STUDIES CONCERNING SPERM TRANSFER IN SOME HIGHER DIPTERA

A thesis submitted by

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July 1971.

CONTENTS

ABSTRACT

A review of the pest status of the flies studied is followed by an appraisal of basic research into the mating behaviour and physiology of higher flies, especially Calliphoridae.

The cumulative mating frequency curves obtained from experiments on caged populations of Lucilia sericata Mg. (Calliphoridae) are interpreted in terms of the density of flies available for mating. A mating rate coefficient is proposed, which should be of value in the study of mating competiveness of different strains.

The alignment of parts during copulation in L. sericata is described and figured. Attention is drawn to lateral ducts in the phallosome which conduct the granular male accessory secretion to the appropriate place in the female. The appearance or non-appearance of this granular secretion in the lateral phallosome ducts is made the criterion in a test for the mated status of males. Lateral phallosome ducts in other calliphorines have been detected and are figured. An account is given of the effect that tepa-treatment of male L. sericata has on some features of mating in this species, and on sterility. Investigations into the nature, fate and function of the male accessory secretion are described.

The functional morphology of the phallosome in some Sarcophaginae is compared with that in Lucilia.

Accounts are given of sperm transfer by spermatophore in Glossina austeni, and of phallosome structure in the same species. A test for the inseminated status of female tsetse flies, without involving dissection, is described. Arguments are put forward in support of the view that Glossina is better regarded as related to the Hippoboscidae than as related to the Muscidae.

Descriptions are given of the co-adapted phallosome and spermathecal ducts of Merodon equestris (Syrphidae). Spermatophores are described from the Bibionidae.

The evolution of sperm transfer mechanisms in the Diptera is discussed.

INTRODUCTION

The mating of flies forms the subject matter of this thesis. It is hoped that the studies reported here will serve the dual purpose of extending our basic understanding of the mating process in the Diptera, and of indicating how such knowledge can be of value in relation to modern methods of pest control, in particular that known as the 'sterile males' method of control.

1. Pest status of the flies studied

Lucilia sericata Meigen (Calliphoridae) is a common insect originally of the Northern temperate - zone. No doubt aided by the trading and colonising activities of man it has become spread over many other parts of the globe, notably South Africa, Australia and New Zealand. It is probably absent from Central and South America, from the S.W. Pacific Islands, and from Madagascar (Zumpt 1965).

It is well known as a pest of sheep, causing "strike", a condition in which fly larvae (maggots), laid as eggs on the soiled or blood-stained fleece, invade the skin and flesh (Cragg, 1955). This causes the sheep to lose condition, and can lead to death by toxaemia or septicaemia. Human myiasis can also occur, though initially maggots will remain restricted to dead tissue of an existing wound (Stewart, 1934). Despite this habit of facultative wound parasitism in sheep the species is predominantly a carcass-feeder, and is easily reared under laboratory conditions on meat or liver (Cousin, 1923; Mackerras, 1933).

Lucilia sericata has at times been confused with a closely related species, Lucilia cuprina (Wiedemann) (Waterhouse and Paramonov, 1950); this however is not native to Europe, but comes from Africa and the Orient, and has also been introduced into Australasia. It has the same tendency to cause sheep strike in those areas in which it occurs.

Calliphora spp. (Calliphoridae) are not able to initiate sheep strike in Britain (Ratcliffe, 1935) but can develop in already existing lesions caused by Lucilia. In Tasmania C. vicina R.D. is important in in sheep myiasis, as is C. stygia; the latter is the most important primary strike-causing fly in New Zealand, being responsible for almost all cases in one census (Macfarlane, 1938). Since Calliphora spp. often feed on faeces and other decomposing matter, as well as visiting domestic food supplies, they are clearly a public health problem, and the same remark applies to many blowflies (Norris, 1965) and flesh flies (Sarcophaginae).

The genus Glossina occupies over four million square miles of Africa, and without doubt has a profound effect on the life of man in this area, either directly as a vector of human trypanosomiasis, or indirectly as a vector of nagana, a similar disease of cattle (Buxton, 1955; Glasgow, 1963; Ford, 1971).

Merodon equestris (Syrphidae) in the larval stage attacks daffodil bulbs and severe infestations are possible (Woodville, 1955). Control has been achieved by the use of dieldrin and aldrin, but the persistence and deleterious effects of these compounds at higher levels in the food chain and the threat of resistance developing in the fly (Woodville, 1965) have stimulated the search for alternative methods of control.

In general, there is a growing desire to replace organochlorine compounds by more specific agents of control. There has also been some concern over the large quantities of organochlorine and organophosphorus chemicals used in sheep dipping, and about the methods adopted for their disposal after use (Leech and Macrae, 1970).

The sterile male technique

Lucilia sericata, Glossina spp and Merodon equestris have all been considered as candidates for control by the sterile male release method. The principles of the technique are as follows. Large numbers of the particular insect are bred (or caught from the field) and the males sterilised by irradiation or by chemical means. The males are then released. They mate with wild females in the field, which consequently produce infertile eggs. That fraction of the female population which chances to mate with wild fertile males produces the next generation, but this will be of reduced numbers, the reduction being proportional to the dilution of the wild male population by the sterile males. Each generation is treated in the same way, the sterile/fertile ratio becoming increasingly favourable and so the pest population rapidly diminishes in size till it becomes extinct. It is clear that this control strategy is quite specific to the insect species attacked (Knipling, 1960). The elimination of the screw worm fly Callitroga hominivorax Coq. (Calliphoridae) by this means from Curacao (Baumhover et al., 1955), and then from most of the infested areas of the United States of America (Knipling, 1964) proves that the method can work in practice. Successful control of some trypetid fly species has also been achieved (Knipling, 1967; Nadel and Guerrieri, 1969; Proverbs, 1969; Steiner et al., 1970). Against this, there have been several failures particularly in attempts at mosquito control (Rai, 1969).

Following the successful screw worm campaign, an attempt was made in this country to attack by the same means a supposedly isolated Lucilia sericata population, on Holy Island, Northumberland, in 1956 and 1957. This trial resulted in no reduction in the wild population and was therefore regarded as a failure by the team in charge (Macleod and Donnelly, 1961). Unfortunately, the cause or causes of failure could not be

identified precisely and the same workers came to the very general conclusion that "either the sterilization was inadequate or ..... the sterilized males were unable to compete with the native males." They went on to recommend that there should be further study of the mating behaviour of sterilised males. Lucilia sericata was recently listed as one of five livestock pests for which there existed "considerable research background", and which "may be amenable to control by the sterile male technique" (I.A.E.A. Vienna, 1969). Active interest in applying the technique to the control of L. cuprina is being shown by Australian workers (Whitten, 1969, 1970).

The research, described in this thesis, into events which take place during mating in higher flies, with special reference to Lucilia sericata, must be seen in the context of this earlier failure of control using the sterile males method, and also in the context of basic studies carried out by other workers on higher flies, which will now be briefly reviewed.

3. Review of basic studies in the mating of higher flies, especially Calliphoridae

i. Anatomy of the reproductive system.

Weismann (1863), Lowne (1870, 1890-95) and Bruel (1897) have described the general anatomy and histology of the reproductive systems of Calliphora, a genus which closely resembles Lucilia in these features. Paired orange-red testes and paired whitish accessory glands are present in the male. The ejaculatory duct, after passing through the ejaculatory bulb or pump, runs the length of the penis (phallosome) and opens at the apex of that organ. The female has large paired ovaries each containing many ovarioles; short lateral oviducts lead to a common oviduct. This widens just before the point where the ducts of the three spermathecae collectively debouche; posterior to this there are two pockets in the

dorsal side of the tract. Lowne (1870) wrote that the function of these pockets was "to receive the male fluid", especially "masses of coagulated albumen".

Lowne (1870) and later Goodliffe (1938, unpublished Ph.D. thesis), thought that the male accessory secretion (the "albumen" of Lowne) served as a medium for the sperm, but this is almost certainly not the case, at least in Lucilia.

Graham-Smith (1939) described in considerable detail the musculature and movements of the male and female reproductive systems in Calliphora. Hori (1960) compared the male reproductive systems of over 70 species of muscoid Diptera, and illustrated how the shape of the testis in Lucilia sericata altered with age, changing from ovoid to dumbell-shaped. Donnelly (unpublished) has studied the anatomy of the reproductive systems of Lucilia sericata, and found that it closely resembles that of Calliphora.

ii. Phallosome structure

The term phallosome will be used in the sense of Christophers and Cragg (1921) to mean the male intromittent organ, regardless of homologies. To avoid all possible ambiguities, it is safer to add that it is the intromittent organ bearing the male gonopore, since in some flies and possibly other insects additional appendages enter the vagina at copulation. (Sections 2 and 6).

The phallosome of Calliphora, a fairly complex structure, has been figured by Lowne (1870, 1890-95), Bruel (1897) and Graham-Smith (1939) as part of their more general studies. Graham-Smith showed how the phallosome articulates with its base, and how it aligns with the other appendages, the anterior and posterior parameres. The Lucilia phallosome has been illustrated in numerous taxonomic works (e.g. Richards, 1926; Aubertin, 1933; Séguy, 1928; Kano et al., 1967). Pollock (unpublished M.Sc. thesis) noted lateral phallosome ducts which will be more fully described later (Section 2).

Zumpt and Heinz (1950) compared the male intromittent organ of Calliphora with those of three species of Sarcophaga, and tabulated their interpretation of the homologies of the different parts. Roback (1954) gave many detailed illustrations of the phallosome in his study of 145 species of Sarcophaginae, but made no observations on the manner in which the phallosome functioned.

iii. Inversion and circumversion of the phallosome in higher flies.

From the researches of Feuerborn (1922), Lamb (1922), Richards (1927), Hardy (1944), Crampton (1944) and others, it is clear that in flies such as the Calliphoridae the male hypopygium is constructed as though twisted around the horizontal axis through 360° (circumversion) so that the male genital tract loops over the rectum. It has to be remembered that some authorities regard the morphological dorsal face of the phallosome as being contiguous with the venter of the vagina at copulation (inverse correlation) rather than with the dorsum of the vagina (direct correlation) (Hardy, 1944). In the present treatment this complication will be ignored, and the terms "upper" and "lower" surface of the phallosome will be used as appropriate for the phallosome in its normal position in the vagina during copulation: i.e. the upper or dorsal surface of the phallosome is that face in contact with the dorsum of the vagina during copulation.

iv. Courtship and pheromones

Courtship in calypterate flies is relatively undeveloped, in comparison with what is to be seen in many drosophilid and trypetid flies, for example. One analysis of premating movements in a calypterate fly is that given by Tauber (1968) on Fannia (F. femoralis Stein and F. caricularis (L.)), in which the effects on mating performance of removing the wings and various legs from the flies were studied. Bartell et al. (1969) gave a brief illustrated description of the simple precopulatory movements and gestures to be seen in Lucilia cuprina (a fly in which overt sexual attacks are

more vigorous and frequent than in L. sericata - personal observation). Theynoted that mounting may take place by a male alighting directly upon another fly, though more often the flies make an ambulatory approach.

Pheromones are known to occur in some calypterrate flies. Fletcher et al. (1966) considered that an excitatory sex pheromone was given off by virgin male screw worm flies (Cochliomyia hominivorax), eliciting a female response. According to Ragoff et al. (1964) female houseflies (Musca domestica) excite males to increased sexual activity by means of a volatile chemical. Mayer and Thaggard (1966) found that there was an olfactory attractant specific for male M. domestica, associated with live virgin, dead and mated females, and house-fly contaminated cages. Parker (1968) could not find any evidence of a volatile sex attractant from either sex in Protophormia terrae-novae; likewise Dean et al. (1969) could find no volatile sex attractant in Glossina. An attractant could not be demonstrated by Bartell et al. (1969) in L. cuprina, but females appeared to elicit increased sexual activity in males held out of contact from them. All these volatile excitatory substances elicit a response of a low order only, and nothing is known of their significance in the field.

v. Mating vigour

A distinction will be made here between mating rate, the rate at which mating pairs form when virgins of the two sexes are put together in a mating cage; mating propensity, the ability of individual flies to mate repeatedly in a given period of time (usually it is the male's ability to mate with a succession of females which is of interest); and mating duration, the time spent by pairs of flies in copula.

Much work has been done on the genetical control of mating rates in Drosophila, particularly by Manning, Speiss and Parsons. Manning (1961, 1963) studied the genetical response of Drosophila to selection

for fast and slow mating rates, and analysed the concomitant alterations in behaviour, not necessarily sexual, shown by the selected lines.

It has been supposed that mating vigour is of prime importance in any sterile male campaign since it is clearly essential for the success of the campaign that at least some released sterile males should mate with nubile (i.e. mature virgin) females before wild males are able to do so; such released males are then said to be "competitive" with wild males.

Baumhover (1965) believed that in the screw-worm fly C. hominivorax a measure of the mating propensity of males (at least under laboratory conditions) was provided by noting the reduction in life span of females caged with these males, due to sexual harassment. This "sexual aggressiveness" test was adapted to select sexually vigorous strains of flies for use in the sterile male release campaign. Recently, however, doubts have arisen as to whether the relatively poor sexual performance of a new Mexican strain of screw-worms under laboratory conditions necessarily indicates that the flies will be poorer performers under Mexican field conditions, compared with the Georgia/Florida strain previously used (Alley and Hightower, 1966; Spates and Hightower, 1967; Hightower and Graham, 1968).

Mating duration shows an astonishing amount of interspecific variation. In species of Simuliidae it may last for 2-3 minutes only (Davies 1965) and in Fannia it can be equally short (Tauber 1968). At the other extreme Glossina may copulate for several hours at a time. In the screw-worm fly (C. hominivorax) mating lasts about 5 minutes (Crystal 1967); Donnelly (unpublished) observed that in L. sericata mating duration averaged about 15 minutes. In Drosophila mating duration is a far less variable attribute than the time taken for individual flies to begin mating after they have been put together as virgin pairs (MacBean and Parsons, 1966).

vi Coitus, the transfer of secretions, and their effect on the female

Graham-Smith (1939) was the first to study the mechanics of copulation in a calliphorid fly, though before him Berlese (1902) had made some observations on the mating of Musca domestica L. Graham-Smith did not have the advantage of dissecting copulating flies and had to infer how the parts functioned from the structure and movements of the separate sets of genitalia. He thought that for the two male products to reach their proper destination in the female it was likely that the phallosome altered its position in the female, first delivering the accessory secretion, and then, by deeper insertion, the sperm. He also believed that the sperm followed the accessory secretion in time of delivery, and that their secretions never mingle. Donnelly (unpublished) agreeing that the secretions in Lucilia sericata remain separate, found that the sperm is transferred first, before the accessory secretion. By analogy, this is probably the true condition in Calliphora.

The act of mating may have diverse effects on female behaviour and physiology. Obviously a female when mated becomes capable of producing fertile eggs for the first time (parthenogenesis is uncommon in the Diptera, but examples are mentioned by Stalker, 1954, and White, 1964). It is common for oviposition to be delayed until mating, as in Oscinella frit (Hillyer, 1964); oviposition may be greatly increased after copulation as in Lucilia sericata (Cousin, 1927), L. cuprina (Mackerras, 1933) and Drosophila (David, 1963; Merle, 1968), or less markedly so as in Cochliomyia hominivorax (Crystal and Meyners, 1965; Crystal, 1967). In many insects the females are monogamous and shun or repulse males after insemination; this is true of Lucilia cuprina (Barton-Browne, 1958) and of C. hominivorax (Bushland and Hopkins, 1951). In studies on monogamy laboratory results can be misleading, since captive mated females may be unable escape from the attentions of virile males and multiple mating

may be forced upon females which if at liberty would remate much less readily. Tsetse flies are perhaps in this category (Dame and Ford, 1968).

Since mating has these and other effects, it is useful to know more about the secretions that are transferred at mating, as these may well be the causative agents. They may be classified as follows:-

(a) Spermatozoa. These are the primary product of the male, though they do not necessarily form the greater part of the male secretions. For example in the male Trichoptera studied by Khalifa (1949) a very large protein mass accompanied the relatively small sperm packet transferred at mating. Smith (1956) found that Drosophila subobscura females seldom remate, and that the act of copulation elicits unreceptive behaviour for the period immediately following mating (up to 24 hours), but that receptivity then returns unless sperm has been successfully transferred. Similar results were obtained by Manning (1962) using D. melanogaster.

(b) Fluid in which the sperm are conveyed. This is presumably derived from the testis in most cases. Lee (1950) tentatively concluded that this fluid was the most likely cause of the "insemination reaction" seen in Drosophila.

(c) Secretion of male accessory glands. This may be mobile or viscous, or may set to a harder mass. In Drosophila it is a sticky fluid with small refractile bodies suspended in it (Nonidez, 1920); that of Lucilia sericata also has granular inclusions (Donnelly, unpublished; see also Section 5). In many insects the male accessory gland secretion forms the spermatophore, a more or less rigid body encapsulating the sperm mass or masses. Roth (1962) showed that in the cockroach Nauphoeta cinerea (Olivier) it was the implantation of the spermatophore by the male that triggered off subsequent non-receptivity of females; receptivity was apparently uninfluenced by the presence of sperm in the spermathecae.

The possibility that the same is true for Glossina (see Section 7) deserves to be investigated. Musca domestica does not have separate accessory glands in the male, but the ejaculatory duct is highly glandular and serves the same purpose. Implantation and extirpation experiments by Riemann et al (1967) indicated that there is a factor in the glandular ejaculatory duct of this species which inhibited female receptivity to males. Male accessory glands may be of more than one type. In Rhodnius prolixus Stal. transparent opaque accessory glands provide the secretions which form the spermatophore, while the secretion of a opaque transparent gland causes the oviduct to contract rhythmically, an action which is said to assist the migration of sperm to the spermathecae (Davey, 1958). No species of calypterate Diptera has more than one type of distinct accessory gland in the male, as far as is known.

(d) Other secretions. Biarati (1968) showed that the ejaculatory bulb of Drosophila contained a secretion which was transferred to the female at mating. Butterworth (1969) independently found that the ejaculatory bulb of Drosophila secretes a substance which is detectable by chromatographic methods in mated females; the substance was identified by Brieger and Butterworth (1970) as cis-vaccenyl acetate. Presumably the secretion discovered by Biarati is identical with this. The ejaculatory duct of Muscidae, and to a lesser extent of Calliphoridae, has glandular walls particularly at the apical end. Very little is known about the secretions of the ejaculatory duct in Calliphoridae.

In this large and rapidly expanding area of research, the following topics have been selected for further study (a) mating rates (b) functional morphology of genitalia (c) indices of mated status (d) some mating attributes of tepa-treated males, and (e) the nature of the male accessory secretion in Lucilia sericata. Finally, an attempt has been made to delineate the evolution of sperm transfer mechanisms in the Diptera.

SECTION 1.

THE CUMULATIVE MATING FREQUENCY CURVE IN *LUCILIA SERICATA*

Analysis of the cumulative mating frequency curve in *Lucilia sericata*

Introduction

Manning (1961) has noted that there is a great variation in the time elapsing before individual mating pairs form, after virgin male and female *Drosophila* are placed together. By selecting *D. melanogaster* for fast and slow matings he was able to establish lines which had different mating rates.

In several other *Drosophila* species the genetical control of mating rates has been studied, especially by Spiess and co-workers, and Parsons and his collaborators.

Despite the widespread interest in mating rates, little analysis of the shape of the cumulative mating frequency curve appears to have been made. The indices and transformations of mating rate data that have been proposed in the literature are of an *ad hoc* nature, not based on any specified model.

In experiments using *Lucilia sericata* Mg reported here, it was observed that the rate at which mating pairs formed in cages declined with time, and an explanation of this has been sought, particularly in terms of the decreasing density of candidate flies. It was hoped by this means to establish a mating rate coefficient, which should be valuable in the comparison of mating vigour in different strains of flies. Such comparisons may help in the selection of strains suitable for sterile-male release work.

Materials and Methods

The Lucilia sericata strain used was derived from a culture maintained at the Central Veterinary Laboratory, Weybridge, Surrey, and at the time of use was probably considerably inbred.

Larvae were fed on excess minced meat or ox liver, and were allowed to migrate from the meat into sawdust for pupation. Before emergence pupae were gently sieved out and placed en masse into cages. Flies that emerged over a 24 hour period (approx.) were caught singly in 2" x 1" tubes, as many as were required for the experiment in hand. They were then sexed, and released into segregated cages. Since flies of this species do not mate before they are three days old (Donnelly, 1965), this ensured that all the flies used were virgins. Defective flies were eliminated when detected.

Following the observations by Cousin (1929) that females require protein for sexual maturation, and by Donnelly (unpublished) that males also require protein to reach full reproductive maturity, adults were fed on ox liver for two days within the first week after emergence. Water bottles fitted with an asbestos wick, and granulated sugar (sucrose) were always available in the fly cages. The age of flies used in experiments ranged between five and nine days (see Table 11); there was no evidence that this range in ages had any effect on the shape of the mating frequency curve. Usually, comparisons made were between flies of similar age, history and origin, except in the "second chance of mating experiments" (Experiment 6). Mating experiments were conducted on a bench with strong overhead fluorescent lighting (4 rose-coloured Phillips 8ft lamps, TLAF 125W/29C5; and 1 white Phillips 8ft lamp TLAF 125W/33 A5). The lamps were 14" above the tops of the fly cages. Observations on mating were carried out on flies confined in

perspex cages measuring 12" x 8" x 7", having two opposite sides of nylon or terylene netting, and a 5" diameter circular hole in one wall for the introduction and removal of flies. The hole was closed by a muslin sleeve. Ambient temperatures are recorded in Table 11.

Preparatory to a mating experiment, all the flies to be used were tubed out and randomly assigned to the various replicate cages. Flies of the other sex were tubed into small plastic containers (4" diameter, 2" high). Each container was placed amongst the flies already in a mating cage, then opened, gently shaken to dislodge any stationary insect , removed, and the timing clock started. As successive pairs mated, the time of onset of mating was taken to the nearest minute. An arbitrary 30-second period was allowed at the start of each experiment for the flies to settle down, and any matings in this period were scored as occurring in the first minute. Mating pairs were not interfered with immediately they formed, but were tubed out by hand after a minute or so, disturbing the other flies as little as possible. Mating tests involved using replicate mating cages; of necessity, these replicate tests had to be started at certain intervals of time, and so were not run quite simultaneously. This was not thought to have affected results. At no stage were flies anaesthetised.

Experiment 1.

The time of formation of successive mating pairs was noted in three replicate cages, each containing 20 males and 40 females at the start of the experiment. The average times of formation of successive pairs were calculated (Table 1) and these were plotted as a cumulative mating frequency curve. (Fig. 1).

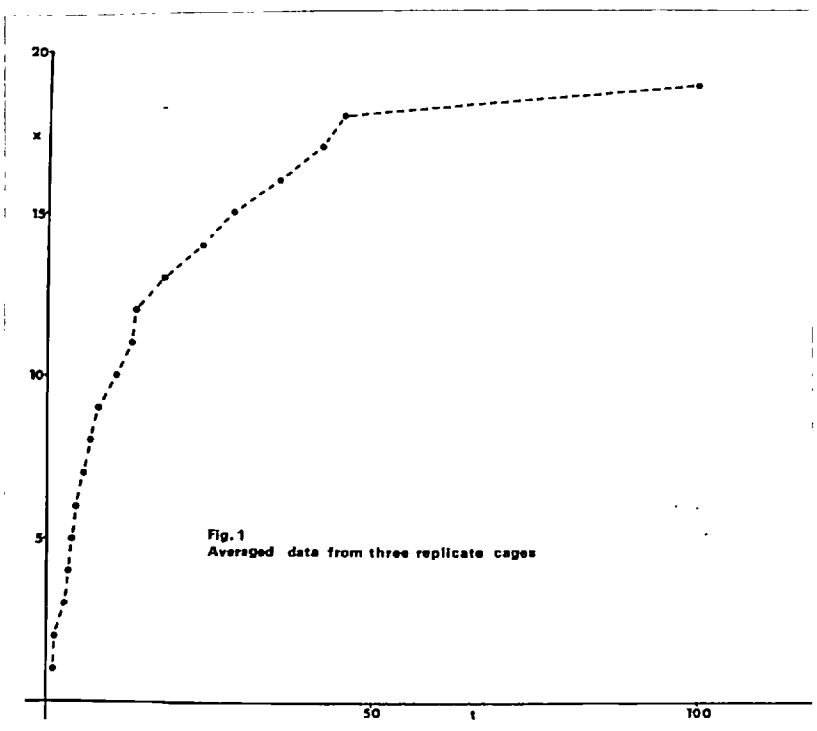


Fig. 1. Cumulative mating pairs (x) slotted against average time (t) to formation of respective pairs (Cages A, B and C, Table 1). t is in minutes.

Experiment 2.

Experiment 1. was repeated, but the sex ratio was reversed, i.e. each cage contained 40 males and 20 females at the outset. The times of onset of mating are given in Table 2, and the cumulative mating frequency curve plotted in Fig. 2. from the averages obtained.

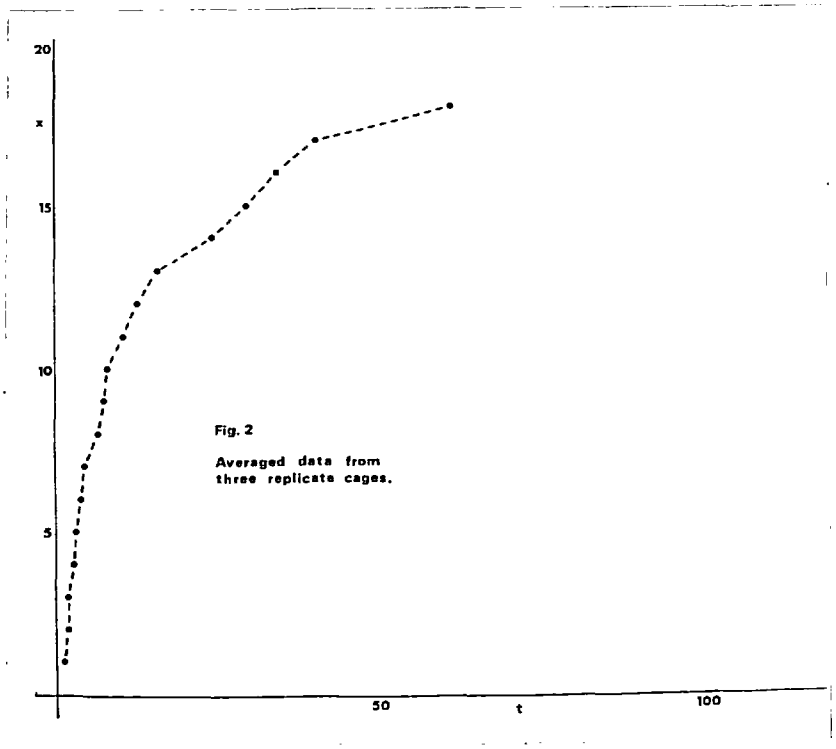


Fig. 2. Cumulative mating pairs (x) plotted against average time (t) to formation of respective pairs (Cages A, B and C, Table 2). t is in minutes.

Experiment 3.

Since the two previous experiments appeared to indicate that reversing the sex ratio in the cages made no appreciable difference to the mating rates, this was tested by running six cages together, three containing 40 males and 20 females each, and the other three 20 males and 40 females each. Cages of each kind were started in alternating sequence. The times of formation of successive pairs in each cage are shown in Table 3; average times of formation of successive pairs in the two series of 3 cages each were calculated. The two sets of averages were plotted as cumulative mating frequency curves, and are shown in Fig. 3.

