CONTROL OF THE REPRODUCTIVE CYCLE IN TSETSE FLIES (*GLOSSINA* SPP.)

by

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Thesis presented for the Degree of Doctor of Philosophy of the University of Edinburgh in the Faculty of Science, 1972.

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SUMMARY

This investigation is concerned with the initiation and maintenance of reproductive processes in two species of tsetse fly; Glossina morsitans and G. austeni.

The first ovulation in both species is dependent on mating. Neither spermatophore construction nor sperm transfer are prerequisites for ovulation. Injection of extracts and tissues from males and mated females into the haemocoel or uterus of virgins does not induce a greater proportion of ovulations than occurred in operated controls. The number of flies ovulating depends on the degree of trauma involved in the operative technique. Isolation of male terminalia within the female tract is the most effective of the surgical procedures but does not result in anything like the almost absolute induction of ovulation resulting from completed mating. It is postulated that a temporal summation of mechanical stimuli is a requirement for the first ovulation. Subsequent ovulations are independent of the means of induction of the first ovulation.

Initiation of ovulation by mating is not species-specific. Wing-beat notes of males are not essential for conspecific matings.

The sizes of follicles in mated and unmated females were measured at intervals during the first sixteen days after eclosion. Follicle growth rates are the same in both groups prior to the first ovulation but thereafter growth was faster in the mated flies. Eggs retained for prolonged periods in virgins lost potency and eventually degenerated. A water- and ethanol-soluble factor, which is present in ovaries containing mature eggs, partially inhibits the growth of developing follicles.

When physical conditions are constant ovarian development is related to concurrent larval development and is independent of events in previous cycles. Missing of cycles results in advancement of ovulation and thus in acceleration of ovarian maturation.

Ovulation occurs between fifteen minutes and one hour after larviposition.

Feeding frequency is directly related to number of full-term larvipositions, and inversely related to number of missed cycles in a fixed time. Virgins feed more frequently than do mated females of the same age which miss all of their cycles. Existing accounts of timing of feeding during interlarval periods are confirmed.

Interactions between components of reproductive physiology are discussed along with hypothetical integrative mechanisms.

INTRODUCTION

The aim of this thesis was to define and to examine some of the "mechanisms" which start and maintain the regular reproductive cycles of tsetse flies. The two species for which the Edinburgh Laboratory was designed, and upon which this work is based are Glossina morsitans Westw. and G. austeni Newst. A brief account of the pertinent aspects of tsetse anatomy, development and physiology are given below and this is followed by a statement of the ideas to be examined in this thesis.

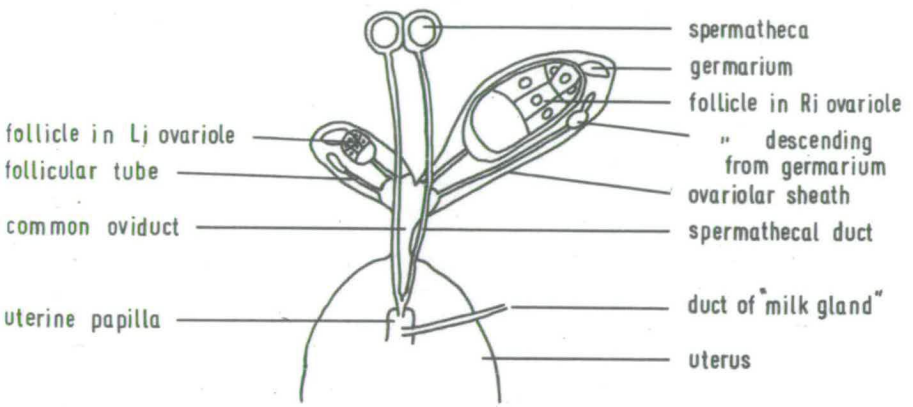
Tsetse flies reproduce by a method known as "adenotrophic viviparity" which is found only in some groups of the Diptera. All stages from the egg to the third-instar larva are retained by the maternal reproductive system in a part of the common oviduct modified to form a uterus. Leading to the oviductal shelf (a flap of tissue between the common oviduct and the uterus) is the duct from the female accessory glands which have been modified to form "milk glands" (Fig. 1). These provide the developing larva with its total food intake between absorption of yolk and ingestion of the first blood-meal in the adult instar. Also leading to the uterus via the oviductal shelf is the common spermathecal duct.

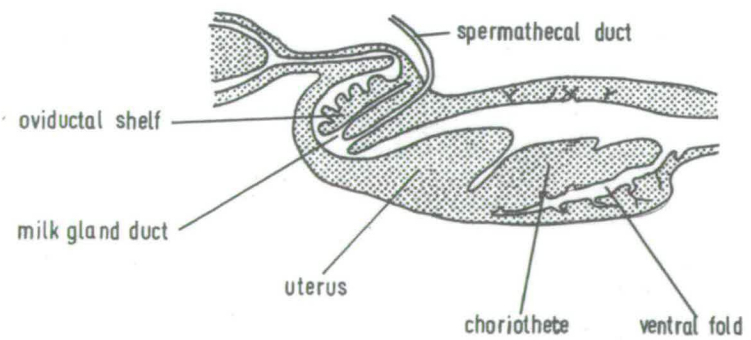
Saunders (1960a, b; 1961) showed that, contrary to previous opinion, each ovary of a female tsetse comprises two ovarioles each of which contains a follicle or egg which is at a stage of development different from those in the other ovarioles. In teneral flies the largest follicle is in the inside right ovariole (Ri), the second largest in the left inside ovariole (Li), the third largest is in Ro (right outside) and the smallest is in Lo.

FIGURE 1 — The reproductive system of a teneral female tsetse fly.
(diagrammatic)

Dorsal view of complete system (x 60)

(after Saunders, 1961)





Median sagittal view of anterior part of uterus

(x 200)

(after Roberts, 1972)

Figure I is a diagrammatic representation of the reproductive system of a teneral female of G. morsitans. Different species show different degrees of follicular development at eclosion - for example, all four follicles have descended from their germaria by eclosion in G. brevipalpis whereas in G. austeni only Li and Ri have descended by this time, (Saunders, I96I). The innervation of the female reproductive system is unknown despite investigations by Minchin (I905), myself and, presumably others. Langley (I965) has described the appearance of the neuroendocrine and stomatogastric systems of G. morsitans females as revealed by staining with chrome haemotoxylin and phloxin. In most respects these systems seem to conform to the pattern exhibited by other Cyclorrhaphan species but the peculiarities which do present themselves may be related to viviparity. The role of humoral factors in the reproductive physiology of tsetse will be discussed later.

The earliest sign of a follicle descending from its germarium can be discerned, in G. morsitans, about twelve days after pupariation - about the time when the eyes of the pupa are becoming pigmented. According to Moloo (I97I) egg development starts when the germarial oocyte divides four times consecutively to form a primary follicle consisting of sixteen nuclei surrounded by follicular tissue. After further differentiation a follicle, consisting

of an oocyte and fifteen trophocytes surrounded by follicular epithelium, is formed. Moloo concludes that the follicle cells and trophocytes transfer protein and RNA to the oocyte during vitellogenesis. The trophocytes effect this transfer, probably through intercellular fusomes. Later the follicular epithelium secretes the chorion and the trophocytes degenerate. In a teneral female yolk synthesis is under way in two follicles. Thus possible hormonal control of the initiation of egg development and vitellogenesis is independent of feeding (Saunders,1961). Egg development is, however, not completed unless feeding occurs. The degree to which follicle growth rate is enhanced by the first blood-meal is, according to Saunders (1961), independent of the size of that meal. Foster and Allingham (1972) found that the longer the interval between eclosion and the first blood-meal, the greater is the initial rate of follicle growth after feeding. In flies starved for the first four days after eclosion ovulation was delayed by about two days compared with the situation in flies starved for only one or two days.

As described above teneral flies have a large, but immature, follicle in Ri in which vitellogenesis is well under way. This follicle matures, becomes chorionated and is ovulated by about day 7 in mated flies in the Edinburgh colony. The other follicles mature and are ovulated in order of size and under normal circumstances each egg is ovulated only when it is mature and when the uterus is empty. Unmated females ovulate their eggs rarely, if at all. Mellanby (1937) found that associated with egg retention by virgins was a reduction in growth rate of the other

follicles, but she did not attempt to quantify this phenomenon. Saunders (1970) found that the reduction of follicle growth in virgins results in eggs reaching maturity 8 or 9 days later than in mated females.

The means whereby egg retention results in reduction of follicle growth is unknown. The component of copulation which initiates ovulation and the means by which it does so are unknown and these problems have been investigated by Saunders and Dodd (1972). My contribution to this paper, a copy of which forms Appendix 2 of this thesis, is discussed in Chapter 3. The method by which eggs pass from the ovaries to the uterus is unknown but is probably a result of muscular activity of the oviducts, such as occurs in Aedes aegypti (Curtin and Jones, 1961).

The uterus, about the time when it receives an egg, becomes distended, probably as a result of contraction of the uterine dilator muscles (Roberts, 1972). The chorion of the developing embryo becomes attached to the choriothete, which is a ventral fold of the uterus composed of columnar epithelial cells. Its function, according to Roberts (1972) is to support the embryo and young larva and this function is later taken over by "uterine ridges" which also serve to retain milk gland secretion in the region of the larval head. Roberts contradicts the view taken by earlier authors such as Bursell and Jackson (1957) and also that of Tobe and Davey (1971) who believe that muscles in the choriothete assist in the exchorionation of the first-instar larva. Jackson (1957) concluded that removal of the first and second larval exuviae is also accomplished by the choriothete.

The cycles of degeneration and regeneration of epithelium and muscles reported by Bursell and Jackson (1957) were not verified by Tobe and Davey (1971) but an explanation based on changes in relative positions of muscles resulting from stretching of the uterus has been given by Roberts (1972). It appears, then, that the choriothete may or may not assist the labral egg-tooth of the first instar larva to burst and remove the chorion and it is probably not involved in further moults.

Diagrams of the external morphology of the non-pharate first, second and third larvae of G. swynnertoni are given by Newstead, Evans and Potts (1924) who describe how the external signs of the respiratory system of the first instar larva consist of a pair of abdominal stigmata separated by a ridge. Small respiratory lobes, separated by a slight depression, are present in the second-instar larva and the third-instar has prominent respiratory lobes separated by a deep furrow. The lobes of the third-instar larva blacken a few days before larviposition. It is mainly upon these criteria that the stages of development of intrauterine larvae are defined during dissection.

It has already been mentioned that food intake in preimaginal stages occurs during absorption of yolk and consumption of "milk gland" secretions. After examining serial sections of G. palpalis females, Hoffmann (1954) came to the conclusion that the milk-gland complex undergoes great morphological and histological changes during the course of pregnancy. He found that in virgins the structure was simple but in pregnant flies

increased ramification of the ducts associated with involvement of tracheoles occurred. He believed that milk glands function only after about the third day of pregnancy and continue for about one hundred hours in primiparous flies and sixty hours in others. The larva feed, he suggested, by rhythmically pressing its pharynx against the papilla (where the milk-gland duct joins the uterus) and ingesting the extruded liquid. Bursell and Jackson (1957) found that feeding occurs mainly during the first and second instars and that little food is taken by the third instar. These authors maintain that "milk" is secreted freely into the uterine cavity from which the larva takes it up. Roberts (1971) found that milk-gland secretion is discharged during all three larval instars and that it is taken in by all three instars. He maintains that the secretion is retained around the anterior end of the larva by "uterine ridges" adpressed to the larva.

Normal parturition is a rapid and consequently rarely observed phenomenon. Larviposition normally occurs in daylight and in this laboratory nearly always in the afternoon. This is in agreement with Jackson (1949) and Nash and Trewern (1972). Foster (unpublished) found that larviposition could be enforced by gently squeezing a fully pregnant female (whose black-lobed larva is visible through the postero-ventral abdomen) between finger and thumb. Burrt (1952) believed parturition to occur mainly as a result of the efforts of the larva. Hoffmann (1954) maintained that some assistance may be provided by the legs of the mother. Roberts (1972) suggests that uterine muscles are

involved in the expulsion of the larva.

The fate of the larva once it is discharged, the course of metamorphosis and eclosion are not directly pertinent to this thesis. It is sufficient to point out that eclosion of the adult occurs, in the Edinburgh laboratory about thirty days after larviposition. The newly-hatched fly burrows up through the substrate by means of the ptilinum in the typical Cyclorrhaphan manner (Jackson, 1948). Hardening and darkening of the cuticle occur after eclosion and "bursicon" may be involved, as in Calliphora (Fraenkel and Hsiao, 1965). Some physiological changes associated with the first few blood meals are reported in tsetse. For example, the thoracic muscles double in weight during the course of the first few meals (Bursell, 1961; Glasgow and Glasgow, 1962). Foster (1972) showed that accessory gland secretion is deficient in teneral males of G. morsitans and that they are unable to inseminate females, whereas all 3-day-old fed males of this species are capable of insemination. The effect of the first few meals on early follicle growth in females has already been discussed.

The precise time at which females copulate varies according to the species. Although females of both G. fuscipes and G. pallidipes are inseminated before they have fed, copulation occurs in the former before emergence from the undergrowth and in the latter while the females are actually seeking their first blood meal (Saunders, 1962; Harley, 1966a, b). Vanderplank (1948) gives diagrams of the topological interrelationships of male and female genitalia in a couple of G. palpalis. From these sketches it appears that the superior claspers of the male grip the female abdomen while the tip of the penis reaches the upper end of the

uterus so that the sperm duct opening lies near the opening of the spermathecal duct dnd the "pneumopophyses" of the male stretch the uterus. This account may have to be reconsidered when the results of Pollock's (1970, 1971) work on the course of copulation and spermatophore construction have been completed. So far Glossina austeni is the only higher dipteran shown to use a spermatophore for sperm transfer. "Mating scars" may occur in mated G. palpalis females (Squire, 1951; Vattier, 1965). Vanderplank (1948) refers to "mating notes" produced by wing beats of flies and he maintains that these are important in determining whether or not copulation occurs. Interspecific copulation is not an infrequent occurrence in the genus Glossina; Vanderplank (1948) gives a detailed account of the results of pairing tsetse of many different species and of combining the gametes of various species. The production of viable larvae by cross-mated females is less frequent than the occurrence of cross-mating and the resulting hybrids are usually either sterile or give rise to sterile progeny.

There is no evidence for the production of pheromone by tsetse. Such sex attractants have been looked for without success in G. morsitans orientalis by Dean, Clements and Paget (1968), who also describe some aspects of the mating behaviour of this species.

Release of spermatozoa from the spermathecae occurs after the egg reaches the uterus (Hoffmann, 1954). Pinhao (1970) describes in detail the course of the later stages of ovulation, including the entry of the egg into the uterus, subsequent release of spermatozoa from the spermathecal duct openings and the relevance of the uterine papilla to these events. Hoffmann (1954) found

that whereas the non-gravid uterus was devoid of sperm, when an egg was present a mass of sperm could be seen forming a cap on it. He proposed that ovulation induces spermathecal duct activity, as in Drosophila (Nonidez, 1920).

Females of both species of tsetse reared in the Edinburgh laboratory are normally allowed to mate on day 2. They ovulate for the first time on about day 6 or 7, deposit their first larva about day 14 and subsequent larvae are dropped at about 8 day intervals by females fed normally. Interference with the feeding of inseminated females usually results in abortion of the embryo or larva, especially if individual meals are interrupted (Mellanby, 1937), or in prolongation of pregnancy when starvation is imposed (Saunders, 1971).

The feeding patterns of eight mated females of G. austeni were analysed by Nash, Jordan and Boyle (1967). These authors found that flies tended to fast before larviposition and to engorge repeatedly after parturition: failure to larviposit did not disrupt the feeding pattern. The mean interval between meals was 2 days. Females entered a post-reproductive period after about 6½ months and would then starve for up to 11 days.

The details of the feeding mechanism, blood concentration, digestion and excretion are not directly relevant to this thesis. The aspects of these subjects pertinent to the reproductive physiology of the flies are considered, in context, in Chapter 2.

Curtis (1968) showed that an abortion induced by mating females with certain irradiated males advanced subsequent ovulation by about 2 days. His estimates of numbers of abortions were

based on the interval between successive successful larvipositions.

The questions which this study is concerned with are:-

What component of copulation initiates ovulation?

How is it mediated?

How does egg retention in virgins result in reduction of follicle growth rate?

What is the fate of eggs retained in the ovary?

What criteria determine the time of expulsion of uterine contents?

How long after larviposition does the next ovulation occur?

Does advancement of ovulation occur after abortion, however caused, and does it affect the relationship between follicle sizes and larval growth in subsequent cycles?

Are the published accounts of feeding patterns during virginity, pregnancy and "missed" cycles accurate?

MATERIALS and METHODS

Source of material.

The species used were Glossina morsitans orientalis Vanderplank and G. austeni Westwood. G. austeni material was obtained from the Langford colony, of Zanzibar origin. Experimental subjects were either laboratory-bred in Edinburgh or were frequently supplied as puparia from Langford. G. morsitans material was principally derived from Langford stock, of Rhodesian origin, but the colony in Edinburgh contained some material, of Mozambique origin, from the Lisbon colony of Professor Azevedo.

Maintenance of flies.

A detailed account of the design and operation of the colony is given by Mews (1969) who describes the apparatus used to control the physical conditions in the fly-room, the source and maintenance of the rabbits etc. The room is kept constantly at 25-26°C and at relative humidity of about 65%. Light is provided for12 hours in every 24 (0600-1800 hrs) by fluorescent strip lights. The breeding stock is maintained in polystyrene lunch boxes whose bottoms have been replaced by terylene netting and whose tops are partly netted. Ten flies of the same sex are kept in each of these colony cages. Experimental flies are kept individually in polystyrene tubes with stoppers (6cm long, 3½cm diameter). These tubes have one end sawn off and replaced by terylene netting. The stoppers are bored through and flies are imprisoned by using the stoppers to hold in place a square of netting at the top of the tube. All containers are washed and rinsed well when the flies are killed. The containers are placed on plastic trays which catch larvae soon after they are born.

Flies are offered food for 5 or 10 minutes daily except Sundays, from the ears of sandy half-lop rabbits (Nash et al 1966). Records are kept of feeding and larviposition performance of each fly maintained individually.

Puparia are kept in polystyrene screw-top containers containing sand and suspended over water. The lids of these containers are partly replaced by terylene netting. Adult flies are transferred from these containers to their individual cages after chilling them at 4°C until they are immobile. Mews (1969) has shown that such chilling has no significant effect on either the longevity or the reproductive performance of the flies.

The day of eclosion of a fly is designated day 0 for that fly. Flies which eclode on Sundays are not distinguished from those which do so on Saturday afternoons and are both given the same date code for their eclosion. Females mated on day 2 are usually no older than 48 hrs at that time.

Mating.

The ability of a male to inseminate a female increases during the first few days after eclosion (Mellanby,K., 1936; Southon and Cockings, 1965; Nash, Jordan and Boyle, 1966) and is dependent on the male feeding (Foster, 1972). There is evidence that young females are more readily inseminated than older ones (Nash, 1955). In Edinburgh females are normally mated on day 2 (ie when they are no older than 48 hrs), although in many experiments the females were left unmated for longer periods. Males were mated only when they were at least 8 days old. Often males were allowed to mate once only before culling and no male was mated more than

3 times. The interval between matings by the same male was not less than 3 days.

Aspermic males. If males are mated frequently they become exhausted of spermatozoa, but remain capable of copulation. The number of matings required to reduce males to aspermicity and the effects of aspermic matings on females are described in Chapter 3.

"Stock 73" males. These were obtained (as puparia) from Dr. Curtis. They are "semi-sterile" in that although their spermatozoa were capable of forming zygotes about 20% of the ensuing embryos were non-viable and were aborted. These males were the offspring of males which had been subjected to low levels of gamma radiation (Curtis 1968a, b).

Some females were mated with males with genitalia mutilated by cautery.

Detection of copulation.

Copulation involves the insertion of the male genitalia into the female tract. It is impossible to determine whether normal penetration and genital expansion by the male has occurred except by dissection. Thus some of the copulations recorded, especially by males with mutilated genitalia, may have been deficient in some respects.

Detection of insemination.

The appearance of the spermathecae of inseminated and uninseminated females have been described and depicted by K. Mellanby (1936). The opacity of intact spermathecae under the stereoscopic microscope is not an infallible indicator of sperm content of these organs

especially when spermatozoa are low in number or are abnormal in some other way (as in "semi-sterile" males). Insemination of almost all females dissected during the present study was checked by crushing the spermathecae under a coverslip and viewing under x250 magnification. Spermatozoa are sometimes readily detected by this method even in flies whose spermathecal translucency suggested that insemination had not occurred. The absence of spermatozoa is, of course, difficult to confirm by either method but examination of crushed spermathecae carries greater reliability than does perusal of intact organs.

Spermatophore detection.

Apart from direct observation of copulation, the only way to determine whether a particular female tsetse has mated without dissecting it is detection of the spermatophore. Pollock (1970) has shown that this structure is built within the uterus (of G. austeni) during copulation and it is rejected after some of the contained spermatozoa have transferred to the spermathecae. Pollock (1970) found that not all inseminated females of G. austeni had expelled their spermatophores a day after pairing. In the present work spermatophore production was detected in each instance by isolating the relevant male and female couple in a glass tube capped with pierced Parafilm, checking for copulation at intervals, removing the male after 1 day and searching for spermatophores 2 days after pairing. The tubes, when devoid of flies were sluiced with water and examined under a stereoscopic microscope for spermatophores which, when present, were very obvious.

Determination of physiological age.

Saunders (1962) described a method for determining the age of a female tsetse fly based on the sequential events occurring within the reproductive system: this technique was improved by Challier (1965). An adult female tsetse has, as described above, two ovarioles in each ovary. The size order of these ovarioles (oocytes, nurse cells, follicle cells) is with very few exceptions Ri largest followed by Li, Ro, and Lo smallest. If the ovarioles are numbered inversely according to size (ie 1 largest to 4 smallest) the configuration (Lo)4, (Li)2, (Ri)1, (Ro)3 defines a nulliparous fly. This series of numbers (4213) is the "nombre repere" for such a fly (Challier, 1965). After the first ovulation Li becomes the largest ovariole and the "nombre repere" (N.R.) is 3142, after 2 ovulations Ro is largest and N.R. is 2431, after the third ovulation Lo becomes the largest and N.R. is then 1324 and after the fourth ovulation the configuration is the same as that of a nulliparous fly, 4213, and the cycle of events is repeated. After ovulation a dilatation is produced in the follicular tube to form an expanded sac which later contracts to form a "follicular relic" (see chapter 1). Flies which have ovulated once have one such relic (in Ri), those which have ovulated 2,3 and 4 times have 2,3 and 4 follicular relics. Thus one can distinguish nulliparous flies from those which have undergone 4 or a multiple of 4 ovulations by the presence of 4 follicular relics in the latter and the absence of relics in the former. Flies with N.R. 4213 and with 4 follicular relics are designated $4n$ (where n is a positive integer), those with N.R. 3142 and 4 relics are designated $4n+1$,

those with N.R. 2431 and exhibiting 4 follicular relics are designated 4n+2 and those whose N.R. is 1324 and which have 4 follicular relics are designated 4n+3. Thus from the relative sizes of the ovarioles and the number of follicular relics one can determine the number of ovulations that have occurred and by consideration of the phase of development reached by the uterine contents one can assign a fly to a particular stage within an ovulatory cycle (Fig. 2). For flies reproducing normally at a constant temperature a correlation exists between "ovarian" and calendar age.

The number of "missed cycles" (ie cycles not resulting in recorded larvipositions, due to oocyte degeneration or undetected abortion) can be derived from the following equation:-

No. of ovulations = No. of recorded larvipositions + No. of "missed cycles" + U.C.

where U.C. is uterine contents (0 if uterus is empty; 1 if any stage is in uterus). It is upon the validity of this relationship that much of this work depends.

Females whose "ovarian" ages were to be determined were killed by immersion in a deep freeze where they were stored until they could be dissected. Killing by this method is rapid. Flies were usually dissected within minutes of death, although those stored for a week or so were preserved well enough for dissection to be successful. The reproductive system was removed by nicking the lateral margins of the abdomen at about the level of the sixth segment with scissors and applying traction along the anterio-posterior axis of the fly. The posterior abdominal segments containing the reproductive system, some hind-gut and fat body were removed by this method and they were

FIGURE 2 — Method for determining "ovarian age" of a tsetse fly.

