | | | | + | | | + | | 2 |
| 16 | 19 | | | | | + | + | | | + | + | 4 |
| 17 | 20 | | | | | + | | | | + | + | 3 |
| 18 | 21 | + | | + | + | | | | + | + | | 5 |
| 19 | 22 | + | | | | | + | + | | | | 3 |
| 20 | 23 | | + | | | | | + | | + | | 3 |

+ signifies infected fly.

stabilates retained their infectivity and viability throughout the period of storage, which in some instances was 1 year.

### 2. Type of infection

In testing for homogeneity of the stabilates, about 500 *G. morsitans* had been infected with population 667-A and any fly in which active trypanosomes were seen was recorded as infected. However, it was observed that in flies killed early, the trypanosomes were confined to the posterior part of the midgut, whereas in those killed later, the trypanosomes had spread forwards in the ectoperitrophic space. In a further series of tests conducted similarly with population 667-B, distinction was made between a fly with trypanosomes in the posterior midgut (type I infection), and one in which the trypanosomes had migrated forward to fill the ectoperitrophic space or had also invaded the foregut (type II infection). The results of these tests, in which 370 *G. morsitans* were dissected, are shown in Fig. 1. Sixty per cent

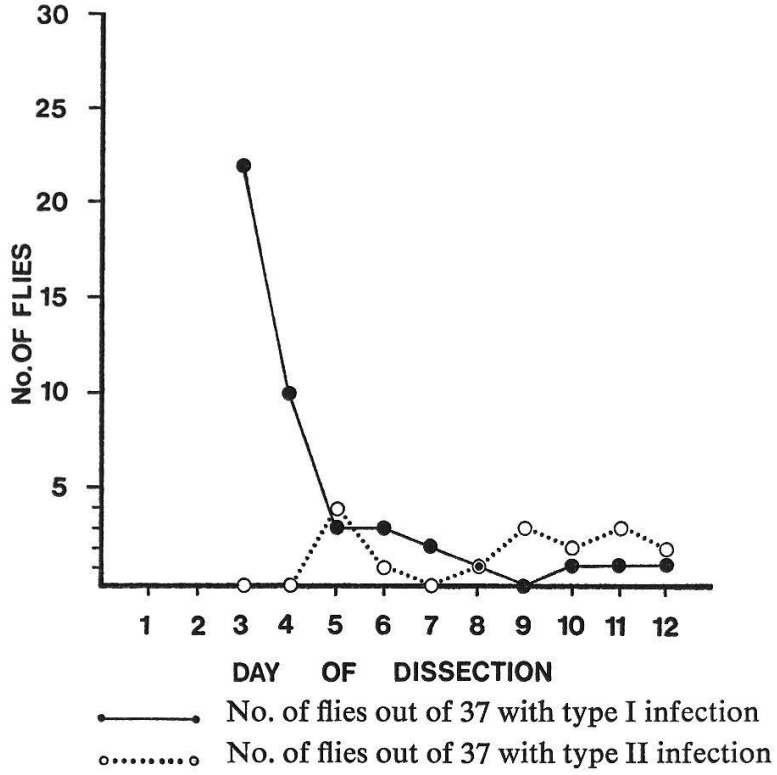


*Fig. 1.* Distribution of types I & II infections of *T. brucei,* 667-B in 370 *G. morsitans.*

of the flies were infected at day 3; all had a type I infection. By the 6th day the proportion of infected flies had fallen to approximately 10 % and it remained at this level. Type II infections were evident by the 5th day and, since it appeared that flies in which the trypanosomes moved forwards in the ectoperitrophic space retained their infections, they were regarded as having an established infection. The tests showed that it would not be necessary to examine flies daily, and that large numbers of flies would be required in order to detect differences between treatments.

### 3. Cyclical development

350 *G. morsitans* were fed on stabilates of 667-B diluted 1:100 with ox blood. Flies were kept in the fly room at 26 °C and were dissected at 3, 5, 10, 20, and 30 days after their infective feed. After day 10 the mice were examined twice weekly for parasitaemia; any fly which infected its mouse was recorded as having a mature infection. This procedure was adopted as the standard.

