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ABSTRACT OF THESIS

Name of Candidate OOLUSEGUN OLADIPUPO DIPEOLU

Address [redacted]

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Title of Thesis The development of trypanosomes in tsetse flies.

The literature since 1895, when the tsetse fly was first shown to be capable of transmitting trypanosomes, has been critically reviewed. Despite numerous studies on the development of trypanosomes in tsetse flies covering this period of 80 years, no firm conclusion can be drawn as to the factors which control or influence the process. Discrepant results can be partly explained by the variability of the material used by different workers or by the same workers in different tests.

In this thesis a standardized method for studying factors which may influence the development of trypanosomes in tsetse flies is described. Flies are infected by feeding through a chicken skin membrane on aliquots, diluted in defibrinated blood, of a stabilated population of trypanosomes. In this way variables, such as changes that take place in the trypanosome population during the course of an infection in an animal or in different animal hosts, and the number of trypanosomes in the host at different times, which have not been controlled in previous studies, have been eliminated.

It has been shown that when flies are fed on stabilated trypanosomes and are subsequently maintained under standard conditions, the results are consistently reproducible. One condition can then be altered and the results compared with the standard. Since the proportion of flies which become infected is always small, large numbers of flies must be used in each test in order to increase the probability of detecting significant differences.

It has been shown that the position of trypanosomes in the alimentary canal of an infected fly is important. Four categories of infection were recognized. A change from Category 1, where the trypanosomes were restricted to the posterior third of the midgut, to Category 2, where the trypanosomes had spread forwards along the length of the midgut but were confined to the ecto peritrophic space, was significant. This change signified that the fly would retain its trypanosomes, the infection then became an established one. In some cases, all established infections became mature, in others, although the trypanosomes persisted in the fly for a long period, the trypanosomes never completed their development cycle by infecting mice.

Among the factors studied which might influence the development of trypanosomes in tsetse flies were: the effect of temperature at which the flies were maintained; the effect of temperature on the pupae; the age of the flies at the time of their infective feed; the species of fly; the species of trypanosome; the strain

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Use other side if necessary.

of trypanosome; the number of syringe passages which the trypanosomes had undergone in mice since cyclical development; the sex of the fly; the food host of the fly.

The development of trypanosomes in tsetse flies

By

O.O. Dipeolu, Diplom Tierarzt, Giessen, (W.Germany)

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SUMMARY

1. The literature on the development of trypanosomes in tsetse flies and factors affecting it was critically reviewed. This revealed that all the information on this aspect of trypanosomiasis had been derived from the following sources; a) the dissection of wild flies collected from different localities in Africa, b) experiments in which flies emerging from wild caught pupae were used, and c) tests in which laboratory bred pupae were used but the emerging flies were fed on different animals at different times during an infection. Since none of these methods could be controlled, the results varied, and there resulted differences of opinion over the relative importance of the factors that facilitate development.

2. The experimental procedures were standardised. Flies were infected by feeding them artificially through chicken membrane on a suspension of trypanosomes in defibrinated blood. In this way, the concentration of trypanosomes in the infective feed was controlled and the uncontrollable variables associated with the animals, such as fluctuation in the number of trypanosomes in the peripheral blood and antibody response, were eliminated. With these standardised procedures, many factors which could influence the development of trypanosomes in flies were tested. The importance of maintaining strict sterile precautions during the infective feed was stressed.

3. The first experiments were directed towards establishing methods for infecting tsetse flies by membrane feeding, maintaining the flies, and following the development of trypanosomes in the

midgut of the flies. The experiments were terminated on the 12th day post infection, flies having been dissected daily from days 3 to 12. These experiments showed that the position of the trypanosomes in the alimentary canal of an infected fly was significant. Consequently, 4 types of infection, termed 'categories' were recognized. It was then evident that the change from a category 1 to a category 2 infection was critical. As a result, all infections of category 2 to 4 were called 'established infections'.

4. A model for the study of the development cycle of trypanosomes in tsetse flies was established. This took into account both the number of days after the infective feed required before the infection in the fly would become mature, and the minimum number of flies required to make valid conclusions.

5. The development cycle of T.brucei and T.congolense in the tsetse is described. The attainment of the ectoperitrophic space through the open posterior end of the tubular peritrophic membrane is not a difficulty for the trypanosomes. The first "barrier" against development is the early mortality of the trypanosomes a few hours after ingestion into the midgut. The second "barrier" is the establishment of the infection i.e. the migration forwards by the trypanosomes from the ectoperitrophic space of the posterior segment into that of the middle segment and beyond. For flies infected with T.congolense and given the standard treatment, there is correlation between the number of established and mature infections. With T.brucei, however, another 'barrier' is interposed which prevents all the established infections from becoming mature.

6. The cyclical development of T.brucei and T.congolense was parallel, up to and including the involvement of the hypopharynx. The metacyclics of T.congolense develop in the hypopharynx and the latter was always invaded in mature infections; the invasion of the labrum epipharynx and hypopharynx was often massive. Metacyclics of T.brucei develop in the salivary glands, but the invasion of the latter was often transitory. When found in the salivary glands, trypanosomes of T.brucei were often scanty, occurring in isolated groups, and leaving long stretches of the glands uninvaded.

7. The advantages of using the membrane feeding technique to infect flies were discussed. Results of experiments in which flies received the standard treatment were reproducible, even though they were perfomed at different times. The longevity of the experimental flies was not adversely impaired.

8. The earlier laboratory observations on the effect of temperature in the development of brucei-group trypanosomes were confirmed. At 31°C the development cycle of T.brucei was shorter and a larger proportion of the flies developed mature infections than at 26°C. The same effect wasobtained when pupae were incubated at 31°C and the infected adults kept at 26°C. The rate of development was decreased at 20°C.

9. Raising the temperature from 26°C to 31°C had no effect on the development of T.congolense in G.morsitans. At 20°C the development cycle was prolonged but not inhibited.

10. When two strains of T.brucei were subjected to identical conditions, their infectivity to G.morsitans, duration of development and of the pre-patent period in mice were found to differ. Similar results were obtained when two strains of T.congolense were compared.

11. Populations of T.congolense, which had undergone 6 or 7 mouse passages after cyclical development differed from one which had undergone only 2 mouse passages. In the former, only a small proportion of the established infections became mature. Failure to recognize such population differences was regarded as one of the handicaps in the studies of trypanosome/fly relationships. A suggestion was also made that such population differences could be responsible for the so called 'non transmissible strains'.

12. A tenfold increase in the concentration of T.brucei and T.congolense in the infective feed did increase the proportion of infected flies initially, but there was no difference in the proportion of established and mature infections in flies killed later.

13. When G.morsitans were infected with culture forms of T.congolense, the proportion of flies with trypanosomes on the 3rd day after infection was higher than when flies were fed on blood forms, but the proportions of flies in which the infections became established did not differ. The development cycle and the prepatent period in mice was prolonged in flies which had fed on culture forms.

14. When G.morsitans were infected with culture forms of T.brucei, the age at which the cultures were harvested determined the initial infection rate in the flies.

15. Fly forms of T.brucei and T.congolense were no longer infective to mice 48 hours after ingestion of the bloodstream forms.

16. The proportion of flies with initial midgut infections of T.brucei and in which the infections became established was lower in G.austeni than in G.morsitans.

17. The proportion of flies with initial midgut infections of T.congolense was lower in G.austeni than in G.morsitans; the duration of the development cycle was prolonged in G.austeni.

18. No difference was found in the susceptibility of male and female G.morsitans to T.brucei, or to T.congolense.

19. The infection rates were the same in flies which had been maintained on rabbits or on mice after their infective feed. Neither did the number of feeds taken by the flies affect the development of T.brucei and T.congolense.

20. The results of this study indicate that the "transmissibility factor" is not only 'fly-linked' or 'trypanosome linked' but may also be the resultant of the interaction between the two organisms.

21. The position of the trypanosomes in the alimentary tract of tsetse flies must be regarded in studies on their cyclical development.

22. The limitations of field results, in which wild flies were dissected and species of trypanosomes identified by their distribution in the fly's alimentary canal, were discussed.

23. The importance of using large number of flies in the experiments for the study of development of trypanosomes in tsetse flies was discussed.

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1. INTRODUCTION

1.10 The development of trypanosomes in tsetse flies.

It was clear from historical accounts that both the Africans and the first European settlers in Africa were conscious of the role of tsetse flies in trypanosomiasis. Eakin (1914) gave a detailed account of the recognition of the tsetse flies as cause of the sleeping sickness by the Efik people in the South eastern state of Nigeria. He reported that they attributed sleeping sickness to the bite of a fly known as Nsung Idap, or sleeping fly. This insect was commonly known to every town in the district as Nsung Obio or town fly. Eakin later identified this fly as Glossina tachinoides. South African natives were reported to have warned the Europeans to avoid certain routes lest their horses and trek oxen died, or advised them to cross a narrow fly belt in the dark when the tsetse flies would be inactive (Nash, 1969).

Bruce (1895) wrote, "the idea that the Fly Disease or Nagana is caused by the bite of the tsetse fly is the European theory and as everyone knows it has been popularly prevalent ever since white men first landed in South Africa". Captain Cornwallis Harris was reported to have recorded on a sketch map of South Africa made while hunting in 1837, "a country abounding in flies destructive to cattle". (Nash, 1969). In fact, the name 'tsetse' comes from a Botswana word which means "a fly destructive of cattle".

At this time the role of insects as carriers of disease was unheard of to both Africans and Europeans and they thought that the tsetse injected a venom. The first person to take scientific interest in tsetse was Major Vardon. Wanting to test the authenticity of the role

ascribed to the tsetse, he put the matter to the test by riding his horse into a tsetse infected locality in Botswana without dismounting or allowing the animal to feed. The horse subsequently died after 20 days. Vardon sent specimens of the flies that had bitten his horse to Professor Westwood, who in 1850 described and named the species G.morsitans. The next scientific interest was shown when, in 1864, Kirk wrote a paper for the Linnean Society. He pointed out that the tsetse disease was not contagious but caused by the bite of the tsetse. Suspecting that the horny dilation at the base of the proboscis might contain some very strong poison, or a gland for its secretion, he dissected it and found nothing but a series of large muscular bundles.

In the ensuing years scientific opinion veered to the idea that insects might carry parasites pathogenic to man and animals. In 1877 Sir Patrick Manson showed that the young of Filaria bancrofti, a small nematode worm which causes elephantiasis in man, are sucked up with blood by mosquitoes in which they grow and develop. Although he did not go on to show that the mosquitoes later infect healthy people with these parasites, nevertheless his work started speculation as to the possibility that insects might be carriers of diseases such as Nagana and Malaria. Shortly after Manson's discovery, in 1880, G. Evans announced the discovery in India of a trypanosome, later named Trypanosome evansi, in the blood of horses and camels which were suffering from a disease known as "Surra". This brought the trypanosome family into prominence and opinions grew among the investigators that Surra might be similar to Nagana. When Manson, working in England in 1890, transmitted malaria disease to volunteers through the bite of anopheline mosquitoes imported from Italy,

speculations mounted as to whether the tsetse flies would transmit Nagana in a similar fashion. It was not until 1895 that the two partners - the tsetse and the trypanosome - became incriminated.

Bruce (1895, 1896) demonstrated that the tsetse fly could act as a carrier of Nagana from infected to healthy animals. He allowed caged tsetse to begin feeding through the netting on heavily infected dogs and then quickly transferred the cages to healthy dogs; he found that in this way he could transmit the disease. He showed that tsetse could transmit the disease to susceptible animals in nature. The three horses, which he took on several occasions to the 'fly area' for a few hours (10 a.m. - 4 p.m.) and permitted to be bitten freely by tsetse flies but not allowed to eat or drink until their return to the top of the hill, were subsequently infected. He also brought up large numbers of tsetse from the fly belt and placed them directly on a healthy horse; it became infected. He also found that the blood of wild game shot in the fly belt caused infection when injected into his experimental animals, thus proving that the game forms a reservoir of the disease.

Before Bruce's work on Nagana, no one suspected any connection between Nagana and sleeping sickness. Neither was the cause of these diseases known. In his report, Bruce had referred to the organisms transmitted by the tsetse as "the Haematozoon or blood parasite of the Fly Disease" and had used the circumstantial evidence that they were found in the blood of every animal suffering from the disease to prove that they are the cause of Nagana. The publication of his works however aroused almost immediately suspicions as to the true nature of sleeping sickness. Blanchard (1904) quoted a sentence from a memoir published by J. Brault in 1898, in which the opinion was expressed

that sleeping sickness was a protozoal disease caused by trypanosomes and transmitted by tsetse flies. When in 1902 Forde discovered trypanosomes in the blood of man - which were later described and named T. gambiense by Dutton (1902) - and in 1903 Castellani (1903) reported that he had found trypanosomes in the cerebrospinal fluid of 70% of sleeping sickness cases, a chorus of prophetic utterances predicting that sleeping sickness would prove to be transmitted by tsetse flies were produced. According to Blanchard (1904) he, Brumpt, and Sambon expressed this view quite independently of one another between June and July of 1903. Nevertheless, it was Bruce once again, who, in collaboration with his colleagues of the Sleeping Sickness Commission, Nabarro and Greig (1903a and b), first supplied the experimental demonstration of the transmission of sleeping sickness by tsetse flies. They succeeded in transmitting trypanosomes to a healthy blackfaced monkey by feeding on it wild flies caught in the vicinity of the hut-tax labourers' camp near Entebbe, Uganda. Although, with the scanty knowledge of trypanosomes at that time, it was impossible to tell if the trypanosomes transmitted by these tsetse flies to monkey were the same as those found in cases of sleeping sickness, the result was striking in that it showed that tsetse flies could transmit trypanosomes. A specimen of the tsetse flies used in this experiment was sent to Mr.Austen who gave the information that it was G. palpalis.

The indications from Bruce's work was that the mode of transmission of trypanosomes by the tsetse flies from an infected to a healthy animal is mechanical and that no previous development of the parasite in the fly was required. In a later publication, Bruce (1903) showed that flies which fed on sleeping sickness cases could

transmit the infection up to at least 48 hours after feeding. Doubts as to the validity of this conclusion were expressed in some quarters, especially the German investigators. They pointed to two aspects of his experimental results which suggested the possibility of a developmental phase in the flies. These are:
(a) Bruce (1896, 1903) examined the proboscis and stomach contents of the tsetse flies at varying intervals after feeding them on an infected animal; the proboscis showed only one or two active trypanosomes up to 46 hours after feeding. The stomach on the other hand contained in some cases blood up to 118 hours after feeding and in rare instances numerous and vigorously active trypanosomes were seen amongst the unchanged red blood corpurscles.
(b) Bruce et al. (1903b), in one of their experiments to demonstrate the transmission of trypanosomes by tsetse flies, showed that freshly caught G. palpalis could convey trypanosomes to healthy animals without previously feeding them on an infected animal in the laboratory. Since Bruce's experiments had been conducted with wild caught flies, there was no evidence that his results had not been influenced by this factor. While not disproving Bruce's theory of mechanical transmission, many investigators turned their attention to studying the possibility of a developmental phase of trypanosomes in tsetse flies.

Nabarro and Greig (1903) dissected G. palpalis at intervals after the flies had fed on infected animals. Active trypanosomes believed to be T. gambiense were seen in the stomach contents up to seventy one hours but not later; trypanosomes believed to be those of Jinja cattle disease, up to one hundred hours. In some flies which had fed on cattle with the Jinja disease parasites were seen in the

proboscis up to three and three quarters hours but not later. Some details of the morphology of the trypanosomes were given. Schaudin (1904) showed that T. nuctuae undergoes a cyclical development in mosquitoes which had been fed on owls infected with the parasite. These results were regarded by many as conclusive proofs that trypanosomes pass through a developmental cycle in their invertebrate hosts. Koch (1905a) was the first person to supply experimental proof of a development stage of trypanosomes in the flies. His first observations were made on G. fusca. He noticed that by pressing the bulb at the base of the proboscis he could cause the extrusion of a drop of clear fluid which, after examination contained very numerous trypanosomes in different stages of development. Since the fluid was quite free from red corpuscles, and the trypanosomes were more numerous than they ever were in the blood and were in various stages of development, he concluded that the parasites go through a course of development in the fly as in the case of Malaria. The stomachs of these flies contained trypanosomes in various stages but no blood; in the intestine they were wanting or present in small numbers only. Koch described their morphology with figures indicating what he believed to be the sexual forms of T. brucei. The males he described as long and narrow and the female as thick and plump. He noted that most of the trypanosomes were of a larger type than in the blood and that small forms with the blepharoplast in front of the nucleus as well as spherical cells occurred. He claimed to have found fertilized females in the lower part of the stomach and he described forms with many nuclei which he believed to be zygotes. He did not succeed in infecting flies by feeding them on animals containing

trypanosomes. When they were fed on recently infected cattle the trypanosomes disappeared as the blood was digested; when he used oxen and mules which had long been infected and contained few parasites there were some successes. His conclusion from this was that flies can be infected only by trypanosomes which are in a certain stage of development and that such a stage occurs in the animals that are little sensitive as big game. He attempted without success to infect rats with trypanosomes from the stomach. In a second paper (1905b), Koch endeavoured to establish differences between T.brucei and T.gambiense based upon morphological variations of the trypanosomes found in wild caught G. morsitans, G. fusca, and G. palpalis. He believed that the first two species are carriers of T. brucei while the latter is the only carrier of T. gambiense. Gray and Tulloch (1905) demonstrated that the trypanosomes of sleeping sickness multiplies in the stomach of G. palpalis. They found that in 10% of wild flies fed on infected monkeys enormous increase occurred in the number of trypanosomes taken in, and in about 5.6% of them this increase was continued at later periods up to 288 hours. 2 of 200 fresh caught flies contained trypanosomes in the same enormous quantity as found in 10% of flies which had been fed on an infected monkey. They gave a detailed morphology of the trypanosomes found in the flies but these were similar to that already described by Koch earlier in the same year. They found the fly forms of trypanosomes no more infective to monkeys 24-120 hours after the infective feed - thus confirming the previous observations of Bruce (1903) and Koch (1905a). This observation was later confirmed by Prowazek (1905), Bouet (1907), and Minchin (1908).

An important progress was made in the investigations into the development of trypanosomes in tsetse flies when Koch (1906) announced his success in breeding flies in his laboratory at Amani, German East Africa, from pupae deposited by wild flies. The observations he made from his subsequent experiments were therefore of a greater value than the earlier ones because the flies he examined were certainly free from infection unless, hereditary transmission could be shown to occur. In an attempt to determine whether G. fusca could serve as a carrier of T. gambiense he fed 42 G. fusca and 11 G. tachinoides, all bred in the laboratory, on rats which had been previously infected with T. gambiense. 10-12 days later it was found that 8 G. fusca and 3 G. tachinoides were infected with enormous number of trypanosomes in their intestine. Koch used this result as a pointer again to a developmental phase of trypanosomes in the flies. The publication of Koch's results led to the creation of two schools of thought on the question of developmental phase of trypanosomes in tsetse flies, the chief protagonists being Koch, Stuhlmann and Kleine and the antagonists Bruce and Novy. Novy (1906b) concluded that the trypanosomes met within the intestine of tsetse flies were 'cultural forms' of harmless non-parasitic flagellates corresponding to the equally harmless Herpetomonas and Crithidia which had been found in mosquitoes by Schaudin (1904) and himself (1906a). Apart from this his view was strenghtened by the following facts i) the trypanosomes found in the fly's intestine are of larger size than those of the blood; ii) they are present in flies which had not fed on infected animals (Novy believed that tsetse flies could exist without a diet

of blood); iii) the failure of the intestinal forms to infect susceptible animals and most especially iv) the failure to secure growths of the trypanosomes, pathogenic or non-pathogenic, in all, or even in a large per cent of the flies fed on infected animals. This was the first time the low rate of infection in flies was being used as an argument against a developmental phase in flies. In a later publication, Novy (1907) showed that T. brucei survived only as late as 30-36 hours in very few mosquitoes; dead forms were found in the stomach of the majority of them after a few hours.

Dutton, Todd and Hannington (1907) published their observations on the fate of T. gambiense after being taken up by various arthropods. In G. palpalis they found unaltered parasites in the alimentary canal up to 48 hours; living but altered up to 72 hours after feeding. In blood ingested for some days parasites approximating to "male and female" types of trypanosomes became relatively much more numerous than at first. They hinted at the possibility of tsetse inheriting trypanosomes from its parents, a possibility already expressed earlier in the same year by Ross (1907). Stuhlmann (1907) published a preliminary account of the results obtained by himself and Kudicke with T. brucei. These investigators worked both with freshly caught flies and with laboratory bred flies. The latter were found to become most easily infected if fed on the infected animal for their first meal after being hatched from the pupa; in 80-90% of the flies so treated, after a short time (2-4 days) the intestine became full of trypanosomes, indifferent forms showing many division stages. They found that the infection spread from the intestine into the stomach, but rarely extended further forwards than this region, unless the flies

were re-fed on healthy animals; when that was done the infection could be traced forward as far as the proventriculus. It was found also that the infection produced in laboratory bred flies gradually died out and only 10% of the flies obtained an infection of the stomach and thoracic intestine. The morphological descriptions of the forms they found in the intestine were similar to those already given by Koch (1905a). However, they presented evidence to show that the long forms are found in the proventriculus and oesophagus, and rarely in the proboscis, while the small forms are abundant almost exclusively in the proboscis. As a result of these observations they suggested the following scheme of development. The infection begins by a multiplication of indifferent forms in the intestine. From this point they spread forwards as far as the proventriculus where they believed conjugation takes place. After conjugation, probably, are produced the small forms, which may be the infective forms which pass back into a new vertebrate host. The nature of the long, slender forms is doubtful; they appear to die out. In no cases did trypanosomes invade the end gut. Neither were they found in the salivary glands, nor in any organs of the fly except the proboscis and the digestive tract. They also believed that trypanosomes are not transmitted by tsetse flies to their posterity. They confirmed Koch's observations that the state of the parasites in the infecting animal seemed to have some influence. All their attempts to infect flies with the blood of a certain goat failed, while those with sheep, calf or dog succeeded. Dealing with the views of Novy (1906) who doubted if the forms described by various observers belong to the cycle of development of vertebrate

trypanosomes, they pointed out that at Amani, where the experiments were conducted, trypanosomes were never seen in laboratory bred G. fusca unless the flies had been fed on an infected animal.

Minchin (1908), describing in a memoir the results of his experiments during his 8 month work as a member of the Sleeping Sickness Commission, was the first to differentiate between the multiplicative and developmental stages in the development of trypanosomes in tsetse flies. Dissecting at daily intervals wild caught flies which he had previously fed on monkeys infected with T. gambiense, he observed that the trypanosomes at first multiplied in the digestive tract of the flies. After 24 hours the developmental stage sets in and he could find 2 types - slender and stout - sharply distinct from one another, with no intermediate forms. At about 48 hours, these two types were succeeded by a more uniform type, so far as structure was concerned, but varying from slender to fairly stout, with all possible transitions, and of considerable length. On the 3rd day, after infection, trypanosomes found were forms of great length, relatively varying from slender to stout, sometimes appearing degenerate in structure and diminished in number, but in other cases numerous, active, and with no signs of degeneration. On the 4th day trypanosomes were rarely seen, and, if present, were very scanty in number and of large size. In no case had he found any signs of T. gambiense in the tsetse fly later than the 4th day after infection. He argued that if the trypanosomes really died out in the fly without completing any life cycle, it would indicate that G. palpalis was not the true host for the trypanosomes; if however the disappearance of the trypanosomes was only apparent, and they really persist, the

possibilities might be that they passed into some organs of the fly, or they had assumed some minute ultra-microscopic form, or they had passed on into the rectum in order to become encysted there. Since in a former publication (Minchin, 1907) he had shown the encystation of T. grayi in the rectum of tsetse flies during the phase of development, he opted for the last possibility. He agreed with Stuhlman's work of 1907 which made it evident that T.brucei goes through a developmental cycle in tsetse flies. He suggested that the G. fusca used by Stuhlman might be the only true agent of transmission of T. brucei and it is only in this species that this trypanosome would undergo such a cycle. He gave the studies of Brumpt (1906 a, b, c) on the transmission of fish trypanosomes by leeches which showed that for every species of fish trypanosomes, there was always what may be termed a right leech.

In December 1908, Kleine (1909) described an experiment conducted in Kirugu, German East Africa which seemed to prove that transmission of trypanosomes occurred after a developmental cycle in the case of T.brucei and G. palpalis. He fed 50 G. palpalis, caught on the Mori river, on three animals suffering from Nagana for 3 days (one mule, two sheep) and from the fourth day onwards they were fed daily on a fresh healthy animal. From the 18th to the 24th day the flies fed on the same sheep, from the 25th to 39th day on the same ox. Both sheep and ox were subsequently infected; the latter 12 days after the last feed of the flies. All the other animals fed upon between the 4th and 17th day remained healthy. By this, Kleine showed that flies, which had fed on blood containing trypanosomes, first remained uninfective for many days after the infective feed before becoming infective again. He attributed this

uninfective period to the development cycle in the fly. Although he was able to isolate 2 infected flies among the 50 G. palpalis used for this experiment, he did not give the results of their dissection. (Kleine 1909b). In his next publication (Kleine, 1909c) he reported using laboratory bred instead of wild G. palpalis to conduct the experiment and he obtained the same result. Of the 410 laboratory bred flies which he used, 22 or 5% were infected. He noticed that those which infected animals amongst them did so after approximately 20 days and he consequently held this to be the duration of the development cycle of T. brucei in G. palpalis. He tried to describe the course of the cycle through the morphology of trypanosomes in his stained preparations. He concluded that it was not necessary for flies which infected animals to possess trypanosomes in the salivary glands since none of his which did so had their salivary glands invaded. He however saw "resting stages" of trypanosomes in the salivary glands of some infected flies and he believed that they must have proceeded from the mouth parts. All the flies which had infected animals had enormous number of trypanosomes in the midgut and proventriculus. Only a few of these had them in the mouth parts as well. He concluded that the infective forms of the development cycle were present only in the midgut since all flies which were dissected 3 weeks after the infective feed, just around the time they infected animals, contained typical blood forms. He was however sure of a connection between these forms and the "resting stages" in the salivary glands, although he could not explain it. He also disproved any idea of hereditary transmission of trypanosomes to offsprings among the tsetse flies. His laboratory bred flies which infected monkeys at least 20 days after the infective

feed came from pupae deposited by flies collected from places of endemic foci. If the transmission were hereditary, these laboratory bred flies could have infected the monkeys almost immediately after hatching. This was confirmed later by Bruce et al (1911b), Fraser and Duke (1912b).

In an experiment conducted in a similar way to the original one of Kleine, Bruce et al (1910a) obtained results which confimmed Kleine's observations. They fed 60 wild caught G. palpalis for 2 days on two monkeys previously infected with T. gambiense and kept them under observation for 86 days before they were dissected. The flies were maintained on a healthy monkey for the first five days after the infective meal, and every successive period of 5 days on a fresh monkey, up to 86 days, when the experiment was terminated. They found that all monkeys used after 25 days were infected with T. gambiense. On dissection, they found only one fly infected with abundant trypanosomes in the midgut and the salivary glands. They considered it a mystery that the salivary glands were invaded since the proventriculus was not infected. A tiny drop of fluid taken from the gut of this fly and injected into a monkey produced infection.

With the idea of a developmental phase of trypanosomes firmly established among the investigators, attention was turned to the course of the development cycle. A new problem however was introduced when Bouffard (1909), working in Bamako, West Africa, with laboratory bred flies, showed that the development of a species of trypanosomes was confined to the proboscis. Although he could not identify the species, he found that the proportion of infected proboscides increased with the duration of the experimental and varied between 11 and 70%. He had fed his flies on a sheep infected with

these trypanosomes and experiments had been conducted at temperatures between 25°C and 28°C and 70% relative humidity. Bouffard's observations were confirmed by Roubaud (1910). In his investigations in Dahomey, in which he used laboratory bred G. palpalis, G. tachinoides, and G. longipalpis, he found that a trypanosome species, which he named T. cazalboui, never showed intestinal forms but its development was confined to the proboscis. He reported that, after the infective feed, some of the parasites fixed themselves to the wall of the labrum and took on the Leptomonas form and multiplied. The hypopharynx was invaded in a short time and there the Leptomonas reassumed a trypanosome form which resembled the blood form. Roubaud went further to compare this mode of development with the results of his experiments with T. dimorphon and T. pecaudi. He found that these species underwent an intestinal developmental phase in the flies, where they multiply rapidly and invade the whole of the anterior midgut as far as the pharynx. Flies were not infective until the intestinal trypanosomes had penetrated into the proboscis, fixed themselves to the wall of the labrum, changed to the leptomonas form, and multiplied. Subsequently, they invaded the hypopharynx where the leptomonad forms re-assumed the blood forms. The works of Bouffard and Roubaud thus showed that different trypanosomes could have different courses of development in tsetse flies; a fact not hitherto suspected.

Bruce et. al (1911a) showed the involvement of the salivary glands in the development cycle of T. gambiense. They found that although the salivary glands could be invaded by T. gambiense by about the 28th day after the infective feed, the flies were not infective to monkeys until about the 34th day. They concluded that there could be no infectivity of flies to susceptible hosts without the invasion of the

salivary glands since 8 of the 9 flies which infected their individual monkeys possessed trypanosomes in the salivary glands. The type of trypanosomes found in the salivary glands when a fly became infective was similar to the short stumpy form found in vertebrate blood and they believed that this reversion to the blood type was a sine qua non in the infective process since the only fly with salivary gland invasion which did not infect monkey had trypanosomes in the salivary glands which were not the typical blood forms. They speculated on how the trypanosomes could have got to the salivary glands. It was improbable, they thought, that they passed from the alimentary canal by way of the salivary ducts; neither could they imagine their entering the abdominal portion of the salivary glands since they had never found trypanosomes in the body cavity of flies. They extended their investigations to T. brucei, T. pecorum, T. nanum and T. vivax. They found that the development of T. vivax was restricted to the proboscis, while that of T. pecorum and T. nanum ended in the proboscis after an intestinal phase. With T. brucei, they obtained negative results.

Fraser and Duke (1912a) confirmed the involvement of the salivary glands in the course of development of T. gambiense in tsetse flies and went further to show that T. brucei has the same pattern of development with the infective forms in the salivary glands. As previously observed by Bruce et.al., (1911a) they produced experimental evidence to show that the development of T. pecorum took place in the midgut, proventriculus and proboscis, the trypanosomes being attached in clusters to the labrum. They also demonstrated that the development of T. uniforme in G. palpalis was similar to that of T. vivax as

described by Bruce et al. (1911a). In the same year, Duke (1912) showed that the development of T. nanum was similar to that of T. pecorum in that it commenced from the midgut and extended forward via the proventriculus until finally the proboscis was reached. In 1913, Rodhain et al. confirmed Roubaud's observations that T. pecaudi has its development cycle limited to gut and proboscis.

One other problem introduced at this time into the studies concerning the development of trypanosomes in tsetse flies was whether the type of development in the fly for a given species of trypanosome is constant, irrespective of the species of tsetse concerned. This had been a contention between the investigators ever since Bruce (1895) published his original work and represented G. morsitans as the only transmitter of Nagana. Kleine at first believed that G. morsitans could transmit T. gambiense and even gave a communication from Taute (Kleine, 1909c) who in Ikoma, (East Africa), was reported to have had in custody a G. morsitans fly which was still infective 83 days after the infective feed; a possibility which was not acceptable to the members of the sleeping sickness commission especially Bruce and Dr. Hodges. Kleine changed his mind when he and Taute (Kleine 1909d) could not obtain transmission after 672 and 120 laboratory bred G. morsitans had fed on a monkey infected with T. gambiense. The view popularly held then was that G. morsitans could only transmit Nagana; and G. palpalis the sleeping sickness. Bouet and Roubaud (1910) however showed the ease with which one species of Glossina can replace another in a successful transmission experiment. In a series of experiments they showed that T. cazalboui frequents the proboscis only of G. morsitans, G. palpalis and G. longipalpis; T. gambiense develops in the midgut and salivary

glands of G. morsitans and G. palpalis; and T. dimorphon occupies both gut and proboscis in G. morsitans and G. palpalis. The same workers (Bouet and Roubaud (1911)) transmitted T. pecaudi by G. tachinoides, G. longipalpis and G. palpalis, and found that, in all the positive flies dissected, flagellates extended throughout the gut and proboscis. They found the highest rate of infection with T. pecaudi in G. longipalpis, higher in G. tachinoides, and least in G. palpalis. They thereby recognized that one species of flies could be a better carrier than the other. Taute (1911) transmitted T. gambiense by G. morsitans and described swarming trypanosomes in the salivary glands of the infected flies. Rodhain and his collaborators (1912) found T. cazalboui transmitted by G. morsitans and flagellates were present only in the proboscis; while T. congolense carried by G. morsitans showed infection of the midgut and proboscis. Bruce et al (1915 b and c) showed that the course of development of T. pecorum and T. caprae in G. brevipalpis is the same.

Two years after Stephen and Fantham (1910) had described T. rhodesiense, Kinghorn and Yorke (1912a) succeeded in showing that its development cycle is very similar to that of T. gambiense but that the duration of the cycle (11-15 days) was shorter at least in G. morsitans. Having confirmed this observation in a later publication, (Yorke and Kinghorn, 1912b), they collaborated with Lloyd to produce evidence that the invasion of the salivary glands with T. rhodesiense was secondary to the intestinal infection. They (Kinghorn, Yorke, and Lloyd, 1912) found that whenever trypanosomes were found in the salivary glands they were always

infective to mice on innoculation, as were also those present in the midgut. They believed that this showed that the invasion of the salivary glands only occur when the trypanosomes in the midgut have reached a certain stage of development.

When by 1913 the development cycle of most of the known species of trypanosomes had been established, it was evident that some species had identical development cycle in the flies. Since at that time there was little or no criterion for the diagnosis of different species of trypanosomes, some investigators thought that a classification of the species of trypanosomes according to their distribution in the alimentary canal of the tsetse flies during the development would be of assistance in diagnosis. Duke (1913) and Roubaud (1913), working quite independently in the same year classified the African pathogenic trypanosomes according to the mode of their development in tsetse flies. They recognised 3 groups:-

1) Trypanosomes in which the midgut, and the salivary glands of the tsetse are involved. They called this "Brucei group", comprising of *T. gambiense*, *T. rhodesiense*, and *T. brucei*.

2) Trypanosomes in which the development in flies is restricted to the midgut and proboscis; known as "congolense group", and consisting of *T. dimorphon*, *T. pecorum*, *T. nanum*, *T. pecaudi* and *T. congolense*.

3) Trypanosomes in which the development is limited to the proboscis only, called the "vivax group" and consisting of *T. vivax*, *T. uniforme*, and *T. cazalboui*.

The components of each of these groups portrayed the handicap suffered by the investigators of those days. Each observer, depending on his origin, named any species without any standard criterion. At this

time, each colonial power was being represented by a Sleeping Sickness Commission in Africa; the British by the Sleeping Sickness Commission of the Royal Society, the Germans by the German Sleeping Sickness Commission, the French by the French Commission for Sleeping Sickness and the Portuguese by the Portuguese Sleeping Sickness Commission. Since there was little or no communication between these workers, the nomenclature of species was bound to vary. It is however to their credit that the main criterion for classification of these trypanosomes still stands today. Only the components of 2 of the 3 groups had to be modified.

Robertson (1913b) was the first person to publish a full account of the development cycle from the early period of infection to the time a fly becomes infective. Using laboratory bred flies which had been fed on animals infected with T. gambiense, she observed that in the early days of the cycle, flies which possessed conditions that would allow establishment of infections had trypanosomes persisting and multiplying vigorously only at any part of the middle or hinder intestine. She never found trypanosomes in the anterior intestine in such flies at an early period. She observed that the infection literally grows forward by sheer force of multiplication till it fills the whole of the midgut. The anterior portion of the intestine and the proventriculus show the typical slender forms and are not invaded until the 10th to 20th day, or thereabouts. She noted that the trypanosomes encounter some difficulty before reaching the proventriculus. She believed that the penetration of the salivary glands by the trypanosomes occurs quite clearly from the hypopharynx and the successive stages of the process can be seen very well in the live state, in careful dissections of the glands at the appropriate

periods. She observed that the slender trypanosomes pass up into the hypopharynx from the proventriculus, come along the narrow duct of the salivary glands and settle down and attach themselves in the slightly broader cellular part of the salivary glands or at the entrance to the glandular part, the rest of the gland being quite free from trypanosomes. They then change to round-ended, crithidial flagellates which multiply and fill up large portions of the gland. Later, the trypanosome forms are produced which are similar to blood forms.

After Bruce and his collaborators (Bruce et. al 1914b) had described the development cycle of T. simiae and showed by it that it belongs to the congolense group, the investigators turned their attention to finding out the rate of infections of flies in nature. A full report of this is given in Section 1.11.

Not until 1929 did the investigators turn their attention again to the development of trypanosomes in tsetse flies. In this year, Wigglesworth (1929) published a paper which showed the method of formation and function of the peritrophic membrane. He showed that the proventriculus does not only act as a sphincter between the fore - and midgut, but it is also responsible for the production of the peritrophic membrane. Wigglesworth showed that this membrane is secreted in the form of a fluid by the ring of large epithelial cells at the base of the proventriculus. The fluid is pressed and condensed to form a uniform membrane by being drawn through the cleft between the wall of the proventriculus and the funnel shaped invagination of the fore-gut.

Although the peritrophic membrane was first described by Stuhlmann (1907) in which he even noted the presence of T. brucei in

the midgut outside the membrane, and although a similar observation was recorded for T. congolense by Johnson and Lloyd (1929) and later by Lloyd (1930), no attempt was made to associate this situation with the life cycle of the trypanosomes. This membrane was ignored in all previous investigations of trypanosomes because the view held, and confirmed by Wigglesworth, was that the membrane is formed anew after each meal, the old one being discarded. It would thus appear that between meals there are periods when a membrane is absent. If that were so, this organ would have no effect upon the developing trypanosomes and could be ignored. Hoare (1931a) was however able to show that the peritrophic membrane is produced in a continuous stream from the anterior end, only its distal part being gradually discarded as it advances down the hind gut. The integrity of this membrane was later confirmed by Taylor (1932).

Hoare (1931a, b, 1933), elucidating the role of the peritrophic membrane in the development of T. grayi in tsetse flies, showed how in the course of the development in the gut of the fly the distribution of T. grayi is determined by the presence of the peritrophic membrane. This naturally drew the attention of other workers to the long neglected question of its influence upon the development of the pathogenic African trypanosomes. Taylor (1932), in his study of the development of T. gambiense in G. tachinoides showed that up to the 4th day after infection of the fly, this trypanosome develops within the endoperitrophic space of the midgut. The flagellates then escape through the open end of the peritrophic membrane into the hindgut and invade the ectoperitrophic space of the midgut, gradually migrating forwards until they reach the proventriculus, where they

find themselves in a cul-de-sac. He was not able to trace the route by which the trypanosomes reached the salivary glands - although he showed that infection extends to the point of origin of the peritrophic membrane between the annular pad and the invaginated fore-gut. This part of the problem was solved by Yorke, and his colleagues (Yorke et.al., 1933), who, working with T.brucei in G.morsitans and G.palpalis, showed that the trypanosomes pass from the ectoperitrophic space of the proventriculus into the endoperitrophic space by penetrating through the least resistant part of the peritrophic membrane, namely through the fluid part at the point where it is being secreted from the epithelial ridge. Having thus found their way into the inner lumen of the proventriculus the trypanosomes proceed forward via the oesophagus into the proboscis, entering the hypopharynx at its open end and again migrating backwards into the salivary glands; where they give rise to the infective metacyclics. Fairbairn (1958) confirmed the ability of trypanosomes to penetrate the fluid portion of the peritrophic membrane in order to gain access to the endoperitrophic space of the proventriculus. Working with T. rhodesiense, he showed microphotographs of T. rhodesiense penetrating the fluid secretion and of the formed membrane in the roof of the proventriculus thus proving that this part of the membrane, having been freshly secreted, is still sufficiently fluid for the trypanosomes to penetrate.

Willett (1964, 1966) showed that so long as the peritrophic membrane in the teneral fly has not reached the hindgut and been ruptured by the spines, its posterior end is completely closed and the membrane forms an unbroken sac, thereby disproving Wigglesworth's statement that the peritrophic membrane in the freshly emerged fly is

ragged and discontinuous. He produced evidence from his observations to support his belief that a fly which takes an infective meal in the first few hours of its life is presented with a unique opportunity to attain the ectoperitrophic space close to the proventriculus instead of having to complete the long migration round the open end of the membrane in the hindgut and forward again to the proventriculus.

Freeman (1970a), although acknowledging the classical concept of the course of development of the trypanosomes in the flies, suggested that passage by the trypanosomes through the soft anterior part of the peritrophic membrane could be another method, since the trypanosomes would then avoid the alien acidic conditions of the rectum, which, with PH. 5.8 had been shown by Bursell and Berridge (1962) to be fatal to trypanosomes. Moreover this method would afford the trypanosomes the shelter of the ectoperitrophic space at an early stage. She believed that the crossing of the peritrophic membrane at this point was feasible since it occurs in the reverse direction later in the life cycle of the trypanosomes. She also suggested that the rapidity with which blood passes into the midgut from both the crop and directly from the oesophagus might produce sufficient pressure to push at least some trypanosomes against and subsequently through the soft anterior part of the peritrophic membrane. She quoted a personal communication from Dr.Vickerman who showed that erythrocytes could be caught up in the peritrophic membrane. In a later publication, (Freeman, 1970b) she claimed to have found trypanosomes embedded in the peritrophic membrane and in the ectoperitrophic space of one out of 20 G. morsitans which she had fed on the day of their emergence on guinea pigs infected with

T.brucei and dissected 30 minutes later. She concluded that the trypanosomes might have penetrated the peritrophic membrane at the soft part or they might have been forced through it. She also reported seeing a liverpool strain of T. vivax in the peritrophic membrane of one G. morsitans and in the ectoperitrophic space of another out of 15 G. morsitans examined 30 minutes after the infective feed (Freeman, 1970c).

Using electron microscopy to study the structure and functions of the peritrophic membrane, Moloo and his collaborators (Moloo et al. 1970) provided some indication of the possible route taken by trypanosomes to cross the "membrane" barrier. They found three cell types (Type I, II and III) in the annular pad of the midgut epithelium. They showed that when the secretion is completed, the membrane consists of two distinct layers; the upper, or the first formed layer, which is electron-dense and 0.1 μ thick and secreted by Types I and II cells, and a second amorphous, electron opaque layer, 3-4 μ thick and secreted by Type III cells. After it has been drawn through the "press" the peritrophic membrane is only 0.35 μ thick. They could find no evidence to support Freeman's suggestion that the trypanosomes could penetrate the membrane not long after the ingestion of an infective blood meal since the membrane is a bilaminate and continuous structure and it was difficult to conceive how trypanosomes could force their way through this barrier except it is shown that they have the necessary enzymatic means of digesting the membrane. They regarded the assumption of Yorke et al (1933) and Fairbairn (1958) that trypanosomes could penetrate the newly secreted fluid part of the peritrophic membrane as partially correct since although the second layer of the membrane is semifluid

when newly secreted, the electron dense first formed layer is by then already partly polymerised. It is however very likely, they explained, that the trypanosomes cross the membrane barrier in the region of the Type II cells where the first layer is still in an unpolymerised granular form.

Quite recently, Mshelbwala (1972) indicated that the haemocoele of tsetse flies could be involved during the course of development of trypanosomes. Dissecting 1,285 flies which had earlier been fed on animals infected with T.brucei, he found that 262 possessed established infections. Of these, 40 or 15.27% were found with trypanosomes in the haemocoele, especially that of the thoracic haemolymph and the legs. Forms seen included trypomastigotes, epimastigotes, amastigotes and metacyclics. The latter were infective to mice on innoculation.

1.11 The rates of infection of tsetse flies in nature.

Even before scientific interest was shown in trypanosomiasis, historical accounts had shown several occasions when indirect allusions were made to the low rate of infection in tsetse flies. David Livingstone mentioned an occasion during his Missionary Travels of 1857 when he suffered a loss of 43 of his oxen and was quoted (Nash, 1969) as saying that he was puzzled because "not a score of flies were ever upon them".

Although the earlier investigators were engrossed in the task of working out the course of development of trypanosomes in the fly, they often made references to the low rate of infections in the flies. Kleine (1909 a and b), in his original experiments which proved

conclusively that a development cycle of trypanosomes takes place in the tsetse flies, had only 2 out of 50 G. palpalis infected. In a subsequent similar experiment with laboratory bred G. palpalis, 22 or 5% of the 410 flies were infected. (Kleine 1909c). In the experiments set up by Bruce and his collaborators to confirm or disprove Kleine's observations (Bruce et.al. 1910a) only 1 out of the 60 wild caught G. palpalis was infected. By the time Duke (1913) and Roubaud (1913) classified the African pathogenic trypanosomes according to their modes of development in the flies, it was a common knowledge among the workers that the rate of infections was highest in the vivax group of trypanosomes, less in the congolense group and least in the brucei group. Since most of this information came from results conducted in the laboratory, it was not known whether the facts would agree with the situation on the field. Hence, most of the investigators turned their attention to studying the rate of infection of tsetse flies under natural conditions. The methods used by most of the workers were similar. Flies were often collected in endemic areas and dissected. If trypanosomes were found only in the proboscis, the fly was said to be infected with vivax group of trypanosomes; if the trypanosomes were found in the gut and proboscis, the infection was said to be of congolense group; infections involving the midgut and saliva were referred to as brucei group.

The accompanying table shows the record of dissections of wild flies by various workers between 1914 and 1932. The pattern of the infection rate conforms to that observed in the laboratory. Except in a few cases, the infection rate decreases from the vivax group through the congolense group to the brucei group.

OBSERVER	DATE	PLACE	SPECIES OF FLY	NO. DISSECTED	TOTAL INFECTIONS PER CENT	+GROUP ANALYSIS PER CENT
Robertson	1914	Masindi, Uganda	*G. morsitans*	445	9.4	1 = 3.8 2 = 2.9 3 = 0 4 = 2.7
Duke	1914	Masindi, Uganda	*G. morsitans*	1,117	13.4	1 = 8.5 2 = 3.1 3 = 0.2 4 = 1.6
Duke	1914	Fajao, Uganda	*G. morsitans*	606	9.6	1 = 7.0 2 = 1.8 3 = 0 4 = 0.8
Duke	1914	Ngussi River, Uganda	*G. pallidipes*	65	18.4	1 = 1.5 2 = 6.3 3 = 3.0 4 = 7.6
Johnson and Lloyd	1922-23	N. Nigeria	*G. palpalis*	552	5.6	1 = 2.2 2 = 0.5 3 = 0 4 = 2.9
			G. tachinoides	1,500	11.3	1 = 5.4 2 = 1.1 3 = 0 4 = 4.8
			G. morsitans	500	26.4	1 = 15.2 2 = 6.0 3 = 0 4 = 5.2
Duke	1923	Mwanza, Tanzania	*G. swynnertoni*	2,206	9.3	1 = 6.5 2 = 1.9 3 = 0.13 4 = 0.70
Lloyd and Johnson	1923-24	N. Nigeria	*G. morsitans*	448	9.6	1 = 6.5 2 = 3.1 3 = 0
			G. tachinoides	1,093	2.6	1 = 2 2 = 0.6 3 = 0
			G. palpalis	534	1.7	1 = 1.5 2 = 0.2 3 = 0

(continued)

OBSERVER	DATE	PLACE	SPECIES OF FLY	NO. DISSECTED	TOTAL INFECTIONS PER CENT	+GROUP ANALYSIS PER CENT
Lloyd, Johnson, Young, Morrison	1924-25	N.Nigeria	G.morsitans	5,891	18.84	1 = 15.1 2 = 3.7 3 = 0.04
			G.tachinoides	10,799	5.43	1 = 4.2 2 = 1.2 3 = 0.03
Chorley	1929	Lomagundi, S.Rhodesia	G.morsitans	1,523	11.101	1 = 4.7 2 = 6.4 3 = 0.001
Duke	1932	Fajao, Uganda	G.morsitans	735	11.9	1 = 3.5 2 = 1.4 3 = 0 4 = 3.0
Duke	1932	Ngussi River, Uganda	G.brevipalpis	121	0.8	-
Duke	1932	Nsongezi, Uganda	G.morsitans	109	5.5	1 = 0.9 2 = 1.9 3 = 0 4 = 2.7
Duke and Wallace	1932	Bugoto	G.pallidipes	231	6.4	-
Whitnall	1934	Umfolosi, Zululand	G.pallidipes	2,735	4.13	1 = 2.06 2 = 1.09 3 = 0.98

+ 1 = Vivax group
2 = Congolense group
3 = Brucei group
4 = Gut only

1.12 Factors affecting the rate of infections in tsetse flies.

Since trypanosomiasis was so widespread, it was very difficult to see how it could be sustained in nature with such infection rates observed in the laboratory and on the field, except that the small percentage among the population which are capable of being infected are the gifted ones which are also very efficient at effecting transmission. For example, Duke (1919), quoting figures from his collections of wild flies collected near Entebbe, Uganda, in 1903 where the natives were considered to be infected with trypanosomes in the proportion of 1 in every 3 or 4, found only 0.3% of all the flies infected. Summarising the results he obtained by dissecting wild flies caught at Mwanza fly belt, Duke (1923b) commented that the percentage of wild flies capable of cyclically carrying the brucei group of trypanosomes appeared to be about 0.2 and that this percentage was responsible for the infection of at least 16-20% of the local game. It was therefore essential that attention should be turned to those factors to which the trypanosomes and flies are exposed before and after they have come into contact with each other.

A great majority of the information obtained from the laboratory are on the brucei group. This is because the laboratory animals are easily susceptible to infections with it. Less is known of the congolense and vivax groups because laboratory animals are less susceptible to infections with them.

i) Temperature:- This is one of the extrinsic factors that had attracted the attention of several workers. Fraser and Duke (1912a), working as members of the Sleeping Sickness Commission of the Royal Society, were the first to record temperature experiments.

With the aim of investigating whether temperature affects the rate of infection in flies, they kept laboratory bred G. palpalis, which had been previously fed on monkeys infected with T. gambiense, in an incubator at 25°C and set up a control at their laboratory temperature of 18°C. Their results were however inconclusive since a great majority of the flies kept at 25°C died. Bruce et al. (1914b) noticed that they could not effect transmission of T. simiae with G. morsitans until after 50 days in the coldest time of the year on Kasu Hill (temperature 16.6°C). When a control was set up by putting G. morsitans, which had fed on animals infected with T. simiae, in an incubator at 28.3°C, transmission was effected within 20 days.

Many investigators had studied the effect of temperature on the field. Kinghorn and Yorke (1912 a & b), in their reports to the British South African Company remarked that all the 3 transmission experiments they conducted in the dry, and commencement of the rainy seasons, were successful, while only 2 of the 5 carried out during the rains proper were positive. Furthermore, in the rainy season, only 3 of 231 flies, or 1.29%, proved to be transmitting the trypanosomes, as against 4-8% in the dry season. In the same year, the same authors (Kinghorn and Yorke (1912c)) demonstrated the influence of temperature on the development of trypanosomes in tsetse flies with a series of experiments. They noted that T. rhodesiense was successfully transmitted in the experiments with laboratory bred flies set up at Luangwa valley where the temperature was between 67.4 - 84.6°F. All efforts in this direction however failed on the Congo-Zambesi watershed where the temperature was 60.5-68.0°F. In order to ascertain whether the temperature difference alone was the main cause, they kept flies in Congo Zambesi in an incubator at the

temperature approximating to that of the Luangwa valley. Although many flies had died by the end of the second week, yet 3 of the remaining 58 flies had become infective. They corroborated this evidence further by an analysis of the transmission experiments in Luangwa valley. They found thus that the largest number of infective flies was obtained at the hottest season of the year, 6 having been found infective when the mean temperature was 83.8°F and 1 when it was 75-78°F. Comparing also the results obtained by feeding fresh caught wild flies on healthy monkeys, they found that 1:534 G. morsitans were infective in the Luangwa valley while in Congo Zambesi, the ratio was 1:1,260. In a series of investigations in Northern Nigeria which involved dissection of wild flies collected from various areas at different seasons of the year, Lloyd and Johnson (1922-23, 1923-24) and Lloyd, Johnson, Young and Morrison (1924-25) observed that the proportions of flies infested with T. vivax increases as the rains progresses, and falls to a minimum at the hottest months of the year. They also found that the infection rates of wild flies with T. congolense was constant all year round except for a well marked fall during the hottest months of the year. This observation for T. vivax was confirmed by Squire (1951-52) who dissected wild flies collected at Njala, Sierra-Leone. Leggate (1962), basing his observations on the dissection of wild G. morsitans and G. pallidipes caught at the Rekomitjie Research Station, in the Zambesi valley of Southern Rhodesia, confirmed that the vivax type of infections were higher in the cooler months for both species of flies. Analysis of his figures further showed that the infection rate of G. morsitans with T. vivax was significantly lower during the peak of

rainfall than at the onset of rainfall: an observation which was in contrast to those of the previous observers. Leggate also observed that the congolense and brucei groups were more frequent in the hotter months. Chorley (1929), dissecting *G. morsitans* collected from Lomagundi area, Southern Rhodesia, noted seasonal rise in the hot weather of the flies infected with *T. congolense* and *T. vivax*. Lloyd, Lester, Taylor and Thornewill (1933) recorded monthly infection rates in *G. morsitans* over several years and demonstrated an annual cycle in rate of infection with *T. vivax* and *T. congolense* varying between approximately 20 percent in March and September and 40 percent in January and July.

Burtt (1942) found that the proportion of wild flies with infected salivary glands was greater in samples from the Masherwa locality than in those from Sigi and the Old Road. This corresponds with the fact that Masherwa has a lower altitude and therefore presumably a warmer climate. However, the highest rate of infected flies was found in September, when the temperatures were almost the lowest of the season. Fairbairn and Burtt (1946) noted at Tinde, in the north of Tanzania that cyclical transmission of *T. rhodesiense* by *G. morsitans* was obtained very rarely in the cooler season, whereas it was very frequent in the hot time of the year.

Ford and Leggate (1961) examined all available infection rate data from tsetse flies of the *G. morsitans* group from 1916 to 1961 in relation to the geographical position and climates of the localities from where they came. Scrutiny of their tables suggested that there is a relationship between the gross infection rate and distance from the equator. There was clearly a tendency for infection rates to increase, the further the departure from the middle of the Glossina zone,

which was taken as Latitude 7°S on which the median of the Glossina distribution in Africa lies. Since in the tropics there is a tendency for the mean annual temperature to increase with distance from the equator, it was apparent that temperature was the factor responsible for this phenomenon. Results from Zululand did not fit into the trend, and this was explained by the fact that Zululand lies in the furthest south with climate bordering on the temperate zone and the mean monthly temperature is comparatively low.