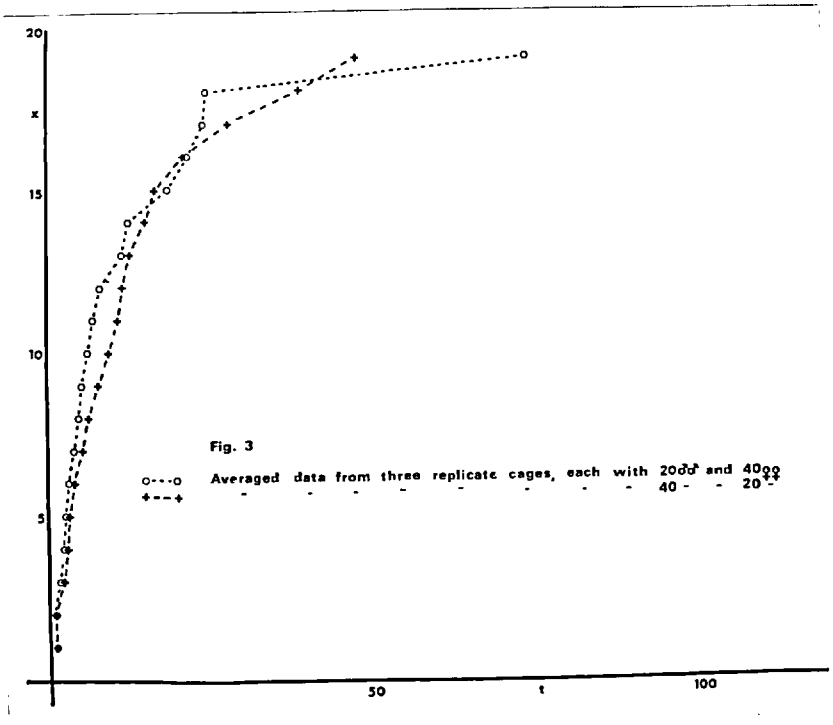


Fig. 3. Cumulative mating pairs (x) plotted against average time (t) to formation of respective pairs (Cages A, C and E; B, D and F, in Table 3). t is in minutes.

Experiment 4.

More mating cages were set up with an initial population of 40 males and 20 females. Results from these, and the results of Experiments 1, 2, 3 and 6 (total of 19 cages and 1140 flies) in which at least 18 out of a possible 20 pairs of flies per cage were observed to form, were pooled. Data from all cages in which this condition was met were used. Table 4 shows the results of tests not included under other experiments, and the average times to mating of successive pairs taking all 19 cages together. These averages are plotted as a cumulative mating frequency curve in Fig. 4.

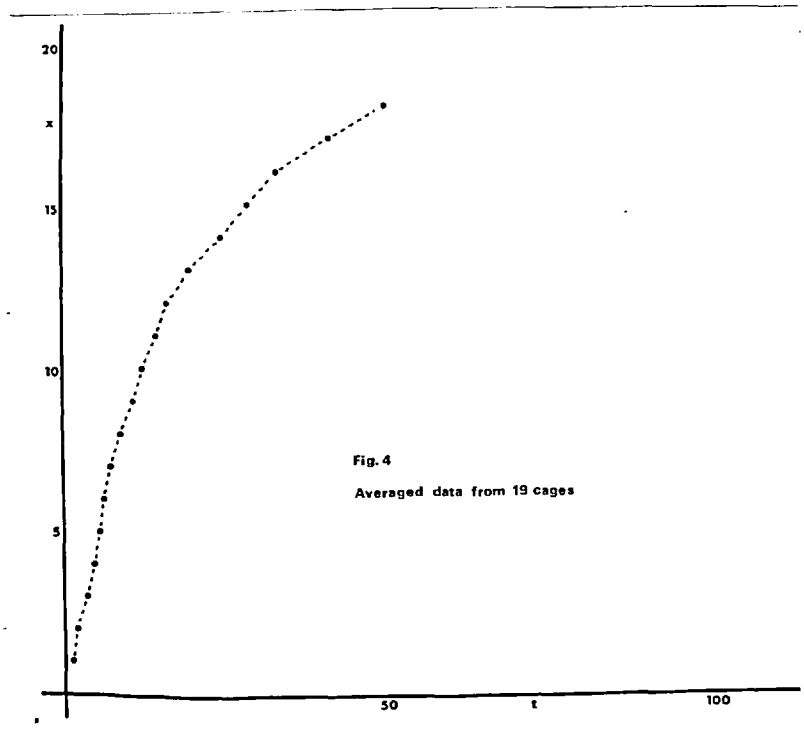


Fig. 4. Cumulative mating pairs (x) plotted against average time (t) to formation of respective pairs (19 cages listed in Table 4). t is in minutes.

Experiment 5.

Mating experiments were conducted using cages each having 30 males and 30 females at the start of the experiment. The timings of successive matings are shown in Table 5, together with the average of the results for the seven cages. These averages are plotted as a cumulative mating frequency curve in Fig. 5.

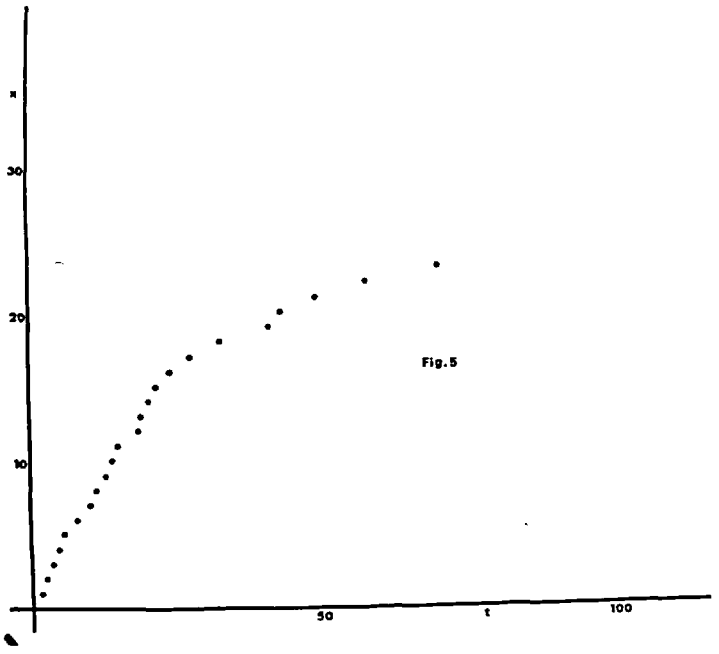


Fig. 5. Cumulative mating pairs (x) plotted against average time (t) to formation of respective pairs. (7 cages listed in Table 5). t is in minutes.

Experiment 6.

To test whether or not the remaining unmated flies of the majority sex left over from a 40:20 ratio mating experiment had different mating rate characteristics from the flies which actually mated, the following experiments were conducted.

(a) Two cages, each containing 40 males and 20 females were run as in previous experiments. The times of onset of mating of successive pairs were noted in the usual way. At the end of the observation period, any unmated females in a cage were removed, as were an equal number of unmated males, leaving a residue of 20 unmated males in each cage. On the next day, the two lots of residual males were combined in a single cage, and 20 virgin females was run at the same time. The three lots of 40 males used in this experiment were all of the same brood and had been randomly assigned on the first mating day, and the four lots of females had been treated likewise.

The results are shown in Table 6, and displayed in Fig. 6. The experiment was repeated, and the results given in Table 7, and Fig. 7.

(b) The same experiment was repeated, but the females formed the majority sex, so that each mating cage initially contained 40 females and 20 males.

The results are shown in Table 8, and Fig. 8.

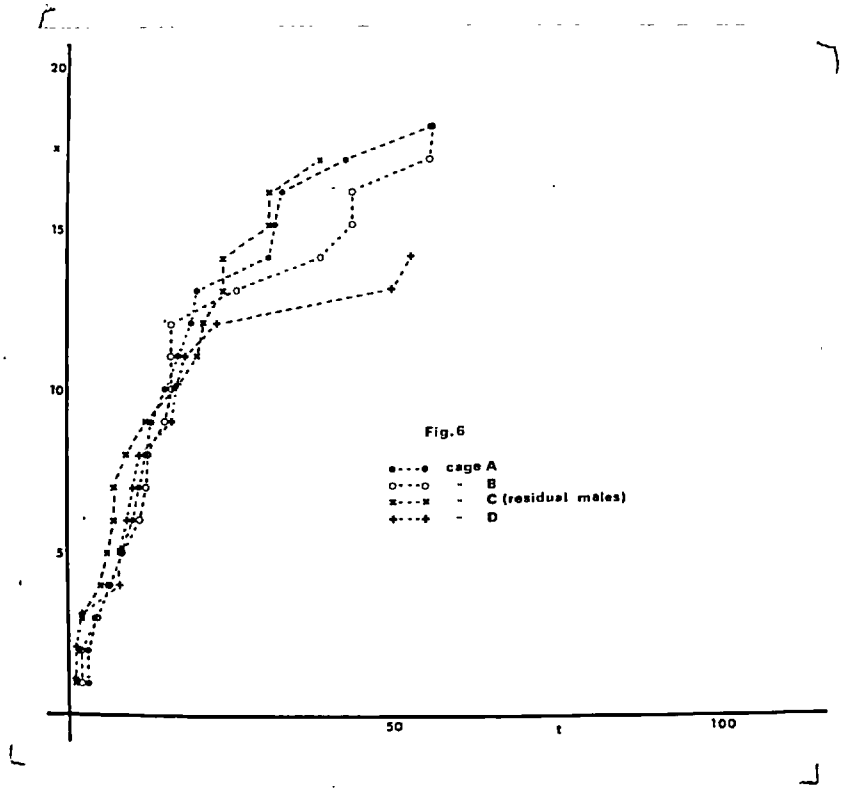


Fig. 6. Cumulative mating pairs (x) plotted against time (t) to formation of respective pairs. t is in minutes. (Table 6).

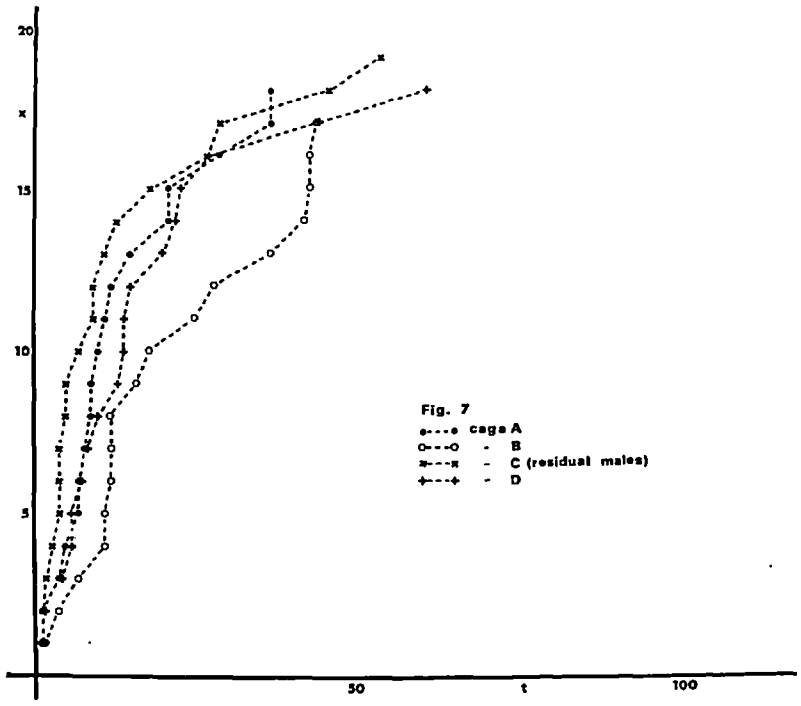


Fig. 7. Cumulative mating pairs (x) plotted against time (t) to formation of respective pairs. t is in minutes. (Table 7).

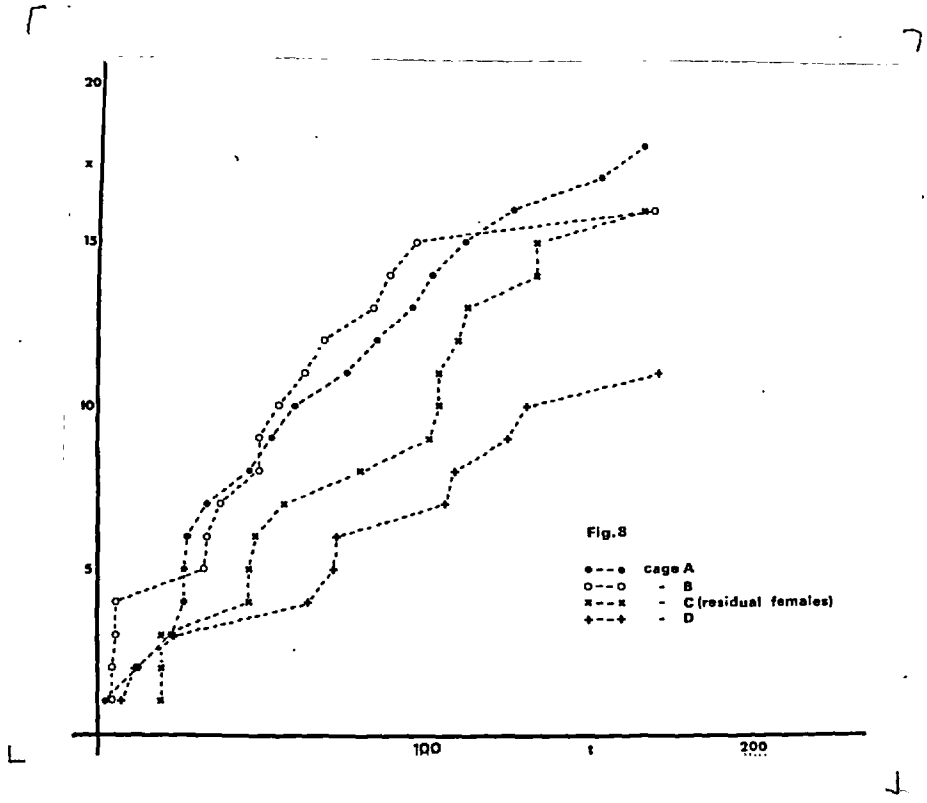


Fig. 8. Cumulative mating pairs (x) plotted against time (t) to formation of respective pairs, t is in minutes. (Table 8).

Experiment 7.

(a) To test if the residual majority sex failed to mate because of a tiring effect, from which they may have recovered by means of the day's interval between tests as conducted in Experiment 6, 80 virgin females were put with 20 virgin males for one hour, and the time of onset of successive matings noted. Mating pairs were removed; so were unmated males left after the end of the hour, and an equal number of females. Then 20 more virgin males were put into the cage (now containing 60 females) and matings timed and pairs removed as before, for one hour.

Results are shown in Table 9, and Fig. 9.

(b) The experiment was repeated, but with the sex ratio reversed, so that successive lots of 20 virgin females were put with 80 virgin males.

Results are shown in Table 10, and Fig. 10.

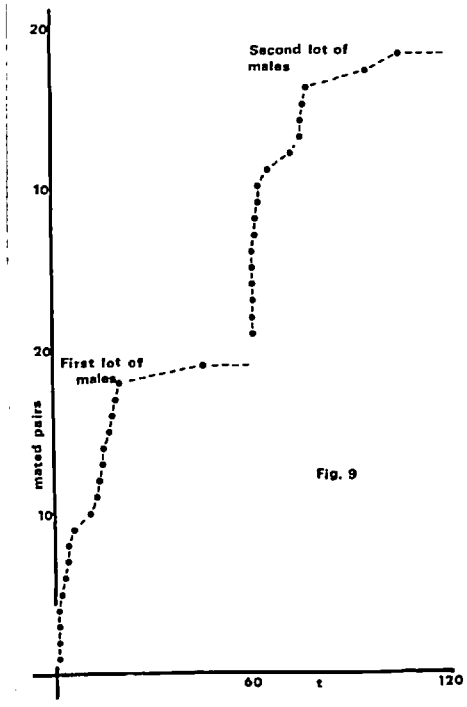


Fig. 9. Cumulative mating frequency curve obtained by placing two successive lots of 20 males with 80 virgin females. t is in minutes. (Table 9).

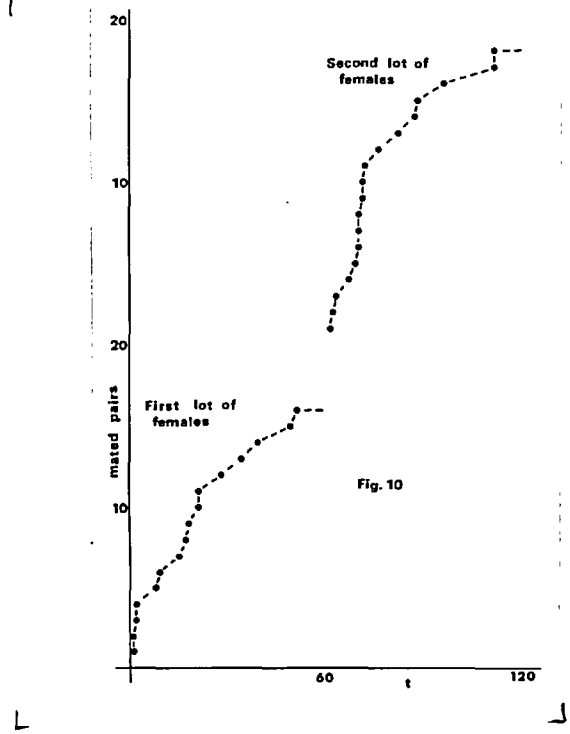


Fig. 10. Cumulative mating frequency curve obtained by placing two successive lots of 20 virgin females with 80 virgin males. t is in minutes. Table 10).

Transformation of the mating rate data

(a) The model

Since it is here conjectured (see discussion) that the characteristic decline in observed mating rate in a cage is determined mainly by the progressively decreasing density of candidate flies, an attempt is made here to set up a model of the mating cage situation.

Suppose that initially there are m males and f females in the mating cage, and that by time t, x mated pairs have formed and have been removed. At time t, there will be (m - x) males and (f - x) females left in the cage.

Since in this model only density effects are being considered, suppose that at time t all males have a uniform probability of mating, and likewise all females. The inate ability of a given fly to act as a partner in a mating pair is assumed to remain unchanged throughout the experiment, unless and until that fly mates, and is therefore removed.

If the mating rate (rate of formation of mating pairs) is proportional to the density of both the male flies and the female flies,

then $\frac{dx}{dt} = k(m - x)(f - x)$

Where k is a constant, to be known as the mating rate coefficient.

Whence $k = \frac{1}{t(m - f)} \ln \frac{f(m - x)}{m(f - x)}$

$= \frac{2.303}{t(m - f)} \log \frac{f(m - x)}{m(f - x)}$

$\therefore \quad t = \frac{2.303}{k(m - f)} \log \frac{f}{m} \frac{(m - x)}{(f - x)}$

$= \frac{2.303}{k(m - f)} \log \frac{f}{m} + \frac{2.303}{k(m - f)} \log \frac{(m - x)}{(f - x)}$

This has the form of an equation for a straight line, so that plotting t against $\log \frac{(m - x)}{(f - x)}$ should give a straight line, with $\frac{2.303}{k(m - f)}$ as gradient.

If at the outset the density of males is the same as that of the density of the females (as in Experiment 5), then m = f and the previous equations do not apply.

Instead, $\frac{dx}{dt} = k(m - x)(f - x)$

becomes $\frac{dx}{dt} = k(m - x)^2$

whence $k = \frac{1}{t} \frac{x}{m(m - x)}$

$= \frac{1}{t} \left(\frac{1}{m - x} - \frac{1}{m} \right)$

$\therefore \quad t = \frac{1}{k(m - x)} - \frac{1}{km}$

This is a linear equation, so that t plotted against $\frac{1}{(m - x)}$ should give a straight line, with slope $\frac{1}{k}$.

(b) Applying the transformation

Taking cages with a sex ratio of 40:20 or 20:40, the plot of log $\frac{40 - x}{20 - x}$ against t should give a straight line (approximately) if the assumptions behind the hypothesis are correct. The graph is shown in Fig. 11; a straight line has been drawn through the points by eye, and the t values on this line corresponding to integral values of x have been read off and replotted in Fig. 3 against x + 1 values. The reason for using x + 1 here is that each experimentally obtained t value in Fig. 3 relates to each successive mating, 1 to 18, whereas in Fig. 11 they relate to the conditions immediately before each successive mating. The fit of the model curve to the experimentally obtained one is evidently good, and a value for k can be calculated from the slope (Fig. 11, slope = 82.64, k =·00139).

Similarly, taking cages with an initial population of 30 males and 30 females, the plot of $\frac{1}{m - x}$ against t should give a straight line (approximately). The averaged data are plotted in Fig. 12, and a straight line fitted by eye. K has been calculated from the slope (Fig. 12, slope 751.9, k = .00133).

It should be noted that the original cage data are of a cumulative frequency distribution type. Therefore, even after pooling the time of onset of successive matings from several (19) cages, the points in Graph 11 are not independent of each other and so many of the usual line-fitting techniques are not immediately available. For instance, should random effects displace one point out of line, subsequent points would also fall off the line to a similar degree even if all the assumptions of the model were valid; thus discrepancies will tend to perpetuate themselves in points higher up the line. To avoid this difficulty, the experimentor may restate his expectations in a way that facilitates later analysis,

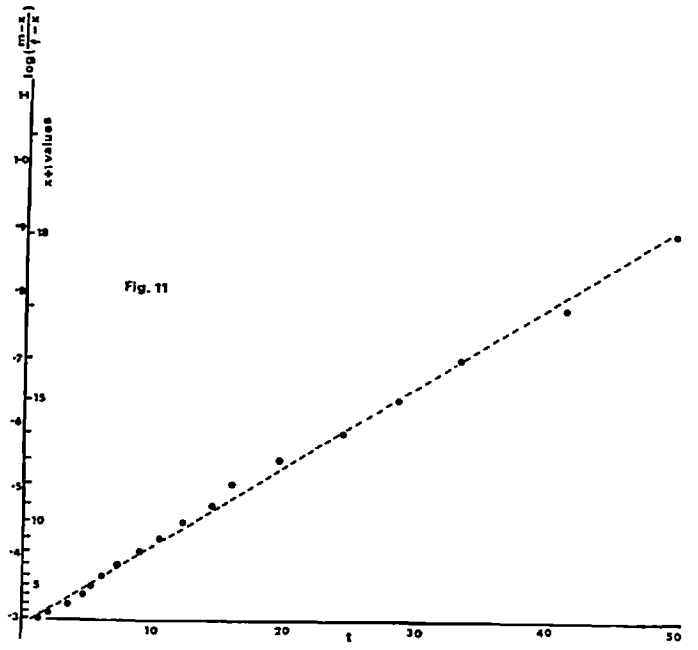


Fig. 11. $\log \frac{(m - x)}{f - x}$ values plotted against t. m is no. of virgin males at the beginning of the experiment f the no. of virgin females, x the number of pairs mated by the beginning of successive time intervals between matings. t is the time of onset of successive matings. The dashed line is filled by eye to the points. (t values from Table 4; $\log \frac{(m - x)}{f - x}$ values from Table 13).

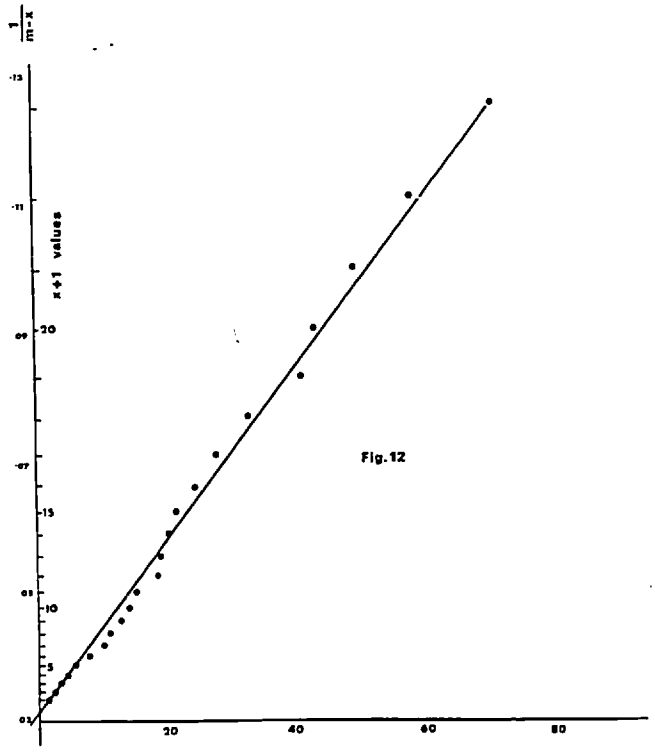


Fig. 12. $\frac{1}{m - x}$ values plotted against t. Symbols as for Fig. 11 (t values from Table 5).

namely, that the intervals between successive matings (t - intervals) should be proportional to the intervals on the log $\frac{(m - x)}{(f - x)}$ scale. This can be analysed by computing the regression of t-intervals on log $\frac{(m - x)}{(f - x)}$ intervals. This has been done and the steps in the computation are set out in Table 12. The F-test shows that a highly significant proportion of the variance of the data is accounted for by the regression line, and this success may be taken as evidence strongly in favour of the proposed model's validity. (See Fig. 13).

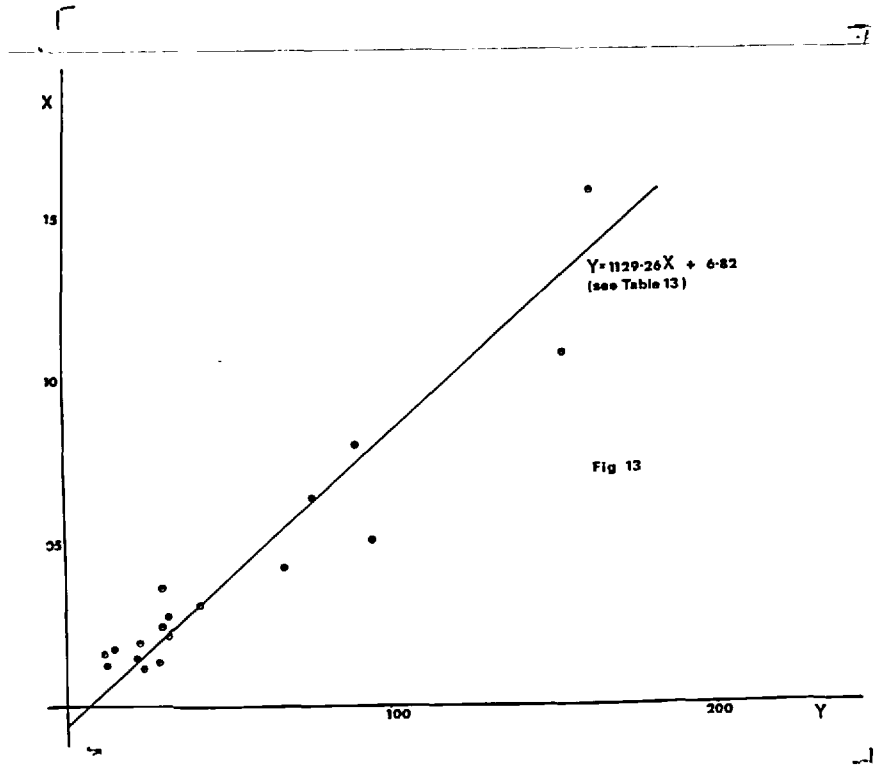


Fig. 13. Regression line of Y on X. X is successive intervals between log $\frac{(m - x)}{(f - x)}$ values. Y is successive intervals between the total values shown in Table 4. Values for X and Y are shown in Table 13.