Table 2 shows that of the flies killed on day 3, 50 % were infected; by the 5th day the proportion had fallen to 14 % and thereafter was unchanged. All but 2 of the infections which persisted up till day 5

*Table 2.* Infections of *T. brucei* in tsetse flies *

| Treatments | Dissection on | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | day three | | | | day five | | | | day ten | | | | day twenty | | | | day thirty | | | |
| | | I | EI | MI | | I | EI | MI | | I | EI | MI | | I | EI | MI | | I | EI | MI |
| 1 Standard | (50) | 50 | 0 | 0 | (50) | 14 | 10 | 0 | (50) | 8 | 8 | 0 | (100) | 12 | 12 | 1 | (100) | 2 | 8 | 3 |
| 2 Flies maintained at 31 °C | (50) | 78 | 0 | 0 | (50) | 20 | 14 | 0 | (50) | 8 | 8 | 0 | (50) | 10 | 10 | 8 | (50) | 14 | 14 | 14 |
| 3 Flies maintained at 20 °C | (50) | 12 | 0 | 0 | (50) | 6 | 0 | 0 | (50) | 12 | 6 | 0 | (50) | 6 | 6 | 0 | (50) | 2 | 2 | 0 |
| 4 Pupae at 31 °C, flies at 26 °C | (50) | 56 | 10 | 0 | (50) | 30 | 16 | 0 | (50) | 8 | 8 | 0 | (50) | 8 | 8 | 8 | (50) | 12 | 12 | 10 |
| 5 Flies older than two days | (50) | 40 | 0 | 0 | (50) | 16 | 4 | 0 | (50) | 8 | 4 | 0 | (50) | 8 | 8 | 0 | (50) | 10 | 10 | 2 |
| 6 *G. austeni* | (50) | 24 | 0 | 0 | (50) | 16 | 0 | 0 | (50) | 2 | 2 | 0 | (50) | 2 | 2 | 0 | (50) | 0 | 0 | 0 |
| 7 Strain 1096-A | (100) | 36 | 0 | 0 | (100) | 16 | 5 | 0 | (100) | 10 | 4 | 0 | (100) | 6 | 4 | 2 | (100) | 3 | 3 | 3 |
| 8 Strain 1096-B | (100) | 30 | 0 | 0 | (100) | 25 | 7 | 0 | (100) | 8 | 6 | 0 | (100) | 4 | 4 | 2 | (50) | 10 | 8 | 2 |

( ) Number of flies infected.
I Infected per cent.
EI Established infection per cent.
MI Mature infection per cent.
Flies were infected with *T. brucei* 667-B, except for numbers 7 and 8.
* Flies were *G. morsitans* except for No. 6.

were established infections; the proportions of flies with established infections on days 10, 20 and 30 did not differ significantly from that on day 5. However, not all of the established infections became mature, possibly because insufficient time had been allowed for the trypanosomes to complete their development. A further 100 flies were, therefore, infected and kept for 50 days. Again, of the 6 flies which had established infections when they were finally dissected, only 2 had infected mice.

The single fly in the 20-day group with a mature infection was detected by a probe test; it was kept for a further 26 days during which time it fed on 6 clean mice. In one of the mice the prepatent period was 5 days, in another it was 7 days and in the remaining 4 mice it was 6 days. By examination of the feeding records of the 3 flies with mature infections in the 30-day group, the prepatent periods were found to be 7, 7 and 5 days.

### B. Modification of the standard procedure

In the following experiments the procedure was modified and the results were compared with those obtained by the standard treatment. The significance of the difference was determined by calculating $\chi^2$ by means of a 2 by 2 contingency table. When the total numbers for comparison were less than 200, Yates' correction was applied (FISHER, 1941).

#### 1. Effect of temperature on flies

After flies had taken their infective feed, they were divided into 2 groups; one group was kept in an incubator at 31 °C, the other at 20 °C. The results of these tests are presented in table 2. The pattern of infection in the flies kept at 31 °C was the same as that of the flies kept under standard conditions, with a significantly higher proportion of flies being infected at day 3 ($P < 0.05$). The proportions of flies with established infections did not differ significantly from each other from day 5 onwards nor did they differ from those of the flies maintained under standard conditions. However, the proportions of established infections which became mature on days 20 and 30 were higher in the flies maintained at 31 °C ($P < 0.01$). By examination of the feeding records of the flies which infected mice, it was estimated that the average time for the development cycle was 12 days. Hence, raising the temperature from 26 °C to 31 °C accelerated the development and increased the proportion of flies in which the infection became mature.