In the laboratory it has been shown that the effect of temperature is not only upon the speed at which the trypanosome may complete its cycle in the fly, but also upon the proportion of flies in which salivary infections develop. Kinghorn and Yorke (1912c) and Kinghorn, Yorke and Lloyd (1913) showed that at a temperature of 15.5°C the development of T. rhodesiense in G. morsitans could proceed up to a point, that the trypanosomes could persist in this stage for at least 60 days without the salivary glands being invaded, and that it was only when the flies were placed in an incubator, at 26.7°C that the salivary glands were invaded and the cycle was completed. Lloyd (1930) incubated G. tachinoides at 29.4°-36.1°C for a few days during their infecting feed. In this way he obtained greatly increased transmission rates of two old laboratory strains of T. brucei. Although he was not able to repeat his results, he was certain that there was an optimum temperature favouring development, and a critical temperature both above and below it. Duke (1930a) discussed Lloyd's work and was of the opinion that the temperature factor was not important in Entebbe. Three years later, he (Duke 1933b) also obtained increased transmission rates of T. gambiense and T. rhodesiense

by exposing G. palpalis to a temperature of 35°C-36.7°C. Taylor (1932) studied the development of infections in salivary glands in G. tachinoides infected with T. gambiense and maintained at the laboratory temperature and at 37°C for 16 to 24 hours daily for the 4 days after they received the infecting meal on animals. In all the 26 strains which he tested, he obtained a greatly increased transmission; in the case of one strain, a cyclical development in 73.5% of the flies used was obtained. This high rate could however not be obtained in a repeat experiment. Van Hoof and Henrard (1934) compared lengths of cycle through Glossina and observed that they were shorter in Leopoldville experiments than in Entebbe and probably longer than in Northern Nigeria; Murgatryod and Yorke (1937) analysing Van Hoof's and Henrard's paper remarked that it was interesting to note that the experiments in which the authors obtained the most prolonged developmental cycles were performed during the cold season of the year. Fairbairn and Culwick (1950), working with T. rhodesiense and G. morsitans found that the effect of temperature differed with the sex of the fly and each sex must be considered separately. In the male fly the infection rate was not influenced by temperature to which the adult flies were exposed; in the females the infection rate was governed by the temperature of the fly maintenance. The length of cycle in the female fly was not however influenced by the temperature to which the adult fly was exposed. Desowitz and Fairbairn (1955) found a greater transmission in G. palpalis incubated at 29°C after the infective feed with T. vivax than those incubated at 22°C. The cycle was completed in 5 days and 13 days among the flies incubated at 29°C and 22°C respectively. They also noted during dissection that the majority of the infections found in flies kept at 22°C showed only

scanty trypanosomes in the labrum and hypopharynx whereas in the flies kept at 29°C, trypanosomes were extremely numerous in the mouth-parts. Fairbairn and Watson (1955) investigating the behaviour of <u>T. vivax</u> in <u>G. palpalis</u>, demonstrated that infection rates of this species of trypanosome in the fly were "correlated with temperature and with the mean length of the trypanosomes on which the flies had fed". They found that the infection rate decreased with increasing temperature of fly maintenance.

A different effect of raised temperature was described by Burtt (1946a) who, comparing flies which had emerged from pupae incubated at 30°C with flies which had emerged from pupae kept at normal laboratory temperatures, the flies themselves being maintained at laboratory temperatures, found that in the flies which had emerged from the incubated pupae (a) there was not a single failure to secure a cyclical transmission, (b) there was a significant increase in the salivary glands infections, and (c) there was a significant shortening of the length of development cycle in the fly. The species used was <u>T. rhodesiense</u> and the fly, <u>G. morsitans</u>. Fairbairn and Culwick, (1950) continuing the investigations with the same strain of <u>T. rhodesiense</u> and with <u>G. morsitans</u> found that there was a very significant positive regression of infection rate on pupal temperature in male flies and the infection rate in the females was governed by pupal temperature. They also confirmed Burtt's observation that the length of the development cycle was reduced in males and females by increasing pupal temperature. Fairbairn and Watson (1955) showed that with an increase of pupal temperature from 24-26°C to 28-29°C there was a significant increase in the infection rates of <u>G. palpalis</u> infected with <u>T. vivax</u> at all fly-maintenance temperatures. They

could obtain no transmission from flies which had emerged from pupae kept at 23°C and had subsequently been maintained at 23°C. Consequently, they recommended that for the laboratory transmission of T. vivax by G. palpalis the pupae should be incubated at 28°C and flies maintained at 23°C.

ii) Several syringe passages in the laboratory:

It had been established by many workers that if a polymorphic trypanosome is passaged from animal to animal by syringe, the strain becomes non-transmissible by tsetse flies. Many workers refer to these as "old laboratory strains".

Duke (1923a), conducted studies on the effects produced on a strain of T. brucei maintained for $2\frac{3}{4}$ years by direct transmission through Glossina palpalis and syringe passage from monkey to monkey. He found that the strain had completely lost its power of developing cyclically in the fly from which it was originally recovered. He repeated the experiment with another strain of T. brucei which he had maintained in the laboratory by syringe passage for 2 years and compared his results with those obtained when the strain was originally isolated. In the latter, 0.68% of the 2.32% infected flies possessed salivary glands infections, whereas in the former none of the 2.2% infected flies developed salivary glands infections. He observed no morphological distinction between the two populations. When a year later he (Duke, 1924a) repeated the experiment with the same strain being 3 years old with a further 21 syringe passages, he obtained similar results but then noted that the posterior nuclear

forms which were common when the strain was originally isolated had virtually disappeared. The virulense of the strain to monkeys was also more enhanced with every passage. Duke concluded from these experiments that continued upkeep of a strain by direct transmission or artificial passages leads first of all to the loss by the trypanosomes of the power of invading the salivary glands; this is followed later by the complete loss of the power to develop in any part of the alimentary canal of the insect. He used this to explain a peculiar phenomenon recorded by Miss Robertson (1913b), who found by dissection a fly showing massive gut infection 56 days after its infecting feed; the fly had no salivary glands infection, neither did it infect a susceptible animal. This observation of loss of infectivity to flies after repeated syringe passages in the laboratory was confirmed later by several workers (Duke 1924b, Murgatroyd and Yorke 1937a, 1937b; Roubaud and Colas-Belcour, 1936).

Murgatroyd and Yorke (1937a) not only confirmed Duke's observations but showed further that the animal host in which the trypanosome strain was passaged influenced the behaviour of the trypanosomes as well. A strain of _T. brucei_, isolated fresh from wild fly in Uganda and syringe passaged for 5½ years in mice and guinea pigs showed different behaviours. At the end of this period the mouse-passaged population exhibited changes in morphology; greatly increased pathogenicity for mice and greatly decreased pathogenicity for guinea pigs; and loss of transmissibility to _Glossina_. The guinea-pig passaged population exhibited no change in morphology; they showed a gradual increase in pathogenicity for guinea pigs and mice and loss of capacity of transmission by _Glossina_.

The population of this strain passaged from time to time through Glossina for 4 years retained its infectivity to the fly and its original characters appeared to be preserved practically unchanged. Fairbairn and Culwick (1947) passaged a strain of T. rhodesiense cyclically with G. morsitans through sheep, and by syringe through mice as from 1936. In January 1946theynoted that the 2 populations differed in morphology, the cyclically passaged population being polymorphic and the syringe passaged population being monomorphic. While the length of the long forms in the former had not altered, that of the latter had decreased by about 5μ. Only the Belgian workers gave a report of cyclical infection being lost after a single mechanical passage of T. gambiense in G. palpalis (van Hoof and Henrard, 1934; van Hoof, Henrard and Peel, 1937b). They also recorded another case in which T. brucei was maintained in the laboratory through laboratory animals by unbroken series of mechanical passages for 4 years, at the end of which it was still capable of producing cyclical infection in tsetse. From their experience, these workers believed that a strain which had lost its power of cyclical development never regains it.

Very little is known about the effect of prolonged artificial transmission on the other groups of trypanosomes. Van Hoof, Henrard, and Peel (1937b) reported that a strain of T. congolense which had been passaged more than 200 times through rats and guinea pigs in Europe was sent to the Congo where an attempt was made to infect G. palpalis from it. 2 transmission experiments with 190 and 271 flies failed. The authors compared this with the wild Congo strains of T. congolense which had always produced 1-2% mature infections in their laboratory and concluded that the laboratory

strain of T. congolense, after prolonged passage by syringe, had become incapable of completing its cycle in tsetse. Rodhain (1941) produced some evidence that if T. vivax is mechanically transmitted by other biting flies like Stomoxys or Tabanids, it becomes non-transmissible.

These laboratory observations are thought by various workers to indicate what happens in nature especially if transmission is direct. Only Duke had submitted any evidence to justify this. Contributing to the final report of the League of Nations International Commission on Human trypanosomiasis, he (Duke, 1928) drew conclusions from his experiments and suggested that prolonged sojourn of T. gambiense in an individual, whether man or animal, in which the trypanosomes produces a slowly progressive illness, tends to reduce and eventually to abolish the transmissibility of the strain by tsetse. He believed that it is the prolonged struggle between the infected mammal and the trypanosomes that results in the loss by the parasite of transmissibility by tsetse, a situation exemplified by a chronic patient. Duke (1930) proved his suppositions experimentally when he conducted an experiment in which he fed G. palpalis on trypanosomes derived from chronic patients, and those derived from an acute patient on his death bed. None of the 6885 flies used for the chronic experiments developed a salivary gland infection; neither was any animal on which they had fed infected. 2.3% of the 3.8% among the 855 flies used for acute experiments had salivary gland infections. This observation was confirmed later by the Belgian authors (van Hoof, Henrard and Peel, 1938; van Hoof 1947). They went further to show that virulent strains of T. gambiense which produce heavy blood infections and

rapid clinical progress in man, may also be incapable of producing cyclical infection in G. palpalis: it is the strains of low virulence which most readily produce cyclical infection.

There was also some information on the fate of a strain of trypanosomes which had undergone prolonged cyclical passage. Duke (1928a) showed that after successive cyclical transmissions of T. gambiense in domestic goats, the strain became non transmissible. The same was obtained for domestic pig (van Hoof 1947).

iii) The infective and maintenance hosts.

These factors had attracted the attention of many investigators. Early in the 19th century, Kleine and Fisher (1911) obtained 10% infected flies when monkeys were used as maintenance host throughout the experiment compared with 2.4% when ruminants were employed. Robertson (1912) observed that in many experiments carried out at Mpumu by the Royal Sleeping Sickness Commission the infected buck produced a high percentage of positive flies, but that monkeys infected by means of these flies gave in turn only the usually low percentage characteristic of cycles started from monkeys. Duke (1912a) obtained evidence which suggested that residence of T. gambiense in antelope (bushbuck and reedbuck) resulted in an increased infectivity of the strain to tsetse. The same author (Duke 1913a) maintained flies, after they have been fed on "monkeys infected with T. gambiense, on different animals. For each animal used he set up a control by feeding infected flies on monkeys. His results showed that the percentage of positive flies was considerably greater when they had been fed upon bushbuck (11.5% to 2.6%), or on sheep (20.6% to

3.6%). The flies that had fed on calf showed a lower percentage than those fed on monkeys (0% to 3.3%).

Corson (1935) obtained a very high rate of salivary glands infection (60%) when he fed G. morsitans on a reedbuck which had been previously infected with T. rhodesiense. The experiments were conducted under ordinary climatic conditions. When he repeated the experiment during which he set up a control, he found that he still had the high salivary glands' infection rate of 33.3% with the reedbuck compared with 1.1% with the monkey which had been infected with the same strain of T. rhodesiense as the reedbuck. The author suggested that the special suitability of the reedbuck's blood rather than a selective change in the trypanosomes was responsible. Duke (1935), while agreeing with Corson's observations, disagreed with his conclusions on the ground that he had overlooked the endogenous cycle of T. gambiense in monkeys blood postulated by Robertson. (1912, 1913a). He recorded the observations he had made at Entebbe which showed that the infectivity to flies was always enhanced at the commencement of an infection of an antelope with T. rhodesiense. After prolonged residence in one and the same antelope however, the power of the strain to infect flies was either weakened or suppressed or even destroyed. Corson (1936) repeating his previous experiments of 1935 obtained another high rate of infection in the salivary glands of G. morsitans (6%) after feeding them on reedbuck which had been infected with T. rhodesiense. The control results obtained by feeding G. morsitans on 2 sheep previously infected with the same strain of T. rhodesiense was 2.7%. He suggested that the species of animal might be the determining factor in the rate of infection of the flies.

Van Hoof et al. (1937a, 1938) showed that the monkey Cercocebus galeritus agilis was a host in which T. gambiense exhibited a much higher degree of infectivity for G. palpalis than it did on guinea pig, on various other monkeys and on man. Van Hoof (1947) repeating the experiment with the same species of monkey which had been infected with T. brucei noted a high transmissibility index in G. palpalis infected through the monkey. Corson (1938) demonstrated again the importance of the source of the infective trypanosomes. G. morsitans, fed on Southern Reedbuck (Redunca arundinum) which harboured T. rhodesiense produced 47-60 percent salivary rates; others, whose infective feed was derived from gazelle previously infected with the same strain of T. rhodesiense gave no salivary infections although 150 flies lived for a month. Fairbairn and Burtt (1946) also observed a high rate of gland infections in G. morsitans fed on Bohor Reedbuck (R. redunca) previously infected with T. rhodesiense. Burtt (1946) observed that the degree by which transmissibility of T. rhodesiense was raised varied according to the host species used. Of those he studied, it was least marked in trypanosomes from sheep and most pronounced in those from cercopithecus monkeys. Vanderplank (1941) released figures which showed that an artificially infected ant-bear (Orycteropus) produces a low salivary rate in G. morsitans infected with T. rhodesiense. This was later confirmed by Burtt (1946b).

Investigations on whether the species of the host on which flies are maintained after the infective feed might affect the development of trypanosomes are not numerous. Roubaud (1911) found that feeding G. palpalis on a goat infected with T. cazalboui (T. vivax) and

maintaining some on birds, others on reptiles, have no effect upon the development of the infection in the tsetse. Duke (1921) also showed that the maintenance host has no effect on the development of trypanosomes. Harding (1940) however obtained 3 salivary gland infections out of ten surviving G. tachinoides after they had been fed once or twice on guinea pig previously infected with T. brucei and maintained on man or on citrated human blood. Only one inference had been given on the effect of the source of infective trypanosomes on the duration of the development cycle in flies. At Tinde, the strain from sheep took a significantly longer period than that from Thomson's gazelle; P being under 0.01 (Fairbairn and Burtt, 1946). The same authors showed by the probing technique that the mammal from which a fly acquires its infection does not have a marked influence on the number of metacyclics. (Fairbairn and Burtt, 1946). Quite recently, Geigy et al. (1971) showed that the infection rates of T. brucei in G. morsitans and G. fuscipes are not affected by subsequent blood meals derived from suitable or unsuitable human donor. Feeding the flies on bovine blood however, they obtained increased infection rates for G. fuscipes and decreased ones for G. morsitans compared with those fed on the human blood. They concluded that not the individual but the species of the mammal and the species of the fly that are decisive.

iv) Age of the fly.

Early in the 19th century, Stuhlmann (1907) noted that laboratory bred tsetse flies became most easily infected if fed on the infected animal for their first meal after they have hatched from the pupae. Duke (1935a) observed that if a batch of flies were repeatedly fed on an infecting animal the rate of infection was very little raised. He obtained this information from a series of experiments in which he compared the infection rate in G. palpalis which after hatching, had received single infective feeds on animals infected with T. gambiense and T. rhodesiense and those which had received several infecting feeds. In 1937, van Hoof, Henrard and Peel (1937a) concluded from the figures of their experiments that feeds on non-infected animals previous to the infective feeds diminish notably the aptitude of G. palpalis for the development of T. gambiense. Wijers (1958a) reported an important experiment which demonstrated the influence of the age at which the fly received the infective feed on the infection rate with T. gambiense. He obtained striking difference in the infection rates between G. palpalis which had their infective feed on the morning after emergence and those which had their infective feed when they were older: 7.6 percent of 1st day flies had salivary gland infections, as against 1.10% and 0% of the 2nd and 3rd day flies respectively. Investigating further the effect of the infective feed in the period between 0 and 24 hours after emergence, he (Wijers 1958b) found that 12.5 percent of the 48 flies which were persuaded to feed with 3-19 hours of emergence developed salivary gland infections. Harley's experiments (1971) confirmed this observation. However, Dar (1971) found that the age of G. morsitans did not affect its susceptibility to infections with T. brucei.

Some attention had also been given to the age of the fly in the field. A number of workers had suggested that there is a relationship between the seasonal variations in mean age of flies and trypanosome infection rates (Lloyd et al. 1924; Nash and Page, 1953). Squire (1951, 1954) however concluded from a study of G. palpalis palpalis (R-D) in Sierra Leonethat there was no relation and that the observed seasonal fluctuations in infection rate were the result of other factors, possibly climatic. Harley (1966a) presented figures which suggested that many of the 1445 G. pallidipes females and 892 G. fuscipes fuscipes caught on man and cattle around Lugala in Busoga District of Uganda, must have become infected when more than just a few days old. He made a similar observation later (Harley 1966-1967b). In the same year (Harley 1966b) presented dissection results of females of G. palpalis fuscipes, G. pallidipes and G. brevipalpis caught during 24 hour catches for a period of about one year at Lugala in Uganda. He applied the Saunder's method (Saunders, 1962) to classify them into their physiological ages. He found that over 80% of infections in all the three species were found in category 4+ flies (over 40-50 days old), the percentage of which in the catches varied in much the same way as the total infection rate. This suggested that the fluctuations of infection rate are largely due to changes in mean age. In a later publication the same author (Harley 1966-67a) demonstrated that the incidence of mature vivax and congolense type infections rose with age in all the three species of Glossina, beginning for the most part when flies were about a fortnight old. Brucei-type infections were rare and he found them only in flies more than 35 days old.

The Belgian workers (van Hoof et al. 1937a) had reported that the development of T. vivax (cazalboui) in G. palpalis is not affected by the age of the fly. They fed batches of bred flies on an infected goat as their first meal, or on the same goat after having had a first meal on some other species of clean animal. The rate of infection they obtained was almost identical.

v) The sex of the fly.

The literature on this factor had been well reviewed from the early period to 1950 by Fairbairn and Culwick (1950). Most of the results that had emerged from the laboratories at that time suggested that infection rates in males are higher than in female flies. However, field observations after 1950 had produced a preponderance of reports of higher infection rates in females than males (Squire, 1951; Page and Jordan, 1958; Harley 1966a, 1966-67b). Leggate (1962) found no significant difference between the infection rates in male and female G. morsitans; in G. palpalis however, females showed a significantly higher rate of infection than male flies. Page and Jordan (1958) also found that the infection rates in both sexes of G. palpalis in Ugbobigha, Nigeria, were similar. Results from the laboratory had been more cautious. Fairbairn and Culwick (1950) found the male as a far more efficient transmitter of the disease than the female, except at the highest temperatures. Other workers (Baker and Robertson, 1957; Wijers (1958b) found little or no difference in infectivity between the two sexes.

vi) Morphology of trypanosomes.

Koch (1905a) had noticed as early as 1905 that not all of the blood trypanosomes are adapted for the infection of flies, but only such as occur in a definite, but as yet unknown stage. Robertson (1912, 1913a) recognised a positive and a negative period in the endogenous cycle to which the trypanosomes of brucei group are subjected in the vertebrate host. She found that during one of the periods of this cycle, short forms are prevalent, and if flies were fed on the vertebrate host at such times a high percentage of them was always positive. She therefore called such periods "positive period". She believed that these short forms have become resistant by resisting the destruction of trypanosomes which took place during the "depressed period" of the endogenous cycle. They are consequently the ones capable of carrying on the development in the invertebrate host. This observation was later confirmed by Reichenow (1921), Hoare (1940), Ashcroft (1957) and Wijers (1959) and Vickerman (1965). However, Baker and Robertson (1957) using a strain of _T. brucei_ and one of _T. rhodesiense_ with _G. morsitans morsitans_ found that "neither the morphology nor the intensity of the parasitaemia in the infecting mammal was obviously related to the subsequent infection rates in the tsetse flies". Ward's results (Ward 1968) with _G. austeni_ and _T. brucei_ agreed with these findings of Baker and Robertson. Wijers and Willett (1960) provided experimental evidence that the infection rate in the fly is related to the absolute numbers of short stumpy forms of _T. gambiense_ in the host's blood taken in by _G. palpalis_. Recently, Page (1972) reached the same conclusion with _G. morsitans_ and _T. brucei_. Not long ago however, Mshelbwala (1967) indicated that _G. palpalis_ could become infected

from a blood meal of T. gambiense apparently containing only long-thin forms. More recently, Ormerod and Venkatesan (1971) after reviewing evidence for and against concluded "we consider it unwise, however, to try to reach a conclusion, since the evidence is so scanty and contradictory, as to whether the long-thin or short-stumpy form is infective for the tsetse".

vii) The strain of the trypanosome species.

Robertson (1912) first recognised differences in individual strains of T. gambiense in the infectivity to flies. The literature on this factor has been well reviewed by Buxton (1955) and Baker and Robertson (1957).

In spite of the great attention given to the investigations on African Trypanosomiasis, one of its many unsolved problems is the variable and usually low susceptibility of tsetse flies to infection by trypanosomes. Results of previous works had been so conflicting that it is still not definitely known whether it is factors pertaining to the fly, to the trypanosome or both, which permit or prevent establishment and complete development of the trypanosomes in the tsetse fly. The reasons for this are clear; tests had been first conducted on flies which emerged from wild caught pupae. Even after flies from laboratory bred pupae were being used, they were fed on different animals at different times during an infection. Hence no two experiments are strictly comparable. Only a limited value could be placed on the results of field dissections since it is difficult to distinguish species of trypanosomes in the fly. In

the criterion used for identification, i.e. distribution of trypanosomes in the alimentary canal of the fly, double infections could be missed.

A standard method is therefore required in order to assess factors which may influence the establishment and development of trypanosomes in tsetse flies. Since it is now possible to rear tsetse flies in the laboratory, and to stabilize populations of trypanosomes by low temperature preservation, trypanosome/fly relationships can be studied under controlled conditions. In a mammal host, the trypanosome population is a constantly changing one; thus if flies are fed on animals, an uncontrolled variable is introduced into every test. By feeding flies through membranes on a population of trypanosomes stabilized by low temperature preservations, this variable is eliminated. Further, the number of trypanosomes in the infecting feed can be controlled.

As Dr. Willett suggested to Buxton in a conversation (Buxton 1955) there appear to be three critical stages in the cyclical development of brucei-group grypanosomes in the tsetse fly. These are:

1) escape from the intra- to the extraperitrophic space at the junction of mid and hind guts.

2) penetration of the peritrophic membrane to gain access to the proventriculus with forward migration to the proboscis.

3) back tracking to the salivary glands.

In the development of _T. congolense_ only barriers 1 and 2 have to be circumvented: _T. vivax_ is not subjected to any of these barriers. The use of membrane feeding, stabilized populations of trypanosomes and laboratory reared flies will permit the investigation of each of the critical stages and the factors influencing them. This technique

was adopted by Dar (1971) in his preliminary studies on the development of salivarian trypanosomes in tsetse flies. The aim of this work was to continue and to expand these studies.

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MATERIALS AND METHODS

2.10 The trypanosomes

T.brucei

1) T.brucei, TREU 667. This strain was isolated by the Edinburgh Veterinary expedition to East Africa in 1968. It was designated EVE 10 and stabilated. At the Centre for Tropical Veterinary Medicine of Edinburgh University (CTVM), the stabilates of EVE 10 were named TREU 628, the term "TREU" meaning "Trypanosomiasis Research at Edinburgh University". TREU 628 was injected into two rats; when the parasitaemia was high, after 22 days, it was stabilated in liquid nitrogen and designated TREU 667.

Two stabilates of TREU 667 were injected into 2 mice and given to the Parasitology section of the Zoology Department. Here it was passaged three more times through mice and at the 4th passage, the mice were killed when the parasitaemia was high and 200 stabilates were prepared from the pooled blood. The stabilates were stored in liquid nitrogen. This population was designated T.brucei, A and it had undergone 7 artificial passages after cyclical development. In the course of experiments, one of the flies which had fed on a suspension of T.brucei, A in defibrinated ox blood developed a mature infection and infected a mouse. Blood from this mouse was injected into 6 mice and at high parasitaemia, after 5 days, the mice were killed and 200 stabilates made from the pooled blood. These stabilates were designated T.brucei, B; they had undergone two mouse passages after cyclical development. Haemocytometer counts of the number of trypanosomes in each population were made a few minutes before preservation. Those of

T.brucei, A was 5×10^8 trypanosomes per ml. of blood and T.brucei,B 5.3×10^8 trypanosomes per ml. of blood. No differential count of the pleomorphic forms were made. Fifty microscopic fields of stained slides of each of the populations were inspected; it was observed that there was a greater percentage of long forms than the short 'stumpy' and intermediate forms in both populations.

2) T.brucei, TREU 1096. This strain was originally isolated from cattle at Ibadan, Nigeria, in March 1971. There, it had 7 syringe passages in rats before it was stabilated. Some of the stabilates were sent to the C.T.V.M. where they were designated TREU 1096. Two mice, which had been injected with stabilates of TREU 1096, were given to the Zoology Department, where, after three further passages through mice, 200 stabilates were prepared and preserved in liquid nitrogen. These were designated T.brucei, C and they had undergone 11 artificial passages after cyclical development. Blood from a mouse, which was infected by a fly which had fed on a suspension of T.brucei, C, was injected into 6 mice; at high parasitaemia, after 9 days 200 stabilates were prepared from the pooled blood. These stabilates were designated T.brucei, D and they had undergone two artificial passages after cyclical development in the fly.

Fifty microscopic fields of stained slides of each of the populations were inspected; it was observed that there was a greater percentage of long forms than short 'stumpy' and intermediate forms in both populations. Haemocytometer counts of the number of trypanosomes were also made a few minutes before preservation. The number of trypanosomes of T.brucei, C per ml. of blood was 4.8×10^8 and T.brucei, D, 4.4×10^8.

T. congolense

1) T.congolense, TREU 692. This strain was isolated as EVE 4 by the Edinburgh Veterinary Expedition to East Africa in 1968. The stabilates were sent to the C.T.V.M. where they were designated TREU 623. There, flies were fed on a mouse previously infected with TREU 623 by intraperitoneal innoculation and, 21 days later, the hypopharyngeal eluate of one of the infected flies was injected intraperitoneally into a mouse. The mouse showed high parasitaemia on the 27th day. It was killed and the stabilates made from its blood were designated TREU 692. Two mice were innoculated with 2 of these stabilates and sent to the Zoology Department where further mouse passages were initiated. 200 stabilates were made from the population of the 3rd passage in mice at the Zoology Department. These were designated T.congolense, A and they had undergone 6 artificial passages after cyclical development. Blood from a mouse, which was infected by a fly after it had fed on a suspension of T.congolense,A, was injected into 6 mice. When the parasitaemia was high, after 7 days, the mice were killed, and 200 stabilates were made from the pooled blood. These were designated T.congolense, B and they had undergone 2 artificial passages after the cyclical development. Later, the last stabilate of T.congolense,A was injected into 3 mice and 90 stabilates were made from the pooled blood. These were named T.congolense, C and they had undergone 7 mouse passages since cyclical development.

Microscopical examination of 50 fields in the stained slide of each of these populations did not show any difference between the morphological characters of the populations. The number of trypanosomes per ml. of blood, as counted by the haemocytometer, was

4 x 10^8 for T.congolense, A, 4.4 x 10^8 for T.congolense, B and 4.8 x 10^8 for T.congolense, C.

2) T.congolense, LUMP 92. This strain was originally isolated from a cow at Malakal, Upper Nile province of Sudan on 22.9.69. It was then passaged thrice through mice and then twice through calves. Blood from the infected calf was innoculated into 4 mice at Khartoum, Sudan and sent to the London School of Hygiene and Tropical Medicine, where it was preserved in liquid nitrogen after 2 further mouse passages. 3 stabilates were sent from the London School to the Zoology Department of Edinburgh University. With these, a further 3 passages through mice were initiated before preservation. Ninety stabilates were made and designated T.congolense, D. Blood from a mouse, which was infected by a fly after it had fed on a suspension of T.congolense, D was injected into 3 mice. When the parasitaemia was high, after 12 days, the mice were killed and 90 stabilates made from the pooled blood. These were designated T.congolense, E and they had undergone 2 artificial passages in mice after cyclical development in the fly.

The morphological characters of these populations did not differ. The number of trypanosomes in blood was 3.6 x 10^8 per ml. for T.congolense, D and 4.0 x 10^8 per ml. for T.congolense, E.

Culture forms T.congolense, TREU 261 came into the C.T.V.M. in 1966 as T.congolense, R. le Page. It was then passaged into 2 mice before being deep frozen as TREU 234. One of these stabilates was then injected into mice, and at high parasitaemia, after 27 days, stabilates were made from its blood. These stabilates were designated

TREU 261. Late in 1966, one of these stabilates was injected into a mouse and the strain was maintained thereafter by syringe passage.

Culture forms of T.congolense, B and T.brucei,B were also used.

2.11 Detection of trypanosomes in animals.

A wet film was made by placing a drop of the blood on a microscope slide (Chance, Smethwick, U.K.) and dropping a coverslip gently on it. Examination for motile trypanosomes was carried out under phase contrast microscope (x40 objective, x10 occular). A maximum of 50 fields were inspected. If no motile trypanosome was found, the blood was regarded as negative.

2.12 Preservation of viable trypanosomes in liquid nitrogen.

Mice were always bled at the first peak of parasitaemia. The mouse was killed in "2lb. sweet jar" containing a layer of cotton wool previously soaked with ether. Immediately the mouse stopped breathing it was dissected with sterile scissors and forceps and the heart exposed. A 1 ml. syringe containing 0.05 ml. of heparin solution (1000 units/ml.) and fitted with a 25 x 5/8" needle was inserted into the right ventricle and the plunger withdrawn. Depending on the size of the mouse, 0.5 - 1.0 ml. of blood was collected in this way. The syringe was inverted once to allow the heparin solution to mix with the blood; the volume was read, and the blood was deposited into a sterile bijou bottle which was

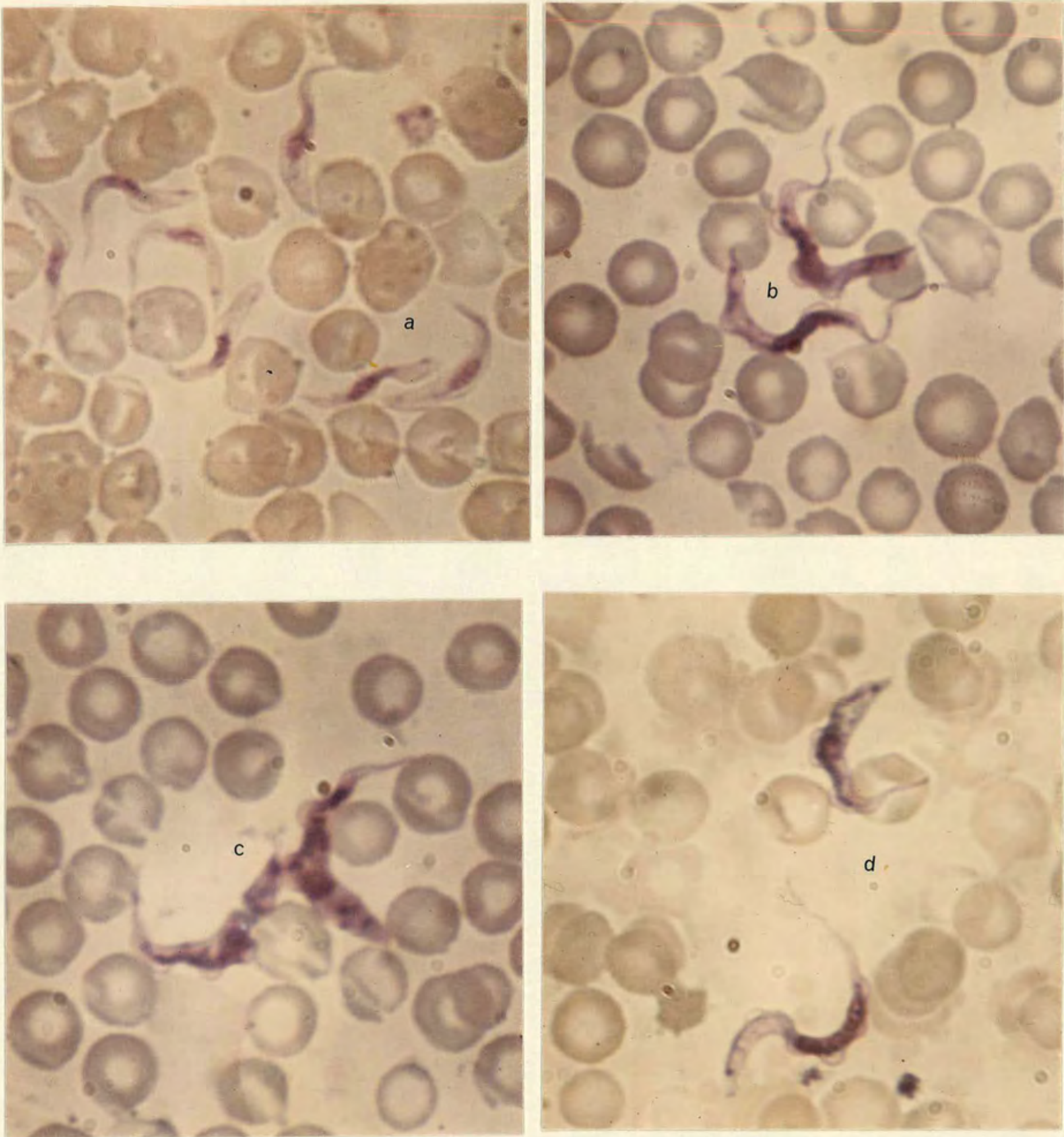


Fig. 1 Blood stream forms of T.congolense(a); and T.brucei (b,c,d)

b,c = few minutes before preservation in liquid nitrogen

d = 12 months after preservation in liquid nitrogen

surrounded by ice in a container. Sterile glycerol was added to give a final concentration of 7.5%. The glycerol was thoroughly mixed with the blood by means of a pasteur pipette, after which a sample was removed so that the number of trypanosomes could be estimated. The blood suspension was then dispensed with a sterile pasteur pipette into the lymph tubes of 1 mm. outside diameter and 4 inches length. These tubes were cleaned before use by overnight immersion in potassium dichromate-sulphuric acid solution and followed by repeated rinsing in tap water. After drying, they were sterilized. During filling, the lymph tubes were carried on a rack similar to that described by Cunningham et.al. (1963). After filling, the tubes were sealed in the microburner flame starting with the end from which they were filled. The sealed tubes were transferred to a paper container of appropriate size which was then put inside a duralmin tube. With a pentel pen the coded words for the species and strain of trypanosomes and the day of preservation were written on the label of the paper container. The duralmin tube was put in a 25 mm. wall insulating jacket which was plugged with non-absorbent cotton wool and left in a dry ice cabinet overnight.

The next day, the insulating jacket was removed from the dry ice cabinet and the paper container was immediately transferred into one of the four compartments of a numbered canister of the liquid nitrogen refrigerator. The day of preservation, the number of stabilates made, the compartment of the canister in which the stabilates were placed, the species, strain and concentration of the trypanosomes were recorded. The liquid nitrogen in the refrigerator was topped up with 40 lbs. liquid

nitrogen collected once a week from the Chemistry Department of Edinburgh University.

Removal of stabilates from liquid nitrogen refrigerator:

The canister containing the stabilates was selected and brought into the neck of the nitrogen refrigerator. It was then clamped in position with the top of the canister not less than 15 cm. from the top of the tank. The paper tube containing the stabilates was selected from the compartment of the canister, held with long forceps, and the required number of lymph tubes was picked out with fine forceps and dropped into a 1000 ml. plastic beaker containing water at laboratory temperature. The paper container was released back into its compartment and the canister returned into the tank.

2.13 Trypanosome counting methods

A neubauer haemocytometer consisting of two counting chambers was used. Each counting chamber was made up of squares, each of which has an area of 1/40 mm^2 and depth of 1/10 mm.

A thin smear of facial sebum was made on the ridges on either side of the counting chamber of the haemocytometer, and a coverglass gently pressed onto the ridges. With the aid of a haemocytometer pipette, a 100 fold dilution of the trypanosome infected blood in a citrated saline was run into both chambers of the haemocytometer. The haemocytometer was retained in an horizontal position for 10 minutes so that the trypanosomes might settle in the counting chambers.

On each occasion, trypanosomes in 100 squares of the counting chambers were counted. The number of trypanosomes per ml. of the suspension was calculated by multiplying the total number of trypanosomes counted in 100 fields by 4×10^6.

2.14 The tsetse flies

Experiments were conducted with _Glossina morsitans orientalis_ Vanderplank, and _Glossina austeni_, Westwood. Puparia were obtained from the tsetse fly colony of the Zoology Department (Edinburgh University) or from the tsetse laboratory in Bristol. Maintenance of flies - A detailed description of the apparatus and technique used to regulate the physical conditions within the flyroom had been given by Mews (1969). A brief account of the system is given below. The fly room was kept constantly at 25-26°C and at a relative humidity of about 65%. Illumination was provided for 12 hours in every 24 (0600-1800 hours) by fluorescent strip lights.

The flies were kept individually in polystrene tubes plugged with polythene stoppers. The closed end of the tube was cut off and replaced by black terylene netting glued to the rim by chloroform. Two sizes of tube were used, 6 x 3.5 cm and 6 x 2 cm (Arnold R. Horwell Ltd.). On hatching, flies were placed in the larger tubes until they had been given their infective feed and mated. They were then transferred to the smaller tubes for the convenience of feeding them on mice. For handling, flies were chilled at 4°C until they were immobile.

Puparia were kept in polystrene screw-top vessels containing 1 cm. of sand. The vessel was suspended over water. The lids of

these vessels were partly replaced by terylene netting. The day of eclosion of a fly was designated day 0 for that fly. Flies which ecloded on Sundays were not distinguished from those which did so on Saturday afternoons and both were given the same date code for their eclosion.

Mating of flies - Flies were mated a day after they have received the infective feed, i.e. when they were 2-3 days old. For each mating, a male was placed in the tube containing the female, the occurrence or non-occurrence of mating within a few minutes was noted, and the male was removed a day later.

2.15 Maintenance of flies on mice and rabbits

The apparatus used to restrain the mice while flies were feeding on them was first described by Cockings et al. (1959). A detailed diagram of the apparatus has been given by Page (1972). Flies were offered food on mice every second day. If, however, a fly did not feed within 10 minutes, it was offered food daily until a meal was taken, when feeding on alternate days was resumed. Records were kept of the feeding, an example of which is given in Appendix 7 Engorgement was represented by 2+ and partial engorgement by +.

A detailed description of apparatus and method for feeding flies on rabbits has been given by Mews (1969). During feeding of the flies, the rabbits were immobilized in boxes with the ears strapped to horizontal supports so that cages containing the flies could easily be placed thereon.

2.16 Preparation of chicken skin membrane

Care was taken to see that the processes of the preparation of chicken membrane, the mechanical defibrination of blood (2.17) and the subsequent feeding of the flies on the infective blood through the membranes (2.18) were performed under sterile conditions. Preliminary work had confirmed Wallace's observations (1931) that bacteria, if experimentally introduced into the gut of flies subsequently fed on infective blood, have an inhibiting effect. Flies which fed on contaminated blood died within 48 hours after the infective feed. The abdomen of such flies almost invariably turned black after death and if dissected, masses of various types of bacteria could be seen.

Killed chickens were thoroughly defeathered, a process facilitated by previously immersing them in lukewarm water for about 5 minutes. After defeathering, each chicken was deskinned under the sterile hood with sterilized scissors and fine forceps. The internal surface of the skin was carefully but thoroughly scraped with a sterilized scalpel or razor blade, so that as much subcutaneous tissues as possible was removed. A second pair of sterilized scissors was used to cut the skin into small circular membranes just wide enough to cover the blood receptacle of the feeding capsule (See 2.18). In this way 20-30 membranes could be cut from the skin, depending on the size of the chicken. The membranes were laid flat, with the internal surface facing upwards, on self closing polythene bags. Eight to ten membranes could be arranged on a polythene bag in this way. The polythene bags with the membranes on them were transferred quickly from the sterile hood into a UV light chamber. The latter was switched on 15 minutes

previously so that the atmosphere in the chamber was already sterile. The polythene bags were placed on a cardboard box with the internal surface of the membranes exposed to the UV light, so that the distance between the membranes and the fluorescent bulbs of the UV light was 38 cms. After 15 minutes exposure, the membranes were transferred back to the sterile hood, where, with sterile fine forceps, each membrane was gently transferred inside the self adhesive polythene bags. The latter were sealed and stored in the deep freeze at -20°C after 8-10 membranes had been placed into each of them.

2.17 Mechanical defibrination of blood

Blood was collected from the abbatoir once a week in a 2 litre flask, and immediately defibrinated shaking vigorously.

Before use, the glass beads were cleaned by overnight immersion in pottasium dichromate-sulphuric acid solution and repeated rinsing in tap water. After drying in an oven, they were poured into a clean 2 litre flask where they formed a layer of about 2 mm. at the base. The latter was corked tightly with cotton wool and autoclaved at 15 lbs./20 minutes.

Fresh blood from the punctured jugular vein of a dying ox was released directly into the 2 litre flask up to a marked level above the glass beads. The flask was immediately corked again with the cotton wool and the contents were vigorously shaken for about 10 minutes. The flask was then placed in a refrigerator for 5 minutes so that the defibrinated blood might settle. Usually, the latter settled between a bottom layer of glass beads and an upper layer of blood foam. The flask was then transferred to the sterile hood,

where the defibrinated blood was decanted into 3 or 4 autoclaved universal bottles. These were stored in a refrigerator at 4°C until required.

2.18 Artificial feeding of tsetse flies

Depending on the number of flies available, one, two, or three stabilates were removed from the liquid nitrogen refrigerator as described in 2.12.

Meanwhile, feeding capsules made of perspex were submerged in tap water contained in a 5 litre beaker. The whole contents were heated until the water had boiled for 30 minutes. The capsules were then transferred to the U.V. chamber, where they were exposed to U.V. light for 15 minutes as described for sterilizing the chicken membranes (2.16). Depending on the number of flies available, a minimum of 6 and a maximum of 12 capsules were used. Each capsule (Fig. 2) possesses an inlet and an outlet, both of which lead into an inner tube of 1.750" diameter. This inner tube is surrounded by a bigger outer tube of 2.500" diameter which acts as an insulator against heat loss when water heated to 38°C was being passed through the inner tube. A hollow of 7/8" radius was made on the surface of the inner tube. This serves as the receptacle for the infective blood.

From the U.V. chamber, the capsules were transferred quickly into the sterile hood where, with a pair of sterilized forceps a thawed chicken membrane was carefully stretched over the blood receptacle of each feeding capsule. The internal surface of the membrane, which would have contact with the infective blood, faced

downwards. The chicken membranes were thawed by removing 1 or 2 polythene bags, in which they were contained, from the deep-freeze and exposing them to laboratory temperatures in the sterile hood for about 10 minutes, before being placed on the feeding capsule.

The feeding capsules with the chicken membranes were transferred to the feeding apparatus shown in Fig. 3. This consists of a waterbath regulated to 38°C. About 30 mm. above the water bath, a circular wooden plate of 44 cms. diameter was fixed in a horizontal position with the aid of three retort stands and clamps. Holes of 6.5 cm. diameter, which are just wide enough to hold the capsules tightly, were bored on the edges of the wooden plate. The capsules were fixed into these holes and connected as shown in Fig. 3 . The first capsule bears a rubber tube from its inlet into the water bath and the last capsule a rubber tube from its outlet into the waterbath. The capsules in between were connected by rubber tubes to one another in series i.e. from outlet of one capsule to the inlet of the next. The waterbath was then switched on and water at 38°C was allowed to circulate for 15 minutes. This was to allow sufficient time for the circulating water to heat up the surface of the blood receptacle to 37°C allowing for a loss of 1°C between the circulating water and the blood receptacle.

After the water had circulated for 15 minutes the capsules were filled with the infective blood. The infective blood was prepared from the stabilates removed from the liquid nitrogen. The stabilates were weighed and the blood contents were introduced into a sterilized bijou bottle with the aid of a specially made fine glass tube attached to a rubber bulb. The bijou bottle served as the container for the infective blood and it was previously immersed up to the neck in blocks

of ice where it remained throughout the experiment. The empty lymph tubes were weighed and from the difference between the filled and empty weights, the volume of the contained trypanosome suspension was calculated. Except otherwise stated, each stabilate was diluted 100-fold with defibrinated ox blood. An even mixture of the trypanosomes in the infecting feed was effected by gentle agitation of the bijou bottle for 2 minutes on an agitator. The infective blood was then introduced into the blood receptacle of the feeding capsule with a 1 ml. syringe fitted with a 25 x 5/8" needle. Each capsule was initially filled with 0.2 ml. of infective blood and successively replenished with 0.1 ml. of the blood after 2 flies had fed. Three lamp housings containing 60 watt bulbs were placed below the circular wooden plate so that the feeding capsules were evenly illuminated from below. This served as a source of attraction to the flies (positive phototropism).

Individual cages of flies were placed on the feeding capsules and allowed to feed for a maximum of 10 minutes. Any fly which did not feed within this time was discarded; the cages of those that fed bore a label on which were recorded the sex of the fly, the species and strain of the infective trypanosomes and the date of the infective feed.

2.19 Probing of tsetse flies on warmed microscope slides

The method of Lewis and Langridge (1947) was modified. With a pentel pen, a circular zone of about 20 mm. was drawn on a clean microscope slide. The latter was placed on an aluminium plate with the surface, on which the circle had been drawn, facing downwards.

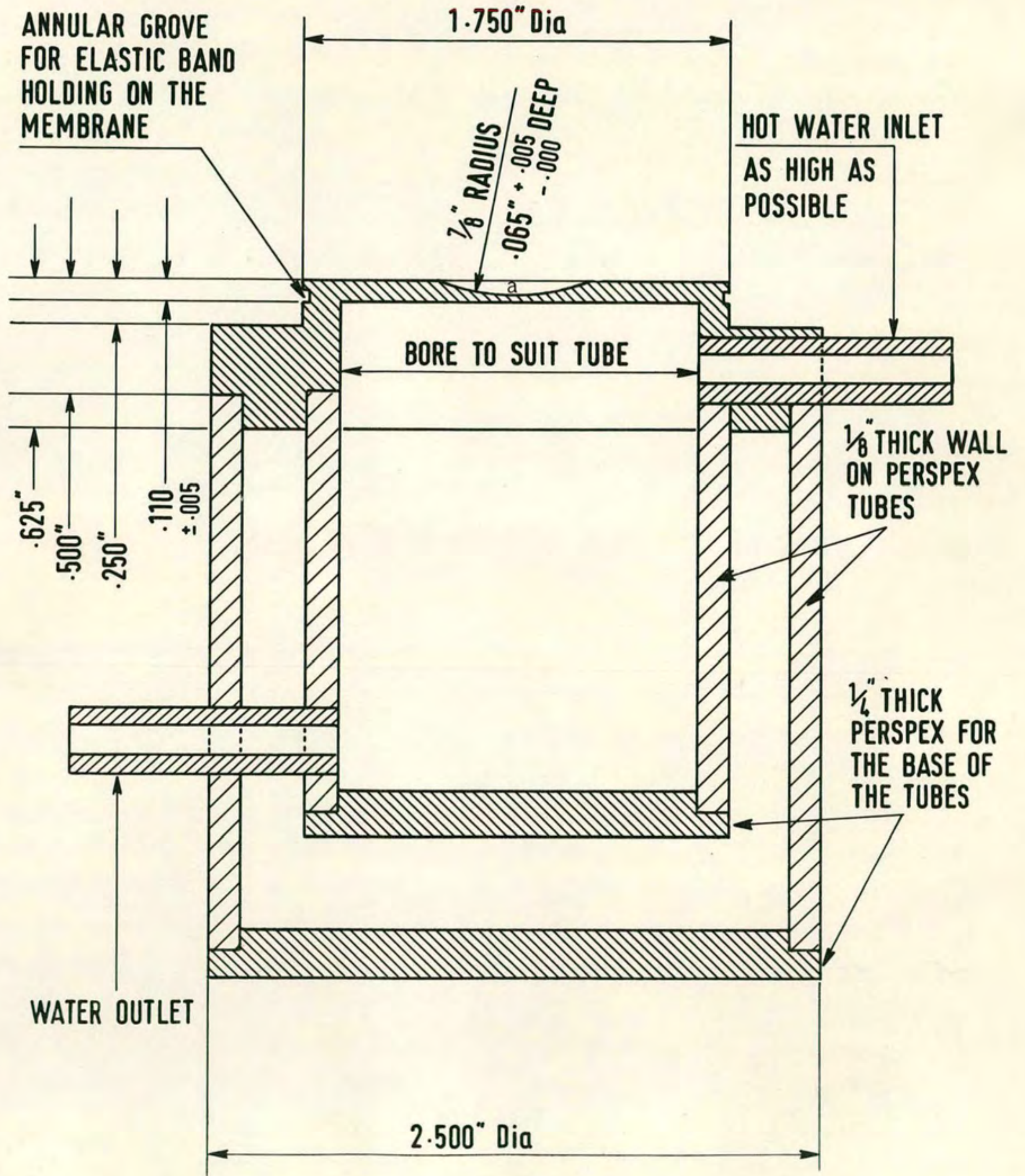


Fig.2 Cross section of a feeding capsule
a) = blood receptacle.

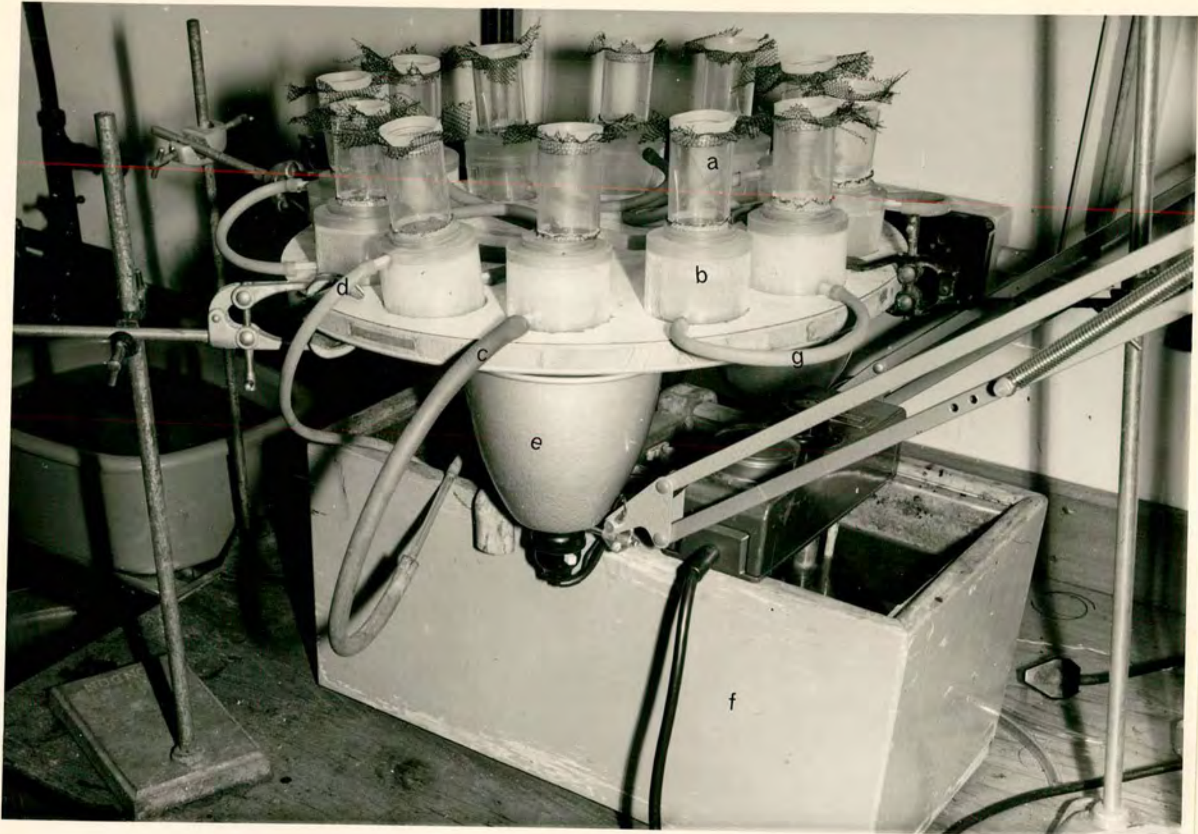


Fig.3 The artificial feeding apparatus

a = Fly tube
b = feeding capsule
c = General water inlet
d = General water outlet
e = Lamp housing
f = Water bath
g = Tube connection

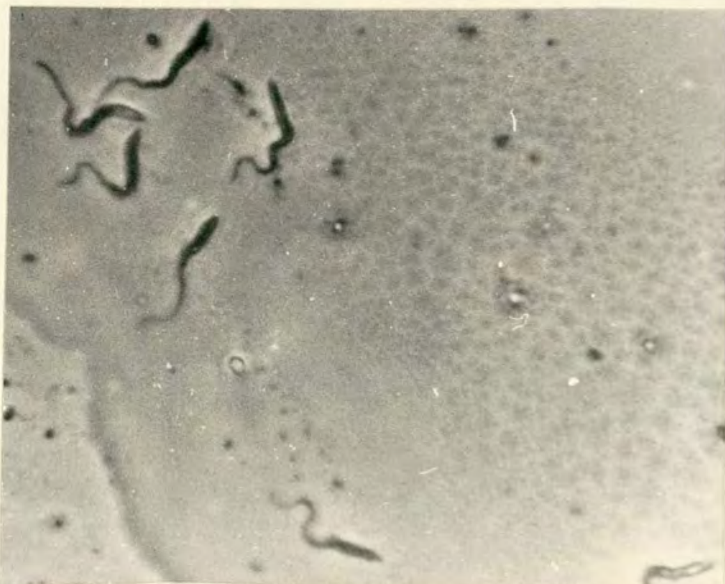


Fig.4 A live salivary track of a G.morsitans extruding T.congolense. 12 days post infection

6 or 7 microscope slides could be arranged on the aluminium plate in this way. The aluminium plate was placed in a thermostatically controlled waterbath at 39°C. Fifteen minutes later, when the microscope slides had warmed up to about 37°C, a fly tube containing a hungry fly was placed on each of the warmed microscope slides with the terylene netting side facing downwards. The fly was so placed that the tube netting rested on the circular demarcated zone. Since the diameter of this tube is equal to that of the circular zone, the fly was exposed only to this zone and the salivary probe was collected on it. The restriction of probing to this circular area limited the area to be inspected under the microscope for the presence of trypanosomes.