Discussion

The experiments show that in a limited caged population there is in general a falling off of observed mating rate with time. While this effect may sometimes be less evident in certain individual cage results, when the data from numerous cages are pooled, as in Graph 4, the effect becomes clear.

Experiments 1 - 3 indicate that reversing the sex ratio makes little difference to the cumulative mating curve. This is understandable, since both males and females contribute equally to mating pairs. The different forms of overt sexual behaviour shown by the two sexes should not lead one to suppose that they necessarily make different contributions to the mating rate. In fact on this evidence they do not.

The observed fall off in mating rates with time could be due to:-

(i) An increase in the proportion of sexually less vigorous flies in the mating cage as mated pairs are tubed out.

(ii) A progressive but temporary exhaustion of sexual activity by the candidate flies ("discouragement" Smith, 1956).

(iii) A decline in density of candidate flies.

(iv) Combination of these factors.

There is no doubt that in some flies the mating rate is under genetical control. Manning (1961, 1963) was able to select Drosophila melanogaster for fast and slow mating rates, by taking from a mating population of 50 pairs the first 10 pairs to mate and the last 10 pairs, and using these to establish his fast and slow lines respectively, repeating this operation in successive generations. Kessler (1968, 1969) was also able to establish fast and slow mating lines in D. pseudoobscura. However, Kosef-Santibañez et al (1967) using D. pavani and the same

method of selection as Manning (1961) failed to get clear divergence in mating rates in their selected lines. All these workers found there was considerable day-to-day variation in observed mating rates, even in the selected lines. Kaul and Parsons (1965) by means of cross-breeding experiments with strains of D. pseudoobscura found evidence of genetical control of mating speed, and in general there is abundant evidence that there are heritable factors governing mating rates (Spiess and Langer, 1964; Spiess et al., 1966; Parsons and Kaul, 1966, in D. pseudoobscura; Prakash, 1968, in D. robusta; Spiess and Spiess, 1969, in D. persimilis).

Since Manning (1961) and Kessler (1969) was able to set up their fast and slow mating lines by selecting fast and slow mating members of a large population of flies, it might be thought that the tailing off in mating rate is simply an effect of genetic diversity in the unselected population. That this is not so even in Drosophila is shown by the shapes of the cumulative mating frequency curves of the selected lines, and of F, hybrids of these lines (Manning, 1961; Kessler, 1969, Fig. 3.), in which a tailing off also appears. Experiment 6. tested whether the flies left over unmated at the end of a mating experiment were markedly poorer in mating rate performance than control flies, and they were found not to be so. The general shape of the curves obtained on the first mating day in Experiment 6. was the same as that obtained on the second day. This is not to say that selection for fast and slow mating rates would be impossible in Lucilia sericata, but simply that in the inbred strain under examination, whatever mechanism causes the tailing-off of observed mating rate within mating cages, completely swamps the slight selection effect which may have been exerted in Experiment 6.

The tailing off is also unlikely to be an effect of various degrees of injury or other disqualifications for efficient mating caused by environmental conditions. In the present experiments care was taken to

eliminate visibly defective flies, and the flies underwent no anaesthesia; moreover in most mating experiments the great majority of the pairs which theoretically could form did so, so that commonly 90% or more of possible pairs formed within the observation period. Experiment 7. showed that there is no sign of sexual exhaustion in umated flies of the majority sex; mating begins again at a high rate as soon as fresh supplies of the minority sex are put into the cage.

Transformation of the data has been recognised as a problem in the genetical studies already cited. Kaul and Parsons (1965) found that "the frequency distribution of numbers mated plotted against mating speed shows extreme leptokurtosis, which is partly but not entirely removed by taking logs." Both the use of a cumulative percentage curve, and a probit transformation plotted against log. of time (Manning, 1961; Spiess and Langer, 1964; Kaul and Parsons, 1965) have been criticised as being unsatisfactory for statistical analysis (Spiess et al., 1966). Spiess et al (1966) found it useful "to weigh the number of matings per 5-minute intervals by the reciprocal of time x 100, plus a weight of 1 for those not mating after 30 minutes" to obtain an index of mating speed. This is a purely arbitrary procedure and is not based on any rational model.

With the more obvious alternatives eliminated, there can be little doubt that the main cause of the dropping off in observed mating rate is the progressive decrease in the density of candidate flies, as has been assumed in the model already presented. If this were the only cause, then the transformed data should fit a straight line when plotted as a graph. Agreement with this theory is good (Figs 11 and 12). It would seem then that the model is both realistic and provides a useful transformation of data. Values for k, which I have termed the mating rate coefficient, can be calculated from the transformed data. (See also Appendix 1.)

If the model is in good agreement with the experimental data then the assumptions behind it are at least supported. These are (a) that the density of females is exactly as important as the density of males, as regards effect on mating rates. This is indicated anyway by the similarity of results obtained on reversing the sex ratio. (b) that all the flies of any one sex in a mating cage have an equal ability to mate. If this were not so, the transformed data would not fall on a straight line when plotted.

The closest approach to the views presented here has been by Spiess and Spiess (1969), using Drosophila persimilis. They tested the effect that reversing the sex ratio had on mating rate, and concluded that "mating is not actually speeded up nor is total mating different for these two ratios". From their discussion it is clear that they are aware that the probability of mating must be increased with increased density, but analysis was taken little further. In particular, the compounded effect of male density and female density was not touched upon, and the mating index devised by Spiess et al. (1966) (see above) was still employed.

If it were true that all flies of any one sex in a mating cage have the same ability to mate, then chance determines which flies mate first, second and so on. Pushed to the limit, this argument would have it that selection for faster or slower mating rates would be impossible. This is known to be untrue for some Drosophila species, and probably would be untrue for Lucilia sericata, though no tests have been carried out. However, the effect this genetic diversity has in determining the shape of the cumulative mating frequency curve is presumably quite small, and it is concluded that chance is indeed the major determinant of the order in which flies mate under these conditions.

This method of analysis may prove to be of value in comparing the mating performance of different strains of flies, and perhaps of other insects, and may with development, assist those workers concerned with

sterile male release studies.

/In Appendix I is described a closely related, but more powerful, form of analysis, based on the same assumptions as made here./

S E C T I O N 2 a

THE ALIGNMENT OF PARTS DURING COPULATION AND THE FUNCTIONAL MORPHOLOGY OF THE PHALLOSOME, IN LUCILIA SERICATA MEIGEN (CALLIPHORIDAE).

The alignment of parts during copulation and the functional morphology of the phallosome, in Lucilia sericata Meigen (Calliphoridae)

Introduction

Structural details of genitalia in insects are often studied for taxonomic purposes, but the exact functions of the parts are much less frequently understood.

Illustrated accounts of the structure of male genitalia in Lucilia and related genera are given by Lowne (1870, 1890-95), Bruel (1897), Richards (1926), Seguy (1928), Aubertin (1933), Patton and Cushing (1934), Graham-Smith (1938), Zumpt and Heinz (1950), among others.

Lowne (1970) described the bursa copulatrix of the female Calliphora vomitoria (L.) as having two dorsally placed sacs (accessory copulatory sacs), which after mating became filled with "two masses of coagulated albumen, one of which occupied each (sac)", the secretion having been contributed by the male. Graham-Smith (1939) also noted that "pregnant" specimens of C. vivina (R-D). had opaque white granular material in the two sacs whereas virgins had not, and he presumed that the secretion was derived from the male accessory glands. He believed that during mating the paraphallic extremeties were inserted into the orifices of the accessory copulatory sacs "presumably to guide the secretion of the male accessory glands into the sacs." In his saline preparations of the male reproductive system, the sperm and the accessory material passed down the male tract at different times, so that there was no mixing of the two products. He supposed that since the two secretions were passed to different destinations in the female, the phallosome must take up two positions at different times during copulation. Donnelly (unpublished) using L. sericata, also found there was no mixing of the two male products, but showed that the sperm preceded the accessory secretion during copulation, contrary to Graham-Smith's belief. He saw that, as for

Calliphora, accessory secretion was placed in the paired accessory copulatory sacs in the female; he also noticed that two brownish marks develop there within 24 hours of mating, persisting indefinitely thereafter.

The following account describes how the male and female parts are aligned during copulation in L. sericata, and how the sperm and the male accessory secretion are conducted to their appropriate places in the female. Some of what follows is based on Pollock (1968, unpublished M.Sc. thesis), and a statement to this effect is made where appropriate.

Materials and Methods

Flies were cultured and mated as described in Section 1. Mating pairs were thrown into cardice/acetone mixture, in which they froze instantly without disengaging. Later they were removed and placed in Bouin's fluid for fixation and preservation. Dissections were carried out under water in a wax dish. Some specimens were held on a "Pelcool" freezing plate, by the coupled flies being painted with successive layers of 5% sucrose solution, and sectioned with a razor blade. Others were wax-embedded in a vacuum oven, microtome sectioned at 10μm and stained with haematoxylin. Specimens for stereoscan microscopy were prepared by affixing them to standard metal "stubs" with "Durofix", and coating them with metallic film under vacuum before examination by the Cambridge Stereoscan microscope (Imperial College, Metallurgy Department).

The alignment of parts during copulation

Fig 14 (modified from Pollock, 1968, unpublished M.Sc. thesis) shows the position taken up by the male genitalia in relation to the female sexual structures, during copulation. The terminal segments of the female are held in the copulatory pouch of the male. The phallosome lies along the female tract as far as the spermathecal duct opening. The anterior parameres lie alongside the phallosome within the vagina, with their apical hairs reaching to about the spiny ridges of the hypophallus (Fig. 15 and 16). It is not known if the anterior parameres have any further function beyond giving some support to the phallosome. The spine of the phallosome lies outside the vagina, abutting against the midline of the ovipositor, ventrally; the posterior parameres lie outside the ovipositor, laterally. Both sets of structures hold the terminal part of the ovipositor in position. The inferior and superior

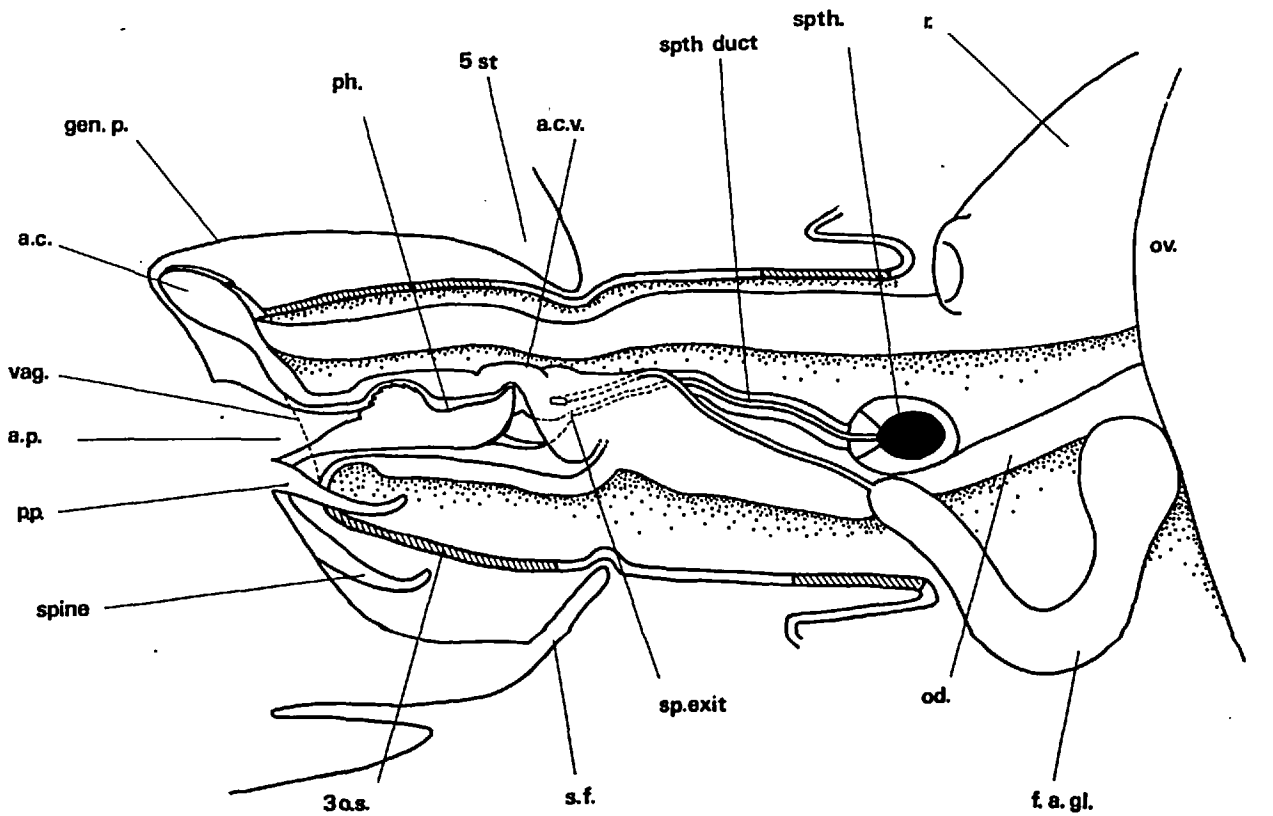


Fig. 14. Semi-diagrammatic view of parts interlocked during copulation in Lucilia sericata, as shown by the dissection of mated pairs. For abbreviations, see over.

(Modified from J. N. Pollock, unpublished M.Sc. thesis 1968)

Fig. 14. Abbreviations used:-

a. c.	aral cerci
a. c. v.	accessory copulatory vesicle or sac
a. p.	anterior paramere
f. a. gl.	female accessory gland
gen. p.	genital pouch of male
od.	common oviduct
ov.	ovary
ph.	phallosome
p. p.	posterior paramere
r.	rectum
s. f.	superior forceps
sp. exit	sperm exit on phallosome apex
spth.	spermatheca
spth. duct	spermathecal duct
vag.	vaginal opening
3 o. s.	sternum of 3rd ovipositor segment of female
5 st.	fifth sternum of male abdomen

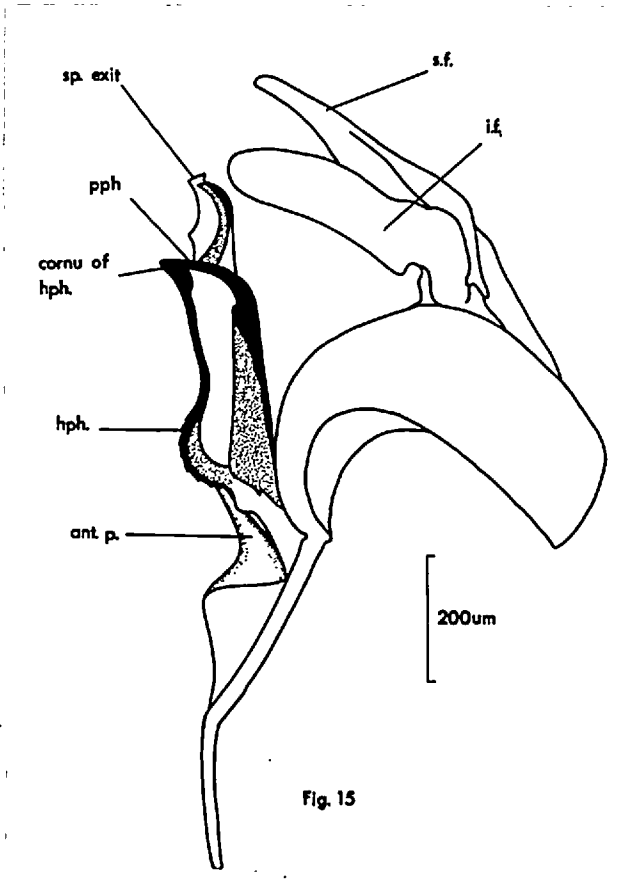


Fig. 15. Lateral view of male genitalia of Lucilia sericata, in copulatory position. ant. p., anterior parameres, hph., hypophallus; i. f. inferior forceps; pph, paraphallus; Other abbreviations as in Fig. 14.

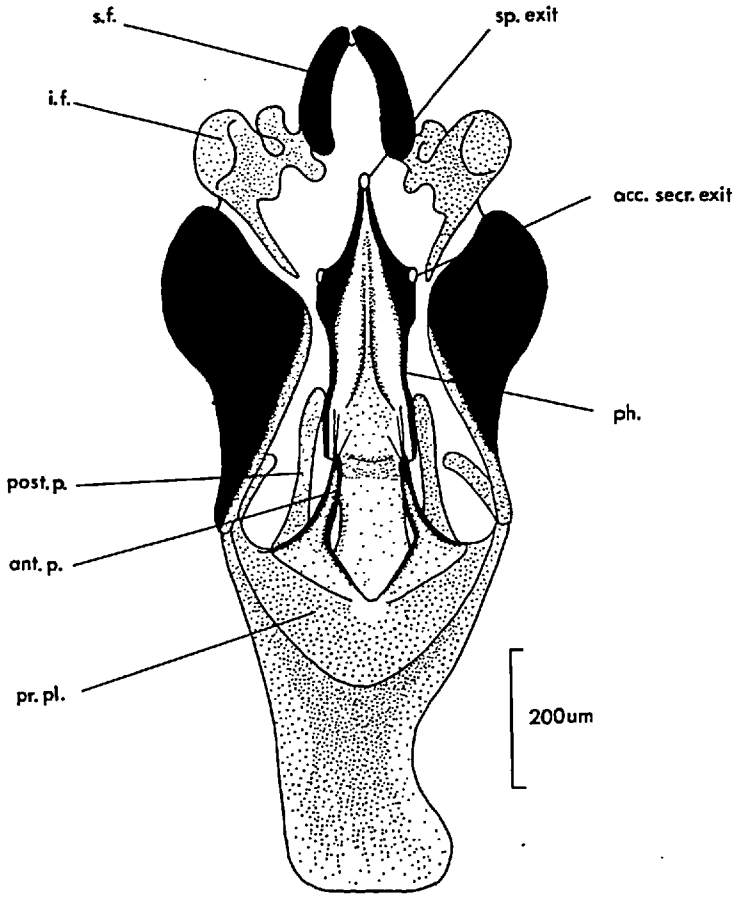


Fig. 16

Fig. 16. Ventral view of male genitalia of Lucilia sericata, in copulatory position. acc. secr. exit, accessory secretion exit; post p., posterior paramere; pr. pl., progenital plate. Other abbreviations as in previous two figures.

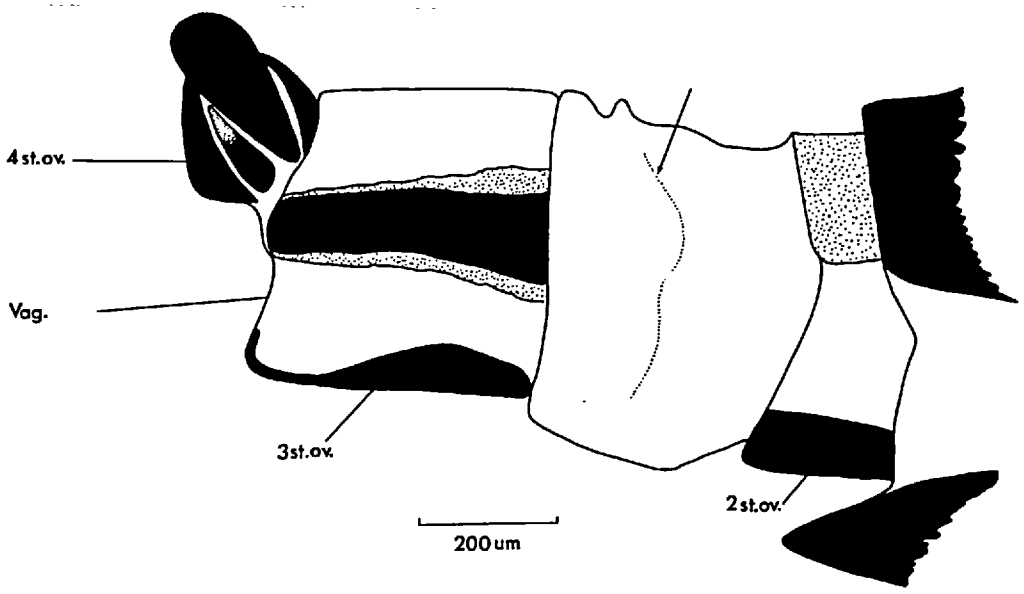


Fig. 17

Fig. 17. Lateral view of female Lucilia sericata ovipositor in copulatory position. The arrow indicated the constriction of the intersegmental membrane made by the 5th abdominal sternite and the superior forceps ofthe male. vag., opening of vagina; 2 st. ov., 3 st. ov., 4 st. ov., sternites of 2nd, 3rd and 4th ovipositor segments.

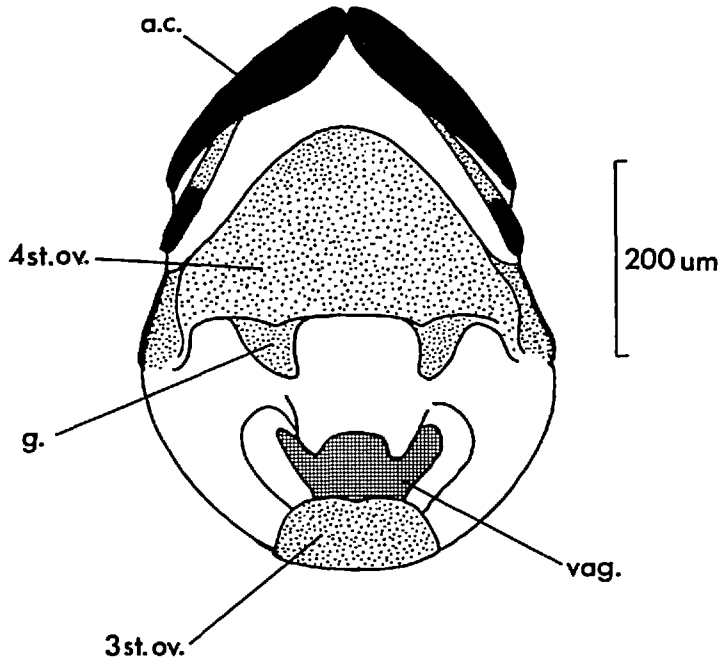


Fig.18

Fig. 18. Posterior view of female Lucilia sericata ovipositor, in copulatory position. Abbreviations as before; for g see text.

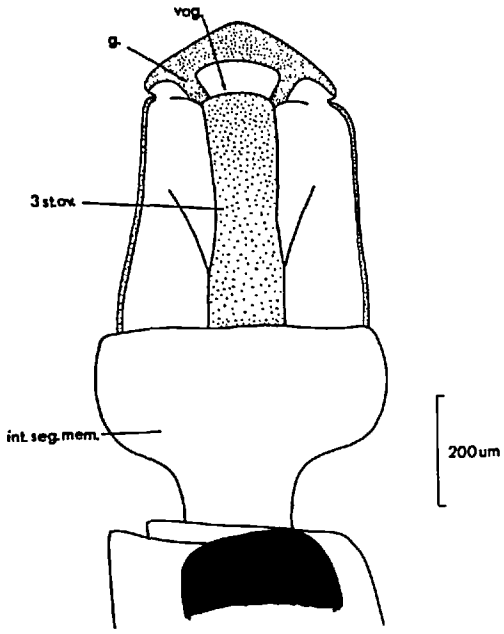


Fig. 19

Fig. 19. Ventral view of female Lucilia sericata ovipositor, in copulatory position. The constriction of the intersegmental membrane (int. seg. mem.) is clear.

forceps below, and 5th sternum above, hold the ovipositor in a firm pincer grip, acting on the membrane between the second and third ovipositor segments. The ovipositor becomes constricted in this region (Figs 17 and 19).

Coupled pairs frozen within two minutes of the onset of mating sometimes had the anterior parameres held at 90° or more to the phallosome, and not yet entering the vagina but pressing against two chitinous areas dorsal to the vagina (the areas marked g in Figs 18 and 19). Some of these specimens had the spine of the phallosome just within the vagina. This position of the parameres was never seen in the scores of dissected pairs frozen at later stages of mating, and can perhaps be regarded as a step in the adoption of the full copulatory posture, and the same may be true of the abnormal position of the spine.

Phallosome functional morphology

Longitudinal sections of pairs frozen and fixed in copula show that the gonopore of the male is applied to the common opening of the female spermathecal ducts (Fig 20). Sperm can therefore be passed directly from the male ejaculatory canal to the very short common duct. Males fixed and dissected during mating show sperm within the ejaculatory canal massed together and haphazardly arranged, not uniformly aligned as they would be if they were being transferred under the control of their movements. It is reasonable to suppose that sperm reach the male genopore by the work done by the ejaculatory pump, and the subsequent migration into the spermathecae is assisted by individual sperm movements.

It was argued (Pollock,1968, unpublished M.Sc. thesis) that the deposition of male accessory gland secretion at the appropriate point in the female could be achieved without any shift in position of the genitalia

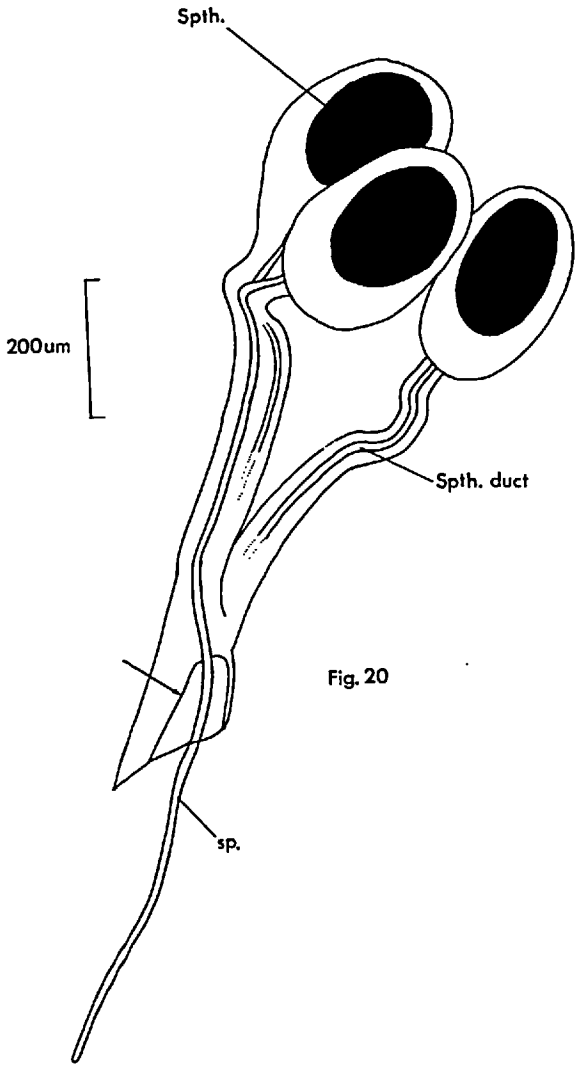


Fig. 20. Spermathecae dissected from a mating female Lucilia sericata. The arrow indicates the impression made by the phallosome, removed during dissection. The sperm rope (sp) from the ejaculatory duct of the male is intact and is continuous with the sperm in the spermathecal ducts.

from that shown in Fig. 14, if there were apertures at the cornua of the hypophallus distinct from the sperm exit, through which the secretion could pass. Accordingly, lateral openings and ducts were looked for and found to be present. Surprisingly, the ducts run from a point close to the sperm exit and open at a place more remote from it. Both the ducts and the lateral exits are visible by x 100 microscopical examination of water mounts: some pressure has to be applied to the coverslip on such a preparation, in order to bring the lateral openings into view. The ducts are somewhat less visible if the specimen is cleared; clearing also removes any granular accessory secretion from the lateral ducts. Some published figures show the ducts more or less distinctly, though their significance has not apparently been appreciated (e.g. Lowne, 1870; Bruel, 1897).