When the temperature for fly maintenance was lowered to 20 °C,

fewer flies were infected on day 3 ($P < 0.01$) and development was delayed since it was not until day 10 that the infections became established. None of the infections became mature. Similar effects of temperature have been demonstrated previously (*loc. cit.* p. 185).

### 2. Effect of temperature on pupae

Newly deposited pupae were collected daily and placed in an incubator at 31 °C. The time required for the development of these pupae was 23 days as opposed to 28 days for pupae kept in the fly room. After eclosion, the flies were transferred to the fly room and treated as for the standard. As shown in table 2, the distribution of infection in these flies resembled that in flies kept at 31 °C. Nine of the 10 flies with established infections on days 20 and 30 infected mice. A difference was the early forward movement of the trypanosomes in the midgut since infection had become established by the 3rd day.

The high temperature for pupal development was evidently injurious to the flies since subsequent tests showed that 90 colony reared flies deposited 57 pupae in a 30-day period, whereas 92 flies which emerged from pupae incubated at 31 °C deposited only 9 pupae. BURTT (1946) and FAIRBAIRN & CULWICK (1950) found that raising the temperature for pupal development accelerated the development and increased the infection rate of *T. rhodesiense* in *G. morsitans.*

### 3. Age of flies

Flies were fed once or twice on rabbits and were 5 or 6 days old when they were given their infective feed. The infections in these flies did not differ significantly from the standard (Table 2). WIJERS (1958) and HARLEY (1971a) both found that infection rates were higher in flies infected within 24 hours of eclosion than in flies infected later. However, the species used were respectively *G. palpalis* and *G. fuscipes.*

### 4. Species of fly

To test whether *T. brucei,* 667-B would develop equally well in another species of fly, *G. austeni* was substituted for *G. morsitans.* Fewer flies were infected on day 3 ($P < 0.01$), fewer developed established infections ($P < 0.01$) and none infected mice (Table 2). Field observations have also indicated that species of tsetse differ in their susceptibility to infection by trypanosomes.

*Table 3.* Infections in *G. morsitans* which had been fed on different concentrations of *T. brucei* 667-B

| Day of dissection | Dilution of stabilates | Number of flies | Infected flies per cent | Established infections per cent |
|---|---|---|---|---|
| 3 | 1:10 | 123 | 77 * | 0 |
| | 1:100 | 67 | 51 | 0 |
| | 1:10,000 | 25 | 48 | 0 |
| 10 | 1:10 | 48 | 8 | 6 |
| | 1:100 | 67 | 7 | 6 |
| | 1:10,000 | 50 | 4 | 2 |

* $P < 0.01$.

### 5. Concentration of trypanosomes in the infective feed

The effect of feeding the flies on different numbers of trypanosomes is shown in Table 3. A tenfold increase in the number of trypanosomes significantly increased the proportion of flies infected at day 3, but by the 10th day the proportion in each group was similar. By weighing flies before and after feeding, it was found that a fly ingested about 20 mg blood. When the stabilates were diluted 1:10,000, it was estimated that each fly would ingest about 80 trypanosomes.

### 6. Strain of trypanosome

*G. morsitans* were infected with 2 different populations of a strain of *T. brucei* which originated in Nigeria. The flies were kept under standard conditions and the results are shown in Table 2. The infections produced by both populations of 1096 in *G. morsitans* were similar. One fly, which was found to be infected by a probe test on day 16, was isolated and fed on 3 clean mice. The prepatent periods in the mice were 8, 9 and 9 days. By examination of the feeding records of the 5 flies with mature infections, the duration of the development cycle was found to be between 15 and 18 days, and the prepatent period in mice, 9 days. Strain 1096 differed from 667 in (1) the smaller number of flies infected on day 3 ($P < 0.01$), (2) the faster development cycle, and (3) the longer prepatent period in mice. It is likely that these differences were inherent to the strain since the same results were obtained with 2 populations of each strain. One population had been syringe passaged once in mice since cyclical transmission, the other, 11 times.