The whole apparatus was illuminated from above through a lamp housing containing a 60 watt bulb. In this way, any fly which had probed was easily identified. Any fly which did not probe after 10 minutes was removed and tried the next day. The salivary probe of any fly was inspected immediately under the phase contrast microscope (x20 objective, x10 occular). If trypanosomes were found along the salivary tracks (Fig. 4) the slide was fixed in formol saline and stained in Giemsa after details of the fly, the species and strain of the trypanosomes and the age of the infection in the fly, had been recorded.

2.20 Dissection of tsetse flies for the location of trypanosome infection

The fly to be dissected was killed with chloroform. Holding the fly between the thumb and the next finger of the left hand and with the dorsal surface upward, the legs and wings were pulled off with the

right hand. Still holding the fly in this position, a longitudinal incision was made from the base of the neck to the posterior end of the last thoracic segment, followed by three lateral incisions across the thorax from the origin of the legs of one side to those of the other side. This was to loosen the strong thoracic muscles surrounding the proventriculus and the anterior segment of the midgut.

The fly was placed in 2-3 drops of BSI-buffer solution on a clean microscope slide. The salivary glands (Fig. 5) and the proboscis with its components, i.e. labrum, labrum epipharynx and hypopharynx were dissected out according to the method described by Lloyd and Johnson (1924).

The midgut was dissected thus: Working under a dissecting microscope with a pair of fine dissecting needles, the thorax was gently pulled away from the abdomen, so that the proventriculus and the crop protruded out, still attached to the abdomen (Fig. 6). The constriction between the thorax and the abdomen was loosened through this process so that, with the dissecting needles, the whole of the alimentary canal could be pulled out with the proventriculus and the crop still attached. The complete alimentary canal was lifted from the slide to another one containing 2 or 3 drops of BS-1, laid along its length (Fig. 7) and scanned under a x20 objective of a phase contrast microscope for trypanosomes. If trypanosomes were seen, the midgut was 'cut' into three segments, i.e. anterior, middle and posterior segments. Wigglesworth's identification criteria (1929) were used. As soon as the segments were separated, each was quickly transferred to a separate clean slide containing 1-2 drops of BS-1.

To distinguish between the endo- and the ectoperitrophic infection in any of the segments, the midgut wall was carefully punctured with a fine dissecting needle. The peritrophic membrane then bulged out as a tubular 'loop'. This was gradually eased out of the 'hole' in the midgut segment. The latter, without the endoperitrophic membrane was then transferred to another slide containing 1 or 2 drops of BSl, in which it was teased out. The endoperitrophic membrane need not be teased; the trypanosomes were clearly seen within the membrane under a phase contrast microscope.

2.21 Preparation of saturated solution for the control of relative humidity

At various times in the course of this work flies and pupae were incubated at 31°C and 20°C (flies only) instead of at 26°C. Supersaturated solutions of potassium tartrate were used on these occasions.

The solution was prepared by dissolving enough potassium tartrate to saturate at boiling in a 100 ml. beaker. This was allowed to cool partially, at which time a small amount of potassium tartrate was added again. After the mixture had cooled, considerably more solid was added. A solid mass of the salt was thus formed at the base of the beaker with the solution on top of it. The solution was decanted into clean petri-dishes under the sterile hood. Each petri-dish was 5 cm. in diameter and 2 cm. high. After all the solution had been decanted, the solid was distributed, with the aid of a clean spatula, into each of the petri-dishes where it immediately sank and formed the bottom layer to the solution. Care was taken to see that the overlying solution occupied about 1 cm. of the height of

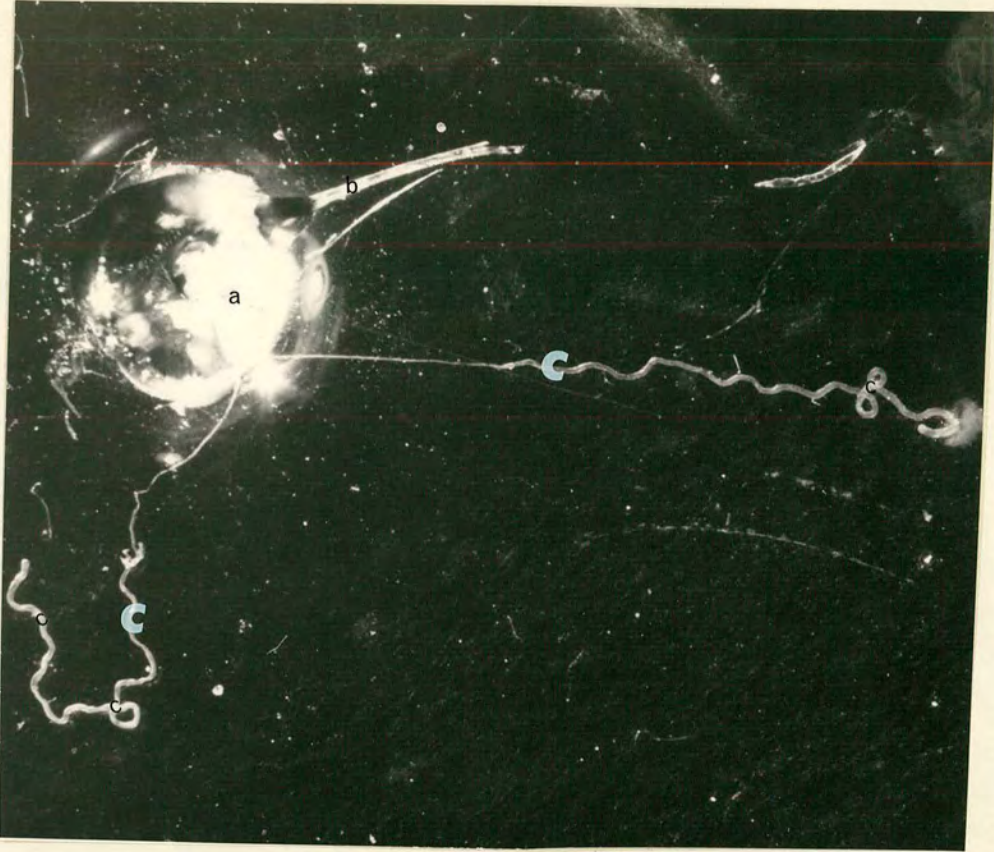


Fig.5 Wet preparation of a fly's head (a); showing the mouthparts (b); and the attached salivary glands(c).

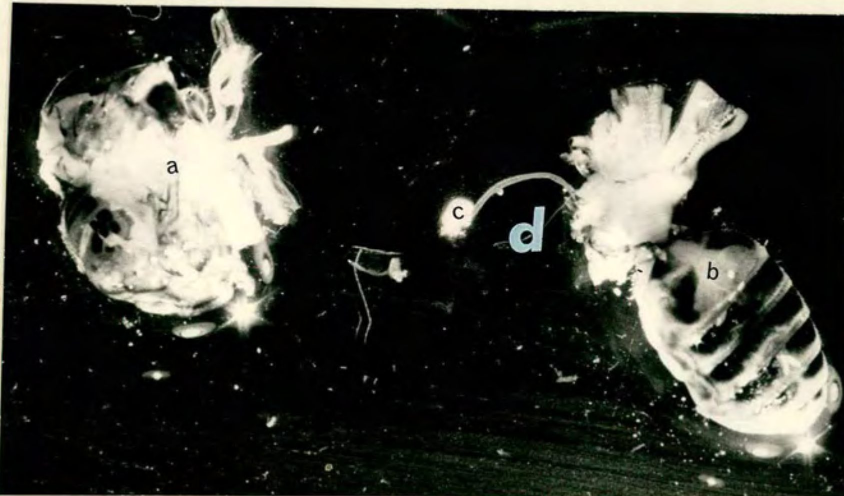


Fig.6 Wet preparation showing the separation of the fly's thorax (a); from its abdomen (b); thereby leaving the proventriculus (c); and the crop duct (d) protruding out into the dissecting medium

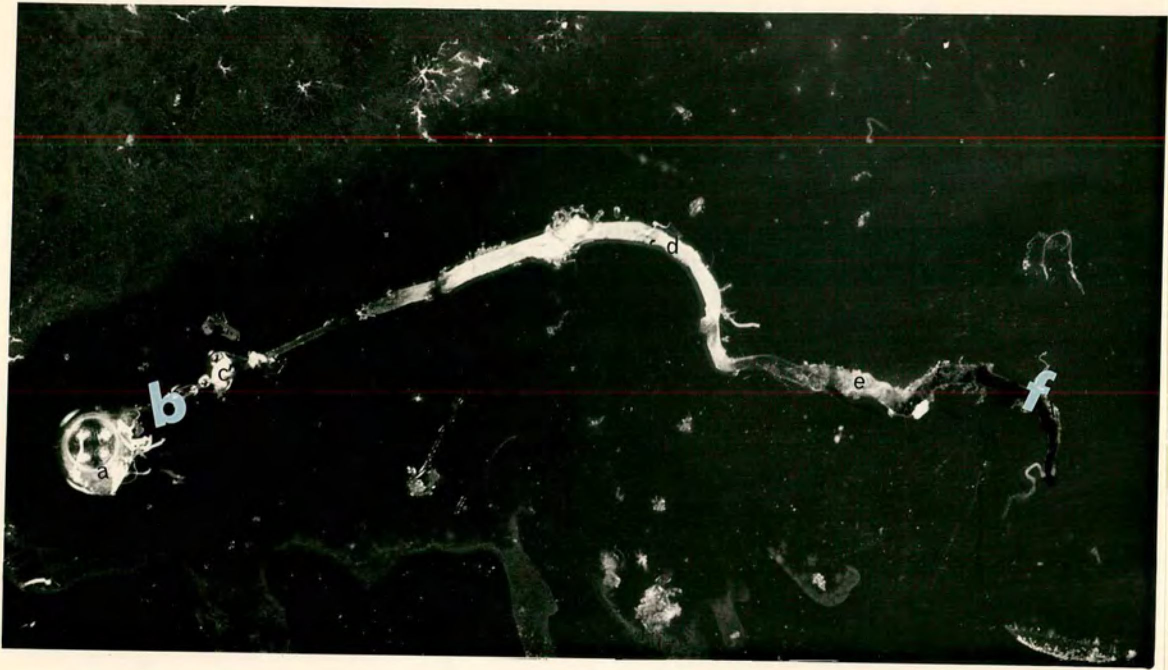


Fig.7 A wet preparation of midgut dissection showing a distended crop (a); crop duct (b); proventriculus (c); anterior segment (d); middle segment (e); and posterior segment (f)

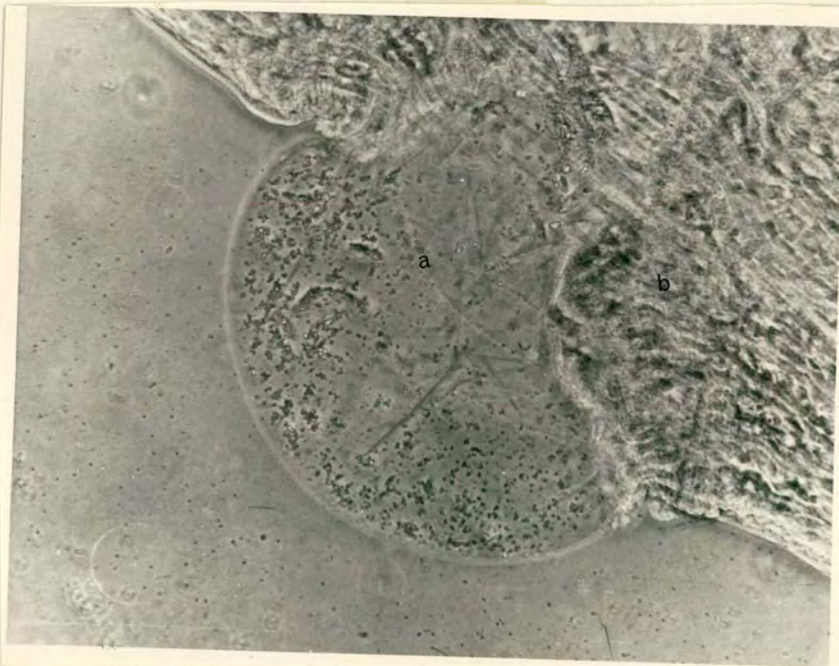


Fig.8 Tubular loop of the peritrophic membrane (a) bulging out of the midgut (b) after puncture

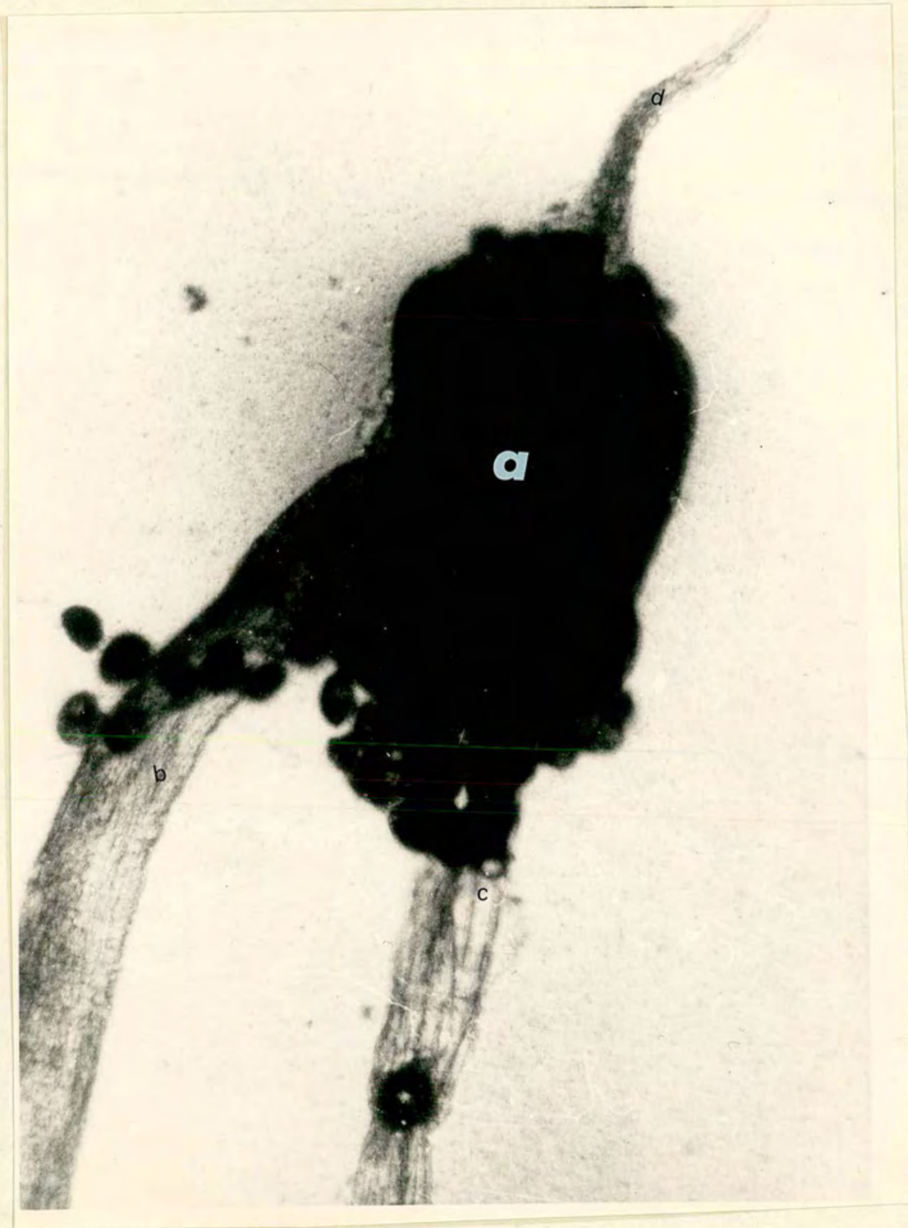


Fig.9 A wet preparation showing the size relationship of proventriculus (a); anterior segment of the midgut (b); crop duct (c) and Oesophagus (d)

the petri-dishes so that about 1 cm. was left free from the top. Care was also taken to see that there was not much liquid over the solid so that the diffusion within the solution might not be too slow.

The petri-dishes with their contents were gently lowered with a pair of strong forceps into "2 lbs. sweet jars, each of the latter accommodating two of the former. With the same forceps, a perforated wire gauze, previously cut to the size of the diameter of the bottom of the jar, was lowered into the jar so that it rested on the pair of petri-dishes. The jars were tightly screwed and transferred into the incubators regulated at 31°C and 20°C. The relative humidity of this solution at 31°C is 74.0% and at 20°C 75.0%. (Winston and Bates, 1960). The level of the solution in the petri-dishes was constantly maintained by replenishing it weekly with a saturated solution of potassium tartrate.

2.22 Laboratory Animal maintenance

A Q strain of mice, bred in a closed colony in this laboratory, was used throughout this work. Mice, at least 6 weeks old, were collected from the breeding room and maintained on Edinburgh University Diet (Appendix 2) in the animal house. Six mice were kept in a cage and, since one fly was fed on its own mouse in most of the experiments, the mice in each cage were differentiated by ear-puncture. Both sexes of mice were used and whenever possible, mice of the same sex were placed in the same cage.

Half-loped rabbits, bred in a closed colony at the Zoology Department and kept in the rabbit room were used for the experiments. They were fed on diet S61. (Appendix 1).

2.23 Statistical calculations

The aim of this work was to standardise the methods of studying the development of trypanosomes in tsetse flies. It was therefore necessary to set up control experiments under standard conditions, the results of which would serve as models. Whenever the condition of the experiment was altered so as to test for a factor, the results had to be compared with those of the standard in order to test whether a significant difference was produced.

The attribute tested within the population of flies was the variation in the frequency of their infection with trypanosomes when different treatments were applied. For each treatment therefore, there was always a number of flies in the population which possessed the attribute being tested, and a number of them which did not. Since such two items were to be compared, the two-by-two table was used to calculate the value of X^2. If the total number of flies were less than 200, Yates correction was used. (Snedecor). The same calculation was later used to test the significance of variations in the frequency of established and mature infections within the population of flies when subjected to different treatments apart from the standard. A worked example is shown in Appendix 3 .

In the present investigation, stabilated populations of trypanosomes were diluted with a feeding diluent and used to infect flies through the membrane. Since the number of flies available every day was variable, different numbers of flies were fed on different stabilates at different times. It was therefore necessary to find out whether the distribution of these trypanosomes in the stabilates is homogenous. Since small number of flies was involved, Fisher's formula (Fisher) for the test of homogeneity in small samples was applied to test for this factor. An example is given in Appendix 4 .

EXPERIMENTAL

3.10 To establish methods for infecting tsetse flies by membrane feeding, for maintenance of the flies, and for following the development of trypanosomes in the flies.

In assessing factors which may influence the establishment and development of trypanosomes in tsetse flies under controlled conditions, both the trypanosomes and the tsetse flies will be subjected to different experimental conditions. It is therefore essential to develop a procedure which is reproducible and gives satisfactory results. This procedure will then be regarded as the standard treatment with which other treatments can be compared. In the search for a standard treatment, the following must be considered.

1) the age of the fly at the time of the infective feed
2) the method of infecting the flies
3) the method of maintaining the infected flies
4) the preparation of the infective blood meal
5) the dissection of the flies

Dar (1971) infected 1-day old flies by feeding them through mouse skin membranes on trypanosome stabilates which had been diluted with inactivated horse serum. The infected flies were maintained on rabbits. One randomly selected fly out of each group of 10 flies was killed and dissected daily, starting from day 1 post-infection. He chose 10 days as the period for which information was required since it appeared that midgut infections became established between days 5 and 10 post infection.

Preliminary tests showed that chicken skin could be substituted for mouse skin as a feeding membrane. Killed chickens were available every week as the end product of other experiments being conducted in

this laboratory. Twenty membranes could be obtained from one chicken as opposed to two from a mouse and the flies fed readily through the chicken skin membrane.

Horse serum was found to be unsatisfactory as a feeding diluent; the flies were reluctant to feed except after the addition of adenosine triphosphate (ATP). The flies fed readily through a membrane on fresh or stored blood without ATP.

Both sexes of fly would be used for experiments. The female flies would have to be mated, and the practice in this laboratory is to mate the females 48 hr. after emergence. The following routine was adopted:

the flies were given their first feed, which was the infective feed, within 36 hr. of emergence, on the following day, females were mated, that is when the flies would be 2 or 3 days old, on the next day, the flies were offered food on the maintenance host, and on the day after that, examination of the infected flies was started. Hence, dissections were always started on the third day after the flies had taken their infective feed and when the flies would be 4 or 5 days old.

3.11 To test the effect of defibrinated rabbit blood as diluent for the trypanosomes in the infective feed.

Procedure Tests were conducted with T.brucei, A and T.congolense, A and with G.morsitans and G.austeni. The stabilates were always diluted 1:100. The estimated concentration of T.brucei in the infective feed was 5×10^6/ml and of T.congolense 4×10^6/ml.

Blood was obtained with aseptic precautions from the ear of a rabbit; it was defibrinated and stored at 4°C until required.

A minimum of 10 flies was required for any experiment; if more flies were available, then two or more groups of ten could be infected at the same time. One fly from each group was dissected daily starting from the 3rd day after the infective feed. The sex of the fly was noted, the midgut was then removed and thoroughly searched for the presence of trypanosomes. If any trypanosomes were seen, their morphology and position in the gut was recorded. The flies were fed on rabbits every second day, but any fly that failed to feed was offered food again next day. The feeding record of each fly was kept.

Results The results are shown in tables 1, 2, 3 and 4. The column headed group number indicates the order in which the stabilates were removed from the cold store for use. Age of blood (days) indicates the interval between bleeding the rabbit and use of the blood in the experiment. When less than 10 trypanosomes were seen in the entire midgut, the infection was scored as light (+); in all other cases (++) at least 100 trypanosomes were seen. Willett (1955) noticed that trypanosomes survived in flies which had died overnight and were dissected the following morning. In the present experiments, flies

must have died between approximately 6 p.m. and 9 a.m. so it was likely that if they had been infected the trypanosomes would have been seen. Indeed, active trypanosomes were found in a proportion of the flies found dead when due to be dissected.

Altogether 67 out of 250 G.morsitans were infected with T.brucei and 14 out of 120 G.austeni. The figures for T.congolense were 41 out of 150 (27%) G.morsitans and 32 out of 180 (18%) G.austeni, infected. With both species of trypanosome the higher proportion of G.morsitans that were found infected suggest that this species of fly is a more susceptible host for the trypanosomes than G.austeni.

Although the groups of flies had been fed at different times on different samples of the stabilated population of trypanosomes, the distribution of infected flies in each group appeared to be random. This implies that the distribution of trypanosomes in the lymph tubes is equal and homogeneous. This was confirmed by applying the Fisher's formula for the index of dispersion to these results (Experiment 3.45). The other variable, the age of blood used as diluent for the infective feed, did not appear to affect either the viability of the flies or the number of flies infected in each group. These results were important since the experiments were designed on the assumption that the distribution of trypanosomes in the stabilates would be equal, and that the characters of the trypanosomes in the stabilates would be preserved during long periods of storage.

Table 1 The results of the dissection of 150 G.morsitans which were infected by feeding them on a suspension of T.congolense, A in defibrinated rabbit blood. The flies were maintained on rabbits.

GROUP NO.	STABILATE NO.	AGE blood (days)	DAYS 3	4	5	6	7	8	9	10	11	12	No. Infected
25	5	1	M D	M ++	F ++	F	M	M	M	F ++	F	M	3
26	6	1	M D	M D	M	M	M ++	M	F	F	F	M ++	2
29	7	1	F	F	F ++	F	F ++	M D	F	F	F ++	F	3
30	8	2	M D	F D	F	F D	F +	M	F	F	M +	M +	3
32	9	2	F	F	F D	F ++	M	F	M ++	M ++	F	M	3
35	10	3	M ++	M	M	M	F ++	M	M	F	F	M +	3
37	11	3	M +	M +	F	F	M	M	M	M	F	M ++	3
39	12	3	F	F	M	F	F	M ++	M	F	M	F	1
58	13,14,15	12	F	F ++	F ++	M	M	M	M +	M	M	M	3
59	13,14,15	12	F ++	M	M	M	M	M	M ++	M	F	M	2
60	13,14,15	12	M	M	F ++	M	F	F	M ++	M ++	M	M	3
72	16,17	17	M	F ++	M	M	M ++	M	M	F	M	M	2

Table 1 (continued)

GROUP NO.	STABILATE NO.	AGE blood (days)	DAYS 3	4	5	6	7	8	9	10	11	12	No. Infected
73	16,17	17	M	M +	M	M	M	M ++	F	F	M	M	2
95	18	4	M ++	F	F	M	F ++	M	F	F	F ++	M	3
96	19	4	M ++	F	M	M	F	M +	M +	F ++	M ++	F	5
	TOTALS		5	5	4	1	6	3	5	4	4	4	41

PERCENTAGE INFECTED 27%

M = Male

F = Female

D = Fly dead

+ = Light infection of the midgut

++= moderate or heavy infection of the midgut

This key will be used for Tables 1-4.

Table 2 The results of the dissection of 180 <u>G.austeni</u> which were infected by feeding them on a suspension of <u>T.congolense</u>,A in defibrinated rabbit blood. The flies were maintained on rabbits.

GROUP NO.	STABILATE NO.	AGE blood (days)	DAYS 3	4	5	6	7	8	9	10	11	12	No. Infected
24	5	1	M ++	F ++	F	F	F	F	M	F	M	F +	3
27	6	1	F	F	M	M	F	F	F	M	F	F +	1
28	7	1	F ++	M	F ++	M ++	M D	F	F	F	F	F ++	4
31	8	2	F D	F	M ++	F	F	M	F	M	M	F	1
33	9	2	F D	M	M ++	F	M	M	F	M ++	F ++	F	3
34	10	3	M	M	M	F	M	M	M	M	M	M ++	1
36	11	3	F	M ++	M	M	M	M	M	M	M	M ++	2
38	12	3	M ++	M +	M	M	M	M	M	M	M	M	2
55	13,14,15	12	F ++	F ++	F ++	F	F	F	F	F	F	M	3
56	13,14,15	12	M	M	M	F	M	F	F	F	F	F	0
57	13,14,15	12	M	M	M	F ++	F	M	M	F	M	M	1
74	16,17	17	F	F	F	M	F	F	F	M	F	F	0
75	16,17	17	F	M +	M	F	M	M	M	M	M	M ++	2
76	16,17	17	M	M ++	M	M	M	M	M	M	F	F +	2

Table 2 (continued)

GROUP NO.	STABILATE NO.	AGE blood (days)	DAYS 3	4	5	6	7	8	9	10	11	12	No. Infected
77	16,17	17	M	F	M	M	F	M	M	M	F	F +	1
78	16,17	17	F	M	M	F	M	M	F	F	F	M	0
102	20	1	F	M	F ++	F	M	F +	F	F	F	F	2
103	20	1	F	F	F	F	M +	F ++	M +	M +	M	M	4
	TOTALS		4	6	5	2	1	2	1	2	1	8	32

PERCENTAGE INFECTED 18%

Table 3 The results of the dissection of 250 *G.morsitans* which were infected by feeding them on a suspension of *T.brucei*,A in defibrinated rabbit blood. The flies were maintained on rabbits.

GROUP NO.	STABILATE NO.	AGE blood (days)	DAYS 3	4	5	6	7	8	9	10	11	12	No. Infected
47	8,9	10	M	M	M	M	M	M ++	M	M	M	F ++	2
48	8,9	10	M ++	F	F	F ++	M ++	F	F	M	F	M	3
49	8,9	10	M	M	F	M +	M ++	M	M	M	M	M	2
50	8,9	10	M	F	F	F +	M ++	F ++	F ++	F	M ++	M ++	6
51	8,9	10	M	M	M	M	M	M	M	F	M	M	0
52	8,9	10	M	F	F	M	M D	M	F	F	F	F	0
69	11,12	14	M	M	F ++	M ++	F	M +	F	F	M	F	3
70	11,12	14	M ++	F	F	M	M	M	M	F	M	F	1
71	11,12	14	F	F	M	F	M	M	M	M	M	F	0
79	13,14	1	M	M	F	M	F	F ++	M	F	M +	M	2
90	16,17	4	M	M	F ++	M	M	F	F	F	M	M	1
91	16,17	4	F	F ++	F ++	F	F	M ++	M	M	M	M	3
92	16,17	4	M	M	M	F	F	M ++	F	M	M	M	1
93	16,17	4	F	F	M	F ++	M	M	M +	F	M +	M	3

Table 3 (continued)

GROUP NO.	STABILATE NO.	AGE blood (days)	DAYS 3	4	5	6	7	8	9	10	11	12	No. Infected
94	16,17	4	M	M ++	F	M	M ++	M	M	M +	F	M +	4
97	18	1	F	M	M	M	M ++	M ++	F	F	F ++	F ++	4
98	19	1	F	M	M	M	M ++	M	M	M	M +	F +	3
99	20	1	M ++	M	M	F	F +	M +	M	F ++	M +	M	5
100	21	1	F ++	M	M	M	F	M	M ++	M	F ++	M	3
101	22	1	F	M +	F	F	F	M ++	M ++	M	F	M	3
105	23	2	F +	F +	M D	M ++	M D	F	M	M	F ++	F	4
106	24	2	M +	F ++	M +	F	M	M	M	M	F	F ++	4
107	25	2	M	F ++	M +	F	F D	M	M	M ++	M	F ++	4
108	26	3	M ++	M ++	M +	M ++	M +	M	M	M	F	F	5
109	27	3	F	M	M ++	M	M	M	F	M	M	M	1
	TOTALS		7	7	7	7	8	9	4	3	8	7	67

PERCENTAGE INFECTED 27%

Table 4 The results of the dissection of 120 G.austeni which were infected by feeding them on a suspension of T.brucei, A in defibrinated rabbit blood. The flies were maintained on rabbits.

GROUP NO.	STABILATE NO.	AGE blood (days)	DAYS 3	4	5	6	7	8	9	10	11	12	No. Infected
44	7	8	F	F ++	F	F	F	F ++	M ++	M ++	F	F ++	5
45	8,9	10	M	F	M	F	F	M	F	F	M	F D	0
46	8,9	10	M	F	F ++	M	M ++	M	F ++	M	M	M ++	4
53	10	10	F	M	M	M	M	M	F	F ++	M	M	1
54	10	10	M	F	F + D	F	M	M	F D	F	M	M	1
66	11,12	15	M	F ++	M	M	M	M	M	F	M	M	1
67	11,12	15	M	M	M	M	M	M	M	M	F	F	0
68	11,12	15	M	M	F	M	F	M	M	M	M	M	0
80	13,14	1	M	M	M	M	M	M	M	M	M	M	0
81	13,14	1	F	M	F	M	M	M	M	M	M	M	0
82	13,14	1	F	M	M	M	F	M	M	F +	F	M	1
83	13,14	1	M +	M	M	M	M	M	M	M	M	M	1
	TOTALS		1	2	2	0	1	1	2	3	0	2	14

PERCENTAGE INFECTED 12%

3.12 To test the effect of substituting mice for rabbits as the food hosts of the flies, and of using ox blood instead of rabbit blood as diluent for the trypanosomes in the infective feed.

In the previous experiments rabbits were bled from the ear at intervals of 2 weeks to obtain blood for the preparation of the infective feed. It would be more convenient to use ox blood as the feeding diluent since it could be obtained ad lib from the slaughter house. The ox blood was handled as described (Methods, 2.17).

At the conclusion of Experiment 3.11, the two rabbits which had been used as food hosts for the infected flies were found to be infected with trypanosomes. Therefore it would not be possible to use rabbits as maintenance hosts for infected flies even in experiments scheduled to last no longer than 12 days. The same feeding routine was followed as in Experiment 3.11, but mice were used as food hosts instead of rabbits. From day 7 on, the blood of the mice was examined for trypanosomes and any infected mice were replaced with fresh ones.

Since in Experiment 3.11 some flies had become infective by the 12th day, a more extensive examination of the distribution of the trypanosomes in the fly would be necessary. Hence, in these tests, the midgut, proventriculus and the mouth parts were examined for the presence of trypanosomes.

Results The results are presented in tables 5,6,7 and 8. 17 out of 100 G.morsitans and 13 out of 110 (11.8%) G.austeni were infected with T.brucei, and 11 out of 60 G.morsitans (18%) and 13 out of 90 G.austeni (18%) were infected with T.congolense. The two-by-two table with

Yates correction was applied to test whether there was a significant difference between the infection rates of this experiment and those of Experiment 3.11 where the flies were maintained on rabbits. For G.morsitans fed on T.congolense the calculated value of X^2 is 2.954 and $P < 0.10$ and for G.austeni X^2 is 1.310, $P < 0.30$. With T.brucei and G.morsitans X^2 is 4.912, $P < .05$ and for G.austeni X^2 is 0.140, $P < 0.70$. There was therefore no significant difference between the infection rates of both experiments. It was observed that the flies did not feed so well on mice as they did on rabbits. However, the use of mice as food hosts was unavoidable.

In one G.austeni infected with T.congolense (Table 6) trypanosomes were found in the midgut as well as the labrum epipharynx as early as the 5th day after the infective feed; in three other flies in the same table, although their labrum epipharynx were infected, no trypanosomes were seen in their midguts. The earliest invasion of the labrum epipharynx by T.brucei was on the 11th day. No bias could be detected in the distribution of infected flies between the groups.

<u>Table 5</u> The results of the dissection of 60 <u>G.morsitans</u> which were infected by feeding them on a suspension of <u>T.congolense,</u>A in defibrinated ox blood. The flies were maintained on mice.

GROUP NO.	STABILATE NO.	DAYS 3	4	5	6	7	8	9	10	11	12	No. Infected
20	4	M	M +	M	M	M	M	M	M -	M	M	2
24	5	F	M	M	M ++	F	M	M *	M	F	F	2
27	6	F	F	M +	M	F	F	M	F	M	F	1
28	7	M +	M	F	M	M *	M	M	F	M	F -	3
41	14	F	M	M	F	M	M *	F ++	F	M	M	2
42	15	M	F	M	F *	M	F	M	M	M	M	1
	TOTALS	1	1	1	2	1	1	2	1	0	1	11

PERCENTAGE INFECTED 18%

M = Male

F = Female

D = Fly dead

+ = Light infection of midgut

++= Moderate or heavy infection of midgut

* = Midgut and Proventriculus infected

- = Labrum epipharynx, Proventriculus, and Midgut infected

()= Labrum epipharynx infection with no trypanosomes in the midgut

This key will be used for Tables 5-8.

Table 6 The results of the dissection of 90 G.austeni which were infected by feeding them on a suspension of T.congolense, A in defibrinated ox blood. The flies were maintained on mice.

GROUP NO.	STABILATE NO.	DAYS 3	4	5	6	7	8	9	10	11	12	No. Infected
12	1	M	M	(M)	M	M	M	M	M	M	M -	2
13	2	M	M	M	M	F	M *	M	M	M	M	1
14	2	M	M	M -	M +	(F)	F	M	M	M	F	3
15	2	M ++	M	M	M	F	F	M	M	M	M	1
23	4	M	M	M	F	F	M *	F	F	M	M	1
25	5	M	M	M ++	F	M	M	M	F	M	F +	2
26	6	M	M	F	M	F	F	M	M	M	M -	1
33	8	M	(F)	M	M	M	M	M	F -	F	M	2
39	13	M	M	M	M	M	M	F	F	M	F	0
	TOTALS	1	1	3	1	1	2	0	1	0	3	13

PERCENTAGE INFECTED 18%

<u>Table 7</u> The results of the dissection of 100 <u>G.morsitans</u> which were infected by feeding them on a suspension of <u>T.brucei</u>,A in defibrinated ox blood. The flies were maintained on mice.

GROUP NO.	STABILATE NO.	DAYS 3	4	5	6	7	8	9	10	11	12	No. Infected
1	28	M	M	F	M	M	M	F	M	M	F	0
2	29	F +	M	F	M ++	M ++	M	M	M	M	M	3
4	30	M	M	M + D	M	M	M	F	M	M	M	1
6	31	M	F	F	F	M	F	M	M	M	M +	1
7	31	M	M	F	M	M	M	M	M +	M +	M *	3
14	32,33	M	M	M	M	M	M	M	M	F	M ++	1
15	32,33	F	M	M	M	M	M	M	M	M ++	M	1
21	35	M	M +	M	F	M ++	M ++	M	F	M	M	3
23	35	M	M ++	M	M	M	F	M	M	M −	F	2
25	36	M	M	F	M	F	F	M	M	M −	M +	2
	TOTALS	1	2	1	1	2	1	0	1	4	4	17

PERCENTAGE INFECTED 17%

Table 8 The results of the dissection of 110 G.austeni which were infected by feeding them on a suspension of T.brucei, A in defibrinated ox blood. The flies were maintained on mice.

GROUP NO.	STABILATE NO.	DAYS 3	4	5	6	7	8	9	10	11	12	No. Infected
3	29	M	F	F	M	M	F	M	M	M	F	0
5	30	F	M	M	M	F	F	M	M ++	M ++	F *	3
8	31	F	F +	F	F	F	F	F	M	M	F *	2
9	31	F	F	F	F	F	F	M	F	F +	F	1
16	32, 33	F	M	M	F	F	F	M	F	M	F *	1
18	34	F	M	M	M	F	M	F	F	M	M	0
19	35	M	F	F	F	F ++	F	F	F ++	F	M	2
20	35	F +	M	F	F	F	F	F	F	F	M	1
22	35	F	M	M	M	M	M ++	M	M ++	M	M ++	3
24	35	M	M	M	M	F	M	M	M	F	M	0
26	36	M	F	F	F	M	M	M	F	F	M	0
	TOTALS	1	1	0	0	1	1	0	3	2	4	13

PERCENTAGE INFECTED 11.8%

3.13 To test the effect of the age of the flies at the time of the infective feed on the development of trypanosomes in tsetse flies.

Procedure. Tests were conducted with T.congolense,A and T.brucei,A. The flies were fed on clean rabbits about 36 hours after emergence. Two days later they were offered another feed. After being starved for further three days the flies were offered the third and infective feed through the membrane. Each fly was thus approximately 6½ days old at the time of the infective feed.

Results. The results are shown in Tables 9, 10 and 11. With T.congolense (Tables 9 and 10) 16 of the 100 G.morsitans and 6 of the 60 G.austeni (10%) were infected. With T.brucei (Table 11) 9 or 18% of the 50 G.morsitans were infected. By applying the two-by-two table with Yates correction to test the significance of the difference in the rates of infection in this treatment and the standard (Experiment 3.12) the value of X^2 for G.morsitans with T.congolense is 0.359, $P < 0.70$ and for G.austeni, X^2 is 1.097, $P < 0.80$. With T.brucei and G.morsitans X^2 is 0.006, $P = 0.95$. There was therefore no significant difference in these rates of infection. Two of the G.morsitans infected with T.congolense and dissected on days 4 and 7 (Table 9) had labrumepipharyngeal infections without trypanosomes in the midgut.

These results suggest that the older flies are as good intermediate hosts as the teneral flies but a larger number of flies would be required to confirm this conclusion.

Table 9 The results of the dissection of 100 G.morsitans which had been fed twice on clean rabbits before infecting them with T.congolense,A.

GROUP NO.	STABILATE NO.	DAYS 3	4	5	6	7	8	9	10	11	12	No. Infected
16	2	M	M	M	M	M	M	M	M	M	M	0
17	2	M	M	M +	M	M	M	M D	M	M	M -	2
18	3	M	M	M	M	M	M	M	M	M -	F	1
21	4	F	F	M	M	F -	M	M *	F	M	M +	3
29	7	M	F -	M	M	M	M	F	M	M	M	1
30	8	M	M	M	F +	M	M	F	F	F -	M	2
34	10	M	M	F	M	(M)	M	F	F	M	M	1
35	11	M ++	M	M	F	F	F	F	F -	F	F	2
36	12	F	(F)	F	F	M	F	F	F	M	M	1
37	12	F ++	F	F	F	M	M +	F	M	M ++	M	3
	TOTALS	2	2	1	1	2	1	1	1	3	2	16

PERCENTAGE INFECTED 16%

M = Male

F = Female

D = Fly dead

+ = Light infection of midgut

++ = Moderate or heavy infection of midgut

* = Midgut and Proventriculus infected

- = Midgut, Proventriculus and Labrum epipharynx infected

() = Labrum epipharynx infection with no trypanosomes in the midgut

This key will be used for Tables 9-11.

Table 10 The results of the dissection of 60 G.austeni which had been fed twice on clean rabbits before infecting them with T.congolense, A.

GROUP NO.	STABILATE NO.	DAYS 3	4	5	6	7	8	9	10	11	12	No. Infected
19	3	M	M	M	M	M	M	M	M	M	M	0
22	4	F	M	M	M	F	F	M +	F	M	M	1
31	7	M	F	F -	F	M	M	M	M -	F	M	2
32	8	M ++	M	M	M	M	M	F	M	M	M	1
38	11	M	M ++	M	M	F	F +	F	M	M	F	2
40	13	M	M	F	F	F	F	F	F	F	F	0
	TOTALS	1	1	1	0	0	1	1	1	0	0	6

PERCENTAGE INFECTED 10%

Table 11 The results of the dissection of 50 G.morsitans which had been fed twice on clean rabbits before infecting them with T.brucei, A.

GROUP NO.	STABILATE NO.	DAYS 3	4	5	6	7	8	9	10	11	12	No. Infected
10	32,33	M	M	M	M	M	M	M +	M	M	M	1
11	32,33	F	F	F	F	F	M	M	M	M	F -	1
12	32,33	M	M	M	M	F	M	M +	M	F	M	1
13	32,33	M	M	M	F	M	M	M -	M	F	M ++	2
17	34	F +	M +	M ++	M +	M	F	M	M	M	F	4
	TOTALS	1	1	1	1	0	0	3	0	0	2	9

PERCENTAGE INFECTED 18%

3.14 To test the influence of temperature on the development of trypanosomes in tsetse flies.

Procedure. T.brucei,A was used to conduct this test. After flies had taken their infective feed, they were divided into two lots. One lot was kept in an incubator maintained at 25°C and the other in an incubator at 36°C over a saturated solution of potassium tartrate. The object of keeping the flies in an incubator at approximately the same temperature as that of the fly room (26°C) was to determine whether or not this method of maintenance had an adverse effect on the flies.

Results. Table 12 shows the results of the flies kept in the incubator at 25°C. Of the 150 flies kept in this way 25 or 17% were infected. This rate of infection was not significantly different from that of the standard where flies were kept in the fly room (26°C). When the significance of the difference of both rates of infection was tested by applying the two-by-two table with Yates correction the value of X^2 is 1.717, $P < 0.20$. Moreover, the longevity of flies kept in this way was not adversely affected and they fed as regularly as the flies kept in the fly room. This result is important because it shows that incubators could be used in future experiments to test the effect of different temperatures on the development of trypanosomes in the flies.

The flies kept at 36°C showed a greater rate of midgut infections since 53 or 35% of the 150 G.morsitans were infected. However, 47 or 31% of all the experimental flies died during the experiment. Of these, 14 were infected. A temperature of 36°C was hence too high for the flies.

Table 12 (continued)

GROUP NO.	STABILATE NO.	DAYS 3	4	5	6	7	8	9	10	11	12	No. Infected
13	43,44	M	M ++	F	M	M	F *	F	F	F	M	2
14	45	F ++	F	F	F	M	M	F	F *	M	F	2
15	45	M	M	M	M	M	M	M	M	M	M	0
	TOTALS	6	2	3	1	4	2	1	1	2	3	25

PERCENTAGE INFECTED 17%

M = Male

F = Female

D = Fly dead

+ = Light infection of midgut

++ = Moderate or heavy infection of midgut

* = Midgut and Proventriculus infected

- = Midgut, Proventriculus, and Labrum epipharynx infected

3.15 To test whether there is a correlation between the infection rate in flies infected with trypanosomes and killed at 5,10 and 40 days after their infective feed.

The results of previous experiments (3.11-3.14) had shown that the trypanosomes first establish in the midgut and later migrate to the proventriculus and the mouth parts. For *T.congolense* it is usually assumed that the infection in a fly is mature when its hypopharynx is colonized. In case of *T.brucei* the criterion for maturity of infection is the invasion of the salivary glands. This experiment was therefore designed to see whether there was a correlation between the number of flies with trypanosomes in the midgut at 5 and 10 days and those kept for 40 days.

Procedure Tests were conducted with *T.congolense*,A. and *T.brucei*,A.

Groups of 16 flies were fed on the same stabilates; 4 of the flies were dissected on day 5, 4 on day 10 and the remaining 8 on day 40 or later. Each of the flies which were maintained for 40 days was fed on three mice in rotation. This was to reduce the chance of infection failing to become patent in the mice. The mice could vary in their susceptibility to infection. The mice were examined for trypanosomes twice a week. Wilson (1968) showed that *T.congolense* could take up to 6 weeks to become patent in mice. When a fly was found to be infected on dissection, the mice on which it had fed were kept for 6 weeks before being regarded as uninfected. All the 40 day flies were probed regularly after the 10th day post infection.

Results and Conclusions The summaries of the results are shown in Tables 13 and 14.

For T.congolense (Table 13) it appeared that there was good correlation between the number of flies found infected when killed at 5 or 10 days (11/80) and at 40 days (12/80). In the early period, one fly carried trypanosomes in the hypopharynx. This fly was dissected on day 10, but had not fed since day 7. Invasion of the hypopharynx probably occurred subsequent to day 7. If kept longer this fly would probably have infected mice.

Of the 10 mouth part infections in the 40 day flies only one had an hypopharyngeal infection and infected all the three mice on which it had fed. The remaining 9 flies did not infect mice. Although there were abundant trypanosomes in the midgut, proventriculus, and the labrum epipharynx of each of these 9 flies on dissection, the hypopharynx of none of them was invaded. Only the single fly with hypopharyngeal infection extruded trypanosomes in the saliva on several occasions during probing. The probes of the other flies contained no trypanosomes.

For T.brucei (Table 14) it appeared that the presence of trypanosomes in flies at 5 and 10 days after their infective feed does not indicate that the infection will persist. Whereas 14 out of the 80 flies killed at 5 and 10 days were infected, only 6 out of 80 killed at day 40 were infected. Of these 6 flies, only 3 infected mice. Further, when these 3 flies were dissected, only one showed a small number of trypanosomes in the salivary glands. Either, the salivary gland infections in two of the flies were transitory, and it was at this time that the mice became infected, or metacyclic

trypanosomes can develop without invasion of the salivary glands.

It was remarkable that in both species of trypanosomes, the trypanosomes can be retained in the midgut or remain in the proventriculus for long periods without completion of the development cycle.

Table 13 The results of the dissection of 160 G.morsitans which were infected with T.congolense,A and killed 5, 10 and 40 days later.

TREATMENTS	TOTAL NO. OF FLIES	TOTAL NO. INFECTED	DISTRIBUTION OF TRYPANOSOMES IN THE FLIES			NO.OF MICE INFECTED OUT OF 3
			+M	++M,P,L	+++M,P,L,H	
Early Dissections (5 and 10 days)	80	11	5	5	1	0
Late Dissections (40 days or later)	80	12	2	9	1	3

(M)+ = Midgut only

(M,P,L)++= Midgut, Proventriculus and Labrum epipharynx

(M,P,L,H)+++ = Midgut, Proventriculus, Labrum epipharynx and Hypopharynx

Table 14 The results of the dissection of 160 G.morsitans which were infected with T.brucei,A and killed 5, 10 and 40 days later.

TREAT-MENTS	TOTAL No. OF FLIES	TOTAL NO. INFECTED	MIDGUT AND PROVENTRICULAR INFECTIONS	MATURE INFECTIONS	NO. OF MICE INFECTED OUT OF 3
Early Dissections (5 and 10 days)	80	14	14	-	-
Late Dissections (40 days or later)	80	6	3	+3	++i)2 ii)1 iii)1

\+ = The three flies infected mice. Their infection was therefore classified as mature although only one of them had trypanosomes in the salivary glands.

++ = i, ii, iii, represent the 3 flies which infected mice.

<u>3.16</u> To test the effect of using defibrinated ox blood to maintain the flies subsequent to their infective feed.

If experimental flies could be maintained on defibrinated ox blood by membrane feeding isolation of flies with mature infection would be facilitated. Such flies would deposit metacyclics in the pool of blood on which they had fed and the trypanosomes could be detected microscopically. Secondly the number of trypanosomes ejected when a fly feeds could be estimated by counting the trypanosomes in the blood residue on which the fly had fed.

<u>Procedure</u> The test was conducted with <u>T.congolense</u>, A. The defibrinated ox blood was used both as a feeding diluent for the infective feed and as a maintenance medium.

The feeding capsules were filled with defibrinated ox blood, covered with chicken skin membrane in the usual way (Methods 2.18). Flies were placed on the capsules to feed every other day. Those that failed to feed were offered food on the following day.

Groups of 10 <u>G.austeni</u> were dissected from days 3 to 12. Groups of 16 <u>G.morsitans</u> were fed on the same stabilates and kept for 5,10 and 40 days. 4 of the flies were dissected on day 5, 4 on day 10, and the remaining 8 were scheduled for dissection on day 40. As from the 10th day post infection, the pool of blood on which each of the 40 day flies had fed was injected into three mice in rotation after a drop had been inspected microscopically for the presence of trypanosomes.

Results and Conclusions Of the 110 G.austeni (Table 15) 13 were infected (12%). Applying the two-by-two table with Yates correction to test the significance of the difference between the infection rate of this treatment and that of the standard (Experiment 3.12) the value of χ^2 is 0.122, $P < 0.80$. Hence, the rates of infection of both treatments are similar. The longevity of the G.austeni was not adversely affected at least up to 12 days post infection.

The attempt to keep the G.morsitans for 40 days before dissection had to be abandoned. The flies started to die after 14 days and none lived longer than 30 days. Before the 14th day, these flies fed as regularly as those maintained on mice. There was however a reluctance to feed after the 14th day. Almost every dead fly had a solidified black brownish mass in the posterior segment of the midgut. The dead flies contained no trypanosomes. Neither was any trypanosome seen in the blood on which they had fed before death. No mouse was infected.

These results suggested that after repeated feeds on defibrinated ox blood, flies were unable to digest the blood meal.

Table 15 The results of the dissection of 110 G.austeni which were infected with T.congolense, A and maintained by feeding them on defibrinated ox blood through a membrane.

GROUP NO.	STABILATE NO.	DAYS 3	4	5	6	7	8	9	10	11	12	No. Infected
1	1	M	M	F	M	M ++	M	M	F	F	M	1
2	1	F	M	F +	M	M	F	M	M	F	M	1
3	2	F	F	F	F +	M	F	F	F	F	F	1
4	2	M	F	F	F	F	F	F	M	M	F	0
5	3	M ++	F	F	F	M	M	M	M ++	F	F	2
6	4	M	F	M	M	M	M	F ++	F	M ++	M	2
7	4	M	M +	M	M	M	M	M ++	M	M	M	2
8	5	F	F	F	F	M	M	M	F	M	F	0
9	5	F ++	M	M	M ++	M	F	F	M	M	F	2
10	5	F	M	M	M	M	M	F	F	F	M ++	1
11	5	M	M	M ++	M	F	F	F	M	M	F	1
	TOTALS	2	1	2	2	1	0	2	1	1	1	13

PERCENTAGE INFECTED 12%

M = Male

F = Female

\+ = Light infection of midgut

++ = moderate or heavy infection of midgut

3.17 To test the infectivity of Trypanosoma congolense, B to tsetse flies under standard conditions.

All the stabilates of T.congolense,A had been used. One mouse had become infected after a fly had fed on it (Experiment 3.15). Blood from this mouse was passaged into 6 further mice. When the parasitaemia was high, after 7 days, the mice were killed, and about 200 stabilates were prepared from the pooled blood. These stabilates were designated T.congolense,B.

It was necessary to find out if the new stabilates were similar to those that had been used for previous experiments and which were designated T.congolense,A and how many of the infected flies would develop mature infections.

Procedure After any G.morsitans that were available had fed on blood containing T.congolense,B, they were divided into two batches. The flies of one batch were dissected, as previously, on days 3-12 after infection. The flies of the other batch were dissected on days 13-22 after infection. All the flies of both batches were fed on a group of 12 mice for 9 days. From the 10th day on three clean mice were allocated to each group of 10 flies. The mice were inspected daily before commencement of feeding and any infected mouse was replaced. When a fly was found to be infected on dissection, the mice on which it had fed were kept for 6 weeks before being regarded as uninfected.

Another change was introduced at this stage of the work. It seemed that the position of the trypanosomes in the gut during the first few days after the infecting feed was indicative of the subsequent events. The position of the trypanosomes in the alimentary tract of the fly was therefore recorded in greater detail. 4 types of

infection were recognised. A fly in which the trypanosomes were restricted to the posterior segment of the midgut was designated a Category 1 infection (Fig.10). In this segment the trypanosomes occur both within and outside the peritrophic membrane. Identification of the midgut segments was as described by Wigglesworth (1929). In a category 2 infection (Fig.11), the trypanosomes had moved forwards in the extraperitrophic space. They are confined to this space except in the posterior segment of the midgut. In a category 3 infection (Fig.12) the trypanosomes were found in the proventriculus, and in category 4, they had reached the proboscis (Fig.13).

<u>Results</u> These are summarised in Tables 16, 17 and 18.

In Table 16, we see that on the 3rd to 5th day after the infective feed, 16 of the 30 flies were infected but in only one of these had the trypanosomes moved forwards into the extraperitrophic space (Category 2). After the 5th day only 1 out of each group of 10 flies was infected but now the infections were of category 2, 3 or 4. The total number of flies with such infection, that is, those in which the trypanosomes had moved forwards into the extraperitrophic space, was 6 (6%).