Recently mated males show obvious traces of the granular accessory secretion remaining within the lateral ducts (Fig. 21), whereas virgin males hardly ever show this (Pollock 1969; see also Section 3). When pressure is applied to the coverslip on a specimen with fully charged ducts, it usually happens that the secretion oozes from the lateral exits. Fig. 22 shows the phallosome of a virgin male, upper surface; the lateral exits are patent. In Fig. 23 the specimen is a phallosome dissected from a mated male fly, and male accessory secretion can be seen both at the lateral exits, and on the main shaft of the phallosome. There can be no doubt therefore that the secretion is able to pass out of these lateral openings to the female.

How the accessory secretion gets into the lateral ducts from the ejaculatory duct is less clear. Close to the origin of the lateral ducts, and just behind and below the gonopore there is a small "window" (Fig. 24) in the phallosome. It might be thought that the accessory secretion passes out of the sperm exit, entering the lateral ducts through the

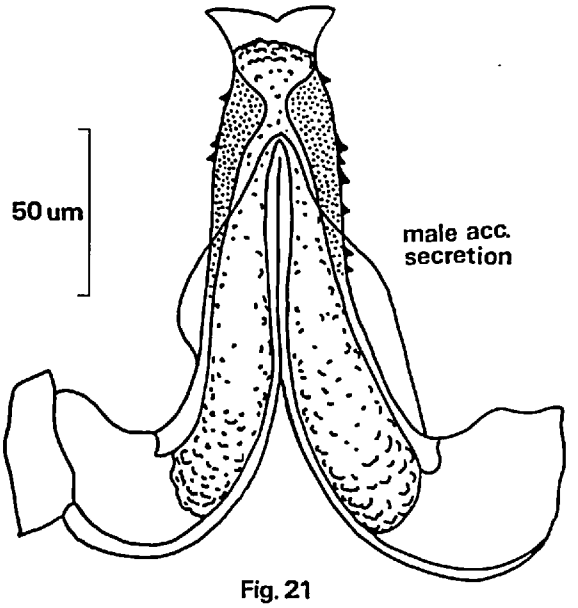


Fig. 21

Fig. 21. Terminal part of phallosome of a mated Lucilia sericata male, dorsal view, showing granular secretion within the lateral phallosome ducts. (From J. N. Pollock, unpublished M.Sc. thesis).

Fig. 22. Steroscan (x 754). Lucilia sericata phallosome dissected from a virgin male. The lateral phallosome duct apertuns are patent.

Fig. 23. Steroscan (x 580). Terminal view of Lucilia sericata phallosome from a mated male. The lateral phallosome duct exits are occluded with accessory secretion, some of which also lies on the outer surface of the phallosome.

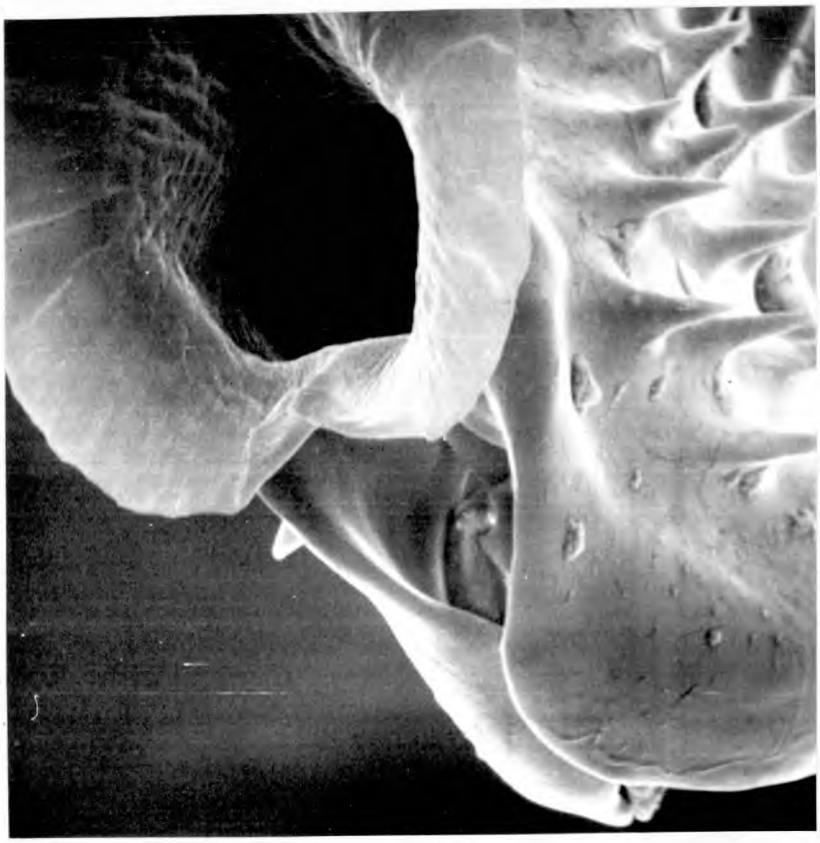


Fig. 24. Steroscan (x 1740). The subterminal "window" on the Lucilia sericata phallosome.

"window". The fact that in dissection of the male reproductive system in saline, accessory secretion appears to pass out of the terminal aperture, might seem to give support to this idea. However, this interpretation is thought to be incorrect, for the following reasons. (i) The terminal aperture is not found to be clogged up with accessory secretion after mating, in the manner of lateral duct openings. (ii) The "window" does not have accessory secretion adhering to it. (iii) The lower lip of the sperm exit is well developed and does not appear adapted to conducting any secretion to the "window". One can only suppose that the lateral ducts lead off from the ejaculatory duct immediately before the sperm exit is reached. Probably the structures to be seen through the "window" are cuticular linings to the lateral ducts, but this is not certain. The absence of any "leaking" of accessory material through the window (except under pressure) supports this view.

As Fig. 23 shows, the tip of the paraphallus is serrated. From its relation to the lateral exits it is clear that this tip lies exactly where it will be surrounded by male accessory secretion in the event of mating. Since it is in that position that the dark granules of Donnelly make their appearance about a day after mating (Donnelly, unpublished) it would appear very likely that the serrated paraphallic tips are responsible for the development of the granules. In a later section (Section 5) it will be shown that male accessory secretion persists in the female bursa at least a week after mating; an alternative hypothesis is that the paraphalli pierce the intima of the bursa, so allowing ingress of the accessory gland material into the haemocoel. By this argument, the brown granules are scar-tissue. This is supported by the observation that rather similar brown spots sometimes occur further down the female tract, at a position where the strong barbs of the hypophallus work against the female intima. Also small brown marks have been observed in the bursa of mated female

Eroischia brassicae (Muscidae), which seem to correspond to sharp prominences on the phallosome of the same species. Owing to the small size of the structures, it is not possible to remove a paraphallus tip from a male Lucilia without crushing the fly, so the obvious experiment of seeing whether such a fly produces only one dark granule in its mating partner cannot be performed. Donnelly (unpublished) found that females did not develop granules if their mating was interrupted before the transfer of male accessory secretion. This does not necessarily invalidate the scar tissue hypothesis, as it may be that only during the later stages of mating is the saw-edge of the paraphallus applied with any power.

Indirect support for the foregoing interpretation comes from the spring-like nature of the paraphalli. Graham-Smith (1939) noted the absence of muscles from the phallsome (of Calliphora), and presumed that "the parts of the phallosome never change their relative position". However, it is easy to imagine that if the circular muscles of the female bursa contract during copulation, the effect would be to press on the lower curved surface of the paraphalli and so force the tips of the paraphalli upwards guided by the cornua. The bursa wall would then almost certainly be abraded. The hypothesis that injection of male accessory secretion into the female by this means is responsible for the observed inhibition from remating by mated females is discussed later. Artificial injection of male accessory secretion into the haemocoel of virgin females did not completely eliminate the flies' receptivity to mating attempts by males (see Section 5) and there is no direct evidence that injection via the bursa occurs during mating.

Discussion

From the preceding description, it is clear that the phallosome is adapted to the transfer of two separate secretions, namely the fluid which contains sperm, and the male accessory secretion. Only the first of these passes through the terminal male aperture; there is no need to postulate the adoption of two different positions of the phallosome during copulation, as was suggested by Graham-Smith (1939). This appears to be the first report of apertures specialised for the egress of accessory secretion, on the phallosome of a dipteran, or indeed of any insect.

That lateral accessory ducts on the phallosome are permanently open along their length (though possibly not at their point of origin) and lead from the region of the sperm exit, suggests that they are modifications of the margins of an originally single male gonopore. The lower lip of the original gonopore may be imagined as developing paired bays specialised for the conduction of accessory secretion, the bays later becoming distinct apertures and relatively remote from the sperm exit. Later still, in some genera the accessory apertures migrated to the upper surface of the phallosome, a position they now occupy in Lucilia, Calliphora etc. The tips of the paraphalli simultaneously underwent the same migration, giving rise to the strongly curved shaft of the paraphallus in these genera. (See Section 2b).

Edwards (1929) drew attention to the existence of "closed" systems in the Diptera, a "closed" system being one in which the seminal ducts of the male are co-adapted to the spermathecal ducts of the female. The Lucilia system qualifies as a closed one, as the sperm are always within the ejaculatory duct of the male or the spermathecal ducts of the female, during transfer (Fig. 20). Obviously, a closed system of sperm transfer implies a separate phallosome exit for the male accessory

secretion if the two products are not to become mixed, and this is probably the main significance of the accessory ducts and apertures on the Lucilia phallosome. In open systems, as in Aedes (Spielman, 1964), Drosophila (Nonidez, 1920), and Glossina (Pollock, 1970), the sperm and the accessory secretions come into mutual contact within the bursa (uterus), though in all these cases mentioned subsequent sperm migration leads to a separation of the male products. (See also Section 9).

Summary

The alignment of male and female parts during copulation in Lucilia sericata is described. The anterior parameres, as well as the phallosome, enter the female tract. Sperm is passed directly from the terminal phallosome aperture into the very short common spermathecal duct. Male accessory secretion, also passed over during copulation, does not come out of the same aperture, but through paired accessory ducts and apertures on the lateral parts of the phallosome, and is thereby placed in its proper position in the female tract. Only a single position of the phallosome during copulation need therefore be postulated for the transfer of both sperm and male accessory secretion. The lateral accessory apertures are presumed to have been derived from the margins of an originally single male gonopore, It is possible that the serrated paraphalli abrade or pierce the female intima at later stages of copulation, with consequent injection of male accessory secretion, but direct evidence is lacking.

S E C T I O N 2 b

LATERAL PHALLOSOME DUCTS IN SOME CALLIPHORINAE, OTHER THAN *LUCILIA SERICATA*

Lateral phallosome ducts in some Calliphorinae, other than Lucilia sericata.

Introduction

The discovery of lateral phallosome ducts in Lucilia sericata makes it desirable to examine the structure of the phallosome in other calliphorine flies, to see how widespread is the occurrence of the ducts, and hence find out if in principle the same method of assessing the mated status of the male could be used for these flies as is possible for L. sericata (see Section 3). The phallosome will be described and figured in various Lucilia species, and in other genera of the Calliphorinae. Failure to observe lateral ducts in some species is also recorded.

Method and Materials

To obtain native blowflies, minced meat was left for some hours in the open during the summer months, and adults bred from the eggs that were laid in the meat. Lucilia richardsi Collin was captured by net in the field. Exotic species were obtained as dead specimens from workers in various parts of the world: Lucilia cuprina (Wied.) (Cooper Technical Bureau, Berkampsted, Herts), Chrysomyia megacephala (Fabr.) (University of Hawaii, Honolulu), Cochlioyia hominivorax (Coq.) and C. macellaria (United States Department of Agriculture, Mission, Texas), Calliphora stygia (Fabr.) and C. augur (Fabr.) (Melbourne, Victoria, Australia). Dissections were made as described for Section 3 but phallosomes were drawn in lateral view, and with the spine still attached.

Results

Since the general appearance of the phallosome in various Lucilia species and related genera has been illustrated by Richards (1926), Aubertin (1933), Seguy (1928) and others, it is only necessary here to show the position of the lateral ducts, the paraphalli, the sperm exit and the ejaculatory duct (where visible) without paying great attention to details such as the number and position of spiny prominences on the phallosome. In all cases illustrated, the tip of the paraphallus coincides with the opening of the lateral penis ducts (Fig. 25).

Lucilia. Lucilia cuprina is generally agreed to be closely related to L. sericata (Hepburn, 1943; Waterhouse and Paramonov, 1950); the lateral ducts are easily seen, though they are slightly less transparent than those of L. sericata. The lateral ducts of L. richardsi are not very easily visible, but the lower margin can be seen clearly. In both L. caesar (L.) and L. illustris (Mg.) the phallosome is slender; the opening of the lateral duct is well away from the sperm exit and is not set on such a marked prominence as in L. sericata and L. cuprina. Due to sclerotisation, only the middle parts of the lateral ducts of L. caesar and L. illustris are clearly visible, but the exits can be seen in some specimens. L. ampullacea Villeneuve has a heavily sclerotised phallosome, obscuring all but part of the lower margin of the lateral ducts.

Calliphora. The phallosome has the same general shape as that in Lucilia, and in Calliphora vicina Robineau-Desvoidy (= C. erythrocephala (Mg.)) and C. vomitoria (L.) the lateral ducts are even more clearly visible than in L. sericata. As previously mentioned, they have been figured by Lowne (1870, 1890-95) and Bruel (1897), but not identified. C. stygia has also been found to have lateral ducts; those of C. auger are not well defined, and fresh material would be required to verify

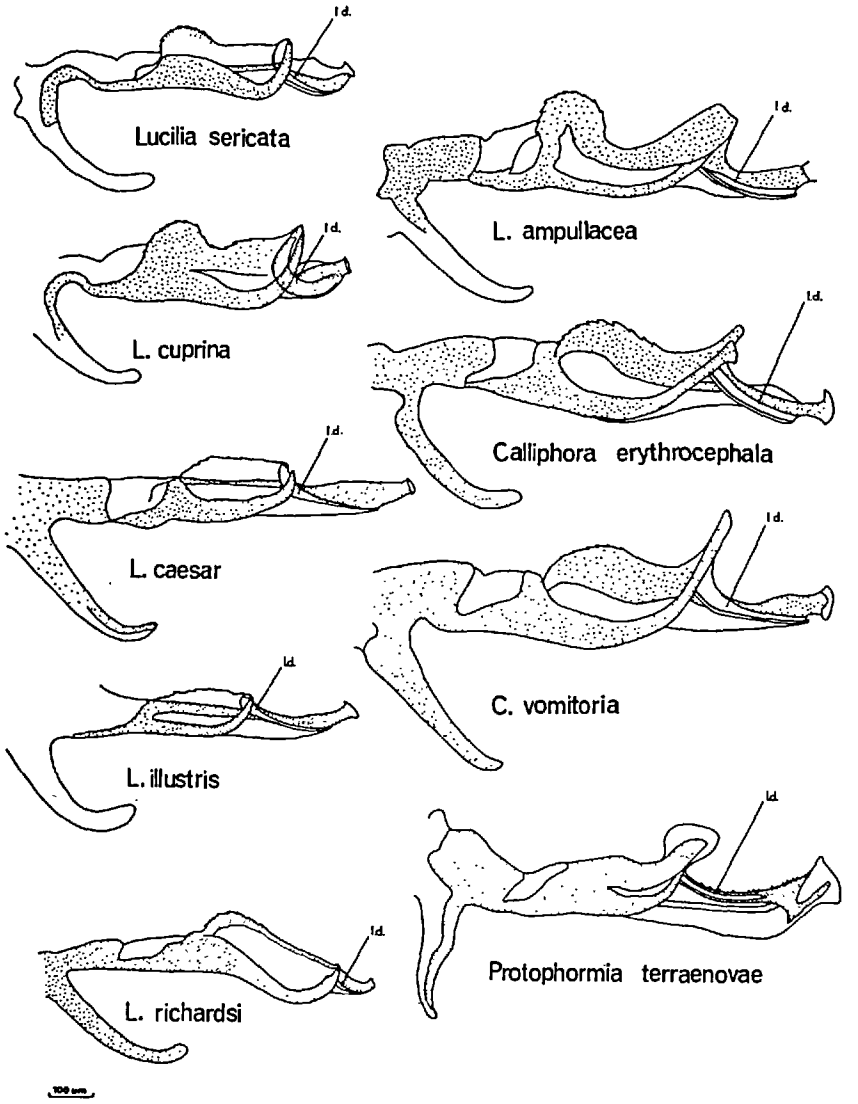


Fig. 25

Fig. 25. Lateral view of the phallosome in 9 species ofCalliphorinae, to show the lateral phallosome ducts.

their presence.

Protophormia. The phallosome of P. terraenovae (Robineau-Desvoidy) is dark and heavily sclerotised, making examination for lateral phallosome ducts difficult. However, by dissection and by using reflected light, the ducts can be seen. Specimens have also been observed having granular secretion within the duct.

Chrysomyia. The phallosome of C. megacephala has been examined, but without fresh material it is impossible to be sure that the lateral ducts are present.

Cochliomyia. Though lateral ducts could not be identified in either C. hominivorax or C. macellaria, probably because of sclerotisation, the phallosome is of interest as the paraphalli are but gently curved, so that the tips lie below the ejaculatory canal and sperm exit, not above them as in the other genera examined. Probably Lucilia spp. evolved via such a stage, since the lateral ducts in those species have their origin below the sperm exit, but deliver their charge by apertures on the upper surface of the phallosome (See Section 2a, Discussion).

Discussion

Lateral phallosome ducts have now been seen in six Lucilia species (including L. sericata). Since the phallosome has the same general form throughout the genus (Aubertin 1933), it is reasonable to suppose that the ducts are universal in the genus.

More species and genera need to be examined, particularly in the fresh state and with the aid of the steroscan microscope, to explore fully the extent to which lateral phallosome ducts occur in the Calliphorinae. Nevertheless, such ducts have now been identified in ten species and three genera. It is too early to state that lateral phallosome ducts are universal in the subfamily, though this is quite possible. The design of the phallosome in those species where the lateral ducts have been seen is sufficiently uniform for one to conclude that the ducts in these species are homologous structures (Fig. 25).

In Calliphora the ducts are even clearer (in three species) than in Lucilia sericata, and it is likely therefore that the mated status test for males (Pollock 1969) would be applicable to these blowflies.

Summary

Lateral phallosome ducts have been observed in several Calliphorine genera, and it is concluded that they are of widespread occurrence throughout the subfamily.

S E C T I O N 3.

TEST FOR THE MATED STATUS OF MALE *LUCILIA SERICATA*

Test for the mated status of male Lucilia sericata

Introduction

The precise cause or causes of failure of attempts to use sterile males for the control of insect pests merit particular study. In the case of the Lucilia sericata project on Holy Island, Macleod and Donnelly (1961) could draw only the most general conclusions that either their released males were not fully sterile, or these males were not equal to native males in competition for native females.

In the latter case, a means of detecting whether a given male has mated or not would provide useful information. Marked sterile and normal males could be released into the field, and on recapture their mated status assessed. Competiveness of these males under field conditions could thereby be measured.

The observation recorded in Section 2, that granular male accessory secretion was visible in the lateral phallosome ducts after mating (Fig 21), appeared to provide a test for the mated status of male Lucilia sericata, and this possibility was investigated further (Pollock, 1969).

Materials and Methods

Stocks of Lucilia sericata were reared as already described. Mating cages were set up, and mating pairs set aside as they formed; they were allowed to terminate copulation naturally. The males were then stored in cages with water and granulated sugar, but given no further access to females. Control virgin males of the same batch were reared in just the same way, but were not given an opportunity to mate unless otherwise stated (see the first experiment). Both virgin and mated males were then dissected to remove the phallosome. For this, flies were caught individually in tubes and the head and thorax crushed with the fingers. The phallosome was dissected out, and the basal part bearing the spine removed from the distal section bearing the lateral penis ducts. This was done to make inspection of both lateral phallosome ducts easier, as otherwise the spine twists the phallosome sideways on the microscope slide. Water mounts were used, and microscopical examination was done at x 100.

In the first experiment, 16/17 day old virgin males were used, and 8/9 day old virgin females.

Three cages A, B and C were set up, each with 20 virgin males at the outset. Cage A contained no females; cage B contained 20 females mated the previous day; cage C contained 20 virgin females. The three cages were placed side by side on the bench under the usual lighting conditions and the flies of Cages B and C observed for one hour for mating. No mating occurred in Cage B. Ten pairs formed in Cage C, and were tubed out as they did so. The ten males left unmated after one hour in Cage C were tubed out individually, to prevent subsequent mating. The resulting four categories of males (a-d, see below) were immediately dissected and examined in the way previously described:-

(a) 10 virgin males left over from mating Cage C.

(b) 10 mated males from Cage C.

(c) 20 virgin males kept as controls (Cage A males)

(d) 20 virgin males from Cage B.

The results are shown in Table 14.

In the second experiment, virgin and mated males were dissected on the following days, taking the day of mating as Day 0:-

(a) 0-1 (b) 7-8 (c) 14 or more.

At each of these time intervals 100 mated males and 100 virgin males were dissected, using a total of 600 males. The results are shown in Table 15a. This is a compilation of results from several batches of various ages but in all cases the virgin flies were of the same batch and age as the corresponding mated males.

In the third experiment, similar time intervals were allowed to elapse between mating and examination, but in this case the flies came from the same batch and were all mated on the same day. This was done in case the result of the second experiment was a sampling artifact, as various batches of flies had been used in that experiment. Flies were 5 days old at mating in the third experiment.

Over 75 mated pairs were obtained within one hour by putting together about 100 virgin males and 100 virgin females in one cage. The pairs were tubed out as they formed and after each pair had disengaged the male was retained as before. The mated males were randomly assigned to three approximately equal lots, and these placed into three cages. At the same time over 75 virgin males of the same batch of flies were also divided into three lots and put into another three cages. Twenty five flies each of the mated males and of the virgin males were examined on days 0, 7 and 14 (day 0 being the day of mating). Results are shown in Table 15b.

TABLE 14. Effect of mating, and association with females without mating, on the presence in the male of granular secretion in the lateral penis ducts.

Category of male:	Secretion present	Secretion absent
(a) 10 virgin males, allowed to associate with females, but which did not mate	0	10
(b) 10 mated males	10	0
(c) 20 virgin males, no contact allowed with females	1	19
(d) 20 virgin males, allowed to associate with mated females (no mating occurred)	0	20

TABLE 15a. Presence (positive) or absence (negative) of granular secretion in the lateral penis ducts of virgin and mated male flies of the same age, dissected at three different time intervals after mating.

	Mated Males		Virgin Males		Total Mated/ Virgin prs
Days after Mating	Positive	Negative	Positive	Negative	Dissected
0,1	96	4	1	99	100
7,8	93	7	3	97	100
14 or more	84	16	6	94	100
Totals	273	27	10	290	300

TABLE 15b. Presence (positive) or absence (negative) of male accessory secretion in the lateral phallosome ducts of mated and virgin male Lucilia sericata, at different intervals after mating, using a single batch of 150 male flies.

	Mated Males		Virgin Males	
Interval in days	Positive	Negative	Positive	Negative
0	25	0	0	25
7	24	1	0	25
14	19	6	1	24

Results

The results of the first experiment (Table 14) showed clearly that mating itself is necessary to cause granular secretions to appear in the lateral penis ducts (except in a single virgin male). Mere association with virgin females (Cage C) or mated females (Cage B) is ruled out as a possible cause. The experiment also shows that under the conditions used mated females did not remate.

Table 15a appears to show that the presence or absence of granular secretion in the penis ducts as an indicator of the mated status of male flies becomes less reliable with longer lapses of time after mating. It was observed that the phallosomes of older males are somewhat darker and the granular secretion rather less distinct than in younger males, and this probably accounts for the decline in accuracy of the test. These changes with age were not quantified, however. The possibility that the decline was due to a sampling artefact was investigated in the third experiment by using a single batch of flies giving data covering three time intervals (0, 7 and 14 days) between mating and examination.

The accuracy of the test up to a week after mating is confirmed by the results shown in Table 15b; a decline in accuracy for longer time in intervals was also indicated.

The following remarks therefore apply to flies given up to 8 days' interval between mating and dissection. Pooling the data from Tables 15a and 15b, it appears that 1-2% of virgin males may show granulations in the lateral phallosome ducts. Presumably these cases arise from the tendency of males confined to all male cages to form homosexual pairings; under field conditions they might be less likely to occur. On the other hand there is no obvious reason why the internal features of heterosexual mating in Lucilia should differ according to whether the act takes place

in the laboratory or the field, and it will be assumed that the results of laboratory tests already given can be applied to samples taken from the field. The pooled data show only about 5% of mated males will fail to show granular lateral ducts. Marked flies could be released and recaptured after a week and examined for granular secretion. By analysis of the recaptured sample an estimate of the ratio of mated to unmated male flies could be made. For example, a ratio of 10 positive:3.5 negative by the test corresponds to a 10 mated:3 unmated male population, and other correspondences can be calculated from the data.

Discussion

Muirhead-Thomson (1968) has drawn attention to the need for more information concerning the ecology and physiology of male insects, especially in view of the possibility of using sterile males for the control of certain pests.

The technique used in the present work of dissecting the male in order to assess its mated status in the first such test to be devised for any insect of medical/veterinary importance. It can be described as a self-marking technique, as the indicator appears automatically as a result of mating without any previous preparation of the flies by the experimenter. However, for applications in the field (not attempted in this work) one may suppose that flies would be marked before release, so that they could be identified on recapture and distinguished for native (i.e. not laboratory-reared) flies. Appropriately marked sterile and fertile males could be released into the field and on recapture their mating successes scored and hence their mating competitiveness assessed. Such information would in itself be of great value. If, in addition, a reliable technique for assessing the age of male blowflies could be devised, then the two techniques used together on native field-caught males would give unique insight into the reproductive life of these insects.

Efficient means of capture of male blowflies is probably the greatest practical requirement to be met before the mated status technique could be adopted for field studies on this mobile, low density pest.

S E C T I O N 4.

TESTS ON TEPA-TREATED MALE LUCILIA SERICATA

Tests on tepa-treated male Lucilia sericata

Introduction

Experience has already been gained in conducting mating experiments using Lucilia sericata, so that normally the majority of pairs which can possibly form do so within a conveniently short observation period. Also, using normal fertile flies, a test by which the mated status of males may be determined by dissection has been established.

Since these techniques were evolved largely in response to needs arising from the Holy Island sterile male release trial (Macleod and Donnelly, 1961), they were here applied to sterilised males. Other related tests were also carried out and reported here.

Materials and Methods

Tepa (tris (1-aziridinyl) phosphine oxide) was injected into male flies as aqueous solution, using a foot operated Burkard micro-applicator. Flies were lightly anaesthetised with CO_2 and held gently in the gloved hand for injection. A small number of flies that were crippled by this treatment (e.g. by the loss of a leg) were destroyed.

Experiment 1.

A pilot experiment was conducted to assess the percent sterility resulting from injection of varying doses of tepa.

Flies were tubed and sexed within 24 hours of emergence (i.e. on day 1). On day 2 they were given fresh liver. On day 4 the remaining liver was removed. The male flies were put randomly into groups of 25; they were injected with 0.3 µl distilled water or tepa solution at various doses (0.135 µg, 0.27 µg, 0.54 µg, 1.08 µg tepa/0.3µl) as appropriate, and stored by groups in separate cages at 27°C. Females were fed on liver on the same days, but were not injected. Flies were mated on day 6. For this, females were randomised and put into small plastic containers, thirty per container, no anaesthetic being used for this operation. Containers were randomly assigned to the cages in which treated males were kept. The number of males per cage was standardised to 20 by removing corpses and by catching any live males remaining in excess of 20.