(after Saunders, 1970)

NR	a	b
4213	0	0
3142	1	1
2431	2	2
1324	3	3
4213	4	4n
3142	4	4n+1
2431	4	4n+2
1324	4	4n+3

NR = nombre repere
a = number of follicular relics
b = " " ovulations
c = follicular relic

placed in a drop of 0.9% saline on a slide under a stereoscopic microscope. Presence or absence of spermatozoa in the spermathecae, contents of the uterus and the nombre repere were noted for each fly. The oocytes were dissected out of their ovarioles with mounted needles and their lengths were measured with a calibrated eyepiece graticule.

Experimental procedures.

Many of the techniques used are self-explanatory. Details of those which require explanation, particularly the surgical manipulations, are given here. The relevance of these operations to existing knowledge and the results which they gave are explained in chapters 3 and 4.

1) Mutilations.

In the experiments concerned with the role of auditory stimuli in mating the antennae and wings were removed. Antennae were amputated with a pair of fine scissors under a stereoscopic microscope and no postoperative treatment was given. Wings were sectioned as near to the thorax as possible, the wing remnants were trimmed off and the wounds were sealed with a thick layer of molten wax. The wax not only prevented fluid loss from the wound but was also assumed to reduce amplitude and frequency of vibrations produced by the wing moving mechanism of the thorax.

The genitalia of some males were mutilated by cautery of one pneumopophysis or of both of them. These organs are thin-walled erectile bulbs which can be projected from the vesica distal to the harpes and are shown stretching the uterus during copulation by Vanderplank (1947). Males of G. morsitans were

held on their backs with Plasticene, their hypopygia drawn back with pins and the pneumopophyses were cauterised with a hot needle. Very few males died as a result of this operation.

2. Transplants of tissues and organs into haemocoel.

Various parts of male and female flies were introduced into the thoracic haemocoel of virgins. Donor tissue was dissected out in saline (Ephrussi and Beadle, 1936) and stored for a few minutes in a refrigerator. Recipient animals were held between finger and thumb and a small incision was made dorso-laterally on the thorax. Donor material was inserted either on a platinum loop or with watchmaker's forceps. Care was taken not to penetrate the thorax deeply but the tissue was slipped under the cuticle and poked well away from the site of entry. After placing a crystal of penicillin and streptomycin on the wound it was sealed with wax. Postoperative survival was usually about 80%. Operated controls were slit, saline was inserted and the wounds were closed after treatment with antibiotics just as in the experimental flies.

3. Transfusions and injections.

Haemolymph for transfusion was obtained by puncturing the abdominal cuticle of the donor, compressing its thorax lightly between finger and thumb and picking up the extruded juice with a 2 ul Microcap. Individuals of both species had under 5 µl of easily accessible haemolymph. The preparation of various extracts and homogenates is described in chapter 3 in the relevant sections. All injections of liquids into the haemocoel were performed through the dorso-lateral wall of the abdomen, about

half way between thorax and vulva with Microcap capillaries. No treatment of the wounds was necessary. Postoperative survival varied greatly according to material injected. Injection of saline produced a death rate of about 20% in 4 days whereas one of the homogenates killed over 60% in this time.

4. Intrauterine insertions.

Glass rods were prepared by drawing out a glass capillary to form a fine tube, breaking this off near the undrawn capillary and melting the end of the fine tube to form a bead of 0.5mm in diameter for nulliparous flies and about 2mm in diameter for parous flies. The recipients of these beads on rods were held between finger and thumb and after dipping the bead in saline insertion was effected easily.

Various male organs were inserted into the uterus through Pasteur pipettes drawn out to form fine tubes.

5. External mechanical stimulations.

There is no a priori reason for excluding a possible induction of ovulation by mechanical stimulation of cuticular sense organs during copulation. The most likely locations for receptors of such stimuli appear to be around the genital orifice and on the thorax of the female. These two regions receive much stimulation during copulation - the former from the abdomen and superior claspers and the latter from the front legs of the male.

Four techniques were used in attempts to induce ovulation in 10-day-old flies by mechanical stimulation. Some females were shaken in their tubes by a "wrist action" mechanical shaker for 2 hrs., thereby receiving quite violent contact all over their

bodies. Others had their dorsal surfaces attached to blocks of paraffin wax so that more or less local stimuli could be applied to their genital regions. One such local stimulus was air-puffs from a glass tube connected to an aquarium pump. The wax blocks with attached flies were clamped horizontally and glass jets attached to the pump were placed a few cms. behind, and in the same axes as, the flies. The setae in the genital regions of these flies were seen to be in constant motion during the 2hr. application of the puffs. Some virgins on their blocks were clamped near a mechanical shaker which held small paintbrushes whose soft ends moved constantly over the posterior ends of the abdomens of the flies for 2 hours. Other blocks with attached flies were clamped over a magnetic stirrer and a mixture of iron filings and syrup was applied to the setae around the external genital region. When the stirrer was switched on the setae were bent in constantly changing directions by the movements of the iron filings. All of these stimuli were applied to flies for 2 hrs. and the flies were killed 5 days later. Mated and unmated controls were also kept and killed on day 15. In another experiment thoracic setae were rendered immobile with molten wax.

6. Enforcement of larviposition and larval retention.

Larviposition is induced within a short time by compressing between finger and thumb the thorax and anterior abdomen of a periparturient female (ie one in which the black respiratory lobes of the intrauterine late third-instar larva can clearly be seen through the postero-ventral abdominal wall), (Foster, unpublished). This technique works well on flies in late pregnancy.

It precludes information on the mechanism of larviposition, since larval movement, uterine contractions, nervous and endocrine systems could equally well respond to the crude stimulation applied. If compression of the female is over-enthusiastic then, on occasion, the postparturient uterus prolapses. In such cases a crumpled chorion can often be seen lying adjacent to the choriothete.

If such preparations could be kept physiologically functional in saline they would provide a useful system for investigating short-term events in the reproductive system. For example, defined stimuli could be applied to regions of the uterus in attempts to cause ovulation. Lack of flies and time has so far prevented my following this up but the technique looks promising because it provides a method of observing otherwise hidden events without disruption of nervous and hormonal links.

Enforced larval retention was ensured by ligation of the vulva around the larval polypneustic lobes with a human hair. This procedure produced a 67% death rate in 4 days.

RESULTS

A INITIATION OF OVULATION.

Since mated female tsetses almost invariably ovulate, whereas virgins rarely, if ever, do so, the mating act must contain one or more component stimuli which initiate the process. An understanding of ovulation, therefore, requires answers to the questions i) what is the stimulus by which copulation induces ovulation? and ii) how is the stimulus mediated?. In other words one must determine both the stimulus involved and its mode of action. Saunders and Dodd (1972) describe experiments whereby certain copulatory events were shown not to be involved in inducing the first ovulation. They suggest that an as yet unidentified "mechanical" factor is involved.

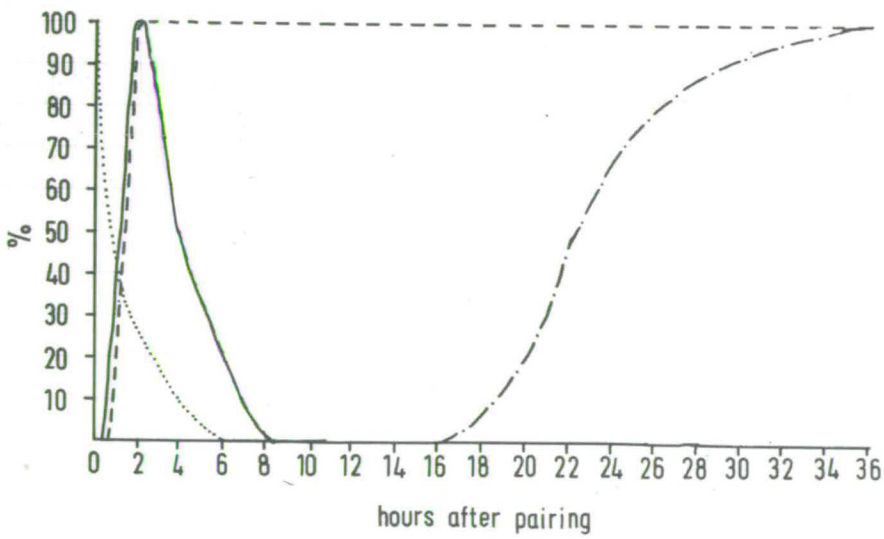
1. The time-course of events in the reproductive systems of flies mated on day 8.

Many of the following treatments given to virgins in attempts to make or prevent them from ovulating were performed on 8-day-old virgins. On day 8 females of *G. morsitans*, provided they have fed normally, have only Ri chorionated. The result of dissecting flies, mated on day 8, at various intervals after pairing are given in Figure 3. All females commenced copulation immediately on pairing. From this figure it is clear that all flies have separated from their mates about 6 hours after copulation started; all have received a spermatophore by 2 hours and expelled it by 8 hours. Ovulation has usually occurred by 24 hours after pairing.

2. Effect of mechanically stimulating virgin females.

Although no records have been found of insects in which ovulation is induced by the purely mechanical components of mating, it is known that in some insects such stimuli promote egg maturation. For example, the spermatophore - induced egg maturation of

FIGURE 3 Time course of events associated with mating 8-day-old females of G.morsitans



the cockroach Diploptera punctata is probably operated by mechanical stimuli only (Engelmann, 1959). In the locust Schistocerca gregaria mating results in acceleration of egg growth as do enforced activity, electrical stimulation and excessive handling: all these treatments are associated with the release of neurosecretory substance from the corpora cardiaca (Highnam, 1961, 1962).

Egg maturation prior to the chorionation of the first follicle is independent of mating in tsetse flies (Saunders, 1970; Dodd, 1971). It is not known, however, whether any mechanical component of mating results in ovulation in these species. Hence, the following experiments were performed in which virgin females were subjected to a variety of mechanical stimulations.

a) Generalised mechanical stimuli.

Table 1 gives the results of treating virgins for 2 hours on a mechanical shaker as described in chapter 2. Virgin controls were kept and none of these ovulated. Nearly all of the flies so treated subsequently retained their eggs whereas all the mated controls ovulated. This provides some evidence that body-surface stimuli detected by females during mating do not cause them to ovulate.

b) Localised cuticular stimuli.

Application of air-puffs, paintbrush strokes and moving iron filings to the external genital region of virgins did not significantly induce ovulation (table 1): only one ovulated out of 36 females so treated, whereas all of the mated controls and none of the virgin controls ovulated. This adds to the evidence that stimuli applied to the external genital region of the female dur-

In the following tables the abbreviations used are defined as follows:-

No = number

S.E. = standard error

ov. = ovulated

ret. = retained

M = G. morsitans

A = G. austeni

FNo. = female number

cop. = copulation

op. = operated

insem. = inseminated

u = uterine contents

a = egg

b = first or second instar larva
C = third instar larva
Cwl = third instar larva (white lobes)

Cbl = third instar (black lobes)

d = uterus empty

LP = number of recorded larvipositions

MC = number of missed cycles

Spth = spermathecae

spp = spermatophore

Foll. = F.D. = follicle designation

NR = nombre repere

MNC = median neurosecretory cells of the pars intercerebralis

TH = tissue from thorax containing corpora allata, corpora cardiaca.

UC = uterine contents

HL = haemolymph

Table 1

Effect of various mechanical stimulations on the ovulation in virgins. Flies were treated on day 10, killed on day 15.

A = G. austeni M = G. morsitans

Treatment	No.op.		No.Died		Ri.ov.		Ri.ret.	
	M	A	M	A	M	A	M	A
Mechanical shaker	12	12	1	1	1	0	10	11
Mated controls	8	8	0	0	8	8	0	0
Unmated controls	5	5	0	0	0	0	5	5
Air puffs	12	12	0	0	0	1	12	11
Mated controls	8	8	0	1	8	7	0	0
Unmated controls	5	5	0	0	0	0	5	5
Paint brush	12	12	0	0	0	0	12	12
Mated controls	8	8	1	0	7	8	0	0
Unmated controls	5	5	0	0	0	0	5	5
Iron filings	12	12	0	0	0	0	12	12
Mated controls	8	8	0	0	8	8	0	0
Unmated controls	5	5	0	0	0	0	5	5

Table 1 (continued)

All experiments were performed on 6-day-old females of C. morsitans which were dissected on day 10.

Treatment	No.op.	No.Died	Ri.ov.	Ri.ret.	No.expelled rod
Glass rod inserted into vagina	10	0	0	10	10*
Rod sealed into vagina	15	1	1	13	2
Rod inserted and sealed after mating	10	0	10	0	4
Mated controls	5	0	5	0	-
Unmated controls	5	0	0	5	-

* by day 2

ing copulation do not cause ovulation.

However, the types of artificial mechanical stimuli applied in these experiments may not have been sufficiently similar to normal mating stimuli to be effective.

c) Intra-uterine mechanical stimulation.

Such stimulation was applied with fine glass rods which had one end melted to form a bead about 1.0mm in diameter. It was found impossible to insert larger beads into the vulvae of nulliparous flies. Such rods were inserted as far as possible into the uteri of 8-day-old virgin females of G. morsitans. They were expelled by most of the females within a day of insertion. None of these flies subsequently ovulated, (table 1). Similar rods were held in the reproductive tracts of other virgins by melting wax around the glass end projecting from each vulva. Some flies expelled their rod but only one fly of the 15 operated on subsequently ovulated (table 1). These results provide evidence that stretching , alone, of the female tract by the male during copulation is probably not the ovulation-initiating stimulus. That ovulation was not inhibited by the rods was shown by waxing rods into the vulvas of ten 8-day-old females which had been mated a few hours previously. On day11 it was found that 4 of these flies had expelled their rods but ovulation had occurred in all 10 flies.

3. Effect of mating females with mutilated males.

Attempts at mimicking the mechanical stimulation of the female received during copulation were not successful in inducing ovulation. Here are described attempts made to eliminate certain

mechanical components of mating by removal or cautery of parts of the male anatomy.

a) Amputation of male genitalia during copulation.

Further evidence concerning the role of mechanical stimuli applied by the penis to the female genital tract during copulation was gained by chilling twenty 8-day-old females of G. morsitans which had been in copula for less than five minutes. Each male was sectioned at the posterior end of the abdomen thereby leaving only the genitalia and some associated tissues penetrating the female. When the flies were returned to room temperature most of the male remnants were released within an hour probably because the male clasping mechanisms were inoperative. None of these females subsequently ovulated (table 2). A repeat of this experiment with the male genitalia sealed into the females with wax and cauterised internally with a hot needle (see chapter 2) resulted in retention of the insert for at least one day by all females and until dissection on day 11 by 3 females. None of these 3 were found to have ovulated but three of the total 11 dissected flies had done so. None of these females contained spermatozoa in their spermathecae and no signs of spermatophores were found in any dissections. Thus removal of all stimulation apart from some of the mechanical consequences of the presence of a penis in the vagina resulted in some increase in ovulation compared with the artificial techniques used previously but did not result in the ovulation rate found after copulation. It is probable that the isolated penes penetrated females to varying extents and certain receptors, particularly those in the anterior

Table 2

The effect of penisectomy on ovulation in 8-day-old females of *G. morsitans*.

Operation	No.op.	No.Died	Ri.ov.	Ri.ret.	No.insem.	No. expelled penis
Penis section only	20	0	0	20	0	20*
Penis waxed in	11	1	3	8	0	8
Penis waxed in and removed on day 10	7	1	1	5	0	0
Mated controls	5	0	5	0	5	-
Unmated controls	5	0	0	5	0	-

* within 1 hour

portions of the uterus, may thus have been missed.

When male terminalia were waxed into twenty-two 8-day-old virgin females fourteen were found to have retained the inserts on day IO. The remaining 8 were dissected and one of them had ovulated. The inserts were removed from the fourteen flies on day IO and these females were dissected on day I2. One had died, one had ovulated and the uteri of 3 others were found to have been removed along with the penis and wax on day IO. These latter three flies were in good condition and had fed well despite the fact that their ovaries were attached only to tracheae and nerves. Of the remaining 9 females one had ovulated. It thus appears that ovulation was not necessarily imminent in those flies in the previous experiment which had retained a penis until dissection.

Of 35 dissected females given a waxed-in penis 6 ovulated. This proportion of ovulations is no higher than that induced by other operational procedures (including "operated controls"). The conclusion one can draw from the results of this technique is that mechanical stimuli applied to the posterior region of the female tract are not alone responsible for ovulation.

b) Ablation of pneumopophyses.

Twenty 8-day-old males of G. morsitans were subjected to cautery of their pneumopophyses. Nineteen survived until the next day, when they were offered 2-day-old virgin females. Mounting was observed in all pairings. Males whose genitalia

did not penetrate those of their mates were attempting copulation every time they were examined during two days whereas those which effected penetration copulated for a few hours only. No spermatophores were recovered from any of these females, none were inseminated and none ovulated (table 3). The uteri of other 8-day-old virgin females mated with these males contained no spermatophores 3 hours after commencement of copulation whereas the uteri of 10 females of the same age mated with normal males each contained a spermatophore at this time.

This experiment shows that the pneumopophyses of G. morsitans males are necessary for successful copulation - their total amputation renders penetration of the vagina by the penis difficult and totally inhibits the construction of spermatophores and the initiation of ovulation. It does not, however, tell us much about the nature of the stimulus which causes ovulation in females.

Ten females of G. morsitans were offered males mutilated by ablation of one pneumopophysis. All these males mounted their mates and most penetrated the vulva. On day 12 one female was found to be inseminated and it had ovulated. Nine females remained uninseminated and of these seven had ovulated and two had not ovulated. No spermatophores expelled by the females were detected and when other females mated with these males were dissected 3 hours after copulation started only one contained a spermatophore. The male which produced this spermatophore was the one which had inseminated its mate in the previous experiment and which on examination was found to be incompletely cauterised. All six females mated with normal males had received spermatophores 3 hours after

Table 3

The effect of cauterising the pneumopophyses of G. morsitans males on their copulatory performance.

Operation	No.op.	No.mounted	spp. insem.	No.Died	Ri.ov.	Ri.ret.
*2P⁻	19	19	0	0	0	19
*1P⁻	10	10	0	1	0	8	2
mated controls	5	5	4	5	0	5	0
virgin controls	5	-	-	-	0	0	5

* i.e. both pneumopophyses cauterised or only one cauterised.

copulation started.

Thus semi-pneumopophysised males can initiate ovulation despite their inability to inseminate. This latter inability is clearly dependent on the failure to form spermatophores. These results add to the evidence concerning the independence of ovulation on insemination and spermatophore construction but do not give much more information on the nature of the ovulation-initiating stimuli in copulation.

c) Removal of male legs.

During normal copulation in G. morsitans and G. austeni the ventral surfaces of the thorax and abdomen of the male are adpressed to the dorsal surfaces of the thorax and abdomen of the female. The prothoracic legs of the male grip the anterior margin of the female thorax, the metathoracic legs of the male normally hold the female wings away from the genitalia and the tarsi of the mesothoracic legs often move over the lateral and antero-lateral aspects of the female thorax. The superior claspers grip the abdomen of the female. To find out whether stimulation of sense organs in the female (thoracic) cuticle by male legs is necessary for ovulation to occur the following experiments were performed.

Two-day-old females of G. morsitans and G. austeni were each placed with a male of their own species and after copulation had occurred for 5 minutes the couples were chilled to immobility. The prothoracic legs were removed from some of the males, the mesothoracic legs from others and the metathoracic legs were amputated from some of the remainder. The couples were then allowed to continue mating and the males were removed from the tubes 2 days later.

Table 4

Effect of removal of legs from 10-day-old males on copulation with 2-day-old females. The females were dissected on day 10.

	MALES		FEMALES							
legs amputated	No.op.		No.cop. after op.		died		Ri.ov.		Ri.ret.	
	M	A	M	A	M	A	M	A	M	A
prothoracic	15	15	1	0	0	1	0	1*	15	14
mesothoracic	15	15	15	15	0	0	15	15	0	0
metathoracic	15	15	15	15	0	0	15	14	0	1
unop. control	10	10	10	10	0	1	10	9	0	0

* the spermathecae of this fly did not contain spermatozoa.

The females were dissected on day 10. Removal of the prothoracic legs rendered normal copulation impossible. It appears that during normal copulation these legs, which are applied to the anterior margin of the thorax of the female, are necessary for maintenance of contact between the thoraces of the male and female. Males whose prothoracic legs were amputated either discontinued copulation immediately or maintained genital contact only for a few minutes. None of the females of either species placed with such males were inseminated and only 1 out of 30 ovulated (table 4). Amputation of the mesothoracic legs affected neither copulation nor ovulation - all the flies mated with males lacking these legs were inseminated and ovulated. The presence of metathoracic legs does not seem to be a prerequisite of ovulation as 14 out of the 15 females of G. austeni and 15 out of the 15 females of G. morsitans mated with males lacking these legs were inseminated and ovulated.

To confirm that ovulation is not caused by stimulation of the female thorax by male legs the thoraces of 10 females each of G. austeni and G. morsitans were coated with wax so the setae thereon were rendered immobile. These flies were alive on day 10 and on dissection all were found to have been inseminated and all had ovulated.* Thus stimulation of the thoracic cuticular sense organs by the legs of the male during copulation is not the stimulus which causes the female to ovulate.

4. Effect of mating females with aspermic males.

Saunders (1960) came to the conclusion that insemination cannot be the factor controlling ovulation on the basis of his finding that certain "virgin" females of G. morsitans (which had,

* Of 10 control flies, whose thoraces had been waxed but which were left unmated, none ovulated.

in fact, copulated but were found to be uninseminated) ovulated. In order to provide stronger evidence concerning the role of spermatozoa in the determination of the first ovulation males of both G. morsitans and G. austeni were mated with many successive females of their own species with the aim of exhausting them of spermatozoa. Spermathecae of mated females were in all cases crushed and examined for the presence of spermatozoa.

Table 2 of Saunders and Dodd (1971) (see Appendix 2) gives the results of an experiment in which 12 males of G. morsitans were were such offered up to 13 females, at intervals of a few days, over a total experimental period of 76 days. The first few matings of each male resulted in insemination and ovulation in their mates, but later pairings resulted in ovulation in the absence of insemination. These results clearly show that insemination is not a prerequisite of ovulation. They also show that males of this species become aspermic between their third and ninth matings. Three males (Nos 10, 11 and 12) gave "aspermic" 4th matings, passed sperm to the next 2 or 3 females and then reverted to aspermicity. For males 1 to 9 the mean number of matings required to exhaust the males' spermatozoa supply was just under 6.

To provide information about the numbers of ovulations undergone in a known time by females mated with "spermic" and "aspermic" males, and about the follicle lengths in such flies, ten males of both G.morsitans and G. austeni were each mated with a series of 2-day-old females of their own species so that each male mated about a dozen times in 40 days. The females were dissected when they were 45 days old. Eight of the G. morsitans males and 7 of the

G. austeni males reached aspermicity and none appeared to recover fertility on later occasions. Examples of the data obtained from series wherein males became aspermic are given in tables 5 and 6 which show that the first few females mated with each male received spermatozoa and that the others were found to have spermathecae in which no spermatozoa were detected. For example, male 1 inseminated 8 females and copulated with, but did not inseminate a further 4 females, and male 3 inseminated 6 and copulated with an additional 7 females without transferring sperm to them. Of the series of females which showed some members to whom sperm had not been passed but which had ovulated the death rates were about 3% for G. morsitans and about 25% for G. austeni. The numbers of females which did not ovulate (and which it is assumed did not complete the full mating process) was low for both species (0 for G. austeni and 3 individuals of G. morsitans of which 2 had been offered males which had reached aspermicity). The estimated mean number of matings required to exhaust the male reproductive system of spermatozoa was 8 or 9 for both species (table 7). Table 8 shows that whereas most of the females (of both species) which had been mated with fertile males were in their 4th or 5th cycle on day 45, those mated with males which were aspermic were mostly in their 5th or 6th cycle at that time. The effect of the repeated early ejection of uterine contents associated with "aspermic" matings on the rate of progression of the ovarian cycle is discussed in a later section.

The results of these experiments confirm the opinion of Saunders (1960) that sperm transfer is not a necessary precondition

Table 5

Examples of numbers of larvipositions, estimated missed cycles, presence or absence of spermatozoa in spermathecae, follicle lengths and uterine contents of series of females of G. morsitans mated consecutively with individual males until the latter were aspermic.