In all the flies which were kept for longer before being examined (Table 17) the infection was category 4. There were 9 or 6% such infections out of 150 <u>G.morsitans</u>. Among the flies dissected on days 3-12 (Table 16) the percentage of flies in which the trypanosomes had moved forwards into the extraperitrophic space was also 6%. This correlation was very important because it showed the significance of the change from a category 1 to a category 2 infection. It seemed that an infection became established once the trypanosomes

moved forwards from the posterior segment into the ectoperitrophic space of the midgut. Hence, it was decided that such an infection should be referred to as an "established infection". In this thesis therefore, an established infection denotes one in which trypanosomes are found throughout the ectoperitrophic space and further forwards. The term "infected fly" will be reserved for any fly in which active trypanosomes are found in any part of the alimentary tract.

As recorded in Table 18, all but one of the infected flies which had been maintained for the longer period were infective to mice. The only fly that did not infect mice was the one dissected on day 13 and had trypanosomes only up to the labrum-epipharynx. Its hypopharynx was not yet invaded. There was little variation in the incubation period in the mice, the average period being 8 days. The feeding record showed that transmission of trypanosomes to mice was effected only when the fly engorged fully. No infected mice were found after partial engorgement. This population of trypanosomes was also observed to be very virulent to mice since the infected mice were dying at about 6 days after the first parasitaemia.

When the results of this experiment are compared with those of Experiment 3.15 it is evident that the two populations of T.congolense, TREU 692 differ in at least two respects. Firstly, the high rate of infection on days 3-5 with T.congolense,B which falls to a low but almost constant number from days 6-12 was not apparent with T.congolense,A. Secondly, 8 out of the 9 flies infected with T.congolense,B infected mice whereas only 1 out of 9 flies infected with T.congolense, A developed a mature infection.

The difference between these two populations of T.congolense TREU 692 seemed to lay in the number of mouse passages before they were stabilated.

Table 16 The results of the dissection of 100 G.morsitans which were infected with T.congolense,B and dissected from days 3 to 12 afterwards.

GROUP NO.	STABILATE NO.	DAYS 3	4	5	6	7	8	9	10	11	12	No. Infected
1	1,2,3	M	M 1	M	M	M	M	M 4	M	F	F	2
2	1,2,3	F 1	M 1	M D	F	M 4	M	F	F	M	M	3
3	1,2,3	M	M	M 1	F 1	M	F	M	M	M	M	2
4	1,2,3	M	F	F 1	M	M	M	M	M	F	F	1
5	4	M 1	M	M 1	M	M	M	M	M	M	M	2
6	5	F 1	M 1	M	M	F	M	M	F	F 2	M	3
7	6	F 1	M	M	F	F	F	F	F	M	F	1
8	7,8	M	M 1	M 1	M	M	F	M	M	F	F 4	3
9	7,8	F	M 1	M	M	F	F 2	M	M	F	F	2
10	9	F 2	F 1	F 1	F	M	F	F	F	F	F	3
TOTAL INFECTED		5	6	5	1	1	1	1	0	1	1	22
CATEGORY 2-4		1	0	0	0	1	1	1	0	1	1	6

M = Male
F = Female
D = Fly dead on the same day due for dissection.
D followed by a figure = Fly dead on the day specified by the figure.
1,2,3,4 = denotes the category of infection and refers to position of trypanosomes in the gut (Text).

This key will be used for Table 17 as well.

Table 17 The results of the dissection of 150 G.morsitans which were infected with T.congolense,B and dissected from 13 to 22 days afterwards.

GROUP NO.	STABILATE NO.	DAYS 13	14	15	16	17	18	19	20	21	22	No. Infected
1	1,2,3	M	F D5	F	F	M	M	M	M	M	M	0
2	1,2,3	F D5	F	F	M	F	F	F	M	M	M	0
3	1,2,3	M	M	M	M	M 4	M	F	F	M 4	M	2
4	1,2,3	F	F	F	M	M	M	M	M	M	M	0
5	4	M	M	M	F	F	M	M	M	M	M	0
6	5	M	F	M	M	M	M	M	M	F	F	0
7	6	F	M	M	M	F	F	F	M	F	F	0
8	7,8	M	M	F 4	M	F	M	F	M	M 4	M	2
9	7,8	F	M	F	F	M	F	F	F	F	F	0
10	9	F	F	F	F	F 4	M	M	F	F	F	1
11	36	F 4	F	M	F	F	M 4	M	F	F 4	F	3
12	36	M	M	M	M	M	F	M	M	F 4	F	1
13	37	F	F	M	F	F	F	F	F	F	F	0
14	37	F	M	M	M	M	M	M	M	M	M	0
15	38	M	M	M	M	M	M	F	F	M	F	0
CATEGORY 4		1	0	1	0	2	1	0	0	4	0	9

Table 18 The results of the dissection and infectivity to mice of the 8 flies with mature infection amongst the 150 G.morsitans which were infected with T.congolense,B.

FLY NO.	DAY DISSECTED	CATEGORY INFECTION	DAY OF LAST FEED	DAY OF FIRST PARASITAEMIA	INCUBATION PERIOD IN DAYS
1	17	4	16	25	9
2	21	4	18	24	6
3	15	4	14	22	8
4	21	4	17	25	8
5	17	4	15	25	10
6	18	4	12	20	8
7	21	4	17	27	10
8	21	4	18	26	8

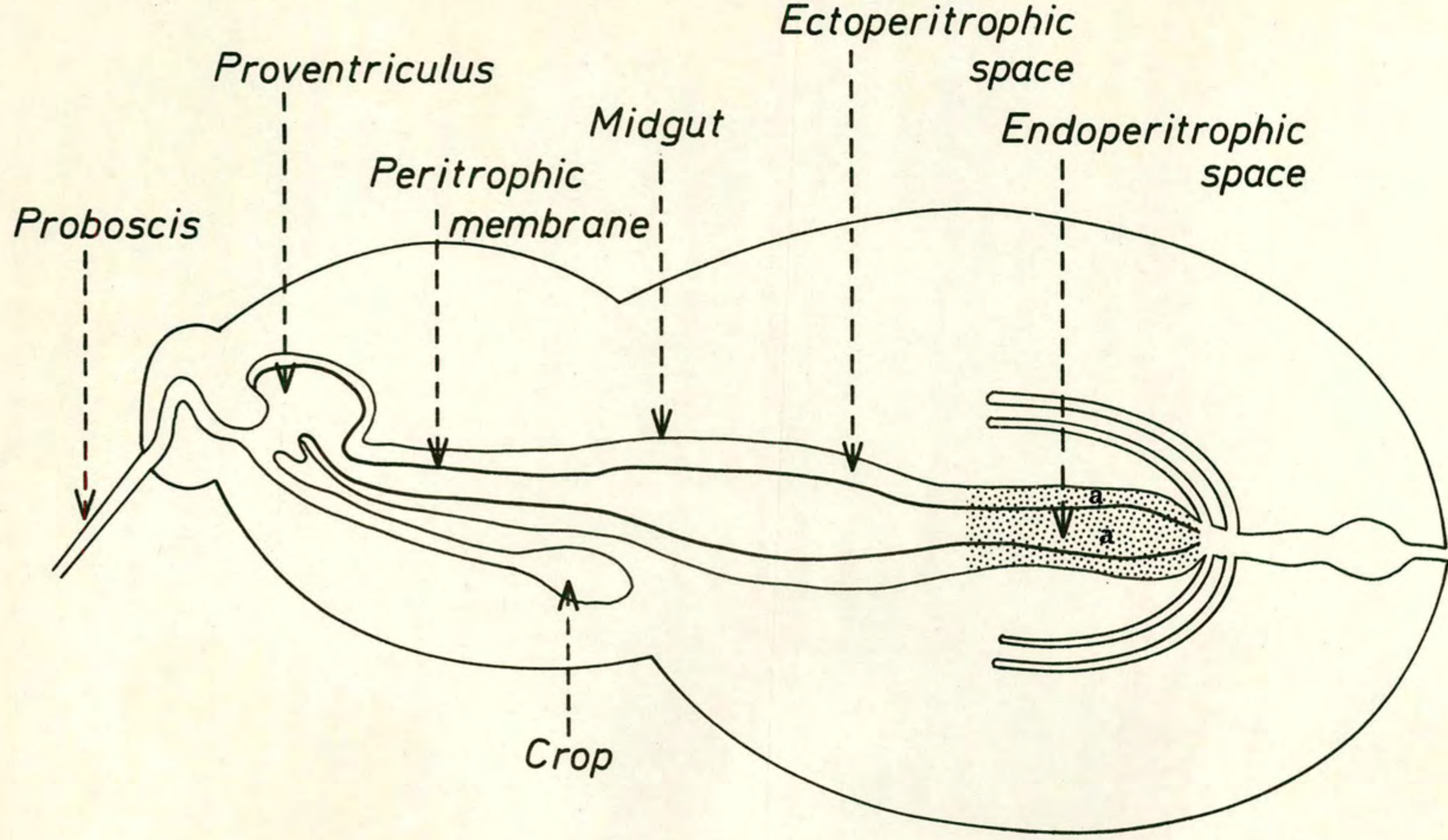


Fig.10 Types of Infection in the fly

a) = Trypanosomes

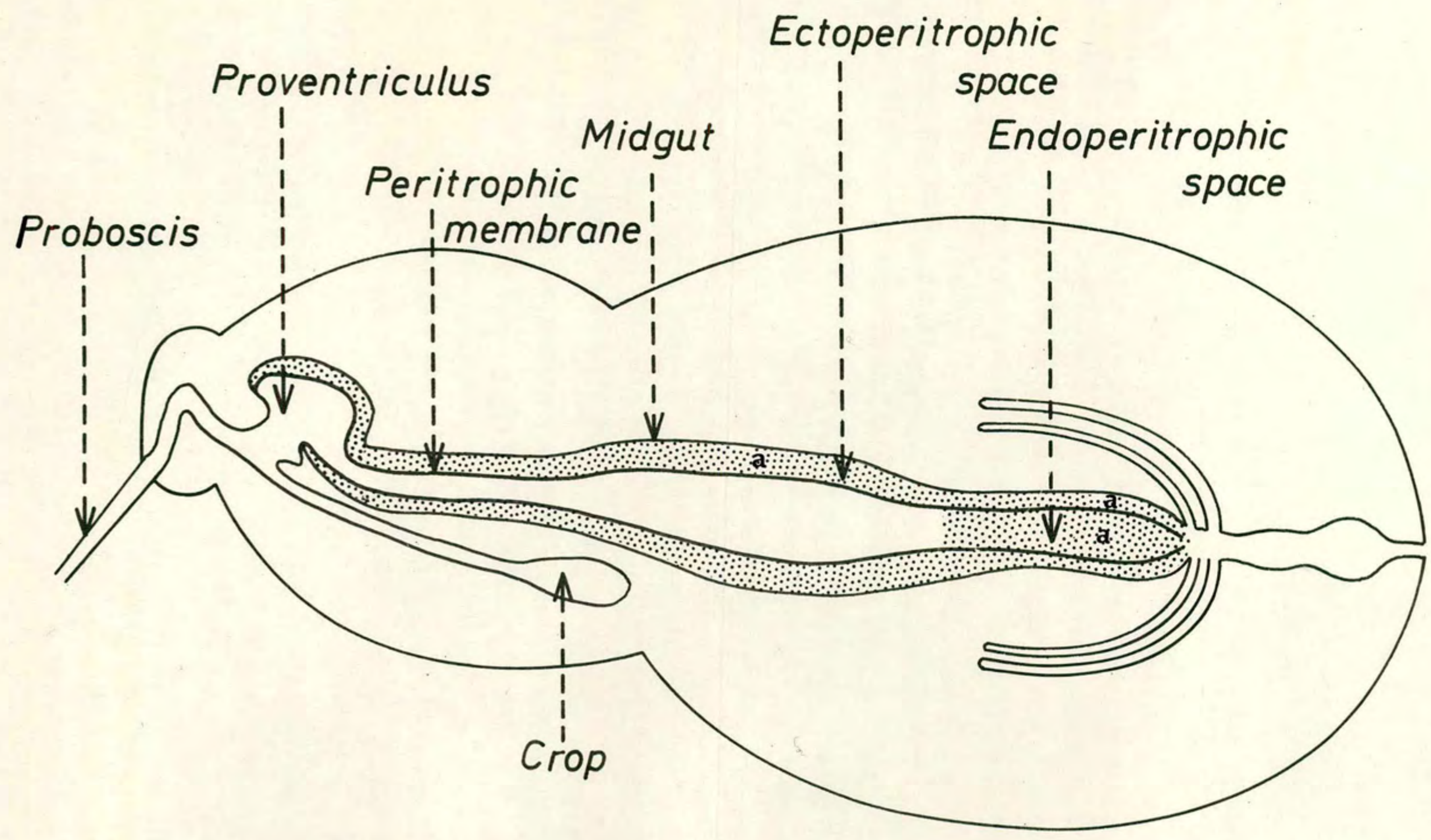


Fig.11 = Types of infection in the fly

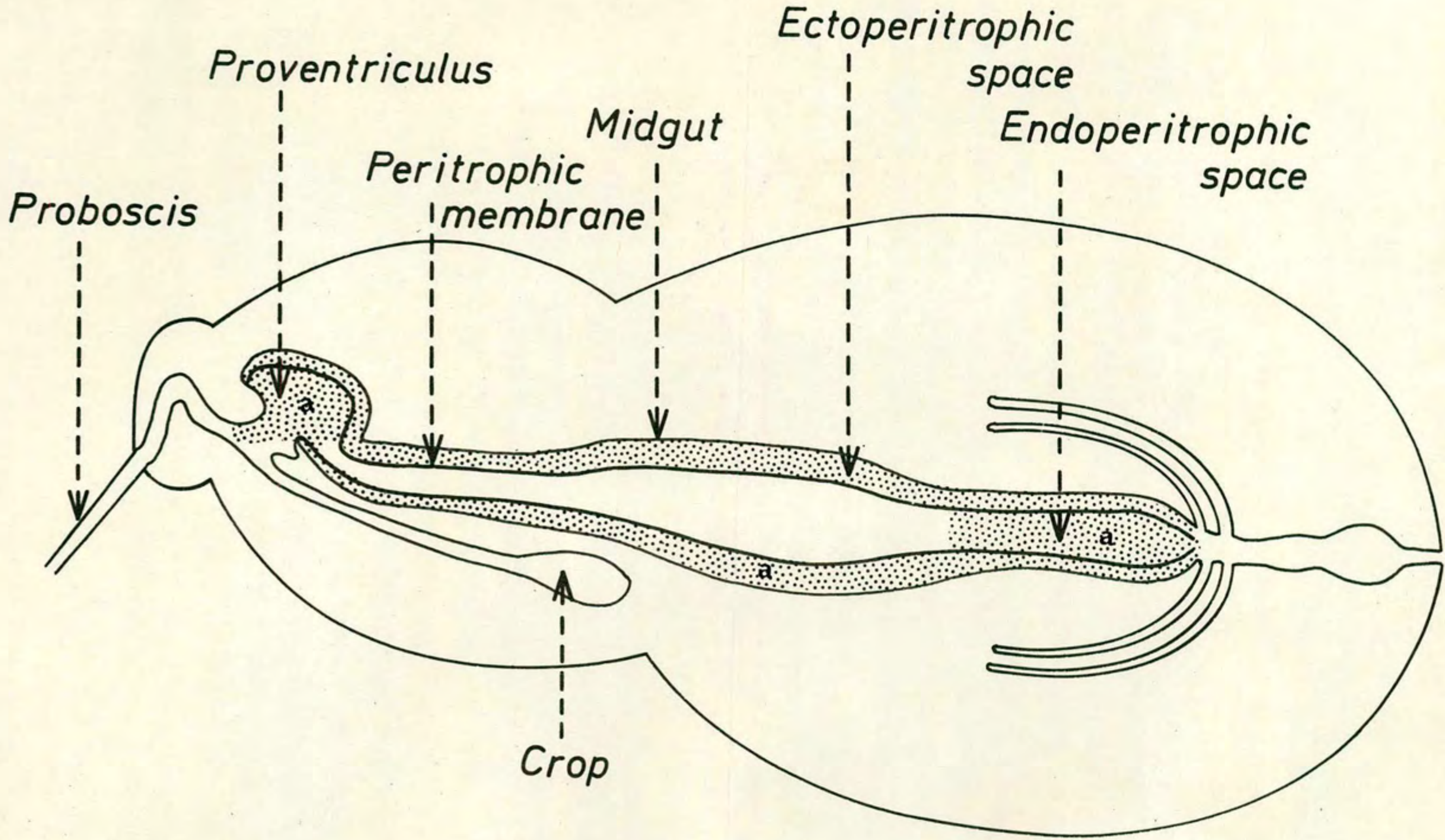


Fig.12 = Types of infection in the fly

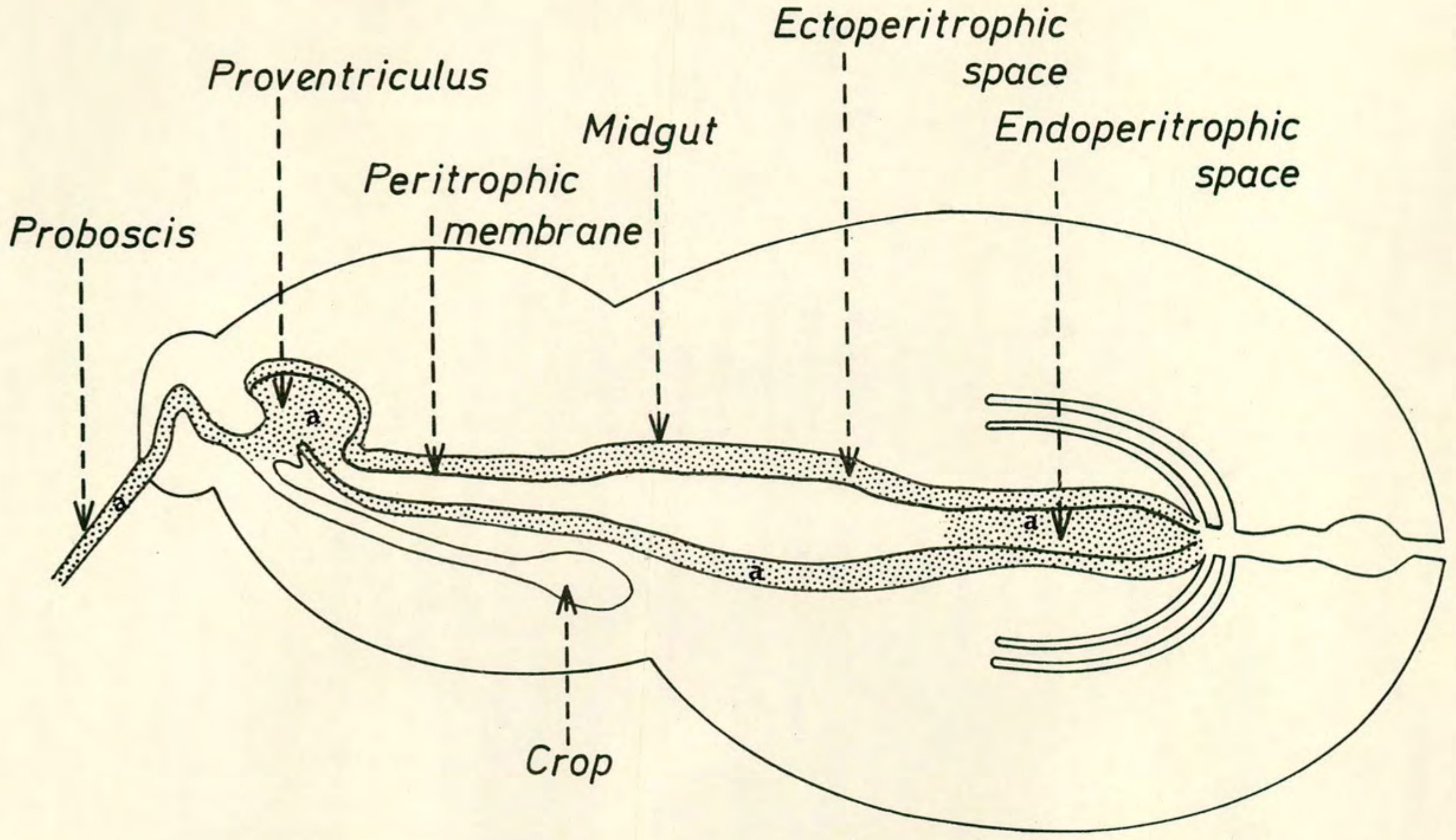


Fig.13 = Types of infection in the fly

a) = Trypanosomes

3.18 To test the infectivity of Trypanosoma brucei,B to tsetse flies under standard conditions.

All the stabilates of T.brucei,A had been used. Blood from one of the mice infected after a fly had fed on it (Experiment 3.15) was passaged into 6 further mice. When the parasitaemia was high, after 5 days, the mice were killed and about 200 stabilates prepared from the pooled blood. These were designated T.brucei,B.

The aims of this experiment were similar to those expressed under Experiment 3.17.

Procedure The details are as described for T.congolense,B in Experiment 3.17.

Results The results are shown in Tables 19, 20 and 21.

11 out of the 30 flies examined on days 3-5 were infected but in only one of them had the infection become established (Table 19). From the 6th to 12th day there were only 6 infected flies of which 4 were established. In all, 5% of the flies had established infections out of a total of 17 infected flies.

All but one of the 9 infected flies among the 150 G.morsitans (6%) kept for longer before being examined possessed established infections (Table 20). The remaining one fly died on day 4 and had a category 1 infection. There was good agreement between the number of flies with established infections whether the flies had been killed before or after 12 days. Recognition of an infection as established for all those flies with infections of categories 2 to 4 was confirmed.

Table 21 gives the details of the 3 flies that infected mice. When these three flies were dissected, only one showed scanty trypanosomes in the salivary glands. This was similar to the dissection results of the 3 flies that infected mice when flies infected with T.brucei,A were kept for 40 days (Experiment 3.15). It appeared that mature infections of both populations of T.brucei, TREU 667 behaved similarly in their infectivity to mice.

It was observed at this stage of the work that there was little difference in the number of infected flies or flies with established infections between the two species of trypanosome. There was a difference however between the two species in the proportion of established infections which became mature.

Table 19 The results of the dissection of 100 G.morsitans which were infected with T.brucei,B and dissected from days 3 to 12 afterwards.

GROUP NO.	STABILATE NO.	3	4	5	6	7	8	9	10	11	12	No. Infected
		DAYS										
1	1,2	M 1	M	M	F 1	M	F	M	M	M	M	2
2	1,2	M	M	M	M	F	M	M	M	M	F	0
3	1,2	M	M	M 3	F	M	M	M	M	M 3	M	2
4	3,4,5	M 1	M D	M	F	F	M	M	M	M	M	1
5	3,4,5	F	M 1	M	M	M	M	M	M	M	M	1
6	3,4,5	M	M	M	F	M	M	M	M	M 4*	M	1
7	3,4,5	M 1	M 1	M	M	M	F	F	M	M	F 1	3
8	6	F 1	M 1	M	F	F	F	F 3	M	M	F 4*	4
9	6	F	F	M	F D	F	M	F	F	M	M	0
10	7	F 1	M 1	F 1	F	M	F	M	M	F	F	3
TOTAL INFECTED		5	4	2	1	0	0	1	0	1	3	17
ESTABLISHED INFECTION		0	0	1	0	0	0	1	0	1	2	5

M = Male
F = Female
D = Fly dead on the same day due for dissection.
D followed by a figure = Fly dead on the day indicated by the figure.
1,2,3,4 = denotes the category of infection.
4* = Trypanosomes found in Labrum-epipharynx.
4^+ = Trypanosomes found in the salivary glands.

This key will be used for Table 20 as well.

Table 20 The results of the dissection of 150 G.morsitans which were infected with T.brucei,B and dissected 13 to 22 days afterwards.

GROUP NO.	STABILATE NO.	13	14	15	16	17	18	19	20	21	22	No. Infected
		DAYS										
1	1,2	M 4*	M	M	M	F	F	M	M	F	M	1
2	1,2	F	M	M	M D5	M	M	M	M	M	M	0
3	1,2	M	M	F	M	M	M	M	M	M	F	0
4	3,4,5	M	M	M	M	M	M	M	M	F	F	0
5	3,4,5	M	M	M	M	M	M	M	M	M	F	0
6	3,4,5	F	F	M D4 1	M	M	M	M	F	F	M	1
7	3,4,5	F	M	M 4*	M	M	M	M	M	M 4*	F	2
8	6	F	M	M	F	F	F	M	M	M	M	0
9	6	M	M	F	F	F	M	M 4+	M	F	M	1
10	7	M	F	M	F	F	M	M	M	M	M	0
11	29	F	F	M	F 4*	F	M	F	F	F	F	1
12	29	F	F	M	F	M	M	M	F	F	M	0
13	30	M	M	M	F	M	M 3	M	F	M	M 3	2
14	30	F	F	M	M	M	M	M	M 3	F	M	1
15	31	F	M	M	M	M	M	M	M	F	M	0
TOTAL INFECTED		1	0	2	1	0	1	1	1	1	1	9
ESTABLISHED INFECTION		1	0	1	1	0	1	1	1	1	1	8

Table 21 The results of the dissection and infectivity to mice of the 3 flies with mature infection among the 150 *G.morsitans* which were infected with *T.brucei*, B.

FLY NO.	DAY DISSECTED	CATEGORY INFECTION	DAY OF LAST FEED	DAY OF FIRST PARASITAEMIA	INCUBATION PERIOD IN DAYS
1	19	4+	18	24	6
2	21	4++	20	25	5
3	22	3	20	26	6

\+ = Scanty trypanosomes found in the salivary glands.

++ = Abundant trypanosomes in the labrum-epipharynx but salivary gland empty.

3.19 To test for the retention of infectivity of the trypanosomes in the infective feed during the time required to feed several groups of flies.

Procedure Both T.congolense,B and T.brucei,B were used to conduct this test.

Ten feeding capsules were filled initially with 0.2 ml of the infective blood. A fly was then placed on each capsule and allowed to feed for 10 minutes. The flies were removed at the end of 10 minutes, each capsule was refilled with 0.1ml infective blood and another group of 10 flies was placed on the capsules. The time taken to complete each of these processes was recorded with the aid of a stop watch.

The groups of flies were numbered in the order in which they were fed. In each group, the first fly taken off the feeding capsule was No.1, the second No.2 et seq. The flies were also dissected according to their serial number. Thus the No.1 of any group was dissected on day 3 and No.10 on day 12.

The total time spent on each procedure was as follows:-

1) Feeding 7 groups (10 mins. per group) = 70 mins.

2) Removing and replacing flies on the capsules = 7 mins.

3) Refilling the capsules = 21 mins.

Total = 98 mins.

Results and Conclusions It can be seen from Tables 22 and 23 that 17 or 24% of the 70 G.morsitans became infected with T.congolense,B while 9 or 13% of the 70 flies fed on T.brucei,B were infected. There were 6 and 2 established infections for T.congolense and T.brucei

respectively.

The distribution of infected flies between the groups was random. The trypanosomes in the infecting feed had retained their viability and infectivity to flies for at least 98 minutes. In none of the experiments reported in this thesis was the time interval between feeding of the first and last flies so long as 98 minutes. Feeding was usually completed within 60 minutes or less.

It was concluded that there was an equal chance for the flies which fed first or those which fed last to become infected.

Table 22 The results of the dissection of 70 G.morsitans which were infected with T.congolense,B. The time interval between the start of feeding group 1 and the end of feeding group 7 was 98 minutes.

GROUP NO.	STABILATE NO.	DAYS 3	4	5	6	7	8	9	10	11	12	No. Infected
1	31 to 35	M 1	F 1	F	M 3	M 4	F	M	F	M	F	4
2	"	M 1	F	M 1	M	M	M	M	F	F	F	2
3	"	M 1	F 1	M	F	F	M	F	M	M	M D	2
4	"	M	M	M	M 1	M	M	F	M	M	F	1
5	"	F 1	M 1	M	M 2	M D	M 4	F	F	F	F	4
6	"	M	M 1	M	F	F	F	M	F	F	M 4	2
7	"	M 1	M	F	M	F	F	M 3	F	F	F	2
TOTAL INFECTED		5	4	1	3	1	1	1	0	0	1	17
ESTABLISHED INFECTIONS		0	0	0	2	1	1	1	0	0	1	6

M = Male

F = Female

D = Fly dead on day due for dissection.

1,2,3,4 = denotes category of infection.

This key will be used for Table 23 as well.

Table 23 The results of the dissection of 70 *G.morsitans* which were infected with *T.brucei*,B. The time interval between the start of feeding group 1 and the end of feeding group 7 was 98 minutes.

GROUP NO.	STABILATE NO.	DAYS 3	4	5	6	7	8	9	10	11	12	No. Infected
1	53-55	M	M	M	M	M	M	M	M	M	M	1
				1								
2	"	F	M	M	M	F	M	M	M	M	M	0
3	"	F	M	M	M	M	M	M	M	F	F	2
		1						3				
4	"	M	M	M	M	M	M	M	M	M	M	2
		1								1		
5	"	F	F	M	F	F	F	F	F	F	F	2
		1							1			
6	"	F	M	M	F	F	F	F	M	F	F	1
							3					
7	"	F	M	M	F	M	F	F	M	F	F	1
		1										
TOTAL INFECTED		4	0	1	0	0	1	1	1	1	0	9
ESTABLISHED INFECTIONS		0	0	0	0	0	1	1	0	0	0	2

<u>3.20</u> To test the effect of concentration of trypanosomes in infecting feed on the infection rate in the flies.

<u>Procedure</u> This test was conducted with <u>T.congolense</u>,B and <u>T.brucei</u>,B.

The concentration of trypanosomes in the infecting feed was increased 10 fold so that the stabilates were diluted 1:10 instead of 1:100 with defibrinated ox blood. Thus the approximate concentration of <u>T.congolense</u>,B was 4.4×10^7 trypanosomes per ml. and that of <u>T.brucei</u>,B 5.32×10^7 trypanosomes per ml.

<u>Results</u> From Tables 24 and 25 we see that each of the species of trypanosome had 20 infected flies of which 7 were established in both cases. Numerically there was little or no difference between the number of infected flies as well as the number of established infections in this treatment and the standard (Experiments 3.17 and 3.18). For <u>T.brucei</u>, however, it seemed there was a slightly higher number of 3rd day infection than that of the standard treatment although the totals of 3rd to 5th day infections for both treatments were almost the same. These results were confirmed by later experiments in which the concentration of <u>T.brucei</u>,B in the infective feed was increased tenfold and 80-90% of the flies were found to be infected on the 3rd day. For <u>T.brucei</u>, the number of flies in which the trypanosomes persist and multiply up to the 3rd day may be directly related to the number of trypanosomes ingested.

Table 24 The results of the dissection of 100 **G.morsitans** which were infected with stabilates of T.congolense,B diluted 1:10 with defibrinated ox blood.

GROUP NO.	STABILATE NO.	DAYS 3	4	5	6	7	8	9	10	11	12	No. Infected
1	10-30	M	M	M	M	F	M	F	F	M	F	3
			1	1		1						
2	"	M	F	F	M	F	F	M	F	M	F	2
		1		1								
3	"	F	F	F	*	F	M	F	F	F	F	1
				1								
4	"	F	M	F	F	F	M	F	M	F	F	3
			2	2	1							
5	"	F	M	F	F	F	M	F	F	M	F	2
		1		2								
6	"	F	M	M	F	M	F	M	F	F	F	2
		1			2							
7	"	M	F	F	F	M	M	M	M	M	F	2
		1						D			4	
8	"	F	F	F	F	F	M	F	M	F	M	2
		1		1								
9	"	M	F	F	M	M	M	M	F	F	F	2
			2			2						
10	"	F	M	M	F	M	F	F	F	F	M	1
			1									
TOTAL INFECTED		5	4	6	2	2	0	0	0	0	1	20
ESTABLISHED INFECTIONS		0	2	2	1	1	0	0	0	0	1	7

M = Male
F = Female
* = Fly escaped
D = Fly dead on day due for dissection
1,2,3,4 = denotes the category of infection
4* = Trypanosomes found in Labrum-epipharynx

This key will be used for Table 25 as well.

Table 25 The results of the dissection of 100 *G.morsitans* which were infected with stabilates of *T.brucei*,B diluted 1:10 with defibrinated ox blood.

GROUP NO.	STABILATE NO.	DAYS 3	4	5	6	7	8	9	10	11	12	No. Infected
1	8-28	F 1	M	F	M	M 1	F	F	F* 4	F	M	3
2	"	F 1	F	M	F 3	F	M	M	M	F	F	2
3	"	M	F 1	F 3	M	M	M	F	F	F	F	2
4	"	M 1	M	M	M	F	M	M	F	M	F	1
5	"	F	F	M	F	F	M	F	F	M	M	0
6	"	F 1	M	F 3	F	M	M	F	F	F	M	2
7	"	M 1	F 1	F	F	F	M	M	M	F	F 4*	3
8	"	M 1	F	M 3	F 1	M	M	M	M	F	M	3
9	"	F 1	M	F 2	M	M	M	M	F	M	F	2
10	"	F 1	M 1	F	M	F	M	F	M	F	F	2
TOTAL INFECTED		8	3	4	2	1	0	0	1	0	1	20
ESTABLISHED INFECTIONS		0	0	4	1	0	0	0	1	0	1	7

3.21 To test the effect of the age of the flies at the time of the infective feed on the development of trypanosomes in tsetse flies.

Procedure The *G.morsitans* used for these experiments were fed on a pair of clean rabbits about 36 hours after emergence. Two days later they were offered another feed. They were then starved for three days after which they were offered the third feed, which was the infective feed. Each fly was thus approximately 6½ days old, at the time of the infective meal. Tests were conducted with *T.congolense*,B and *T.brucei*,B.

Results These are shown in Tables 26 and 27. Twenty flies were infected with *T.congolense* and 3 of these infections were established. These infection rates compare favourably with the standard. The two-by-two table with Yates correction was applied to test the significance of the difference in the established infections of this treatment and the standard. The values obtained, $X^2 = 2.900$, $P < 0.10$ show that the difference is not significant. This indicates that the age of the fly at the time it ingests trypanosomes of *T.congolense* does not affect the subsequent development of the trypanosomes in the fly.

In case of *T.brucei* (Table 27) there was only one established infection out of 12 infected flies. This proportion of established infection is significantly lower than the standard. $X^2 = 6.186$, $P < 0.02$.

Table 26 The results of the dissection of 100 G.morsitans which had been fed twice on clean rabbits before infecting them with T.congolense,B.

GROUP NO.	STABILATE NO.	DAYS 3	4	5	6	7	8	9	10	11	12	No. Infected
1	63-66	F 1	M 1	M	F	M	M 1	M	M	F	M	3
2	"	M 1	M	M	M	F	M	M	M	M	M	1
3	"	M 1	M 1	M	M 2	M	M	M	M	M	M	3
4	"	M	M	M 1	F 1	M 1	M	M	M	M	F	3
5	"	M 1	M	M	M	M	M	M 4	M	F	M	2
6	"	M 1	M 1	M 1	F	F 1	M	M	F 2	F	M	5
7	"	M	M	M	M	F	M	M	F	F	M	0
8	"	M 1	F	M	M	M	M	M	M	M	M	1
9	67	M	M	M	M	F	M	M	M	M	M	0
10	67	M 1	M 1	M	M	M	M	M	M	M	M	2
TOTAL INFECTED		7	4	2	2	2	1	1	1	0	0	20
ESTABLISHED INFECTIONS		0	0	0	1	0	0	1	1	0	0	3

M = Male

F = Female

1,2,3,4 = denotes category of infection.

Table 27 The results of the dissection of 100 G.morsitans which had been fed twice on clean rabbits before infecting them with T.brucei,B.

GROUP NO.	STABILATE NO.	DAYS 3	4	5	6	7	8	9	10	11	12	No. Infected
1	48-52	M 1	F	M	M	M	M	F	M	M	F	1
2	"	M	M	F	M	M	M	M	M	F	M	0
3	"	M 1	M	M	M	M	M	M	M	M	M	1
4	"	M	F 1	M	M	M	F	M	M	M	M	1
5	"	M	M	F	M	M	M 2	M	M	M	M	1
6	"	F	M 1	F	M	M	F	F	F	M	M	1
7	"	M 1	F	M	M	M	F	F	M	M	F	1
8	"	M 1	M 1	M 1	M	M	M	M	M	M	M	3
9	"	M	F	M	F	M	M	M	F	M	M	0
10	"	M 1	M	M 1	F 1	M	F	M	M	M	M	3
TOTAL INFECTED		5	3	2	1	0	1	0	0	0	0	12
ESTABLISHED INFECTIONS		0	0	0	0	0	1	0	0	0	0	1

M = Male

F = Female

1,2,3,4 = denotes category of infection.

3.22 To test the effect of incubating tsetse pupae at 31°C on the infection rate of trypanosomes in the flies.

Procedure Each day the newly deposited pupae from the colony of flies were collected and transfered to an incubator maintained at 31°C and 74% relative humidity. Flies which emerged from these pupae were given the infective feed and then kept in the fly room throughout the experiment.

Records were kept of the number of pupae put in the incubator, the number of flies that emerged, and the time taken for eclosion.

Results It is seen from Table 28 that the emergence rate of the pupae kept at 31°C was about 90% and the time required for their development approximately 23 days. The emergence rate of pupae incubated at the lower temperature of the fly room (26°C) is about 88% and the time required for their development 28 days (Dr.Saunders, personal communications).

27 out of 100 G.morsitans were infected with T.congolense (Table 29) and of these 9 had established infections. Using the two-by-two table with Yates correction to test the significance of the differences in infection rates of this treatment and the standard the values of X^2 are 0.433, $P < .70$ and 0.289, $P < 0.70$ for infected flies and established infections respectively. It appears therefore that incubation of pupae at 31°C had no effect on the development of T.congolense in the flies.

The figures for T.brucei were 22 infected flies, eleven of which had established infections (Table 30). Applying the test of significance in the usual way X^2 is 0.289, $P < 0.70$ and X^2 is 1.087, $P < 0.30$ for infected flies and established infections respectively. None of these values is significant.

Table 28 Record of Eclosions of the pupae incubated at 31 °C.

+DAY PUPAE LAID	++DAY OF ECLOSION	PUPAL DEVELOPMENTAL PERIOD	NO. OF PUPAE COLLECTED	NO. OF FLIES WHICH EMERGED	EMERGENCE RATE IN %
103	126	23 DAYS	33	29	90
110	133	23 "	31	27	90
117	140	23 "	58	51	90
124	147	23 "	31	28	90
131	154	23 "	40	39	90
138	161	24 "	32	28	90
145	168	23 "	38	29	80
152	176	23 "	42	38	90
166	189	23 "	39	37	90
173	197	24 "	43	40	90

\+ = Record of pupal-deposition is kept in the order of the days in a year. Thus January 1 is Day 001 and February 1 is Day 032 *et seq.*

++ = Eclosion usually lasted 3 days. The last day is recorded.

Table 29 The results of the dissection of 100 G.morsitans which, after emerging from pupae incubated at 31°C, were infected with T.congolense,B.

GROUP NO.	STABILATE NO.	DAYS 3	4	5	6	7	8	9	10	11	12	No. Infected
1	39	M 1	M 1	M	M	M	M	M 4	M	M	F	3
2	40	F 1	M	M 1	M	M	M	F	M	M	M D 4	3
3	40	F 1	F	M	F	F 1	M D	F 1	F	M	F	3
4	40	M 1	M 1	M	M	M 2	M	F	F	M	F 1	4
5	40	F 1	M 1	M 1	F	M	M	M	M	F	F	3
6	41,42	M 1	M 2	M	F	M	F	M 1	M	F	M	3
7	48	M 1	F	F	F	M	F	F	F	F	F	1
8	49,50	F 1	M	M	M 1	M	M	F	F	F	M	2
9	51	M	F	M 4	F	F	F	M	F	F 3	F	2
10	52,53	F	F 2	F 4	M	M 4	M	M	M	M	M	3
TOTAL INFECTED		8	5	4	1	3	0	3	0	1	2	27
ESTABLISHED INFECTION		0	2	2	0	2	0	1	0	1	1	9

M = Male
F = Female
D = Fly dead on day due for dissection
1,2,3,4 = denotes category of infection
4* = Trypanosomes found in the Labrum-epipharynx

This key will be used for Table 30 as well.

Table 30 The results of the dissection of 100 _G.morsitans_ which, after emerging from pupae kept at 31°C, were infected with _T.brucei_, B.

GROUP NO.	STABILATE NO.	DAYS 3	4	5	6	7	8	9	10	11	12	No. Infected
1	32,33	F 3	F	F	M	M	M	M	F	F 4*	F	2
2	32,33	M	F 2	F 2	F	F	F	F	M	F	F	2
3	32,33	F	M	M 2	M	F	M	F	F	F	F	1
4	34	F	M	F	F	F	F	F	M	F	F 3	1
5	35	M	M 1	F	F 2	F 2	M	M	F	F	F	3
6	35	M 1	M	M	M	M	M	F	F	F 3	F	2
7	36,37	F 1	F 1	F 1	M	F	F 3	F	F	F	F	4
8	36,37	F 1	M	F	M	M	F 1	F	F	F	F	2
9	38,39	F 1	F 1	F 1	F	F	F	F	F	M	F	3
10	38,39	M 1	M	M	M	F	M	F	M 3	M	F	2
TOTAL INFECTED		6	4	4	1	1	2	0	1	2	1	22
ESTABLISHED INFECTIONS		1	1	2	1	1	1	0	1	2	1	11

3.23 To test the effect of temperature on the development of trypanosomes in tsetse flies.

Procedure This test was conducted with T.congolense,B. Whenever at least 9 flies were available, they were fed on stabilates in the usual way and then divided into 3 batches. One batch was maintained in the fly room, and served as controls for the experiment. The other two batches were placed in incubators maintained at 20°C and at 36°C.

Results As expected, 19 flies were infected in the control group (Table 31) and 6 had established infections. Of the 100 flies kept at 20°C 24 were infected but only 2 had established infections (Table 32). When the significance of the difference between the number of established infections at this temperature and that of the control was tested by applying the two-by-two table with Yates correction the value of X^2 is 4.687, $P < 0.05$. This indicates that the difference is slightly significant.

25 of the 100 G.morsitans kept at 36°C died during the experiment. No useful information could be obtained from this part of the experiment.

Table 31 The results of the dissection of 100 G.morsitans which were infected with T.congolense,B and kept in the fly room (26°C).

GROUP NO.	STABILATE NO.	DAYS 3	4	5	6	7	8	9	10	11	12	No. Infected
1	41,42	M 1	M 1	M	M	M	M	M	M 4	M	F	3
2	49,50	F 1	M	F	M	M	F	F 4	F	F	M	2
3	54,55	M 1	M	M 1	M	M	M	M	M	M	M	2
4	56	M	F 1	M	F	F	F 2	M	F	F	M	2
5	57-61	M 1	M	M	F	M	M	M	F	M 1	M	2
6	"	M	M	M	M 2	M	M 1	M	M	M	M	2
7	"	F	F	F	M	F 1	M	M	F	F	F	1
8	"	F	M	M	F	M	F	F	F 2	F	F	1
9	"	F 1	M	F 1	M	M	M	M	F	F	F 4	3
10	62	M	M	M	M	M	M	M 1	M	M	M	1
TOTAL INFECTED		5	2	2	1	1	2	2	2	1	1	19
ESTABLISHED INFECTIONS		0	0	0	1	0	1	1	2	0	1	6

M = Male

F = Female

1,2,3,4 = denotes category of infection.

This key will be used for Table 32 as well.

<u>Table 32</u> The results of the dissection of 100 <u>G.morsitans</u> which were infected with <u>T.congolense</u>,B and kept in an incubator at 20°C.

GROUP NO.	STABILATE NO.	DAYS 3	4	5	6	7	8	9	10	11	12	No. Infected
1	41,42	M	M 1	M 1	M 1	M	F	F	F 1	M	M	4
2	49,50	M	M	M	F	F	M	F	M	F	M	0
3	54,55	F	F 1	M	M	F	M	M	M	F	F	1
4	54,55	M 1	F	F 1	F 1	F	F	M	F	F	M	3
5	56	M	M 1	M 1	M	M	M	M	M	M	M	2
6	57-61	F 1	M	M	M	M 1	M	M	M	F	M	2
7	"	F	M 1	F 1	M 1	F	M	F 2	F	F	F	4
8	"	F	M 1	M	F 1	F	M	F	F 3	F	F	3
9	"	M 1	M	F 1	F	F	M	F	F	F	F	2
10	62	M 1	M 1	M 1	M	M	M	M	M	M	M	3
TOTAL INFECTED		4	6	6	4	1	0	1	2	0	0	24
ESTABLISHED INFECTIONS		0	0	0	0	0	0	1	1	0	0	2

3.24 To test the susceptibility of G.morsitans and G.austeni to infection with trypanosomes when the flies were maintained on rabbits.

When the flies which had been infected with T.brucei,A and T.congolense,A were maintained on mice (Experiment 3.12), the proportion of flies infected was slightly less than when they were maintained on rabbits. (Experiment 3.11); but the difference was not significant. Secondly, results of tests conducted with T.brucei,A and T.congolense,A in which both G.morsitans and G.austeni were used, showed that the infection rates were higher in the former than the latter. It is now to be seen whether similar results would be obtained with the cyclical populations of these trypanosomes.

Procedure T.brucei,B was used to conduct this test. After the infective feed, the flies were maintained on a pair of clean rabbits and dissected as usual from days 3 to 12.

Results These are summarised in Table 33. Of the 15 G.morsitans infected, 3 had established infections; and of the 9 G.austeni infected, 1 had established infection. It is evident from the values of X^2 calculated by applying the two-by-two table with Yates correction that the infection rate in G.morsitans was not influenced by maintaining them on rabbits instead of mice and that the G.austeni is as susceptible to infection with T.brucei as G.morsitans.

Table 33 Summary of results of the dissection of 100 G.morsitans and 100 G.austeni which were infected with T.brucei,B and maintained on rabbits.

TREATMENT	TOTAL FLIES	TOTAL INFECTED	FLIES WITH ESTABLISHED INFECTIONS	VALUES OF X^2, P	REMARK
G.morsitans with T.brucei	100 (100)	15 (17)	3 (5)	0.130 P<0.80	Not significant
G.austeni with T.brucei	100	9	1	+0.255 P<0.70	Not significant

\+ = The number of established infections in G.austeni in this experiment is compared with that of G.morsitans in the same experiment.

() = shows the corresponding figures for the standard.

3.25 The Infection rates of male and female flies.

At this stage of the work, it was necessary to take the infection rates of male and female flies into consideration. The supply of flies was irregular and variable. Because the majority of the females were required to maintain the breeding colony, there was always a greater supply of males than female flies.

From all the experiments conducted with T.congolense,B and T.brucei,B, the number of male and female flies infected and with established infections were counted and are recorded in Tables 34 and 35 respectively. The proportions of infected males and females were then calculated and are given in Table 36.

As seen in Table 36, the female flies have higher percentages of established infections than males for both species of trypanosomes. When the significance of these differences in the number of established infections was tested by applying the two-by-two table, the calculated value of X^2 for T.congolense is 4.490, P 0.05 and for T.brucei, 4.169, P 0.05. These differences are significant. The proportion of infected flies was the same for both sexes with T.congolense, but with T.brucei the females have a higher proportion than the male flies. This difference is significant as the value of X^2, calculated in the usual way, is 6.350, P 0.02.

All these indicated that the female flies are more susceptible to infections with trypanosomes than males.

Table 34 Susceptibility of male and female G.morsitans to infection with T.congolense, B.

Expt. No.	MALE FLIES				FEMALE FLIES			
	Total Flies Used	Total Infected	Total Established	Total Not Infected	Total Flies Used	Total Infected	Total Established	Total Not Infected
3.17a	60	11	2	49	40	11	4	29
3.17b	82	4	4	78	68	5	5	63
3.19	39	14	6	25	31	3	3	28
3.20	38	7	2	31	61	13	5	48
3.21	84	16	2	68	16	4	1	12
3.22	58	17	6	41	42	10	3	32
3.23a	66	10	2	56	34	9	4	25
3.23b	58	14	0	44	42	10	2	32
TOTALS	485	93	24	392	334	65	27	269

3.17a = Standard treatment (3-12 days)

3.17b = Standard treatment (13-22 days)

3.18a = Standard treatment (3-12 days)

3.18b = Standard treatment (13-22 days)

3.19 = Retention of infectivity during the period of infective feed

3.20 = 10 fold concentration of trypanosomes in infective feed

3.21 = Flies fed at least once before their infective feed

3.22 = Incubation of tsetse pupae at 31°C

3.23a = Effect of temperature; flies kept in the fly room

3.23b = Effect of temperature; flies kept at 20°C

3.24 = Susceptibility of G.morsitans when maintained on rabbits

Table 35 Susceptibility of male and female *G.morsitans* to infection with *T.brucei*,B.

Expt. No.	MALE FLIES				FEMALE FLIES			
	Total Flies Used	Total Infected	Total Estab-lished	Total Not Infected	Total Flies Used	Total Infected	Total Estab-lished	Total Not Infected
3.18a	70	10	3	60	30	7	2	23
3.18b	104	8	7	96	46	1	1	45
3.19	43	4	1	39	27	5	1	22
3.20	48	6	1	42	52	14	6	38
3.21	78	10	1	68	22	2	0	20
3.22	34	5	2	29	66	17	9	49
3.24	50	8	2	41	50	7	1	43
TOTALS	427	51	17	376	293	53	20	240

3.17a = Standard treatment (3-12 days)

3.17b = Standard treatment (13-22 days)

3.18a = Standard treatment (3-12 days)

3.18b = Standard treatment (13-22 days)

3.19 = Retention of infectivity during the period of infective feed

3.20 = 10 fold concentration of trypanosomes in infective feed

3.21 = Flies fed at least once before their infective feed

3.22 = Incubation of tsetse pupae at 31°C

3.23a = Effect of temperature; flies kept in the fly room

3.23b = Effect of temperature; flies kept at 20°C

3.24 = Susceptibility of *G.morsitans* when maintained on rabbits

Table 36 Proportions of male and female G.morsitans infected with trypanosomes, compiled from data in Tables 34 and 35.

SPECIES AND DESIGNATION OF TRYPANOSOME	SEX	TOTAL USED	FLIES INFECTED		ESTABLISHED INFECTIONS	
			TOTAL	PERCENTAGE	TOTAL	PERCENTAGE
T.congolense,B	MALE	485	93	19.2	24	5
	FEMALE	334	65	19.5	27	8
T.brucei,B	MALE	427	51	12	17	4
	FEMALE	293	53	18	20	6.9

3.26 The establishment of a model for the study of the development cycle of trypanosomes in tsetse flies.

Up till now most of the experiments had terminated on the 12th day post infection. The position of the trypanosomes in the alimentary canal of an infected fly had been shown to be important. Consequently, 4 types of infection termed "categories" were recognized. Further, the results showed the significance of the change from Category 1 to Category 2 infection. As a result, all infections of Categories 2 to 4 were called "established infections".

In order to complete the development cycle the trypanosomes must not only invade the hypopharynx (T.congolense) or salivary glands (T.brucei), they must also be transmissible to a mammalian host when the fly feeds. Such an infection in the fly is then said to be

mature. In other words, the development cycle of the two species of trypanosome being used in this work is completed when their infection in the flies is mature. It is necessary to extend the observations to find out whether all the established infections will become mature infections.

The results of experiments with T.congolense,B and T.brucei,B indicated that flies would have to be kept longer than 12 days for the study of the complete development. Although it had been shown in Experiment 3.17 that T.congolense,B could invade the hypopharynx as early as the 7th day, a fly might not feed between the 7th and the 12th day. If such a fly were to be dissected on the 12th day post infection, it would not be known whether the fly would infect mice, if it had been kept longer before being killed.

From an examination of the accumulated results of Experiments 3.17 to 3.24 (Appendix 5a,b) the histograms in Fig. 14 were constructed. In these histograms the unshaded blocks represent the number of infected flies. For T.congolense, we see that 39 out of the 67 flies or 58% of the flies examined on day 3 were infected. The number of the infected flies decreases with time until about the 7th day when it begins to level off. By the 10th day the numbers of infected flies and established infections are almost the same. Flies infected with T.brucei show a similar behaviour. 35 flies or 52% of the 67 flies examined on day 3 were infected. This proportion decreases with time and levels off at about the 6th day. Again, the numbers of infected flies and established infections are almost the same as from the 10th day. The shaded blocks represent flies with established infections. We see that for T.congolense

the number of flies with established infections is relatively constant from about the 4th day and for T.brucei from about the 6th day.

It is therefore obvious that the trypanosomes survive for a few days in about 50% of the flies; but the proportion of flies which provided a suitable environment for further development of the trypanosomes is much less. Since we are now interested in the proportion of established infections that will support mature infections, it is no more necessary to dissect the flies daily. For T.congolense, days 3, 5, 7, 14 and 21 after the infective feed were chosen as the days for dissection of the experimental flies. Since this population of T.congolense invades the hypopharynx around the 7th day, any fly with mature infection would have ample opportunity to feed between the 7th and 21st day. Since, as shown in the histogram, the number of established infections becomes relatively constant as from the 4th day post infection, there should be no significant difference between the number of established infections on day 5 and mature infections on day 21 if all established infections will support mature infections.