*Table 4.* Comparison of established infections

| Treatment | Number of flies | Established infections number | per cent | $\chi^2$ | P |
|---|---|---|---|---|---|
| *Strain 667* | | | | | |
| Standard | 300 | 29 | 9.66 | | |
| Flies at 31 °C | 200 | 23 | 11.5 | 0.915 | > 0.30 |
| Flies at 20 °C | 150 | 7 | 4.66 | 2.174 | > 0.10 |
| Pupae at 31 °C | 200 | 22 | 11.0 | | |
| Flies older than 2 days | 200 | 13 | 6.5 | 0.849 | > 0.30 |
| | 1,050 | 94 | 8.95 | | |
| Strain 1096 | | | | | |
| Standard | 750 | 42 | 5.6 | 1.44 | > 0.20 |

### 7. Infection of salivary glands

*T. brucei* completed its development cycle in a total of 39 *G. morsitans* since all these flies infected mice. When the flies were dissected, only 15 had trypanosomes in the salivary glands and in only 2 of these were the trypanosomes numerous. In the remaining 24 flies, not a single trypanosome could be seen despite a careful search of the entire glands by phase contrast microscopy. All the flies which infected mice had numerous trypanosomes in the midgut and proventriculus; in some the labrumepipharynx was also invaded.

### 8. Established infections

The proportions of flies *(G. morsitans)* with an established infection on days 5, 10, 20 and 30 did not differ significantly in any of the experiments. The values for each experiment (Table 2) were therefore summed and compared with that of the standard. None of the treatments significantly altered the proportion of flies with an established infection (Table 4); nor was there any difference between the proportions of flies with an established infection of strain 1096 and 667.

## Discussion

We have shown that when tsetse flies were kept under standard conditions, the number of infected flies and the type of infection did not differ significantly when the flies were fed through a membrane on

samples of a stabilated population of trypanosomes that had been preserved in liquid nitrogen for varying periods. This technique allows comparisons to be made between populations of trypanosomes, between flies and between conditions under which infected flies are kept. MSHELBWALA (1967) first reported that tsetse flies could be infected by feeding through a mouse skin membrane on trypanosomes that had been preserved by freezing. DAR (1971) extended the application of this method.

In the course of this work, the alimentary tracts of hundreds of *G. morsitans* have been examined at intervals after the flies had ingested infective *T. brucei*. A consistent pattern of the development of the infection has emerged. At 73 hrs after the infective feed, about 50 % of the flies had trypanosomes in the posterior segment of the midgut (as defined by WIGGLESWORTH, 1929) both within and outside the peritrophic membrane. The trypanosomes had evidently multiplied in these flies since in flies killed after 24 hrs very few trypanosomes could be detected. The morphology of the trypanosomes was varied, but many were long and rather broad with the kinetoplast about half way between the posterior end and the nucleus. We refer to this early multiplication and change of form of the trypanosomes as the initial development. By the 5th day after infection, the proportion of infected flies had fallen and in an average of 9 %, the trypanosomes had moved forwards so that the infection extended into the middle or anterior segments of the midgut; in these sites the trypanosomes were restricted to the ectoperitrophic space. This forward movement of the trypanosomes indicated that the infection had become established. The proportion of flies with established infections was the same at days 5, 10, 20 and 30 after the infective feed; from the 10th day, the number of infected flies and the number with established infections was the same. Penetration into the proventriculus did not seem to present an obstacle since, given sufficient time, trypanosomes were always present in the proventriculus as well as in the midgut. Hence to determine the infection rate of *T. brucei* in *G. morsitans* it would only be necessary to examine flies 10 days after they had taken their infective feed.

This pattern of the development of *brucei*-group trypanosomes in tsetse flies confirms that described by the early investigators. ROBERTSON (1913) did daily dissections of *G. palpalis* which had been fed on monkeys infected with *T. gambiense*. She noted that for the first few days after the infective feed the trypanosomes were restricted to the posterior part of the midgut, and that a high proportion of the flies were infected. The infection then "grows forwards by the sheer force of multiplication" although it was only in a small proportion of flies that this process occurred. STUHLMANN (1907) also commented on this early multiplication of the trypanosomes which gradually died out. In

the 10 % of flies in which the infection persisted, the trypanosomes had spread anteriorly in the midgut.