Females were then released into the male cages and the time of formation of successive pairs of mating flies taken to the nearest minute. These experiments were conducted at 27°C, and all cages were observed for at least one hour. Mating pairs were tubed out, and the females stored at 27°C with access to sugar and water.

On day 10 fresh liver was offered to the females for oviposition, and after 4-5 hours the resulting eggs were removed (by forceps) and soaked in 1% sodium hydroxide solution for 10 minutes to separate individual eggs from the masses. The separated eggs were pipetted out into water for rinsing, and pipetted once more on to damp black filter paper, labelled according to the male parentage of the eggs. The filter papers were stored overnight in 100% humidity at 27°C. Hatch

was assessed on day 11. Another cycle of eggs was taken on day 13, and assessed for hatch on day 14.

Results

Results for experiment 1 are shown in Table 16.

Table 16.

Cage	Dosage/fly	% Sterility at 1st oviposition		% Sterility at 2nd oviposition		N	No. of pairs mating by 60 minutes
		Actual	Corrected	Actual	Corrected		
1.	Water	24.13	-	24.73	-	15	11
2.	0.135 µg tepa	23.93	0	27.45	3.6	14	8
3.	0.27 µg tepa	32.74	11.3	32.75	10.7	12	11
4.	0.54 µg tepa	80.40	74.2	71.11	61.6	15	15
5.	1.08 µg tepa	86.42	82.1	87.98	84.0	15	15

Table 16. Sterility and mating performance of lots of 20 male flies treated by injection of the stated doses, and put with 30 virgin females. N is the number of mated females obtained in each mating cage, from which the eggs were obtained for sterility assessment.

Experiment 2.

The number of mated females obtained per dosage was rather low in Experiment 1 and sterility of controls was rather high. In Experiment 2 the same procedure was followed as in Experiment 1, but all cages were run for at least 160 minutes. In addition, males were examined on day 7 (the day after mating) for accessory secretion adhering to the lateral penis ducts. Sterility results and the number of pairs mating by the end of 160 minutes are shown in Table 17. Times of formation of successive pairs of flies in the five cages are shown in Table 18. Results from the examination of males for the mated status test are shown in Table 19. Figure 26 displays the probit sterility/log dose relationship for the flies of the 5 cages. Figure 27 displays the data of Table 18 in graphical form.

Table 17.

Cage	Dosage/fly	% Sterility at 1st oviposition		% Sterility at 2nd oviposition		N	No. of pairs mating by 160 minutes
		Actual	Corrected	Actual	Corrected		
1.	Water only	8.1	-	2.5	-	19	19
2.	0.135 µg tepa	26.3	19.8	15.7	13.5	17	17
3.	0.27 µg tepa	40.1	34.8	31.6	29.8	19	18
4.	0.54 µg tepa	55.1	51.1	52.2	51.0	16	16
5.	1.08 µg tepa	71.2	68.7	59.3	58.6	20	18

Table 17. Sterility and mating performance of lots of 20 male flies treated by injection of the stated doses, and put with 30 virgin females. N is the number of mated females obtained in each mating cage, from which the eggs were obtained for sterility assessment.

Table 18. Times to the formation of successive mating pairs in the five cages of Experiment 2. The bar represents the 160 minute mark.

Cage	1	2	3	4	5
Pair	Watering				
1	5	19	2	2	1
2	9	21	6	2	1
3	9	24	8	6	10
4	23	25	9	6	12
5	25	32	10	8	13
6	27	41	20	9	15
7	28	54	24	23	22
8	33	54	38	25	32
9	36	75	41	26	40
10	36	76	47	28	41
11	37	82	51	30	60
12	40	89	53	32	122
13	52	91	55	46	123
14	53	95	92	56	142
15	56	109	95	64	143
16	64	112	114	97	150
17	64	149	138	-	152
18	76	-	153	-	159
19	89	-	229	-	180
20	-	-	-	-	230

Table 19. Results of examination of males on day 7 for accessory secretion adhering to the lateral penis ducts.

Cage	Positive (Secretion visible)	Negative (No secretion visible)	Total
1	18	1	19
2	17	0	17
3	18	1	19
4	16	0	16
5	20	0	20
Uninjected virgin males	0	9	9

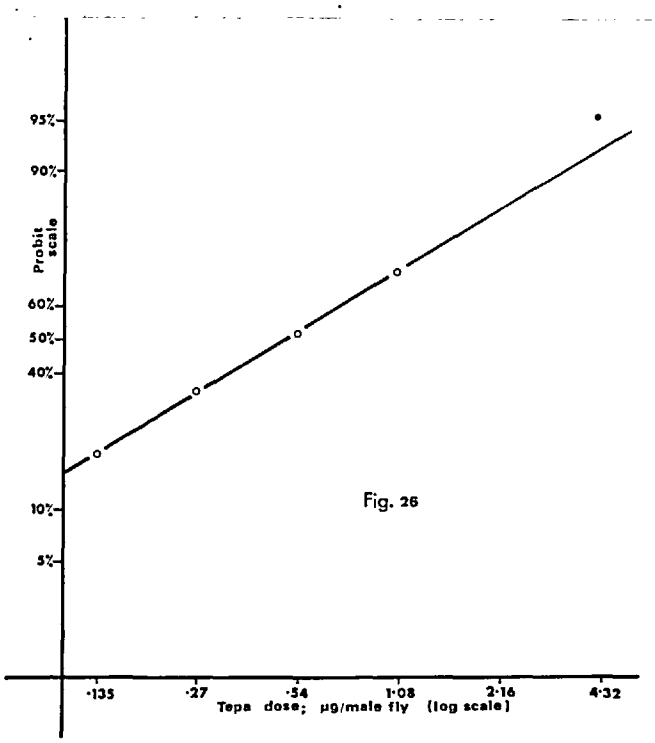


Fig. 26. Probit sterility/log dose relationship, for male flies injected with aqueous solutions of tepa. Open circles represent the data from Experiment 2 (first oviposition), to which the line is fitted. Solid circle represents the sterility obtained in Experiment 3.

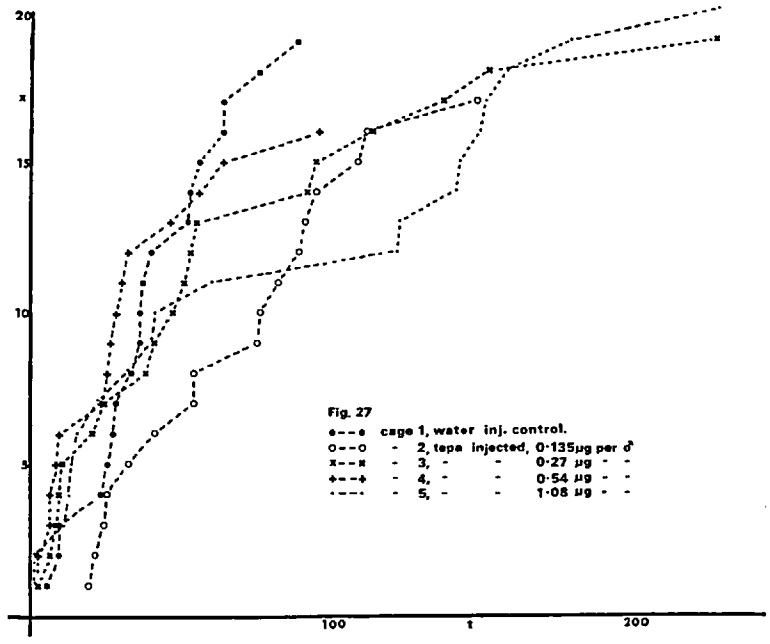


Fig. 27. Cumulative mating frequency curves for cages 1-5.

Experiment 3.

From the data of Experiment 2 it was calculated that a dose of 4.32 µg per male fly should give 90-100% sterility and that dose was used in Experiment 3. The volume of tepa solution per fly was increased to 0.6 µl from 0.3 µl, in order to reduce any error arising from possible unequal droplet size from the micro-applicator. Control males were injected with 0.6 µl distilled water. Flies were reared as in Experiments 1 and 2, but were injected on day 7 and mated on day 9. Onset of mating of successive pairs was timed to the nearest minute, and for some pairs the time of cessation of mating was also noted. (Results for mating duration are shown in Table 22).

Males were dissected to determine their mated status on day 10. In this experiment, three kinds of males were dissected, namely

(a) Virgin, tepa-injected males

(b) Mated, tepa-injected males

(c) Mated, water-injected males.

For each male fly the assessment was carried out without prior knowledge of the mated status of the given fly, by mounting the penis on a slide which was appropriately labelled, and then concealing the label. Before microscopic examination the slides were randomly switched around by an assistant. After each assessment had been recorded, the label on the slide was uncovered and compared with that assessment. Results are shown in Table 20.

For sterility studies, females mated with tepa-injected males were put into one cage, and likewise females mated with water-injected males were put into another, as controls. Oviposition was induced with liver on day 11, and eggs were treated as in previous experiments and

put out on to damp black filter paper overnight. Hatch was assessed on day 12. Oviposition was again induced on day 14 and assessed for sterility on day 15. Results are shown in Table 21.

Table 20. Test for mated status of males, conducted "blind", on 20 mated tepa-injected males, 20 mated water-injected males, and 20 virgin tepa-injected males.

Of 20 mated tepa-injected males, 19 were correctly diagnosed, 1 was not.
Of 20 " water-injected males, 18 " " " , 2 were not.
Of 20 virgin tepa- injected males, all 20 were correctly diagnosed.

Table 21.

	Treatment of male	Sterile eggs	Fertile eggs	Total	% sterility	Corrected % sterility
1st ovi-position	Tepa-injected	431	19	450	95.78	95.07
	Water-injected	37	217	254	14.55	
2nd ovi-position	Tepa-injected	217	7	224	96.8	96.1
	Water-injected	36	184	221	16.2	

Table 22. Duration of mating by tepa-injected and water-injected males.

Tepa-injected: 16, 16, 14, 14, 23, 10, 15, 13, 11, 12. Total = 144, Ave = 14.4

Water injected: 14, 13, 16, 15, 15, 14, 14, 16, 13, 18, 13, 10, 13, 14, 14, 15, 5. Total = 232, Ave = 13.6.

/If the last mating, which was abnormally brief, is omitted, the total becomes 227 and the average 14.2, for water-injected males./

Discussion

Yeoman and Warren (1965) and Miller (1965) have shown that apolate is an effective male chemosterilant for Lucilia sericata, by oral administration. Mating competitiveness was not reduced.

Tepa has not previously been tested against Lucilia sericata. The results of thepresent experiments show that it is highly effective as a male chemosterilant, and the degree of induced sterility is dose dependent. Injection was used to administer the tepa, so the amount used per fly is uniform and accurately known, which is not necessarily the case for oral administration.

The test for mated status was as effective with tepa-injected males (Cages 2-5 in Table 19, and Table 20) as it was with water injected males (Cage 1 in Table 19, and Table 20). Untreated virgin males were all negative (Table 19), and tepa-treated virgin males were negative (Table 20), proving that tepa injection was not itself responsible for granular secretion appearing in the lateral penis ducts. The test for mated status conducted "blind" in Experiment 3, fully confirmed the claims previously made (Section 3) concerning the accuracy of the test. The fact that only very few mated males were negative allows one to infer that nearly all the mated females received some male accessory secretion (as in normal matings), but this was not checked directly by dissecting the females. This conclusion is supported by the observation that the period spent by tepa-injected males in copulation is not reduced compared with that spent by water injected males (Table 22). It has already been mentioned that Donnelly (unpublished) found that male accessory secretion is passed over into the female after sperm, so that a large reduction in mating duration might have been expected to prevent the transfer of accessory secretion.

The number of mated pairs (out of a total possible of 20) which formed during the 160 minute observation period in the five cages of Experiment 2 did not bear any relation to the amount of tepa injected, although the water-injected control insects did form one more pair than the highest scoring tepa-treated flies. This is not thought to be a significant difference.

Summary

Sterility induced by the injection of tepa into male Lucilia sericata was found to be dose dependent, but mating rate was not dose dependent. The mated status test was as applicable to sterilised males as to normal fertile males.

S E C T I O N 5.

INVESTIGATIONS INTO THE NATURE, FATE AND FUNCTION OF THE MALE ACCESSORY GLAND SECRETION IN LUCILIA SERICATA

Investigations into the Nature, fate and function of the male accessory gland secretion in Lucilia sericata.

I. Appearance

The male accessory glands are paired and somewhat banana-shaped. They have been figured by Hori (1960). Their secretion is colourless, appearing white or pearly when viewed by reflected light. By transmitted light, the secretion can be seen to consist of a large number of small transparent but refractile bodies, suspended in a structurless liquid. It is the refractile bodies which give the secretion its granular appearance. When glands are broken open in a drop of water or saline on a microscope slide, the secretion falls to the glass and spreads, and is therefore denser than water. The disperse phase appears miscible with water, however, and is more mobile in such a drop of saline than the granules. Though the secretion emerges from a cut in the gland as a ribbon, this moulding is quickly lost, and no coagulation takes place.

Water mounts of freshly dissected, whole male reproductive systems show differential auto-fluorescence when illuminated by ultra-violet light. The following list shows the degree of auto-fluorescence shown by different tissues of the male reproductive system (Blue/green filters were used; assessment was by direct observation): Muscle - moderate; nerve - a little; male accessory gland secretion and cells - little; trachea - little or none; fat body - strong; sperm - little or none; reddish pigment on testis - none; sclerotised chitin - little or none; intima - strong.

Rhodamine is a vital stain which shows a strong rusty-orange

fluorescence when viewed with ultra-violet light. Donnelly (unpublished) has found that rhodamine fed to males which are then mated, can be detected in the mass of male accessory secretion in the female immediately after mating.

The male reproductive system placed in rhodamine B solution (5% aqueous) takes up the stain, and when viewed under U.V. light (no coloured filters) the degree of rusty-orange fluorescence of some of the tissues was as follows:- Muscle - strong; nerve - strong; male accessory gland secretion - strong; male accessory gland cells - moderate; intima - little or none.

Attempts to use rhodamine to stain the male accessory secretion in the phallosome of mated males with or without ultra-violet light gave far less satisfactory results than direct viewing using ordinary light without any staining.

II. Biochemical tests

Some simple biochemical tests were carried out on the accessory secretion. For each test, one or two male accessory glands were dissected out from a male fly, and placed in a drop of distilled water on a cover slip. The glands were punctured and the contents allowed to spill out into the water. The nearly empty glands were discarded. The drop of distilled water was gently warmed over a flame until the water had evaporated, leaving a fixed residue of secretion.

1. Xanthoproteic test. The coverslip was dipped into concentrated nitric acid, then washed in water, and finally placed in ammonia fumes. A bright yellow colour resulted (tyrosine, tryptophan, phenylalanine).

2. Mercury-bromphenol blue. The coverslip was dipped into mercuric chloride solution and bromphenol blue, after which it was

rinsed in 0.5% acetic acid. A strong blue colour resulted (protein).

3. Millon's reagent. The coverslip was covered by a drop of Millon's reagent. The deposit slowly (15 minutes) turned brownish or reddish pink (tyrosine).

4. Nigrosin. The coverslip was placed in a dilute solution of nigrosin for approximately two hours, and then rinsed in nigrosin decoloriser. The material was stained a strong blue colour, though penetration into the larger granules of the material was poor. (protein).

5. "Labstix" reagent. The contents of two glands were allowed to leak into a small droplet of saline, and the drop was then placed on the protein test square of a "Labstix" reagent strip. The test square went from yellow to greenish-blue, a positive reaction indicating protein. A control strip treated with an equal size droplet of saline did not turn greenish-blue.

These tests indicated that protein was present in the male accessory secretion, at least in the clear liquid. The nature of the granules was not determined.

III. Immunological tests

Introduction

Since the above tests showed that protein was present in the male accessory secretion, an attempt was made to develop an antiserum against the secretion, so that the antiserum could be used to detect male accessory secretion in the female after mating.

Procedure and results

Sixty five male accessory glands were dissected into physiological saline; the glands were broken open, the cellular material discarded, and the granular secretion taken up by syringe and put into a collecting tube. The volume of the mixture was made up to 0.9 ml with saline.

An equal volume of Freund's Bacto Adjuvant complete was added, and the whole thoroughly mixed by pumping it in and out of a syringe. 0.5 ml (25 mg) of terramycine was also mixed in, giving 1 ml final mixture. 0.25 ml of this mixture was injected into each of the four axillary and inguinal lymph nodes of a New Zealand White rabbit. Three weeks later, the rabbit was bled to obtain 10 ml of blood. For this the rabbit was restrained in a small wooden box with its head and ears protuding. A large vein was located on one ear, near the hind margin of the ear on the upper surface. An area was rubbed with alcohol, then shaved of hair using a sterilised scalpel. The surface was again rubbed with a pad of cotton wool soaked in alcohol, and a small nick in the vein made with the pointed tip of a sterilised scalpel. Blood flow (in drops) was slow at first, but was hastened by rubbing Xylene on the tip of the ear. When enough blood had been collected, further blood flow was stopped by pressing the vein. The serum was prepared by allowing the blood to clot and stand for one hour, after which it was centrifuged, leaving the serum as the supernatant fluid.

Antigen solution was prepared by dissecting four male flies and putting the contents of four pairs of accessory glands into 0.1 ml of saline with thorough mixing. Serial dilutions of the antigen were made in a series of twelve tubes. 0.9 ml of saline was placed in the first tube, and in the second and subsequent tubes 0.5 ml of saline. Then 0.1 ml of antigen solution was mixed with the 0.9 ml saline in tube 1; half that mixture was transferred to tube 2 and mixed, and so on down the series. Using an automatic dispensing machine (Weitz, 1957), the twelve dilutions were simultaneously sucked up into precipitin tubes, followed by a quantity of the serum as previously prepared, taking care to keep the area of contact between the two solutions a distinct interface.

After one hour, the tube containing the most concentrated antigen solution showed a precipitin band at the interface; after two hours the next two tubes also showed precipitin bands, but the rest of the tubes did not.

Six weeks after the original injection of antigen into the rabbit, a booster injection was made up by mixing secretion from 65 male accessory glands with 1.0 ml of saline and 1.0 ml of Freund's adjuvant. This was injected intramuscularly. About two months after the booster injection a 20 ml sample of serum was taken from the rabbit in the manner already described, and kept in a deep-freeze as stock serum.

About two weeks later, from female flies mated the previous day, ten were taken at random and the bursae(i.e. the part of the female tract containing the accessory copulatory vesicles) were dissected out. Spermathecae and female accessory glands were excluded. The bursae were ground in a minimum of saline; the extract was filtered using Millipore 0.45 μm, in a Swinex 13 filter holder, giving a few drops of clear extract as filtrate. The operation was repeated using ten virgin female flies. The "mated female" extract was tested against accessory gland antiserum, and against normal rabbit serum (NRS); the "virgin female" extract was similarly treated. A precipitin band formed in the "mated female" extract used versus the antiserum, but not in any of the other tubes. This proved that the bands were genuine precipitin bands, and not artifacts due for instance to the granular parts of the male accessory secretion.

On another occasion, the same procedure was repeated, using (a) 10 virgin female flies (b) 10 females mated the day before and put into the deep freeze (c) 10 females mated the day before but kept alive until required for testing. Extracts from the flies in the three categories were tested against antiserum and against NRS. Only the

"mated female" extracts tested against antiserum showed precipitin rings; all other combinations failed to develop preciptin rings after the lapse of two hours. These tubes were independently examined by technical staff trained in immunological techniques, and their report on the presence and absence of precipitin rings agreed with the information just given.

On a further occasion, male and female flies (both six days old) were mated and the mated females kept alive for a week. Virgin females of the same batch were also kept. Extracts were made of these flies in the usual way and eight precipitin test tubes were set up. Some turbidity in the serum was previously removed by filtering through 0.22 µm Millipore (Table 23).

Table 23

	Result of precipitin test	
	1st replicate	2nd replicate
1. "Virgin female" extract vs antiserum	-	-
2. "Virgin female" extract vs NRS	-	-
3. "Mated female" extract vs antiserum	+	+
4. "Mated female" extract vs NRS	-	-

Discussion.

From the results shown in Table 23, it was concluded that the bursae of females mated seven days previously contained antigenic material derived from the male accessory glands.

It was not surprising to obtain evidence by the preciptin technique that male accessory secretion remains in the bursae of females mated a week previously as traces of this secretion are often

visible in the accessory copulatory vesicles. Nevertheless the success of the technique is interesting, and means that a method now exists for tracing male secretions within the female tract, and presumably it could be used in instances where the secretion contains protein, but does not remain visible in the female. The nearest parallel to this work concerns Drosophila, in which it is known that males and mated females show a specific ninhydrin-positive component lacking in virgin females, as revealed by chromatography (Fox, 1956; Chen and Diem, 1961). This component is derived from the male accessory glands of Drosophila (Chen and Bühler, 1970). In another study involving Drosophila, Butterworth (1969) has shown that a lipid in the ejaculatory pump of the male is to be found in mated female (in trace amounts) but not in virgin females. Brieger and Butterworth (1970) identified the material as cis-vaccenyl acetate, but its function remains unknown.

The studies on L. sericata reported here were not extended to investigate the possible presence of male accessory secretion in the haemolymph of mated females, as tests described in the next subsection indicated that the injection of male accessory secretion did not cause virgin females to repel male advances in all cases, and so the "monogamy" factor is probably not simply the secretion circulating in the haemolymph. It may conceivably be a stimulus to increased oviposition (Garcia-Bellido, 1964; Leahy, 1966; Merle, 1969) but this has not been investigated.

IV. Effect of male accessory secretion on female mating behaviour

Introduction

Injections of male mosquito accessory secretion into virgin female mosquitoes (Aedes aegypti) has the effect of preventing normal insemination on subsequent matings (Craig, 1967; Spielman et al, 1967). The active principle consists of two proteins, neither of which is effective on its own (Fuchs, Craig and Hiss, 1968; Fuchs, Craig and Despommier, 1968; Fuchs and Hiss, 1970). Transplants of the glandular duct of male house flies into virgin females inhibits subsequent mating by those flies (Riemann et al, 1967). Extracts of male ejaculatory ducts of house flies inhibited mating by virgin females (Adams and Nelson, 1968). Of various sections of the male reproductive system of Drosophila implanted into virgin females only the accessory glands had much effect in preventing subsequent mating by the treated females (Merle, 1968).

In the work reported here, Lucilia sericata was used to test the effect of implanting male accessory glands, and injecting male accessory secretion, into virgin females. An alteration in the sexual behaviour of the recipient females was expected, in the direction of increased repulsion of male mating attempts.

Materials and Methods

Stocks of Lucilia sericata were reared as previously described (see Section 1).

Test 1. (a) Three lots of about 12 virgin females 7/8 days old were operated on in the following ways (A - C).

Group A: Each female received one male accessory gland previously heated to 60°C for 10 minutes.

Group B: Each female received one male accessory gland transferred directly from the dissected male without heating.

Group C: Each female was sham-operated by means of a cut in the dorsal thoracic cuticle.

Group D: In addition, females were left unoperated, as controls.

For the operation, each fly was anaesthetised with CO_2 and placed on a platform of perspex. The fly was kept in position on this platform by passing two strips of masking tape over the abdomen and wings, sticking these down on either side of the fly. The thorax was left clear for the incision. The mesonotum was pierced by an injection needle and a male accessory gland previously prepared inserted into the cut. The cuticle was allowed to spring back into place, and the fly released from the masking tape. Flies suffering unintended injury were destroyed. The various lots of flies (A - D) were placed in separate cages overnight to recover from the anaesthetic and operation. On the following day, 160 males (9/10 days old) were randomly segregated into 4 lots of 40, and each group put into a separate cage. Ten females of each kind (A - D) as previously prepared were placed into the male cages and the times of onset of mating by successive pairs noted. Mating pairs were removed as they formed. The remaining females were dissected after 18 hours and examined for sperm in the spermathecae.

(b) The same test was repeated, using 30 males per cage instead of 40, and omitting the sham operated controls.

Test 2. 36 4/5 day old virgin females were randomly divided into 3 lots of 12, and treated thus.

Group A: Untreated

Group B: CO_2 anaesthetised, injected in thorax with male accessory secretion in distilled water.

Group C: CO_2 anaesthetised, injected in thorax with distilled water only.

For the treatment given to Group B above, the male accessory secretion was obtained by puncturing a male accessory gland in a drop of distilled water, and discarding the nearly empty gland. The secretion plus some distilled water was sucked up in a syringe and then injected. The volume of water taken up each time was not carefully controlled, but that injected into Group B flies was approximately the same as that put into Group C flies.

The following day 10 female flies of each of the 3 groups A-C previously prepared were put with 40 males, in 3 separate cages, and the times of onset of successive mating noted.

Results

Previously mated females only rarely remate, at least under the conditions used in these experiments. This is shown by the first experiment in Section 3 in which no remating of mated females was observed within the observation period, whereas 10 virgin flies mated. On another occasion, twenty males were put in each of two cages, together with (i) thirty females mated the previous day, and (ii) thirty virgin females of the same age as the mated ones. There was one remating in the first cage, over the same period (one hour) as 15 matings occurred in the second. In the following experimental results, a comparable low level of mating, i.e. down to less than 10% that of untreated virgin flies, was selected as the criterion of mating inhibition.

Test 1 (a) Results are shown in Table 24.

Table 24. Time in minutes to the formation of successive mating pairs in four cages A - D. Each cage observed for at least 90 minutes. T = 25°C.

Cage	A	B	C	D
Pair	Sham op.	Implant heated	Unop. Control	Living implant
1	1	4	2	12
2	15	9	3	35
3	18	16	5	60
4	22	29	5	+
5	27	34	6	+
6	36	38	17	-
7	36	42	25	-
8	70	76	28	-
9	+	+	32	-
10	-	+	33	-

+ indicates that mating at some unspecified time (but within 18 hrs) had taken place, as judged by spermathecal examination.

- indicates that the females were still virgin, after 18 hours.

Heating the male accessory gland before implantation eliminates any inhibitory effect such as implantation might otherwise have (compare results of cages B and D).

Test 1. (b). Results are shown in Table 25.

Table 25. Time in minutes to the formation of mated pairs in Exp. 1.(b). Each cage was observed for at least 1 hr.

Cage	A	B	C
Pairs	Implant heated	Living implant	Unop. Control
1	2	5	1
2	9	6	1
3	11	8	1
4	12	42	2
5	26	-	2
6	39	-	15
7	41	-	15
8	51	Died } by	31
9	+	Died } next	45
10	+	Died } day	69

+, -, symbols as in Table 24.

From the mortality in Table 25, Cage B, one cannot discount the possibility that implantation of live accessory glands does some non-specific injury, which had as one of its side effects the inhibition of mating, and as another, a higher mortality rate.

Test 2.

Table 26. Time in minutes to the formation of successive mating pairs in 3 cages. (T = 26/28.5°C).

Cage	A	B	C
Pair	Control	Distilled water injected	Accessory secretion injected
1	1	1	7
2	1	1	13
3	2	2	16
4	3	14	50
5	4	32	.
6	5	52	.
7	5	109	.
8	13	.	.
9	15	.	.
10	18	.	.

Observations on mating were stopped after 2 hours. Cages B and C performances are well down on that of Cage A, showing that the operation has seriously interfered with mating. Nevertheless, despite the injury caused by the operation and injection, 4 of the flies receiving male accessory secretion mated within 2 hours.