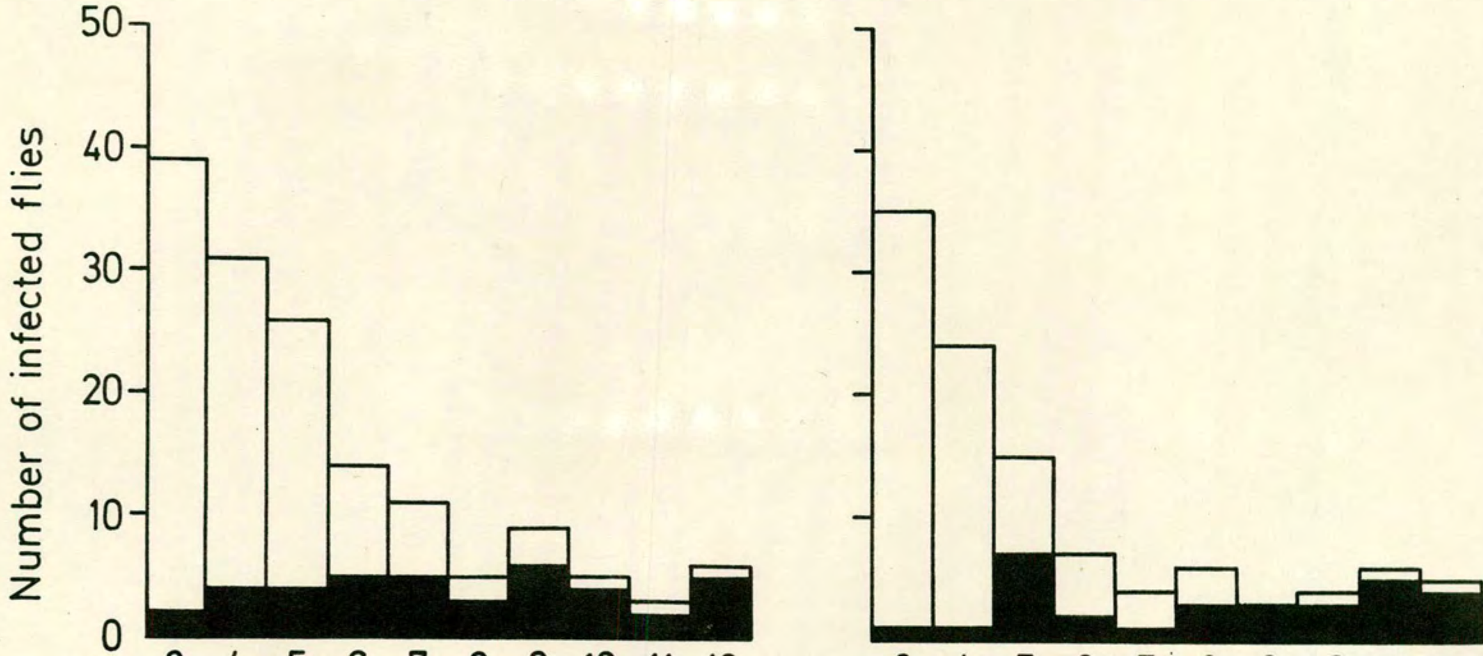
Because the cycle takes longer to be completed in flies infected with T.brucei, the experiments with this species will have to last longer. Hence, flies are to be dissected on days 3, 5, 10, 20 and 30 after the infective feed. Provided that all established infections go on to mature, there should be no significant difference between the number of established infections on day 5 and mature infections on day 30.

The number of flies to be used in each experiment must also be taken into consideration. The results so far had demonstrated the low rate

Fig.14 The variations in the number of infected flies and established infections among the population of flies examined 3-12 days after the infective feed. Histograms constructed from figures in Appendix 5a and 5b.

GLOSSINA MORSITANS

TRYPANOSOMA CONGOLENSE. *TRYPANOSOMA BRUCEI.*



of infection in flies. To make valid conclusions therefore, larger numbers of flies would have to be devoted to each experiment. It was decided that at least 50 flies should be dissected for each of the chosen days.

3.27 To find out whether flies with established infections of *T.brucei* will ultimately become infective to mice.

Procedure The test was conducted with *T.brucei*, B. After flies had taken their infective feed, they were divided into five batches. The flies of the different batches were dissected on days 3, 5, 10, 20 and 30 after the infective feed.

The flies for the 3 and 5 day dissections were fed on a group of 12 mice. Each fly of the other batches was maintained on a single clean mouse. The mice were inspected thrice a week and any infected one was replaced. Commencing from the 10th day post infection, the experimental flies were probed daily on microscope slides warmed to 37°C (Methods 2.19). Metacyclic trypanosomes were seen in the probe of one fly on day 23 after its infective feed. This fly was isolated and kept for 46 days before being dissected. It was fed on six occasions on a clean mouse and the incubation period of the infection in the mice was determined.

Results In Table 37 we see that the proportion of flies with established infections on days 5, 10, 20 and 30 appeared to be similar. When the two-by-two table with Yates correction was applied to the highest (12%) and lowest (8%) proportion, the difference was found not

to be significant, the value for X^2 being 3.781, $P < 0.10$. All the flies which were infected on days 10, 20 and 30 possessed established infections. Only 1 of the 12 and 3 of the 8 established infections on days 20 and 30 respectively were mature and infected mice. For this species of trypanosome therefore, there appeared to be no correlation between the number of established and mature infections. When the four flies with mature infections were dissected, 3 of them had scanty trypanosomes in the salivary glands as well as abundant trypanosomes in the midgut and proventriculus. The remaining one had trypanosomes only in the midgut and proventriculus.

The feeding records of flies which infected mice in this experiment are shown in Tables 38 and 39. One fly, found positive by probe test on day 23 was kept for 46 days. During this time it fed on 6 mice, and as shown in Table 38, the average prepatent period was 6 days. The feeding record of the other 3 flies which infected mice is given in Table 39. By subtracting 6 from the day on which each mouse became parasitaemic, the probable day on which each fly infected its mouse was calculated. In fact all the flies did take a feed on that or on the adjacent day. It is also evident that the duration of the development cycle in the fly was between 20 and 30 days.

Since only 3 of the 8 flies with established infections dissected on day 30 had infected mice, it was possible that if these flies had been kept for longer, their infections would eventually become mature. A further batch of 100 G.morsitans was infected with T.brucei,B and kept under observation for 50 days. Each fly fed on a single mouse.

As in the first test, 6 of the 100 G.morsitans possessed established infections but only 2 of these had mature infections. As judged by the feeding records one of the 2 flies with a mature infection

probably infected its mouse on the 24th day and the other on the 27th day post infection. When they were killed and dissected on the 30th day, both of these flies had scanty trypanosomes in the salivary glands as well as abundant trypanosomes in the midgut and proventriculus. The other 4 flies, which were dissected on day 50, had abundant trypanosomes in the midgut and proventriculus, but their salivary glands were not invaded. Neither did they infect mice.

It was concluded that, under standard conditions, not all flies infected with T.brucei, B will develop mature infections. In flies that can support mature infections, the cycle of development will be completed within 30 days.

Table 37 The results of the dissection of 350 G.morsitans which were infected with T.brucei, B and killed 3, 5, 10, 20 and 30 days later.

Day Dissected	Total Flies	Flies Infected		Flies with established infection		Flies with mature infection	
		Total	Percent	Total	Percent	Total	Percent
3	50	25	50	-	-	-	-
5	50	7	14	5	10	-	-
10	50	4	8	4	8	-	-
20	100	12	12	12	12	1	1
30	100	8	8	8	8	3	3

Table 38 Determination of the pre-patent period of T.brucei, B in mice after fly transmission.

+ Day Fly Fed on mouse	Day Mouse Found Parasitaemic	Incubation Period in days
300	305	5
304	311	6
307	313	6
311	317	6
315	321	6
320	326	6

Dissection Result: Scanty Trypanosomes in salivary glands, swarming trypanosomes in Midgut and Proventriculus.

+ = The days are numbered as they occur in the year, January 1 being day 001

Table 39 The feeding record of the 3 flies which developed mature infections with T.brucei, B.

Fly No.	Feeding Record	Day Dissected	Day Mouse found Parasitaemic	Dissection Result in Category
1	3, 6, 9, 14, 16, +(19)	20	26	++4
2	4, 10, 16, 18, 22 (29)	30	37	3
3	2, 7, 9, 16, 21 (26)	30	31	++4

+ = Figures in brackets represent the probable day of infective feed.

++ = Scanty trypanosomes in the salivary glands.

A series of tests were now carried out with T.brucei, B in which the treatment was modified to see if the development cycle of the trypanosomes or the susceptibility of the flies could be altered.

The experimental procedure was as described in Experiment 3.27. Fifty flies were killed and dissected on days 3, 5, 10, 20 and 30 after their infective feed. The number of infected flies, and the number of flies with established and with mature infections were recorded. The proportions of flies with infections in these different categories were compared with the proportions found when flies received the standard treatment. Whenever the difference seemed large, its significance was tested by calculating the value for chi-square by means of a two-by-two table. If the total numbers were less than 200, Yates correction was used.

3.28 To test the effect of temperature on the development of T.brucei, B in G.morsitans.

Procedure Flies, which had taken their infective feed were divided into two groups; the flies of one group were kept in an incubator at 31°C over a solution of potassium tartrate to give a relative humidity of 74%, while those of the other group were kept in an incubator at 20°C over the same solution to give a relative humidity of 75.0%. "2lbs. sweet jars", each 16 cm. high and 10 cm. wide were used to accommodate the flies in the incubators. Two small petri-dishes, each of diameter 5 cm. and height 2 cm. were filled with the saturated solution of potassium tartrate and lowered gently into the base of each jar. A perforated wire gauze was then placed on the pair of petri-dishes inside the jar. A maximum of 10 flies were placed

in each jar and the polystrene tubes in which they were kept were bounded together by an elastic band. Inside the jar, the tubes rested on the perforated wire gauze.

Results The results of the experiment of keeping flies at 31°C after their infective feed are shown in Table 40. All the flies in which trypanosomes were present on days 10, 20 and 30 had established infections. The proportions of flies with established infections on days 5, 10, 20 and 30 appeared to be similar, X^2 for the highest and lowest proportions on these days being 0.408, $P < 0.70$. 11 of the 12 established infections on days 20 and 30 were mature. From the feeding record of the flies, the average time required for completion of the development cycle was found to be 12 days. Hence, the duration of the development cycle was shorter at this temperature than at 26°C. Only 2 of the 11 flies with mature infections had trypanosomes in the salivary glands as well as in the other parts of the alimentary canal when they were dissected. Two flies had trypanosomes in the midgut, proventriculus and labrum-epipharynx, the remaining 7 flies had had trypanosomes only in the midgut and proventriculus. One of the flies with mature infections deposited metacyclics in its probe on the 12th day post infection. This fly was kept to see how long infected flies could survive at 31°C. It died on day 36.

In flies kept at 20°C (Table 41) there was no correlation between the number of infected flies and flies with established infections until the 20th day, suggesting that the establishment of an infection is prolonged at this temperature. Consequently, there was a significant difference between the proportions of established infections on days 5 and 20; $X^2 = 5.500$, $P < 0.02$. There was no mature infection

even on day 30 and the single fly with an established infection on this day contained trypanosomes in the midgut and proventriculus only.

A comparison of the results of this treatment with the standard will be presented jointly with others in Table 45.

3.29 To test the effect of incubating tsetse pupae at 31°C on the infection rate of T.brucei,B in G.morsitans.

Procedure Each day the newly deposited pupae from the colony of flies were collected and transferred into a polystrene screw-top container containing sand. The lid of the container was partly replaced by terylene netting. The container was then placed in the 2 lb. sweet jar, described in Experiment 3.28, in an incubator at 31°C. The time required for eclosion and the emergence rate of pupae incubated at this temperature have already been given in Experiment 3.22. On hatching, adult flies were transferred from the container to their individual tubes after chilling them at 4°C until they were immobile. They were kept in the fly room after their infective feed.

Results These are shown in Table 42. The difference in the numbers of flies found with established infections on days 5, 10, 20 and 30 was not significant, $X^2 = 2.369$ $P < 0.20$. All except 1 of the 10 flies found infected on days 20 and 30 infected mice. The feeding record of the flies showed that the duration of the development cycle was 12-15 days. Although all the 9 flies with mature infections possessed trypanosomes in the midgut and proventriculus, only 2 of them had

trypanosomes in the labrum epipharynx and in only 2 were a few trypanosomes seen in the salivary glands. A comparison of these results with those of the standard will be presented among others in Table 45.

3.30 To test the effect of the age of the flies at the time of the infective feed on the development of T.brucei,B in G.morsitans.

Procedure The details had been described in Experiment 3.21. All flies used in the experiment were approximately 6½ days old at the time of the infective feed, and they had all fed at least once on a clean rabbit, the infective feed being the 2nd or 3rd feed.

Results Table 43 shows the results of this experiment. The day 10 results, in which there was no established infection, could be attributed to a chance variation in the population of flies dissected on this day, or to some fault in the technique. Apart from this, there was no significant difference between the proportions of established infections on days 5, 20 and 30, $X^2 = 2.458$, $P < 0.20$. The single fly with a mature infection had numerous trypanosomes in both salivary glands. It was observed from the feeding record that the probable day on which this fly first infected a mouse was day 23 post infection.

3.31 To test the susceptibility of G.austeni to infection with T.brucei,B.

Procedure The deviation from the standard was that G.austeni was used instead of G.morsitans

Results These are shown in Table 44. As observed in Experiment 3.24, the infection rate in G.austeni was generally low. A comparison of these results with those of G.morsitans will be shown in Table 45.

To determine whether a treatment had an effect, results must be compared with the standard. The results of Experiments 3.28 - 3.31 are set out together in Tables 45a and 45b so that the difference between the modified and the standard treatments may be readily seen.

The proportion of flies which had trypanosomes in the gut when killed on the 3rd day after infection was significantly increased when the flies were maintained at 31°C and decreased when the flies were kept at 20°C. Also, the number of G.austeni found infected on day 3 was significantly less than the number of G.morsitans. It was only in the flies emerged from pupae which had been incubated at 31°C, that some infections had already become established by day 3.

Of the flies dissected at day 5, the flies whose pupae had been incubated at 31°C showed a difference of borderline significance while the proportion of established infections in the flies kept at 20°C was significantly lower than the standard. Also the number of G.austeni with established infections on the 5th day was significantly lower than that of G.morsitans.

The mature infections were compared on the 20th and 30th days. In order to obtain more numbers for the calculations for X^2, the results of the mature infections on days 20 and 30 were pooled together. As shown in Table 45b, the numbers of flies with established

infections among the flies kept at 31°C and flies whose pupae were incubated at 31°C which supported mature infections on days 20 and 30 were significantly higher than those of the standard. Flies kept at 20°C also had a ~~significantly~~ lower proportion of mature infections than the standard; and the number of mature infections in *G.austeni* was ~~significantly~~ lower than in *G.morsitans*.

Since it had been shown that there was no significant difference in proportions of flies with established infections on days 5, 10, 20 and 30, the groups of flies dissected in each experiment on these different days were regarded as samples of the same population. An addition of all the established infections of these days in each of Experiments 3.28 - 3.31 was made and compared with that of the standard. This is shown in Table 46. Flies kept at 20°C as well as flies whose infective feed was the 2nd or 3rd feed had totals which were significantly lower than that of the standard. This is not unexpected of flies kept at 20°C, since, as previously observed, there appeared to be a prolongation of the establishment of infection at this temperature. The total established infections of *G.austeni* were also lower than those of *G.morsitans*. The other treatments had no influence on the proportions of flies that developed established infections.

Table 40 The number and type of infections in G.morsitans which were maintained at 31°C after being infected with T.brucei, B.

Day Dissected	Total Flies	Infected Flies		Established Infections		Mature Infections	
		Total	Percent	Total	Percent	Total	Percent
3	50	39	78	-	-	-	-
5	50	10	20	7	14	-	-
10	50	4	8	4	8	-	-
20	50	5	10	5	10	4	8
30	50	7	14	7	14	7	14

Table 41 The number and type of infections in G.morsitans which were maintained at 20°C after being infected with T.brucei, B.

Day Dissected	Total Flies	Infected Flies		Established Infections		Mature Infections	
		Total	Percent	Total	Percent	Total	Percent
3	50	6	12	-	-	-	-
5	50	3	6	-	-	-	-
10	50	6	12	3	6	-	-
20	50	3	6	3	6	-	-
30	50	1	2	1	2	-	-

Table 42 The number and type of infections in G.morsitans whose pupae had been incubated at 31°C before the adults were infected with T.brucei,B.

Days Dissected	Total Flies	Total Infected		Established Infections		Mature Infections	
		Total	Percent	Total	Percent	Total	Percent
3	50	28	56	5	10	-	-
5	50	15	30	8	16	-	-
10	50	4	8	4	8	-	-
20	50	4	8	4	8	4	8
30	50	6	12	6	12	5	10

Table 43 The number and type of infections in G.morsitans which had been fed at least once on rabbits before infecting them with T.brucei,B.

Day Dissected	Total Flies	Infected Flies		Established Infections		Mature Infections	
		Total	Percent	Total	Percent	Total	Percent
3	50	20	40	-	-	-	-
5	50	8	16	2	4	-	-
10	50	1	2	-	-	-	-
20	50	4	8	4	8	-	-
30	50	5	10	5	10	1	2

Table 44 The number and type of infections in G.austeni which were infected with T.brucei, B.

Day Dissected	Total Flies	Flies Infected Total	Flies Infected Percent	Established Infections Total	Established Infections Percent	Mature Infections Total	Mature Infections Percent
3	50	12	24	-	-	-	-
5	50	8	16	-	-	-	-
10	50	1	2	1	2	-	-
20	50	1	2	1	2	-	-
30	50	0	0	0	0	0	0

Table 45a,b Comparison of the results of the standard treatment with those of the modified treatments (Experiments 3.28-3.31).

Experiment Numbers	TREATMENTS	Total Flies	Flies Infected Total	Flies Infected Percent	X^2 P	Established Infections Total	Established Infections Percent	++X^2, P
				DISSECTION AT 3rd DAY				
3.18, 3.19	Standard	67	34	51		-	-	
3.28	Flies at 31°C	50	36	+72	4.533 $P < 0.05$	-	-	
3.28	Flies at 20°C	50	6	+12	20.865 $P < 0.01$			
3.29 3.22	Pupae incubated at 31°C, flies kept at 26°C	60	34	57		6	+10	23.264 $P < 0.01$
3.21 3.30	Flies fed at least once before infective feed	60	25	42				
3.31	*G.austeni*	50	12	+24	9.742 $P < 0.01$			
				DISSECTION AT 5th DAY				
3.18, 3.19 3.27	Standard	67	10	15		6	9	
3.28	Flies at 31°C	50	10	20		7	+14	0.315 $P < 0.70$
3.28	Flies at 20°C	50	3	+6	3.301 $P < 0.10$	0	0	
3.22 3.29	Pupae incubated at 31°C flies kept at 26°C	60	19	+32	4.129 $P < 0.05$	10	+17	1.080 $P < 0.30$
3.21 3.30	Flies fed at least once before infective feed	60	10	17		2	+3	2.781 $P < 0.10$
3.31	*G. austeni*	50	8	16		0	0	

Table 45a (continued)

Experiment Numbers	TREATMENTS	Total Flies	Flies Infected Total	Percent	X^2P	Established Infections Total	Percent	++X^2P
				DISSECTION AT 10th DAY				
3.18, 3.19, 3.27	Standard	67	5	7		4	6	
3.28	Flies at 31°C	50	5	10		4	8	
3.28	Flies at 20°C	50	6	+12	0.261 $P<0.70$	3	6	
3.22 3.29	Pupae incubated at 31°C Flies kept at 26°C	60	5	8		5	8	
3.21 3.30	Flies fed at least once before infective feed	60	1	+2	3.825 $P<0.05$			
3.31	*G. austeni*	50	1	+2	3.058 $P<0.10$	1	+2	2.287 $P<0.20$

\+ = Test of significance applied.

++ = Values of X^2 and P obtained in each test by comparing with the standard.

+++ = Results of mature infections of 20th and 30th days pooled together.

Table 45b

Expt. No.	Total Flies	FLIES INFECTED Total	Percent	X^2,P	ESTABLISHED INFECTIONS Total	Percent	X^2,P	MATURE INFECTIONS Total	Percent	X^2,P
								DISSECTION AT 20th DAY		
3.18, 3.27	115	13	11		13	11		1	1	
3.28a	50	5	10		5	10		4	8	
3.28b	50	3	+6	1.807 P<0.20	3	+6	1.807 P<0.20	0	0	
3.29	50	4	8		4	8		4	8	
3.30	50	4	8		4	8		0	0	
3.31	50	1	+2	5.175 P<0.05	1	+2	5.175 P<0.05	0	0	
								DISSECTION AT 30th DAY		
3.27	100	8	8		8	8		3	3	
3.28a	50	7	+14	0.750 P<0.50	7	+14	0.750 P<0.50	7	14+++	13.445 P<0.001
3.28b	50	1	+2	3.324 P<0.10	1	+2	3.324 P<0.10	0	0	
3.29	50	6	+12	0.246 P<0.70	6	+12	0.246 P<0.70	5	10	+++ 11.243 P<0.001
3.30	50	5	10		5	10		1	+2	+++ 1.467 P<0.30
3.31	50	0	0		0	0		0	0	

3.18, 3.27 = Standard
3.28a = Flies kept at 31°C
3.28b = Flies kept at 20°C
3.29 = Pupae incubated at 31°C, flies kept at 26°C
3.30 = Flies fed at least once before infective feed
3.31 = G.austeni

Table 46 Total established infections on days 5, 10, 20 and 30 in each of the modified treatments (Experiment 3.28-3.31) compared with those of the standard.

Experiment Numbers	TREATMENTS	Established Infections, Days 5,10,20,30 Total Flies	Total Established	Percent	++X^2,P	Remark
3.18, 3.19 3.27	Standard	349	31	9		
3.28	Flies at 31°C	200	23	11.5		
3.28	Flies at 20°C	200	7	+3.5	7.510 P<0.01	Significant
3.29 3.22	Pupae incubated at 31°C flies kept at 26°C	200	25	11		
3.21, 330	Flies fed at least once before infective feed	220	11	+5	4.219 P<0.05	Margin of Significanc
3.31	G. austeni	200	2	+1	16.912 P<0.01	Significant

\+ = Test of significance applied.

++ = Calculations based on comparison with the standard.

3.32 To test the infectivity to mice of the fly forms of T.brucei,B.

Procedure On each of four different occasions, designated Periods 1, 2, 3 and 4, all the available flies were fed on a suspension of T.brucei,B in defibrinated ox blood at a concentration of 1:10 instead of 1:100. The flies of periods 1 and 2 were divided in each case into three batches and the flies of the different batches were dissected on days 1, 2 and 3 post infection. On each of days 2 and 3, trypanosomes from 8 infected flies among those dissected were pooled together and injected into 2 mice. All the other infected flies on day 3 were used for agglutination test as part of a joint experiment. All flies of period 3 were dissected on day 3 post infection, and the infected ones were used for the agglutination test. The flies of period 4 were dissected on days 3 and 10; the infected flies were again required for agglutination tests.

Results These are summarised in Table 47. It will be seen from the table that the infection rates range from 95-100%, 75-80% and 73-84% on days 1, 2 and 3 respectively. The infection rate is hence highest on day 1 but it has already diminished by day 2. Quantitatively however, it was observed that the number of trypanosomes increased progressively from day 1, when very few trypanosomes were seen in any of the infected flies, through day 2 when appreciable numbers were seen, to day 3 when the trypanosomes were found in very considerable numbers. The mice, into which the fly forms were injected on days 2 and 3 post infection, were discarded after 6 weeks since they had not become infected.

Comparison of these results with those in which the flies received the standard treatment showed that a tenfold increase in the concentration of trypanosomes in the infective feed did affect the proportion of infected flies at day 3. (Table 48). There was no difference in the proportion of infected flies or in the type of infection in flies killed at day 10.

Table 47 Results of the dissection of *G.morsitans* which were infected with stabilates of *T.brucei*, B diluted 1:10 with defibrinated ox blood.

Period	Day Dissected	Total Flies	Flies Infected Total	Flies Infected Percent	Established Infections Total	Established Infections Percent
	1	8	8	100		
1	2	8	6	75		
	3	8	6	75		
	1	20	19	95		
2	2	20	+16	80		
	3	30	+22	73		
3	3	25	22	84		
4	3	50	37	74		
	10	38	3	8	2	5

+ = The trypanosomes from 8 of these flies were injected into 2 mice.

Table 48 Comparison of the infection rates in flies receiving the standard treatment with those of flies which had fed on blood containing ten times more trypanosomes.

Experiment Numbers	TREATMENTS	Day Dissected	Total Flies	Flies Infected Total	Percent	X^2,P	Established Infectio Total	Percent	X^2,P
3.18, 3.19 3.27	Standard	3rd DAY	67	34	51				
3.20 3.32	Tenfold Concentration		123	95	77	12.773 $P<0.01$			
3.18, 3.19, 3.27	Standard	10th DAY	67	5	7		4	6	
3.20 3.32	Tenfold Concentration		48	4	8		3	6	

3.33 To test the infectivity to flies of the culture forms of *T.brucei*.

Procedure Mice were infected with T.brucei, Treu 667. When the parasitaemia was high, they were given to Miss Cunningham who prepared the culture forms by introducing the blood stream forms from the infected mice into hanging-drop cultures of complete alimentary tract of *G.morsitans* pupae, which were older than 21 days. Subcultures of the parasites were prepared every 4 or 5 days. This method of culturing trypanosomes has been described (Cunningham 1971).

Cultures harvested on the 3rd and on the 11th day after they were

initiated were used in these experiments. The trypanosomes in the culture medium were diluted with defibrinated ox blood to give the required concentration of about 4×10^6 organisms per ml. The number of trypanosomes was thereby comparable with the numbers in the infective feed of a standard experiment.

After flies had fed on the infective suspension, they were divided into 2 batches. Flies of one batch were dissected on day 3 and the other on day 30 post infection. Each of the flies kept for 30 days was maintained on a single mouse.

Results The results of these experiments are given in Table 49. The difference in the proportion of flies found infected when dissected on the 3rd day after ingestion of culture forms harvested after a shorter or longer interval, was significant, $X^2 = 6.095$, $P < 0.02$. The single fly which infected its mouse had scanty trypanosomes in the salivary glands; it probably infected the mouse on day 25 as judged from the feeding record of the fly.

The comparison of the results of feeding flies on culture forms with those of the standard is shown in Table 50. In flies dissected on the 3rd day, the infection rate of the flies fed on 3rd day culture forms did not differ from that of flies fed on blood forms, but prolonging the period in culture has reduced the infectivity of the trypanosomes to the flies. Few of the flies fed on culture forms had retained their infections when dissected on day 30.

Table 49 The number and type of infections in *G.morsitans* which were infected with culture forms of *T.brucei*, B.

Day Dissected	Designation	Total Flies	Flies Infected Total	Flies Infected Percent	Established Infections Total	Established Infections Percent	Mature Infections Total	Mature Infections Percent
3	3rd Day culture	25	11	44				
	11th Day culture	25	4	16				
30	3rd Day culture	75	1	1	1	1	1	1
	11th Day culture	75	2	3	1	1		

Table 50 Comparison of the infection rates in flies fed on culture forms of *T.brucei*, B with those fed on blood forms.

Expt. No.	Total Flies	INFECTED FLIES Total	Percent	X^2, P	ESTABLISHED INFECTIONS Total	Percent	X^2, P	MATURE INFECTIONS Total	Percent	X^2, P
					DISSECTION AT 3rd DAY					
3.18, 3.19 3.27	67	34	51							
3.33a	25	11	44							
3.33b	25	4	+16	++ a) 10.556 $P < 0.01$ b) 6.095 $P < 0.02$						
					DISSECTION AT 30th DAY					
3.27	100	8	8		8	8		3	3	
3.33a	75	1	+1	5.410 $p=0.02$	1	+1	5.410 $p=0.02$	1	1	
3.33b	75	2	+3	1.945 $P<0.20$	1	+1	5.410 $P=0.02$	0	0	

\+ = Test of significance applied

++a = 11th day culture compared with the standard

b = 11th day culture compared with 3rd day culture

3.18, 3.19, 3.27 = Standard

3.33a = 3rd Day culture

3.33b = 11th Day culture

3.34 To compare the infectivity to flies of two populations of T.brucei which had been passaged once and 11 times in mice.

T.brucei,C is the designation given to T.brucei TREU 1096 which had undergone 10 syringe passages before being stabilated on the 11th passage (Methods 2.10). The cyclical population of this strain which had been passaged once from mouse to mouse is designated T.brucei,D.

Procedure After flies had taken their infective feed on the suspension of T.brucei,C in defibrinated ox blood, they were divided into 5 batches. The flies of the different batches were dissected on days 3, 5, 10, 20 and 30 after the infective feed. The flies scheduled for dissection on days 3 and 5 were fed on a group of 12 mice while each of the other flies was maintained on a single mouse. The experimental flies were probed from the 10th day post-infection. One fly, which deposited metacyclics in its saliva on the 16th day, was isolated and kept for 35 days before being dissected. During this time it was fed on three occasions on a clean mouse and the prepatent period of the infection in the mice determined.

Blood from one of the mice infected by the flies with mature infections of T.brucei,C was passaged into 6 other mice. When the parasitaemia was high, after about 7 days, the mice were killed and about 200 stabilates were made from the pooled blood. These stabilates were designated T.brucei,D and they were used to conduct an experiment as described for T.brucei,C.

Results These are shown in Tables 51-55. With T.brucei,C (Table 51) there was good correlation between the number of established infections and infected flies on days 20 and 30. As has been shown for T.brucei,B, the number of flies with established infections at day 5 was indicative of the proportion of flies which would retain their infections. The single fly, which was isolated by probing fed on days 17, 21 and 26 on a clean mouse. Trypanosomes were seen in the mouse on which the fly had fed on day 17, 9 days later, while those on which it fed on days 21 and 26 became parasitaemic 8 and 9 days later respectively. The average prepatent period of this strain of T.brucei in mice was therefore 9 days. The feeding records of the other 4 flies which infected mice are given in Table 52. By subtracting 9 from the day on which each mouse became parasitaemic, the probable day on which each fly infected its mouse was calculated. All the flies took a feed on that day or on the day adjacent to it. It is evident from the table that the duration of the development cycle in the fly was between 15 and 18 days. Indeed, the three flies with mature infection scheduled for dissection on day 30 had to be killed on days 24, 25 and 26 post-infection when the mice on which they had fed became parasitaemic. This was to prevent congestion of the overpopulated mice room. On dissection, all the 5 flies with mature infection on days 20 and 30 had trypanosomes in the midgut and proventriculus, but in only 2 of these were the salivary glands invaded, one having numerous trypanosomes, the other few.

Table 53 shows the results for flies infected with T.brucei,D. It is evident from the table that this population behaves similarly

as T.brucei,C. Of the 8 established infections on days 20 and 30, only 3 infected mice and none of these possessed trypanosomes in the salivary glands. Their midguts and proventriculi were however infected.

Table 54 gives the comparison of the proportions of infections on the days in which flies were dissected. On all these days the infection rates of T.brucei,C and T.brucei,D were almost similar. Comparing the two cyclical populations, the proportions of infected flies on day 3 and of established infections on day 20 are significantly lower in T.brucei,D than in T.brucei,B. The proportions of established infections which became mature however are similar in both cases.

In Table 55, additions were made of all the established infections of T.brucei,C and T.brucei,D on days 5, 10, 20 and 30 and these were compared between themselves and with T.brucei,B. There was no significant difference between the total established infections of T.brucei,C and T.brucei,D; neither was the difference between those of T.brucei,D and T.brucei,B significant.

Table 51 The number and types of infections in G.morsitans infected with T.brucei,C.

Day Dissected	Total Flies	Total Infected	Established Infections	Mature Infections
3	100	36		
5	100	16	5	
10	100	10	4	
20	100	6	5	2
30	100	3	3	3

Table 52 The feeding record of the 4 flies which developed mature infections with T.brucei,C.

Fly No.	Feeding Record	Day Dissected	Day Mouse Found Parasitaemic
1	3, 6, 10, 15, +(18)	20	26
2	2, 4, 8, 12 (15), 20, 22	24	24
3	2, 5, 7, 9, 13, (17), 21, 24	25	25
4	3, 5, 8, 10, 14 (17), 21, 23	26	26

+ = Figures in brackets represent the probable day of infective feed.

Table 53 The number and types of infections in G.morsitans infected with T.brucei,D.

Day Dissected	Total Flies	Total Infected	Established Infections	Mature Infections
3	100	30		
5	100	25	7	
10	100	8	6	
20	100	4	4	2
30	50	5	4	1

Table 54 Comparison of infection rates in flies which received the standard treatment after infection with T.brucei, B, T.brucei, C and T.brucei, D.

Expt. No.	Total Flies	Flies Infected Total	Flies Infected Percent	Flies Infected X^2, P	Established Infection Total	Established Infection Percent	Established Infection X^2, P	Mature Infections Total	Mature Infections Percent	Mature Infections X^2, P
DISSECTION AT 3rd DAY										
3.18, 3.19 3.27	67	34	51							
3.34a	100	36	36							
3.34b	100	30	30	8.209 $P<0.01$						
DISSECTION AT 5th DAY										
3.18, 3.19, 3.27	67	10	15		6	9				
3.34a	100	16	16		5	5				
3.34b	100	+25	25	1.887 $p<0.20$	7	7				
DISSECTION AT 10th DAY										
3.18, 3.19 3.27	67	5	7		4	6				
3.34a	100	10	10		4	4				
3.34b	100	8	8		6	6				
DISSECTION AT 20th DAY										
3.18, 3.27	115	13	11		13	11		1	1	
3.34a	100	6	6		5	5		2	2	
3.34b	100	4	+4	6.182 $p<0.02$	+4	4	6.182 $p<0.02$	2	2	
DISSECTION AT 30th DAY										
3.27	100	8	8		8	8		3	3	
3.34a	100	3	3		3	* 3	3.165 $p<0.10$	3	3	
3.34b	50	5	10		4	8		1	2	

3.18, 3.19, 3.27 = Standard
3.34a = T.brucei, C
3.34b = T.brucei, D

+ = Test of significance applied
* = T.brucei, C and T.brucei, D compared

Table 55 Total established infections on days 5, 10, 20 and 30 in flies fed on T.brucei,C and T.brucei,D compared with those fed on T.brucei,B.

Experiment Number	Total Flies	Established Infections Days 5,10,20,30 Total Established	Percent	X^2,P	Remark
3.18,3.19 3.27	349	31	9		
3.34a	400	17	+ 4.25	* 2.027 p 0.20	
3.34b	350	21	+6	3.029 p 0.10	

3.18, 3.19, 3.27 = Standard

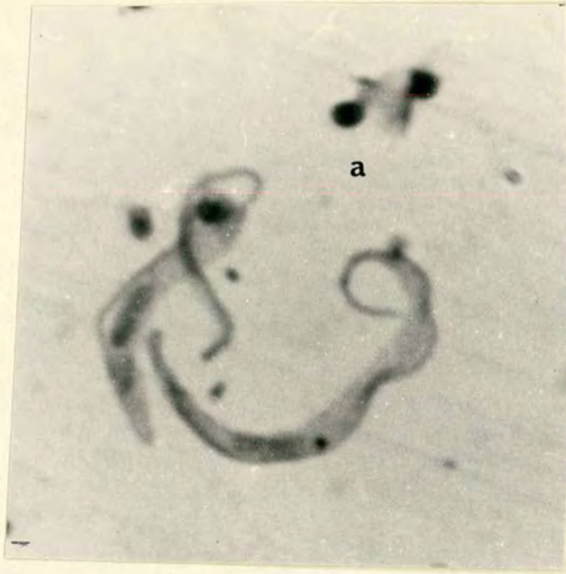
3.34a = T.brucei,C

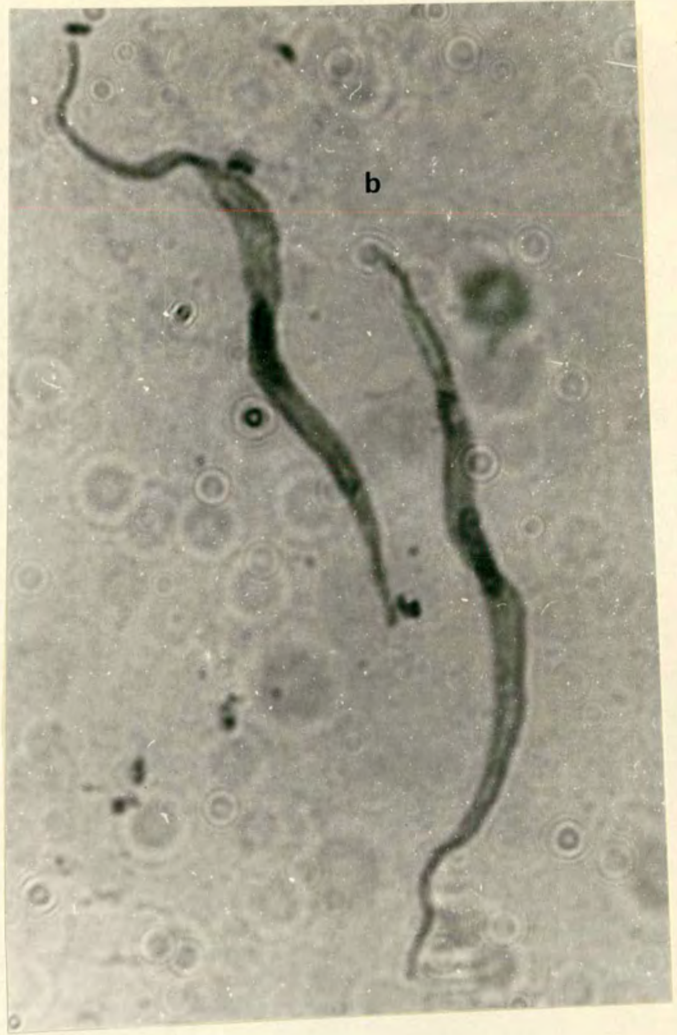
3.34b = T.brucei,D

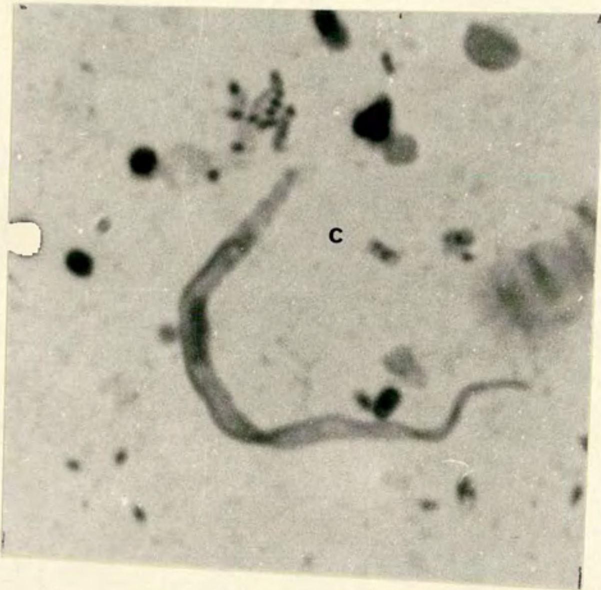
+ = Test of significance applied

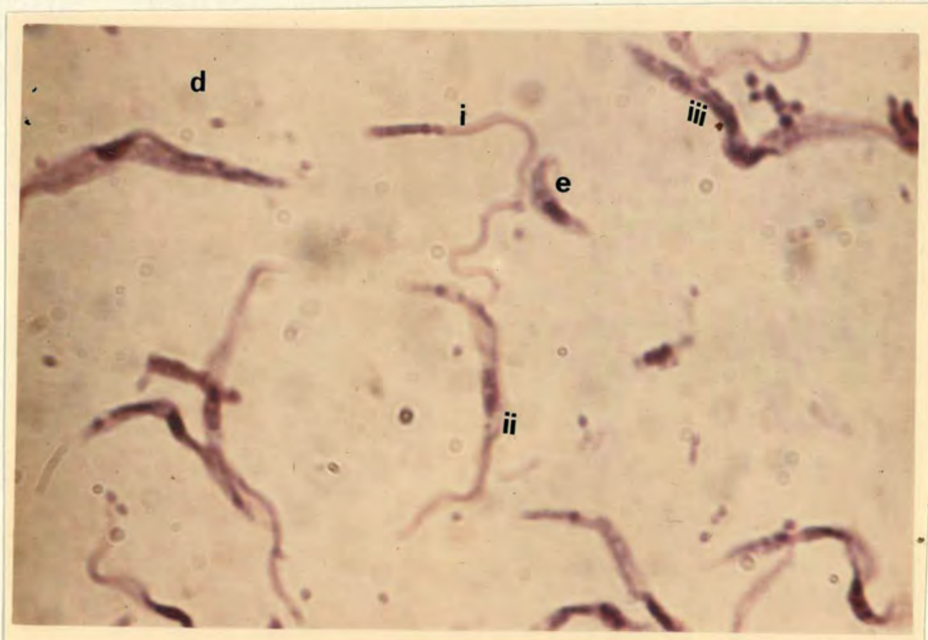
* = T.brucei,C and T.brucei,D compared

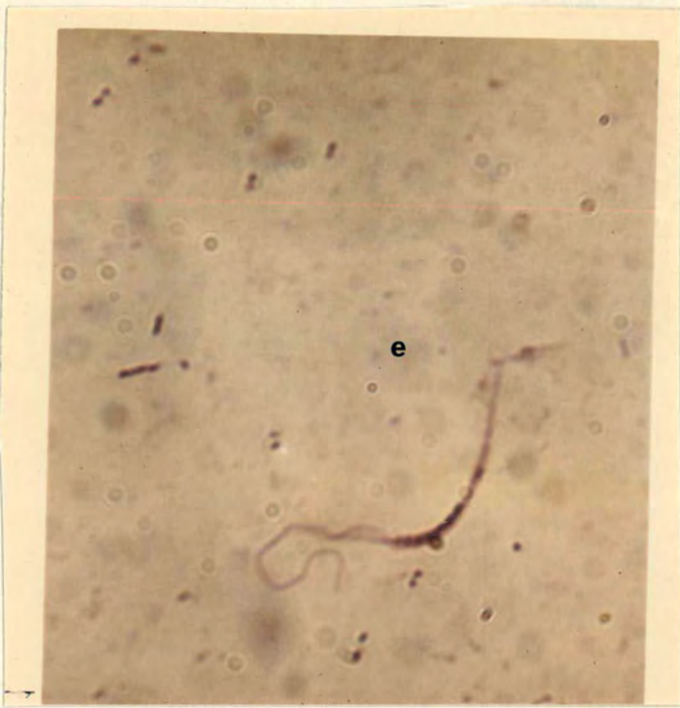
Developmental stages of T.brucei







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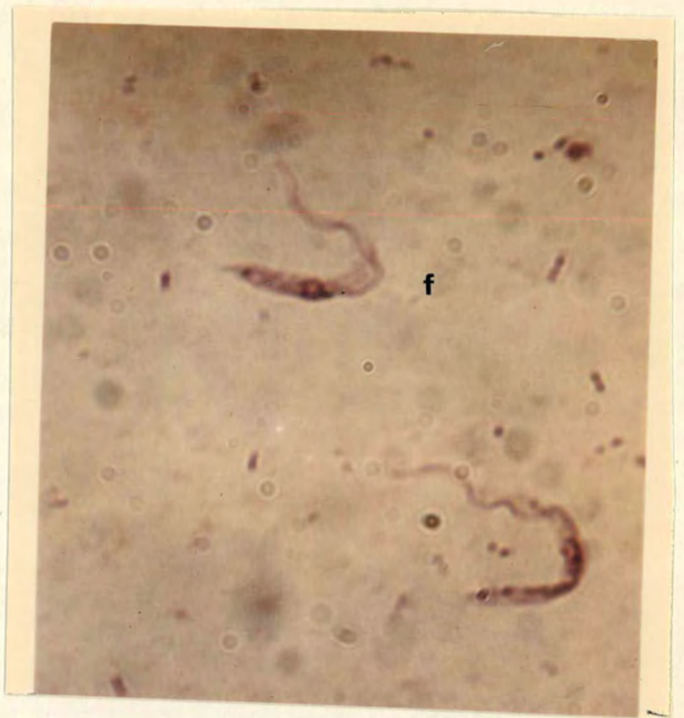
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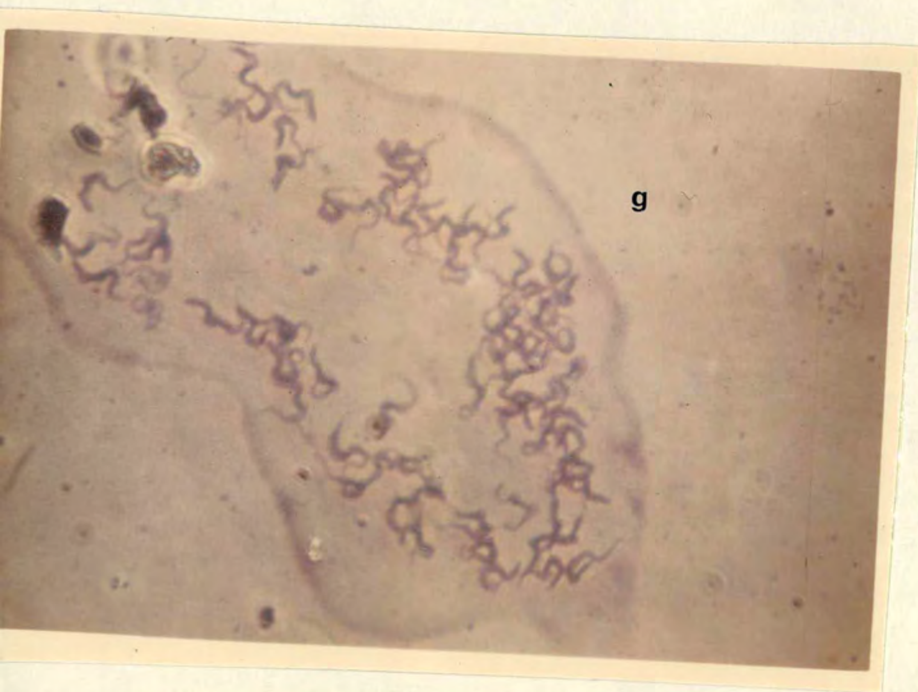
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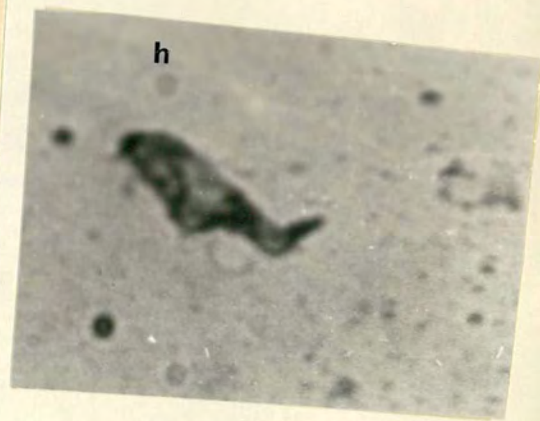
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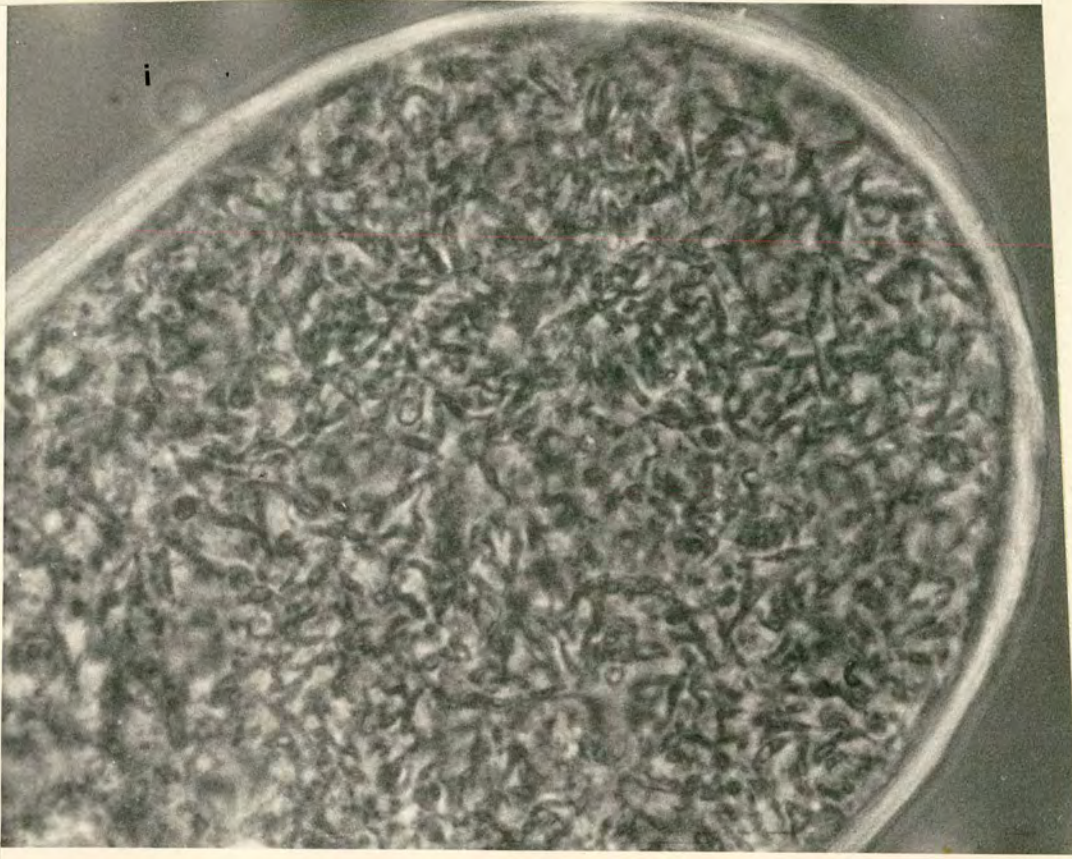


Fig.15 Developmental forms of T.brucei in the tsetse fly. All photographs magnified 400 times.

a) Typical midgut 'broad' form. Ectoperitrophic space, posterior segment. 3 days post infection.

b) Midgut 'long thin' form. Ectoperitrophic space, posterior segment. 5 days post infection.

c) Midgut 'long thin' form. Ectoperitrophic space, middle segment. 10 days post infection.

d) Anterior segment of midgut, ectoperitrophic space showing (i) 'proventricular' form; (ii) long thin form iii) 'broad' form. Note (e) which looks like an epimastigote.

e) 'Proventricular' form in the proventriculus. 10th day post infection.

f) Trypomastigotes in the labrum. 23 days post infection. The trypanosomes had become thicker and shorter.

g) Salivary probe. 25 days post infection.

h) A metacyclic from a teased salivary gland. 30 days post infection.

i) Trypanosomes in the enlarged base of the salivary glands. 30 days post infection. Such heavy infections of salivary glands with <u>T.brucei</u> are rare.

3.35 To find out whether flies with established infections of T.congolense will ultimately become infective to mice.

Procedure The test was conducted with T.congolense, B. After flies had taken their infective feed, they were divided into five batches. The flies of the different batches were dissected on days 3, 5, 7, 14 and 21 after the infective feed.

The flies for the 3 and 5 day dissections were fed on a group of 12 mice. Each fly of the other batches was maintained on a single clean mouse. The mice were inspected thrice a week and any infected one was replaced. Any fly, whose mouse was found infected before the period scheduled for its dissection, was immediately killed and dissected. This was to prevent the congestion of the mice room because many mice were being kept simultaneously for other experiments.

Commencing from the 7th day post infection, the experimental flies were probed daily on microscope slides warmed to 37°C. Metacyclic trypanosomes were seen in the probe test of one fly on the 14th day post infection. This fly was isolated and was kept for 60 days before being dissected. It was fed on six occasions on a clean mouse and the incubation period of the infection in the mice was determined.

Results Tables 56, 57 and 58 show the results of this test.

As shown in Table 56 there was good agreement between the number of flies with established and mature infections. On dissection, it was found that trypanosomes were present in the hypopharynx and in the other parts of the alimentary canal of the flies with mature infections. The single fly without a mature infection on day 21 had trypanosomes still persisting in the posterior segment of the midgut. The

proportions of flies with established infections on days 5, 7, 14 and 21 were similar; X^2 for the highest and lowest proportions was 0.250, $P < 0.70$.

The feeding records of flies which infected mice in this experiment are shown in Table 57 and 58. One fly found positive by probe test on day 14 was kept for 60 days. During this time it fed on six mice, and, as shown in Table 57, the prepatent period was the same, 8 days, in all the mice. The feeding record of the other 11 flies which infected mice is given in Table 58. By subtracting 8 from the day on which each mouse became parasitaemic, the probable day on which each fly infected its mouse was calculated. In fact all the flies did take a feed on that or on the adjacent day. It is also evident that the duration of the development cycle in the fly was 7-10 days.

Table 56 The number and type of infections in G.morsitans which were infected with T.congolense,B.

Day Dissected	Total Flies Used	Flies Infected		Flies with established infections		Flies with mature infections	
		Total	Percent	Total	Percent	Total	Percent
3	50	32	64	-	-	-	-
5	50	20	40	5	10		
7	100	14	14	9	9	7	7
14	100	7	7	7	7	6	6
21	100	7	7	6	6	6	6

Table 57 Determination of the prepatent period of T.congolense, B in mice after fly transmission.

+ Day Fly Fed on Mouse	Day of First Parasitaemia	Incubation Period (Days)
214	222	8
220	228	8
224	232	8
227	235	8
232	240	8
237	245	8

\+ The days are numbered as they occur in the year, January 1 being day 001.

Table 58 The feeding record of the 11 flies which developed mature infections with T.congolense, B.

Fly No.	Day on which fly fed	Day Fly Dissected	Day Mouse found Parasitaemic	Dissection Result in Category
1	3, 5, +(10), 13	14	18	4
2	2, 5 (7) 9	14	15	4
3	2, 7, (10) 13	14	18	4
4	2, 6, (9) 12	14	17	4
5	2, 5, (9) 12	14	16	4
6	2, 5, (8) 12	16	16	4
7	3, 7, (10) 12, 15	18	18	4
8	2, 4, 8, (10) 14, 17	18	18	4
9	2, 5, (9) 13, 16	17	17	4
10	3, 5, (7) 10, 12, 15	16	16	4
11	3, 5, (7) 9, 13	15	15	4

\+ Figures in brackets represent the probable day of infective feed.

A series of tests were carried out with T.congolense, B, in which the treatment was modified to see if the development cycle of the trypanosomes or the susceptibility of the flies could be altered.

The experimental procedure was as described in Experiment 3.35. Fifty flies were killed and dissected on days 3, 5, 7, 14 and 21 after their infective feed. The number of infected flies, and the number of flies with established and with mature infections were recorded. The proportions of flies with infections in these different categories were compared with the proportions found when flies received the standard treatment. Whenever the difference seemed large, its significance was tested by calculating the value for Chi-square by means of a two-by-two table. If the total numbers were less than 200, Yates correction was used.

3.36 To test the effect of temperature on the development of T.congolense, B in G.morsitans.

Procedure The details were the same as those given for T.brucei, B in Experiment 3.28.

Results In flies kept at 31°C (Table 59), there was good correlation between the numbers of established and mature infections from day 7 on. X^2 test showed that there was no significant difference between the proportions of flies with established infections on days 5, 7, 14 and 21. It was observed from the feeding record of the flies with mature infections that the duration of the development cycle was the same as that of flies kept at 26°C. All flies with mature infections possessed

trypanosomes in the hypopharynx and in other parts of the alimentary canal.

None of the flies kept at 20°C developed a mature infection even on day 21 (Table 60); neither had any infection become established by day 5. Only 1 of the 5 infected flies on day 7 possessed an established infection. As observed with T.brucei, therefore, the establishment of an infection was prolonged at this temperature. Consequently, there was a significant difference between the proportions of established infections on days 5 and 21; $X^2 = 4.582$, $P < 0.05$.