MALE 1

FNo	LP	MC	NR	Spth	Lo	Li	Ri	Ro	U
1	3	0	4213	+	-	0.36	1.36	0.20	Cwl
11		died prematurely							
21	2	1	4213	+	-	0.34	1.34	0.22	Cwl
31	4	0	3142	+	0.16	0.70	-	0.24	a
41	3	0	4213	+	-	0.36	1.40	0.24	b
51	4	0	3142	+	-	0.48	-	0.16	a
61	4	0	3142	+	-	0.48	-	0.20	a
71		not mated							
81	2	1	4213	+	-	0.34	1.40	0.20	Cbl
91	1	3	3142	+	0.22	0.80	-	0.24	a
101	0	5	2431	-	0.30	-	0.20	1.20	a
111	0	4	3142	-	0.16	1.34	-	0.34	a
121	0	5	2431	-	0.18	-	-	0.54	a
131	0	5	2431	-	0.20	-	-	0.62	a

MALE 2

FNo	LP	MC	NR	Spth	Lo	Li	Ri	Ro	U
2	3	0	4213	+	-	0.32	1.28	0.18	Cwl
12	3	0	4213	+	-	0.38	1.42	0.20	Cwl
22	3	0	4213	+	-	0.40	1.56	0.22	Cwl
32	3	1	3142	+	-	0.50	-	0.22	a
42	4	0	3142	+	0.18	0.30	-	0.28	a
52	4	0	3142	+	-	0.52	-	0.22	a
62	3	1	3142	+	0.20	1.04	-	0.50	a
72		not mated							
82	3	1	3142	+	0.16	0.86	-	0.24	a
92	2	1	4213	+	-	0.36	1.34	0.20	Cwl
102	0	4	3142	+	0.20	1.38	-	0.32	a
112	0	5	2431	-	0.18	-	-	0.40	a
122	0	5	2431	-	0.20	-	-	0.50	a
132	0	5	2431	-	0.20	-	-	0.72	a

Table 5(continued)

MALE 3

FNo	LP	MC	NR	Spth	Lo	Li	Ri	Ro	U
3	2	1	4213	+	-	0.32	1.36	0.22	Cwl
13	2	1	4213	+	-	0.42	1.56	0.20	Cbl
23	4	0	3142	+	-	0.56	-	0.24	a
33	2	1	4213	+	-	0.44	1.50	0.20	Cbl
43	2	1	4213	+	-	0.42	1.62	0.20	Cwl
53	3	0	4213	+	-	0.36	1.60	0.20	Cbl
63	0	4	3142	+	0.16	0.76	-	0.28	a
73	not mated								
83	0	5	2431	+	0.18	-	-	0.48	a
93	0	5	2431	+	0.20	-	-	0.66	a
103	0	5	3142	+	0.20	1.50	-	0.42	d
113	0	4	3142	+	-	0.68	-	0.24	a
123	0	5	2431	+	0.22	-	-	0.68	a
133	0	5	2431	+	0.30	-	-	0.72	a

MALE 6

FNo	LP	MC	NR	Spth	Lo	Li	Ri	Ro	U
6	2	1	4213	+	-	0.26	1.08	0.18	b
16	3	0	4213	+	-	0.56	1.46	0.22	Cwl
26	3	1	3142	+	-	0.56	-	0.24	a
36	4	0	3142	+	-	0.88	-	0.28	a
46	died prematurely								
56	3	0	4213	+	-	0.40	1.60	0.24	Cbl
66	2	1	4213	+	-	0.38	1.58	0.18	Cbl
76	not mated								
86	4	0	3142	+	0.16	0.60	-	0.22	a
96	1	3	3142	+	-	0.48	-	0.22	a
106	0	4	3142	+	0.20	1.58	-	0.44	a
116	virgin								d
126	0	5	3142	+	0.20	1.44	-	0.42	d
136	0	virgin							d

Table 6

As table 5 for G. austeni males and females.

MALE 1

FNo	LP	MC	NR	Spth	Lo	Li	Ri	Ro	U
1	3	0	4213	+	-	0.36	1.56	0.16	Cbl
11	4	0	3142	+	0.16	0.70	-	0.22	a
221		died prematurely							
31	3	0	4213	+	-	0.38	1.54	0.16	Cbl
41		died prematurely							
51		died prematurely							
61		not mated							
71		died prematurely							
81	0	4	4213	+	-	0.38	1.50	0.20	d
91	0	4	3142	-	-	0.68	-	0.18	a
101	0	5	2431	-	0.14	-	-	0.46	a

MALE 3

FNo	LP	MC	NR	Spth	Lo	Li	Ri	Ro	U
3	4	0	3142	+	-	0.44	-	0.20	a
13	4	0	3142	+	0.16	1.18	-	0.28	b
23	2	1	4213	+	-	0.42	1.50	0.18	Cbl
33	3	1	4213	+	-	0.40	1.60	-	d
43	4	0	4213	+	-	0.40	1.58	0.18	d
53	3	1	3142	+	0.18	1.10	-	0.26	b
63	2	2	3142	+	-	0.32	-	0.18	a
73	4	0	3142	+	0.18	L.46	-	0.30	Cwl
83	2	2	3142	+	0.14	1.26	-	0.32	b
93	0	5	3142	-	0.16	1.42	-	0.32	d
1o3	0	5	3142	-	0.18	1.60	-	0.46	d
107		died prematurely							
113	0	5	2431	-	0.22	-	0.14	0.50	a

Table 6(continued)

MALE 4

FNo	LP	MC	NR	Spth	Lo	Li	Ri	Ro	U	U
4	1	3	4213	+	-	0.48	1.58	0.18		d
14	3	1	3142	+	-	0.48	-	0.18		a
24	3	1	3142	+	-	0.36	-	0.16		a
34	3	1	3142	+	-	0.56	-	0.18		a
44	3	1	3142	+	0.14	0.96	-	0.22		b
54		died prematurely								
64		died prematurely								
74	0	4	3142	-	-	0.64	-	0.18		a
84	0	4	3142	-	-	1.06	-	0.22		a
94		died prematurely								
104		died prematurely								
109	0	5	3142	-	0.14	1.40	-	0.28		d
114	0	4	3142	-	-	0.40	-	0.14		a

MALE 5

FNo	LP	MC	NR	Spth	Lo	Li	Ri	Ro	U	U
5		died prematurely								
15	1	1	4213	+	-	0.26	0.78	0.14		b
35	3	1	3142	+	-	0.46	-	0.16		a
45	4	0	3142	+	-	0.42	-	0.16		a
55		escaped								
65	4	0	3142	+	-	0.46	-	0.18		a
77		died prematurely								
85	4	0	3142	+	-	0.72	-	0.20		a
95	0	4	3142	-	0.18	1.08	-	0.22		a
105	0	4	4213	-	-	0.40	1.50	0.16		d
115	0	4	3142	-	-	0.48	-	0.22		a

Table 7

Results of experiment in which males were repeatedly mated until some became aspermic.

G. morsitans			G. austeni	
No.	%*	Category	No.	%*
99		females offered mates	79	
3	3.3	died prematurely	20	25.3
3(a)	3.3	not ovulated (virgins)	0	0
26	26.3	uninseminated + no. larvipositions + ovulated	16	20.3
65	65.7	inseminated + at least one larviposition	43	54.4
2	2.0	inseminated, no larvipositions, ovulated	0	0
8		no. of males reached aspermicity	7	
		estimated mean no. of matings before male reached aspermicity		
8.8		min.	8.0	
		max.	8.7	
6-11		range - min.	7-10	
		max.	5-11	

* - of no. of females offered mates

(a) - 2 of these had been offered males which had reached aspermicity.

Table 8

Results of experiment in which males were mated repeatedly showing numbers of females inseminated and uninseminated in each ovarian cycle.

G. morsitans			G. austeni		
no. ovs.	insem.	uninsem.	no. ovs.	insem.	uninsem.
4	35	0	4	16	1
5	30	9	5	29	11
6	2	7	6	0	2

for ovulation in G. morsitans and show that the same is true in G. austeni.

5. Effect of inserting male reproductive organs into virgin females.

Implantation of paragonia of Drosophila melanogaster males into the haemocoel stimulates virgins to lay eggs (Leahy, 1966) as does transplantation of male accessory glands in Aedes aegypti (Leahy and Craig, 1965). To discover whether any part or group of parts of the male reproductive system of G. morsitans produces a factor capable of causing females to ovulate the following experiments were performed.

a) Insertion of parts of male reproductive system into the thoracic haemocoel of virgin females.

The following male organs were implanted as described in chapter 2: accessory glands, testes, ejaculatory ducts and complete male reproductive systems. Experimental, operated control (into which only saline was inserted) and mated and virgin control flies were killed on day 18 and dissected. The results are shown in table 9 and in Saunders and Dodd (1972) (Appendix 2). Operated experimental flies showed no greater a proportion of ovulations than operated controls ($\chi_1^2 = 1.57$). The operated controls, however, had a significantly higher ovulation rate than the unoperated controls ($\chi_1^2 = 4.04$, $P<0.05$). This indicates that the operationally induced ovulation in the experimental group was due rather to surgical shock than to the nature of the implant. One may conclude tentatively from this experiment that substances secreted by the male reproductive system and passed to the female during copulation are not responsible for the onset of ovulation. Nevertheless, such substances would be released naturally within the uterine

Table 9

The effect of insertion of tissues from donor flies into the haemocoels of 9-day-old virgin females of *G. morsitans*.

IMPLANTED ORGAN		No. females	No. died	No. diss.	Egg(Ri) ov.	Egg(Ri) ret.
Inseminated spermathecae	(a)	19	4	15	3	12
Virgin spermathecae	(b)	20	7	13	2	11
Ejaculatory duct	(c)	16	5	11	1	10
Testis	(d)	18	0	18	3	15
Accessory gland	(e)	18	2	16	3	13
Complete male reproductive system	(f)	15	2	13	2	13
Operated control		27	0	27	6	21
Unoperated control		38	1	37	2	35
Mated control		6	0	6	6	0

lumen, hence the operational procedure used above would miss the appropriate receptors. Consequently the following technique was tested.

b) Injection of homogenates of male reproductive system into the uteri of virgin females.

Females of G. morsitans were 6 days old when operated and were held for 6 days after operation, then dissected. Injection was effected through fine glass tubes (Pasteur pipettes drawn in a flame). The transfer of material was verified by swelling of the abdomen and movement of the tracheae as seen from the ventral surface, and by the extrusion of homogenates from the vulva upon withdrawal of the pipette. It is probable that injected substances reached all parts of the uterus. Table 10 and Figure 4 shows that injection of homogenated parts and complete male reproductive systems did not result in a large increase in number of females ovulating. This experiment strengthens the evidence that male secretions are not responsible for ovulation, either by stimulation of chemoreceptors or by stimulation of mechanoreceptors.

6. Effect of squeezing males after 5 minutes copulation.

This experiment is reported separately because the nature of the stimulation given to the female is not precisely defined - some male secretions could be passed to the female and/or climactic movements (if such occur) by the male may be brought about prematurely. Sixteen 2-day-old females of G. morsitans were allowed to copulate for 5 minutes. The pairs were then chilled and the thorax and abdomen of each male was pinched firmly between finger and thumb. The

Table 10

The effect on ovulation of injecting homogenated male organs into the uteri of 6-day-old G. morsitans virgin females. Flies were dissected on day 12.

MALE ORGANS	No. op.	No. died	Ri ov.	Ri ret.
Reproductive system	20	1	1	19
Reproductive system + glass rod*	10	0	1	9
Accessory gland	10	2	0	7
Testis	10	1	0	8
Ejaculatory duct	10	0	1	10
Fat body	10	1	0	9
Operated control	10	1	0	9
Mated control	5	0	5	0
Unmated control	10	1	0	9

* 8 of these expelled their rod including the 1 which ovulated.

males were discarded as were females in which the penis did not maintain penetration throughout the pinching. The result of this procedure was that 2 of the 20 females subsequently ovulated and none of them contained sperm in their spermathecae. Eight unmated and mated controls retained their eggs and ovulated respectively. Further females were examined after operation and no evidence of secretion within the genital tract was found in any of them.

This experiment provides no useful information about how males cause females to ovulate. If a large increase in the proportion of females ovulating had resulted from the procedures described then one would have reason to suggest that some "climactic" movement by the male had been brought about prematurely and it was this that caused ovulation, although the role of male secretions would not have been clarified by this experiment alone.

7. Effect of mating females with teneral males.

Foster (1972) stated that accessory gland secretion in G. morsitans males remain very sparse until after the first meal, nevertheless, unfed males will almost invariably copulate. The effect of copulation in the comparative absence of accessory gland activity was examined. Twenty G. morsitans males were left unfed for 3 days after emergence, then paired with 2-day-old virgin females of the same species. Four males were observed to mount immediately and

penetration occurred in 3 of these. On dissection (day 10) 5 females were found to have ovulated but none were inseminated. No evidence of spermatophores was found in any dissected female. Mating females with teneral males provides one more method of inducing ovulation in the absence of sperm transfer and spermatophore construction (in G. morsitans). If the accessory glands in these teneral males are almost inactive, as suggested by Foster 1972, then these results provide evidence against involvement of accessory gland secretion in ovulation.

8. Effects of interspecific mating.

Vanderplank (1948), in his experiments on cross matings, found that G. morsitans males would copulate with G. austeni females and G. austeni males would copulate with G. morsitans females. His records of these pairings are confined, however, to only four females. In order to gain further information of these cross-matings the following experiments were performed.

Insemination and ovulation data derived from females of G. morsitans each placed for 24 hrs. with a potent male of G. austeni and for females of G. austeni placed with potent males of G. Morsitans when the females were 2 days old and which were dissected when 10 days old are given in tables 11 and 12. Eight of the G. morsitans females and 5 of the G. austeni females ovulated. Of these 4 of the G. morsitans and 1 of the G. austeni females were inseminated.

Twenty 10-day-old males of G. austeni were each placed with a 2-day-old female of the same species and whether or not the couples were copulating was recorded at intervals for 24hrs. The males were removed when they were 11 days old and mated 2 days later with other 2-day-old females of G. morsitans and a copulation record was kept as before. Spermatophores were sought in the tubes of the females to

Table 11

Results of experiment in which 2-day-old females of G. morsitans were placed with 10-day-old males of G. austeni. Females were dissected on day 15.

Group	Ri ovulated		Ri retained	
	insem.	uninsem.	insem.	uninsem.
"Cop"*	2	2	0	7
"Not cop."*	2	2	0	41

Table 12

Results of experiment in which 2-day-old females of G. austeni were placed with 10-day-old males of G. morsitans. Females were dissected on day 15.

Group	Ri ovulated		Ri retained	
	insem.	uninsem.	insem.	uninsem.
"Cop"*	1	0	0	2
"Not cop."*	0	4	0	68

* "Not cop." and "Cop" - the couples were examined at 5 mins. and 1 hour after pairing. "Not cop." means that the flies were not observed to be in copulation. "Cop." means that the flies were observed in copulation.

provide an independent check of the completion of copulation (see chapter 2). Females were killed and dissected when they were 15 days old. Table 13 gives the results for these pairings. The males inseminated all the conspecific females and spermatophores were recovered from 16 out of 20 of them. During their first allospecific pairing four G. austeni were observed to copulate and after two of these matings spermatophores were recovered. Three of the matings resulted in insemination and subsequent ovulation. During a second allospecific pairing two couples were observed to copulate and both of the females were inseminated and ovulated. No male gave two consecutive allospecific copulations. Out of 34 interspecific pairings 7 couples were observed to copulate, 5 females were inseminated and ovulated and two spermatophores were recovered.

A similar experiment was performed using twenty 10-day-old males of G. morsitans, placing them first with females of their own species and then twice with females of G. austeni: the results for the interspecific pairings are given in table 14. Nineteen out of twenty males of G. morsitans copulated with females of their own species. All of the females which copulated ovulated and 15 out of 19 spermatophores were recovered. Of the 17 males offered G. austeni females for the first time none were observed to copulate and only one female was found to be inseminated and to have ovulated. Of the 16 G. morsitans males offered G. austeni females for the second time one was seen to copulate (the female was inseminated and later ovulated) and one female ovulated although it had not been observed to mate, and its spermathecae were devoid of spermatozoa.

No evidence of pregnancy or even of embryogenesis was observed

Table 13

Results of experiment in which G. austeni males were mated first with G. austeni females (Mate 1) and then twice successively with G. morsitans females (Mates 2 and 3). Females were mated on day 2 and killed on day 15.

G. aust. male	Mate 1 G. aust.				Mate 2 G. morsit.				Mate 3 G. morsit.			
	Cop*	Spp	Spth	Ov	Cop*	Spp	Spth	Ov	Cop*	Spp	Spth	Ov
1	+	+	+	+	-	-	-	-	-	-	-	-
2	+	+	+	+	-	-	-	-	-	-	-	-
3	+	+	+	+	+	+	+	+	-	-	-	-
4	+	+	+	+	-	-	-	-	-	-	-	-
5	+	-	+	+	male dead							
6	+	+	+	+	-	-	-	-	-	-	-	-
7	+	+	+	+	-	-	-	-	+	-	-	+
8	+	+	+	+	male dead							
9	+	+	+	+	-	-	-	-	-	-	-	-
10	+	+	+	+	-	-	-	-	-	-	-	-
11	+	-	+	+	-	-	-	-	+	-	+	+
12	+	+	+	+	+	-	-	-	-	-	-	-
13	+	+	+	+	-	-	-	-	male dead			
14	+	+	+	+	-	-	-	-	-	-	-	-
15	+	-	±	+	-	-	-	-	-	-	-	-
16	+	+	+	+	+	-	-	-	-	-	-	-
17	+	-	+	+	-	-	-	-	male dead			
18	+	+	+	+	+	+	+	+	-	-	-	-
19	+	+	+	+	-	-	-	-	-	-	-	-
20	+	+	+	+	+	-	+	+	-	-	-	-

* Cop - couples were observed at 5 mins., ½ hr., 1 hr., 6 hr., and 24 hrs. after pairing. A negative sign in this column does not mean that a couple did not copulate but that is was not observed to do so.

Table 14

Results of experiment in which G. morsitans males were mated first with G. morsitans females (mate 1) and then twice successively with G. austeni females (Mates 2 and 3). Females were mated on day 2 and killed on day 15.

G. morsit. male	Mate 1 G. morsit.				Mate 2 G. aust.				Mate 3 G. aust.			
	Cop*	Spp	Spth	Ov	Cop*	Spp	Spth	Ov	Cop*	Spp	Spth	Ov.
1	+	+	+	+	male dead							
2	+	+	+	+	-	-	-	+	-	-	-	+
3	+	-	+	+	-	-	-	-	-	-	-	-
4	+	+	+	+	male dead							
5	+	+	+	+	-	-	-	-	-	-	-	-
6	+	+	+	+	-	-	-	-	-	-	-	-
7	+	+	+	+	-	-	-	-	-	-	-	-
8	+	+	+	+	-	-	-	-	-	-	-	-
9	+	-	+	+	-	-	-	-	male dead			
10	+	+	+	+	-	-	-	-	-	-	-	-
11	+	+	+	+	male dead							
12	+	+	+	+	-	-	-	-	-	-	-	-
13	+	+	+	+	-	-	-	-	+	-	+	+
14	+	+	+	+	-	-	-	-	-	-	-	-
15	+	+	+	+	-	-	-	-	-	--	-	-
16	-	-	-	-	-	-	-	-	-	-	-	-
17	+	+	+	+	-	-	-	-	-	-	-	-
18	+	-	+	+	-	-	+	+	-	-	-	-
19	+	+	+	+	-	-	-	-	-	-	-	-
20	+	-	+	+	-	-	-	-	-	-	-	-

in any of the cross-paired females. These experiments show that the mating stimulus which initiates ovulation is not specific and they confirm that ovulation can occur in the absence of insemination. Under the conditions in the Edinburgh laboratory the insemination rates of G. morsitans females by G. austeni males (9 out of 90) and of G. austeni females by G. morsitans males (3 out of 108) appear to be lower than those found by Vanderplank (2 out of 3 and 1 out of 1 respectively).

9. Effect of copulation duration and of multiple copulation on ovulation.

Work on this aspect of mating has been reported in Saunders and Dodd (1972) but was not performed by myself. When mating couples of G. morsitans were separated at various times after initiation of copulation the following results were obtained.

Nearly all of the females separated manually within 1 hour of the beginning of copulation were not inseminated nor did they ovulate. After 2 hours of coitus about one quarter of the females were inseminated and ovulated, another quarter were not inseminated and ovulated and the remainder were not inseminated and did not ovulate. After longer periods of copulation the proportion of females inseminated increased up to 6 hours copulation but in every case a small proportion of flies remained uninseminated but ovulated. The experiment shows that insemination does not have to precede ovulation and that increasing copulation time is associated with increasing chance of ovulation.

All but one of the females which separated themselves, even those which did so after only one or two hours of copulation, ovulated and the majority of these were inseminated.

When males were mated once for 45 minutes for 2, 3 or 4 consecutive 45 minute periods with different females it was found that the proportion of the females ovulating in each group were not significantly different. In other words the females which had mated for 45 minutes with previously unmated males did not show ovulation rates different from those females which had mated for 45 minutes with males which had mated once, twice or thrice before.

In another experiment about 25% of uninseminated females which had been given one 45 minute mating ovulated. Of those given two consecutive 45 minute matings with different males 35% ovulated. Forty-nine per cent of those given 3 and 80% of those given 4 consecutive matings ovulated. Control flies which were chilled and handled 4 times but not mated had a 24% ovulation rate. This is significantly less than that of the females mated 4 times. None of these experimental flies expelled a detectable spermatophore. This experiment shows that females summate the mating stimuli provided by different males but does not give information about the nature of the stimulus except that the construction of spermatophore is not a prerequisite of ovulation and neither is sperm transfer.

10. The role of auditory stimuli in mating.

The wing-beat frequency of a courting male of Drosophila species is an important factor in determining whether or not the courted female will copulate with that male (Bennet-Clark and Ewing, 1970). The main female receptors for such vibratory stimuli are situated in the aristae of the antennae (Manning, 1967). Vanderplank (1948) working on interspecific pairings of Glossina species stated "Unless the mating notes (wing-beat, flying or stationary) of the two species

to be crossed overlap in their frequency range, no attempt to mate will under normal conditions, be made by the two species." To find out whether the wing-beat notes of male tsetse influence female sexual receptivity the wings and/or antennae of G. morsitans were amputated (see chapter 2) prior to pairings. The results of these treatments on copulation insemination and ovulation are given in table 15. It is clear that neither the antennae nor wings of either sex are necessary for copulation to occur and so if "mating songs" are emitted by male tsetse they are not necessary for copulation to occur.

11. Effect of mutilating copulating females.

In order to investigate possible blood- and nerve-borne factors controlling ovulation the following experiment was performed. Females of both G. morsitans and G. austeni were divided when they were 10 days old into two groups. One group, containing two-fifths of each day's batch, was left unmated and the flies in the remaining group were mated on day 10. Five minutes after the commencement of copulation in the latter group all the flies in both groups were chilled to immobility and the "neck" of some flies in both groups were ligatured, the heads of some flies in both groups were amputated and the "waists" of some flies in both groups were ligatured. None of the males was operated upon. Some females in both groups were left unoperated. Thereafter the flies were allowed to regain room temperature (25°C). All surviving flies were killed on day 13 and dissected. The results of this procedure are shown in table 16. The death rates of flies in the operated groups are high - about 75% of those mated and about 50% of those unmated died in the 3 days

Table 15

Effect of removal of wings or antennae on mating and subsequent events in G. morsitans.

M*	F*	No	Cop	insem.	Ov.	
-	-	10	10	10	10	
W	-	10	10	10	10	
-	A	10	10	10	9	(one died)
W	A	10	9	9	9	
A	W	10	9	9	9	

*M - male
*F - female

W - wings amputated)
A - antennae amputated) as described in chapter 2.

Table 16

Data for 10-day-old virgins of G. morsitans (M) and G. austeni (A) to some of which amputations and ligatures were applied after commencement of copulation.