In more recent work interest has centred exclusively on infection rates; criteria for infection have been the presence of metacyclic trypanosomes in a probe or in the salivary glands, or transmission of infection to laboratory animals. Differences in the infection rates have been attributed to differences in the number of trypanosomes in the blood, or to the proportions of long, intermediate and stumpy forms at the time when the flies were fed (VAN HOOF, 1947; PAGE, 1972; WIJERS & WILLET, 1960). Elsewhere these correlations have not been confirmed (BAKER & ROBERTSON, 1957).

Our results suggest that it is a property of this closed colony of *G. morsitans* which determines the infection rate. In none of our experiments was it possible to change significantly the proportion of flies with established infections. By contrast, the proportion of flies in which initial development occurred varied from 20 to 80 %, and the proportion of established infections which became mature varied from 0 to 100 % depending on the conditions to which the flies were subjected. DUKE (1933), in a review of the early work on factors which may influence the development of trypanosomes in tsetse flies, concluded that it was only in a certain proportion of any fly population that trypanosomes developed. What he described as the "trypanophil" quality resided in the entire digestive tract and affected all species of trypanosome with a developmental phase in the gut. The trypanosomes that develop initially may correspond with the Type A culture forms of BROWN & EVANS (1971). Possibly only flies with the "trypanophil quality" provide conditions suitable for type A to change to type B forms. Type B forms could be maintained in culture indefinitely; by analogy, the trypanosomes in an established infection would be of Type B.

Since the proportion of flies in which an established infection became mature varied from 0 to 100 %, it appears that certain conditions are required to induce the final phase of the development. HARLEY (1971) fed batches of flies which emerged from pupae collected in the same area, at the same time on a rat infected with *T. rhodesiense.* Although 32 % of the males and 15 % of the female *G. brevipalpis* had gut infections when dissected 35 days later, none had salivary gland infections. By contrast, about 15 % of the *G. pallidipes* and of the *G. fuscipes* did have salivary gland infections. AMREIN & HANNEMAN (1969) found that when cultures were inoculated with samples from the same population of *T. brucei,* the development of forms infective to the mammal depended on the type of blood in the medium. Hence it would appear that the development of a mature infection is independent both of the quantity and form of trypanosomes ingested or introduced into a culture medium.

### *Salivary gland infection*

The usual criterion for distinguishing *brucei*-group trypanosomes in wild caught flies, namely, the presence of trypanosomes in the salivary glands as determined by microscopic examination, is of doubtful value. In only 15 of the 39 *G. morsitans* which infected mice were trypanosomes seen in the salivary glands although they were present in the labrum epipharynx of some of these flies. Similar observations have been made previously (BURTT, 1946; BAKER & ROBERTSON, 1957). On 2 or 3 occasions, flies with mature infections were offered food daily on clean mice; all the mice became infected and the prepatent periods were similar. These observations, although few, suggest that mature infections are not transitory. Where, then, do the metacyclics develop, or is it only the metacyclic form which can establish in a mammal?

ROBERTS et al. (1969) described the formation of chancres in cattle bitten by tsetse flies infected with *T. congolense*. Most of the trypanosomes in these nodules were typical blood forms, but some with the appearance of proventricular forms were also present. GORDON & WILLET (1958) described similar chancres in rabbits and in man when bitten by flies infected with *T. rhodesiense*. The trypanosomes in these lesions were predominantly long, slender blood forms. These authors also state that while feeding a tsetse fly sometimes regurgitates and a "vomit drop", which probably contains proventricular forms, is passed into the host's tissue. The apparent absence of salivary gland infections in flies which have transmitted trypanosomes suggests that proventricular forms might undergo change to infective forms in mammalian tissue. This assumption should be tested by injecting separately into mice and rabbits by the subcutaneous route, the triturated labrum, proventriculus and salivary glands of flies which had already infected mice while feeding. In the present experiments the trypanosomes frequently appeared in the mice subsequent to dissection of the flies.

This interpretation may explain why it is rare to find more than 1 or 2 per thousand flies with salivary gland infections although 20 to 30 % of the game from which the flies derive their food may prove to be carrying *brucei*-group trypanosomes (DUKE, 1933).