Discussion

The male accessory secretion passed over to the female at copulation does not come into contact with the sperm, and is transferred after sperm ejaculation (Donnelly unpublished). It does not seem therefore that the accessory secretion aids the sperm directly, and Donnelly (unpublished) has also shown that normal fertile eggs are laid by females interrupted in mating, before the transfer of accessory secretion. It is highly unlikely that the secretion, channelled through special ducts for delivery by the male, and stored in special pouches in the female, could be functionless. Since mated females either do not remate under the experimental conditions used in these tests or do so only rarely, it seemed likely that the secretion might alter the behaviour of the female, causing it to reject further male advances. One possibility might be that the secretion, stored in the accessory copulatory vesicles of the mated female, is released slowly into the haemocoel, exerting a long term effect on the mated female resulting in monogamy. Supporting this supposition is the evidence already given, namely that the antigenic properties of the secretion remain even after a week in the female. Moreover, the paraphalli apparently damage (possibly pierce) the vesicle wall at mating (Section 2), leaving scars. If this hypothesis be correct, then introduction of secretion into the haemocoel of virgin females directly should elicit non-receptive behaviour in the flies so treated.

All three tables in this subsection show that implantation of male accessory glands, or injection of accessory gland secretion, into the thoraces of virgin female flies decreases the mating rates of these flies compared with uninjected or sham operated controls; but these tests were unable to differentiate between (a) a possible "monogamy effect"

that injection of secretion might have had on virgin females, and (b) a non-specific debilitating effect on the organism as a whole resulting in lower vitality, and hence lower mating rates (Table 25). However, the important point is that despite the possible existence of both these effects, some mating in fact took place (at least 40% in each test). It was concluded therefore that the monogamous nature of mated females could not be wholly ascribed to the possible presence of circulatory male accessory secretion in the female haemocoel, attractive though the hypothesis might seem.

By contrast, Fuchs et al (1970) working with Aedes aegypti found that extracts equivalent to as little as $\frac{1}{80}$ male when injected into the thoraces of virgin females causes them to become unable to accept sperm (< 10% insemination), even in the company of virile males for 24 hours. Riemann et al (1967) found reduction in receptivity by virgin females given two male ejaculatory ducts as implants, but these workers do not discuss the possible non-specific debilitating effect such implants might have induced. Implanting heated male accessory glands is not a serious handicap to the mating ability of females so treated, and rules out any possibility of a heat-stable monogamy factor being present in these glands.

It was concluded from the present work that the monogamous nature of mated females could not be wholly ascribed to circulating male accessory secretion in the female haemocoel. Attempts were made to remove the testes from males, so that the males could subsequently be used to mate with females without inseminating them, as Riemann (1969) has done with male houseflies. This proved impossible, due to the great injury done to the blow-flies and their subsequent high mortality. Riemann (personal communication) also had the same trouble using blowflies. Perhaps the most likely mechanism in Lucilia sericata is that the

accessory secretion acts via receptors at the site of deposition in the females, and from here the stimulus acting on the central nervous system may be hormonal or nervous. It may be also that this is only one of several mechanisms all reinforcing one another.

S E C T I O N 6

THE PHALLOSOME OF SARCOPHAGINAE

The phallosome of Sarcophaginae

Introduction

Lateral penis ducts have been found in several genera of the Calliphorinae (Section 2b) and may be characteristic of many, if not all, members of this subfamily. The flesh flies, Sarcophaga and its allies, are often considered as another subfamily within the Calliphoridae (e.g. van Emden, 1954), though some authorities give them family status (e.g. W. L. Downes, 1955). A study of the genitalia of these flies was therefore undertaken to see if structures comparable to the lateral penis ducts were to be found, and if so, to discover what function the ducts might have.

Materials and Methods

Sarcophagine specimens were obtained either by capture of the living insects in the field, or by receiving consignments of flies from cultures maintained by research workers elsewhere. Agria affinis Fallén was obtained from Dr. H.L. House, Belleville, Ontario, Canada; Sarcophaga barbata Thomson from Dr. D. S. Saunders, Edinburgh University; S. bullata Parker from Dr. G. S. Fraenkel, Illinois, U.S.A. Nine other species of Sarcophaga were captured in the field, but of these only six gave rise to observations recorded hereafter; these were S. carnaria L., S. subvicina Rohdendorf, S. "vulgaris" (see later), S. melanura Mg., S. aratrix Pandellé, and S. incisilobata Pandelle. Males were identified with the help of the key in van Emden (1954). Sometimes the male structures were examined by first crushing the thorax of the live fly and then dissecting out the genitalia under water without clearing. At other times the fly was kept in a glass tube till it died, after which it was dissected.

A culture of S. subvicina was set up by dissecting larvae from the incubatory pouch of a captured female, and rearing them on fresh ox liver placed on damp saw-dust. Mating pairs were removed from the cage, frozen in cardice-acetone mixture, preserved in Bouin's fixative, and later dissected. A mixed culture of Sarcophaga carnaria group flies was also set up, using females captured in the field. Mating pairs from this culture were treated as above, but the males only of each couple identified.

For the naming of parts of the male genitalia, the scheme of Roback (1954) has been followed, except that the terms superior forceps and inferior forceps have been preferred to anal forceps and anal plates respectively, and the term phallosome has been used as throughout this thesis.

The functional morphology of the male genitalia in three species of the Sarcophaga carnaria group of species.

Since most of the observations made concerned S. carnaria and closely related species, the phallosome morphology of these flies is dealt with first. This account concerns three taxonomic units, of which two (S. subvicina and S. carnaria) are generally recognised as good species, which the third is regarded by van Emden (1954, p. 116, footnote) as "almost certainly" a good species, and here treated as simply a third species, S. "vulgaris". All three will be referred to collectively as S. carnaria group.

1. Description of some phallosome features common to all three S. carnaria group species.

The ejaculatory duct is broad and easily dissected from the sheathing corpus. At the level of the base of the lateral filaments, it sends off a branch to each side, into the lateral filaments. (Fig. 28). The ejaculatory duct does not continue further in the midline, and although it seems to be attached to the cuticle at this point, there is apparently no median opening. This is contrary to the statement of Patton (1934).

The lateral filaments are tubular, as shown by the steroscan photographs (Figs 29, 30). The openings of the ejaculatory duct at the free end of the filaments are approximately semi-circular, and together the two form what is effectively a nearly circular orifice with a median septum.

2. Sperm and accessory secretion outlets from the phallosome of S. carnaria group spp.

During the dissection of uncleared material, observations were

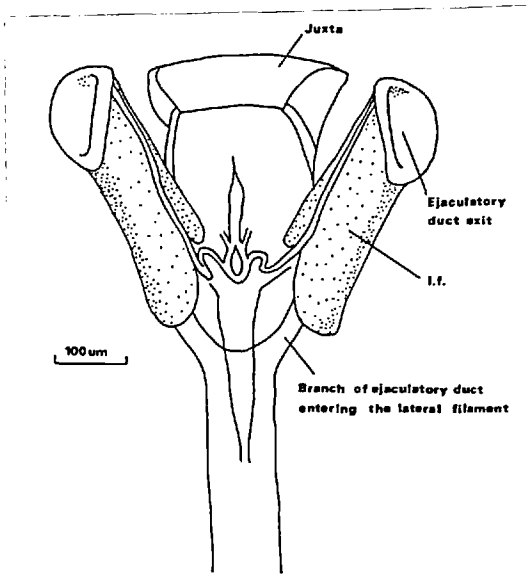


Fig. 28

Fig. 28. Dissection of phallosome of Sarcophaga subvicina

Fig. 29. Lateral view of phallosome of Sarcophaga subvicina (x 110)

Fig. 30. Paired sperm exits (lateral filaments) of Sarcophaga subvicina (x 330)

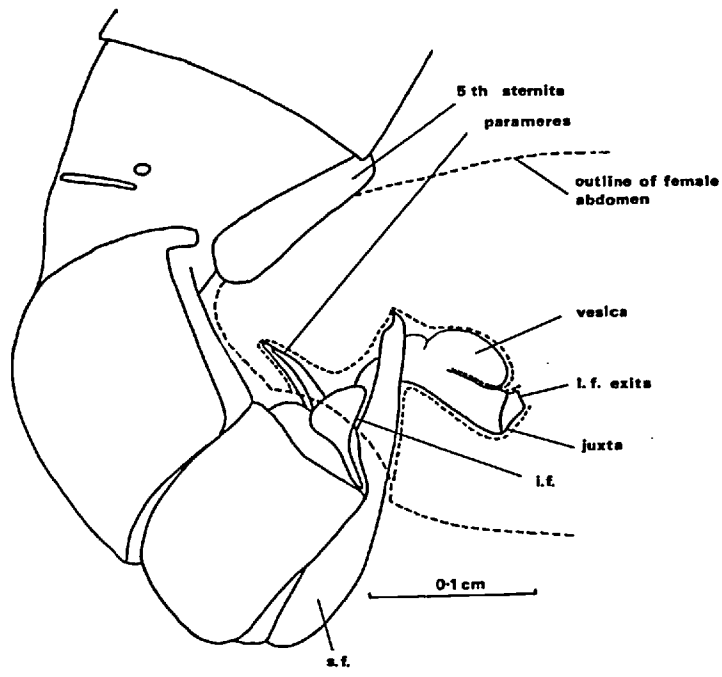


Fig. 31

Fig. 31. Copulation posture of Sarcophaga subvicina

made on the point of exit of the sperm and accessory secretions from the phallosome of the three S. carnaria group species. Both male products were observed coming from the lateral filaments, though never mixed together. Specimens killed immediately before dissection usually showed accessory secretion actively flowing from the lateral filament orifices; specimens which died in the tubes before dissection often had dead sperm lying at the exit of the lateral filaments. These observations were contrary to expectations, as it had at first been supposed that a median sperm exit would be found, analogous to the arrangement in Lucilia. However, after examining many flies neither sperm nor accessory secretion was ever found coming from any other area of the phallosome, confirming that no other exit exists.

Where accessory secretion was abundant, it appeared to be a white emulsion (i.e. droplets of one liquid were suspended in another) and was immiscible with water. The fate of the male accessory secretion in the female has not been studied.

3. The posture of the male genitalia during copulation

Thomas (1950) examined pairs of Sarcophaga (probably S. knabi) in cop., and came to the following conclusions:-

(a) The superior forceps lie against the ventral wall of the female canal.

(b) The anterior and posterior parameres lie against the dorsal wall of the female tract.

(c) The phallosome lies between the prongs of the superior forceps.

(d) The inferior forceps hardly enter the female canal. Observations similar to (a) and (c) above were made on S. rosellei by Patton and Ho (1938).

From dissections of copulating Sarcophaga carnaria group pairs

made during the present work, points (b), (c) and (d) were confirmed. To points (a) and (c) should be added that the sharp tips of the superior forceps push against the dorsal wall of the female tract. A more significant point is that in most of the dissections made of copulating pairs, the lateral filaments were applied to the openings of the spermathecal ducts, and sperm was present as a continuous "rope", extending from the lateral filaments to the spermathecal ducts. Hence the function of the lateral filaments as sperm conduits, inferred from the examination of individual males, was fully confirmed by dissection of pairs. All these observations were repeated using pairs taken from the pure S. subvicina culture. (Fig. 31).

Phallosome functional morphology in other sarcophagines

1. Agria affinis Fallen (Agriini).

Fixed copulating pairs were dissected and the alignment of parts and the method of sperm transfer were compared with those of Sarcophaga carnaria group. The following main differences were noted:

(i) Sperm pass over to the female via a median gonopore, found at the end of the elongated phallic tube in Agria affinis.

(ii) There was no evidence of any other opening on the phallosome, and neither lateral filaments nor median filaments (see Discussion) occur on the phallosome.

(iii) The inferior forceps are well developed (unlike those of Sarcophaga carnaria group) but neither the inferior nor the superior forceps enter the vagina. This in marked contrast to what happens in S. carnaria group.

2. Sarcophaga melanura Meigen.

In this species (the commonest in the field collections) the lateral filaments are large with a wide mouth. Several specimens were found to have sperm issuing from the lateral filaments.

3. Sarcophaga aratrix Pandellé, and S. incisilobata Pandellé.

In these two species a granular secretion was observed issuing from the lateral filaments during dissection of fresh material under water. Exit of sperm was not observed.

Discussion

The observations described in the present section show that the lateral filaments, of S. carnaria group at least, are the route for the egress of sperm from the phallosome into the female, and no median opening exists. In Agria affinis on the other hand, the route is via a median gonopore, and lateral openings are apparently absent.

It is safe to assume that the mode of sperm transfer in Agria affinis is of a more generalised type than that of Sarcophaga carnaria group, since a median male gonopore is normal for higher Diptera, as for other insects. There is as yet insufficient evidence to be certain how the S. carnaria system evolved, though it was possibly via a system in which the phallosome had three openings, a single median and paired lateral ones. The figures of male sarcophagine genitalia in Roback (1954) show that Rafaelia rufiventris Tris. (Roback's fig. 311) has a phallosome apparently of the triple aperture type, and this may represent an intermediate stage.

The selective advantage of these alternatives in the route of sperm transfer is obscure. That is not to say there are no such advantages. Roback (1954) described the median filaments of the subtribe Sarcodexiina as greatly resembling the lateral filaments, but thought that their origin was different. If their functions are the same as those of the lateral filaments, then this pattern of evolution has occurred more than once in the sarcophagines.

In many Sarcophaga species, including S. bullata and S. barbata examined during this work, the lateral filaments are slender and tapering (Roback 1954). It might have been thought that in such cases the lateral filaments were inserted directly into two of the spermathecal ducts at copulation. However, published figures of the spermathecae

of Sarcophaga and related species show no instance of a reduction of three spermathecae to two, nor of a specialisation of spermathecae into one large and two small ones (as in Merodon, Section 8), as might be expected on such a hypothesis. Spermathecal structure has been illustrated in 71 species of sarcophagines dealt with by Patton and Wainwright (1935), and Kano et al (1967), and many of these species have narrow lateral filaments in the male. There is therefore no reason for supposing that those species in which narrow lateral filaments occur use them differently from the broader ones in S. carnaria group. It must be pointed out however that the present studies have not shown that sperm does in fact emerge through the slender lateral filaments, nor that in such species there is no median phallosome opening.

Some Sarcophaga species therefore have twin spermatic ducts, but unlike many Calliphorinae these species do not have apertures on the phallosome specialised for the transfer of accessory secretion alone. A mated status test exactly analogous to that devised for Lucilia is therefore not possible.

S E C T I O N 7

STUDIES ON THE MATING OF *GLOSSINA* WIEDMANN

Studies on the mating of Glossina Wiedmann

Introduction

Initially, Glossina was studied to see if lateral ducts or the equivalent were present on the phallosome, these structures in Lucilia sericata having provided the basis for a test for the mated status of male flies. As will be shown, certain channels are present on the tsetse phallosome, but though their function has not yet been fully worked out, it is clear that the test used for Lucilia sericata cannot be applied directly to Glossina. However, this study of the phallosome and its functions has brought to light the totally unexpected fact that Glossina, unique amongst flies higher than the Nematocera, uses a spermatophore during sperm transfer. An account of the spermatophore, and a description of the functions of the male structures, are given.

Materials and Methods

The species used were Glossina austeni Newstead and G. morsitans Westwood, supplied by the Tsetse Research Laboratory, Langford, Bristol. Unless otherwise stated, the descriptions in this account apply to G. austeni. Pairs of copulating Glossina were frozen in cardice-acetone mixture, fixed and sectioned as previously described for Lucilia sericata. Females 2-4 days old were used, and males were 10 days old or more, an age at which they are efficient at mating. Flies were dissected fresh or after fixation, with watchmakers forceps and under x 50 dissecting microscope. Material for stereoscan microscopy was treated in the usual way (as described for Lucilia sericata), but since the phallosome of G. austeni and G. morsitans has an articulating part, care was taken to set the material in various postures, before coating with metallic film.

The phallosome

The phallosome of Glossina is a highly complex structure, and there is little to be gained from attempting here to homologise the parts with phallic structures in other flies. This remark does not apply to the gonopore, the ejaculatory duct, the ejaculatory pump and its sclerite, all of which are here assumed (in the absence of evidence to the contrary) to be homologous with structures given the same name in other flies.

The ejaculatory duct leads to a wide terminal male gonopore (Fig. 32). Close to the gonopore, and within the phallosome, is the ejaculatory pump and sclerite. The latter is small, rod-like and

Fig. 32. Male gonopore of Glossina austeni (Stereoscan x 702)

asymmetrically placed. It is attached by one end to part of the phallosome cuticle, from which it is probably originally derived. The muscular part of the ejaculatory pump is not as well demarcated from the ejaculatory duct as it is in calliphorid flies.

An important feature of the phallosome is that it has a transverse hinge, so that the end piece bearing the gonopore articulates with the larger basal section containing the ejaculatory pump. Consequently the gonopore may be at the apex of the phallosome, or flexed back to lie near the basal section (Fig. 33). In the flexed position, a strong ridge on the end piece forms the apex of the phallosome. The hinge itself is formed by the articulation of sclerotised areas on the end piece and basal part respectively. Two membranous flaps, studded with small spines, arise in the lateral areas of the join between the main sections of the phallosome. Between the membranous flaps and the end piece run a pair of sunken channels (Figs 34, 35), first noticed in water mounts of fresh material under the light microscope, but studied in more detail by means of the stereoscan microscope. Great importance is attached to these channels, as they probably serve as conduits for one or other of the male secretions. When the phallosome is in the flexed position, the channels are covered by the membranous flaps which fit over "shoulders" on the end piece, so that essentially they become tubular ducts. The orifices of the ducts then lie close to the apex of the phallosome (Fig. 35). When the phallosome is not flexed, the membranous flaps can be manipulated with fine forceps away from the shoulders of the end piece, and the channels are then seen to run from the dorsal face of the phallosome (Figs 34, 36). The exact point of origin of the channels has not been located, so that the question of whether they come from the gonopore or from other exits from the phallosome (c.f. Lucilia, Sarcophaga) has not been settled.

Fig. 33. Lateral view of male genitalia of Glossina austeni.
The terminal part of the phallosome is flexed (stereoscan x 60)

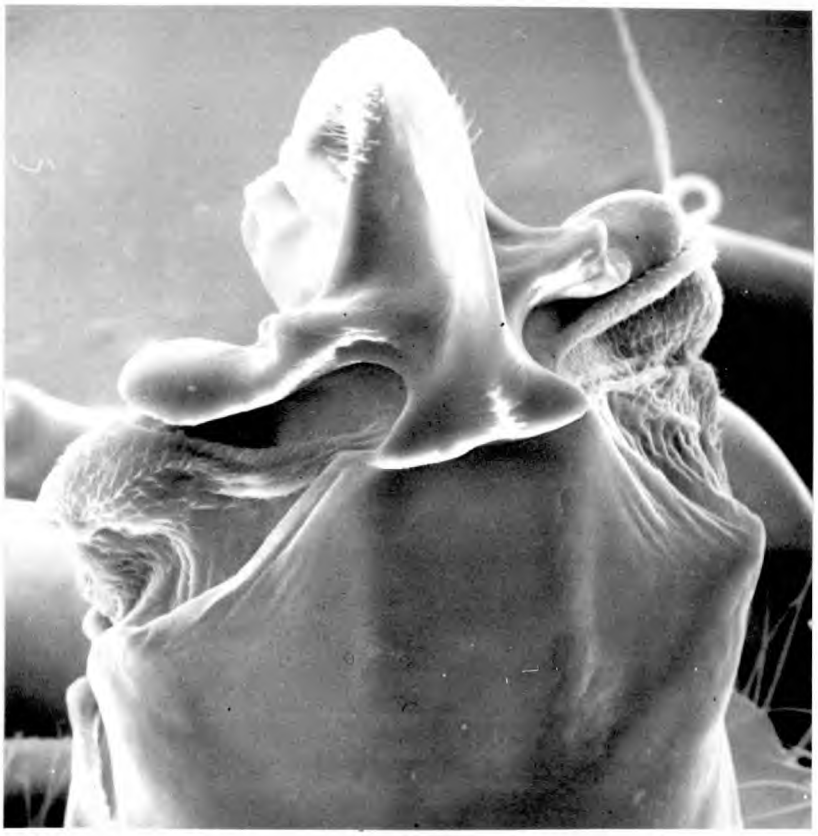


Fig. 34. Dorsal view of phallosome of G. austeni showing a pair of sunken channels. (Stereoscan x 137).

Fig. 35. Nearly terminal view of phallosome of Glossina austeni, showing orifices of lateral channels. (Stereoscan x 130).

Fig. 36. Ventral view of phallosome of Glossina austeni, with the terminal part extended. (Stereoscan x 120).

Structures at the base of the phallosome

Of the other male genitalial structures, the superior claspers are so prominent and well known that little need be added here. Vanderplank (1948) has illustrated how they grip the underside of the female abdomen during copulation in Glossina tachinoides Westwood; Squire (1951) has also described their action in G. palpalis Robineau-Desvoidy. Challier (1968) has reported on the "cicatrices copulatrices" on the female abdomen produced by the superior claspers of the male.

On the other side of the phallosome away from the superior claspers there are two main structures, the ninth tergosternum (Patton, 1934), and the inferior claspers. (Figs 33, 37). The ninth tergosternum reaches its greatest development in the morsitans group, and the interspecific variation in shape of this plate and its median cleft or slot has been used by Jackson (1952) for taxonomic purposes. The function of the cleft, not previously reported, is described under the next heading. The inferior claspers are less robust than the ninth tergo-sternum, and are somewhat leaf-like. Probably they act in the same way as the corresponding structures in G. palpalis, namely to widen the aperture of the vulva. (Squire, 1951) (Fig. 37).

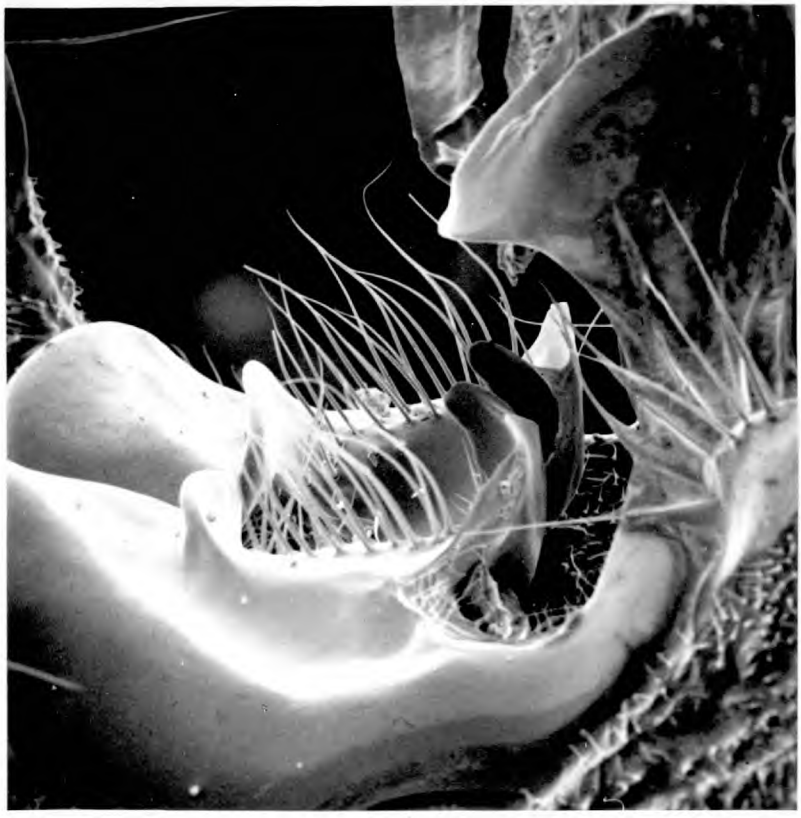


Fig. 37. Inferior claspers and surrounding structures in Glossina austeni. (Stereoscan x 100)

The mechanics of coupling

The phallosome is thrust deeply into the uterus at mating (Fig. 38). The uterus in the virgin female is only a small fraction of the size that it attains in the pregnant female. Consequently structures at the anterior end of the uterus are, in the virgin female, relatively close to the vulva. The gonopore of the copulating male lies at the spermathecal duct papilla, or very close to it. Dissected pairs of copulating flies have shown the end piece of the phallosome in both the flexed and the unflexed positions.

In frozen and fixed males disengaged from the females, the membranous flaps are somewhat irregularly shaped, but cover over the lateral channels except at the anterior exits. The latter are directed apically and open on either side of the crest of the end piece.

During the dissection of some frozen and fixed mating pairs (G. morsitans) twin streams of a whitish material were found to be issuing from the phallosome in the region of the lateral channels previously described. This material was probably the product of the male accessory glands, but further observations are required on this matter.

Thick transverse sections of copulating pairs (Fig. 39) show that the faeces produced by the female during the mating period, which may last for many hours, pass through the cleft in the ninth tergo-sternum of the male. Nash and Kernaghan (1965) have remarked on the fact that hardened faeces at the anus can be the apparent cause of death in tsetse flies, and adaptations to avoid blocking the anus are clearly advantageous.

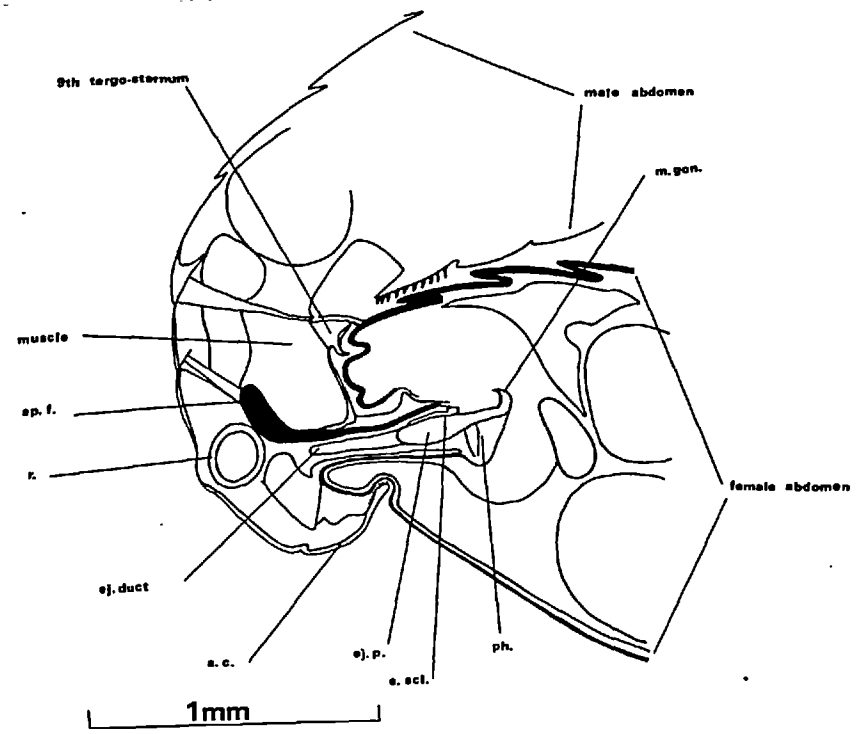


Fig. 38

Fig. 38. Alignments of male and female parts during copulation in Glossina austeni. Abbreviations:- ap.f., apodema of furca; ej. duct, ejaculatory duct; ej.p., ejaculatory pump; e.scl, ejaculatory sclerite; m.gon., male gonopore; ph. phallosome; r, rectum; s.c., superior clasper.