Operation (day 10)	No.op.		died		lived to day 13		Ri ov.		Ri ret.	
	M	A	M	A	M	A	M	A	M	A
Neck ligt + mated	20	20	11	15	9	5	0	0	9	5
Neck ligt unmated	20	20	10	7	10	13	0	0	10	13
Mated control	10	10	0	0	10	10	10	10	0	0
Unmated control	10	10	0	1	10	9	0	0	10	10
Head amptd + mated	20	20	13	14	7	6	1(a)	0	6	6
Head amptd unmated	20	20	11	8	9	12	0	0	9	12
Mated control	10	10	1	0	9	10	10	10	0	0
Unmated control	10	10	0	0	10	10	0	0	10	10
Waist ligt + mated	20	20	15	16	5	4	0	0	5	4
Waist ligt unmated	20	20	12	18	8	2	0	0	8	2
Mated control	10	10	0	0	10	10	10	10	0	0
Unmated control	10	10	0	0	10	10	0	0	10	10

(a) - this fly had no spermatozoa in its spermathecae.

between operation and sacrifice. Possibly the persistent and sometimes violent attempts by the males to mate with operated females resulted in the higher death rate in those which had been placed with males. Nearly all of the mated controls ovulated, practically none of the unmated controls ovulated, none of the unmated operated flies ovulated and only 1 out of the 60 mated operated flies ovulated. None of these latter flies (mated, operated) had received spermatozoa. Males were observed to resume mating readily with those unoperated females with which they were placed but they were seen to be actively rejected by the operated females, who curled their wings in the manner typical of mated females. Removal of the wings from such flies did not result in successful mating although some ephemeral penetration of the male genitalia into those of the females were observed. The results show that these were insufficient to result in either sperm transfer or in ovulation. It appears that copulation cannot be completed in the absence of nervous and humoral connection between abdomen and thorax or between the abdomen and head and it is possible that nerve and/or blood-borne factors operating between the head or thorax and abdomen are involved in the control of the mating act. This experiment does not provide any information about the relationships between mating and ovulation.

12. <u>The effect on ovulation of inserting parts of mated flies into virgins.</u>

After a female has "registered" a completed copulation at least some hours elapse before ovulation occurs. The route by which the information that mating has occurred reaches the ovulation effector could be nervous, humoral or a combination of the two. To test for

possible humoral effects, spermathecae, homogenates and extracts of mated flies were inserted into virgin females of G. morsitans.

a) Spermathecae.

Transplantation of spermathecae from mated females into virgin females of Rhodnius prolixus results in an increased egg production (Davey, 1965). To what extent the effect was on egg maturation, ovulation or just oviposition was not specified. Davey suggested that a blood-borne factor released by the inseminated spermathecae stimulates egg production in Rhodnius although nervous control of oviposition was not excluded. Table 10 shows that insertion of inseminated spermathecae no more ovulations in virgins of G. morsitans than did insertion of virgin spermathecae. Hence spermathecae probably play no role in the control of the first ovulation. This is in agreement with the finding that ovulation can occur in the absence of insemination.

b) Homogenate of head and thorax.

The following experiment was designed to find out whether substances capable of initiating ovulation are present in the neuro-endocrine systems of mated flies. Eight-day-old females of G. morsitans usually ovulate within 24 hours of mating. Ninety 8-day-old females of G. morsitans were mated and 30 of them were killed after 6 hours in copula, 30 were killed 14 hours later (ie within a few hours of ovulation) and the remainder (30) were killed 2 days after pairing. As each group was killed homogenates of brain and the contents of the anterior half of the thorax were prepared in a little saline. The homogenates were centrifuged and then injected into 8-day-old virgin females, 1 µl per fly. The flies were dissected

on day 12. Table 17 and Figure 4 shows that the injection of homogenate induced no more ovulations than did injection of saline. Thus the induced ovulations were probably due to the surgical procedure rather than to the nature of the injected material.

c) Alcoholic extracts.

Forty whole mated females of G. morsitans were ground in 95% ethanol in a tissue grinder. The preparation was filtered and the filtrate was evaporated to dryness. The filtrate was dissolved in 0.9% saline and the solution was injected, in 1 µl aliquots, into twenty 8-day-old virgins. A similar preparation derived from virgin flies was injected into another twenty 8-day-old virgins. Table 17 and Figure 4 show that both extracts induced ovulation to the same extent (1 fly in 13) which was no greater than that resulting from injection of saline. Thus the induced ovulations were probably due to operational shock. "Ovulation hormone", if such exists, however, may be neither alcohol soluble nor heat stable.

d) Substances from post-parturient females.

Nayar (1958) showed that a hormonal interaction between the MNC and the ovaries was responsible for oviposition in Iphita limbata by assessing the effects on egg laying of injecting into virgins blood, aqueous ovarian extracts and neurosecretory cells from mating and mated females.

In order to find out whether similar interactions are involved in ovulation in tsetse flies a similar series of injections was performed. Ovulation occurs within an hour of larviposition in Glossina (see section D of this chapter) even when the parturition is enforced by squeezing (see chapter 2). Thus any humoral factor

Table 17

The effect on ovulation by G. morsitans females of injecting extracts of mated and unmated flies.

Injected material	No treated	No died	Ri ov.	Ri ret.
H* 4hrs after cop	20	8	-	12
20hrs after cop	20	1	2	17
48hrs after cop	20	14	0	6
unmated	20	3	1	16
A*44 mated females	20	6	1	13
40 virgin females	20	6	1	13
control (saline)	20	2	2	16

*H - brain and thorax homogenate of 10 females in each group.

*A - aqueous solution of alcoholic (95% EtOH) extract.

influencing ovulation must be present in the endocrine system, blood and ovaries at some time during that hour. In an attempt to detect any such substance the median neurosecretory cells (with some associated tissue) of pre-parturient flies and of flies which had, within the previous half hour, been induced to larviposit, were inserted separately into the thoraces of 8-day-old virgins of G.morsitans. Iul of haemolymph was drawn from each of 20 post-parturient females and these aliquots were inserted separately into each of 20 recipients. The reproductive systems of the 20 donors were washed with 20ul of 0.9% saline and 20 recipients each received a Iμl aliquot of the extract. Operated control flies received 0.9% saline only. Flies were dissected on day I2.

Table I8 shows that the injections did not evoke a significant number of ovulations. This provides some evidence against direct neuroendocrine instigation of ovulation in tsetse.

An experiment was performed to test for possible humoral control of larviposition. A group of flies was observed during the first pregnancy and on the day following that on which the polypneustic lobes first became visible through the female abdomen some of the flies were induced to larviposit by applying gentle pressure to the thorax. Half an hour after larviposition the MNC with adjacent tissue, the block of tissue in the antero-dorsal thorax containing the remainder of the central neuroendocrine system, and Iul of haemolymph were removed and inserted into separate recipients drawn from the remaining parturient flies. Some recipients received these implants from parturient flies which had not been induced to drop their larvae. Operated control flies received 0.9% saline

Table 18

The effect on ovulation of injecting substances from pre-parturient (P) and post - parturient (PP) females of *G. morsitans* into virgin females.

Injected material	Source	No treated	No died	Ri ov.	Ri ret.
MNC	P	20	5	1	14
MNC	PP	20	4	0	16
haemolymph	PP	20	4	0	16
ovary extract	PP	20	2	1	17
saline	-	20	4	1	15

and unoperated controls were left unmolested.

Table 19 shows that unoperated flies usually dropped their larvae between three and four days after the black lobes became apparent (ie 2 or 3 days after operation of recipient flies) and that this pattern was not altered by injecting any of the materials mentioned: it also shows that material from parturient females dissected two days after the appearance of the black lobes did not induce larviposition. The results argue against direct hormonal control of larviposition in tsetse.

13. A summary of the results of attempting to stimulate the first ovulati

Figure 4 shows the percentage of females of G. morsitans induced to ovulate by use of the techniques described in this chapter, along with the responses of operated and unoperated controls. Copulation, even when deficient, produced more ovulations, than any other technique tested. None of the surgical procedures induced such high rates of ovulation and ovulations resulting from insertion of material or foreign bodies into the uterus were attributable largely to operational trauma. Although ovulation could not be experimentally induced at a high level it is felt that the evidence justifies, mainly by systematic exclusion of other possibilities, the conclusion that ovulation is brought about by a mechanical stimulus applied to the uterus by the male. Simply stretching the uterus has no effect. Whether special movements of parts of the male genitalia are involved or whether the effect is due to a complex of mechanical stimuli is not known.

14. The effect of artificial stimuli on later ovulation.

In order to find out whether artificially induced ovulations

FIGURE 4 Effects of attempts to stimulate ovulation

Treatments:—

1 mated normally
2 unmated unoperated
3 external mechanical stimuli
haemocoelic implants of :—
4 a ♂ reproductive system
b spermathecae
5 homogenate of mated ♀ brain etc.
6 alcoholic extract of mated female
c " " unmated "
d saline
7 material from post-parturient ♀♀
intra-uterine implants:
8 glass rods
9 penis
10 ♂ reproductive organs
11 ♂ squeezed after 5mins cop.
12 chilled in cop.
♀♀ paired with :—
13 semi-pneumopophysectomized ♂♂
14 × males lacking legs II or III
15 " " " I
16 " " wings
17 aspermic ♂♂
18 teneral "
♂♂ of another species
19 G. aust. ♂ G. mors. ♀
20 G. " ♀ G. " ♂

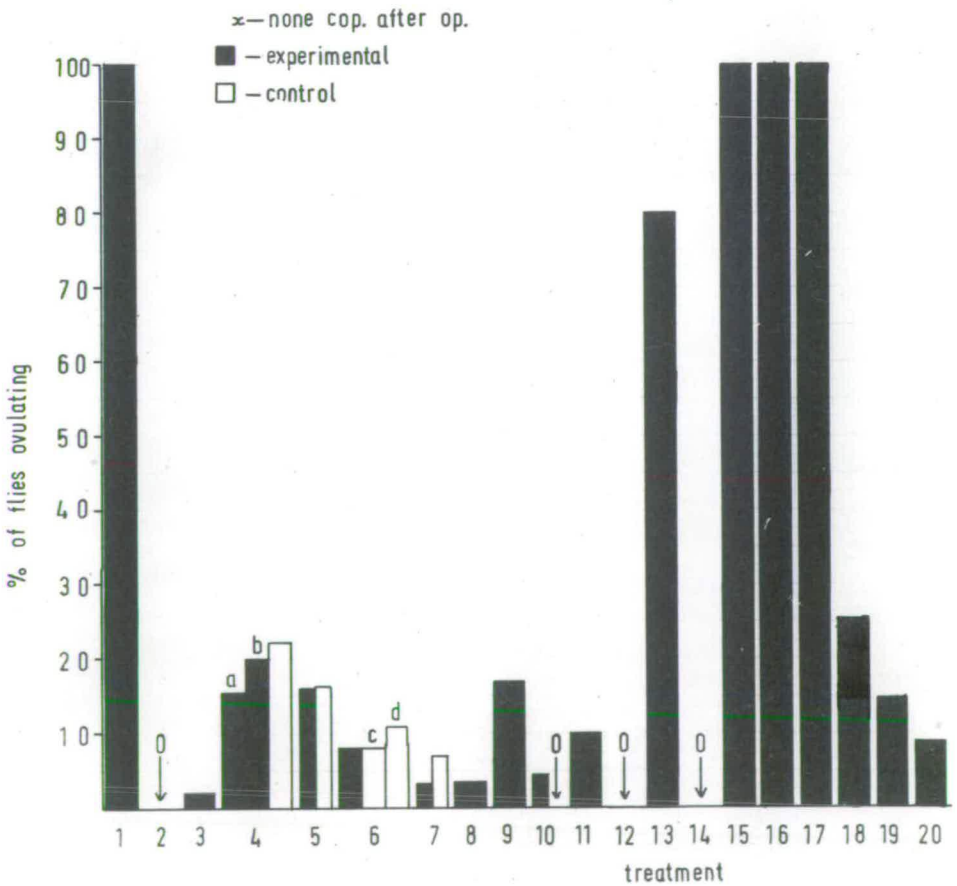


Table 19

The effect on time of larviposition by G. morsitans females of injecting substances into parturient females.

Treatment	No. which larviposited on various days after operation				No op.	No died.
	*1 day	2 days	3 days	4 days		
Unop. control		5	6	1	13	1
Op. control		6	3		9	0
MNC		5	3		8	0
TH A	1	4	3	2	12	2
HL		7	5	1	14	1
MNC		7	3		10	0
TH B	1	2	8	1	13	1
HL		3	5		8	0
MNC		3	8		13	2
TH C		7	5		13	1
HL	1	3	5		11	2

* - after operation of groups A, B and C.

A - from females induced to larviposit

B - from females 1 day after appearance of black lobes

C - from females 3 days after appearance of black lobes.

are followed by subsequent ovulations the following experiment was performed. Twenty 8-day-old virgins of G. morsitans were chilled, and received saline in the thoracic haemocoel. They were then chilled and handled three more times and left unmated until day 23. Of the 18 survivors four were found to have ovulated twice, one had ovulated once and the remainder had not ovulated. It thus appears likely that once the first has been accomplished, other ovulations follow normally.

B. PHENOMENA ASSOCIATED WITH EGG RETENTION.

1. Egg retention and follicle growth rate.

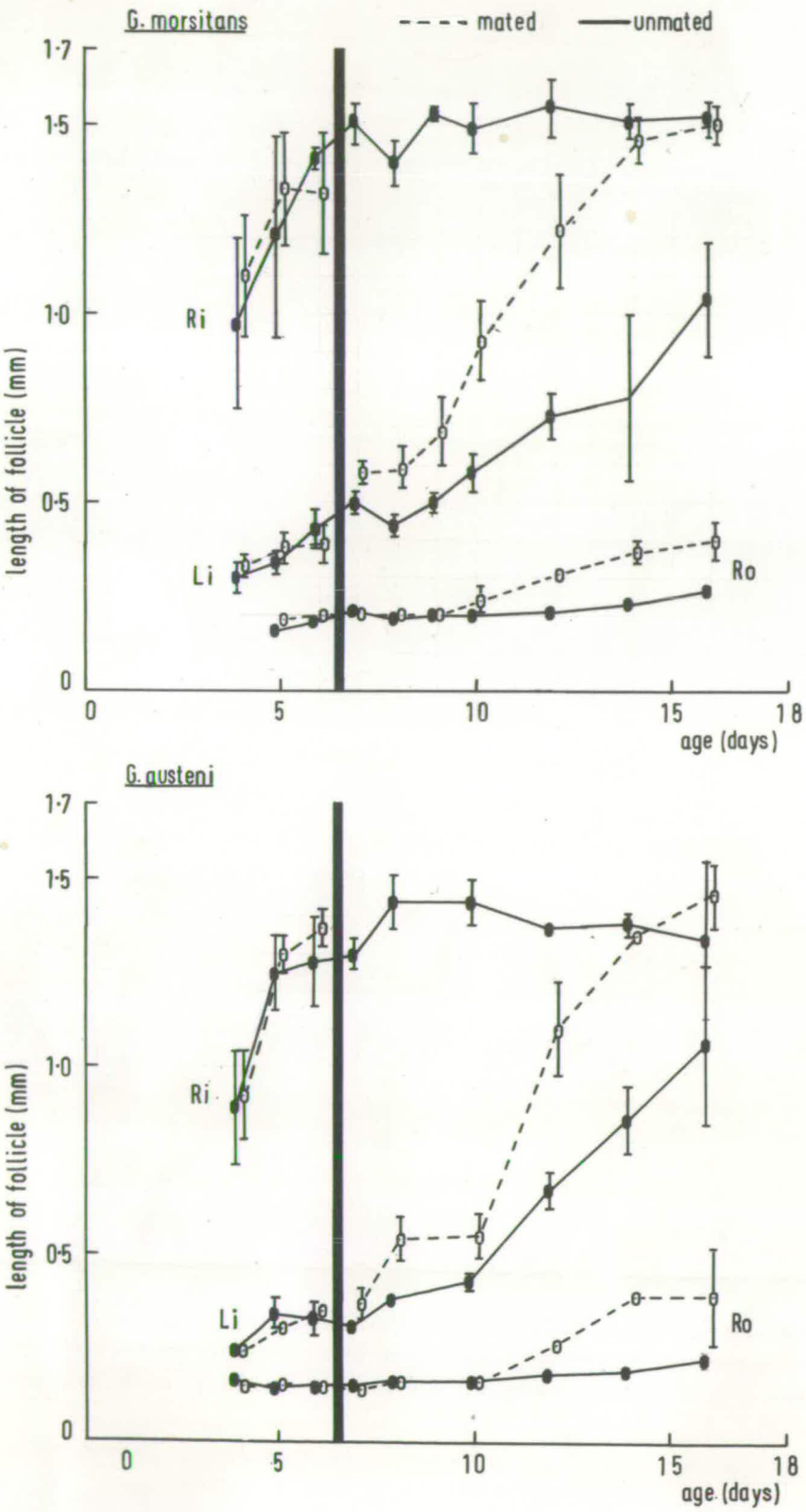
Saunders (1970) and Dodd (1971) found that the sizes of follicles in mated flies prior to the first ovulation are similar to those of follicles in unmated flies of the same age. Unmated female tsetse retain chorionated eggs within the ovaries and associated with this retention there is a reduction in the growth rates of the ovarian follicles. This inhibition of follicle growth was first mentioned by Mellanby (1937) who gave no measurements of the phenomenon. Saunders (1960) measured follicle lengths of 9- to 21-day-old mated and unmated females of G. morsitans and found that the pregnant flies (which, of course, had ovulated) had larger follicles than virgin flies of the same age. Saunders (1970) mentions this inhibitory phenomenon but he gives no data. His statement (personal communication) was based on follicle measurements made on days 5, 13, 22 and 31 of the adult lives of mated and unmated females of both G. morsitans and G. austeni. These days were chosen to coincide with the approximate mid-points

of the first 4 reproductive cycles. Saunders found that in both species the follicle lengths of 5-day-old mated females were not significantly different from those of unmated flies of the same age, but that marked differences existed between the follicle sizes in the two groups at other ages. Li did not become chorionated in the virgins until 8 or 9 days after it had done so in pregnant flies. The data on which the confirmation of this inhibitory phenomenon as given by Dodd (1971) is based are given in figure 5. The follicles of females of both G. morsitans and G. austeni, mated and unmated, were measured at two day intervals throughout the first 16 days following eclosion. Over half of the mated flies of both species dissected on day 7 and on subsequent days had ovulated whereas less than half of those dissected before had done so. In figure 5 the end of day 6 has been adopted as a "standard" for the time of ovulation and this is the significance of the vertical bar at this point. To the left of this bar data for non-ovulated flies have been considered and to the right of the bar are depicted results derived from mated flies which have ovulated and from virgins. The error bars indicate the fiducial limits($P < 0.05$).

Before day 6, the mean follicle lengths are not significantly different in either species as between mated and conspecific virgins of the same age. Thus the growth rates of comparable follicles before the first ovulation are the same in mated and unmated flies. By the second day after ovulation the mean length of Li had become significantly larger than in the virgins and within 4 days of ovulation a difference existed between the mean lengths of Ro of the two groups.

These results provide evidence that the growth rate of follicles

FIGURE 5—Follicle lengths in young flies (mated and unmated)



throughout the period before the first ovulation is independent of mating. They also confirm that acceleration of follicle growth is apparent soon after the first ovulation has occurred.

Egg retention occurs also during the last few days of pregnancy. To ascertain whether artificial prolongation of pregnancy results in prolonged follicle inhibition the following procedure was adopted. Mated females of G. morsitans were observed daily during their first pregnancy until the black respiratory lobes of the late first instar larva were seen through the postero-ventral cuticle. Parturition normally occurs two or three days after these lobes become visible according to Newstead, Evans and Potts (1924) or 1½ days according to Potts (1933). On the day following such blackening the vulvae of some flies were ligatured tightly around the larval respiratory lobes with human hair. Of the parturient flies ready for operation on each day about one half were ligatured (E), one quarter were killed immediately (C1) and the remainder were killed at the time of death of the surviving operated flies (C2); ie 4 days after the ligatures were applied. None of the flies were fed after the day of operation because flies in late pregnancy are reluctant to feed. Follicle lengths of dissected flies are given in table 10. Data are considered only from batches in which all three groups were represented at the time of sacrifice.

The death rate in experimental flies (E) was high (two-thirds of the flies in E groups considered died within the 4 days between operation and sacrifice). One out of 43 control flies (C2) died within this period. All E and C1 flies had NR of 3142 and a black-lobed 3rd instar larva in the uterus and all C2 flies had NR of 2431

Table 20

Effect of enforced larval retention on growth of Ro in *G. morsitans* females.

	E	C1	C2
No considered	60	38	43
Deaths	40	-	1
Mean length of Ro	0.56	0.57	0.71
Max. length of Ro	0.64	0.80	0.84
Min. length of Ro	0.48	0.48	0.56

E - experimental flies ligatured during 1st pregnancy (see chapter 2)

C1 - control flies killed on day of operation of E.

C2 - control flies killed on day of death of E.

and an egg in the uterus on dissection. The means of the lengths of Ro in each group were 0.56mm for E, 0.57mm for C1 and 0.71mm for C2. Analysis of variance showed that growth of Ro in the E flies was significantly inhibited ($P<0.05$). That this inhibition was almost absolute was almost unexpected; it was probably associated with the high mortality rate of the operated flies. This experiment shows that oostasis resulted from artificially prolonged pregnancy but the degree to which this inhibition was due to operational shock cannot be discerned.

2. The nature of oostasis in tsetse.

Adams, Hintz and Pomonis (1968) showed that extracts of the females reproductive system of Musca domestica containing mature eggs when injected into test females, cause a greater inhibition of follicle growth than extracts from females with only immature eggs. These authors proposed that an "oostatic hormone", produced by females with eggs in stages 4 to 10, probably operating by inhibition of corpus allatum, is responsible for oostasis in Musca. It was suggested that the hormone inhibited maturation of immature eggs completely at stage 4. The following experiment was designed to find out whether a similar factor could be detected in tsetse.

A group of G. morsitans females were mated on day 8, by which time Ri is chorionated and capable of ovulation. On day 9 some of the flies were dissected and all were found to have ovulated. On day 10 the follicles of some flies (UC1) were measured and from the reproductive systems of 25 of the others an alcholic (95%ethanol) extract was prepared, filtered and evaporated to dryness. The filtrate was dissolved in 25 µl of 0.9% saline and 1 µl of this solution was injected into each of 20 remaining females (EM). A similar extract, prepared from the reproductive systems of twenty-five 25-day-old virgins, was injected into 20 of the remaining flies (EV) and the rest of the females were left unoperated (UC2). All survivors were dissected on day 13, and the sizes of their two largest

follicles were measured. Thus flies were treated when their ovaries contained relatively immature eggs - one set received a preparation from females with two chorionated eggs (in which oostasis was, presumably, operative), another set received extract from females whose eggs were in the same immature state as themselves and the remainder were untreated. Measurements of follicle sizes were thus available for flies at the beginning and at the end of the experiment and for the two groups of experimental flies.