Further support for this view comes from the recent surveys for sleeping sickness in the Serengeti which were carried out by a team from the Swiss Tropical Institute, Basle (GEIGY *et al.*, 1971; MOLOO et al., 1971; GEIGY & KAUFFMANN, 1973; ROGERS & BOREHAM, 1973). In the first survey 12 *T. brucei* subgroup isolates were made from 115 wild mammals and in the second survey, 40 isolates from 95 mammals. Dissections of 7,000 and 3,500 tsetse flies caught during the surveys failed to reveal a single salivary gland infection. However, when mice

were inoculated with triturated *G. swynnertoni,* some became infected with *T. brucei.*

This study has shown that cyclical development of *T. brucei* in tsetse flies may be influenced by the species of fly, the strain of trypanosome, the prevailing and the past temperatures. In the field, all these factors and others, such as the availability of infected hosts and host preferences of the flies (JORDAN, 1965), interact and so complicate the interpretation of data on infection rates in flies. Conflicting results obtained by laboratory studies may also be due to variation in the organisms and the methods used. In studying the cyclical development of trypanosomes, it is essential to consider the development as represented by the three types of infection, initial, established and mature. Since infection rates are always low, the technique of feeding flies through a membrane on stabilated populations of trypanosomes overcomes the difficulty of obtaining sufficient flies.

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## *Zusammenfassung*

Es wird eine Methode zum Infizieren von Tsetsefliegen beschrieben, bei welcher die Fliegen durch eine Hühnchenhaut-Membran auf definierten Trypanosomenpopulationen gefüttert werden. Diese Trypanosomen, die bis zu einem Jahr in flüssigem Stickstoff aufbewahrt wurden, behielten ihre Infektiosität für die Fliegen bei.

Es sind 3 Infektionstypen in den Fliegen festgehalten worden, nämlich ein initieller, ein etablierter und ein reifer Typus. Etablierte Infektionen wurden 5 Tage nach der infektiösen Blutmahlzeit gefunden. Die Anzahl der Fliegen mit einer initiellen Infektion war abhängig 1. von der Temperatur, bei welcher die Fliegen gehalten wurden, 2. von der Anzahl der Trypanosomen im infektiösen Blut und 3. vom verwendeten Trypanosomenstamm. Die Zahl der Fliegen, die eine reife Infektion aufwiesen, variierte mit der Temperatur, bei welcher (1.) die Fliegen und (2.) die Puppen gehalten wurden. Keine dieser verschiedenen experimentellen Bedingungen beeinflussten in signifikanter Weise die gefundenen etablierten Infektionsraten.

Von 39 *G. morsitans,* die nachgewiesenermassen infektiös waren, da die Kontrollmäuse, auf welchen sie gefüttert worden waren, infiziert wurden, zeigten nur 15 Fliegen Trypanosomen in den Speicheldrüsen.

*Résumé*

On décrit dans le présent travail une méthode pour infecter des mouches tsé-tsé à travers des membrane de peau de poussins sur des populations de trypanosomes conservées sous forme de stabilats. L'infectiosité des trypanosomes pour les mouches a été prouvée, ces protozoaires ayant été maintenus congelés dans de l'azote liquide pendant une année.

Les auteurs reconnaissent trois types d'infection dans les mouches, soit une «initiale», une «établie» et une «mûre». Les infections devinrent «établies» 5 jours après la nutrition des mouches sur une suspension de trypanosomes. La proportion des mouches présentant une infection «initiale» dépend: 1) de la température à laquelle les mouches furent maintenues; 2) du nombre des trypanosomes ingérés pendant la nutrition; 3) de la souche de trypanosomes utilisée.

Quant à la proportion des mouches présentant une infection «mûre», elle depend: 1) également de la température à laquelle les mouches sont maintenues; 2) de la température à laquelle les pupes furent incubées. Aucun de ces traitements n'altéra de manière significative la proportion de mouches présentant une infection «établie».

Il fut possible de détecter des trypanosomes dans les glandes salivaires de 15 *G. morsitans* seulement sur les 39 infectées avec sûreté puisque les souris sur lequelles elles furent toutes nourries présentèrent une parasitémie.