A comparison of the results of this treatment with the standard will be given together with others in Table 62.

3.37 To test the susceptibility of G.austeni to infection with T.congolense, B.

Procedure G.austeni was used to conduct the test instead of G.morsitans.

Results As shown in Table 61 there was no correlation between the numbers of established and mature infections until the 21st day. It was observed from the feeding records of the 2 flies with mature infection that the probable days on which they first infected mice were days 18 and 19 after their infective feed. The duration of the development cycle of this strain of T.congolense is hence longer in G.austeni than in G.morsitans.

To determine whether a treatment had an effect, results must be compared with the standard. The results of Experiments 3.36 and 3.37 are set out together in Table 62 so that the difference between the

modified and the standard treatments may be readily seen.

The proportions of flies which had trypanosomes in the gut when killed on the 3rd and 5th days after infection were significantly lower in G.austeni than G.morsitans. It is evident from the table that raising the temperature from $26^{\circ}C$ to $31^{\circ}C$ had no effect on the development of T.congolense in G.morsitans. When the temperature was reduced to $20^{\circ}C$ however, the proportions of mature infections on days 7, 14 and 21 were significantly lower at this temperature than at $26^{\circ}C$. Since the development cycle of this strain of T.congolense is not completed in G.austeni until 18-19 days after the infective feed, only the 21st day mature infections could be compared. As shown in the table, there was no significant difference in the proportions of mature infections on day 21 between G.austeni and G.morsitans. This suggests that the infectivity of T.congolense to G.austeni is similar to that of G.morsitans although the developmental cycle takes longer to be completed, in the former than in the latter.

Since it was shown that there was no significant difference in proportions of flies with established infections on days 5, 7, 14 and 21, the groups of flies dissected in each experiment on these days were regarded as samples of the same population. An addition of all the established infections of these days in each of Experiments 3.36 and 3.37 was made and compared with that of the standard. This is presented in Table 63. Only the flies kept at $20^{\circ}C$ had totals which were significantly lower than those of the standard. The other treatments had no influence on the proportions of flies that developed established infections.

Table 59 The number and type of infections in G.morsitans which were maintained at 31°C after being infected with T.congolense,B.

Day Dissected	Total Flies	Infected Flies Total	Infected Flies Percent	Established Infections Total	Established Infections Percent	Mature Infections Total	Mature Infections Percent
3	50	30	60	-	-	-	-
5	50	21	42	4	8	-	-
7	50	7	14	5	10	3	6
14	50	4	8	3	6	3	6
21	50	3	6	3	6	3	6

Table 60 The number and type of infections in G.morsitans which were maintained at 20°C after being infected with T.congolense,B.

Day Dissected	Total Flies	Infected Flies Total	Infected Flies Percent	Established Infections Total	Established Infections Percent	Mature Infections Total	Mature Infections Percent
3	50	34	68	-	-	-	-
5	50	25	50	-	-	-	-
7	50	5	10	1	2	-	-
14	50	3	6	2	4	-	-
21	50	2	4	2	4	-	-

Table 61 The number and type of infections in G.austeni which were infected with T.congolense,B.

Day of Dissection	Total Flies	Flies Infected Total	Flies Infected Percent	Established Infections Total	Established Infections Percent	Mature Infections Total	Mature Infections Percent
3	50	18	36				
5	50	10	20	2	4		
7	50	9	18	3	6		
14	50	2	4	2	4		
21	50	2	4	2	4	2	4

Table 62 Comparison of the results of the standard treatment with those of the modified treatments (Experiment 36 and 37)

Expt. Nos.	Total Flies	INFECTED FLIES Total	Percent	X^2,P	ESTABLISHED INFECTIONS Total	Percent	X^2,P	MATURE INFECTIONS Total	Percent	X^2,P
					DISSECTION AT 3rd DAY					
3.17, 3.19 3.35	77	47	61		1	1				
3.36a	50	30	60							
3.36b 3.23	60	38	63							
3.37	50	18	+36	8.641 P$<$0.01						
					DISSECTION AT 5th DAY					
3.17,3.19 3.35	77	28	36		5	6				
3.36a	50	21	42		4	8				
3.23,3.36b	60	31	+52	2.622 P$<$0.20						
3.37	50	10	+20	4.69 P$<$0.05	2	4				
					DISSECTION AT 7th DAY					
3.17,3.19 3.35	127	17	13		11	9		7	6	
3.36a	50	7	14		5	10		3	6	
3.23,3.36b	60	6	10		1	+2	4.586 P$<$0.05	0	0	
3.37	50	9	+18	0.296 P$<$0.70	3	6		0	0	

Table 62 (continued)

Expt. Nos.	Total Flies	INFECTED FLIES Total	Percent	X^2,P	ESTABLISHED INFECTIONS Total	Percent	X^2,P	MATURE INFECTIONS Total	Percent	X^2,P
				DISSECTION AT 14th DAY						
3.17,3.35	115	7	6		7	6		6	5	
3.36a	50	4	8		3	6		3	6	
3.36b	50	3	6		2	4		0	0	
3.37	50	2	4		2	4		0	0	
				DISSECTION AT 21st DAY						
3.17,3.35	115	11	10		10	9		10	9	
3.36a	50	3	6		3	6		3	6	
3.36b	50	2	+4	2.352 $P<0.20$	2	+4	1.953 $P<0.20$	0	0	
3.37	50	2	4		2	4		2	+4	1.953 $P<0.20$

+ = Test of significance applied

3.17, 3.19, 3.35 = Standard

3.36a = Flies kept at 31°C

3.36b, 3.23 = Flies kept at 20°C

3.37 = G.austeni

Table 63 Total established infections on days 5, 7, 14 and 21 in each of the modified treatments (Experiments 36 and 37) compared with those of the standard.

Experiment Nos.	TREATMENT	Total Flies	Established Infections days 5, 7, 14, 21			Remark
			Total Established	Per-cent	X^2, P	
3.17, 3.19 3.35	Standard	434	33	7.6		
3.36	Flies at 31°C	200	15	7.5		
3.36, 3.23	Flies at 20°C	220	5	+2	9.654 P 0.01	Significant
3.37	Experiment with G.austeni	200	9	+4.5	3.253 P 0.10	Not significant

+ = Test of significance applied.

3.38 To test whether flies infected with T.congolense, B and kept at 20°C will develop mature infections.

In Experiment 3.36, it was observed that there was a prolongation of the establishment of an infection in flies infected with T.congolense and kept at 20°C. The purpose of this experiment, therefore, was whether mature infections would develop if flies were kept for an extended period.

Procedure As soon as the flies had fed on the suspension of T.congolense, B they were divided into two batches, designated A and B. The flies of both batches were kept for 21 days in an incubator at 20°C and relative humidity of 75%. After 21 days the flies of batch B were transferred to the fly room (26°C) and kept there for a further 21 days while the flies of batch A were retained in the incubator at 20°C for the same number of days.

All the flies were fed on a group of 12 mice for the first period of 21 days. From days 22 to 42, each fly was maintained on a single clean mouse. Mice were inspected, as usual, thrice a week.

Results As shown in Table 64 all the 8 infected flies out of the 100 G.morsitans kept continuously at 20°C had established infections and 6 of these were mature. All the 6 infected flies of the 160 G.morsitans kept for 21 days at 20°C before being transferred to 26°C carried established infections and 5 of these were mature. The numbers of established and mature infections were therefore similar in flies of both batches.

Table 65 shows the feeding record of the flies that infected mice among those kept continuously for 42 days at 20°C. The probable day

on which each fly infected its mouse was calculated by subtracting 8 from the day on which each mouse first became parasitaemic. It will be seen from the table that this varies from 24-36 days, and the calculated average was 31 days. This was 3-4 times longer than the duration of the development cycle when flies were kept at 26°C (7-10 days). Furthermore, the group of 12 mice on which all the experimental flies had fed for the first 21 days were not infected until discarded after 6 weeks. This confirmed the earlier observation that the infections at 20°C were not yet mature by the 21st day.

Although, on dissection, all the infected flies possessed trypanosomes in the midgut and proventriculus, only those which infected mice had their hypopharynx invaded. The conclusion drawn from this experiment is that the developmental cycle of T.congolense in G.morsitans is not inhibited but is prolonged.

Table 64 Dissection results of 200 G.morsitans infected with T.congolense,B. Flies of one batch were kept continuously at 20°C for 42 days and those of the other batch were transferred to 26°C after 21 days.

TREATMENT	Total No. of Flies	No. of Infected Flies	No. of Established Infections	No. of Mature Infections
Flies kept continuously for 42 days at 20°C	100	8	8	6
Flies kept at 20°C for 21 days, and then at 26°C for 21 days	100	6	6	5

Table 65 The feeding record of the 6 flies which infected mice among the 100 G.morsitans kept continuously for 42 days at 20°C.

Fly No.	Days on which the Fly Fed	Day Fly Killed or Died	Dissection Result in Category	Day of First Parasitaemia in mice
1	13, 15, 23 +(31), 39, 41	42	4	39
2	8, 16, 23, 27 (30) 38, 41	42	4	43
3	13, 17, 19 (33), 38	42	4	42
4	10, 21, (24) 40	42	4	35
5	9, 14, 19, 21, 26 (29), 31, 40	42	4	36
6	9, 12, 14, 21, 28 (36) 40	42	4	42

+ = Figures in brackets represent the probable day of infective feed.

3.39 To test the infectivity to mice of the fly forms of T.congolense,B.

Procedure After all the available flies had fed on a suspension of T.congolense,B in defibrinated ox blood at a dilution of 1:10, they were divided into 5 batches. The flies of the different batches were dissected on days 1, 2, 3, 5 and 7 after the infective feed. On each of days 2 and 3, trypanosomes from 10 infected flies among those dissected were pooled together and injected into 2 mice. All the other infected flies on these days as well as those of days 5 and 7 were used for immunofluorescent test on the fly forms as part of a joint experiment.

Results All the flies examined on days 1 and 3, and 23 of the 25 (92%) examined on day 2 were infected (Table 66). As observed with T.brucei, the number of trypanosomes seen in the flies on day 1 was very few. In squash preparations of the midgut there were less than 10 trypanosomes in 50 microscope fields. The number had increased on day 2 to about 20 trypanosomes per 50 fields and by the 3rd day 3-10 trypanosomes were seen in each field. The mice, into which the fly forms were injected on days 2 and 3 post infection, were discarded after 6 weeks since they had not become infected.

Comparison of these results with those in which the flies received the standard treatment (Table 67) showed that a tenfold increase in the concentration of trypanosomes in the infective feed did affect the proportion of infected flies at day 3. There was no difference in the proportion of infected flies or in the type of infection in flies killed at days 5 and 7.

Table 66 The results of dissection of G.morsitans which were infected with stabilates of T.congolense,B diluted 1:10 with defibrinated ox blood.

Day of Dissection	Total Flies	Flies Infected		Established Infection	
		Total	Percent	Total	Percent
1	25	25	100		
2	25	23	92		
3	20	20	100		
5	20	9	45	1	5
7	27	4	16	2	8

Table 67 Comparison of the infection rates in flies receiving the standard treatment with those of flies which had fed on blood containing ten times more trypanosomes.

Experiment Number	TREATMENTS	Total Flies	Infected Flies			Established Infections		
			Total	Percent	X^2,P	Total	Percent	X^2,P
			DISSECTION AT 3rd DAY					
3.17, 3.19 3.35	Standard	77	47	61				
3.20, 3.39	Tenfold concentration	30	25	83	3.914 $P<0.05$			
			DISSECTION AT 5th DAY					
3.17, 3.19 3.35	Standard	77	28	36		5	6	
3.20, 3.39	Tenfold concentration	30	15	50	2.700 $P<0.1$	3	10	
			DISSECTION AT 7th DAY					
3.17, 3.19 3.35	Standard	127	17	13		11	9	
3.20, 3.39	Tenfold concentration	37	6	16		3	8	

3.40 To test the infectivity to flies of the culture forms of T.congolense.

Procedure The method for the preparation of trypanosome cultures has been described in Experiment 3.33.

On three different occasions, designated Periods 1, 2 and 3, G.morsitans were fed on suspensions of culture forms of T.congolense in defibrinated ox blood. In period 1, the trypanosomes in the culture of T.congolense TREU 261 were diluted 1:30 with defibrinated ox blood and fed to 60 G.morsitans. The flies were then maintained ona group of 12 mice and dissected between days 3 and 12. In period 2, the culture of T.congolense, B which had been established for 9 days from blood of infected mice was diluted 1:100 with defibrinated ox blood and used to infect flies. All the flies were dissected on days 3 and 5. For period 3, stabilates of T.congolense,B were injected into 2 mice. When the parasitaemia was high, a group of 75 flies were fed on them. Immediately afterwards the two mice were used to set up cultures. Cultures harvested on the 3rd and 9th days were diluted with defibrinated ox blood to give concentrations of 4.8×10^6 trypanosomes per ml and 4.4×10^6 trypanosomes per ml respectively. These numbers of trypanosomes were comparable with those in the infective feed of the standard experiment.

After flies had fed on the infective suspension, they were divided into 2 batches. Flies on one batch were dissected on day 3, and the other on day 21 post infection. Each of the flies kept for 21 days was maintained on a single mouse.

Results

a) Period 1. As shown in Table 68, all flies examined on days 3 and 4 were infected. As observed with the blood forms, the number of infected flies dropped from the 6th day on to a range of 1-3 per day. In all, 29 of the 60 flies were infected (50%) and 3 of these had established infections.

b) Period 2. All flies dissected on day 3 were infected (Table 69). 20 of the 25 flies (80%) dissected on day 5 were infected and 3 of these possessed established infections.

c) Period 3. The proportion of infected flies in the control experiment on the 3rd day was significantly lower than that of the culture experiments, $X^2 = 9.232$, $P < 0.01$; the proportions of mature infections were however similar (Table 70). It was observed that the infections in the control experiment were mature in 7-10 days while the maturity of infections in the flies that had fed on the cultures was not accomplished until at least 19 days. The prepatent period in mice was also observed to fall between 11 and 13 days compared with the 7-8 days of the control experiment.

The comparison of the results of feeding flies on culture forms with those of the standard is shown in Table 71. In flies dissected on days 3 and 5, the infection rate of the flies fed on 3rd and 9th day culture forms did not differ from each other, but were significantly higher than the standard. The proportions of mature infections on the 21st day were however not affected.

Table 68 The results of dissection of 60 G.morsitans which were infected with culture forms of T.congolense, TREU 261.

Group No.	DAYS 3	4	5	6	7	8	9	10	11	12	No. Infected
1	M	M	M	M	M	M	M	M	M	M	5
	1	1			1	1			1		
2	M	M	M	M	M	M	M	M	M	M	4
	1	1		1		1					
3	M	M	M	M	M	M	M	M	M	M	8
	1	1	1	1	1	1	1			4	
4	M	M	M	M	M	M	M	M	M	M	4
	1	1		1	1	D2	D3				
5	M	M	M	M	M	M	M	M	M	M	4
	1	1	1						3	D2	
6	M	M	M	M	M	M	M	M	M	M	4
	1	1	1	4							
Total Infected	6	6	3	4	3	3	1	0	2	1	29
Total Established				1					1	1	3

M = Male

F = Female

1,2,3,4 = denote category of infection

D followed by a figure = Fly dead on day indicated by the figure.

Table 69 The results of dissection of 45 G.morsitans which were infected with culture forms of T.congolense,B and killed on days 3 and 5.

Day Dissected	Total Flies	Flies Infected Total	Flies Infected Percent	Established Infections Total	Established Infections Percent
3	20	20	100		
5	25	20	80	3	12

Table 70 The results of the dissection and infectivity to mice of G.morsitans which were infected with culture forms of T.congolense,B. The control flies were fed directly on two parasitaemic mice.

Designation	Day of Dissection	Total Flies	Infected Flies Total	Infected Flies Percent	Established Infections Total	Established Infections Percent	Mature Infection Total	Mature Infection Percent
Control	3	25	15	60				
	21	50	4	8	4	8	4	8
3 Day Old	3	25	25	100				
Culture	21	50	3	6	3	6	3	6
9 Day Old	3	25	21	84				
Culture	21	75	3	4	3	4	3	4

Table 71 Comparison of the infection rates in flies fed on culture forms of T.congolense,B with those fed on blood forms.

Expt. Nos.	Total Flies	INFECTED FLIES			ESTABLISHED INFECTIONS			MATURE INFECTIONS		
		Total	Percent	X^2,P	Total	Percent	X^2,P	Total	Percent	X^2,P
					DISSECTION AT 3rd DAY					
3.17,3.19 3.35	77	47	61							
3.40a	25	15	60							
3.40b	25	25	+100	11.985 $P < 0.01$						
3.40c	45	41	+91	11.324 $P < 0.01$						
					DISSECTION AT 5th DAY					
3.17,3.19 3.35	77	28	36		5	6				
3.40c	25	20	+80	12.725 $P < 0.01$	3	12				
					DISSECTION AT 21st DAY					
3.17,3.35	115	11	16		10	9		10	9	
3.40a	50	4	8		4	8		4	8	
3.40b	50	3	6		3	6		3	6	
3.40c	75	3	4		3	4		3	+4	1.953 $P < 0.20$

+ = Test of significance applied

3.17, 3.19, 3.35 = Standard

3.40a = Control experiment

3.40b = 3rd DAY culture

3.40c = 9th DAY culture

3.41 To test the effect of mouse passage in the development of *T.congolense* in *G.morsitans*.

In Experiment 3.17 it was observed that there was a difference in the proportions of mature infections in flies which had fed on two populations of *T.congolense* TREU 692. It was concluded then that the difference could be attributed to the number of mouse passages the strain had undergone before being stabilated. The purpose of this experiment was to confirm this observation.

Since all but one of the stabilates of *T.congolense*, A had been used, the last one was diluted with citrated saline and injected into one mouse. When the parasitaemia was high, after 10 days, the mouse was killed and about 30 stabilates were prepared from the blood. These stabilates were designated *T.congolense*, C. They had undergone 7 mouse passages since cyclical infection before being stabilated.

Procedure After flies had taken their infective feed on a suspension of *T.congolense*, C in defibrinated ox blood, they were divided into two lots. The flies of one lot were dissected from days 3 to 12 and maintained on a group of 12 mice. The flies of the other lot were kept under observation for 30 days before they were dissected. Each of them was maintained on a single mouse and the mice were inspected thrice a week. Any infected mouse was immediately replaced.

Results The results of the dissections carried out on days 3-12 are shown in Table 72. Thirty per cent of the flies were infected but only 9% had established infections. The high rate of infection on days 3-5 which falls to a low but almost constant number from days 6-12 was also observed in the experiments conducted with *T.congolense*, B

but missed in all those conducted with T.congolense,A.

Of the 50 G.morsitans kept under observations for 30 days, 6 had established infections and 2 of these infections were mature. When these flies were dissected, only the 2 flies which infected mice had trypanosomes in the hypopharynx as well as the other parts of the alimentary canal. The remaining 4 flies with established infections harboured trypanosomes in the labrum epipharynx and the other parts of the alimentary canal; but the hypopharynx was not invaded.

The three populations of T.congolense TREU 692 are compared in Table 73. The flies which had fed on populations A and C had higher proportions of infected flies and established infections. However, in populations A, which had undergone 6 mouse passages since cyclical development, only 1 out of 10 established infections became mature. In population C, which had undergone 7 mouse passages since cyclical development, 2 out of 6 established infections became mature. In population B, which had undergone 2 mouse passages since cyclical development all the 6 established infections became mature.

Table 72 The results of the dissection of 100 G.morsitans which were infected with T.congolense, C.

Group No.	Stabilate No.	3	4	5	6	7	8	9	10	11	12	No. Infected
1	1,2,3,4	M 1	M 1	M	M	M	M	F	F	F	M	2
2	"	M 1	M 2	F	F	F D	F	F 2	M	M	F	3
3	"	M 1	M	M 1	F 1	M	M	F 2	F	F	F	4
4	"	F 1	M	M	F 2	F 1	F	M	F	F	F	3
5	"	F 1	M 1	F	F	M 3	M	F	M 4	M	F	4
6	"	F	F 1	F 2	F 1	F	F	F	M	M	F 4	4
7	"	M 1	F 1	F 1	F	M	F	M	M	M	F	3
8	"	M	F 1	F 1	F	F	F 2	F	F	F	M	3
9	"	M 1	F 1	F	F	F	F 1	F	F	M	F	3
10	"	M 1	F	M	F	M	F	M	M	F	F	1
Total Infected		8	7	4	3	2	2	2	1	0	1	30
Total Established		0	1	1	1	1	1	2	1	0	1	9

Percentage Infected 30%

M = Male
F = Female
1,2,3,4 = denotes category of infection
D = Fly dead on day due for dissection

Table 73 Comparison of the proportions of mature infections in the populations A, B, C of the T.congolense TREU 692.

Experiment Numbers	Designation of Population	Total Flies	Infected Flies Total	Infected Flies Percent	Established Infections Total	Established Infections Percent	Mature Infections Total	Mature Infections ++ Per cent	+ X^2
3.15 Table 13 (Day 40)	A	80	12	15	10	12.5	1	10	16.2 P 0.
3.35 Table 56 (Day 21)	B	100	7	7	6	6	6	100	
3.41 (Day 30)	C	50	6	12	6	12	2	33	9.37 P 0.

+ = X^2 calculated using B population as the standard.

++ = Percentage calculated from the number of established infections.

3.42 To compare the infectivity to flies of two populations of *T.congolense*, LUMP 92, which had been passaged once and 11 times in mice.

T.congolense, D was the designation given to *T.congolense*, LUMP 92, after it had undergone 11 mouse passages (Methods 2.10). When the parasitaemia of the 11th passage was high, the mice were killed and about 90 stabilates made from the pooled blood. Blood from one of the mice which became infected from a fly which had fed on *T.congolense*, D was passaged into 3 other mice. After the parasitaemia was high, the mice were killed and about 90 stabilates made from the pooled blood. These stabilates were designated *T.congolense*, E. As *T.congolense*, LUMP 92 was a new strain from West Africa, it was also the aim of this experiment to compare its infectivity to flies with that of *T.congolense*, TREU 692 which originated from East Africa.

Procedure *T.congolense*, D was first used to conduct the test. Flies, which had taken their infective feed were divided into five batches. The flies of the different batches were dissected on days 3, 5, 7, 14 and 21. Probing of the experimental flies commenced as from the 7th day post infection. One fly, which deposited trypanosomes in its saliva on the 14th day was isolated. It was not killed until day 60 during which time it was fed on clean mice on three different occasions in order to determine the prepatent period of this strain in mice. Flies scheduled for dissection on days 3 and 5 were fed on a group of 12 mice while each of the other flies was maintained on a single mouse.

After the stabilates of T.congolense, E had been prepared as described, they were used to conduct a test, the details of which were similar to those described for T.congolense, D.

Results These are shown in Tables 74, 75, 76 and 77.

It will be seen in Table 74 that there is only one established infection on each of days 5, 7, 14 and 21. The only fly which infected a mouse was the one isolated by probing on the 14th day. This fly was scheduled for dissection on the 14th day, but it was kept until day 60. The mouse on which it had fed until the 14th day was not infected; but those on which it fed on days 17, 21 and 25 post infection became parasitaemic 17, 19 and 16 days later respectively. The average prepatent period was, therefore, 17 days. It was also evident that the development cycle was not completed until the 17th day. When this fly was dissected, it had abundant trypanosomes in the hypopharynx as well as the other parts of the alimentary canal.

Table 75 shows the results of similar tests conducted with T.congolense, E. The proportions of established infections on days 5, 7, 14 and 21 were similar; X^2 for the highest and lowest proportions is 2.400, $P < 0.20$. There was no correlation between the established and mature infections until the 21st day and the two flies with mature infections had trypanosomes in the midgut, proventriculus, labrum epipharynx and hypopharynx.

Table 76 shows the comparison between the infection rates of T.congolense, B (standard), T.congolense, D, and T.congolense, E. The infection rates in T.congolense, D and E were similar on days 3 and 5. On almost all days in which flies were examined, the proportions

of established infections in T.congolense, D were lower than those of T.congolense, E. On the 21st day, the proportion of mature infections was also significantly lower in the former than the latter. Comparing the cyclical population of both strains, it will be seen that the infection in T.congolense, E was significantly lower on days 3 and 5 than that of T.congolense, B. The proportion of mature infections was also significantly lower in the former than the latter.

Table 77 shows the additions of the established infections on days 5, 7, 14 and 21. The proportion of established infections in T.congolense, E was significantly higher than that of T.congolense, D, while it is significantly lower thanthat of T.congolense, B.

Table 74 The number and type of infections in G.morsitans which were infected with T.congolense, D.

Day Dissected	Total Flies	Total Infected	Established Infections	Mature Infections
3	100	16		
5	100	5	1	
7	100	3	1	
14	100	4	1	1
21	100	5	1	

Table 75 The number and type of infections in G.morsitans which were infected with T.congolense, E.

Day Dissected	Total Flies	Total Infected	Established Infections	Mature Infections
3	100	18		
5	100	7	3	
7	100	10	5	
14	100	4	3	
21	100	4	2	2

Table 76 Comparison between the infection rates in T.congolense,B T.congolense,D and T.congolense,E.

Expt. Nos.	Total Flies	INFECTED FLIES Total	Percent	X^2,P	ESTABLISHED INFECTIONS Total	Percent	X^2,P	MATURE INFECTIONS Total	Percent	X^2,P
					DISSECTION AT 3rd DAY					
3.17,3.19 3.35	77	47	61							
3.42a	100	16	16							
3.42b	100	18	18	36.555 $P<0.001$						
					DISSECTION AT 5th DAY					
3.17,3.19 3.35	77	28	36		5	6				
3.42a	100	5	5		1	1				
3.42b	100	7	7	25.531 $P<0.001$	3	3				
					DISSECTION AT 7th DAY					
3.17,3.19 3.35	127	17	13		11	9		7	6	
3.42a	100	3	3		1	1	* 6.200 $P<0.02$			
3.42b	100	10	10		5	5				
					DISSECTION AT 14th DAY					
3.17,3.35	115	7	6		7	6		6	5	
3.42a	100	4	4		1	1	* 4.00 $P<0.05$	1	1	
3.42b	100	4	4		3	3		0	0	

Table 76 (continued)

Expt. Nos.	Total Flies	INFECTED FLIES Total	Percent	X^2 P	ESTABLISHED INFECTIONS Total	Percent	X^2,P	MATURE INFECTIONS Total	Percent	X^2,P
				DISSECTION AT 21st DAY						
3.17,3.35	115	11	10		10	9		10	9	
3.42a	100	5	5		1	1		0	0	* 8.00 $P < 0.01$
3.42b	100	4	4		2	2	7.446 $P < 0.01$	2	2	7.446 $P < 0.01$

* = T.congolense,D and T.congolense,E compared. All other comparisons are for T.congolense,B and T.congolense,E.

3.17,3.19,3.35 = Standard

3.42a = T.congolense,D

3.42b = T.congolense,E

Table 77 Comparison of the additions of established infections of T.congolense,B, T.congolense,D and T.congolense,E.

Experiment Numbers	Total Flies	Established Infections Days 5,7,14,21 Total	Percent	X^2,P
3.17,3.19 3.35	434	33	7.6	
3.42a	400	4	1	* 7.513 P < 0.01
3.42b	400	13	3.25	9.333 P < 0.01

* = T.congolense,D and T.congolense,E compared. The other comparison is for T.congolense,B and T.congolense,E.

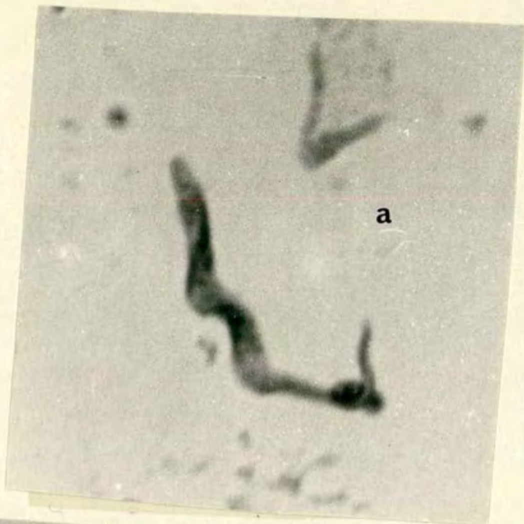
3.17,3.19,3.35 = Standard

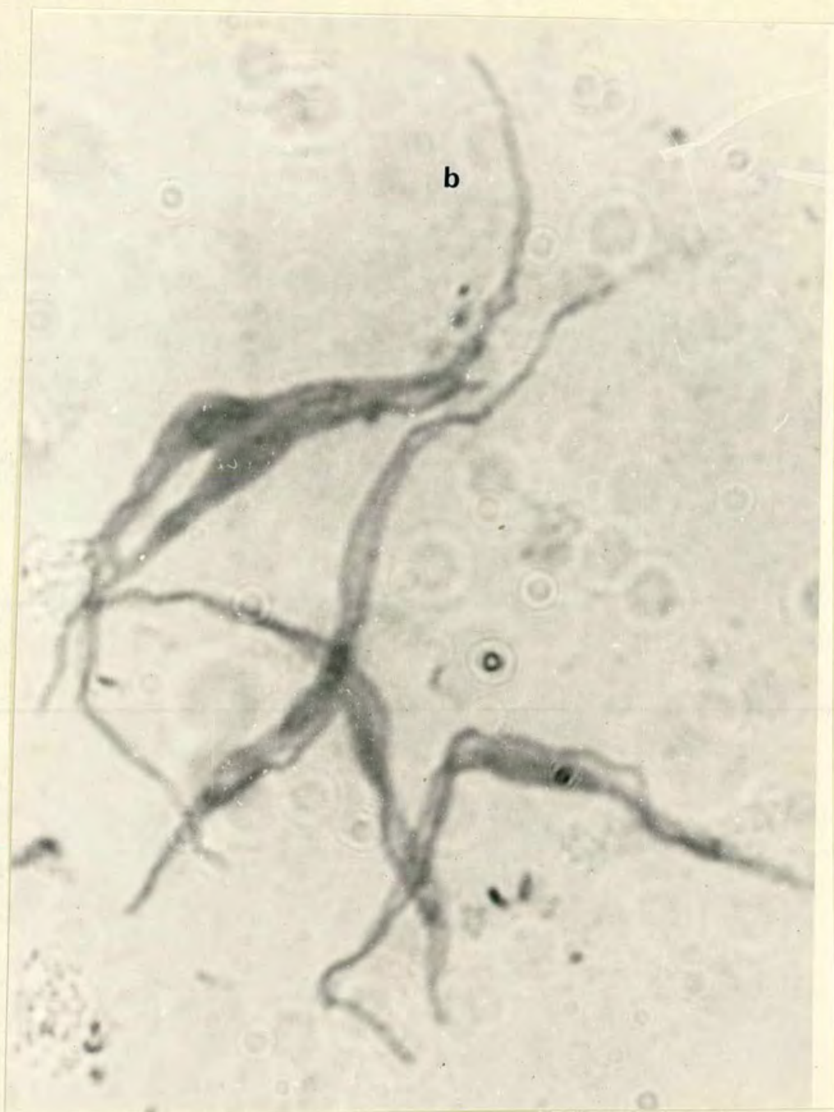
3.42a = T.congolense,D

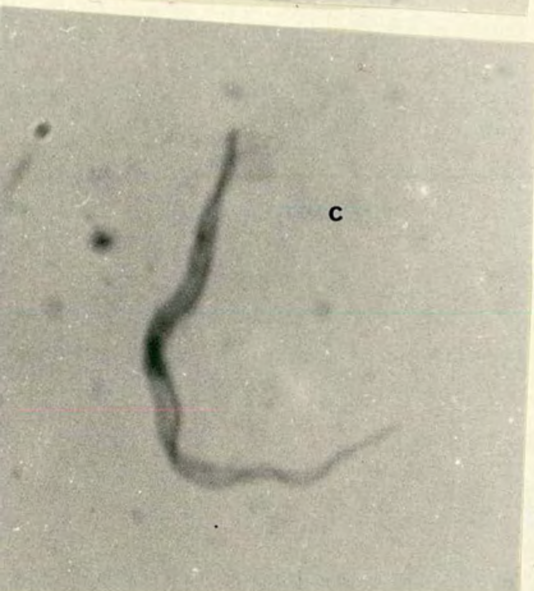
3.42b = T.congolense,E

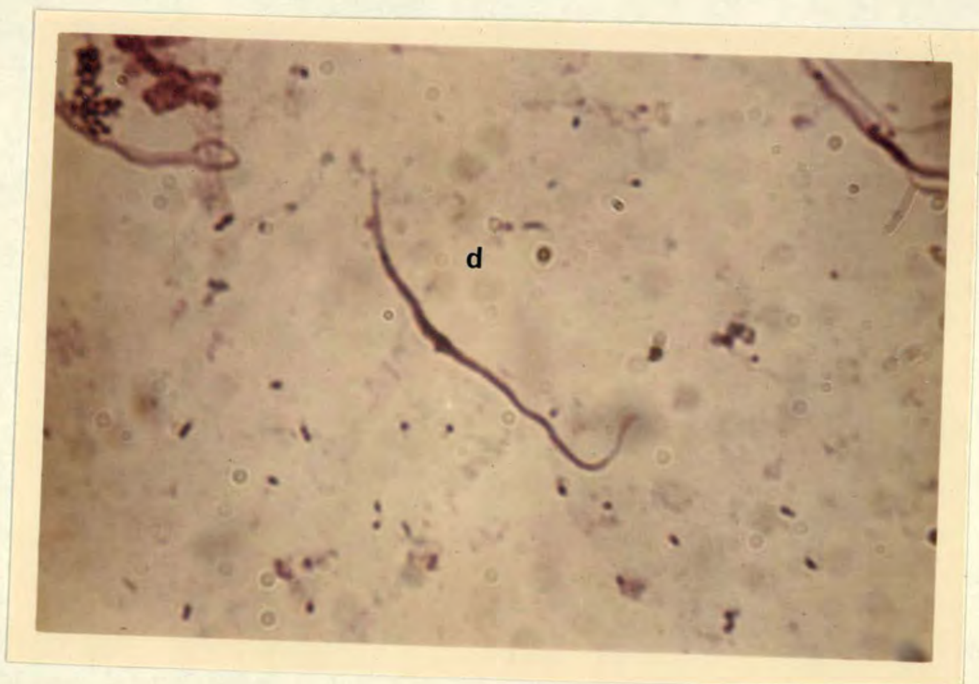
Fig.16 Developmental forms of T.congolense in the tsetse fly.

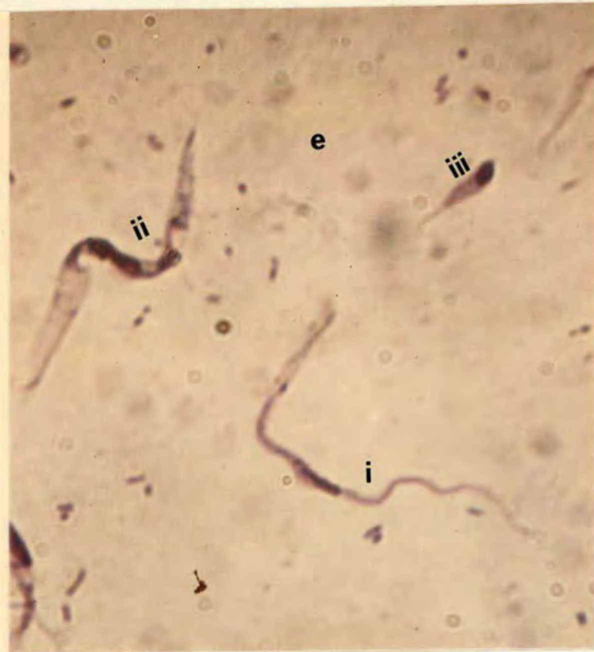
All photographs magnified 400X.







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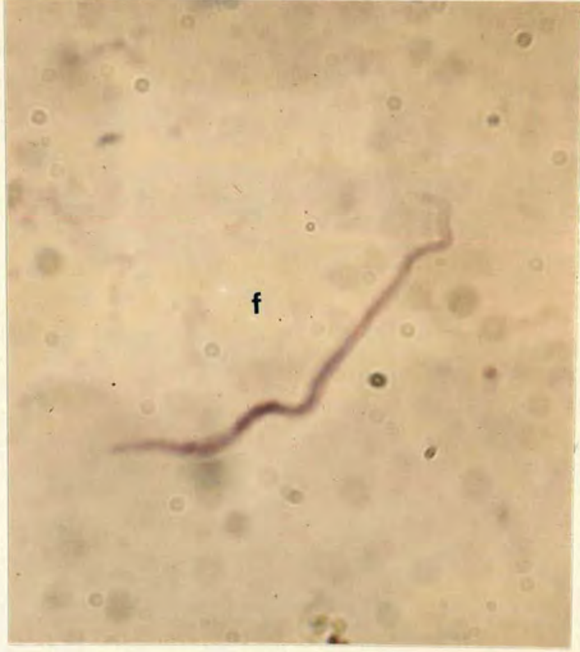
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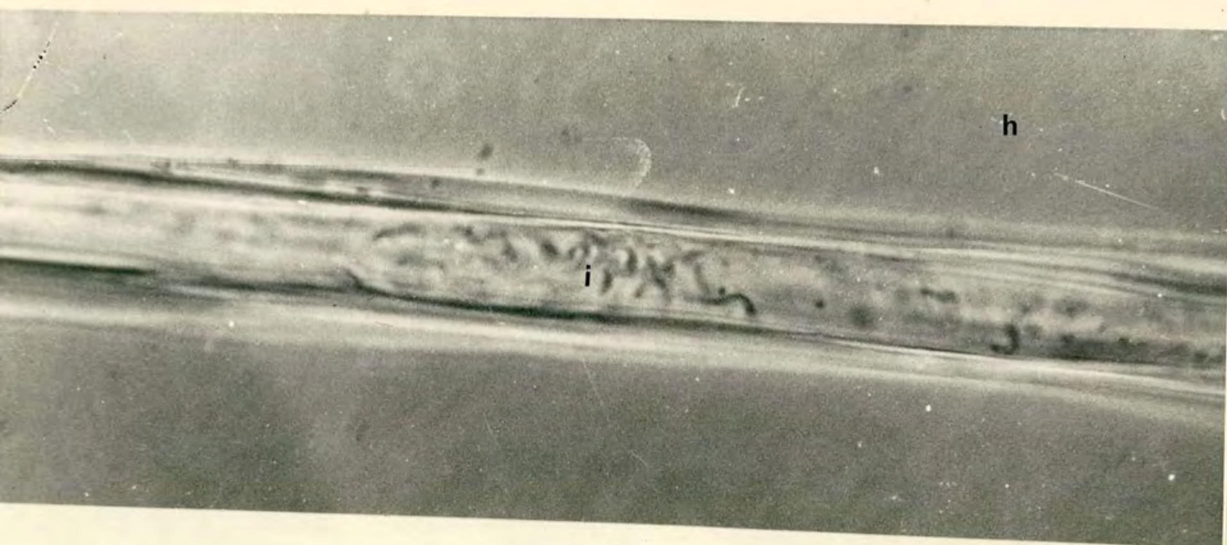


Fig.16 Developmental forms of T.congolense in the tsetse fly. All photographs magnified 400 times.

a) Typical midgut 'broad' form. Ectoperitrophic space posterior segment. 3 days post infection.

b) Midgut 'long thin form'. Ectoperitrophic space, posterior segment. 5 days post infection.

c) Midgut 'long thin form'. Ectoperitrophic space, middle segment. 7 days post infection.

d) 'Proventricular' form in the middle segment, ectoperitrophic space; 6 days post infection.

e) Anterior segment of midgut, ectoperitrophic space showing (i) proventricular form, (ii) broad form. Note (iii) which looks like an epimastigote. 7 days post infection.

f) 'Proventricular' form in the proventriculus. 10 days post infection.

g) Labrum-epipharynx infection. 14 days post infection. Note diffuse nature of infection and rosettes.

h) Infection of hypopharynx, 'live'. Note the metacyclics (i). 14 days post infection.

3.43 The infection rates of male and female flies.

In Experiment 3.25, it appeared that the female flies are more susceptible to infection with trypanosomes than males. In the calculations that led to this observation, the two by two table was used and the figures for male flies were placed as the nominator. Later, it was realised that if the order were reversed by placing the figures for females as the nominator, the values of X^2 showed no significant difference between the infection rates of both sexes.

In this section, the numbers of male and female flies infected, or with established and mature infections from all experiments under standard treatment conducted with T.congolense and T.brucei were counted and are recorded in Tables 78 and 79. The proportions of infected males and females were then calculated and are given in Table 80. The same recordings and calculations were made for all the experiments in which the treatments were modified and these are given in Tables 81 and 82.

As seen in Table 80, there is neither a significant difference in the proportions of infected flies nor in those of established and mature infections between the male and female flies which received the standard treatment. The proportions of male and female flies infected, or with established and mature infections are also similar in each of the modified treatments (Tables 81 and 82).

Table 78 Susceptibility of male and female G.morsitans to infection with T.brucei, under standard treatment.

Expt. Nos.	Infected Flies Sex	Total	No. Infected	Per Cent	Established Infections Total	No. Established	Per Cent	Mature Infections Total	No. Mature	Per Cent
3.18a	M	70	10	14.3	70	3	4.3			
	F	30	7	23.3	30	2	6.7			
3.18b	M	104	8	7.7	104	7	6.7			
	F	46	1	2.2	46	1	2.2			
3.19	M	43	4	9.3	43	1	2.3			
	F	27	5	18.5	27	1	3.7			
3.27	M	326	43	13.1	295	28	9.5	214	4	1.9
	F	124	18	14.5	105	7	6.7	86	2	2.3
3.34a	M	249	44	17.7	197	7	3.6	97	2	2.1
	F	251	27	10.8	203	10	4.9	103	3	2.9
3.34b	M	190	32	16.8	130	8	6.2	42	1	2.4
	F	210	35	16.7	170	9	5.3	58	1	1.7
TOTALS	M	982	141	14.4	839	54	6.4	375	10	2.7
	F	688	93	13.5	581	30	5.2	265	6	2.3

Key for Tables 78-82

3.17a = Standard (3-12 days) T.congolense
3.17b = Standard (13-22 days) T.congolense
3.18a = Standard (3-12 days) T.brucei
3.18b = Standard (13-22 days) T.brucei
3.19 = Retention of infectivity during period of infective feed
3.23 = Effect of temperature. Flies kept at 26°C
3.27 = Standard (T.brucei)
3.28a = Flies kept at 31°C (T.brucei)
3.28b = Flies kept at 20°C (T.brucei)
3.29 = Incubation of pupae at 31°C
3.30 = Flies fed at least once before infective feed
3.33 = Culture forms (T.brucei)
3.34a = T.brucei,C
3.34b = T.brucei,D
3.35 = Standard (T.congolense)
3.36a = Flies kept at 31°C (T.congolense)
3.36b = Flies kept at 20°C (T.congolense)
3.38 = Flies observed for 42 days
3.39 = Infectivity of fly forms
3.40 = Culture forms (T.congolense)
3.41 = Effect of several mouse passages
3.42a = T.congolense,D
3.42b = T.congolense,E
M = Male
F = Female

Table 79 Susceptibility of male and female G.morsitans to infection with T.congolense, under standard treatment.

Expt. Nos.	Sex	Infected Flies			Established Infections			Mature Infections		
		Total	No. Infected	Per Cent	Total	No. Infected	Per Cent	Total	No. Infected	Per Cent
3.17a	M	60	11	18.3	60	2	3.3			
	F	40	11	27.5	40	4	10.0			
3.17b	M	82	4	4.9	82	4	4.9	82	4	4.9
	F	68	5	7.4	68	5	7.4	68	4	5.9
3.19	M	39	14	35.9	39	6	15.4			
	F	31	3	9.7	31	3	9.7			
3.23	M	58	14	24.1	58	0	0			
	F	42	10	23.8	42	2	4.8			
3.35	M	272	52	19.1	238	17	7.1	200	11	5.5
	F	138	28	20.3	112	10	8.9	100	8	8
3.42b	M	216	17	7.8	177	5	2.8	44	1	2.3
	F	284	26	9.1	223	8	3.6	56	1	1.8
TOTALS	M	727	112	15.4	654	34	5.2	326	16	4.9
	F	603	83	13.8	516	32	6.2	224	13	5.8

Table 80 Proportions of male and female G.morsitans infected with trypanosomes compiled from data in Tables 78 and 79.

Species	Sex	Infected Flies			Established Infections			Mature Infections		
		Total	No. Infected	Per Cent	Total	No. Estab.	Per Cent	Total	No. Mature	Per Cent
T.brucei	M	982	141	14.4	839	54	6.4	375	10	2.7
	F	688	93	13.5	581	30	5.2	265	6	2.3
T.congolense	M	727	112	15.4	654	34	5.2	326	16	4.9
	F	603	83	13.8	516	32	6.2	224	13	5.8

Table 81 Susceptibility of male and female G.morsitans to infection with T.brucei under modified treatments.

Expt. No.	Sex	Infected Flies			Established Infections			Mature Infections		
		Total	No. Infected	Per Cent	Total	No. Estab.	Per Cent	Total	No. Mature	Per Cent
3.28a	M	159	50	31.4	120	13	10.8	63	6	9.5
	F	91	15	16.5	80	10	12.5	37	5	13.5
3.28b	M	178	15	8.4	133	5	3.8	73	0	0
	F	67	4	5.9	62	2	3.2	27	0	0
3.29	M	119	31	26.0	119	13	10.9	37	3	8.1
	F	131	26	19.8	131	14	10.7	63	6	9.5
3.30	M	156	28	17.9	123	8	6.5	65	1	1.5
	F	94	11	11.7	77	3	3.9	35	0	0
3.33	M	145	13	8.9	110	1	1.00	110	0	0
	F	55	5	9.00	40	1	2.5	40	1	2.50
3.34a	M	249	44	17.7	197	7	3.6	97	2	2.1
	F	251	27	10.8	203	10	4.9	103	3	3.0
3.34b	M	190	32	16.8	130	8	6.2	42	1	2.4
	F	210	35	16.7	170	9	5.3	58	1	1.7

Table 82 Susceptibility of male and female G.morsitans to infection with T.congolense under modified treatments.

Expt. Nos.	Sex	Infected Flies Total	Infected Flies No. Infected	Infected Flies Per Cent	Established Infections Total	Established Infections No. Estab.	Established Infections Per Cent	Mature Infections Total	Mature Infections No. Mature	Mature Infections Per Cent
3.36a	M	176	33	18.8	138	11	7.9	114	6	5.3
	F	124	32	25.8	62	4	6.5	36	3	8.3
3.36b	M	173	41	23.7	121	3	2.5	92	0	0
	F	127	28	22.0	79	2	2.5	58	0	0
3.38	M	92	8	8.7	92	8	8.7	92	6	6.5
	F	108	6	5.6	108	6	5.6	108	5	4.6
3.40	M	212	78	36.8	104	14	13.5	99	6	6.1
	F	143	62	43.4	86	5	5.8	76	4	5.3
3.41	M	61	15	24.6	49	6	12.2	21	1	4.8
	F	89	21	23.6	81	9	11.1	29	1	3.4
3.42a	M	209	12	5.7	170	0	0	61	0	0
	F	291	21	7.2	230	4	1.7	39	1	2.5
3.42b	M	216	17	7.9	177	5	2.8	44	1	2.3
	F	284	26	9.2	223	8	3.6	56	1	1.8

3.44 Does the number of feeds taken by flies influence the cyclical development of the trypanosomes?

In this section, the feeding records of all the flies used in Experiments 3.27-3.42 were examined. This was to ascertain whether the number of feeds taken by the flies on the maintenance host (mice) influences the infection rates in the flies. The number of feeds taken by all the experimental flies before they were dissected were recorded. A table was constructed in which flies were grouped according to the number of feeds taken. In each group, the number of uninfected flies, the number infected, the number with established infections and the number with mature infections were recorded (Appendix 6a,6b). From these data the histograms (Fig. 17a and 17b) were constructed.

It appears from these histograms that the number of feeds taken by a fly has no effect on its susceptibility to infection by T.congolense or T.brucei nor on the cyclical development of these trypanosomes. Some flies which had not fed since the infective feed were nevertheless infected. A small proportion of these possessed established infections of T.congolense. For T.brucei, the least number of feeds before an infection became mature was 4, and for T.congolense, 2. There is no evidence that very many feeds are necessary for the establishment or maturity of infections with either species of trypanosome. Only few flies had more than 8 feeds and the proportion infected amongst these was not higher than expected.

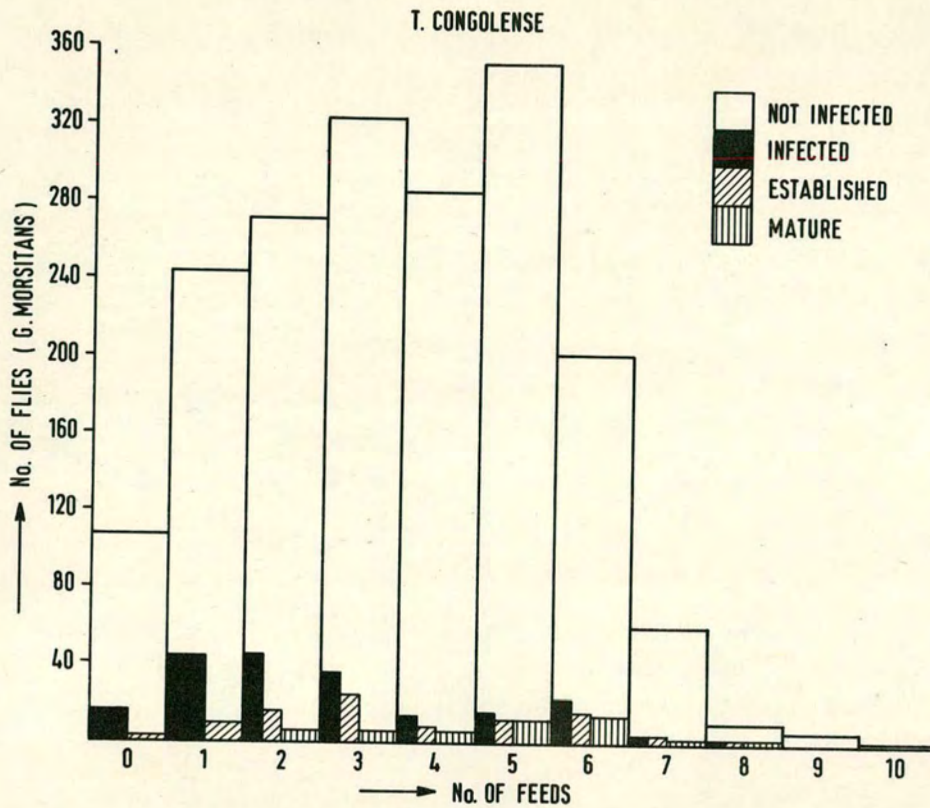


Fig.17a

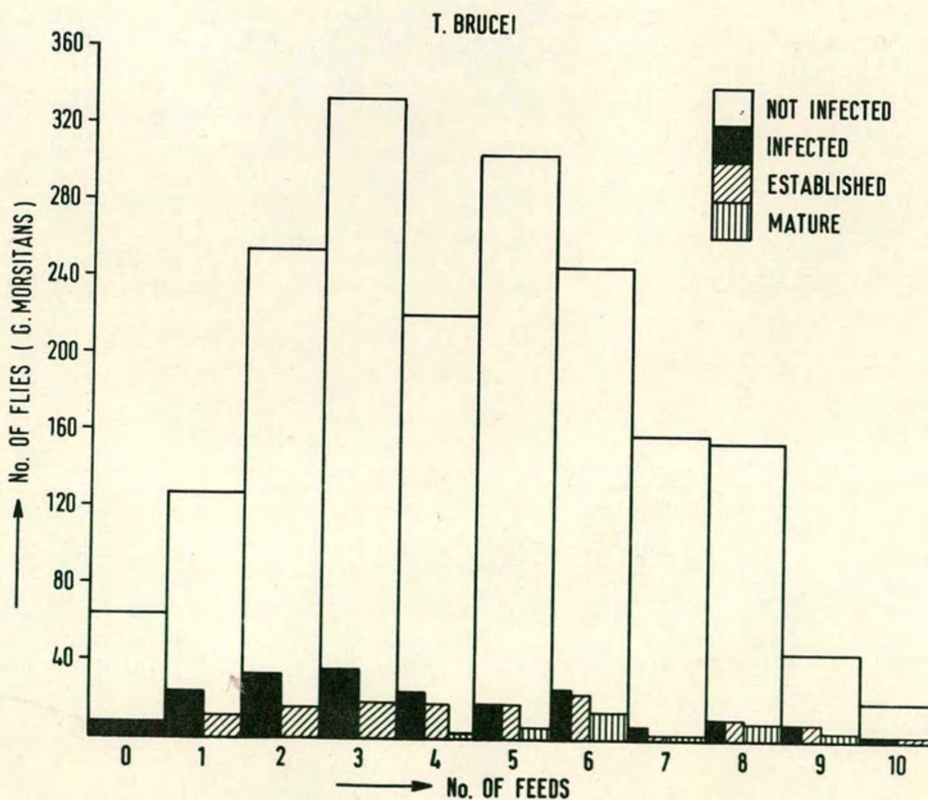


Fig.17b

The number of feeds on the maintenance host (mice) taken by _G.morsitans_ before they were dissected. Histograms constructed from Appendix 6a, b.

3.45 Is the distribution of the trypanosomes in the stabilates equal and homogenous and is the viability of the trypanosomes still maintained after a long period of preservation?

It was indicated in Experiment 3.11 that, although groups of flies (10 flies in each group) had been fed at different times on different samples of the stabilated population of trypanosomes, the distribution of the infected flies in each group appeared to be random. In this section, attempt is made to ascertain whether the distribution of the trypanosomes in the stabilates is uniform.

All the groups of flies that had fed on the same stabilates in each experiment in which the treatment was standard, were recorded and constituted a sample. The mean of the infected flies in each sample was calculated and X^2 obtained by applying Fisher's formula for index of dispersion. The X^2 of as many samples as possible within each standard experiment were thus calculated and added together. Fisher's formula was applied to the summed X^2 to test whether it accords with the binomial distribution. An example of these calculations is shown in Appendix 4 and the results of the calculations for the experiments in which flies received the standard treatments are summarised in Table 83.