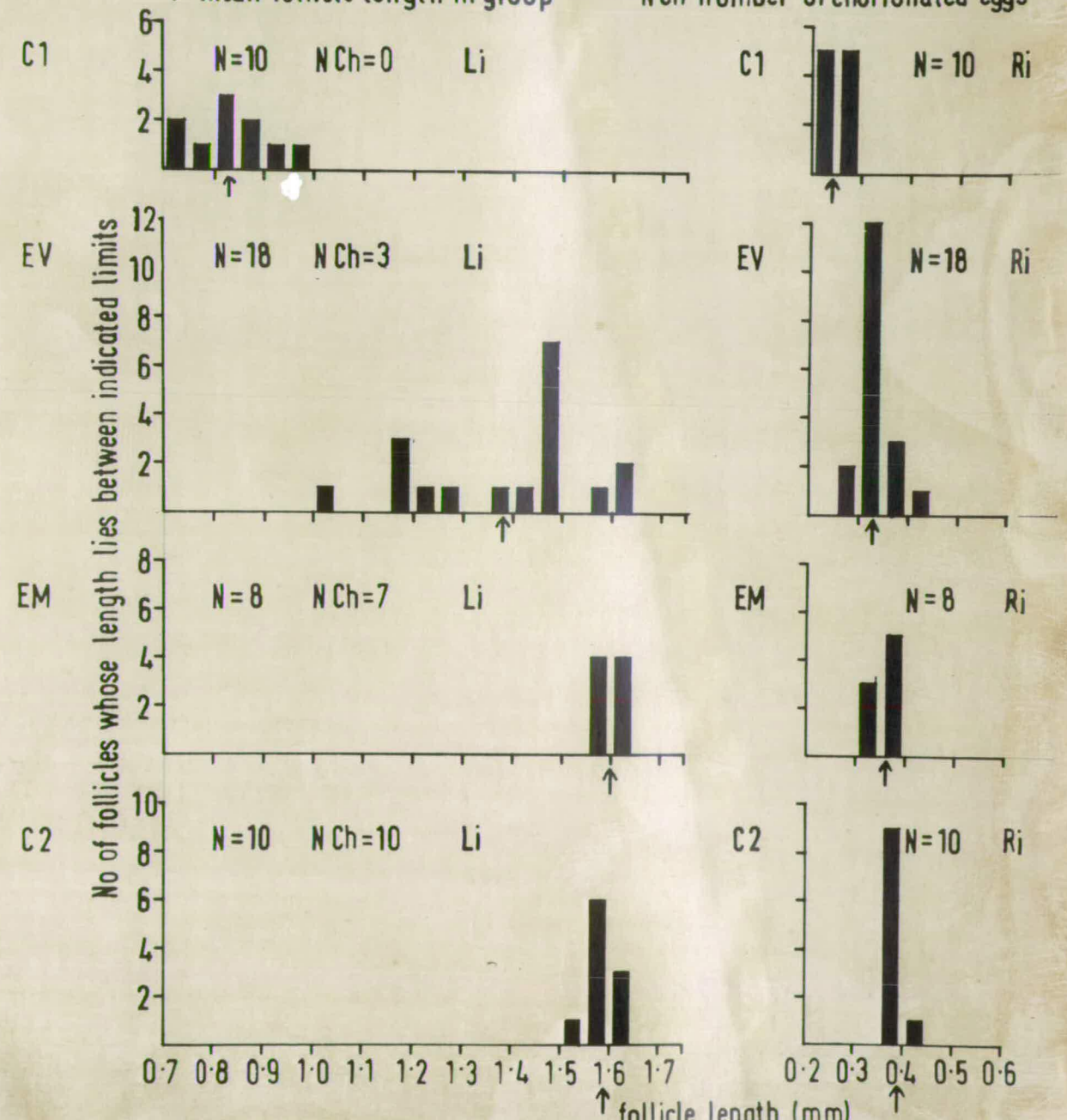
Figure 6 shows the distribution and mean values of the lengths of Ri and Li in the four groups. Each bar represents the number of follicles whose length lies between the limits indicated by the edges of the bar. The width of each bar represents 0.05mm, although a small space has been left between bars to increase graphical clarity. The figure shows that the distribution of follicle lengths and their mean value were similar for groups EM and C2. The distribution of follicle lengths in group EV was, however, much wider than that of any other group. Moreover, the mean lengths of Ri and Li in the EV group were lower than those of corresponding follicles in EM and C2 but higher than those of CI. Thus injection of extract from reproductive systems containing small follicles did not markedly affect follicle growth after operation but the result of injecting extract from reproductive systems containing chorionated eggs was a partial inhibition of follicle growth during the post-operative period. More EM flies than EV flies died and so the apparent follicle inhibition is not related to mortality.

Li in all C2 flies and in 7 out of the 8 EM flies dissected was chorionated whereas only 3 out of the I8 EV flies dissected had Li chorionated. All flies fed 2, 3 or 4 times between operation and sacrifice and no differences existed in feeding frequency between the groups. Some of the flies which died prematurely had fed, others had not. The stage of development of offspring in the

FIGURE 6 Effect of extracts of reproductive systems on follicle length distribution and mean follicle length in G morsitans females. Flies were injected on day 10, dissected on day 13 except for C1.

C1 control flies killed at beginning of experiment
C2 " " " " same time as experimental flies
EV experimental flies injected with extract from r.s. of virgin females
EM " " " " " " " " mated "

↑ mean follicle length in group NCh number of chorionated eggs



uterus did not appear to be affected by the operation although a great variation existed within each group, from 1st instar larva in the chorion to white-lobed third instar larva. One of the operated controls had aborted and its uterus contained an egg.

These results suggest that an alcohol and water soluble follicle inhibiting factor ("FIF") may be present in the reproductive systems of flies containing chorionated eggs in their ovaries. This possible factor is, however, different from that postulated by Adams et al in that it does not prevent vitellogenesis and does not totally inhibit egg maturation at any stage - virgin females do develop 2nd and subsequent eggs but at a reduced rate.

3. The effect of prolonged egg retention on egg viability.

In order to determine how long eggs can remain in the ovaries of virgins before they become functionally inviable (ie before they lose the ability and/or the opportunity of forming embryos) female tsetse flies were mated after various periods of virginity and were dissected at various intervals thereafter.

Virgin females of G. morsitans and G. austeni were mated at ages 2, 5, 10, 15, 20 and 45 days and dissected at 2, 5, 8, 12, 16 and 20 days after mating. There were 10 flies in each category and so 360 females of each species were used. The categories into which individual females from each day's eclosion batch were placed were decided by reference to a random-number chart. The numbers of flies which died prematurely and of those which did not ovulate are given in tables 24 and 25. It is seen that the longer the delay in mating the greater the tendency to failure to ovulate. Tables 21 and 23 give the number of eggs ovulated or degenerated at intervals

Table 21

Ovarian age categories of females of G. morsitans mated at various ages and dissected at various times thereafter.

No. of ovs. and degs.	UC	Age when mated 2 days						5 days						10 days					
0	d	8	8					9	1					3					
1	a		2	10					9	3				7	6				
	b				1					2					2				
	c				6	4	1			5	6	1	1			6	3		
	d				1		1									1			
2	a				1	5	3				4	4			1	1	6	2	
	b						2					2	1					3	
	c											1	4					1	3
	d												1				1		1
3	a												3					2	4
	b																		
	c																		
	d																		
4	a																		
	b																		
	c																		
	d																		
5	a																		
	b																		
	c																		
	d																		
Days after mating		2	5	8	12	16	20	2	5	8	12	16	20	2	5	8	12	16	20
Age (days)		4	7	10	14	18	22	7	10	13	17	21	25	12	15	18	22	26	30

Table 21 (continued)

No. of ovs. and degs.	UC	Age when mated 15 days						20 days						45 days					
0	d													1					
1	a	10	2					4	5						1				
	b		1																
	c			6															
	d		1	1					2										
2	a		1	3	3			6	2	2									
	b									1									
	c				4	5	2			1	5	2							
	d				1	2	1				1				1				
3	a				1	3	3			5	3	3	2	3*	3				
	b						1				1	1	1						
	c				1		1					1	1						
	d														2	1			
4	a												1	1		2	2(a)	1	
	b															1	1	1	
	c																1		1
	d															1			
5	a															1	2	2	3
	b																		
	c																		3
	d																		1
Days after mating		2	5	8	12	16	20	2	5	8	12	16	20	2	5	8	12	16	20
Age (days)		17	20	23	27	31	35	22	25	28	32	36	40	47	50	53	57	61	65

* 1 had 3a in uterus

(a) 1 had 2a in uterus

Table 22

Ovarian age categories of females of G. austeni mated at various ages and dissected at various times thereafter.

No. of ovs. and degs.	UC	Age when mated 2 days						5 days						10 days					
0	d	7	7					7						1					
1	a		3	8				2	9	1				8	5				
	b				3					2					4				
	c				5		1			5	2					6			
	d															1	1		
2	a					5	2				4	1				3	6	3	
	b						3					3						1	
	c											4	4					3	
	d																		
3	a												3					2	7
	b																		
	c																		
	d																		
4	a																		1
	b																		
	c																		
	d																		
5	a																		
	b																		
	c																		
	d																		
Days after mating		2	5	8	12	16	20	2	5	8	12	16	20	2	5	8	12	16	20
Ages (days)		4	7	10	14	18	22	7	10	13	17	21	25	12	15	18	22	26	30

Table 22 (continued)

No. of ovs. and degs.	UC	Age when mated 15 days						20 days						45 days					
0	d																		
1	a	8	2					1	1										
	b		1																
	c			2															
	d		2	2				2	1	1									
2	a		1	2	1			4	1	1	1								
	b		1			1				1									
	c			1	1	1				2		1							
	d				1						1								
3	a				1		2			2	3		1	2	2				
	b					1					1								
	c					3	2					2							
	d											1	2		2				
4	a					1	1				1		1	1*		1	1		
	b																1	1	
	c															1		1	
	d													1	1	1		1	
5	a														1	1	1	2*	3*
	b																		
	c																		
	d																		

Days after mating	2	5	8	12	16	20	2	5	8	12	16	20	2	5	8	12	16	20
Age (days)	17	20	23	27	31	35	22	25	28	32	36	40	47	50	53	57	61	65

* one fly in each of these groups had 2a in the uterus.

Table 23

Numbers of flies which died prematurely in experiment where females of G. morsitans and G. austeni were mated at various ages and dissected at various times thereafter.

Days after mating	Age when mated (days) 2		5		10		15		20		45	
	M	A	M	A	M	A	M	A	M	A	M	A
2	0	2	1	1	0	1	0	2	0	2	1	4
5	0	0	0	1	0	0	4	2	0	4	2	4
8	0	0	0	2	1	0	0	3	1	3	0	5
12	1	1	0	4	0	2	0	0	0	2	1	6
16	1	3	2	2	2	1	0	3	2	4	1	4
20	2	3	0	3	3	1	2	5	2	3	0	4

Table 24

Numbers of virgins (females with spermathecae devoid of spermatozoa and which did not ovulate) in experiment where females of G. morsitans and G. austeni were mated at various times thereafter.

Days after mating	Age when mated (days) 2		5		10		15		20		45	
	M	A	M	A	M	A	M	A	M	A	M	A
2	1	0	0	0	0	0	0	0	0	0	4	0
5	0	0	0	0	0	1	1	0	1	1	1	0
8	0	0	0	0	1	0	0	0	0	0	3	1
12	0	0	0	0	0	0	0	1	0	1	3	1
16	0	1	0	0	0	0	0	0	1	2	5	1
20	0	1	0	0	0	1	0	0	3	2	2	1

M - G. morsitans A - G. austeni

after mating females at various ages. The time after mating at which the first ovulation occurred decreased with increasing age of female. Flies mated on day 2 ovulated between days 5 and 8. A greater number of first ovulations occurring two days or less after mating were found in females mated on days 10 and 15 than in those mated on day 5. In many in which mating was delayed to day 20 or 45 at least two ovulations had occurred two days later. Second and third ovulations were also accelerated by delay in mating. Pregnancies often resulted from the first ovulation in flies mated within 15 days of eclosion. In groups mated on days 20 and 45 no pregnancies resulted from the first ovulation, and of the 4 G. morsitans and 6 G. nusteni females of these groups which had larviposited (tables 26 and 27) none had ovulated less than twice. It appears that Ri loses the ability to develop if retained in the ovaries as a chorionated egg for 15 days before ovulation, assuming that it is chorionated by day 5. Where mating is delayed for 40 days Li and Ro and often Lo are also non-viable so that pregnancy does not result until at least the fourth ovulation is reached.

In order to find out whether the non-production of larvae by eggs retained in the ovaries for long periods was due to their extrusion from the uterus or whether they were, in fact, incapable of producing embryos the following experiment was conducted. Virgins of various ages were mated and the vulva sealed with wax (see chapter 2). The number of eggs ovulated and trapped in the sealed uteri which had undergone embryonation at various times after operation were then counted.

Twenty to 25-day-old virgin females were found to have Ri and

Table 25

Number of dissected G. morsitans females which had deposited at least one third-instar larva catagorised as in table 21.

Days after mating	Age when mated (days)					
	2	5	10	15	20	45
2	0	0	0	0	0	0
5	0	0	0	0	0	0
8	0	0	0	0	0	0
12	1	1	6	2	1	0
16	3	6	3	4	0	0
20	5	7	4	3	3	0

Table 26

Number of dissected G. austeni females which had deposited at least one third-instar larva catagorised as in table 22.

Days after mating	Age when mated (days)					
	2	5	10	15	20	45
2	0	0	0	0	0	0
5	0	0	0	0	0	0
8	0	0	0	0	1	0
12	0	3	6	1	1	0
16	3	5	5	6	0	1
20	5	5	6	3	2	1

Li chorionated in their ovaries. In many virgins of this age the egg in Ri was in the process of degeneration. Three days after mating many of them had two eggs in the uterus. Of these intra-uterine eggs one (presumably from Ri) was usually obviously degenerating and the other (from Li) was usually undergoing embryonation. Some other flies treated similarly had one egg in the uterus at this time and this was developing. In these flies the egg in Ri was usually degenerating while still retained in the ovary. It appears that degenerating eggs sometimes lose the ability to be ovulated, perhaps because they do not stretch the ovary as much as potent eggs and therefore are no longer able to inform the ovulatory mechanism that they are ready for ovulation; or perhaps because the presumably peristaltic ovulatory process cannot expel non-turgid eggs. These observations confirm that the first egg (in Ri) is non-viable by about day 20.

Some 35-day-old virgin females of G. morsitans were dissected. Others were mated and the vulvas sealed with wax; they were dissected 5 to 8 days later. None of the virgins had ovulated and all had 3 chorionated eggs in the ovary (Ri, Li, Ro). The egg in Lo was not chorionated. The second follicle in Ri was about 0.30mm long and that in Li was just discernable. The chorionated egg in Ri was often obviously degenerating. Five days after mating Ri and Li were usually in the uterus, neither had begun development and both appeared degenerate. Some had ovulated Li only (which was degenerate) and in these Ri was degenerating in the ovary. Thus in flies so treated the first egg in Ri was degenerate and sometimes not ovulated, that in Li was invariably ovulated and degenerate,

that in Ro was chorionated but not ovulated and may have been viable.

Of the flies dissected 8 days after mating several had lost the wax plug over the vulva and eggs had been expelled. Where the seals were intact up to two eggs were found in the uterus along with chorion remnants of other eggs. Never more than one egg showed signs of embryonation and no exchorionated larvae were found. The first eggs from Ri and Li were either degenerating in the ovary or they had been ovulated, that from Ro had invariably been ovulated and was found in the uterus, that from Lo was sometimes ovulated but even when retained it was always chorionated. The intact eggs found in the uterus were probably from Ro and Lo and it is probable that when both were present the one showing development was from Lo.

These experiments show that chorionated eggs have a limited viability. In old virgins some eggs degenerate in the ovaries and may become incapable of ovulation and others remain able to be ovulated although they lose the ability of forming embryos. Impotent eggs are expelled from the uterus soon after ovulation in females mated when old. How the uterus can distinguish between potent and impotent eggs is not known.

C. EFFECT OF MISSED CYCLES ON REPRODUCTIVE EVENTS.

A "missed cycle" is a failure in the normal reproductive rhythm of a mated female. It can result from failure of egg development or ovulation or from an abortion. Ovarian malfunction and consequent missing of cycles can be induced in the laboratory by maintaining flies at temperatures around 29° - 30°C (Mellanby, 1937, Saunders and Hill, personal communication) but no attempts to investigate this phenomenon have been made during the course of

the present work. The results which follow were derived from females mated with aspermic males or with "semi-sterile" males as well as from females which were mated with normal males but nevertheless missed cycles.

1. Evidence for the view that under constant physical conditions ovarian development is related solely to concurrent larval development and is independent of previous events.

Figure 6 (which is a rearrangement of the data given in Figure 5) shows the relationship between follicle sizes and stage of pregnancy of primiparous G. morsitans females. From Figure 6 it is clear that follicles are larger in flies containing the more mature larvae. No fly whose uterus contained an egg had a chorionated egg in the ovaries. Some (about 25%) of the females containing "b" stage larvae had a chorionated egg in the ovary while all females containing 3rd instar larvae had chorionated ovarian eggs. Thus, eggs become chorionated at about the time when the larva is undergoing its second ecdysis. Table 28 shows a similar increase in follicle lengths associated with increasing stage of development of the larva. These results were derived from 45-day-old females of G. austeni and G. morsitans which had not missed any cycles and which had produced 3 or 4 full-term larvae.

That the positive correlation between larval and ovarian development is retained when missed cycles have occurred in previous cycles is demonstrated in Figure 7. This figure shows the follicle sizes and uterine contents of 45-day-old G. austeni females which had been mated with "semi-sterile" males (some of which, incidentally, had been mated to aspermicity) and had missed in one or more cyles.

FIGURE 7 Relationship between follicle lengths and stage of development of intrauterine offspring.

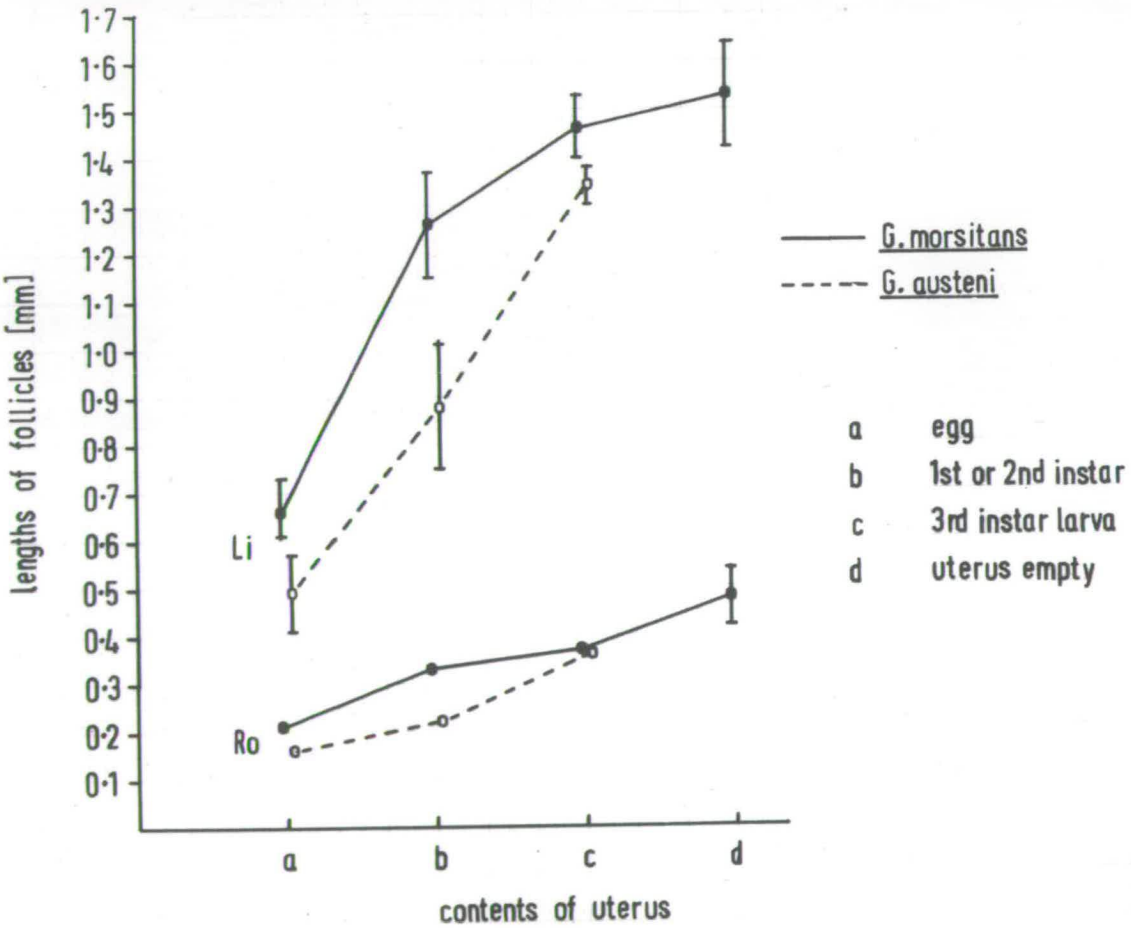


FIGURE 8 Relationship between stage of development of intrauterine offspring and lengths of follicles in 45-day-old females of G.austeni which have missed various numbers of cycles

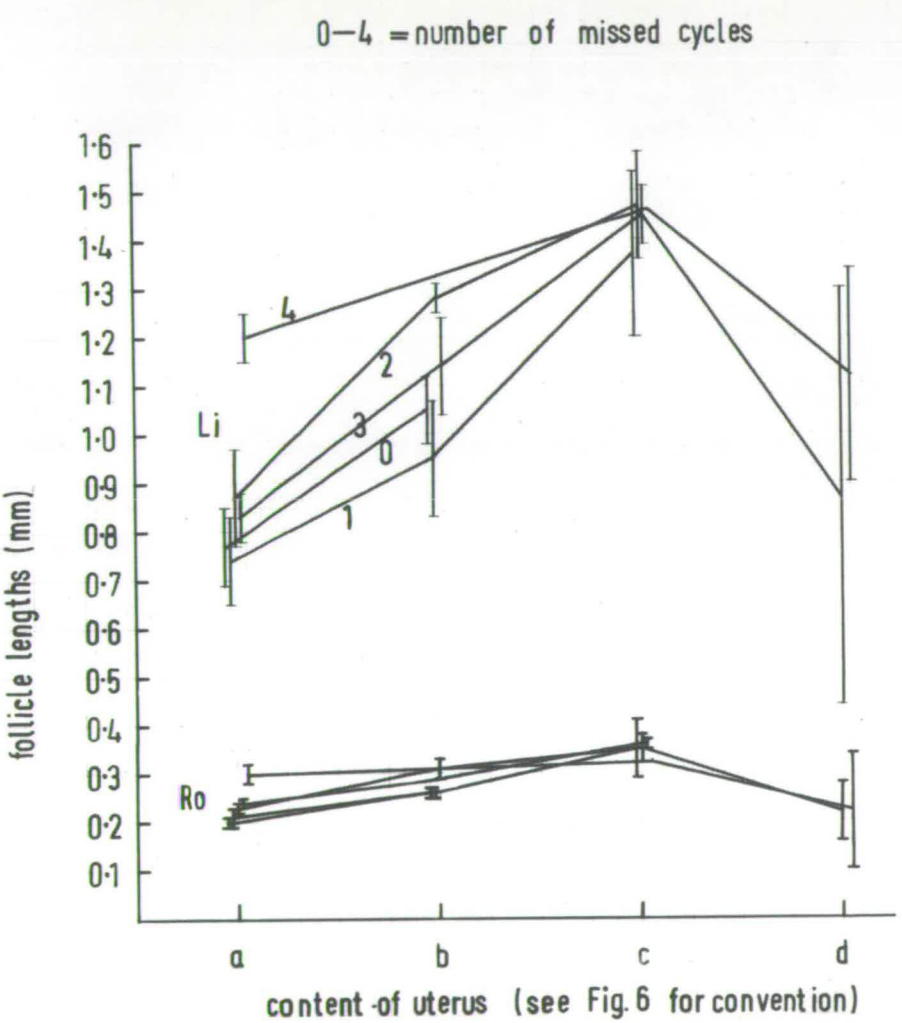


Table 27

Uterine contents and lengths of ovarian follicles of 45-day-old females of G. morsitans and G. austeni which have undergone no missed cycles.

Expt.	No. ovs	foll.	Contents of uterus a N	a mean	a S.E.	b N	b mean	b S.E.	c N	c mean	c S.E.	d N	d mean	d S.E.
G. aust.1	4	Li							10	0.37	0.01	1	0.40	
		Ri								1.49	0.02		1.58	
	5	Li	19	0.54	0.03	1	1.18		2	1.32	0.14			
		Ro		0.18	0.00		0.28			0.26	0.04			
G. aust.2	4	Li							5	0.46	0.02			
		Ri								1.49	0.07			
	5	Li	15	0.77	0.08	7	1.05	0.07						
		Ro		0.20	0.01		0.26	0.01						
G. mors.	4	Li				1	0.36		18	0.42	0.01			
		Ri					1.40			1.55	0.03			
	5	Li	16	0.62	0.05				1	1.64				
		Ro		0.22	0.00					0.40				

G.aust.1 - from experiment in which males were mated to aspermicity

G.aust.2 - from experiment in which some males were semi-sterile

G.mors. - from experiment in which males were mated to aspermicity

The greater variation in follicle lengths of flies with empty uteri was due to the fact that many females, especially those mated with aspermic males had aborted eggs and thus had small follicles and empty uteri, whereas others had undergone full pregnancy and thus, for the short period in which the uterus was empty, had a chorionated egg in the ovaries. It is clear from Figure 7 that the relationship between follicle lengths and stage of intrauterine development is independent of previous abortions and egg degenerations. This is because all normal reproductive cycles start in the same way, with an egg in the uterus and ovarian follicles of minimal sizes: a missed cycle cannot alter the qualitative features of the initial point in the cycle, it can only bring forward its onset.