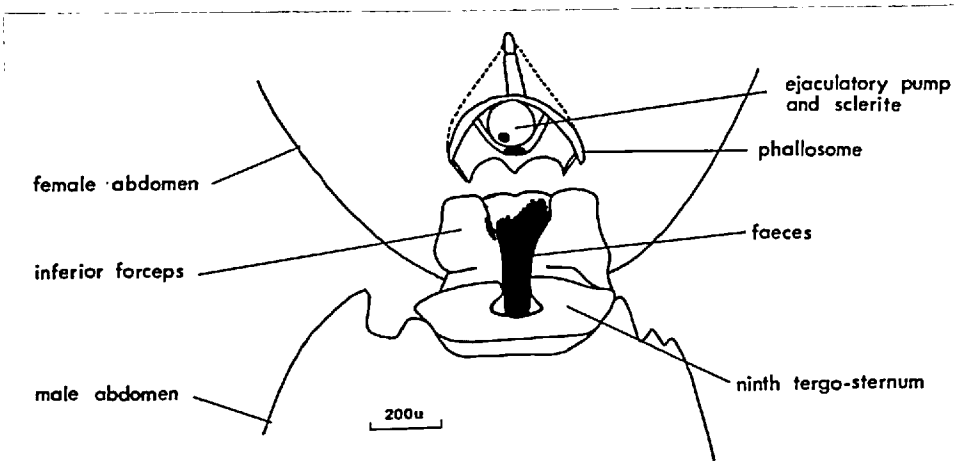


Fig. 39

Fig. 39. Transverse section through copulatory pair of Glossina austeni, showing faeces of female being conducted away via the cleft in the ninth tergo-sternum of the male.

Sperm transfer by means of Spermatophore in Glossina austeni

Some frozen and fixed pairs showed on dissection an accumulation of material at the anterior end of the uterus, taking the form of a transparent gelatinous body with an opaque centre. At first the transparent body was interpreted as a fossa, which is a gelatinous lining known to be present at the anterior end of the uterus in all fusca group flies (Jordan, 1963; Canelhas, 1965), and the sperm was believed to be an adventitious deposit. Further dissections corrected this initial impression, and it became clear that the transparent body was in fact a spermatophore, containing a very large quantity of sperm. The spermatophore is a prominent colourless object in the uterus of recently mated females (Figs 40, 41). A typical example measured 0.7 x 0.5 x 0.4 mm, but the size varies. Microscopical examination of fresh and fixed specimens reveals a single closely packed sperm mass within the transparent body of the spermatophore; the mass can be dissected out whole from the surrounding material even when fresh, as the sperm hold together, like the fibres in a fresh clot of blood. When viewed through the microscope, sperm can be seen to be active inside spermatophores placed in a saline solution. The part of the spermatophore in immediate contact with the rounded sperm mass appears more transparent than remoter parts, and is firmer in consistency. It keeps its shape even after the sperm mass has been dissected out, but splits easily on compression. As shown in Fig. 40, the spermatophore and sperm mass are visible through the uterus wall.

Spermatophores are found adhering to the antero-dorsal wall of the uterus, and project backwards into the uterine cavity to an extent depending on spermatophore size. At this point on the wall is the opening of the fused spermathecal ducts. Some dissections of fixed,

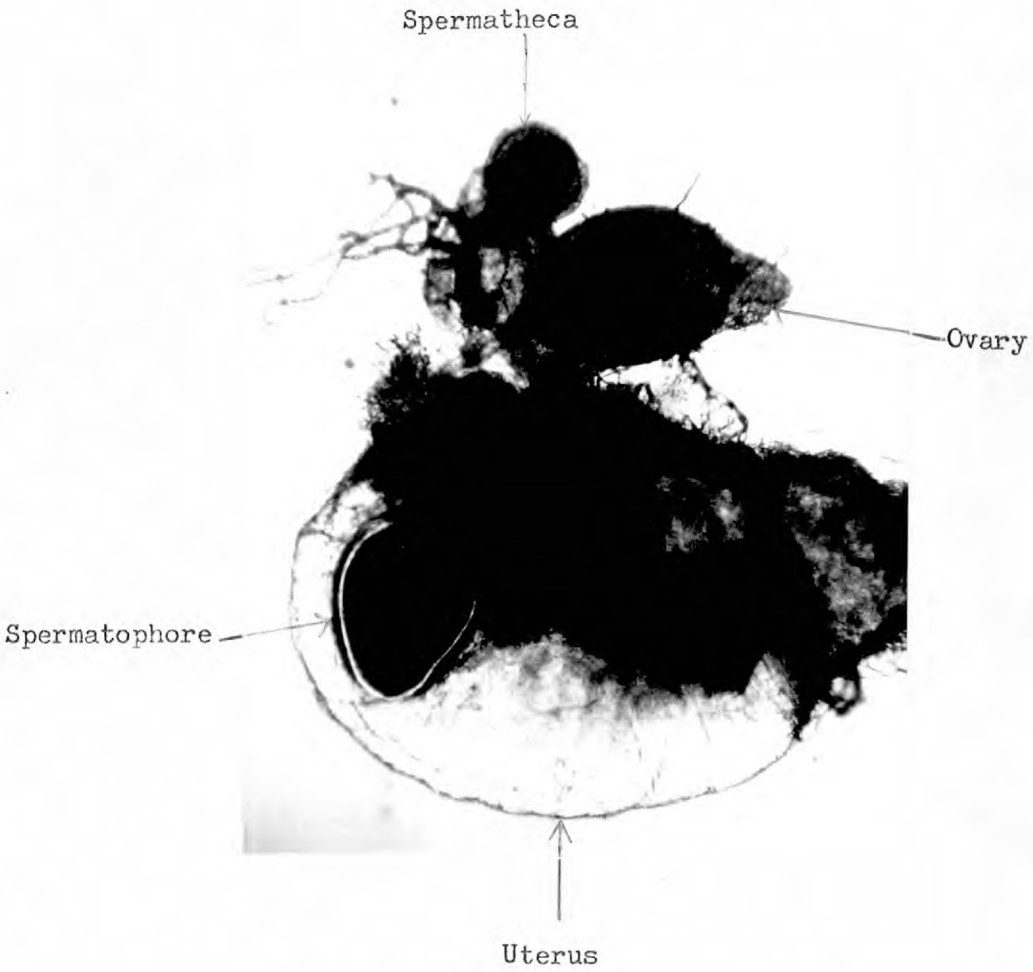


Fig. 40. Reproductive system dissected from a recently mated female Glossina austeni, showing a spermatophore within the uterus. Lateral view of water mount (x 35).

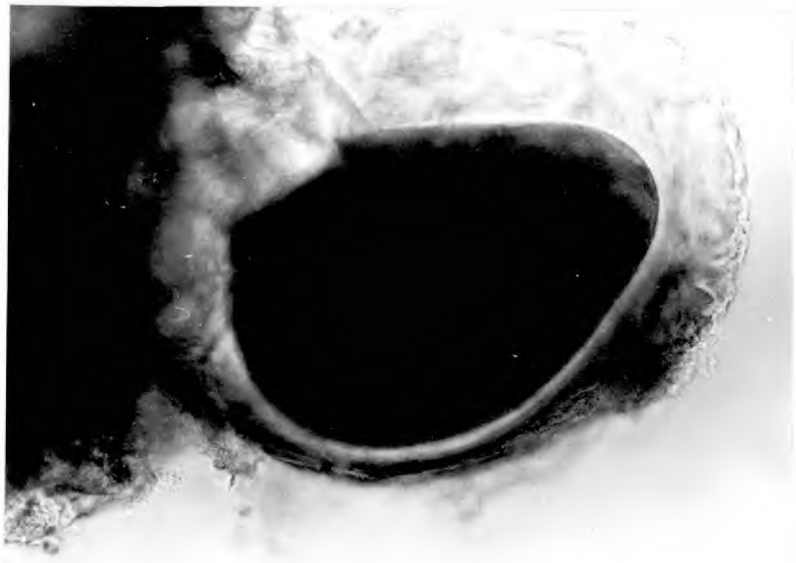


Fig. 41. Spermatophore of Glossina austeni dissected from the uterus of a recently mated female fly. On the left is part of the uterine wall to which the spermatophore is still attached (Saline mount, x 80).

recently mated female flies have yielded a sperm mass bearing a bifid process that has precisely the shape of the spermathecal ducts, showing that the sperm mass, though otherwise completely enclosed within the spermatophore, is placed exactly at the entrance to the common spermathecal duct. Whether the bifid process is made of the cuticular lining of the ducts or of fixed material taken intact from within them is not yet known.

Because the accessory glands of the male are nearly exhausted after mating, it is reasonable to assume that at least the bulk of the spermatophore is of male accessory gland secretion, though the possibility that the female makes some contribution cannot be ruled out. For instance, pairs frozen and fixed before spermatophore transfer have revealed on dissection a roughly triangular, completely clear material at the anterior end of the uterus, apparently secreted by the female. It is not known whether in unfixed flies this is liquid, gelatinous, or solid, but its presence is interesting as it may throw light on the means by which the spermatophore is gelled following ejaculation.

Most inseminated females dissected within a few hours of mating were found to have a spermatophore in the uterus, but some had none. To find out what happens to a spent spermatophore, fifty pairs of flies were set up in clean glass tubes and examined the next day, when twenty nine tubes were found to contain expelled spermatophores. Such spermatophores were somewhat shrunken but rapidly swelled to nearer normal size when placed in water. They contained considerable quantities of sperm; it was also noticed that spermatophores dissected from the uterus often contained more sperm than could evidently be accommodated within the spermathecae of the recipient female.

Whether the presence of an expelled spermatophore showed that the female concerned had been inseminated was investigated by dissection.

It was found that all twenty nine female flies had sperm in the spermathecae. It is worth recording that in two of these females spermatophores were also found in the uterus, showing that sperm transfer may occur twice between the same pair of flies within twenty four hours. The other twenty one females were also dissected; eleven had spermatophores in the uterus, and of these ten had sperm in the spermathecae, with one doubtful case. The remaining ten females had neither a spermatophore in the uterus, nor sperm in the spermathecae. The relatively low insemination rate compared with the results of Nash et al (1968) for mating in tubes is no doubt due to the flies having been mailed for use here, and is in itself of little interest. The results (Table 27) show that there is an excellent correlation between the presence of a spermatophore in either the tube or the uterus, and the presence of sperm in the spermathecae. This can be regarded as good preliminary evidence that the presence of the expelled spermatophore can be used to show that the female has been properly inseminated. On the other hand, the non-appearance of an expelled spermatophore after a day or two does not necessarily show failure of sperm transfer. Females tubed with males for twenty four hours sometimes retained the spermatophore throughout the second period. Probably even these spermatophores would be expelled ultimately, but this point has not been verified.

Five females dissected within 15 minutes of the natural termination of mating had very few sperm in the spermathecae, but a large sperm-filled spermatophore was present in the uterus.

It was found that Glossina morsitans also uses a spermatophore.

TABLE 27.

	No. with sperm in spermathecae	No. without sperm in spermathecae	Presence of sperm in spermathecae doubtful
Pairs with expelled spermatophore in tube; none in uterus.	27	0	0
Pairs with spermatophore in uterus; none expelled	10	0	1
Pairs showing spermatophore in uterus and in tube.	2	0	0
Pairs with no spermatophore, either in tube or in uterus.	0	10	0
	Total 50 pairs		

The presence or absence of spermatophores in the uterus or expelled in the mating tube, correlated with presence or absence of sperm in the spermathecae.

Discussion

Though a very large number of tsetse flies have been dissected by various workers (e.g. at least 12000 were recorded by Vanderplank, 1946), the spermatophore seems to have escaped notice despite its large size. The reasons for this fact may be that (a) the spermatophore is usually expelled within 24 hours after mating (b) only relatively recently have ample supplies of tsetse flies been available for breeding and study under controlled conditions (c) even if previously seen, the spermatophore might possibly have been mistaken for a deformed egg or larva, and so escape notice. This is also true of expelled spermatophores, which might possibly have been discounted as abortions.

An interesting aspect of the discovery of the spermatophore is that it offers an alternative explanation for the observation by Mellanby (1936) that sperm are found to be absent from the spermathecae when pairs are forced apart before the natural termination of the mating period. Mellanby's conclusion was that sperm were passed just before the end of copulation; it can now be seen that almost certainly some of the prematurely parted flies must have formed a spermatophore, from which the sperm had not yet started to migrate. It has already been noted that even in normally mated females, relatively few sperm have reached the spermathecae within 15 minutes of the end of mating. Nevertheless, it may be true that sperm are passed into the spermatophore during the second half of the mating period.

For Glossina nigrofusca Newstead it has been denied that the phallosome could reach the anterior end of the uterus (van Emden, 1944). Vanderplank (1948) nevertheless presented a schematic figure of normal coitus in Glossina tachinoides Westwood, with the male gonopore at the

oviducal entrance. Spermathecal ducts were not shown in this figure. Machado (1959) and Jordan (1963) both agree that in fusca group flies the signum probably protects the uterus from damage by the phallosome at mating. Machado (1964) gave strong evidence on morphological grounds that the phallosome fits accurately into the signum of Glossina fusca congolensis Newstead and Evans. The present observations on the penetration of the phallosome therefore agree with most published remarks on the subject, though these have referred to other species.

The observations on Glossina spermatophores have some practical value. First, the only current method for detecting whether a female has been inseminated (other than by breeding) is to dissect and examine the spermathecae for sperm content. (Mellanby, 1936). Empty spermathecae are taken to show that the fly is virgin. As already noted, the presence of an expelled spermatophore in the mating tube shows that sperm transfer has taken place, so that dissection is not necessary, and the female can be used for further studies. This could be particularly useful in crosses involving sterilised males (Curtis, 1968; Dame and Ford, 1968), from which offspring are in any case not expected.

Second, a very few sperm were detectable in spermathecae immediately after mating, though a full spermatophore was present in the uterus, and there can be little doubt that in such cases sperm had not had time to migrate to the spermathecae. Evidently, dissection of the females to determine their mated status would yield more information if the uterus were inspected as well as the spermathecae, especially when the latter appear to be empty. This inspection might be of particular value in control operations where the wild tsetse population has become very sparse, at which point it is essential to know if females are meeting males for insemination.

Third, if dissections of parous but non-pregnant females caught

in the wild should show spermatophores in the uterus, this would prove that mating could take place more than once by female tsetses, a matter which is of importance in sterile male release work.

As mentioned earlier, the spermatophore was at first mistaken for a fossa. After this impression had been corrected the possibility was considered that the fossa in fusca group females might in reality be a retained spermatophore. Jordan (1963) has remarked that "opaque concretions" made on the signum (the hardened surface of the fossa) as a result of mating in Glossina tabaniformis Westwood, "in some ways ..... are reminiscent of the plugs of albumin-like material which are a secretion of the male found in the oviducts of the female Anopheles gambiae Giles (Gillies, 1956).". However these concretions are persistent throughout the life of the female tsetse, and signa (and hence presumably fossae) are present in the virgin female G. tabaniformis (Jordan 1963). The relationship, if any, between the concretions and the spermatophore (assuming that G. tabaniformis uses one) has yet to be worked out. More information about the mechanism of sperm transfer in fusca group flies (regarded by some authorities as the most primitive of the tsetse flies) would be of great interest.

This report brings the total of dipterous families in which spermatophores are known for certain to occur to four, namely Ceratopogonidae, Simuliidae, Chironomidae, and the family to which Glossina belongs. In Appendix III evidence will be presented to support the contention that the genus Glossina is properly placed near the Hippoboscidae as the Glossinidae and it is this latter family rather than the Muscidae which should be regarded as showing spermatophores. In addition, Appendix 4 describes spermatophores in a fifth family, the Bibionidae (Nematocera). Downes (1968) has suggested that a sixth, the Thaumaleidae (Nematocera) may also use them. The significance of

of spermatophores in the evolution of the Diptera is discussed in Section 9.

Certain comparisons between Lucilia and Glossina with respect to their methods of sperm transfer and to the structures concerned with this operation, will now be made.

(a) Glossina uses a spermatophore; Lucilia has a closed system, without a spermatophore.

(b) The full quota of sperm have entered the spermathecae of Lucilia by the end of mating, since no sperm can be seen elsewhere in the reproductive tract; in Glossina entry into the spermathecae has only just begun at that time.

(c) The secretions of the male accessory glands of Glossina are chiefly devoted to forming the spermatophore; in Lucilia the corresponding secretion has no contact with the sperm.

(d) Special lateral penis ducts and orifices are present on the Lucilia phallosome for the conveying of male accessory secretion; in Glossina there are lateral channels of uncertain function on the phallosome.

(e) The ejaculatory sclerite of Lucilia is large, and placed within the abdomen, not in the phallosome; The ejaculatory sclerite of Glossina is small and rod-like, very close to the gonopore and within the phallosome. The ejaculatory pump of Glossina is not nearly as well differentiated from the ejaculatory duct as is the case in Lucilia.

(f) A test has been devised to assess the mated status of male Lucilia; no such test exists for Glossina, but a different test can be applied to Glossina austeni females to indicate whether they have been inseminated. This latter test does not require dissection, but does require the females to have been mated in tubes.

Summary

Some anatomical details of the male genitalia in Glossina are described, in particular paired channels on the phallosome. The function of the cleft in the ninth tergo-sternum is shown to be the conduction away of any faeces expelled by the female during copulation.

The mechanics of coupling in Glossina is described. Sperm transfer is shown to be effected by means of a spermatophore. The spermatophore is described. This is the first time a spermatophore has been observed in any fly outside the Nematocera. Usually, the spermatophore is expelled within 24 hours of the onset of mating, and the presence of an expelled spermatophore in a tube housing a female fly is shown to be a reliable indicator of the inseminated condition of that fly.

A comparison of the sperm transfer mechanism in Lucilia with that of Glossina is made.

SECTION 8

PHALLOSOME STRUCTURE IN THE MALE, AND THE CO-ADAPTED SPERMATHECAL DUCTS OF THE FEMALE, IN MERODON EQUESTRIS (F.) (SYRPHIDAE)

Phallosome structure in the male, and the co-adapted spermathecal ducts of the female, in Merodon equestris (F.) (Syrphidae)

Introduction

The functional morphology of the phallosome has already been examined in some detail in the calliphorid Lucilia sericata (Mg.) (Section 2a). The main findings were that the male genitalia in this species were adapted for the transfer of sperm and of male accessory secretion to different destinations in the female, by means of special ducts and openings on the phallosome. For comparative purposes, and because it is a species against which the sterile male release technique might be employed for control, Merodon equestris F. (Syrphidae) from the Cyclorrhapha Aschiza was selected for study. This is a fairly large distinctive species, seasonally abundant in the neighbourhood of daffodills, of which it is a major pest in the larval stage.

Materials and Method

Attempts were made to rear flies by catching females in the field and placing eggs that they laid on to daffodil bulbs (untreated with pesticides), and growing these through to the next season. Though about 300 bulbs were so prepared, less than 10 flies emerged next Spring. Most specimens used in the work were therefore caught from the field. Mating pairs could not be obtained for dissection as pairs put into cardice-acetone had parted before making contact with the freezing mixture. Measurements of female parts were made using a microscope with a calibrated eye piece; male parts were measured from the stereo-scan photographs.

The terminology employed for the male parts is that of Metcalf (1921), except for the term phallosome, used here as defined in the General Introduction.

Male Structures

The phallosome is a long slender but fairly rigid body (Figs 42, 43). Towards the distal end it bifurcates, each gently curving ramus bearing a duct and orifice (Figs 44 and 46). Flanking the phallosome is a pair of appendages (internal lobes of Metcalf, 1921). Each of these consists of a basal stalk bearing a flattened more or less oval plate; the inner and outer faces of the plate are roughened with denticles. Sheathing all these appendages so far mentioned is a pair of robust, partially fused, structures, forming the penis sheath. Each has at the apex a strong comb-like structure (fig 45) with basally directed teeth: this is the superior lobe.

Female Structures

There are three spermathecae of which one is larger than the other two. The ducts from the two smaller spermathecae join about halfway along their length; the remaining two ducts join immediately before the common exit is reached. For a short way up the two ducts from the common exit the lumen is relatively wide. There is then an abrupt narrowing of the lumen, and at this point there are blackened patches on the duct walls.

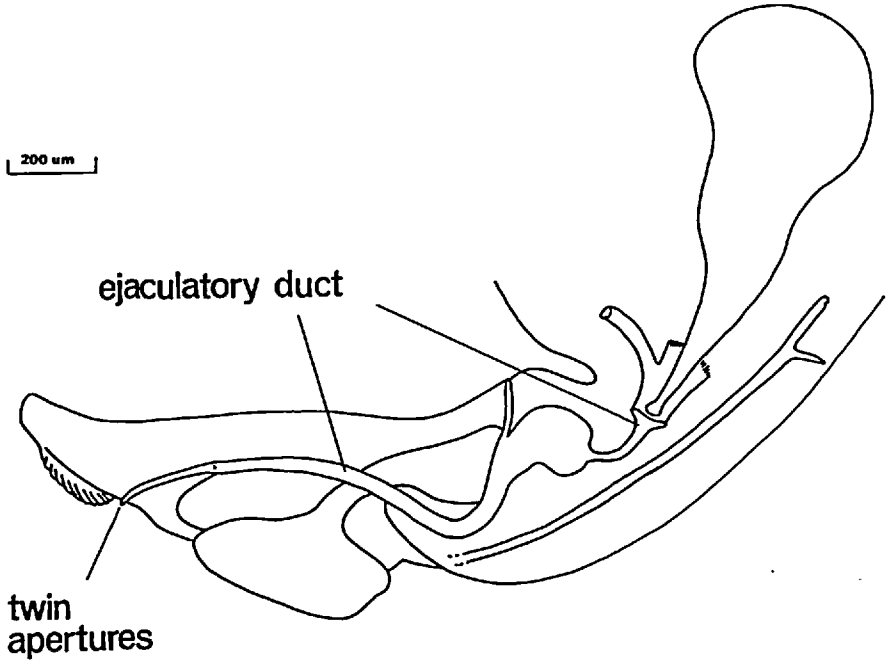


Fig. 42

Fig. 42. Cleared male genitalia of Merodon equestris, with the LHS members of paired structures removed, showing the course of the ejaculatory duct to the paired apertures at the apex of the phallosome.

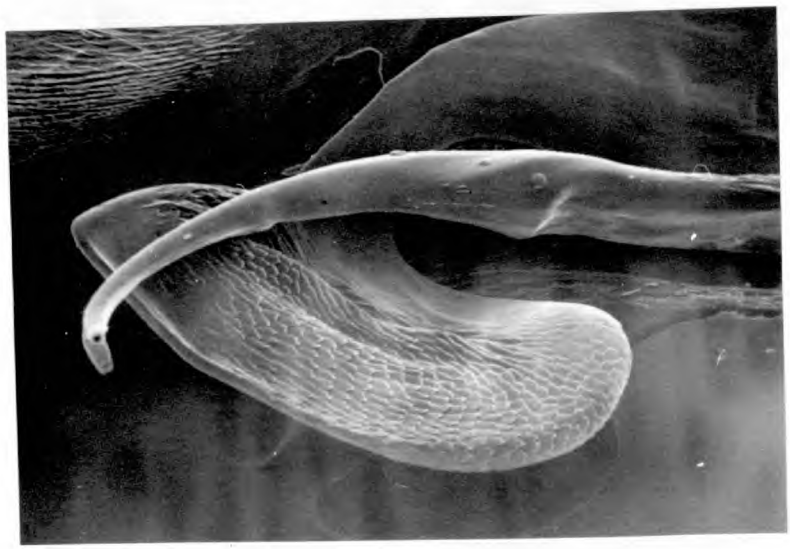


Fig. 43. Phallosome of Merodon equestris, showing twin spermatic apertures at apex. Right internal lobe also shown. (Stereoscan x 180).

Fig. 44. Paired spermatic apertures on phallosome of Merodon equestris (Stereoscan x 1440).

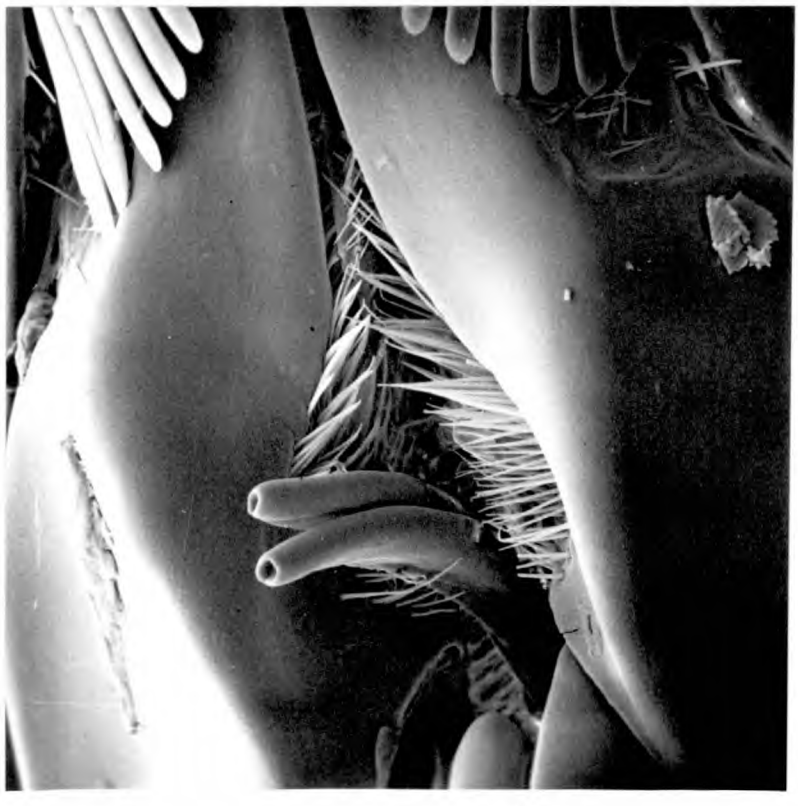


Fig. 45. Phallosome in situ, Merodon equestris (Stereoscan x 360)

Fig. 46. Penis sheath, Merodon equestris (Stereoscan x 120)

Correspondence in dimensions of the male and female parts

Since the male reproductive apparatus is equipped with a pair of seminal ducts and apertures, and the female correspondingly has two broader parts to the spermathecal ducts, it seemed probable that the one fitted into the other at copulation. The sizes of the various parts were measured with the following results:-

TABLE 28.

	Male System	Female System
1.	Greatest internal diameter at seminal orifice ..... 5µm Least internal diameter at seminal orifice 3µm	Internal diameter of upper (narrow) portion of spermathecal duct ...3µm
2.	Greatest external diameter at seminal orifice .....11µm Least external diameter at seminal orifice 9µm	Internal diameter of lower (wider) portion of spermathecal duct ..10µm
3.	Length of free ramus of phallosome 220µm	Length of lower (wider) portion of spermathecal duct 120µm
4.	---	Transverse diameter of small spermatheca ..... 10 units Transverse diameter of large spermatheca .....12.5 units

The correspondence between the diameters of the ducts is good. The ramus tips of the phallosome are narrow enough to be inserted into the broader parts of the spermathecal ducts, and the internal diameter of the seminal orifices agrees closely with the internal diameter of the narrower lengths of the spermathecal ducts, implying that the phallosome rami and the spermathecal ducts would make a continuous tube of nearly uniform bore on insertion.

The rami of the phallosome are longer than the wide parts of the spermathecal ducts, hence penetration right up to the darkened area should be possible. Since the two male orifices are identical in size, one may assume that each delivers the same quantity of sperm. The ratio of the width of a small spermatheca to the width of the large one is $\frac{1}{1.25}$. The cube of this is $\frac{1}{1.953}$ i.e. very close to $\frac{1}{2}$. The volume of the two small spermathecae combined is therefore approximately the same as the volume of the larger one. This is further indirect evidence that the two rami of the phallosome are inserted into the two main spermathecal ducts during mating.

As mating pairs were not available for study, no direct observations were made on how the other male genitalia function during copulation. No doubt both the denticles on the inner lobes, and the tine-like structures on the superior lobes, assist in holding the sexes together at mating.