Data were also collected to show whether the infectivity of the trypanosomes to the flies was maintained after a long period of preservation. The number of infected and uninfected flies among those which had fed at various times on the same stabilated population of trypanosomes under the standard conditions were recorded. These are shown in Tables 84a, b and 85 a, b. The standard result was taken as that obtained from the first stabilates to be used after preservation. Whenever the difference in the number of infected flies with any stabilate seemed large compared with the standard, the

2 x 2 table with Yates correction was used to test its significance.

It is evident from tables 83, 84a,b, and 85a,b, that the distribution of the trypanosomes in the stabilates is uniform and that the infectivity of the stabilated population of the trypanosomes to the flies is maintained after a long period of preservation.

Table 83 Summary of the calculations to show uniformity in the distribution of trypanosomes in the stabilates.

Experiment No.	No. of Table	Species of Trypanosomes	Species of Flies	Value of $\sqrt{2X^2} - \sqrt{2n-1}$	Remarks
3.11	2	*T.congolense,A*	*G.austeni*	- 2.242	Less than 1, binomial distribution
3.11	3	*T.brucei,A*	*G.morsitans*	- 2.721	Less than 1, binomial distribution
3.11	4	*T.brucei,A*	*G.austeni*	- 1.962	Less than 1, binomial distribution
3.17	16	*T.congolense,B*	*G.morsitans*	- 2.096	Less than 1, binomial distribution
3.18	19	*T.brucei,B*	*G.morsitans*	- 1.734	Less than 1, binomial distribution

Tables 84 a, b Number of flies infected and uninfected when fed on the stabilated population of *T.congolense*,B at various times.

Experiment No.	Stabilate No.	Preservation Period/Days	Total Flies	No. Infected	Percent
3.17	1,2,3	9	40	8	20
3.17	7,8	29	20	5	25
3.19	31-35	39	70	17	24
3.23	57-61	43	50	9	18

Experiment No.	Day of Dissection	Stabilate Number	Preservation Period/Days	Total Flies	No. Infected	Per Cent	X^2,P
3.17	3rd DAY	1-9	9-29	10	5	50	
3.35		81-84	145	50	32	64	1.706 $P < 0.20$
3.17	5th DAY	1-9	9-29	10	5	50	
3.35		85-88	165	50	20	40	

Tables 85 a, b Number of flies infected and uninfected when fed on the stabilated population of *T.brucei*,B at various times.

Experiment No.	Stabilate No.	Preservation Period in Days	Total Flies	No. Infected	Percent
3.18	1,2	6	30	4	13.3
3.18	3,4,5	11	40	6	15
3.18	6	26	20	4	20
3.19	53-55	79	70	9	13

Experiment No.	Day of Dissection	Stabilate No.	Preservation Period/Days	Total Flies	No. Infected	Percent
3.18	3rd DAY	1-9	6-26	10	5	50
3.27		70-73	195	50	25	50
3.18	5th DAY	1-9	6-26	10	2	20
3.27		74-76	205	50	7	14

The following experiments were carried out at various times and are reported concisely.

3.46 To test for the infectivity to tsetse flies of different antigenic variants of T.brucei,A.

Procedure A rabbit was infected with T.brucei,A. On the 5th, 10th, 15th and 20th days after infection as many flies as were available were allowed to feed on the rabbit. Flies were then separated into groups of ten and each group was dissected from days 3-12 after the infective feed.

Immediately after the flies had fed, about 5 ml. of blood was obtained from the rabbit's ear. 0.3 ml. of the blood was innoculated into each of 3 mice, the remainder was allowed to clot to obtain serum. When the trypanosomes became numerous, the mice were killed and stabilates prepared. Some of the stabilates prepared from mice, which had been infected with rabbit blood taken on the 5th and 20th day of its infection, were used to infect further groups of flies. Stabilates were also made from the rabbit's blood on the 20th day of infection.

Flies were dissected as in previous experiments from day 3-12 after their infective feed.

Results As shown in Table 86, the infection rate was highest among the flies which fed directly on the rabbit 5 days after it had been infected with T.brucei. When the stabilates of the 5th day population in mice were fed to flies through the membrane and their results were compared with the stabilates of the 20th day

population in mice, no significant difference was observed either in the number of flies infected or in flies with established infection. (Table 87).

Stabilates made directly from rabbit blood on the 20th day post infection did not infect flies when fed to them through the membrane. This could be a genuine effect or be due to a faulty technique of preservation.

Table 86 Results of the dissection of G.morsitans and G.austeni fed on various days on a rabbit which had been infected with T.brucei,A.

Treatment	Fly Species	Total Flies	Total Infected	Percent Infected	$^{+}X^2$, P
1) 5 days after infection	G.morsitans	30	8	26.7	
	G.austeni	60	8	13.33	
10 days after infection	G.morsitans	60	8	13.33	3.430 $p < 0.10$
	G.austeni	10	0	0	3.110 $p < 0.10$
15 days after infection	G.morsitans	40	4	10	4.628 $p < 0.05$
	G.austeni	50	3	6	2.546 $p < 0.20$
20 days after infection	G.morsitans	70	9	13	3.901 $p < 0.05$
	G.austeni	20	1	5	2.044 $p < 0.20$

\+ Comparison made with the proportions of flies which fed on the rabbit 5 days after its infection.

Table 87 Results of the dissection of flies fed on stabilates of 5th and 20th day populations of T.brucei,A in mice.

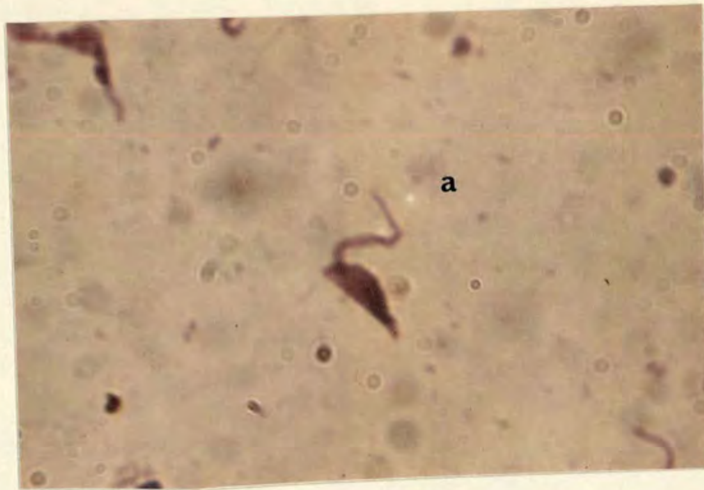
Treatment	Fly Species	Total Used	Total Infected	Percent Infected	Total Estab.	Per Cent	$^{+}X^2$, P
5th Day mice population	G.morsitans	50	19	38	6	12	
20th Day mice population	G.morsitans	50	16	32	3	6	1.953 $p<0.20$
20th Day rabbit population	G.morsitans	50	0	0	0	0	

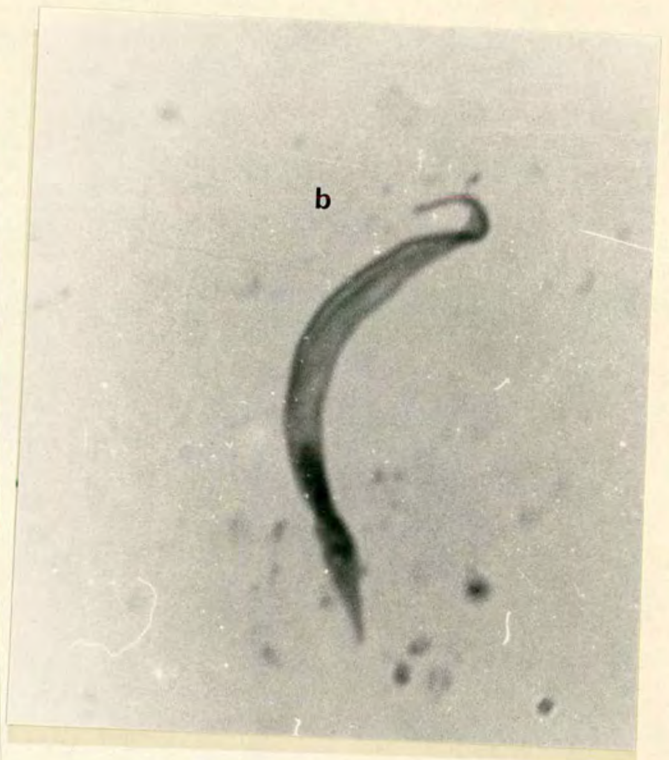
+ Comparison made with the 5th day mice population.

3.47 To test for the infectivity to tsetse flies of the first cyclical population of T.brucei.

Procedure Stabilates were prepared directly from a mouse infected by a G.morsitans which had fed on a suspension of T.brucei,A in defibrinated ox blood. A few minutes before the stabilates were prepared, blood from this mouse was injected into 6 other mice in order to obtain the T.brucei,B (Experiment 3.18). The stabilates were fed to G.morsitans, the concentration of the infective feed being 5.28×10^6 trypanosomes per ml. Flies were dissected from days 3-12 and they were maintained on mice.

Results   Of the 50 flies used 11 or 22% were infected.   4 or 8% of these posessed established infections.   These figures are similar to those obtained in the standard treatment for T.brucei,B. (Experiment 3.18).





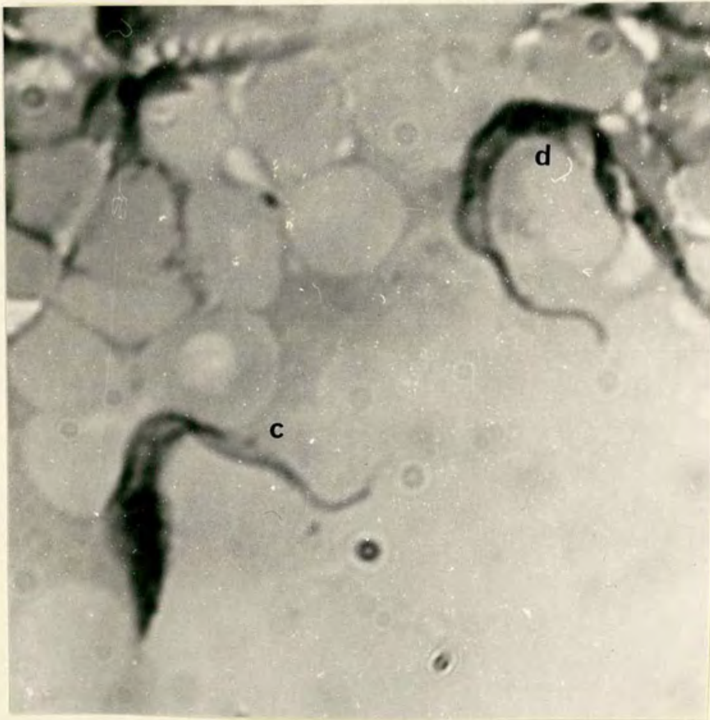


Fig.18 'Aberrant' forms of (a) T.congolense (5 days post infection); (b) T.brucei (7 days post infection found in the ectoperitrophic space. (c) is also an 'abberant' form of T.brucei in the endoperitrophic space, 5 days post infection, posterior segment. Note the typical broad midgut form (d).

DISCUSSION

4.10 Artificial feeding of tsetse flies.

Rodhain et al. (1912) were probably the first to feed tsetse flies on blood through a membrane. The benefits of this technique have been indicated by various authors (Cockings, 1960; Southon and Cockings, 1963; Lumsden, 1964; De Azevedo et.al., 1968) and it has been used to maintain large colonies of tsetse flies in the laboratory. Few attempts have been made to use this method in the study of fly/trypanosome relationship. Thus, in their pioneer work, Rodhain et.al. (1912) fed flies on blood through a guinea pig membrane, centrifuged the blood and counted the number of motile trypanosomes. This process was repeated later by van Hoof et.al. (1937c). Cunningham (1960) infected G.pallidipes by feeding them through a membrane on a suspension of T.brucei, in defibrinated ox blood with the aim of investigating if any antigenic variation takes place in trypanosomes during and after the cycle in Glossina. Wilson et.al. (1966) fed G.morsitans previously infected with T.congolense into blood through a membrane, and used the infectivity titration technique to calculate the numbers of infective metacyclic trypanosomes of T.congolense ejected by G.morsitans during feeding. The same procedure was repeated by Harley and Wilson in 1968 for G.morsitans, G.pallidipes and G.fuscipes. The membrane feeding technique was recently used by Dar (1971) in his preliminary studies on the development of trypanosomes in tsetse.

The membrane feeding technique was used throughout the present studies. Instead of infecting flies by feeding them on animals which had been infected with trypanosomes, flies were infected by

feeding them through chicken membrane on suspensions of trypanosomes in defibrinated blood. The advantages of the latter over the former method are numerous. Firstly, a stabilated population of a species of trypanosome can be used for a conveniently long time to perform various experiments. In this way, factors affecting the fly/trypanosome relationship could be investigated by subjecting the trypanosomes and the flies to different treatments. The results obtained from these treatments can be compared with those of the experiments which received the standard treatment since the same population of trypanosomes had been used to infect the flies. Secondly, the uncontrollable factors associated with feeding the flies on infected animals are removed. The concentration of the trypanosomes in the infective feed can be controlled. This is a constantly changing variable in the animal and cannot be controlled. It was shown for T.congolense (Experiment 3.41) that different populations of the same species of trypanosomes behave differently in flies. For T.brucei, it was shown that flies fed directly on rabbit 5 days after it was infected with this species of trypanosomes had a significantly higher infection rate than those fed on the same rabbit 15 and 20 days after its infection (Experiment 3.46). Consequently, results of experiments in which flies are fed on infected animals on different days are not comparable with one another. Failure to take this fact into account is partly responsible for the conflicting results obtained by the previous workers. Throughout this investigation, however, the results of experiments in which the flies received the standard treatment were reproducible even though they were performed at different times. Thirdly, it allows for a greater scope of experimental work on

trypanosomes. For example, culture forms of trypanosomes are known to lose their infectivity to animals about 4 days after their initiation. (Thomson and Sinton, 1912; Cunningham and Harley, 1961 ; Dar, 1971). Their infectivity to flies and relationship to the fly forms of trypanosomes could only be tested by feeding them to flies through the membrane (Experiments 3.33 and 3.40).

There is little or no information on the longevity of flies which obtained their infective feed through the membrane. In the present studies, it was observed that, if strict sterile procedures are maintained during the infective feed, the longevity of the flies are not adversely affected. The average rate of mortality of the experimental flies kept in the fly room ($26^{o}C$) was 3%. Moreover, flies were kept for as long as 50 days (Experiment 3.27) and some infected ones, isolated by probing, were not killed until 46 and 60 days after their infective feed (Experiments 3.27, 3.34, 3.35 and 3.42). For the purpose of investigating the development of trypanosomes in the tsetse, this longevity is adequate. It is even compared to the observations of Jack (1939) who, in a series of experiments with G.morsitans found that the mean length of life of the fly was 2 or 3 months.

Flies maintained on mice after the infective feed did not engorge regularly not because they had been previously fed through the membrane but because of their preference for rabbit as feeding host. When maintained on rabbit after the infective feed, they fed regularly. If however the infective feed was contaminated a great majority of the experimental flies died within 48 hours. Those that lived longer showed a reluctance to feed. When they eventually died, trypanosomes were absent and various types of bacteria could be

seen in their midgut. Some investigators had noticed that bacterial infection of the gut is a potent factor in determining the rate of infection in the flies. Carpenter (1912) noted in his dissection of wild flies that those with a large number of bacteria rarely contained trypanosomes. Wallace (1931) observed that bacteria, if experimentally introduced into the gut of flies subsequently fed on trypanosome infective blood, have an inhibiting effect. Johnson et.al., working at Sherifuri in northern Nigeria (1928) found that if tsetse infected with T.congolense are fed through membrane on bacterially contaminated meals, the gut trypanosomes die out. Lester, (Johnson et.al. 1928) cultivated the gut forms of T.congolense up to the 8th day, and T.brucei up to the 4th day until bacterial contamination destroyed the trypanosomes. For the success of the membrane feeding technique in the study of fly/trypanosome relationship, it is essential that sterile conditions, such as those described in Sections 2.16, 2.17, and 2.18, are strictly maintained.

The chicken skin membrane is thin, transparent, and easily penetrable by the fly's proboscis. Preliminary tests with mouse skin membrane showed that flies invariably exerted much energy in trying to penetrate the membrane with the proboscis even though the skin was shaved and the subcutaneous fatty tissues were thoroughly scraped. A similar observation was made with shaved rabbit skin by Langley and Maly (1969) and these authors consequently introduced the agar membrane.

Another advantage of the chicken skin membrane is that as many as twenty to thirty membranes could be 'cut' from a chicken and preserved for use. Only 3 or 4 membranes could be obtained from the mouse. The rabbit skin, from which many membranes could also be 'cut' is

more difficult to obtain, and the membranes more difficult to prepare. The other types of membranes, i.e. Agar membrane (Langley and Maly, 1969); Guinea pig, sheep, lamb intestine (Southon and Cockings, 1963); Sausage skin (Cockings, 1960); fibrin membrane (Kimber and Harley, 1965); and ox colon (Rogers, 1971) have not been tried in this work. Azevedo (1968) considered the fibrin membrane easy to prepare and easily penetrated by the flies. Although the agar membrane can be made very thin, it might not be convenient for offering the infective feed to flies because blood could soak through it.

The ox colon membrane recently used by Rogers (1971) has all the advantages of the chicken membrane in that many membranes could be prepared at once and stored for use.

No attempts had been made to infect flies with stabilated populations of trypanosomes in such a large scale as done in this work. It is known that trypanosomes retained their infectivity to animals after a long period of preservation. That their infectivity to flies is also retained was shown in the present studies (Experiment 3.45). Furthermore, it was shown that the trypanosomes are equally distributed in each lymph tube (Experiments 3.11 and 3.45). Consequently engorged flies have an equal chance of ingesting the same number of trypanosomes provided the infective feed is thoroughly mixed. It was further shown in Experiment 3.19 that the trypanosomes in the infective feed retained their infectivity to flies during the time required to feed several groups.

During the present studies no evidence emerged to show that the preservation of trypanosomes itself adversely affected their development

in the flies. Flies fed directly on mice infected with T.congolense,B produced similar results to those fed on the stabilated population of the same trypanosome. (Experiment 3.40, Table 71). It is however not known why the number of T.congolense in the stabilates were halved after a long period of preservation. The evidence for a faulty preservation is very slim since this was observed in all the populations of the two strains of T.congolense; and these were prepared on different occasions. It was however consistently noted that the number of active T.congolense in the thawed stabilates was few; counts showed that the post-freeze numbers were about half that of the pre-freeze. This loss of the trypanosomes in preservation did not affect their infectivity to flies. Flies fed directly on mice infected with T.congolense,B, produced similar results to those fed on stabilates made from the mouse blood immediately after the flies had fed. It would appear from Wilson's figures (1968) for the infectivity of preserved T.congolense to mice, that part of the stabilated populations did not survive.

One of the uncontrollable factors introduced into this system is the blood diluent for the infective feed. As shown in Experiment 3.11, the age of the blood, at least up to 17 days after collection from the abbatoir, does not affect the viability of the flies. Neither is the number that became infected affected. For the purpose of investigating the development of trypanosomes in flies therefore, the collection of blood once a week is within acceptable limits, although fresh blood, obtained daily, was preferred for the maintenance of large colonies of flies through membrane feeding. (Azevedo, 1968). The blood was collected from different ox each week. This, however, does not appear to have any influence since

the results of experiments which received the standard treatment were similar throughout this work, even though the experiments were set up with blood obtained from different oxen at various times. For future work, this system can be made more sensitive by obtaining blood from the same animal throughout the experiments.

As mentioned earlier (Section 2.17), it was essential that blood should be collected from the abbatoir under aseptic conditions.. Azevedo (1968) gave a long list of the bacteria found in the blood of animals collected in the abbatoir without aseptic precautions, and the role of the bacteria in inhibiting the development of trypanosomes in the flies had been pointed out earlier in this section. As for feeding stimulants, Galun and Margalit (1969) found that ATP served as a feeding stimulant for the tsetse fly. Preliminary tests with horse serum in the present studies confirmed this observation. The flies however fed readily through the membrane on fresh or stored blood without ATP provided it was warmed up to about 37°C.

Among all the types of blood that could be used as feeding diluent, the defibrinated one was chosen. In spite of its known disadvantages, it appears that for the present at least, it is the only type of blood that can be used without supplementing the infective feed. Azevedo et.al. (1968) found that glucidolytic, lipolylitic and proteolytic enzymes were missing in the defibrinated pig blood, which he had used to maintain his colony flies. They consequently had difficulty in digestion and their longevity was low. When they added these enzymes to the defibrinated pig blood the longevity of his flies increased from a maximum of 24 days to a maximum of 79 days. The recent attempt of Geigy et.al. (1971)

to maintain G.morsitans and G.fuscipes on defibrinated bovine blood after they had been infected with T.brucei was accompanied with a high mortality among the flies. Similarly the attempt to maintain infected flies on defibrinated ox blood in this work failed and had to be abandoned (Experiment 3.16). It was found that the flies were unable to digest the blood meal after repeated feeds on defibrinated ox blood. The results of the present studies however showed that as long as the flies take only the infective feed on defibrinated blood, their longevity was not impaired. No enzymes were added to the defibrinated blood to correct the enzymatic imbalance because this will introduce foreign elements into the infective feed. For the same reason, heparinized or citrated blood was avoided. For the future, lyophilized blood should be tried as a feeding diluent. If it could be proved to contain those enzymes lacking in the defibrinated blood, it will be very suitable as a feeding diluent.

4.11 The development of trypanosomes in tsetse flies.

The course of cyclical development of the brucei and congolense group of trypanosomes as described by Hoare (1933) and further elaborated by him in 1949 (Hoare, 1949), still remains partly hypothetical, although consistent with the facts of the anatomy of the fly. Another object of this study was to redefine, if necessary, the association of the different stages of trypanosome development with the site of infection.

It was shown in Experiments 3.32 and 3.39 that a high percentage of the trypanosomes ingested by the tsetse flies die within the first

24 hours. At this time, trypanosomes could be found in the midgut of all the flies that had taken the infective feed, but the number seen in each fly was small. The surviving trypanosomes multiplied and by the 3rd day post infection, a considerable number of them could be seen in the midgut of about 60-80% of the flies that had originally taken the infective feed. This shows that initially all flies are susceptible to infections with T.brucei and T.congolense; but the proportion of flies in which further development of the surviving trypanosomes takes place is less. This mortality of trypanosomes a few hours after they have been ingested by tsetse flies had been observed as early as 1903 by Nabarro and Greig (1903). Dar (1971) made the same observation when he was studying the transformation of blood stream forms of T.brucei after ingestion by the tsetse flies. He dissected flies at 0, 1, 4, 8, 24 and 30 hours after the infective feed. In this way, he was able to conclude that the long slender blood stream forms are the ones killed off rapidly in the midgut, while the surviving intermediate and 'stumpy' forms assume the typical midgut form and multiply. Since no flies were dissected before 24 hours post infection in this work, no light could be thrown on the 'form' of T.brucei that survived in the early stage. There is agreement, however, with Dar's observation that the surviving trypanosomes multiply.

Assuming that the intermediate and the 'stumpy' forms were the surviving ones which multiply, it is not known why there should be a decrease in the proportion of flies which possessed midgut infections on the 3rd day, since each of the examined flies ought to have ingested a certain amount of all forms of T.brucei.

Furthermore, T.congolense, which behaves similarly, had not been shown to possess 'stumpy' or intermediate forms. Only standardised experiments with a large number of flies dissected at early intervals as Dar did could resolve this problem.

As stated earlier, the surviving trypanosomes had multiplied to a considerable number by the 3rd day post infection. As shown in Experiment 3.17, Fig. 10 , they are restricted at this time to the posterior segment of the midgut occurring both within and outside the peritrophic membrane. This sort of trypanosome distribution we called 'category 1 infection' and is possessed by 50-60% of the flies on the 3rd day after they had ingested T.brucei or T.congolense.

Three distinct morphological forms could be distinguished at this time. These are the 'typical' midgut form, which, forming about 85-90% of the population, dominates; a small percentage of long thin forms, and a few typical blood forms. The 'typical' midgut form of both T.brucei and T.congolense is long and has a subterminal kinetoplast. The kinetoplast of the former is central; that of the latter is marginal. Dar (1971) described the 'typical' midgut form of T.brucei and T.congolense as aflagellate. In the present studies, the 'typical' midgut form of T.congolense was always found to be aflagellate, that of T.brucei was variable, some being aflagellate, others with a distinct flagellum and the rest with a small flagellum. Compared with the long thin forms, the typical midgut form is broad. The long thin forms are narrow and could be threadlike. Almost invariably, their subterminal kinetoplast lies halfway between the nucleus and the posterior end. These forms are the 'proventricular forms' of some other authors. Dar (1971) called them 'posterior long forms'. The fact that trypanosomes are found both within and outside the peritrophic membrane suggests that the

route of ectoperitrophic infection is from the open end of the tubular peritrophic membrane.

The 'crossing over' into the ectoperitrophic space seems to present no difficulty.

By about the 5th or 6th day, the typical midgut form multiplies and moves forward in the ectoperitrophic space, where they are confined except in the posterior segment of the midgut. Such an infection we termed a 'category 2' infection. The importance of the attainment of this category had been demonstrated in this work (Experiments 3.17 and 3.18). In only about 10% of the flies which had ingested trypanosomes of T.brucei or T.congolense is this category of infection attained. In the other flies, the trypanosomes soon die out or persist only in the posterior segment. As shown in the histograms accompanying Experiment 3.26 (Fig. 14), these 'persisting' trypanosomes die off progressively so that by the 10th day, post infection, any infection is almost invariably one in which the trypanosomes had moved further forwards from the ectoperitrophic space of the posterior segment. In very rare cases, as that of a fly infected with T.congolense and dissected on day 21 in Experiment 3.35, trypanosomes could be found persisting in the posterior segment for a long time. Because of the correlation between the proportion of flies in which the trypanosomes had moved forward when the flies were dissected on days 3-12 and that in which the trypanosomes had reached the mouth parts when the flies were dissected on days 13-22, the term 'established infection' was introduced to characterise any infection in which trypanosomes are found throughout the ectoperitrophic space and further forwards.

Observations made on the morphology of the trypanosomes at this time show that the midgut still contains two forms i.e. the

long thin forms and the typical midgut form. Theproportion of the long thin form was found to have increased compared with that of the 3rd day. The typical midgut form had lost the uniformity which it exhibited at the 3rd day and now consists of types varying from slender to fairly stout, with all possible transitions between them. The narrowness of the long thin form was always so distinct that the slender typical midgut form could not be confused with it.

Similar observations had been made by the earliest investigators on this field. Gray and Tulloch (1905), Koch (1905a), Minchin (1908), Roubaud (1910) and Bruce et.al. (1911a), had observed that the trypanosomes disappeared from the midgut of a great majority of the flies a few days after their infective feed. They found that in a small percentage, this initial disappearance is followed by a renewed development. Almost all of them found the period of disappearance to be 4th or 5th day after the infective feed. One of them, Roubaud (1910) found that T.dimorphon multiplied in the intestine up to 48 hours after ingestion in a modified form which he called intestinal trypanosome form. He believed this to be an ordinary temporary culture which soon died. In certain cases, however, he found the intestinal trypanosome forms of T.dimorphon multiplied rapidly and in 7-9 days invade the whole of the anterior midgut as far as the pharynx. This, he called 'infection totale' of the digestive tube, and equated it to a permanent culture. Robertson (1912), observing that there was a serious difficulty in the way of the trypanosome in its attempt to establish itself in the Glossina considered that T.gambiense might be held to be established if the gut shows trypanosomes after the 5th day in flies which have had at least one feed of clean blood subsequent to the

infecting feed. Later, the same author (Robertson, 1913b) observed that in the early days of the cycle, flies which possessed conditions that would allow establishment of infections had trypanosomes persisting and multiplying vigorously at any part of the middle or hinder intestine. She never found trypanosomes in the anterior intestine at an early period. As a result she differentiated between trypanosomes "developing" or just merely "persisting" in the midgut of flies. These observations are in full agreement with those made in the present studies except that the establishment of infections was not found to be hindered by subsequent feeds after the infective one (Experiment 3.44).

The earliest workers had also observed two distinct morphological forms of developing trypanosomes in the midgut of an infected tsetse fly. Koch (1905) distinguished a 'thick and plump' type or 'female' and the 'long narrow type' or 'male'. His descriptions fitted with the typical midgut and the long thin form recognized in the present studies. Koch had borrowed these terms (male and female) from Schaudin's work (Schaudin 1904), who had described male, female, and indifferent trypanosomes which he had found in the stomach of mosquitoes which fed on owls previously infected with T.noctuae. Koch believed that there was conjugation between the sexes in the posterior segment of the intestine. British and American investigators especially Novy (1906b) and Robertson (1912, 1913b) disagreed with this view. In the present investigation, there was no evidence of conjugation between the midgut forms of trypanosomes.

Dar (1971) also recognized the typical midgut form and the 'long posterior form' (= long thin form). He found that they are both

present in the endo- and ectoperitrophic space in the posterior region of the midgut and in the digested blood meal in the hind gut for up to 12 days post infection. He found that these long thin forms appear simultaneously as the intermediate and 'stumpy' blood forms are assuming the typical midgut form. He drew the conclusion from their presence in the endo- and ectoperitrophic space as meaning that they (the long thin forms) are the only morphological forms capable of infecting the ectoperitrophic space from the nearby open end of the tubular peritrophic membrane. Observations from the present studies however indicated that both the broad typical midgut form and the long thin form are capable of infecting the ectoperitrophic space. Indeed, as early as the 3rd day post infection, both forms are found intermingled in the ecto- and endoperitrophic space.

As stated earlier, the route of the ectoperitrophic infection appeared to be from the open end of the tubular peritrophic membrane. This is in agreement with the findings of Taylor (1932), Yorke et.al. (1933) and Dar (1971). These authors found that the ectoperitrophic infection commences at the posterior end and then spreads forward along the midgut towards the proventriculus. Even before the role of the peritrophic membrane was elucidated by Hoare (1931a and b), Robertson (1913b) had already noted that the infection in a fly generally develops in the hinder intestine from where it grows forward towards the proventriculus. There was no evidence in the present studies to show that the ectoperitrophic space was infected soon after the infective feed as Freeman (1970) had shown through histological studies. She had found trypanosomes embedded in the peritrophic membrane soon after the infective feed and suggested that

trypanosomes could penetrate the peritrophic membrane not long after the ingestion of an infective blood meal. Moloo et.al. (1970) in their recent electron microscopy study of the structure and functions of the peritrophic membrane pointed to the impossibility of trypanosomes penetrating the peritrophic membrane at the early stage except it is shown that they have the necessary enzymatic means of digesting the membrane. Dar (1971) also made histological sections of the midgut and found trypanosomes and erythrocytes 'penetrating' the peritrophic membrane. He regarded this however as an artefact of the sectioning technique owing to the considerable infolding of the membrane. In the present studies, trypanosomes were very rarely found in the anterior segment of any infected fly until about the 5th day post infection. Even then, such infections are subsequent to infections of the posterior segment and the ectoperitrophic space of the middle segment.

The importance of established infections had already been referred to. Many previous investigators in this field had used the term 'established infection' without actually defining what was meant by it. Dar (1971) was the first to propose that the criterion for the establishment of the midgut infection should be the presence of trypanosomes in the ectoperitrophic space. The results of the present studies do not agree with this criterion since it was found that the establishment of an infection is not only linked up with the invasion of the ectoperitrophic space by trypanosomes but also by the "breaking out" of the latter from the posterior segment into the ectoperitrophic space of the middle segment and further forwards.

The trypanosomes in the midgut of a fly with established infection multiply rapidly and fill up the ectoperitrophic space. It was observed from the present studies that trypanosomes of both T.congolense

and _T.brucei_ could be found in the proventriculus as from the 5th or 6th day post infection. It was observed that the long thin forms (proventricular forms) could be found in a great number in the anterior segment of the midgut prior to the infection of the proventriculus. When the proventriculus is newly infected, there is little to differentiate between the morphology of the trypanosomes found there and those of the anterior segment of the midgut except that in the latter, the long thin forms are intermingled with the typical midgut forms. The typical midgut forms are found to vary in their stoutness depending on the segment of the midgut. The most apparent variation is in the anterior segment where very many of the typical midgut forms could be almost as thin as the long thin forms. Only few stout forms are found. The proportion of the stout forms increases at the expense of the long thin forms in the middle segment; in the posterior segment the stout forms dominate, the long thin forms are fewer. In flies whose proventriculus had been infected for a long time, the proportion of the long thin forms had increased throughout the ectoperitrophic space at the expense of the stout typical midgut forms. It was observed during the dissection of flies infected with _T.brucei_ and kept for 50 days (Experiment 3.27) that the trypanosomes found in the proventriculus and in the whole length of the ectoperitrophic space of the midgut of the infected flies were almost all uniformly long thin forms.

Although dividing forms of the typical midgut forms were seen in stained preparations, the long thin forms were never seen to divide. It is therefore probable that the long thin forms are produced from the divisions of the typical midgut forms. It is probable that the variations in the stoutness of the typical midgut

form are the successive stages of these divisions until the long very thin proventricular forms are produced, which, as it appeared, are the only forms capable of invading the proventriculus. Hence, it is proposed that only those forms which are found in the proventriculus and beyond should be called 'proventricular' forms while all the various stages in the midgut could be referred to as the 'midgut forms'. This of course will still not solve the problem of differentiation as the 'proventricular' forms could move freely both ways between the midgut and the proventriculus.

These observations are in agreement with those of Robertson (1912, 1913). She noted that in T.gambiense cycles in the Glossina very slender elongated forms are produced from the broad forms, and, as in most other trypanosomes life histories, they are naturally present together in the posterior part of the midgut. She noted that the elongated slender forms are destined to pass forwards and form the overwhelming majority of the individuals in the proventriculus and thoracic intestine.

Yorke et.al. (1933) was the first to show the mechanism of the invasion of the proventriculus by the trypanosomes. He showed that they pass from the ectoperitrophic space of the proventriculus into the endoperitrophic space by penetrating through the least resistant part of the peritrophic membrane, namely through the fluid part at the point where it is being secreted from the epithelial ridge. This observation, which had been confirmed by Lewis and Langridge (1947) and Fairbairn(1958) is compatible with the histological studies of Wigglesworth (1929), and the electron microscopic studies of Vickerman and Pery (1968) and Moloo et.al. (1970). Dar (1971) suggested the possibility of the trypanosomes gaining entrance into

the endoperitrophic space through a simple 'overflowing' of the ectoperitrophic infection either at the open posterior end of the tubular peritrophic membrane, or through a break in the membrane, since in older flies, the membrane was frequently found to be broken or discontinuous. No observation could be made in the present investigation as to the method by which the trypanosomes resume their endoperitrophic position in the proventriculus since histological sections were not made. However, dissection results in the present studies did not agree with Dar's 'overflowing' possibility. Co-existing infection of the endo- and ectoperitrophic space was found only in the posterior segment of the midgut. The endoperitrophic space of the other segments were always found uninfected. The cause of the avoidance of these sections by the trypanosomes is unknown. Little is known of the nature of the chemical environment provided by the organs with which the trypanosomes get into contact during the phase of development in the fly. Wigglesworth (1929) found only proteolytic enzymes in the middle segment of the midgut. These are made up of a highly active tryptase, peptidase, and a weak amylase. Dar (1971) found the pH of midgut acid (6.0 - 6.5). It appeared that the environment of the endoperitrophic space of the middle and anterior segments is lethal to the trypanosomes. It will be of interest if more physiological work could be done on the alimentary canal of the tsetse flies so that plausible explanations of this phenomenon could be found.

The trypanosomes migrate forward from the proventriculus into the labrum via the oesophagus. If flies infected with T.congolense

are dissected at appropriate times, trypanosomes could be seen extending further from the proventriculus and lining the oesophagus. The development of *T.congolense* and *T.brucei* is parallel until the mouthparts are reached. A similar observation was made by Dar (1971). It is generally believed that trypanosomes of *T.congolense* group attach themselves to the wall of the labrum, assume the epimastigote form, multiply, some later migrating into the hypopharynx where the cycle is completed after they have changed to the metacyclics (Buxton, 1955). Observations from the present studies confirmed this belief.

It was found that the proventricular thin forms invade the labrum initially from the proximal end, from where the infection extends to the tip of the labrum. As correctly described by Lloyd and Johnson (1924) the labrum often shows a very heavy infection, the trypanosomes being loose and profuse or forming rosettes. In most cases, the infection is so heavy that the identity of each trypanosome in the labral tube could not be discerned after staining with Giemsa. But if the labrum was teased before being stained, forms resembling the epimastigote forms as described by Lloyd and Johnson (1924) are seen intermingling with the proventricular forms. When the hypopharynx was invaded, forms resembling the short epimastigotes and metacyclics were found there. Since Giemsa stain was unable to enter the narrow lumen of the hypopharynx, it is not known which of these two forms dominates in the hypopharynx. Since the epimastogotes were also seen in the labrum, it is suggested that these forms invade the hypopharynx where transformation into the infective metacyclics takes place.

It was observed that the initial invasion of the labrum from the proventriculus was very rapid. There was almost no case of a

proventricular infection of T.congolense not accompanied by a labral infection. Infection of the hypopharynx was observed to follow a heavy infection of the labrum. This confirms the findings of Clarke (1965) that there is a correlation between the extent of labrum infection and the presence of trypanosomes in the hypopharynx; the hypopharyngeal infections tending most frequently to accompany labral infections which had spread farthest down the labrum towards the distal end. Since infection of the hypopharynx was observed to proceed from the tip to the proximal end, it appeared that the invasion of the organ is accomplished after the trypanosomes had reached the tip of the labrum. Since, as earlier stated, the infection of the labrum is always heavy, it appears that this invasion is effected by "spilling" over of the trypanosomes into the lumen of the hypopharynx at its tip. Trypanosomes were never found in the salivary glands of flies infected with T.congolense. It was found that in flies which received the standard treatment after feeding them on T.congolense, there was always a correlation between the numbers of flies with established and mature infection. This suggests that once the 'barrier' against the establishment of infection is overcome in flies infected with T.congolense, the infection will probably mature. An exception however is provided by this species which had undergone several syringe passages (4.13).

As for T.brucei, it is believed that the infective metacyclics are resident in the salivary glands of the fly. Robertson (1913b) gave a detailed analysis of her observations as to the invasion of the salivary glands. She believed that the penetration into the salivary glands occur quite clearly from the hypopharynx, from where the 'slender' trypanosomes pass into the narrow duct of the salivary glands. In these organs, they settle and attach themselves

in the slightly broader cellular part or at the entrance to the glandular part, the rest of the gland being quite free from trypanosomes. Eventually they change to round-ended crithidia (= epimastigotes), which multiply and fill up large portions of the gland. Later, the trypanosome forms (= metacyclics) are produced which are similar to the blood forms. Other investigators (Lloyd and Johnson, 1924; Taylor 1932; Yorke et.al. 1933; Fairbairn and Burtt, 1946, Lewis and Langridge, 1947; Baker and Robertson, 1957; and Dar, 1971) agreed basically with this observation. Lewis and Langridge (1947) and Baker and Robertson (1957) however found that all the premetacyclic stages of T.brucei develop before the invasion of the salivary glands. The former authors described various 'post ventricular' forms of T.brucei and showed that the earliest forms to appear in the anterior station infection were thin long trypanosomes which divided unequally to produce long and short epimastigotes. The latter entered the salivary glands to produce metacyclic trypanosomes. Dar (1971) however found that the first morphological forms to appear in the salivary glands are the 'anterior long forms' and that both the epimastigotes and the metacyclics later develop there.

The observations made in the present studies are in agreement with those of Dar. As observed for T.congolense, the trypanosomes of T.brucei in the mouthparts at the initial phase of the infection from the proventriculus are proventricular forms. Later, forms which vary from the long thin proventricular forms to short forms were seen but they were always trypomastigotes. It is probable that the 'shortening' of the proventricular forms starts in the mouthparts while the actual changes into the epimastigotes and finally into the

metacyclics take place in the salivary glands. Not much information was obtained on the morphology of the trypanosomes in the salivary glands in the present investigation. A great majority of flies which infected mice did not possess trypanosomes in their salivary glands. The regularity, however, with which trypanosomes were found in the labrum of flies infected with T.brucei shows that this is not as uncommon as suggested by Kinghorn and Yorke (1912a) for T.rhodesiense. Burtt (1942) had also found trypanosome colonies in 43 of the 105 flies with infected salivary glands. Quite recently, Page (1972) made a similar observation. Unlike T.congolense, the labral infection with T.brucei was not always heavy. They do not attach themselves to the wall of the labrum and they are freely and actively motile along the labral tube.

It was found during the present studies that only a small proportion of flies with proventricular infection of T.brucei developed mature infections. This was repeated in all the four populations of the two strains of T.brucei used in this work. Even when flies were kept for 50 days after their infective feed, the proportion with mature infection had not increased. Only 2 flies developed mature infection while the infection in the other 4 flies were not mature (Experiment 3.27). Unlike T.congolense therefore, there appears to be another "barrier" for T.brucei interposed between the establishment and maturity of infections. Since, as earlier stated, the development of both species of trypanosomes is parallel up to the establishment of infections, this "barrier" must be responsible for the difference in the rate of mature infections of T.congolense and T.brucei. Taylor (1932) evaluated his results by

including the infection of the proventriculus as an indication of a maturing infection and held the view that it was not involved in transitory infections. Duke (1933c), however, disagreed with Taylor's view, and argued that there would always be some flies in which however long the trypanosomes stayed in the midgut, the salivary glands would never be invaded. Murgatroyd and Yorke (1937b), van Hoof et.al. (1937d), Burtt (1946c) all reached similar conclusions as Duke. Quite recently, Dar (1971) and Page (1972) made the same observations.

Although all flies with scanty trypanosomes of T.brucei in the salivary glands infected mice, a great majority of flies which infected mice did not possess trypanosomes in the salivary glands. This suggests that the mature form of T.brucei is resident in the salivary glands; but that in majority of flies the invasion of these organs is transitory. Failure to find trypanosomes in the salivary glands of flies which had infected animals had been recorded by Duke (1921) and recently observed by Page (1972). In the course of transmission experiments with G.palpalis, van Hoof et.al. (1944) differentiated the salivary gland infections of the brucei group of trypanosomes through their distribution, relative numbers and dimensions of the various developmental forms. They found that in T.brucei and T.rhodesiense the organisms are relatively rare and may be restricted to certain parts of the glands; they are frequently isolated rather than grouped. A similar observation for T.brucei had already been made in 1924 by Lloyd and Johnson. However, Hoare (1945) in a review of the publication of van Hoof and his colleagues, did not accept the author's conclusions because the difference was relative, and because exceptions were recorded by the authors themselves. The observations

made in this investigation are in agreement with the descriptions of van Hoof et.al. (1944). Whenever trypanosomes were found in the salivary glands of flies infected with T.brucei they were usually scanty, occurring in isolated groups and leaving long stretches of the salivary glands free in between.

Some investigators had observed visible changes in the appearance of the salivary glands of flies infected with brucei group of trypanosomes. Thus, Burtt (1942) noticed that the invaded salivary glands of G.brevipalpis assumed chalky appearance; the heavier the infections, the chalkier the appearance. Under the low microscope power, a large proportion of the infected salivary glands appeared Vandyke brown or black. Later, the same author (Burtt, 1945) made the same observations with flies infected not only with T.brucei, but also with T.congolense and T.vivax. An illustration of the difference in appearance between the 'normal' and infected glands was later given by the same author (Burtt, 1950). In the present studies no visible difference was found between the invaded and uninvaded salivary glands of G.morsitans and G.austeni.

Several figures had been given by various authors for the length of the cyclical development of brucei group of trypanosomes in the flies and had been summarised by Harley (1966-67b). These vary from 10 days for T.gambiense (Taylor, 1932) to 48-80 days for the same species of trypanosome (Murgatroyd and Yorke, 1937b). Not much is known of the development time for congolense group of trypanosomes. A development time of 21 days had been recorded for T.pecorum by Bruce et.al. (1911a). The same authors gave 20 days for T.simiae. More recently, Godfrey (1957) gave 21 days for T.dimorphon. Wilson (1968) found the cycle completed in 17 and 34 days in two strains of

T.congolense. In the present studies, the time of the cyclical development was found to vary with the strain of the species of the trypanosome and with the treatment to which the fly is subjected after the infective feed.

The results of this work cannot throw any light on the recent findings of Mshelbwala (1972) that the haemocoele of the tsetse flies could be invaded by all the developmental forms of the trypanosomes including the metacyclics. No attempt was made to look for trypanosomes outside the midgut, since by dissection at daily intervals, the sequence of the development of T.congolense and T.brucei could be progressively and systematically followed from the time of the infective feed to the time when the infection became mature. A study of the role of the haemocoele is however desirable as it might throw light on some unexplainable aspects of the development of trypanosomes in the tsetse flies.

Willett's idea of the difficulties confronting the ingested trypanosomes in the alimentary canal of a tsetse fly (Buxton 1955) had been referred to in Section 1.12. The observations made in the present investigation show that the attainment of the ectoperitrophic space through the open posterior end of the tubular peritrophic membrane is not a difficulty for the trypanosomes. The first "barrier" against development is the early mortality suffered by the trypanosomes a few hours after ingestion into the midgut. The second 'barrier' is the migration by the trypanosomes from the posterior segment forward into the ectoperitrophic space of the midgut. For T.brucei the third 'barrier' is in the attainment of maturity in the salivary glands of the flies.

4.12 The influence of temperature on the infection rate of tsetse flies.

The literature on the effect of temperature on the development of trypanosomes in tsetse flies was reviewed in Section 1.12. The results obtained in the present studies confirmed the earlier laboratory observations that the effect of temperature on T.brucei is not only upon the speed at which the trypanosomes may complete their development cycle in the fly, but also upon the proportion of flies in which salivary infections develop. Thus, as shown in the group comparisons of Table 46, there was no difference between the proportion of flies with established infections when flies were kept at 26°C and 31°C. Among the flies kept at 31°C, 11 of the 12 established infections on days 20 and 30 became mature, whereas a significantly lower proportion (4 out of 21) were mature on the same days among the flies kept at 26°C (Table 45b). It appears therefore that the temperature effect is directed at the processes of maturity of infections and not at their establishment. The extent of invasion of the salivary glands was not affected by the higher temperature since in only 2 out of the 11 flies with mature infections were trypanosomes found in the salivary glands.

The establishment of infections was prolonged in flies kept at 20°C. The duration of the development cycle in flies kept at 31°C was reduced to 12 days compared with 20-30 days of flies kept at 26°C.

Similar observations of increased rate of salivary gland infection in brucei group of trypanosomes had been made by some workers; but the temperatures at which this was noticed was higher. Lloyd (1930) noticed it at temperatures varying from 29.4°C - 36.1°C, Duke (1933) at 35 - 36.7°C, and Taylor (1932) at 37°C. The fact that a temperature rise of 5°C from 26°C - 31°C had created such a significant

difference in the proportion of salivary gland infections, and that a similar effect was observed in higher temperatures of 36° - 37°C by other workers lends support to the suggestion of Lloyd (1930) that there is an optimum temperature favouring development, and a critical temperature both above and below it. That this might be so is further shown by the recommendation of Fairbairn and Culwick (1950). As a result of their observations with T.rhodesiense and G.morsitans, they suggested that flies should be maintained at 28°C and approximately 80% relative humidity, when about 17% of both sexes should become infected; in the male flies the development cycle would take 12 days. Similarly, Fairbairn and Watson (1955) recommended maintenance of flies at 23°C for successful laboratory transmission of T.vivax. It is probable that the optimum temperature for transmission of the T.brucei,B is around 31°C, hence the upshot of the transmission rate. It will be interesting to know whether the optimum and critical temperature is a species, strain, or population property. Standardised future experiments should be able to show this.

The present results of keeping flies at 20°C are partially in agreement with those of Kinghorn and Yorke (1913). These authors showed that at a temperature of 15.5°C, the development of T.rhodesiense in G.morsitans proceeded up to a point, that the trypanosomes could persist in this stage for at least 60 days without the salivary glands being invaded, and that it was only when the flies were placed in an incubator at 26.7°C that the salivary glands were invaded. In this work also, only established infections were obtained at 20°C up to 30 days when the experiment was terminated (Experiment 3.28 Table 41).

It was shown in Experiment 3.28, (Table 45a) that the proportion of flies which had trypanosomes in the gut when killed on the 3rd day

after infection was significantly increased when flies were maintained at 31°C and decreased at 20°C. This suggests that temperature also influences the early mortality of the trypanosomes, which had been shown to take place within the first 24 hours of their ingestion into the fly's midgut (4.11). The next phase of the development, which is the establishment of the infection, is not influenced at 31°C but prolonged at 20°C. There was no evidence from these results to support Fairbairn and Culwick's conclusions (1950) that the effect of temperature differs with the sex of the fly. These authors had found that the infection rate of the adult male flies is not influenced by the temperature to which they are exposed while that of the female flies is governed by the temperature of the fly-maintenance. As shown in Experiment 3.43, Table 81 , the temperature effect was exerted both on the male and female flies.

With T.congolense, it was found that raising the temperature from 26°C to 31°C had no effect on its development in G.morsitans. Neither was its speed of development altered. Unlike T.brucei, the number of infected flies on day 3 post infection was not influenced. At 20°C, it was found, as observed for T.brucei, that the establishment of infections was prolonged and there was no mature infection by day 21. When flies were kept at this temperature for 42 days, it was found that the same proportion of flies as those kept at 26°C were mature, but the duration of the development cycle was prolonged 3 to 4 times. (Experiment 3.38). This shows that at low temperatures the development of T.congolense in G.morsitans is not inhibited but only prolonged.

Since little or nothing is known of the effect of temperature on the infection rates of T.congolense, the results of this work cannot

be discussed within the context of the observations of other workers. It is not known why this species of trypanosome behaved differently from T.brucei at 31°C but almost similarly at 20°C. It is probable that, as suggested for T.brucei, an optimum temperature also exists for T.congolense which, in this case, is higher than 31°C.

Flies, whose pupae were incubated at 31°C, and subsequently maintained at 26°C after the infective feed with T.brucei behaved exactly in a similar way to the adults whose pupae were incubated at 26°C and subsequently maintained at 31°C after the infective feed with T.brucei (Experiment 3.29, Tables 45a, b). This was not the case when such flies were infected with T.congolense. The effect of incubating pupae at 31°C confirmed Burtt's original observation (1946a). He was the first person to notice an increased salivary gland infection in the G.morsitans whose pupae he had incubated at 30°C and which had fed on T.rhodesiense. He also noticed that the flies extruded unusually high numbers of trypanosomes in their saliva during probing. In the present studies, however, little or no difference was found quantitatively in the number of trypanosomes ejected by flies whose pupae were incubated at 31°C and those incubated at 26°C. Fairbairn and Culwick (1950), continuing the investigations with the same strain of T.rhodesiense and G.morsitans, confirmed the shortening of the development period in flies whose pupae were incubated at 30°C, but observed that it is only in female flies that the infection rate is governed by the pupal temperature. In the present studies however, the infection rate of both male and female flies was the same. (Experiment 3.43, Table 81).

Burtt (1946a) quoted Vanderplank's unpublished independent observations indicating that if puparia of G.morsitans or G.swynnertoni are exposed to high temperatures, the resulting flies are exceptionally easy to infect with T.congolense. He thereby concluded that the principle entailed in the incubation of pupae at higher temperatures is applicable to transmissibility of trypanosomes in tsetse flies generally. As indicated above, this effect of increased transmission rate was not obtained with T.congolense. Since Vanderplank did not eventually publish this work, the methods he used are not known. The reason for the non-manifestation of this effect in T.congolense can be explained within the context of the other observations made in this work. The effect of increased pupal temperature is manifested in the increase of mature infections or salivary gland infections in the brucei group of trypanosomes. The proportion of established infection is not influenced. Since T.congolense infections do not mature in the salivary glands but in the mouthparts, the temperature factor may not apply to T.congolense. It is suggested that this could also be responsible for the difference in behaviour of adult flies infected with T.congolense and kept at 31°C.

Burtt (1946a) concluded that the effect is mainly on the tsetse pupae and not on the soft bodied young flies when they emerge. Willett (1964, 1966) suggested that the incubation of tsetse pupae may exert its effect in increasing infection rates in the emerged flies by depleting their fluid or nutritional reserves and so forcing them to feed earlier if they are to survive. During the present studies, attempts to give the infective feed to flies from

the incubated pupae within 24 hours of their emergence was abandoned because of their reluctance to feed. When the infective feed was offered within 36 hours - the routine adopted in all other experiments - the flies fed. It appeared therefore that the reason could not lie in the 'early feeding' suggestion of Willett.

The fact that similar effect of increased proportions of mature infections is produced when the pupal temperature or the temperature of maintenance of adult flies after infective feed is raised, shows that the effect is exerted on the processes that produce mature infections. Some abnormalities had been recognized by several workers on flies maintained at high temperatures or whose pupae were incubated at high temperature. Jack (1939), studying the effect of pupal conditions on the adult flies, found that flies which emerged from pupae kept at 30°C and 80% relative humidity show a higher water content than normal, the accelerated metabolism causing a greater rate of production of water. He also found that adult flies show a definite reduction in the water content even in a saturated atmosphere at 30°C probably due to increased excretion. He noticed that this greater loss of water is probably due to the production of metabolic water from the accelerated metabolism of fat, which takes place at this temperature. Potts (1940) noted that non-viable flies emerge from pupae kept at 31°C. Mellanby (1937) found that the ovaries of G.palpalis fuscipes bred at 30°C were permanently sterilized. An experiment set up during the present studies showed that the reproductive capability of the G.morsitans which emerged from pupae incubated at 31°C was impaired. Flies, which had hatched from incubator at 31°C were mated

and subsequently maintained on mice in the fly room. A control was set up consisting of flies whose pupae were incubated in the fly room and maintained on mice after being mated. The control flies reproduced normally, all the females amongst them depositing pupae at intervals of 8-9 days. Very few females amongst those whose pupae were incubated at 31°C ever deposited pupae. The few that did, did so only once within the 30 days for which they were kept.

All these facts suggest that there are stresses on various physiological systems of such flies; they are not physiologically 'normal'. It is possible that such flies are physiologically weak, hence the ease with which the maturity of trypanosome infections in the salivary glands is achieved. That they are weak is shown again by field reports of seasonal longevity of flies which is said to decrease during the dry season (Jackson, 1942; Nash, 1935, 1936; Buxton and Lewis, 1934). Nash (1942) had reported that puparia of both <u>G.morsitans</u> and <u>G.tachinoides</u> were particularly healthy in February in Northern Nigeria when the mean temperature was 72°F (22.2°C) and the mean range temperature 63.3 - 80.9°F (17.4 - 27.2°C).