2. Effect of missed cycles on rate of "ovarian ageing".

"Ovarian age" is defined in terms of the status in the current cycle plus the number of completed ovulations.

a) G. austeni.

Table 29 shows the lengths of the two largest follicles in 45-day-old females some of which had been mated with "Stock 73" males and the rest of which had been mated with normal males. Males of both types were mated several times after they had become aspermic. Thus a good range of numbers of missed cycles was produced - some spontaneous, some resulting from mating with the "semi-sterile" males and some resulting from aspermic matings. It is clear that associated with this increasing number of missed cycles is an increase in number of ovulations occurring in the 45 days. Figure 8 depicts the increase in ovarian age associated with

FIGURE 9 — Length of Li in females of G.austeni, in their 6th ovarian cycle, which have "missed" in various numbers of cycles.

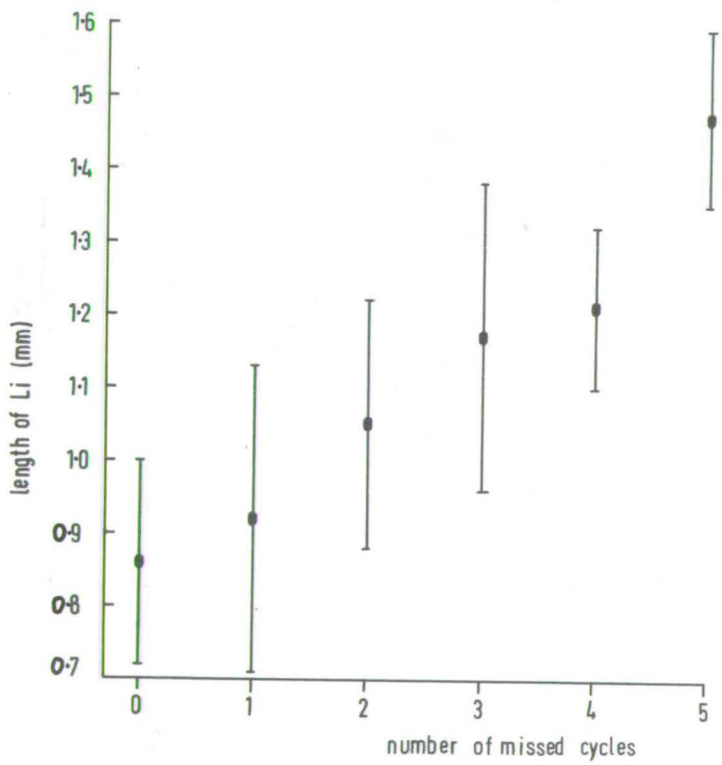


Table 28

Relationship between number of missed cycles and number of ovulations in 45-day-old females of G. austeni mated with "stock 73" semi-sterile males or with normal males of their own species.

No. of missed cycles	Number of ovulations 4				5				6				No. of flies
	N	Foll.	L	S.E.	N	Foll.	L	S.E.	N	Foll.	L	S.E.	
0	5	Li	0.46	0.02	21	Li	0.86	0.07					26
		Ri	1.49	0.07		Ro	0.22	0.01					
1	3	Li	0.39	0.05	14	Li	0.92	0.10					17
		Ri	1.43	0.05		Ro	0.25	0.02					
2					21	Li	1.05	0.08					21
						Ro	0.26	0.01					
3					11	Li	1.17	0.09	3	Lo	0.17	0.01	14
						Ro	0.30	0.02		Ro	0.48	0.01	
4					13	Li	1.21	0.05	6	Lo	0.16	0.01	19
						Ro	0.29	0.02		Ro	0.51	0.04	
5					10	Li	1.47	0.05	13	Lo	0.18	0.00	23
						Ro	0.42	0.07		Ro	0.59	0.04	
6									1	Lo	0.16		1
										Ro	0.64		

missing of cycles. It shows that the lengths of Li, in 45-day-old flies which have ovulated five times, increases with increasing number of missed cycles.

Forty-five-day-old females which missed no cycles ovulated 4 or 5 times in that period (ie about 9 - 11 days per cycle) whereas those which missed 5 or 6 cycles ovulated 5 or 6 times (ie about 7½ - 9 days per cycle). Curtis (1968) estimate that one missed cycle brought forward ovulation by about 2 days which is confirmed by the present findings. The reason why an abortion in early pregnancy does not result in immediate ovulation is that eggs descend from the ovaries only when chorionated. There is no evidence to suggest that follicle growth rate is directly affected during the time between an abortion and the subsequent ovulation. It is probable that an abortion results in the next egg to be ovulated descending as soon as it is capable of doing so instead of being retained in the ovaries for an additional two days under the influence of the intrauterine larva (Saunders, 1970; Pinhao, 1970; Dodd, 1971, Roberts, 1972). A missed cycle results in earlier ovulation and therefore an earlier release of the inhibition of follicle growth rate imposed by the presence of a chorionated egg in the ovaries. Follicle growth rate is increased by missing of cycles through advancement of ovulation.

b) G. morsitans.

Table 30 shows that the increase in the number of ovulations undergone in 45 days, associated with increasing number of missed cycles occurs in G. morsitans females in a similar manner. These data for G. morsitans flies were derived from the experiment in

Table 19

Numbers of ovulations, numbers of missed cycles and follicle lengths of 45-day-old G. morsitans females used in experiment where males were mated to aspermicity.

No. of missed cycles	Number of ovulations 4				5				6				No. of flies
	N	Foll.	L	S.E.	N	Foll.	L	S.E.	N	Foll	L	S.E.	
0	16	Li	0.41	0.01	15	Li	0.68	0.08					31
		Ri	1.52	0.03		Ro	0.24	0.01					
1	15	Li	0.40	0.02	9	Li	0.91	0.13					24
		Ri	1.47	0.04		Ro	0.30	0.03					
2					3	Li	0.63	0.09					3
						Ro	0.23	0.02					
3					2	Li	0.70	0.22	2	Lo	0.26	0.00	4
						Ro	0.24	0.02		Ro	0.70	0.14	
4					4	Li	1.21	0.18	2	Lo	0.19	0.00	6
						Ri	0.35	0.03		Ro	0.58	0.04	
5					4	Li	1.47	0.05	15	Lo	0.24	0.01	19
						Ro	0.40	0.02		Ro	0.76	0.07	

which males were mated repeatedly until they were aspermic (see chapter 2 and section A4 of this chapter).

Table 31 shows that the mean length of Li and Ro in 45-day-old females in their 6th ovarian cycle mated with aspermic males is greater than that in females mated with spermic males. The mates of aspermic males missed all of their cycles and thus had ovaries which were more advanced than those of the females in the same cycle mated with spermic males.

Thus the relationship between ovarian ageing and missing of cycles found to exist in G. austeni females is also demonstrable in G. morsitans females.

D. TIME AFTER LARVIPOSITION WHEN NEXT OVULATION OCCURS.

Females of G. morsitans and G. austeni were killed at various times after depositing their first larvae. Table 32 shows the numbers of females which had ovulated and which had not ovulated the egg from Li by various times after larviposition. All of the G. morsitans females dissected at one hour or more after larviposition had ovulated whereas none had done so 15 minutes after larviposition. of the 10 dissected 15 minutes after the first larviposition 6 were found to have ovulated and 4 were found not to have ovulated the egg from Li. Similar results were obtained from the G. austeni females. It appears that ovulation occurs sometime between about 15 minutes and 1 hour after larviposition in these species.

E. FEEDING AND REPRODUCTION.

Feeding records were routinely kept of all female flies used

Table 30

Sizes of the two largest follicles in females of G. morsitans in their 6th ovarian cycle 45 days after mating with spermic and aspermic males.

Follicle	Egg in uterus						t	p
	spermic mate			aspermic mate				
	N	mean	S.E.	N	mean	S.E.		
Li	26	0.65	0.04	5	1.10	0.17	3.93	0.001
Ro	26	0.25	0.01	5	0.32	0.03	2.56	0.001 to 0.025

Follicle	Egg in uterus						t	p
	spermic mate			aspermic mate				
	N	mean	S.E.	N	mean	S.E.		
Li	29	0.75	0.06	9	1.26	0.13	4.04	0.001
Ro	29	0.26	0.01	9	0.36	0.02	3.59	0.001

Table 31

Time after 1st larviposition when the next ovulation occurs.

Time after 1st larviposition	Li ovulated		Li retained	
	M	A	M	A
15 mins.	0	1	10	9
30 mins.	6	4	4	6
1 hr.	10	10	0	0
3 hrs.	6	6	0	0
24 hrs.	6	6	0	0
48 hrs.	6	6	0	0
68 hrs.	6	6	0	0

as experimental subjects: all were offered a chance of feeding every day, except Sunday. No records were kept of meal sizes. No attempt is made below to examine critically the role of feeding in reproductive physiology - I am just making full use of the feeding data collected while monitoring the effects of various procedures on reproductive events.

1, Feeding frequency and reproductive efficiency.

By reproductive efficiency is meant the proportion of full-term larvae to missed cycles. Figure 9 depicts the relationship found between the number of blood meals taken and the number of third-instar larvae deposited by 45-day-old females of G. morsitans (solid circles and unbroken lines) and of G. austeni (open circles, broken lines). This Figure shows that there is a direct relationship between fecundity and frequency of feeding. The mean feeding frequency of G. morsitans is greater than in G. austeni for individuals which produce 3 or more full-term larvae in a 45 day period, but there is no significant difference between them when only 2 or less larvae are brought to full-term in the same period. Virgin females of G. morsitans took more meals than did comparable females of G. austeni. Langley (1970) suggested that the metabolic demands of G. austeni are less than those of G. morsitans females (under the same conditions) and that the former species converts a greater proportion of ingested protein to larval protein than does the latter species. Figure 9 also shows that virgins of both species took more meals than did females which missed all of their cycles. This is because virgins take smaller meals than do mated females (Langley, 1968) and therefore, as neither requires extra food for larval development,

FIGURE 10- Number of full-term larvipositions and number of meals taken by 45-day-old females.

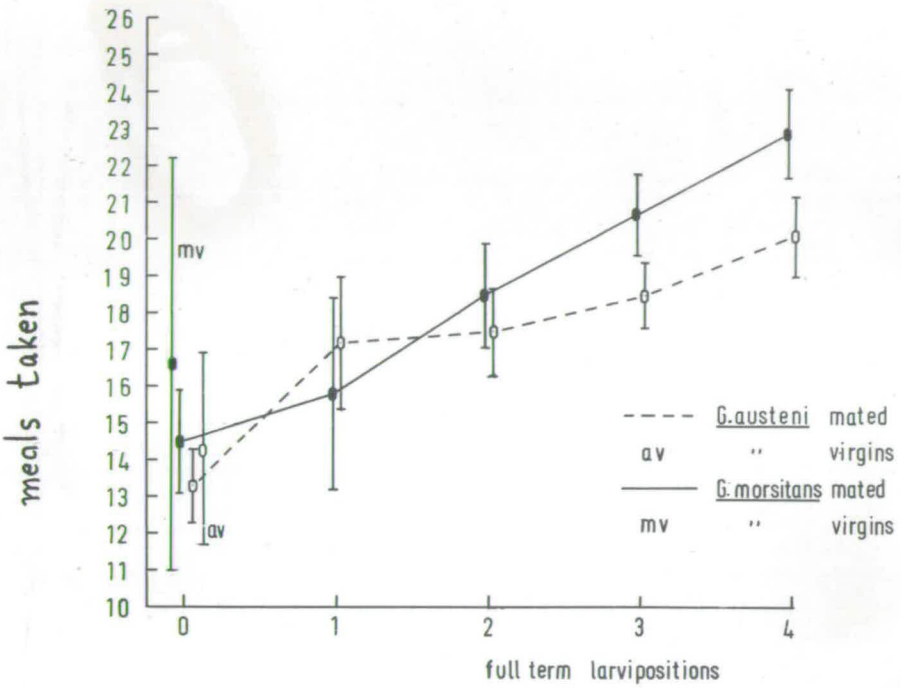
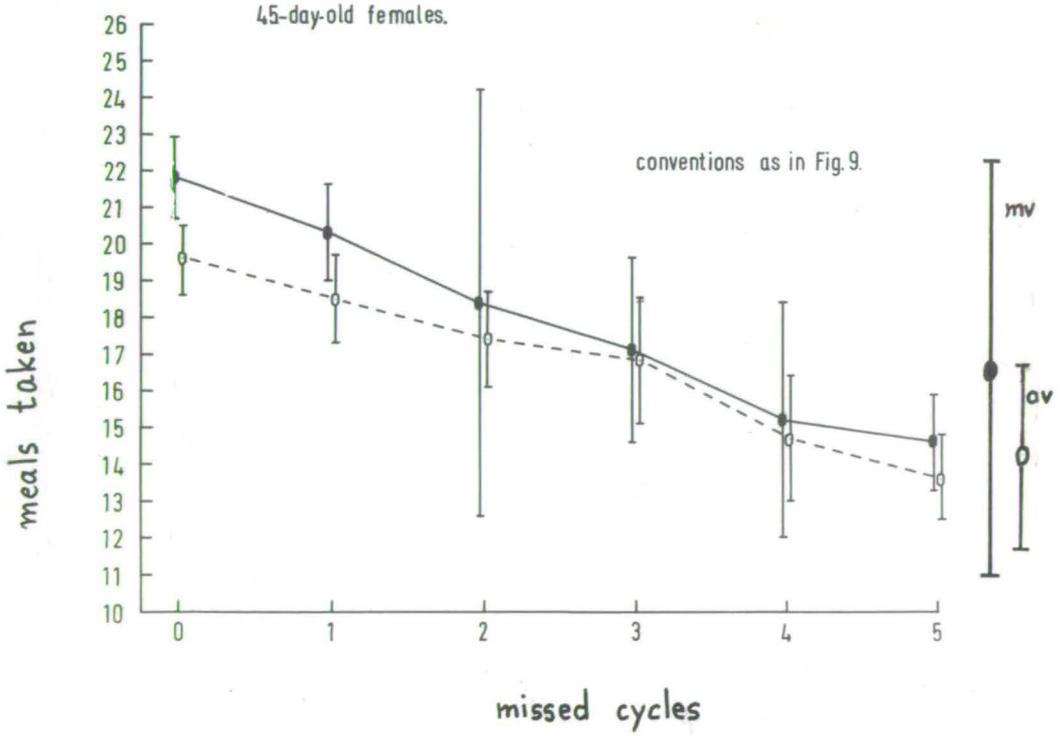


FIGURE 11- Number of missed cycles and number of meals taken by 45-day-old females.



the latter need feed less frequently than the former.

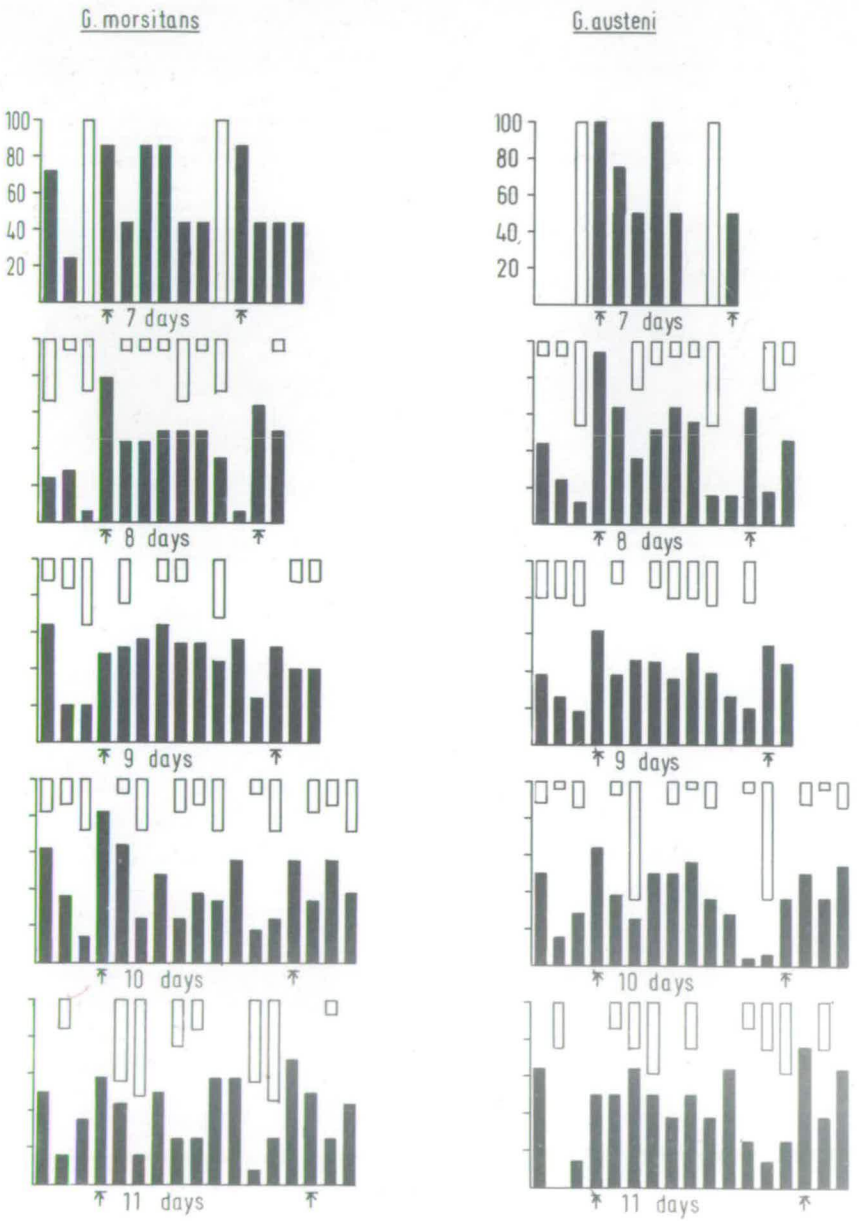
Figure 10 shows the relationship between the number of cycles missed by 45-day-old females of both species and the number of meals taken. In both species it was found that the relationship was inverse. This is because the food demands on a female by its larva are reduced to zero when the latter is aborted. The difference between the two species is significant only when no cycles are missed.

2. Timing of feeding during pregnancy.

Figure 11 shows the proportion of flies feeding on successive days throughout pregnancies lasting from 7 to 11 days. These results were obtained by taking all females (of each species) which missed no cycles in 45 days and deposited 3 or 4 third-instar larvae in that time. The second and subsequent pregnancies of such flies were grouped according to the interlarval periods and the percentages of flies which fed on each day throughout pregnancies of different lengths were obtained. These percentages are shown for the various days before and after larvipositions in figure 11. The proportions of Sundays occurring throughout the cycles are shown because on these days no food was offered to the flies. Thus two factors are to be considered when interpreting the feeding patterns - Sundays and larvipositions.

No obvious differences appear between the two species; both showed similar ranges of pregnancy lengths and their feeding patterns are similar. All of the larvae of both species resulting from pregnancies with 7 day interlarval periods were deposited on Mondays. It is not possible to determine whether the high proportions

FIGURE 12 FEEDING PATTENS DURING PREGNANCY. Each block represents the sum total of individuals with equal interparturient periods-from 7–11 days. The interpretation is complicated by the fact that food was not offered on Sunday. Where Sunday coincided with given points post-partum the proportion of individuals affected is represented by the open suspended column.



feeding on the days after larviposition in these flies are due to their not being fed on the day before or to the fact that they larviposited sometimes between the Saturday afternoon and the Monday mornings. In the results for many of the other interlarval periods it appears that a high proportion of flies feed after a larviposition and fewer flies feed for the few days before a larviposition.

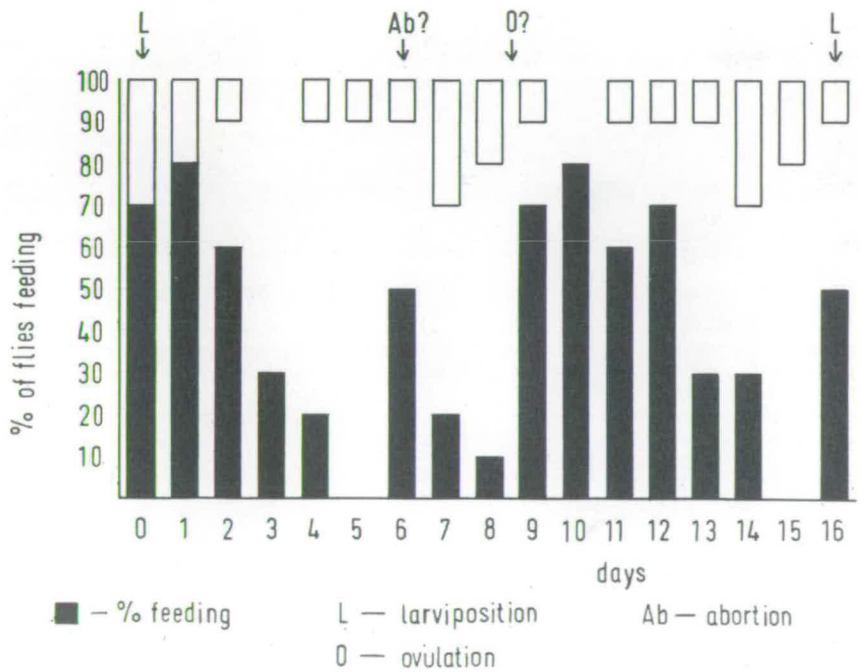
These findings are in accordance with those of Nash, Jordan and Boyle (1967) who analysed the feeding records of 8 G. austeni females. They found that flies tended to fast before a larviposition and engorge repeatedly afterwards. Mellanby (1937) reported that the females of G. palpalis which took a really good meal about three days before larviposition produced the largest "pupae". Figure 11 shows that a greater proportion of females of G. morsitans and G. austeni tended to feed on the third or fourth day before larviposition than did on days nearer that event.

3. Feeding pattern during a missed cycle.

Figure 12 shows the proportion of flies accepted food daily throughout an interlarval period of 16 days which included a missed cycle. This length of interlarval period was chosen because it was the most common. The data were provided by 20 females of G. austeni which had been mated with Curtis' "Stock 73" males.

The most likely account of the events shown by Figure 12 is as follows. The first few days after a larviposition mark the beginning of the next pregnancy and during this period most flies fed daily. The proportion of flies feeding on each day

FIGURE 13— Feeding pattern of G.austeni females during a missed cycle.



drops after about day 3 as is normal during pregnancy. Abortion probably occurred on about day 5 or 6 and this is marked by an increase in the proportion of flies feeding on day 6. It is probable that ovulation occurred on about day 8. The next pregnancy, and the associated repeated engorgement, is apparent from days 9 - 12. Thereafter the proportion of flies feeding drops and larvipoaition was recorded on day 16. Associated with parturition is the expected increase in feeding. Abortion disrupted the feeding pattern in that it evoked engorgement by most flies but the normal pattern of feeding during pregnancy was rapidly established after the next ovulation.

These findings are in agreement with Nash et al (1967) who gave the feeding records of three flies which each missed a cycle. That confirmation of results based on only three flies can be so easily obtained reflects on the strictness of the control exerted on feeding frequency in these flies.

DISCUSSION

Many interactions occurring between the major components of tsetse reproductive physiology have been described. For example, oocyte growth is inhibited by egg retention, ovulation is prompted by mating, completion of pregnancy is dependent on correct feeding, parturition and ovulation are associated etc. The modes of operation of these interactions are, however, almost totally unknown. Possible mechanisms are examined in this chapter.

The control of egg maturation in insects is a complex process: different insects respond differently to the same operation. Nevertheless some important generalisations can be made. It is generally accepted that the corpus allatum (CA) controls vitellogenesis in most insects and that this organ is often under the control of the brain or sub-oesophageal ganglion either through the neurosecretory cells or by means of nervous connections. I do not propose to review the lengthy evidence for this statement but a comprehensive review of the subject is given by Engelmann (1970). Wigglesworth (1936) was the first to suggest that CA was involved in egg maturation and since then the number of papers published on this subject has become almost overwhelming. The activity of the corpus allatum can be influenced by mating, which stimulates egg maturation in Schistocerca gregaria (Highnam and Lusis, 1962) and by feeding. It is not always clear whether feeding has a direct nutritional effect on follicle growth or whether an indirect effect involving release of gonadotrophic material from the corpus allatum is involved. In Rhodnius prolixus food intake probably activates the corpus allatum via stretch receptors in the gut wall (Detinova, 1953). In

Calliphora erythrocephala it appears that the neurosecretory cells provide a substance which is necessary for protease production in the gut. As a result of its release protein digestion ensues and the consequent rise in concentration of metabolites in the haemolymph stimulates corpus allatum activity and hence egg maturation (Strangways-Dixon, 1961). It is possible that the synthesis of some of the proteins necessary for vitellogenesis in Schistocerca gregaria is controlled by the neurosecretory cells of the brain (Hill, 1962). In Glossina vitellogenesis is well advanced before eclosion (Saunders, 1960) and so mating and feeding are irrelevant to the early stages of this process. Follicle growth continues in the ecloded starved fly but at a lower rate than in fed flies. The effect of feeding on follicle growth may be a directly nutritional one but it is more likely that corpus allatum activity is implicated. Females of Oncopeltus fasciatus (Johansson, 1954) and Leucophaea maderae (Johansson, 1955 and Engelmann, 1965) which have been starved do not develop eggs unless active corpora allata from fed females are implanted into them. The effect on follicle growth (especially on later vitellogenesis and chorionation) of implanting corpora allata from fed to starved females of Glossina has not been reported. The role of neurosecretory secretions on egg maturation in Glossina is equally unknown. The interrelationships between nutrient and endocrine materials and egg maturation in the first ovarian cycle are likely to occur also in subsequent cycles in tsetse although the nutritive demands of the larva result in a greater food intake and possibly, therefore, a greater load on the endocrine system.

Endocrine patterns may be complicated by possible interactions between larval and maternal hormone mechanisms.

All one can say with certainty at this stage about the control of egg maturation in the first cycle in Glossina is that enough nutrients and humoral factors are present to enable vitellogenesis to occur throughout pharate adult life and for a few days afterwards and that the later stages of egg maturation depend upon blood-meals. The maturation rates of subsequent follicles are influenced by the presence or absence of chorionated eggs in the ovaries. It is possible that a soluble factor (FIF), present in ovarioles containing chorionated eggs, reduces the growth of unchorionated follicles. This factor is similar in some respects to the "oostatic hormone" in Musca postulated by Adams et al (1968) but the tsetse system differs from that of Musca in that a total inhibition of vitellogenesis does not occur. Another difference between the two systems is that in Musca the interaction is between follicles of successive oogenic cycles whereas in tsetse flies, in which ovulation occurs singly, it occurs between follicles of the same oogenic cycle in different ovarioles. It is probable, however, that in old virgin tsetse the presence of a chorionated egg in one ovariole may inhibit the growth of the next follicle in the same ovariole.

It is not known how the follicle-inhibiting factor affects the endocrine system in tsetse. Its site of origin may either be in the stretched ovarian wall or in the follicle itself. In order to locate the source of this substance it would be necessary

to assay the degree to which extracts of isolated parts of the female reproductive system inhibit follicle growth.

Virgin female tsetse do not ovulate until they are very old and even then egg descent is probably the result of eggs being pushed out of oviducts by the follicles growing behind them. Retained eggs in virgins become incapable of further development within 20 days of chorionation and, after delayed mating, either remain in the ovaries and degenerate or else are ovulated and expelled from the uterus. How it is determined whether or not eggs are to be ejected is unknown: it is possible that ovulated eggs continue to be aborted as long as entire chorionated eggs remain within the ovaries.

One may expect the amount of inhibitory substance to depend on the number of ovarioles containing mature eggs and thus the growth rate of follicles in old virgins with four retained eggs to be lower than that in follicles in flies whose ovaries contained one or two only. That such a differential does not occur (Saunders, 1971) may well be due to the commencement of degeneration in all but one of these retained eggs. By using histological techniques Odhiambo (1971) showed that degeneration of a retained egg has started by the time of chorionation of the next egg. This sequential degeneration, which results in the presence of only one potent egg in the ovaries at any time, may account for follicle growth continuing independently of the total number of retained eggs.

Saunders (1971) found that the second and subsequent eggs in virgins of G. morsitans took 8-9 days longer to develop than

did their counterparts in mated flies. Odhiambo (1971) stated that the second and subsequent oocytes in mated G. pallidipes females mature in 12 or 13 days and in unmated females the process takes about 16 days. Thus mating accelerates egg maturation by 3-4 days. His figure 4 shows that in mated females the first egg is mature, some 25 days after eclosion, but in virgins only 15 days after eclosion. The second egg has matured by about 30 or 35 days in mated flies (2 mean values are given) and by 30 days in virgins. The times for the third egg are 50 days for mated and 60 days for virgin flies, and for the fourth about 62 days for mated and 80 days for virgins. Thus the first egg is mature 10 days earlier in virgins than in mated flies, the second matures 0 or 5 days earlier in virgins than in mated flies, the third matures first in mated flies (by 10 days) and the fourth egg matures first in mated flies (by about 20 days). It is difficult to see how these figures relate to the 3-4 day difference mentioned above but if Odhiambo's figures and their interpretation are correct then the reproductive physiology of G. pallidipes is quite different from that of G. morsitans. It is possible, however, that Odhiambo's technique for determining the number of ovulations undergone by a fly was not precise.

Follicle growth rate appears to be unaffected by pregnancy. Saunders (1960, figure 4) shows that the growth curve of follicle A (Li) after eclosion is not markedly different from that of later follicles even though the latter are growing while larvae are developing in the uterus.

Ovulation in Glossina is dependent on copulation. The rate of follicle growth until the chorionation of Ri is independent of mating. The first ovulation occurs in females of G. morsitans and G. austeni on day 6 or 7 if they are mated during the first 5 days after emergence. The results of experiments reported above suggest that the stimulus in mating which causes ovulation is not a chemical passed to the female by the male, nor is it the transfer of a spermatophore or of spermatozoa. External mechanical stimulation of the female by the male does not appear to be the relevant stimulus nor does simply stretching the uterus, but the roles of other intrauterine stimulations are not clear. Males whose genitalia had been mutilated by total pneumopophysectomy did not complete mating and did not induce ovulation. Males with only one pneumopophysis cauterised caused their mates to ovulate but did not pass spermatophores or sperm to them. It appears that a certain mechanical stimulus applied by the penis to the uterus is required but that this stimulus has not yet been mimicked in the laboratory.

Saunders and Dodd (1972) show that duration of copulation by a female is an important factor determining whether or not ovulation occurs. Females which separated voluntarily from their mates almost invariably ovulated but of those which were manually separated the proportion which ovulated increased with increasing period of coitus even if this was provided by several consecutive males. It thus appears that a certain period, which varies from one female to another but is usually

at least 45 minutes, of the "correct" mechanical stimulus induces ovulation. The nature of the stimulus which identifies it as "correct" is unknown. The receptors for the ovulation-initiating stimulus are probably in the anterior portion of the uterus, possibly in the region of the uterine papilla. It is probable that movement of parts of the phallosome against the unidentified receptors stimulate ovulation but no movements of the male terminalia are visible during copulation.

It is noteworthy that almost all of the surgical procedures used in attempts to mimic components of mating resulted in ovulation by some virgins. The proportion of ovulations induced varied with the site of operation, although no technique resulted in a significant difference between the effect in experimental and operated control flies. Generalisation is rendered more difficult by the low numbers of flies operated on, and the low proportion of these which subsequently ovulated. Thoracic surgery appeared to be more effective than injection of material into the abdomen. Insertion of material into the uterus was least effective. It appears that the proportion of induced ovulations is a function of shock: the more severe the injury the greater the chance of ovulation. These "shock-induced" ovulations are similar to those caused by normal copulation in that they are followed by subsequent ovulations.

Certainly the mating stimulus is not species-specific as evidenced by the findings of chapter 2, section A8 of this thesis, and of Vanderplank (1948). The latter author reports on a large range of interspecific pairings and discusses the taxonomic

significance of his results. His work is open to a few criticisms, for example he states that some G. palpalis fuscipes which had been paired with males of their own sub-species for less than one minute reproduced while others which had remained in coitus for 24 hours or more remained apparently uninseminated. Although one cannot exclude the possibility except by experiment, it appears unlikely that the processes of spermatophore construction and sperm transfer could be completed in such a time especially in view of the relatively long copulation time possible in this species. Spermatophores have never been found in G. morsitans females where copulation has been restricted to 15 minutes. It is possible that the pregnancies observed by Vanderplank in flies paired for less than one minute resulted from previous unobserved copulation, possibly amongst teneral flies, although he gives no details of fly rearing techniques. This suggestion casts some doubt over the results of his cross-pairings, the apparent results of which may have been due to earlier undetected intraspecific pairings. Foster (1972a) found that unfed G. morsitans males copulate readily but rarely inseminate whereas unfed G. austeni males which copulated always inseminated. However, his males were placed with fed females. The pairing behaviour of tsetse flies when both sexes are teneral is not known for any species. Vanderplank (1948) appears to have judged positive insemination solely on the opacity of the intact spermathecae. This is not an infallible method as virgin females not infrequently show cloudiness of the spermathecal contents as do some females mated with

aspermic males. Thus his figures for the percentage of inseminated females which produced larvae may be underestimates: this may account, in part, for the higher fertilisation rates provided by his *in vitro* gamete mixing. Vanderplank's finding that *G. morsitans* hybrids are sterile has obvious potential importance for tsetse control, especially if the hybrids can compete well against normal males for female mates. Curtis (1972) is following this up.

The degree to which interspecific mating occurs in tsetse is likely to be due at least partially to their reduced courtship behaviour, as exemplified by the reduced role of auditory stimuli in mating. That male wings are irrelevant to intraspecific copulation in *G. morsitans* weakens Vanderplank's claim that male wing-beat frequency is important in determining which interspecific pairings result in copulation.

The role of the endocrine system in the regulation of ovulation in insects is not yet clear. Certainly in some mammals ovulation is dependent on copulation, some component stimulus of which is transmitted nervously to the anterior pituitary lobe . Resultant increased production and release of LH and FSH stimulates the final stages of follicle growth and discharge (Nelson 1953). However, it is risky to make comparisons between physiological processes as they occur in different phyla unless they are of a very general nature (Davey, 1967).

The evidence concerning involvement of the central endocrine system in ovulation in tsetse is sparse. The interval

between copulation and ovulation is obviously dependent on the degree of development of the largest follicle but it is never shorter than a few hours. Females of G. morsitans paired with males of their own species on day 8 (when Li is competent for ovulation) do not ovulate until at least 16 hours after pairing (see fig. 3), although they have normally disengaged from their mates by 6 hours. The interval is longer than would be expected for the involvement of a purely nervous mechanism. That the process of ovulation is a lengthy one is unlikely because in no female dissected has an egg been found in transit along the oviduct.

Foster (1972b) found that, in G. austeni, extirpation of the median neurosecretory cells of the pars intercerebralis (MNC^-) after eclosion did not affect egg maturation but it did inhibit ovulation. Ovulation was also inhibited when the operation was performed after larviposition. The operation when performed during pregnancy resulted in prolonged larval retention, "pupation" in utero and inhibition of subsequent ovulation. Deeply incised sham-operated controls often behaved like females which had suffered MNC^-. Foster could come to no conclusions concerning the brain hormone requirements of developing eggs because of the possibility of storage of material by the corpora cardiaca. He concluded that ovulation, larval development and larviposition are to some extent dependent on MNC or nervous tissue closely associated with them. This work has shown the approximate anatomical location of a site probably responsible for some regulation of reproductive functions but has not defined

the nature of the regulatory activity.

Langley's (1967) finding, that paraldehyde fuchsin staining reveals no cyclical change in the neuroendocrine system related to feeding cycles or reproductive cycles, does not necessarily exclude endocrine control of these functions. The histological technique employed gives no information about rates of synthesis or releases of material but only gives a picture of the net contents of parts of the system at a fixed time. It is possible that no overall cycle was detectable because of the interrelationships of several cycles of unequal and asynchronous periodicities associated with different physiological processes.

Whenever a female tsetse is mated during the first few days of adult life ovulation occurs on about day 6 or 7, under the conditions of this laboratory, even though chorionation has occurred a day or two before this event. It is likely that the final stages of egg maturation are incomplete at chorionation and that ovulation is instigated by some stimulus from the egg or associated tissue when maturity has been attained. This stimulus is unlikely to be directly associated with the stretching of the ovarian wall because this does not change greatly during the last few days preceding ovulation and because the sizes of mature eggs vary. It is much more likely that the release of a substance peculiar to fully mature eggs is responsible for activating the ovulatory mechanism. This hypothetical substance will be designated MEF ("mature egg factor").

MEF may act directly on the ovarian wall, which may have

been preconditioned to respond to it by mating. That insertion of extracts and tissues likely to contain MEF do not cause virgins to ovulate is explained by assuming that mating lowers the threshold for response to this factor by the ovarian wall receptors. The finding that once ovulation has been initiated it is usually followed by subsequent ovulations is explicable by the assumption that mating causes a permanent lowering of this threshold so that whenever MEF is liberated ovulation follows.

The central nervous or neuroendocrine system may operate, however, through an indirect control mechanism. For example, MEF sensitive nervous receptors in the ovarian wall could respond to the substance by eliciting a nervous reflex response in the muscles of the ovaries either via the fused thoracic ganglion, or the brain. Again mating could be envisaged as acting through lowering the response threshold of MEF receptors in the ovarian wall or alternatively it could facilitate neuromuscular transmission. A third possibility is that mating initiates cyclical release of an ovulation hormone stored in brain or corpus cardiacum. The ineffectiveness of implanting portions of a presumably active endocrine system can once again be explained by the non-preconditioning of the ovarian receptors for either MEF or "ovulation hormone" in such insects. Further possibilities are that MEF in the haemolymph directly stimulates the release of "ovulation hormone" or that MEF acts on nervous receptors distant from the ovaries and evokes a complex nervous initiation of ovulation.

The results of Foster's (1972b) operations on MNC and

associated tissues may provide evidence for the involvement in ovulation of either nervous activity of the brain or of some humoral activity of the MNC. On this basis it is proposed that the hypothesis that MEF acts directly on ovarian receptors is less tenable than the more indirect mechanisms outlined above. A more precise understanding of this problem is unlikely until the nervous supplies (afferent and efferent) to the female reproductive system have been worked out. Progress has been delayed by the fact that tsetses do not survive open surgery of the abdomen. Moreover, the gross anatomy of this portion of the nervous system which is relatively much more difficult to elucidate than others remains totally unknown despite efforts by, among others, Minchin (1905) and myself.

It is not impossible that MEF and the follicle-inhibiting factor (FIF) are the same substance. Thus follicle-inhibition could remain independent of the total number of chorionated eggs (ripe and degenerating) in the ovaries. In old virgins, eggs which are degenerating would not be expected to have the same metabolic products as "ripe" eggs and MEF/FIF would be produced by the one or more viable chorionated egg in the ovaries.

Nayar (1958) found that blood, MNC and ovarian extracts from females which were about to or had started to lay eggs would induce oviposition and associated behaviour in virgins of Iphita limbata. He postulated that the ovarian wall, in a gravid female, releases a substance into the haemolymph which results in both inhibition of transfer of neurosecretory material into the corpus allatum (thereby eventually stopping yolk

deposition in further follicles) and release of material from the MNC which may provoke oviductal contractions and therefore oviposition.

No evidence was found for direct humoral control of larviposition in tsetse when endocrine tissue, blood or ovarian extracts were injected into parturient females. This leads to one of two conclusions. One is that larviposition is nervously controlled - movements or some other activity of the larva resulting in uterine contraction. The other is that humoral restraint of larviposition is operative in pregnant tsetse. In the pregnant rabbit myometrial activity is restrained by the influence of progesterone. When the progesterone titre of the blood decreases below a threshold the reproductive system comes under the domination of oestrogen and uterine contractions commence. As the young reach the cervix then oxytocin release is stimulated, reinforcing the contractions of the uterus and vagina. Positive feedback from movements of the offspring and of the female abdomen strengthen uterine and vaginal contractions. Although the reproductive physiology of a pregnant rabbit is vastly different from that of a parturient tsetse fly it is possible that broadly analogous processes may occur in them. For example, it may be suggested that in Glossina the uterus normally expels its contents, perhaps as a reflex response to mechanical stimuli applied to the uterine wall and that a humoral factor or nervous pathway inhibits this expulsion under certain circumstances, especially during pregnancy. The restraint

could be removed when mature eggs (producing MEF) occur in the ovaries, or as a result of faulty maternal feeding or starvation. Expulsion could be permitted if an intrauterine larva dies or gives other evidence of inviability or fails to give evidence of viability. Parturition may occur as a result of MEF liberation, as suggested above, or when larval movements lift the restraint, or changes in larval feeding may inhibit release of the restraining factor or nervous transmission. Transfer of substances from immediate pre- and post-parturient females into pregnant flies would not, on the basis of this hypothesis, result in larviposition because the restraint on uterine contraction would not have been lifted thereby.

It has been suggested that parturition in tsetse may be almost entirely due to the efforts of the larva (Burrt, 1952) perhaps, assisted by the female legs (Hoffmann, 1954). That the uterus plays a more direct role in voiding its contents is indicated by the fact that non-motile objects such as eggs and dead larvae are extruded on occasion. Likewise glass rods are expelled within a day or so of insertion, even when waxed in, irrespective of whether the flies are unmated or parous. It is clear that receptors exist in the uterus which distinguish between eggs and larvae to be retained and objects to be expelled. The suggestion that stimuli provided by the feeding of the intrauterine larva are responsible for retention within the uterus leaves unexplained the non-rejection of the egg prior to this. It is possible that ovulated eggs are aborted if further chorionated eggs occur in the ovaries, that embryos and

larvae are aborted if they fail to continue stimulating the uterine receptors. The relationship between intrauterine larval movements and the mechanical stimuli in mating which cause ovulation remains speculative.

Curtis (1968) deduced that early death of an embryo in the uterus (followed, presumably, by abortion) brought forward the next ovulation by about two days. Non-viable embryos were produced as a result of mating normal females with irradiated males and the number of "unsuccessful" cycles (ie "missed" cycles) was computed from the intervals between successive parturitions of viable third-instar larvae. Using the more precise "ovarian ageing" technique to calculate exactly the number of cycles missed by a fly and by inducing abortions by mating females with either aspermic or with semi-sterile males it was found possible to confirm Curtis' results, extend them to G. morsitans and to demonstrate the corollary that the ovarian age of a female which has missed some cycles is greater by a predictable amount than that of a mated female of the same calendar age, which has experienced no missed cycles.

Early expulsion of eggs, embryos and larvae from the uterus may result not only from faulty development due to inherent deficiencies but also from inappropriate feeding by the mother. Mellanby (1937) showed that interruption of individual meals in G. palpalis resulted in abortion. Whether faulty feeding has its primary effect on the offspring, perhaps killing it or in some other way causing it to stimulate expulsion from the uterus, or on the mother, causing abortion

to occur as a nutritional economy measure is unclear. Saunders (1972) found that when females of G. morsitans are starved during pregnancy abortion often occurred but prolongation of pregnancy was also common. He suggested that competition between developing eggs and larvae may occur during starvation. It is possible that when the metabolic demands of essential activities such as muscular activity, osmoregulation etc. are threatened then expulsion of uterine contents may occur because it is the nutrient drain most easily disposed of. It would be worthwhile finding out when larvae are aborted as a result of faulty feeding what proportion of them is dead on expulsion. H. Mellanby (1937), Nash et al (1967) and Saunders (1972) have pointed out that blood-meal timing is an important factor controlling the occurrence or non-occurrence of abortion, the duration of pregnancy and, perhaps, the activity of the accessory ("milk") glands.

The feeding data presented in this thesis, and the conclusions it is possible to draw from them are in accordance with the findings already in the literature. This may be a demonstration of the relatively invariable nature of so crucial a physiological process, or it may result merely from the relative crudeness of the parameters measured. Certainly, there is still much scope for research into the basic physiology of feeding, blood concentration, digestion, excretion etc. as they relate to reproductive processes despite recent advances in this field. The role of endocrine activity in some of these processes has been discussed by some authors. For example, Langley (1967)

suggested that the lower rate of digestion of blood by laboratory-reared flies compared with that in "wild" flies is due to a lower rate of neurosecretory activity in the former. This would be difficult to check because, as the author points out, no cyclical changes in the histochemical appearance of the neurosecretory system have yet been associated with any physiological process.

In summary, this thesis has concentrated on some of the problems of the reproductive physiology of female tsetse which have not, until recently, received much attention. The conclusions reached are mostly derived by exclusion of alternatives based on "negative" results. Such a procedure is justified if, as occurs in the present case, the validity of the experimental techniques used has been well proved in other insects.

ACKNOWLEDGEMENTS

I thank the Wellcome Trust for financial support during the course of this project.

My thanks are due also to Dr. D.S. Saunders for supervising the work, to Dr. J.A. Campbell for his invaluable criticism of the manuscript, to my wife for many hours of typing, to the technical staff for routine maintenance of the tsetse colony and to Mr. D. Cremer for producing the photographs.

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APPENDIX

Reprinted from

PERGAMON PRESS
OXFORD NEW YORK LONDON PARIS

J. Insect Physiol., 1972, *Vol.* 18, *pp.* 187 *to* 198. *Pergamon Press. Printed in Great Britain*

MATING, INSEMINATION, AND OVULATION IN THE TSETSE FLY, *GLOSSINA MORSITANS*

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(*Received* 7 *May* 1971; *revised* 13 *August* 1971)

Abstract—Virgin females of the tsetse fly, *Glossina morsitans orientalis*, retain their first egg within the right ovary whereas mated females ovulate. The component of the mating act which causes ovulation and thereby initiates the ovarian cycles which follow, is not insemination, the construction of a spermatophore in the uterus, or a humoral factor associated with the testes, accessory glands or ejaculatory ducts of donor males, or 'full' spermathecae of donor females. The ovulation rate increases with copulation time and females are shown to 'add up' their 'sexual experience' independently of that of the males. Inter-specific matings between *G. morsitans* and *G. austeni* also result in ovulation, but an unidentified factor associated with the completion of the mating act was also apparent. It is suggested that a 'mechanical' factor probably with nervous and endocrine components is responsible for the release of the egg from the ovary. Observations on the construction of empty spermatophores by aspermic males of *G. morsitans* are included.

INTRODUCTION

TSETSE flies are viviparous insects which produce a single egg at a time. In inseminated females the first egg is ovulated from the right ovary about 7 to 9 days after emergence; it passes into the uterus and develops in a further 8 to 9 days into a fully developed third instar larva which is deposited on the ground to pupate. After the first larviposition reproductive cycles follow each other without further insemination, ovulation of the next ripe egg occurring within 24 hr of each parturition. Sperm remains viable within the spermathecae for up to 200 days or more, and as many as 20 larvae may be produced in this time (AZEVEDO and PINHAO, 1964). In unmated females, however, eggs fail to be ovulated and old virgins may contain several chorionated eggs within the ovaries unless they are forced into the uterus by what is presumably mechanical pressure from the overcrowded ovaries (MELLANBY, 1937). It is clear, therefore, that some component of the mating act controls at least the first ovulation and thereby initiates the reproductive cycles. This paper describes attempts to identify this component.

MATERIALS AND METHODS

These experiments were conducted using specimens of *Glossina morsitans orientalis* Vanderplank from three sources. Some were drawn from a population

maintained in the laboratory in Edinburgh, others were obtained as puparia from the Tsetse Research Laboratory, Bristol (Dr. T. A. M. Nash) or from the School of Public Health and Tropical Medicine, Lisbon (Professor J. Fraga de Azevedo). All three, however, although originating from independent isolations, came from similar areas of Africa (Rhodesia or Mozambique), and the present investigation revealed no appreciable differences between them. A few males and females of *G. austeni* were used in the inter-specific matings; these were also obtained from Dr. T. A. M. Nash, and originated from Zanzibar.

Apart from those kept as virgins, females were mated with older males 2 days after emergence. They were then kept at 25 to 26°C and at about 65% r.h. in small polystyrene cages in a specially constructed fly-room (Mews, 1969). They were offered food daily (except Sundays) from the ears of lop-eared rabbits (Nash *et al.*, 1966) and dissected in 0·9% saline 10 to 18 days after emergence (in different experiments) to ascertain whether insemination and ovulation had occurred. Eighteen days after emergence most inseminated females had ovulated twice and produced their first larva (Saunders, 1962, 1971). These inseminated females also showed the presence of sperm in the spermathecae as a dark mass (Mellanby, 1936) and contained an egg or a larva (their second) within the uterus. The production of a larva was important evidence of fecundation, particularly in cases where little sperm had been passed.