The results of the effect of temperature on the infection rates in flies, as obtained in the present studies, are consistent with field observations. A seasonal increase in the infection rate of flies with brucei group of trypanosomes in the hotter months of the year had been frequently reported. (Kinghorn & Yorke, 1912 a, b, c; Burtt, 1942; Fairbairn and Burtt, 1946; Leggate, 1962). This seasonal increase in the hotter months was bound to be tempered by the increased mortality of flies. Jackson

(1942) found in Rhodesia that life of flies is shortest in the hottest and driest time of the year, when the average male may live only 2 weeks, and females probably twice as long. Nash (1935, 1936) showed that the hottest season, despite optimum humidity, is least favourable to long life because the maximum temperature is most unfavourable. These conclusions were earlier reached by Carpenter (1912) and Buxton and Lewis (1934). In the present studies about 8% of the experimental flies kept at 31°C died during the experiments and almost all between 20 and 30 days post infection. In fact, attempts to incubate flies at 36°C produced no useful information because of the high mortality. It seems however that this increased mortality is compensated for on the field by the increased speed of development in the fly.

On the pupal side, there is evidence that the mortality may not be high provided the temperature is not too high. Buxton (1936) in his studies on soil in relation to the biology of tsetse flies found that the soil atmosphere in northern Nigeria, even under extreme conditions of heat and drought, may not be so dry as to be unfavourable to the puparia of G.submorsitans and tachinoides. Nash (1942) showed in northern Nigeria that G.morsitans and G.tachinoides annually shift their breeding ground in March from the edge of a residual forest island (mean maximum temperature 32.4°C) to the centre (mean maximum temperature 30.3°C) in order to avoid increased pupal mortality. As shown in Experiment 3.22, Table 28, the incubation of pupae at 31°C made no difference to the pupal mortality; neither was the longevity of the adult flies adversely affected. However, the exposure of the pupae to these temperatures in the field is intermittent and it remains to be seen whether it will have the same effect as exposure to a constant

temperature.

The effect of temperature is bound to have affected the results of various investigators who had worked in several parts of Africa. Since laboratory temperatures could be regulated, this will mostly affect those cases in which wild flies were dissected in any locality, or in which experiments were set up with pupae of unknown history collected from the bush. This effect had been amply shown by Ford and Leggate (1961) in their examination of available data of 45 years in relation to the geographical position and climates of the localities, from where they came. Their results show that there is a relationship between the gross infection rate and distance from the equator. This demonstrates once again the need for controlled and standardised experiments on this aspect of trypanosomiasis.

4.13 The effect of repeated syringe passages on the development of trypanosomes in tsetse flies.

The review of literature on this aspect in Section 1.12 shows that there is abundant experimental evidence to show that cyclical development of brucei group of trypanosomes exerts a steadying influence on the trypanosomes, checking the tendencies towards variation and keeping it true to type. Thus, passage through the fly had been shown to stabilize a strain's infectivity to flies, (Duke, 1923, 1924a and b; Murgatroyd and Yorke, 1937a and b, Roubaud and Colas-Belcour, 1936), morphology (Fairbairn and Culwick, 1947), antigenicity (Broom and Brown, 1946) and pathogenecity (Murgatroyd and Yorke, 1937a; Schilling and Schreck, 1930;).

In the present studies the established fact that the infectivity of T.brucei to flies is lost after several syringe passages from

animal to animal was not confirmed. The populations of the 7th and 2nd passages of T.brucei, TREU 667 in mice after cyclical development in flies produced similar results when fed to flies. (Experiments 3.15, 3.18, and 3.27). The same similarity was noticed in the populations of the 2nd and 11th passages in mice of T.brucei TREU 1096 (Experiment 3.34). It appears that T.brucei requires a large number of repeated syringe passages before its behaviour to flies is altered. Thus, in the original Duke's experiment (1923) the T.brucei strain was maintained artificially for 2¾ years before the effect was manifested. Murgatroyd and Yorke (1937a) used for their comparison a T.brucei strain under artificial passages for 5½ years after isolation. Only the number of passages of the strains used in this work could be accurately estimated. Their period of continuous passages in animal could not be accurately determined because this was intermittently broken through low temperature preservation in different laboratories. (Section 2.10). Moreover, the morphological changes which are known to accompany such populations of the strain of T.brucei (Duke, 1924a, Fairbairn and Culwick, 1947;) were not observed in the different populations used in this work. It is very probable that the effect of loss of infectivity to flies could have been manifested if these strains were maintained longer in mice.

Unlike T.brucei, a population of T.congolense which had undergone a small number of syringe passages after cyclical development, had become almost incapable of invading the hypopharynx. As shown in Experiment 3.15 only 1 of the 10 flies with mouthpart infections had hypopharyngeal infection and infected mice. The hypopharynx of the other 9 flies had not been invaded even after

40 days. These flies were infected with T.congolense,A which is a population of T.congolense TREU 692 which had undergone 6 syringe passages after cyclical development. When the population of the 2nd syringe passage after cyclical development of the same strain of T.congolense (T.congolense,B) was fed to flies, almost all mouthpart infections were mature (Experiments 3.17, 3.35). This loss of invasion of hypopharynx after few passages was confirmed again in Experiment 3.41 with flies fed on the 7th mouse passage of T.congolense TREU 692 (T.congolense,C) after cyclical development. In the accompanying table to Experiment 3.41, a comparison of mature infections of populations A, B, and C of T.congolense TREU 692 was made. It was shown that the proportion of flies with mature infections were significantly lower in flies infected with populations A and C which had undergone 6 and 7 mouse passages respectively than those infected with population B which had only 2 mouse passages after cyclical development (Table 73). The 2nd and 11th populations after cyclical development of T.congolense LUMP 92 also behaved similarly. (Experiment 3.42). As stated in Section 2.10 no morphological difference was found between the populations of earlier and later passages of the two strains.

These results confirmed the only known report of the effect of prolonged artificial passage on T.congolense (van Hoof et.al. 1937b). These authors failed to obtain the transmission through G.palpalis of a strain of T.congolense which had been passaged 200 times through rats and guinea pig in Europe before being sent to the Congo. They found only scanty infections in the midgut of 190 and 271 flies fed on this European strain. In the present studies, infections were found up to the labrum epipharynx, only

the hypopharynx was not invaded. This tends to support the conclusion made by Duke for brucei group of trypanosomes(Duke, 1924a) that continued upkeep of a strain by direct transmission or artificial passages leads first of all to the loss by the trypanosomes of the power of invading the salivary glands; this is followed later by the complete loss of the power to develop in any part of the alimentary canal of the insect. It appears that this is also the case with T.congolense and it is evident from these results that the loss in the ability to infect flies is faster than in T.brucei.

Duke (1923) had shown that successive mechanical transmissions produce the same effect. Rodhain (1941) had shown that T.vivax 'mechanically' transmitted by other biting flies (Stomoxys, Tabanidae), becomes non-transmissible. If this effect is general for all species of trypanosomes, the explanation of the cases of 'non transmissible' strains which abound in literature without reference to their histories, could be found. In a localized endemic area, where Glossina and other blood sucking flies especially Tabanids are in close contact with infected animals, a situation like the 'old laboratory strain' may occur through repeated mechanical transmission. T.congolense and T.vivax are also known to produce chronic wasting disease in the animals. It is however not known whether such a long sojourn of trypanosomes in an infected animal is equivalent to repeated syringe passage in the laboratory. That this is so had been shown for T.gambiense by Duke (1930) and confirmed by van Hoof et.al. (1938) and van Hoof (1947). But their observations were made from uncontrolled experiments. Only controlled standardised experiments can resolve this question.

4.14 The age of the fly at the time of the infective feed.

Many investigators had observed that flies, which take their first meal in the first or second day after hatching, are much more easily infected by a trypanosome of the brucei-group than older flies (Stuhlman, 1907; van Hoof et.al., 1937a; Wijers, 1958a; and Harley, 1971). However, Dar (1971) could not find any difference between the older and teneral flies in their susceptibility to infections with T.brucei. Observations had not been made on this effect in flies infected with T.congolense.

In the present studies, the indication is that, for T.congolense, the older flies are as good intermediate hosts as the newly emerged flies, since as recorded in Experiments 3.13 and 3.21, the proportions of flies infected and flies with established infection were similar in both. Since no experiment was carried out to see whether the mature infections would also not be affected, only a cautious conclusion could be drawn.

When young and older flies were fed on T.brucei a significantly lower proportion of established infections was produced in the latter than the former; but the proportions of established infections which became mature in both first feed and older flies were the same (Experiment 3.30 Table 45b). This suggests that this influence is exerted on the processes leading to the establishment of infections and not to those leading to mature infections. This observation is not in agreement with that of Dar (1971) who could find no difference in the infection rates of teneral and older flies fed on T.brucei. Two reasons might be responsible for this discrepancy. Firstly, he used only 50 older flies for his comparison. In the present studies 370 older flies

were used. Secondly Dar's criterion for establishment of infection was the attainment of ectoperitrophic infection. In the present investigation, an infection is only established if the trypanosomes had invaded the ectoperitrophic space of the middle segment of the midgut or further beyond. Hence, fewer flies are likely to be counted as established in these studies than in Dar's.

Willett (1966), having found in his studies that the secretion of the peritrophic membrane of a newly emerged fly is extensive, rapid, and soft at the time of its first meal, suggested that brucei group of trypanosomes may be able to penetrate this soft part of the membrane and gain entrance into the ectoperitrophic space in which they develop. The present work had shown however that the attainment of the ectoperitrophic space through the tubular opening of the peritrophic membrane is easily accomplished. The explanation of Willett can only be adjusted within the context of the present results to mean that trypanosomes which succeed in 'penetrating' the peritrophic membrane of the newly emerged fly into the middle segment or anterior segment of the midgut could initiate an established infection. As earlier indicated however, only very rarely were trypanosomes ever found in the middle or anterior segments earlier than the 5th day post infection. Furthermore, Willett's explanation could not be used to explain the fact that this effect is not manifested when T.congolense is fed to older flies.

It is difficult to estimate the influence of the age at which the wild flies obtained their infective feed on the infection rate on the field in view of the other factors involved. Jackson

(1946) found that in nature, most of the freshly emerged flies remain inactive for a day or two on the underside of small branches with their heads directed outwards; but in the laboratory they will feed on the day after emergence. Hence, there is a possibility that the observed higher infection rate in newly emerged flies might not occur in the field. The same author, (Jackson, 1942) found that at the height of the dry season, a just emerged fly has no great expectation of life. He also found that the cause of high mortality at this period was due to the failure to meet with food sufficiently often. Hence, a newly emerged fly might be disposed to easier infection with brucei trypanosomes, but the chances of its being manifested on the field are slim.

4.15 The influence of the number of trypanosomes in the infective feed.

There are conflicting views on the role of the level of parasitaemia of the infective feed on the infection rate in flies. While Baker and Robertson (1957), working with T.rhodesiense and T.brucei in guinea pigs and G.morsitans and Ward (1968) working with G.austeni and T.brucei observedthat the infection rate was not correlated with the intensity of the parasitaemia of the infecting mammal; Dar (1971) and Page (1972), each working with T.brucei and G.morsitans found that the infection rate of flies was significantly increased when the concentration of trypanosomes in the infective feed was increased.

In the present studies, it was found for both T.brucei and T.congolense that a tenfold increase in the concentration of

trypanosomes in the infective feed did increase the proportion of infected flies at day 3, but there was no difference in the proportion of infected flies or in the type of infection in flies killed later at the 10th day post infection. (Experiments 3.20, 3.32 and 3.39). The disagreement between these results and those of Dar, (1971) who also obtained his information from a controlled experiment with a standardised infective dose, is probably due to the difference in the criterion for recognizing established infections. Since in his experiment, Dar had dissected his 50 flies 10 days after the infective feed, it was probable that he had counted as 'established' all infections that will be regarded only as 'infected' in the present work; hence the difference in the conclusions reached. Page's results (1972) were obtained by feeding flies on mice during different waves of parasitaemia; hence the experiment was uncontrolled. Dar (1971) conducted a similar experiment on rat infected with T.brucei. He found no difference in the infection rate of flies which fed on different occasions although the parasitaemia had fluctuated during the various feeding periods.

Wijers and Willett (1960), working with T.gambiense and G.palpalis had stated that it was the absolute numbers of short stumpy forms in the infective feed, and not the total numbers of trypanosomes in the blood that influences the infection rate. This was a confirmation of views first made by Robertson (1912, 1913a) and subsequently confirmed by some other investigators (Reichenow, 1921; Hoare, 1940; Ashcroft, 1957, Wijers, 1959; Vickerman, 1965; and Page, 1972). However, Baker and Robertson, (1957), Ward, (1968), van Hoof (1947) and Dar (1971) found that the infection rate is not linked up with the morphology of the parasites

in the blood. Mshelbwala (1967) had indicated that G.palpalis could become infected from a blood meal of T.gambiense apparently containing only long thin forms.

In the present work, differential counts of the various forms of trypanosomes in the strains of T.brucei used were not made; hence this factor was not tested. It was however observed that the two strains were pleomorphic almost to the same degree with a greater proportion of long thin forms than the stumpy and intermediate forms. (Section 2.10).

4.16 The strain of the trypanosome species.

It is now a well established fact supported by experimental evidence, that there are differences between strains within every species of trypanosome as firstly observed for T.gambiense by Robertson (1912). Van Hoof et.al. (1938) found that about 5% of the 100 strains of T.gambiense, which they tested, were not transmissible by G.palpalis. The same authors van Hoof et.al. (1938) compared the infectivity to G.palpalis of various strains of T.gambiense and found considerable differences between them, their 'index of cyclical transmissibility' varying from 0 to 10.2. In only 12 out of 19 strains which Duke (1928) tested were the development cycle completed. Taylor (1932) however was successful in transmitting all the 26 strains of T.gambiense through G.tachinoides in Northern Nigeria and he recorded infection rates in the salivary glands ranging from 0.6 to 9.8%.

In the present studies, it was found that the 2 strains of each of the species used behaved differently when fed to G.morsitans. For T.congolense, the number of infected flies and of established

and mature infections were significantly higher in one than the other (Experiment 3.42). More pronounced was the difference in the duration of the development cycle in the flies and the prepatent period in mice. In _T.congolense_ TREU 692, the cycle was completed in 7-10 days and the prepatent period in mice was 8 days; with _T.congolense_ LUMP 92, the cycle was completed in 17 days and the prepatent period was also 17 days. The cyclical population of one strain was very virulent to mice, (Experiment 3.17), that of the other was not. However, the populations of both strains, stabilated after several passages through mice, behaved similarly as regards inability to produce mature infections.

The two strains of _T.brucei_ were transmissible, but the proportions of flies infected, and flies with established infection were higher in one than the other. The proportions of the established infection which became mature were similar in both strains. As observed for _T.congolense_, there were differences in the duration of the developmental cycle, being 20-30 days for one and 15-18 days for the other (Experiments 3.27 and 3.34); the prepatent period in mice was also different. The mode of invasion of the salivary glands was similar in both strains, few salivary glands with scanty trypanosomes having been obtained with both strains.

It is evident from the above comparisons that the character of a strain is determined to a great extent by the 'population' of that strain. While such characters as the length of the developmental cycle in flies and the prepatent period in animals reside in the strain, the ability to produce mature infections in flies appears to be resident in the population. Failure to recognize

this fact must have been a handicap in the studies of trypanosomiasis. It is almost certain that the so called non-transmissible strains were just populations of originally transmissible strains. A particular population might have become non-transmissible through a combination of factors while other populations of the same strain may still be transmissible. This brings out again the essence of a full knowledge of the history of the trypanosomes being used in an investigation.

4.17 The infectivity to flies of the culture forms of trypanosomes.

Very little investigation had been made on the infectivity to flies of the culture forms of trypanosomes. Thomson and Sinton (1912), Reichenow (1939) Gordon and Miller (1961) and Dar (1971) all reached the conclusion that although cultures of trypanosomes are capable of undergoing complete development cycle in Glossina, the infection rates in the flies are not increased. The results of the present studies, as shown in Experiments 3.33 and 3.40, agree with this conclusion. But their observation that the culture forms produce an initial higher rate of midgut infections was only confirmed for T.congolense and not for T.brucei.

For T.congolense, it was found that flies, which had been fed on the cultures harvested 3 and 9 days after initiation and dissected on days 3 and 5 after the infective feed, showed infection rates which were significantly higher than flies fed directly on infected mice (control) or flies which had fed on the stabilated population of the same strain. (Experiment 3.40, Table 71). With T.brucei however, it was found that the proportion of flies infected on the 3rd day after ingestion of culture forms was related to the interval

of harvesting. The proportion of infected flies with cultures harvested 3 days after initiation was similar to that of flies fed on blood forms and given the standard treatment. With cultures harvested 11 days after initiation, the proportion of infected flies was significantly lower. ($P < 0.02$). This suggests that for T.brucei, the age at which the cultures is harvested is one of the determining factors of its infectivity to flies. It was further observed that the development cycle was prolonged and the prepatent period in mice became longer in flies which had fed on the cultures of T.congolense. These were not manifest in T.brucei.

Many previous workers had assumed that the culture forms and the fly midgut forms of salivarian trypanosomes are similar. The recent studies of Dar (1971) however had contradicted this assumption. Vickerman (1969) reported on the ultra-structural similarities between the midgut and culture forms. Dar (1971) however showed that the midgut forms are morphologically a more homogenous population than the culture forms, the homogeneity of form of the latter being strictly confined to the trypanosome population in the log phase of growth. It was also assumed that the similarity of culture and midgut's trypanosome forms is borne out by their non-infectivity to mice; but as Dar (1971) again showed, their respective loss of infectivity had a variable time scale. He found that while the infectivity of midgut forms to mice is lost within 4-8 hours post ingestion, that of the culture forms is retained for 4 days post isolation in vitro. This observation, which had been made before by Thomson and Sinton (1912), Cunningham and Harley (1961), was recently confirmed by Miss Cunningham (personal communication). Evidence of indirect

relationship between the two forms of trypanosome was also provided by Reichenow (1939) who found that *G.gambiense* and *T.congolense* strains with long animal passage histories could not be isolated in culture media and were not transmissible by the tsetse fly. Lwoff and Ceccaldi (1939) showed however that a *T.gambiense* strain, although non-transmissible after 2 years of passage in animals, could still be cultured in von Razghas medium four years later.

The results of the present investigation confirmed Dar's conclusion (1971) that the similarities between the culture and midgut forms of trypanosomes are not as rigid as assumed by the previous workers. If both forms are so rigidly similar, it should be expected that the culture forms are 'pre-adapted' to development in the fly and that the duration of the developmental cycle should be shortened. It should also be expected that the midgut infection rate would be high. The latter expectation was fulfilled in *T.congolense* but not in *T.brucei*. The former expectation was not fulfilled at all and in fact, the duration of development cycle of culture forms of *T.congolense* was prolonged.

As earlier stated, the observation made in this investigation that the proportion of flies infected on the 3rd day after ingestion of culture forms of *T.brucei* was related to the interval of harvesting is in conflict with that of the previous investigators. The experiment from which the observation was made was not repeated. It may therefore be a chance result or be due to a faulty technique. More future work should therefore be devoted to this aspect of trypanosome/fly relationship, using perhaps different types of culture media.

4.18 The infectivity to mice of fly forms.

Many of the earliest investigators in the field of trypanosomiasis had observed that fly forms of trypanosomes are no more infective to animals (Bruce, 1903; Koch, 1905a, Gray and Tulloch, 1905; Prowazek, 1905; Bouet, 1907, and Minchin, 1908). They gave times of loss of infectivity varying from 24-120 hours after the infective feed. This observation had been confirmed more recently by Broom and Brown (1937) and Dar (1971).

In the present studies, fly forms of T.brucei and T.congolense were found to be no more infective to mice 48 and 72 hours after ingestion of the blood stream forms (Experiments 3.32 and 3.39). Dar (1971), who had innoculated midgut forms into mice at intervals of 1, 4, 8, 24 and 30 hours post ingestion, found that the midgut forms lose their infectivity to mice within 4 hours, while those in the crop may remain infective for up to 8 hours. Since midgut forms were not innoculated into mice before 48 hours after the infective feed in the present investigation, Dar's observation could not be confirmed.

Experimental evidence had established the fact that this loss of infectivity of fly forms is due to the loss of 'proteinaceous' surface coat found on the infective bloodstream and metacyclic trypanosomes as a result of the action of the proteolytic enzymes in the fly's midgut. Wigglesworth (1929) had shown that the middle segment of the midgut contains some active proteolytic enzymes which makes the environment markedly acid in pH. Lumsden et.al. (1965) demonstrated a rapid loss of infectivity of bloodstream trypanosomes in vitro at pH 6.5. Vickerman (1969) in his studies on the ultrastructure of trypanosomes showed that the 'proteinaceous' surface coat found on the infective bloodstream and metacyclic

trypanosomes was absent in the non-infective culture and fly midgut forms. He also showed that the coat on the infective forms could be removed by proteolytic enzymes *in vitro*. It appears therefore that the pH effect in the midgut was operative in the removal of the 'proteinacious' surface coat and possibly in the blocking of synthesis of a new one.

The observation of Broom and Brown (1937) that infectivity to mice was retained in the midgut for 48-72 hours could not be confirmed in this work. These authors linked the loss of infectivity of the midgut forms with the acquisition of a negative surface charge by the trypanosomes. In one instance, a mouse into which they have innoculated a 3 week old midgut infection became parasitaemic! The fact that their results had not been confirmed suggests that the cause of the loss of infectivity may not lie in the loss of the positive surface charge by the midgut forms.

4.19 The infectivity to flies of different antigenic variants of *T.brucei*.

No attempt had been made by previous workers to correlate the infection rate of a species of trypanosomes with its antigenic variants in the blood of the animal host. The results obtained for this treatment in this work can therefore not be discussed within the context of those of other investigators.

As shown in Experiment 3.46, the infection rate was highest in flies which had been fed directly on rabbit five days after it was infected with *T.brucei*. When the rabbit's blood was injected into mice and stabilates made from the parasitaemic blood, no

difference was found between the infection rates of flies which fed on the 5th and 20th day trypanosome population. This discrepancy in the results of both procedures is probably due to the difference in the method used in infecting the flies. The variables associated with the rabbit, such as the fluctuations in the number of trypanosomes in the peripheral blood and the rabbit's antibody response could not be controlled when the flies were fed on the rabbit. These factors were eliminated when the flies were fed on the stabilated population of the same antigenic variants as those in the rabbit blood through the membrane.

Gray (1962) and Wilson (1968) had shown that different antigenic variants of T.brucei are produced at intervals of 5-6 days. Hence, the blood on which the flies had fed on the different days probably contained different antigenic variants. Their serological typings could not be accurately determined due to an accident with the liquid nitrogen refrigerator which killed off all the preserved trypanosomes before it could be done. In a future work, more information could be gained on this aspect if standardised experiments are set up which will neutralise the effect of the host's antibodies and the fluctuations of trypanosome concentration in its blood. Membrane feeding system, with a large number of experimental flies may be the only answer.

4.20 Is there a difference in the susceptibility of different species of tsetse flies to trypanosomes?

Information on the differences in the susceptibility of different species of tsetse flies to trypanosome infection had been derived more from speculation than from experimental evidence. Observations made

made on the field had not taken the ecological association, host preferences and the environment of the fly species into account, whereas Leggate (1962) had shown theinfluence of the environmental conditions of the site from which G.morsitans was collected on its infection rate. Moreover, most of the field observations had been made on the morsitans and palpalis groups of Glossina, which by reasons of their habitat are more accessible to the investigators. The fusca group which inhabits the forest had been neglected although it had not been experimentally proved that they are less susceptible to infection with trypanosomes. In fact, Lewis et.al. (1943) had reported the experimental transmission of T.congolense by G.austeni. Vanderplank (1945) had found a female G.austeni infected out of the 1000 wild tsetse he and his African assistant had dissected at Kingolwira, near Morogoro, in Tangayika.

In the present investigation, it was found that when G.austeni and G.morsitans were fed on T.congolense and T.brucei and given the standard treatment, the proportion of flies in which trypanosomes were found on the 3rd day after the infective feed was significantly lower in G.austeni than in G.morsitans (Experiments 3.31, 3.37). With T.congolense, no significant difference was found between the two species of Glossina in the proportions of established and mature infections (Experiment 3.37). The duration of the development cycle of T.congolense in G.austeni (18-19 days) was however about double that in G.morsitans (7-10 days). With T.brucei, the proportion of flies in which infections became established was significantly lower in G.austeni than in G.morsitans. This difference was however not noticed until large numbers of flies were used for comparison (Table 46). The mature infections could not be compared

as none of the 50 G.austeni fed on T.brucei was infected. This could have been probably possible if large number of flies had been used.

Since information on this aspect with T.congolense is very scanty, the results obtained for this species cannot be discussed within the context of the previous works. Even with T.brucei, where more work had been published, most of the comparisons had been made with G.morsitans and G.palpalis. Only Dar (1971) had reported feeding 10 G.morsitans and 10 G.austeni on a mouse infected with T.brucei, and he found no difference in the susceptibility of the two species to infection. Needless to say, these numbers were too few to make valid conclusions. Comparisons made by previous workers on G.palpalis and G.morsitans suggests that the latter is a more efficient vector of trypanosomes of brucei group (Duke, 1933a; 1936). However, Leggate (1962) dissected wild flies in the Rekomitjie Research Station in Southern Rhodesia and found that, although G.morsitans showed a considerably higher rate of mature infections of vivax and congolense group of trypanosomes than G.palpalis, the salivary gland infections in the latter (9.7%) was significantly higher than in G.morsitans (2.8%). The results of the present experiments with T.brucei cannot be compared with these previous observations not only because different species were used but also because different terminologies were used in differentiating 'infected' flies and 'mature' infections.

It was observed that the trypanosomes do not discriminate between species of flies in their sequence and mode of development. Thus, both T.congolense and T.brucei were subjected to the early mortality in both species of the flies and the 'barrier' of establishment of infection was still presented as well. The

lengthening of the duration of the development cycle of T.congolense in G.austeni is probably due to physiological differences between the two species of flies in the mouthparts where maturity of infection is accomplished. It is not likely to be due to any extrinsic or environmental factor as these could have affected both fly species equally, since both were given standard treatments.

The fact that after a simultaneous infective feed on the same pool of infected blood, flies maintained on the same host do not all become infected shows that within the species itself differences in susceptibility to infection exist in individual flies. Even among the flies that eventually developed mature infections, individual differences were noted in their capability to transmit the trypanosomes to animal host and in the number of trypanosomes ejected during probing.

For future work, it is recommended that larger numbers of flies should be devoted to the studies of this factor and standardised procedures should be adopted. The conclusions drawn for T.brucei in this work are uncertain because not enough flies were used to test the differences in mature infections. The conclusions for T.congolense are limited by the fact that only one strain was used. Such studies could be expanded so that many strains could be compared using many different species of tsetse flies. The economic importance of the fusca group of trypanosomes had been shown by Page and Jordan (1958). They found high infection rates of vivax and congolense group of trypanosomes in G.fusca, G.nigrofusca, and G.medicorum in their dissection of wild flies collected at Ugbobigha and Olokemeji in Nigeria. They have found these species to have left their confines of the high forest and to have penetrated

the savannah in many localities.

As for differences in the transmitting power of different races of Glossina, only Duke (1933) had reported no difference. His observation was based on dissection of wild flies collected from two different localities. It is not known whether the same conclusion could be drawn under a controlled experiment.

4.21 The infectivity of male and female flies

It was observed during the present work that there is neither a significant difference in the proportions of infected flies nor in those of established and mature infections between the male and female flies which received the standard treatment after infecting them with T.brucei and T.congolense. In the course of the experiments, it was at first concluded that the female flies are more susceptible to infection with trypanosomes than males. In the calculations that led to this earlier observation, the two by two table was used and the figures for male flies were placed as the nominator (Experiment 3.25). It was however later realised that if the order were changed by placing the figures for females as the nominator, the values of X^2 showed no significant difference between the infection rates of both sexes. It was also noted that the comparisons used then involved treatments other than the standard. In the latter calculations therefore, (Experiment 3.43) only the results of experiments which received the standard treatment were considered and calculations were made with male and female flies as nominator alternatively. In neither case was any significant difference shown between the two sexes.

A great majority of the group of workers who reported greater infection rate in female than males obtained their results from the dissection of wild flies (Squire, 1951; Page and Jordan 1958; Harley, 1966a, 1966-67b, and Leggate, 1962). Each of these results was bound to be influenced by the environmental conditions prevalent in the locality from where the flies were collected. Ford and Leggate (1961) had shown that published results of workers were influenced by the distance of the area of investigation from the equator. They showed that there was clearly a tendency for infection rates to increase, the further the departure from the middle of the *Glossina* zone. Most of the results from the laboratory had suggested a greater rate of infection in male than female flies (Corson, 1935; Burtt 1946; van Hoof 1947). In their experiments, these authors had fed flies on different occasions on infected animals, a procedure which, as previously stated, introduced uncontrollable variables associated with the animal into the experiment.

It is of importance to note that Dar (1971) who used standardised conditions similar to those of the present studies found no difference in the susceptibility of the sexes to infection with trypanosomes. His conclusion, which was limited by the few flies he used, is however now confirmed in the present studies where larger number of flies are used. Fairbairn and Culwick (1950) analysed their results statistically and found a significant difference between the infection rates of male and female flies. The discrepancy of their results with those of the present studies is probably due to the fact that they conducted their analysis on the cumulative data from different experiments, each of which was not controlled.

Comparisons within the modified treatments were also made and, as shown in Experiment 3.43, the infection rates in male and female flies infected with T.brucei and T.congolense are similar. The conclusion of Fairbairn and Culwick (1950) that the effect of temperature differs with the sex of the fly was not confirmed. As shown in Experiment 3.43 (Table 81) the temperature and the incubation of pupae at 31°C exerts similar effect on both male and female flies.

The conclusions drawn from these results are limited by the fact that only one strain each of T.brucei and T.congolense was used to test this factor. Moreover, accumulated data from results of experiments which had received the standard treatment at different times was used because large number of flies of the same sex were not readily available at any time. This probably did not influence the results since, as shown in Section 4.10, the results of experiments which had received the standard treatment are reproducible and the main uncontrollable factor associated with the standard treatment - defibrinated ox blood obtained from different ox - does not influence the results of the experiments. But still, it will be possible to make the results more sensitive if enough flies of both sexes can be devoted at one time to a standardised experiment to test this factor. Only in laboratories, where large quantities of flies are bred can such an experiment be undertaken.

4.22 The influence of maintenance hosts and the number of feeds taken by flies on the cyclical development of trypanosomes.

Investigations were not numerous on the role of the species of the host on which flies are maintained after the infective feed. Roubaud (1911), Duke (1921) and Dar (1971) found that the host on which the flies fed had no influence upon the development of trypanosomes in the tsetse flies. In the present work, no significant difference was found in the susceptibility of *G.morsitans* and *G.austeni* to infection after they have fed on *T.brucei* and *T.congolense* and maintained on rabbit and mice. In an early experiment (Experiment 3.12) a borderline significance was found in the infection rate when *G.morsitans* fed on *T.brucei* were maintained on rabbit and on mice. ($P<0.05$). When this experiment was repeated however (Experiment 3.24) no significant difference was obtained ($P<0.20$).

These results do not agree with the conclusions of Harding (1946) and the recent findings of Geigy et.al. (1971). The former author obtained 3 salivary gland infections out of ten surviving *G.tachinoides* after they had been fed once or twice on guinea pig previously infected with *T.brucei* and maintained on man or on citrated human blood. Geigy et.al. (1971) found that *G.fuscipes* infected with *T.brucei* gave a higher infection rate when maintained on bovine blood, and *G.morsitans* infected with the same strain of *T.brucei* a lower infection rate when maintained on bovine blood, than when both species of flies were maintained on human blood. The discrepancy between these observations and those of the present studies could be attributed to differences in procedures. In the present studies, a standardised technique of membrane feeding was used to infect the flies and they were subsequently maintained on

animals. Harding (1940) and Geigy (1971) infected their own flies by feeding them on infected animals and maintaining them on blood of the different animal hosts through the membrane. Hence, their experiments were beset with a high mortality rate typical of the one that led to the abandonment of such method in the present work (Experiment 3.16). This high mortality rate had hampered a proper evaluation of their results. These discrepancies could be best resolved if the membrane feeding technique could be improved so as to make it possible for flies to be maintained for a long time through it. Together with such an 'improved' membrane feeding technique, a control experiment of maintaining the infected flies on the animals must be set up since the constituent of blood fed to the flies through membrane is always altered to prevent clotting.

Very little is known of the effect of the number of feeds of a fly on the maintenance host on the development of trypanosomes. Robertson (1912) subjected flies, which she had infected with *T.gambiense* to absolute starvation till the 12th day post infection and simultaneously set up a control, feeding them every 2-3 days. She found 22 of the 103 (21.3%) starved flies and only 3% of the control flies infected. Later in 1913, the same author, (Robertson, 1913b) fed flies again on monkey previously infected with *T.gambiense* and maintained them on a clean cock, killing them after a suitable interval without a second clean feed. She found that not only could trypanosomes survive and multiply in the gut in the blood retained from the first feed in some flies, but also that the whole material of the first feed might be displaced from the gut by the 2nd feed, and the trypanosomes still persist.

In the present work, trypanosomes were found in the lumen of the tubular peritrophic membrane for as long as any fly remains infected, and, as shown in Experiment 3.44, Fig.17a,b, the number of feeds taken by a fly has no effect on its susceptibility to infection by T.congolense or T.brucei nor on the cyclical development of these trypanosomes. These conclusions did not agree with the earlier observations of Robertson (1912) but the fact that she could not repeat the results suggests that her earlier observation was based on a chance result. For T.brucei, the least number of feeds before an infection became mature was 4 and for T.congolense 2. This difference was due to the longer period for which flies infected with T.brucei were kept under observation.

The observations made by Taylor (1932) and Yorke et.al. (1933) that the endoperitrophic infection was cleared by the trypanocidal effect of the post infective feeds could not be confirmed in the present studies. Robertson (1912) had made the same observation earlier when she attributed the increased infection rate in starved flies to a "clearing out" effect exerted by the first clean feed on the trypanosomes in the midgut of the fly. The fact that the trypanocidal activity of sera of most laboratory animals and of some wild animals had previously been demonstrated in vitro against the midgut forms of T.congolense by Lloyd (1930) strengthened the arguments in favour of the observations of these authors. In 1931 however, Adams (1931) found the same trypanocidal action of certain sera on brucei group of trypanosomes but also showed that the same sers when de-complemented were no more trypanocidal in vitro, and that the complement activity of the ingested blood was lost within 10 minutes. His conclusion was that the midgut of tsetse flies contains an anti-complementary substance which preserves the midgut infection. Since the midgut of flies used in this work

was not inspected for anti-complementary substance, this point could not be clarified. It will however be of interest if controlled and standardised experiments can be set up to relate the proportion of flies which possess the anti-complementary substance to the rate of trypanosome infection.

4.23 Transmissibility factors and Infection rates in flies.

Several investigators had tried to link the factors which influence the development of trypanosome in tsetse flies either to the trypanosomes or to the flies or to the environment. Emerging terms like 'fly-linked', 'trypanosome-linked', and 'environmental factors' tend to create the impression that these so called 'factors' are independent. The results of this work have shown that these factors are interwoven and agree with a remark by Hoare (1931b), who, while discussing this subject wrote

"Probably however, the insect is not the only factor influencing the development of the trypanosome. It is more likely that its capacity to develop is the result of the interaction of the properties of the parasite itself and of the conditions obtaining in both vertebrate and invertebrate hosts".

In this investigation, all the experiments which received the standard treatment were reproducible for T.brucei and T.congolense. This shows that as long as the trypanosomes retain their properties, their interaction with flies under the same condition will invariably produce identical results. When the system of the experiment is altered to test a factor, either the properties of the trypanosomes or the conditions in the flies, or both are altered, and the result of the interaction between both will depend on the degree of the

alterations.

The effect of temperature on the infection rate in G.morsitans fed on T.brucei can be used as an example. It was shown (4.12) that the effect is threefold. These are i) increase in the number of flies surviving the early mortality at 31°C and a decrease at 20°C; ii) shortening of the duration of the development cycle at 31°C; and iii) increase in the proportion of established infection which became mature at 31°C and at 20°C a prolongation of establishment and probably of maturity. The first effect is probably caused by the trypanosomes finding themselves in a warmer or cooler environment. At 31°C therefore, they multiply rapidly to compensate for the mortality they were subjected to; at 20°C this multiplication was slower. The second effect is probably also caused by an increased metabolism in both flies and trypanosomes at 31°C which increases the migration rate of the trypanosomes in the fly's alimentary canal. At 20°C, this metabolism is decreased and the migration is slower. It was shown that the third effect is probably due to the physiological weakening of the fly. The fact that these effects at 31°C were not manifest in T.congolense only demonstrates the species difference of the two trypanosomes. As suggested in Section 4.12, it is likely that the optimum temperature for T.congolense was not yet reached, and that at 31°C, the trypanosomes still retain the properties they possessed at 26°C and the impact of the alteration in the condition of the fly is not so forceful as to produce a significant change. At 20°C however, the alterations are pronounced enough to create a change. Such explanations could probably be advanced for the effects of all the factors that influence the infection rates in flies.

Since very little physiological work had been done on the trypanosomes and the fly, the critical point for each factor, at which the alteration in the properties of the trypanosomes or in the condition of the fly, produces significant differences from the standard is not known. Since passage through the flies is known to stabilize almost all the characters of a strain of trypanosome (4.13), it is suggested that the most stable or 'standard' properties of a trypanosome strain are those exhibited by the cyclical population.

It is evident that a uniformity is needed among investigators in the characterization of infections. In the present studies, flies were characterized as being 'not infected', 'infected', with 'established infection', or with 'mature infection'. These characterizations represent different stages of development in the fly. Each of these was found to be an entity which can be influenced by different factors. This accounted for the difficulty in comparing the results of the present investigation with those who had characterized the presence of trypanosomes in the midgut, as 'infected' and nothing else. Even Dar (1971), who first categorically defined an established infection, used a criterion with which the results of the present studies differ. This had accounted for differences in the number of established infections that had been counted in both investigations. It is therefore of importance in future work that the characterization of the stages of development of trypanosomes in flies should be standardised so that results of different investigators could be compared on similar grounds.

This work also shows the limitations of those field results in which wild flies were dissected and species of trypanosomes identified by their distribution in the fly's alimentary canal. The disadvantages of this method had been pointed out by various workers (Duke, 1923; Johnson and Lloyd, 1923; Lloyd et.al., 1924; Godfrey, 1964; Fairbairn and Watson, 1955). Not only could double infections be missed, but also the real proportions of mature infections of T.brucei cannot be accurately estimated. As shown in this work, T.brucei invasion of the salivary glands is often transitory. Furthermore, the eventual fate of the midgut infections obtained in such dissections cannot be known, and a neglect of this makes any estimate unreliable. The most important limitation of this method is the lack of knowledge of the history of flies and the trypanosomes found in them. Since a considerable amount of what is known of the factors influencing the development of trypanosomes in tsetse flies had come from such field results, it is not surprising that conflicting results abound in this aspect of trypanosomiasis.

4.24 The limitations of the statistical calculations

Certain observations were made in the course of this work which show the limitations of the two by two table. In a normal calculation (Appendix 3), the 'observed figures' (modified treatment) should be the nominator and the 'expected figure' (standard treatment) the denominator. Where none of the two variables is the standard, the value of X^2 can vary very widely depending on which one is chosen as the nominator. An example of this discrepancy had been referred to in Section 4.21 where a wrong

conclusion was first made as to the susceptibility of male and female flies to infection.

The test of significance also becomes more sensitive the greater the number of flies used for comparison. An example of this was mentioned in Section 4.20 in the case of the established infections in G.austeni compared with G.morsitans. When 100 G.austeni and 100 G.morsitans were used to test for this factor, the difference was not significant. Even when the results of the individual days were compared (Tables 45a and b), the proportion of the established infection in G.austeni rarely showed a significant difference compared with G.morsitans. When however the group comparison was made by comparing the total established infections of days 5, 10, 20, and 30, i.e. a total of 200 G.austeni and 349 G.morsitans, the difference became significant. This clearly shows the importance of large number of flies being devoted to experiments in the study of development of trypanosomes in tsetse flies.

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APPENDIX 1

Diet for Rabbits

DIET S61

Bran	40%
Grass Meal	20%
Middlings	18%
Sussex Ground Oats	12%
Best White Fish Meal	10%

APPENDIX 2

Diet for mice

EDINBURGH UNIVERSITY DIET

Ground Wheat	20%
Ground Oats	17½%
Maize Meal	18¾%
Barley Meal	8¾%
Meat Bone Meal	8¾%
Fish Meal	5%
Dried Skimmed Milk	7½%
Whey Powder	6¼%
Unextracted Dried Yeast	1¼%
Molasses	2½%

APPENDIX 3

Application of the two-by-two table with Yates correction.

The formula is obtained from a four-fold table thus:-

a	b	a+b
c	d	c+d
a+c	b+d	n

where n = a + b + c + d (Snedecor) a is the number of flies in the observed sample which possessed the attribute being tested while b is the number in the same sample which did not possess the attribute. c corresponds to a and d to b in the standard sample. The formula is then written as

$$X^2 = \frac{(ad-bc) - \frac{n}{2})^2 n}{(a+b)(c+d)(a+c)(b+d)}$$

The "$\frac{n}{2}$" is referred to as Yates correction or chi-square adjustment (Snedecor, Fisher) and is only necessary when small numbers are being compared in each sample, otherwise "n" would be substituted in the formula. In this thesis, the Yates correction was used when less than 200 flies were being compared.

Attribute tested:- Whether the difference in the number of established infections on day 5 between flies fed on T.brucei,B and kept at 31°C and those fed on T.brucei,B and given the standard treatment is significant (Table 45a).

The fourfold table is then given by

7	43	50
6	61	67
13	104	117

Applying the formula for X^2, we have

$$X^2 = \frac{\left((7 \times 61) - (6 \times 43) - \frac{117}{2}\right)^2 \times 117}{13 \times 104 \times 50 \times 67}$$

$$X^2 = \frac{(427 - 258 - 58.5)^2 \times 117}{4529200}$$

$$= \frac{1428599.250}{4529200}$$

$$= 0.315$$

Since only 2 samples are being compared, the value for chi-square must be obtained for one degree of freedom. For this example X^2 lies between P = 0.50 and 0.70. Hence P 0.70. The difference between the two samples is not significant.

APPENDIX 4

Is the distribution of the trypanosomes in the stabilates uniform?

Worked example:- *G.austeni* infected with stabilates of *T.congolense*,A (see Experiment 3.11, Table 2).

Sample 1

Stabilate Nos. 13, 14, 15

No. of flies infected in 3 groups of 10 flies which fed on these stabilates = 3 + 0 + 1 = 4

$\bar{x}$ (mean) = 4/3 = 1.33

The squared sum of deviations from the mean is calculated from $S\,(x - \bar{x})^2$ where x is the number of flies infected in each group and $\bar{x}$ the mean.

x	$x - \bar{x}$	$(x - \bar{x})^2$
3 - 1.33	1.66	2.756
0 - 1.33	- 1.33	1.769
1 - 1.33	.33	0.109
Total		4.634

$S(x - \bar{x})^2 = 4.634$

X^2 (the index of dispersion) is calculated from the formula $X^2 = S\frac{(x - \bar{x})^2}{\bar{x}\ g}$ (Fisher)

where g is the proportion not infected in a group of 10 flies and $\bar{x}$ is the mean.

$$X^2 = \frac{4.634}{1.33 \times 8.67}$$

$= 0.402$ Degree of freedom = 2

Sample 2

Stabilate Nos., 16 and 17

No. of flies infected in 5 groups of flies which fed on these stabilates = 0 + 2 + 2 + 1 + 0 = 5

Mean $(\bar{x})$ = 5/5 = 1

By a similar calculation as above,

$X^2 = 0.444$ Degree of freedom = 4

Sample 3

Stabilate No.20

No. of flies infected in the 2 groups that fed on the stabilates = 2 + 4 = 6

$\bar{x}$ = 6/2 = 3

By a similar calculation as above, the value of $X^2 = 0.0833$, the

degree of freedom is 1.

Test for binomial distribution:-

This is tested with the formula $\sqrt{2X^2} - \sqrt{2n - 1}$ where X^2 is the summed total of the X^2 of the samples and n is the total of their degrees of freedom.

For samples 1, 2 and 3 therefore

$X^2 = 0.402 + 0.444 + 0.0833 = 0.929$

$n = 2 + 4 + 1 = 7$

$\sqrt{2X^2} - \sqrt{2n-1} = -2.242$

The difference being less than one, the conclusion is that the variance in the samples shows no sign of departure from that of the binomial distribution. Hence, the distribution of the trypanosomes in the stabilates is equal and homogenous.

APPENDIX 5a Accumulated results of Experiments 3.17-3.24 (T.congolense, B) from which the histogram in Fig. 14 was constructed.

Expt. No.	DAYS 3		4		5		6		7		8		9		10		11		12	
	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b
3.17	5	1	6	0	5	0	1	0	1	1	1	1	1	1	0	0	1	1	1	1
3.19	5	0	4	0	1	0	3	2	1	1	1	1	1	1	0	0	0	0	1	1
3.20	5	0	4	2	6	2	2	1	2	1	0	0	0	0	0	0	0	0	1	1
3.21	7	0	4	0	2	0	2	1	2	0	1	0	1	1	1	1	0	0	0	0
3.22	8	0	5	2	4	2	1	0	3	2	0	0	3	1	0	0	1	1	2	1
3.23a	4	0	6	0	6	0	4	0	1	0	0	0	1	1	2	1	0	0	0	0
3.23b	5	0	2	0	2	0	1	1	1	0	2	1	2	1	2	2	1	0	1	1
TOTALS	39	1	31	4	26	4	14	5	11	5	5	3	9	6	5	4	3	2	6	5
PER CENT	58	1.5	46	6	40	6	21	7.5	16.5	7.5	7.5	4.5	13.5	9	7.5	6	4.5	3	9	7.

NUMBER OF FLIES DISSECTED DAILY = 67

3.17 = Standard (T.congolense, B)

3.19 = Retention of infectivity during period of infective feed

3.20 = Tenfold concentration in infective feed

3.21 = Flies fed at least once before infective feed

3.22 = Incubation of pupae at 31°C

3.23a = Flies kept at 20°C

3.23b = Flies kept at 26°C (fly room)

a = Infected flies

b = Established infections

APPENDIX 5b Accumulated results of Experiments 3.17 - 3.24 (T.brucei,B) from which the histogram in Fig. 14 was constructed.

Expt. No.	DAYS 3		4		5		6		7		8		9		10		11		12	
	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b
3.18	5	0	4	0	2	1	1	0	0	0	0	0	0	1	1	0	1	1	3	2
3.19	4	0	0	0	1	0	0	0	0	0	1	1	1	1	1	0	1	0	0	0
3.20	8	0	3	0	4	4	2	1	1	0	0	0	0	0	1	1	0	0	1	1
3.21	5	0	3	0	2	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0
3.22	6	1	4	1	4	2	1	1	1	1	2	1	0	0	1	1	2	2	1	1
3.24a	5	0	3	0	1	0	1	0	1	0	1	0	1	1	1	1	1	1	0	0
3.24b	2	0	2	0	1	0	1	0	1	0	1	0	0	0	0	0	1	1	0	0
TOTALS	35	1	19	1	15	7	7	2	4	1	6	3	3	3	4	3	6	5	5	4
Per Cent	52	3	28	3	22.5	10.5	10.5	3	6	1.5	9	4.5	4.5	4.5	6	4.5	9	7.5	7.5	6

NUMBER OF FLIES DISSECTED DAILY = 67

3.18 = Standard (T.brucei,B)

3.19 = Retention of infectivity during period of infective feed.

3.20 = Tenfold concentration in infective feed

3.21 = Flies fed at least once before infective feed

3.22 = Incubation of pupae at 31°C

3.24a = G.morsitans fed on rabbits

3.24b = G.austeni fed on rabbits

a = Infected flies

b = Established infections

APPENDIX 6a The number of feeds on maintenance host (mice) taken by the flies before they were dissected. Data compiled from feeding record (T.brucei) and used to construct histogram in Fig.17a,b.

Experiment No.	No. of feeds taken by flies: 0	1	2	3	4
3.27	0	+10	48	55	46
		++4 3 0	4 3 0	5 5 0	3 3 0
3.28a	0	0	3	47	26
			1 1 0	6 4 0	4 3 0
3.28b	24	43	42	34	22
	2 0 0	3 2 0	3 1 0	2 2 0	0 0 0
3.29	10	22	20	51	10
	3 0 0	4 2 0	4 3 0	4 3 0	4 4 0
3.30	30	10	32	34	12
	2 0 0	2 1 0	3 1 0	3 1 0	2 2 0
3.33				1	2
				0 0 0	0 0 0
3.34a	0	16	44	56	43
		5 1 0	8 4 0	10 3 0	4 2 0
3.34b	0	26	65	55	59
		6 2 0	10 3 0	5 1 0	7 4 1
TOTALS: flies	64	127	254	333	220
infected	7	24	13	35	24
established	0	11	16	19	18
mature	0	0	0	0	1

APPENDIX 6a (continued)

Experiment No.		No. of feeds taken by flies															
		5			6			7			8			9			10
3.27		61			42			13			8			5			0
	6	6	2	6	6	2	1	1	0	1	1	0	1	1	0		
3.28		31			18			9			29			12			9
	4	4	0	1	1	1	0	0	0	6	6	6	3	3	3	1	1
3.28		13			15			3			1			0			0
	0	0	0	2	2	0	0	0	0	0	0	0					
3.29		18			35			15			10			4			1
	2	2	2	5	5	5	2	1	1	1	1	1	0	0	0	0	0
3.30		28			16			12			15			1			3
	2	2	0	1	1	0	0	0	0	1	1	1	1	1	0	1	1
3.33		30			29			33			28			12			4
	0	0	0	2	1	1	0	0	0	0	0	0	1	1	0	0	0
3.34a		61			50			50			37			6			2
	2	2	1	4	4	1	2	0	0	1	1	0	1	1	0	0	0
3.34b		62			40			43			27			5			0
	2	2	0	4	3	3	1	1	1	0	0	0	0	0	0		
TOTALS:																	
flies		304			245			158			155			45			19
infected		18			25			6			10			7			2
established		18			23			3			10			7			2
mature		5			13			2			8			3			0

APPENDIX 6b The number of feeds on maintenance host (mice) taken by the flies before they were dissected. Data compiled from feeding record (T.congolense) and used to construct histogram in Fig. 17a, b.

Experiment No.	No. of feeds taken by flies														
		0			1			2			3			4	
3.35		0			25			89			87			61	
				15	6	0	16	8	6	5	4	2	4	3	3
3.36a		0			7			33			56			4	
				3	0	0	11	3	0	13	6	3	1	0	0
3.36b		39			62			33			26			31	
	10	0	0	13	1	0	8	0	0	3	0	0	0	0	0
3.40		0			11			12			7			16	
				7	0	0	2	1	0	1	1	0	1	1	1
3.41		6			19			19			13			3	
	2	2	0	3	1	0	2	2	0	1	1	0	0	0	0
3.42a		38			62			46			60			89	
	1	0	0	0	0	0	3	0	0	3	2	0	5	1	1
3.42b		24			58			39			74			81	
	3	0	0	3	1	0	3	1	8	9	7	0	2	2	0
TOTALS: flies		107			244			271			323			285	
infected		16			44			45			35			13	
established		2			9			15			24			7	
mature		0			0			6			5			5	

APPENDIX 6b (continued)

Experiment No.	No. of feeds taken by flies 5	6	7	8	9	10
3.35	55	25	1	1	0	0
	6 6 6	2 2 2	0 0 0	0 0 0		
3.36	15	53	20	2	5	0
	0 0 0	7 6 6	0 0 0	0 0 0	0 0 0	
3.36	7	3	1	0	0	0
	0 0 0	1 0 0	0 0 0			
3.40	96	57	6	0	0	0
	4 4 4	4 4 4	1 1 1			
3.41	2	13	23	7	1	1
	0 0 0	2 2 1	2 2 0	2 1 1	0 0 0	0 0 0
3.42a	83	28	4	0	0	0
	2 0 0	3 1 0	1 0 0			
3.42b	94	23	4	0	0	0
	3 2 1	3 1 1	0 0 0			
TOTALS: flies	352	202	59	10	6	1
infected	15	22	4	2	0	0
established	12	16	3	1	0	0
mature	11	14	1	1	0	0

Key for Appendix 6a and 6b

3.27 = Standard, T.brucei,B

3.28a = Flies kept at 31°C

3.28b = Flies kept at 20°C

3.29 = Incubation of pupae at 31°C

3.30 = Flies fed at least once before infective feed

3.33 = Culture forms

3.34a = T.brucei,D

3.34b = T.brucei,C

3.35 = Standard, T.congolense, B

3.36a = Flies kept at 31°C

3.36b = Flies kept at 20°C

3.40 = Culture forms

3.41 = T.congolense,C

3.42a = T.congolense, D

3.42b = T.congolense,E

\+ = Total flies

++ = The first figure records the number of infected flies, the second figure, the number of flies with established infections and the third figure, the number with mature infections.

APPENDIX 7 A feeding record on mice of *G.morsitans* infected with *T.congolense*,B and dissected on the 7th day post infection.

Fly No.	Sex	Stabilate Number	Date of Infective Feed	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7 DISSECTION RESULT
1	M	68,69,70	17.8.71	2+		+		2+	-
2	F	"	"		2+		2+		-
3	M	"	"			2+		2+	-
4	M	"	"	2+				2+	-
5	M	"	"	2+			2+		* Category 4 infection
6	M	"	"		2+		2+		-
7	M	"	"	2+				2+	-
8	F	"	"			2+		2+	-
9	M	"	"	+		2+			-
10	M	"	"	+		2+		+	-
11	M	"	"		2+		2+		* Category 1
12	M	"	"	2+		2+			-
13	F	"	"	2+			2+		-
14	F	"	"		2+			2+	-
15	F	"	"	2+		2+		2+	-
16	M	"	"		2+		2+		-
17	M	"	"	2+		2+		2+	-
18	M	"	"	2+		2+			-

M = Male
F = Female
2+ = Engorgement
+ = Partial engorgement
- = Fly not infected when dissected
* = Fly infected with the specified category