Insertion of organs (testes, accessory glands, spermathecae, etc.) from donor flies into the haemocoel of virgin recipients was accomplished by making a dorso-lateral incision in the thorax or abdomen, introducing the tissue, and then sealing the wound with hot paraffin wax. Males were made aspermic by repeated matings with different females until no further sperm was passed.

The effect of copulation time on insemination and ovulation

Pairs of flies were separated manually under cold anaesthesia (4°C) at different times after the onset of coitus. The females were then kept at 25°C until 18 days after emergence before dissection. Control females were either kept as virgins, or allowed full mating time and voluntary separation by keeping the sexes together for about 24 hr. The experimental pairs were observed at 15 min intervals for the first 3 hr, those that parted voluntarily being recorded separately from those pulled apart by hand.

Table 1 shows that all of the virgin females retained their first egg within the right ovary, whereas all of the '24 hr' mated controls ovulated. Of the latter group 45 (94 per cent) were also inseminated as judged by the appearance of their spermathecae and their larval production. None of the females which were separated manually from their mates within 1 hr of the onset of coitus either ovulated or became inseminated, with the exception of two flies which ovulated after mating for only 15 min. Of the 26 females manually separated after 2 hr, however, 7 (27 per cent) were inseminated and all these subsequently ovulated. The 19 uninseminated females in this group included 13 (50 per cent) which retained their first egg and 6 (23 per cent) which ovulated. Similar results were observed after

longer periods *in copula*. This shows that insemination is not a pre-requisite for ovulation, and increasing copulation time may satisfy the requirements for the release of the first egg without sperm transfer taking place.

TABLE 1—THE EFFECT OF COPULATION TIME ON INSEMINATION AND OVULATION IN *G. morsitans orientalis*

Duration of mating	Separated manually					Separated voluntarily				
	No. of females	Inseminated		Uninseminated		No. of females	Inseminated		Uninseminated	
		Ov.*	Ret.*	Ov.	Ret.		Ov.	Ret.	Ov.	Ret.
15 min	27	0	0	2	25	0				
30 min	24	0	0	0	24	0				
45 min	17	0	0	0	17	0				
60 min	15	0	0	0	15	0				
1–1½ hr						10	9	0	0	1
1½–2 hr						9	9	0	0	0
2 hr	26	7	0	6	13	0				
		26·9%		23·1%	50·0%					
2–3 hr						5	4	0	1	0

Duration of mating (hr)	No. of females	Inseminated		Uninseminated	
		Ov.	Ret.	Ov.	Ret.
3†	30	18	0	5	7
		60·0%		16·7%	23·3%
4½†	37	30	0	1	6
		81·1%		2·7%	16·2%
6†	26	18	0	3	5
		69·2%		11·5%	19·2%
24‡ (control)	48	45	0	3	0
		93·7%		6·2%	
Virgin (control)	31	—	—	0	31

* Ov., ovulated; Ret., retained.
† Manually and voluntarily separated flies not differentiated.
‡ Pair left together in tube for 24 hr period.

Between 1 and 2 hr after the onset of coitus some of the pairs separated voluntarily. In all but one instance these females ovulated, and the majority (92 per cent) were found to be inseminated. Clearly, then, this experiment indicates that the minimum time for the successful completion of the mating act is about 1 to 1½ hr, although a later experiment demonstrated insemination in a small proportion of matings after only 45 min.

The fact that practically every female which separated voluntarily from its mate was later found to have ovulated, suggests that a factor associated with the completion of the mating act is at least partly responsible for ovulation.

The effect of mating females with aspermic males

In order to allow full copulation time and voluntary separation of the sexes without sperm transfer, females were mated with males rendered aspermic by repeated earlier matings. In this experiment 12 males were provided with mates on thirteen occasions at intervals of 2 to 13 days over a 76 day period. Normal insemination and subsequent ovulation were observed in those females mated with the males on the first six or seven occasions. In these females sperm was clearly visible in the spermathecae and in 61 out of 64 cases larvae were produced or were present in the uterus (Table 2). After about the seventh mating the males failed to pass sperm, a fact which was confirmed by the empty spermathecae and a complete

TABLE 2—INSEMINATION AND OVULATION IN FEMALES OF *G. morsitans orientalis* MATED WITH POTENT AND ASPERMIC MALES

Mating No.	Male age (days)	Male No. 1	2	3	4	5	6	7	8	9	10	11	12
1	10	IO	IO	IO	IO	IO	IO	IO	IO	IO	IO	IO	IO
2	13	IO	IO	IO	IO	IO	IO	IO	IO	IO	IO	IO	*
3	16–18	IO	EO	IO	IO	IO	*	IO	IO	IO	*	IO	*
4	20	IO	EO	IO	IO	IO	IO	IO	IO	*	EO	EO	EO
5	33	*		IO	IO	IO	EO	IO	IO	IO	IO	IO	IO
6	42	IO		IO	IO	IO	EO	IO	IO	IO	IO	IO	IO
7	48	EO		IO	EO	IO	EO	IO	EO	IO	IO	IO	EO
8	50	EO		EO	EO	EO	EO	IO	EO	EO	EO	EO	EO
9	60	EO		EO	EO	EO	EO	EO		EO	EO	EO	EO
10	63	EO		EO	EO	EO	EO	EO		EO	*	EO	EO
11	66	EO		EO	EO	EO	EO	EO		EO	EO	EO	*
12	69	EO		EO	EO	EO	EO	*		EO	EO	EO	EO
13	76	EO		EO	EO	EO	EO	EO		EO	*	EO	EO

	No. of females	IO	IR	EO	ER	Larvae produced
Virgins (control)	12	—	—	0	12	—
Potent males	64	64	0	0	0	61
Aspermic males	68	0	0	68	0	0

* Female died.

IO—inseminated, ovulated; IR—inseminated, egg retained; EO—spermathecae empty, ovulated; ER—spermathecae empty, egg retained.

failure to produce larvae. All females mated with these aspermic males, however, ovulated normally confirming that insemination is not essential for the release of the egg from the ovary.

The effect of spermatophore formation

Recently it has been shown that sperm transfer in tsetse flies is accomplished by means of a spermatophore which is constructed by the male during coitus from accessory gland secretions (POLLOCK, 1970). Within 24 hr of the start of copulation the sperm in the spermatophore is passed into the spermathecae and after separation of the sexes the spermatophore, usually still containing an appreciable amount of sperm, is discharged from the uterus.

In order to see whether aspermic males make a spermatophore 10 females of *G. morsitans* were mated for 20 hr with the 10 aspermic males remaining from the experiment described above. Nine further females were mated with young virgin males as a control. At the end of the 20-hr period the females were dissected (Table 3). Six of those mated with aspermic males contained a well-formed but

TABLE 3—SPERMATOPHORE FORMATION AND SPERM TRANSFER IN MATINGS BETWEEN FEMALES OF *G. morsitans orientalis* AND ASPERMIC (A) AND YOUNG VIRGIN (Y) MALES

Female No.	Male mate	Spermatophore present	Spermatophore full/empty	Spermathecae full/empty
1	A1	Yes	Empty	Empty
2	A3	Yes	Empty	Empty
3	A4	No		Empty
4	A5	No		Empty
5	A6	Yes	Empty	Empty
6	A7	No		Empty
7	A9	Yes	Empty	Empty
8	A10	No		Empty
9	A11	Yes	Empty	Empty
10	A12	Yes	Empty	Empty
11	Y	Yes	Empty	Full
12	Y	Yes	Full	Full
13	Y	Yes	Full	Full
14	Y	Yes	Full	Full
15	Y	Yes	Full	Full
16	Y	Yes (2)	Full	Full
17	Y	No		Full
18	Y	Yes	Empty	Full
19	Y	Yes (2)	Full	Full

empty spermatophore in the uterus, and in all cases the spermathecae were devoid of sperm. The females mated with potent males comprised 8 with spermatophores in the uterus and all 9 with sperm in the spermathecae. Two of the latter group of females contained two spermatophores, suggesting that successful mating had taken place twice within the 20 hr period. The aspermic males were subsequently

mated for a 6 hr period and then separated manually; 8 of the 10 females mated with them contained empty spermatophores. These observations show that although sperm production ceases after repeated matings, accessory gland secretion and the construction of a spermatophore can continue. Although these spermatophores contain no sperm they possess a well-defined central cavity which is filled with a fluid of unknown origin.

Since the construction of a spermatophore in the uterus might be a factor controlling ovulation the following experiment was performed. Females of *G. morsitans* were mated with potent males for 2 hr. At the end of this period some of the pairs had separated voluntarily but others had to be pulled apart by hand; these are recorded separately in Table 4. The females were then enclosed in 3 × 1 in.

Table 4—The transfer of spermatophores and ovulation in females of *G. morsitans orientalis* mated with young males for 2 hr

	No. flies	Spermathecae empty		Spermathecae full	
		Egg ovulated	Egg retained	Egg ovulated	Egg retained
(a) Flies separated manually					
Spermatophore expelled	5	1	0	4	0
No spermatophore	23	14 (60·9%)	9 (39·1%)	0	0
(b) Flies separating voluntarily					
Spermatophore expelled	23	1	0	22	0
No spermatophore	8	2	0	6	0

glass tubes for 24 hr, then returned to their polystyrene 'colony' cages and the glass tubes washed out with 0·9% saline to recover any expelled spermatophores (Pollock, 1970). They were then maintained for a further 14 days before dissection.

Table 4 shows that spermatophores were recovered from 28 females of which 26 (93 per cent) were found to be inseminated. Of the 31 females without spermatophores, however, only 6 (19 per cent) were found to be inseminated. Clearly the spermatophores in these 6 flies must have been mislaid, but nevertheless a highly significant association was found between finding a spermatophore and sperm in the spermathecae ($\chi^2 = 31{\cdot}94$, $P < 0{\cdot}001$). With this measure of confidence it is safe to assume that those flies without spermatophores and with empty spermathecae never received spermatophores from their mates. However, the results show that 14 (61 per cent) of the 23 females in this category ovulated, so the conclusion must be that spermatophore construction is not a factor causing ovulation. Later experiments in which the respective rôles of the male and female in mating were separated experimentally, support this view.

Inter-specific matings

Females of *G. morsitans* were placed in polystyrene 'colony' cages with males of *G. austeni* (54 pairs) for an 18 hr period. Similarly, females of *G. austeni* were placed with males of *G. morsitans* (17 pairs). In the former crossing 12 pairs were observed to copulate. At the end of the 18 hr period the sexes were separated and the females were kept in glass tubes for a further 24 hr to collect any expelled spermatophores. They were then returned to their 'colony' cages and dissected 10 days after their emergence.

Table 5 shows that 11 (20 per cent) of the *morsitans* females ovulated, 7 (13 per cent) showed sperm in the spermathecae, and in two cases ejected spermatophores were found in the tubes. In the second crossing (*morsitans* male × *austeni* female)

TABLE 5—INSEMINATION AND OVULATION IN INTER-SPECIFIC MATINGS (*G. morsitans* × *G. austeni*)

Mating	No. of females	No. seen to mate	Inseminated		Uninseminated		Spermatophore ejected
			Ov.‡	Ret.‡	Ov.	Ret.	
G. austeni male × *G. morsitans* female	54	12*	6	1	5	42	2†
G. morsitans male × *G. austeni* female	17	0*	1	0	5	11	0†

* Not all matings could be observed in the 18-hr period.
† It must be assumed that all inseminated females received a spermatophore.
‡ Ov., ovulated; Ret., retained.

none of the 17 pairs were observed to mate, but on dissection one female was found to be inseminated and 6 (35 per cent) to have ovulated. In both these experiments little confidence can be attributed to the figures for those pairs seen to mate since the flies could not be observed at sufficiently short intervals during the 18 hr period. Similarly, it must be assumed that all of the females showing sperm in their spermathecae received a spermatophore, but these were not always recovered. These interspecific matings, however, demonstrate that spermatophore construction, insemination, and ovulation can occur in a proportion of *morsitans* × *austeni* crosses, and show that the cause of ovulation is not species specific.

The effect of inserting organs and tissues from donor flies into virgin females of G. morsitans

Various organs and pieces of tissue from donor males and females were inserted into the haemocoel of virgin females of *G. morsitans* 9 days after emergence.

Virgins of this age were chosen because the first egg is fully developed and is presumably competent to descend into the uterus. Tissues and organs inserted were testes, accessory glands, and ejaculatory ducts from donor males, and 'full' and 'empty' spermathecae from inseminated and virgin females, respectively. These were chosen because the insertion of male accessory glands into the haemolymph of *Aëdes aegypti* (LEAHY and CRAIG, 1965) and *Drosophila melanogaster* (LEAHY, 1966) increased oviposition; material from the ejaculatory duct of *Musca domestica* is known to produce unreceptivity in the female (RIEMANN *et al.*, 1967); and 'full' spermathecae in *Rhodnius prolixus* have been shown to produce a humoral factor responsible for increased egg production (DAVEY, 1965). Two groups of control flies were also maintained: some were left unoperated, others (the sham-operated contols) experienced the whole operative procedure but without any tissue insertion. The flies were dissected 18 days after emergence to see whether ovulation had occurred.

In this experiment (Table 6) 2 (5 per cent) of the unoperated controls ovulated, a condition which is unusual, as judged from other results. Nevertheless, a comparison with the operated controls showed that a significantly greater number of

TABLE 6—THE EFFECT OF INSERTION OF TISSUES FROM DONOR FLIES INTO THE HAEMOCOELS OF VIRGIN FEMALES OF *G. morsitans orientalis* 9 DAYS AFTER EMERGENCE

Tissue inserted	No. of females	Egg ovulated	Egg retained
Unoperated control	37	2 (5·4%)	35 (94·6%)
Sham operated control	27	6 (22·2%)	21 (77·8%)
Testis from potent male	18	3 (16·7%)	15 (83·3%)
Accessory gland from male	16	3 (18·7%)	13 (81·3%)
Ejaculatory duct from male	11	1 (9·1%)	10 (90·9%)
Empty spermatheca from virgin female	13	2 (15·4%)	11 (84·6%)
Full spermatheca from inseminated female	15	3 (20·0%)	12 (80·0%)

the latter ovulated ($\chi^2 = 4{\cdot}04$, $P < 0{\cdot}05$). The experimental groups showed ovulation rates of between 9 and 20 per cent. Since these are not significantly different from the operated controls ($\chi^2 = 1{\cdot}05$) the increased ovulation rate is probably due to the mechanical trauma experienced during handling.

Separation of the rôles of the male and the female during coitus

The relative rôles of the male and the female during coitus were examined in the following experiment. Females of *G. morsitans* were allowed to mate with virgin males for 45 min and then separated manually under cold anaesthesia. A second group of females was treated in a similar fashion but allowed to re-mate

immediately with a fresh male for a further 45 min period. Other groups were allowed three or four consecutive 45 min periods of mating. In this manner females experienced up to 3 hr of mating whereas the males never experienced more than 45 min. In the converse experiment, males were allowed up to four consecutive 45 min matings, each with a virgin female. The females in this experiment, therefore, fell into four groups depending on whether their mates had experienced 0, 1, 2, or 3 previous periods of coitus. After separation, each female was enclosed in a glass tube for 24 hr to catch any expelled spermatophores, and then dissected 16 days after emergence to determine whether ovulation had occurred.

Only 10 (1·8 per cent) of 568 such 45 min matings resulted in insemination. This allows modification of the earlier statement that 60 min was probably the minimum time for the completion of a 'successful' mating. These inseminated females are excluded from the results (Table 7).

TABLE 7—THE EFFECT OF MATING FEMALES OF *G. morsitans orientalis* FOR ONE, TWO, THREE, OR FOUR CONSECUTIVE 45-min PERIODS WITH FRESH VIRGIN MALES (A), OR MATING VIRGIN FEMALES FOR 45 min WITH MALES WHICH HAVE EXPERIENCED NONE, ONE, TWO, OR THREE PREVIOUS SUCH MATING PERIODS (B)

(A) No. of 45 min matings	No. of females	Egg retained	Egg ovulated
1	33	25	8 (24·2%)
2	37	24	13 (35·1%)
3	35	18	17 (48·6%)
4	29	6	23 (79·3%)
Chilled and handled four times but not mated	25	19	6 (24·0%)
(B) No. of previous matings by male	**No. of females**	**Egg retained**	**Egg ovulated**
0	29	20	9 (31·0%)
1	28	22	6 (21·4%)
2	27	21	6 (22·2%)
3	27	18	9 (33·3%)
Virgin controls	15	15	0
24-hr* mated controls	16	0	16 (100%)

All matings were performed consecutively and, except for the 24 hr mated controls, only unseminated females are included in the table.

* Pair left together in tube for 24 hr period.

Table 7 shows that whereas all virgin females retained their first egg and all '24 hr' mated controls ovulated, the experimental flies clearly 'added up' their sexual experience and ovulated accordingly, regardless of the fact that the males

mated with them never experienced more than 45 min *in copula*. The differences in ovulation rate in the four groups are significantly different ($\chi^2 = 21{\cdot}11$, $P < 0{\cdot}001$). None of these experimental females expelled a spermatophore, and none of a further 24 females dissected immediately after a similar 45 min period revealed such a structure in the uterus. These observations confirm that spermatophore construction is not responsible for causing ovulation. Control females which experienced four chilling and handling procedures similar to these by the experimental flies, but without the periods of mating, showed an ovulation rate of 24 per cent. This was significantly less than that for the experimental flies mated four times ($\chi^2 = 16{\cdot}40$, $P < 0.001$) and is reminiscent of earlier results for handled flies.

In the converse experiment, in which the males were mated for up to four consecutive 45 min periods with fresh virgin females, the ovulation rates in the four groups of females were not significantly different ($\chi^2 = 1{\cdot}57$) despite the fact that their mates had different amounts of prior sexual experience (Table 7). These results, therefore, show that the duration of mating experienced by the female is an important factor in the control of ovulation.

DISCUSSION

The results of this investigation show that ovulation in the tsetse fly, *G. morsitans*, is not caused by insemination or by the construction of a spermatophore in the uterus. Experiments in which various organs and tissues were inserted into the haemolymph of virgins showed that chemical stimuli associated with testes, accessory glands, and ejaculatory ducts of the male, or 'full' spermathecae of the female, are also not responsible.

The ovulation rate, however, was positively correlated with the duration of mating. Females were shown to 'add up' their sexual experience regardless of the length of time their mates had been in coitus, and to ovulate after a sufficient time had elapsed even if the males mated with them failed to reach the stage of spermatophore construction and sperm transfer. About 25 per cent of the females mated for 45 min subsequently ovulated but less than 2 per cent of such matings resulted in insemination. After 45 min *in copula* there was a steady rise in both ovulation and insemination rates, but even after 6 hr some females failed to ovulate. It would appear from the present data that females of *G. morsitans* kept at 25°C require at least 45 min for a 'successful' mating, and about 3 hr for over 50 per cent of them to become inseminated.

Although spermatophore construction and sperm transfer were shown not to be pre-requisites for ovulation the results of several experiments demonstrated a strong correlation between the completion of the mating act (i.e. voluntary separation of the sexes) and the subsequent release of the egg from the ovary. The data give no clues as to the nature of this reaction.

One of the most interesting aspects of this problem is that mating normally takes place (both in the laboratory and in the field) with females 1 to 3 days after emergence, whereas the first egg is not ovulated until the seventh or eighth day of

post-emergence life. There is, therefore, a 'time lag' of at least 5 days which suggests that the female has a 'memory' of having been mated. The facts that duration of mating was shown to be the important factor, and that this effect can be simulated by handling during the operative procedures, suggest that the stimulus is mechanical and therefore presumably nervous. However, it is difficult to see how a purely 'nervous' mechanism can account for the time lag between mating and ovulation, and a chemical intermediary is perhaps indicated.

The closest parallel to this situation in *Glossina* is probably that in locusts, although in this case the response is ovarian development and not ovulation. For instance, in *Locusta migratoria manilensis*, mating, even with castrated males, accelerated egg maturation, apparently by mechanical stimulation (Quo, 1959). Enforced activity, repeated electrical stimulation, excessive handling (Highnam, 1961, 1962), or slight wounding (Norris, 1954) simulated similar mating effects in *Schistocerca gregaria*. Correlated with mating or artificial stimulation in both these species of locust was a release of neuro-secretory material from the *corpora cardiaca* (Highnam, 1961, 1962; Highnam and Haskell, 1964). It is possible that such a mechanism might explain the control of ovulation in *Glossina* and account for the observed time lag. Subsequent ovulations are probably independent of the mating act and possibly initiated by the larvipositions which precede them.

Acknowledgements—The authors wish to thank Mrs. Pat Smith, Miss Fay Brown, Mrs. Pam Hillier, Mrs. Helen Johnstone, and Mrs. Isa Burden for technical assistance at various times; Dr. T. A. M. Nash and Professor J. Fraga de Azevedo for the provision of tsetse fly puparia; and the Wellcome Trust for financial support.

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Reprinted from
TRANSACTIONS OF THE ROYAL SOCIETY OF TROPICAL MEDICINE AND HYGIENE.
Vol. 65. No. 2. pp. 223, 1971.

Factors regulating ovarian cycle in tsetse flies

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Under normal circumstances mated female tsetse flies ovulate (whether or not sperm transfer occurs) and virgins do not. Thus mating initiates ovarian cyclicity. The sizes of ovarian follicles of mated flies after the first ovulation has occurred are greater than those of comparable follicles of virgins of the same age. Thus the retention of a mature chorionated egg in the ovary of a virgin is associated with a reduction in growth of the other follicles.

A positive correlation was found to exist between number of "missed cycles" (i.e. pregnancies not resulting in independently viable third instar larvae) and the degree of follicular development in mated *G. austeni* killed when 45 days old.

When larval retention was operationally enforced in parturient flies the degree of follicular development was lower than that of non-operated mated controls of the same age.

A hypothesis is proposed which involves:

1. inhibition of follicular growth by chorionated egg in ovaries,
2. prevention of ovulation of mature oöcyte, possibly by purely mechanical means, by uterine contents of parturient females,
3. ovulation occurring earlier after an abortion of egg, 1st or 2nd instar larva than after a pregnancy resulting in an independently viable 3rd instar larva, possibly because the inhibition outlined in (2) has been removed.

Printed in Great Britain by F. J. Parsons, Ltd., London, Folkestone and Hastings.

ABSTRACT OF THESIS

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Degree PhD *Date* June 1972

Title of Thesis Control of the reproductive cycle in tsetse flies (Glossina spp.)

This investigation is concerned with the initiation and maintenance of reproductive processes in two species of tsetse fly; Glossina morsitans and G. austeni.

The first ovulation in both species is dependent on mating. Neither spermatophore construction nor sperm transfer are prerequisites for ovulation. Injection of extracts and tissues from males and mated females into the haemocoel or uterus of virgins does not induce a greater proportion of ovulations than occurred in operated controls. The number of flies ovulating is proportional to the degree of trauma involved in the operative technique. Isolation of male terminalia within the female tract is the most effective of the surgical procedures but does not result in anything like the almost absolute induction of ovulation resulting from completed mating. It is postulated that a temporal summation of mechanical stimuli is a requirement for the first ovulation. Subsequent ovulations are independent of the means of induction of the first ovulation.

Initiation of ovulation by mating is not species-specific. Wing-beat notes of males are not essential for conspecific matings.

The sizes of follicles in mated and unmated females were measured at intervals during the first sixteen days after eclosion. Follicle growth rates are the same in both groups prior to the first ovulation but thereafter growth was faster in mated flies. Eggs retained for prolonged periods in virgins lost potency and eventually degenerated. A water- and ethanol-soluble factor, which is present in ovaries containing mature eggs, partially inhibits the growth of developing follicles.

When physical conditions are constant ovarian development is related to concurrent larval development and is independent of events in previous cycles. Missing ov cycles results in advancement of ovulation and thus in acceleration of ovarian ageing.

Ovulation occurs between fifteen minutes and one hour after larviposition.

Feeding frequency is directly related to number of full-term larvipositions, and inversely related to number of missed cycles in a fixed time. Virgins feed less frequently than do mated females of the same age which miss all of their cycles. Existing accounts of timing of feeding during interlarval periods are confirmed.

Interactions between components of reproductive physiology are discussed along with hypothetical integrative mechanisms.

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