Discussion

Multiple seminal ducts in the Cyclorrhapha are certainly not common, reports being limited to the Syrphidae and to Sarcophaga (Calliphoridae) (Section 6 of this thesis).

In the Nematocera, Sinton (1925) and Hertig (1949) have described and figured the insertion of eversible spermatic filaments of the male Phlebotomus into the paired spermathecal ducts of the female, and Downes (1968) has reviewed the occurrence of multiple seminal ducts in the greater part of the Nematocera. In the Brachycera, multiple seminal ducts are reported in some species of Asilidae (Reichardt, 1929), and may be inferred in others from the structure of female parts (Owsley, 1946).

Metcalf (1921) gave figures of the male genitalia in many Syrphidae, of which Ceriodes tridens, C. abbreviata, and Xylota bicolor, are all shown as having twin ejaculatory processes. These carry spermatic ducts. Microdon tristis is shown as having paired, but unequal, ejaculatory processes. Metcalf states that the male orifice is often difficult to locate, and may be very minute. Glumac (1960) was aware that the spermatic duct was paired in Merodon. He grouped together all those Syrphidae (Syrphoidea of his paper) that have the "spermatophoric tube even" (this is evidently a mistranslation for 'spermatic ducts double') into the family Merodonidae, to include Merodon, Eumerus, Microdon, and four other genera (he does not include Xylota). The rest of his syrphoids have "spermatophoric tube single". Metcalf (1921) implied that Eumerus strigatus has a single male aperture, however. It is clear that Glumac regards the condition of the male genitalia in his Merodonidae as derivative, not primitive.

Gaunitz (1969) has not followed Glumac in setting apart the Merodonidae from the remainder of the Syrphids, but retains Merodon in the Eristalinae. His figures indicate double ejaculatory processes in M. equestris, M. cinereus, and M. rufus; the lack of detail in his drawings prevent identification of these processes in the other Merodon species he dealt with.

The taxonomic value of the presence of paired spermatic ducts in Syrphidae is therefore uncertain; it may well be that the character has arisen independently several times from the simpler unpaired condition.

No accessory apertures could be identified on the phallosome of Merodon equestris. A test for the mated status of the male along the lines already described for Lucilia sericata cannot therefore be applied.

S E C T I O N 9

THE EVOLUTION OF SPERM TRANSFER MECHANISMS IN THE DIPTERA

The evolution of Sperm Transfer Mechanisms in the Diptera

Downes (1968) has argued that the presence of three spermathecae in the female and of a trifid penis in the male co-adapted to the spermathecal ducts, are basic features of the dipteran design. He regards the spermatophore - using families of the Nematocera (the instances of the Ceratopogonidae, Simuliidae, Chironomidae and Thaumaleidae were known to him) as derivative and not contributing further to evolution in the Diptera.

In the writer's view the proposition concerning the three spermathecae must be accepted. The matching assumption that the trifid penis to be seen in many Nematocera is equally basic is not acceptable, since, given that some flies do have spermatophores, one would expect that in the Diptera as for insects generally, the primitive method of sperm transfer must have been by the use of a spermatophore.

It is widely accepted that spermatophores are a primitive feature of insect biology (Khalifa, 1949; Ghilarov, 1959; Davey, 1960; Hinton, 1964; Alexander, 1964; Gerber, 1970). They are present in the more primitive orders (Collembola, Thysanura, Ephemeroptera, Odonata, and the orthopteroid orders) and they occur throughout the Lepidoptera and Neuroptera (except Coniopterygidae). Some orders have lost them altogether, apparently (e.g., Aphaniptera), while in others the occurrence is irregular, and presumably spermatophores have been independently lost in many different lines. There is no evidence from the Insecta that once a spermatophore has been lost it has ever been re-evolved, though this cannot be said to be an impossibility.

According to this view, therefore, the Culiciformia stand closer to the ancestral flies than do the Tipuliformia etc. with respect to

the mechanism of sperm transfer. Incidently this phylogeny is consistent with another proposition supported by Downes (1958), namely that the ancestral flies were biting, at least in the females, a habit retained by most Culiciformia but lost by many other Nematocera (Downes and Colless 1967). In addition, as reported in this thesis, the Bibionidae use spermatophores, though these flies are not blood sucking and therefore the two habits (blood feeding and the use of spermatophores) are not indissolubly linked. That the bibionids have this feature is further proof that this is the primitive method of sperm transfer in the Diptera. In the instance of Glossina it would appear to be much more reasonable to suppose that the spermatophore is a survival rather than a relatively recent innovation, and this view is adopted here. Opponents of this idea must explain a remarkable reversal in evolution that has taken place, namely that the line of flies leading to Glossina had lost the spermatophore and then later regained one of a large and rather conventional type.

It is interesting to note that the placement of the spermatophore in Glossina is deep within the uterus, whereas in the Culiciformia and Bibionidae it is much more superficial (Nielsen, 1959; Davies, 1965; and present thesis).

This inward migration parallels the better studied instance in the cockroaches (Graves 1969) in which the Blattidae have generally superficial spermatophores, whereas in the Blaberidae they are deeply inserted. The latter family also evolved viviparity, making the parallel with Glossina even more striking. Probably viviparity and deep spermatophore placement are linked in some degree by the development of a uterus forcing the spermathecal ducts (on to which the spermatophore is fixed) into an anterior position. It must be remembered however that deep spermatophore placement is also to be found in oviparous insects

e.g. Lepidoptera, selection having presumably favoured the greater protection given to the sperm thereby. Gerber (1970), drawing examples from throughout the Insecta, has argued that there is a tendency in insect evolution to change from the type of spermatophore produced solely under the influence of the male, to the type that is formed (from male secretions) within the female. In the Diptera this appears to be borne out: bibionids use a spermatophore which is possibly retained by the male; in simuliids etc., the females carry the spermatophore rather superficially, Glossina (a higher fly) has its spermatophore well concealed in the female.

There is next the matter of how the habit of spermatophore transfer became lost, as it has done from most flies. There are at least three possible modes of transition from spermatophore transfer to other mechanisms:-

(i) By the accessory material remaining liquid after ejaculation, not forming a semi-rigid container, so that the sperm are discharged free into the female tract, e.g. Drosophila (Nonidez, 1920).

(ii) By the spermatophore simplifying and reducing in size, becoming more of a plug than a container, e.g. Rhodnius (Khalifa, 1950; Davey, 1958).

(iii) By the progressive reduction of the spermatophore into a narrow extension of the penis apparatus, bridging any gap between the male gonopore and the spermathecae, e.g. the arrangement in Locusta migratoria seems to be of this kind (Gregory 1965 ~~a,b~~) though substantial amounts of the long spermatophore remain within the male.

Modes (ii) and (iii) are not yet known for certain from the Diptera, although a semi-solid pellet is found in the ejaculate of some culicids (Gillies, 1956). It so happens that mode (i) includes one of the earliest instances in which sperm transfer was studied in a dipteran

(Drosophila: Nonidez, 1920) and also the family in which most studies of copulation have been carried out (Culicidae: see review in Clements, 1963). It is easy to imagine how alternative (i) could have subsequently given rise to the type of closed system seen in Lucilia, in which the male accessory secretion is no longer directly concerned with sperm transfer, but is still passed over during mating in some quantity. It is not certain that evolution did take this course, however.

Loss of a spermatophore would have been followed (or accompanied) in many instances by the establishment of a closed system of sperm transfer. In this, the male gonopore delivers sperm directly to the spermathecal ducts, with little or no "searching" required on the part of the sperm and hence with less risk of a failure to inseminate. This mode is known from many insects, but notably Lygaeus (Ludwig, 1926) and Oncopeltus fasciatus (Bonhag and Wick, 1953) in the Hemiptera.

In flies the closed system appears to be the commonest method used:-

(a) Nematocera. Downes (1968) has usefully reviewed the closed systems of the Nematocera and states that the mode occurs throughout the psychodiform and tipuliform families that he examined, and persists into the higher flies.

Sinton (1929) and Hertig (1949) showed that in phlebotomines the eversible bifid aedeagus penetrates well up the two spermathecal ducts; in other groups the more usual triple spermathecal ducts are matched by trifid spermatic ducts in the male (Tanyderidae etc., Downes, 1968), suggesting that the same kind of penetration must occur.

(b) Brachycera. Reichardt (1929) described the trifid penis of some Asilidae and gave figures showing the sexes in copula, with the penis rami fitting into the spermathecal ducts. Owsley (1946) described the internal organs of Proctacanthus (Asilidae) in which,

from the structure of the spermathecal ducts alone, one would infer the penetration of paired spermatic ducts to a point about halfway up the spermathecal ducts, during mating.

(c) Cyclorrhapha Aschiza. Merodon clearly has a closed system (see Section 8). It is reasonable to expect most if not all the Syrphidae to have to have closed systems also. The present view taken in this thesis, that having a single phallosome orifice is a more primitive condition than having multiple ones, could perhaps be tested by a comparative study of the phallosome in Merodon and its close relatives.

(d) Cyclorrhapha Schizophora. Lucilia and other Calliphoridae have closed systems, sperm being pumped to the common entrance to the spermathecal ducts. In Agria affinis the male gonopore is inserted into the rather narrow common spermathecal duct. In some Sarcophaga species the male gonopore has given rise to paired orifices, supplied by a branched ejaculatory duct. In Sarcophaga carnaria group, these paired orifices are large, semi-circular and co-adapted to each other, delivering sperm to the three spermathecal ducts as though from a single orifice. In the absence of evidence to the contrary, one may assume that the lateral filaments conduct sperm in all Sarcophaga (s.l.) species.

So far in this discussion little has been said concerning the transfer of male accessory material, but to judge from the frequently large size of these glands, and from what is known about the physiological action of their contents (see Introduction and Section 5), and from the existence of special accessory ducts and orifices on the phallosome in certain species, this must be treated as an important aspect of mating in flies.

Probably most species which use a spermatophore or other forms of "open" sperm transfer, have a single, simple male gonopore, since all the male products are deposited together (as far as is known) in

the female tract. There are apparently no reports of subsidiary male apertures in Culicidae or Drosophila, though there is a suspicion that they may exist in Glossina (See Section 7).

In a closed system, an intimate co-adaptation exists between the sperm conducting routes of the male and the female. There is no room in such a system for the relatively large quantities of accessory material which are also transferred and therefore there would seem to be a need for some mechanism or device by which the accessory secretion is kept apart from the sperm. In Lucilia and at least some other calliphorids this has been achieved by keeping the secretions apart in time and space. Sperm and accessory secretion pass over at different times during the mating act (Graham Smith, 1939; Donnelly, unpublished observations) and in different apertures on the phallosome (Section 2). In Sarcophaga carnaria group the lateral filaments appear to be the only available exits from the phallosome; both sperm and accessory secretions have been observed emerging from the orifices, but never both products together. The fate and function, and placement in the female, of the male accessory secretion in Sarcophaga has not been studied however.

The possible steps in the evolution of lateral ducts in the phallosome of Lucilia have already been discussed. One may suppose that from the margins of the original simple male orifice, specialised bays arose for the guidance of the different secretions to their destinations in the female, and that there developed into the fully developed distinct orifices already described (Section 2).

In Merodon an accessory aperture has not been identified; there are no distinct male accessory glands. It may be inferred that the original gonopore developed two bays in the upper margin for directing sperm into the spermathecal ducts. The presence of seams and sutures running along the ventral mid-line of each ramus of the Merodon phallo-

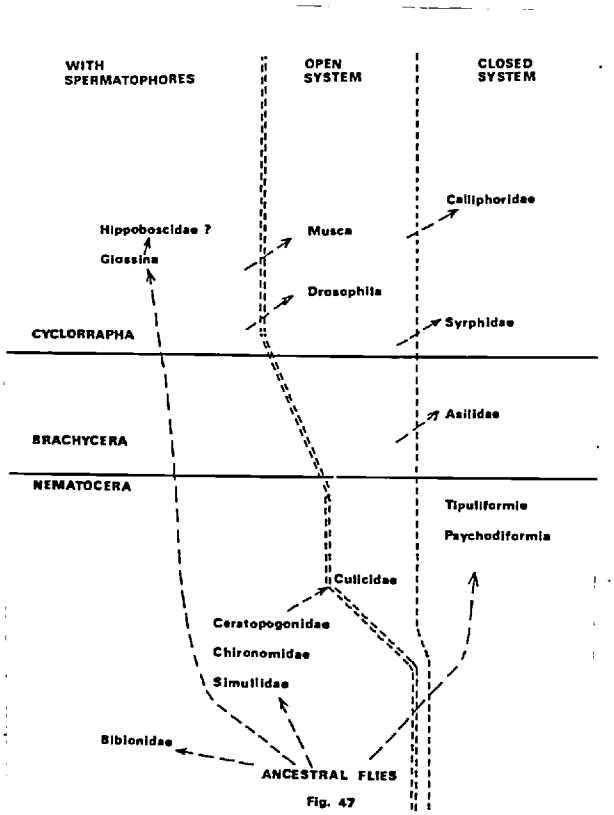


Fig. 47. Tentative scheme showing the evolution of sperm transfer mechanisms in the Diptera.

some, back towards the point of origin of the rami, supports this view.

In the tentative evolutionary scheme given (Fig. 47) the Hippoboscidae have been placed close to Glossina, as there is evidence that the tsetse flies are best regarded as closely related to this family (see Appendix 3 for a discussion of this point) and hence the occurrence of a spermatophore or simple derivative thereof in members of the Hippoboscidae may be predicted.

The evolutionary scheme is provided mainly as a stimulus to the study of sperm transfer mechanisms in the Diptera. The writer is aware that it will have to be revised and perhaps elaborated as more information becomes available.

APPENDIX I

In Section 1 an attempt was made to formulate a model of the mating cage situation. The data was shown to be consistent with the proposed model, and values for K, the mating rate coefficient, could be calculated from the pooled data. An alternative approach to the problem, outlined to the writer by Prof. M. S. Bartlett F.R.S., enables one to test if individual cage results conform to the model. This also leads to the calculation of K values for individual cages. The notes below follow the procedure set out in communications from Prof. Bartlett and his assistant Miss J. Brennan.

The model fitted to each cage was that of random mating where -

Probability (mating in time St) = k. m. f. St.

Where m is the number of virgin males, f the number of virgin females, and k is the mating rate coefficient assumed to be constant.

Cumulative values for m x f x (intervals between matings) are plotted against number of pairs already mated at the beginning of the successive t intervals.

Bounds for the path taken to the end point are obtained by considering the end-point as fixed. 95% confidence bounds are straight lines parallel to the line joining the end-point to the origin and a distance.

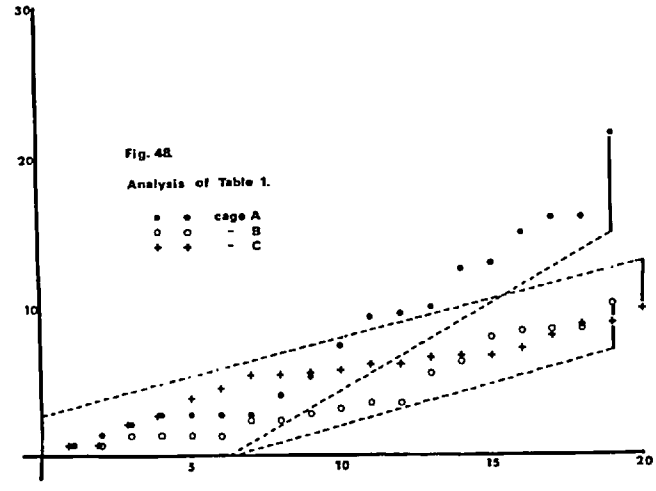
$\pm$ 1.36 s $\sqrt{n}$

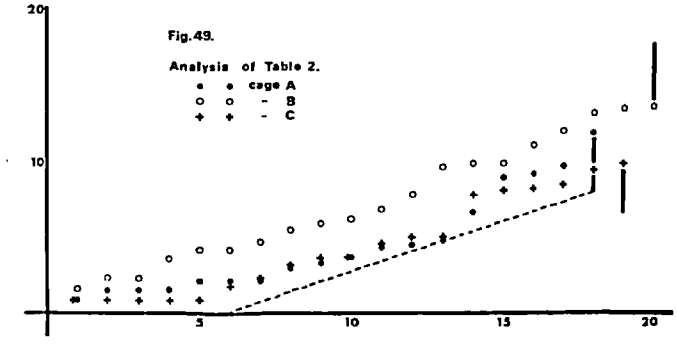
Where s is the value of the slope of the line joining the last point on the graph to the origin, and n is the final number of mated pairs.

The data of all 19 cages has been treated in this way (Figs 48-55) Only one cage (Cage C, Expt 3) has points (two) at or beyond these 95% confidence bounds. Taken as a whole, therefore, the individual cage

results are consistent with the model.

Estimates of k are given by the reciprocal of s. Calculation of confidence bounds for k, the mating rate coefficient, is also possible, but not dealt with here.





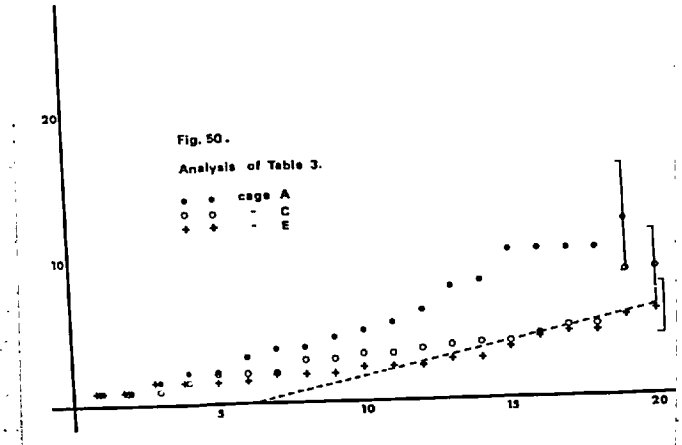


Fig. 50.

Analysis of Table 3.

• • cage A
○ ○ - C
+ + - E

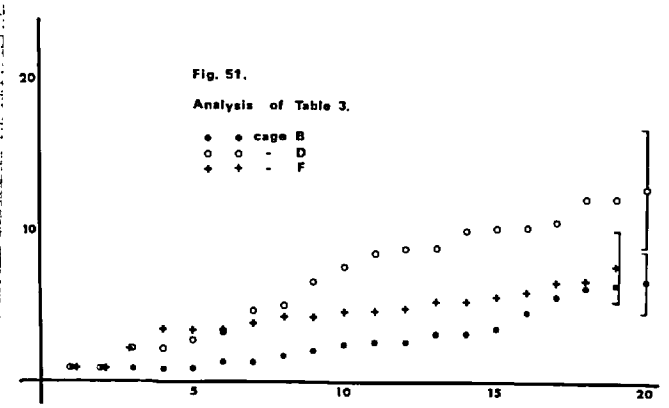


Fig. 51.

Analysis of Table 3.

• • cage B
○ ○ - D
+ + - F

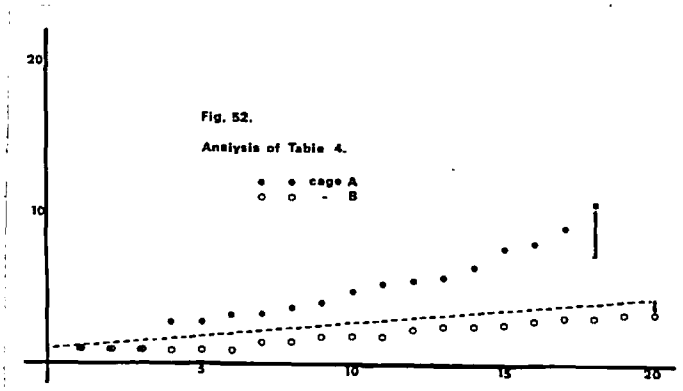


Fig. 52.

Analysis of Table 4.

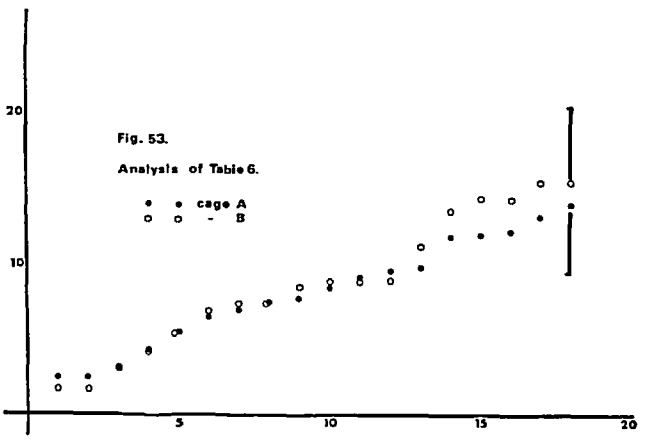


Fig. 53.

Analysis of Table 6.

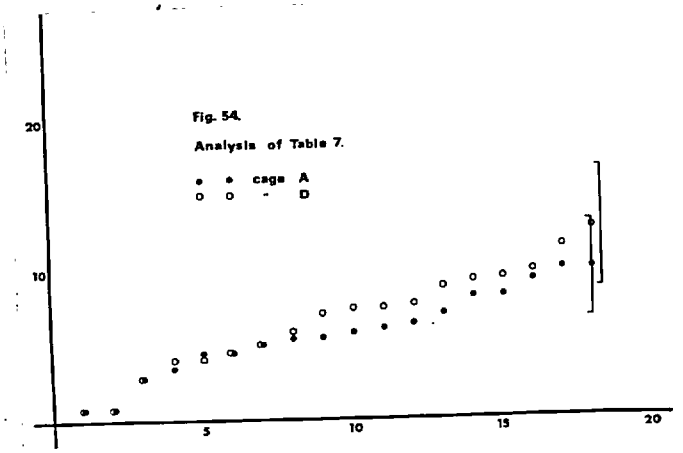


Fig. 54.
Analysis of Table 7.
• • cage A
o o " D

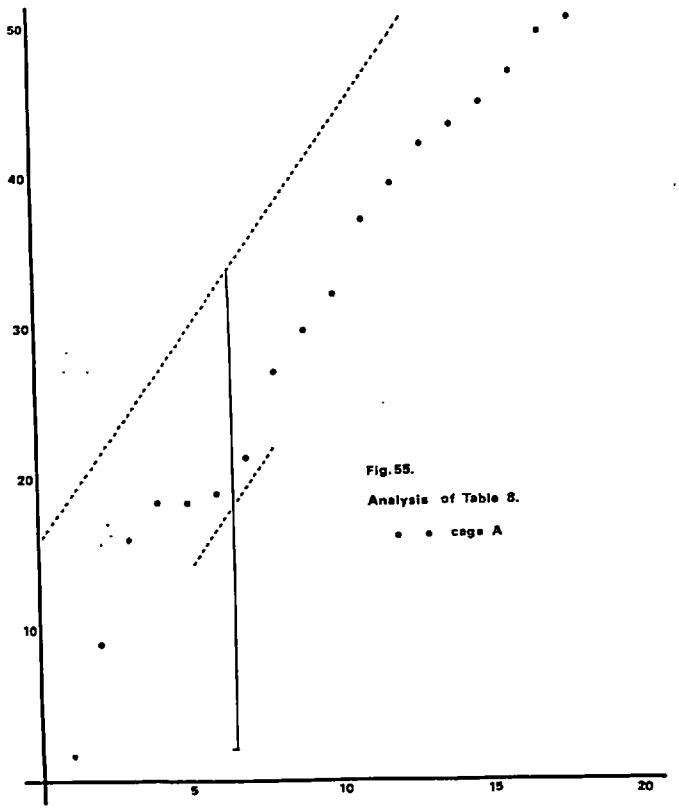


Fig. 55.
Analysis of Table 8.
• • cage A

APPENDIX 2.

Table 1. Times (nearest minute) of onset of mating by successive pairs, in 3 cages (A-C) each containing 20 virgin males and 40 virgin females at the onset

Cage	A	B	C	Total	Average
Pair					
1	1	1	1	3	1
2	2	1	1	4	1.3
3	3	2	3	8	2.7
4	4	2	4	10	3.3
5	4	2	6	12	4
6	4	2	7	13	4.3
7	4	4	9	17	5.7
8	7	4	9	20	6.7
9	10	5	9	24	8
10	16	6	10	32	10.7
11	22	7	11	40	13.3
12	23	7	11	41	13.7
13	25	16	13	54	18
14	38	20	14	72	24
15	41	31	14	86	28.7
16	57	33	17	107	35.7
17	67	34	26	127	42.3
18	67	35	35	137	45.7
19	192	71	38	301	100.3
20	-	-	90	-	-

Table 2. Times (nearest minute) of onset of mating by successive pairs, in 3 cages (A-C) each containing 40 virgin males and 20 virgin females at the onset

Cage	A	B	C	Total	Average
Pair					
1	1	2	1	4	1.3
2	2	3	1	6	2
3	2	3	1	6	2
4	2	5	1	8	2.7
5	3	6	1	10	3.3
6	3	6	3	12	4
7	3	7	4	14	4.7
8	5	9	6	20	6.7
9	6	10	7	23	7.7
10	7	11	7	25	8.3
11	9	13	10	32	10.7
12	10	17	12	39	13
13	11	25	12	48	16
14	21	26	27	74	24.7
15	35	26	29	90	30
16	38	36	30	104	34.7
17	43	46	33	122	40.7
18	74	63	48	185	61.7
19	-	c.70	54	-	-
20	-	76	-	-	-

Table 3. Times (nearest minute) of onset of mating by successive pairs, in 6 cages (A-F), of which A,C, and E each contained 20 virgin males and 40 virgin females at the onset, and B, D and F each contained 40 virgin males and 20 virgin females at the onset.

Cage	A	B	C	D	E	F	Total ACE	Ave ACE	Total BDF	Ave BDF	Total A-F	Ave A-F
Pair												
1	1	1	1	1	1	1	3	1	3	1	6	1
2	1	1	1	1	1	1	3	1	3	1	6	1
3	2	1	1	3	2	3	5	1.7	7	2.3	12	2
4	3	1	2	3	2	5	7	2.3	9	3	16	2.7
5	3	1	1	4	2	5	8	2.7	10	3.3	18	3.0
6	5	2	3	5	2	5	10	3.3	12	4	22	3.7
7	6	2	3	8	3	6	12	4	16	5.3	28	4.7
8	6	3	5	9	3	7	14	4.7	19	6.3	33	5.5
9	8	4	5	13	3	7	16	5.3	24	8	40	6.7
10	9	5	6	16	4	8	19	6.3	29	9.7	48	8
11	11	6	6	19	4	8	21	7	33	11	54	9
12	14	6	7	20	4	9	25	8.3	35	11.7	60	10
13	21	8	8	20	6	11	35	11.7	39	13	74	12.3
14	23	8	9	27	6	11	38	12.7	46	15.3	84	14
15	36	10	9	27	11	13	56	18.7	50	16.7	106	17.7
16	36	19	13	28	16	16	65	21.7	63	21	128	21.3
17	36	30	18	32	19	22	73	24.3	84	28	157	26.17
18	36	39	19	55	19	24	74	24.7	118	39.4	192	32
19	79	42	102	55	42	47	223	74.3	144	48	367	61.17
20	-	55	105	87	62	-	-	-	-	-	-	-

Table 4. Results for two cages, each containing 40 virgin males and 20 virgin females at the start. Total column shows the added results for the following 19 cages: Table 1, A-C; Table 2, A-C; Table 3, A-F; Table 4, A and B; Table 6, A and B; Table 7, A and D; Table 8, A; i.e. all those cages with 40:20 or 20:40 male to female ratio, in which 18 or more pairs mated within the observation period, and which were not subject to any form of selection.

Cage	A	B	Total (19 cages)	Average
Pair				
1	1	1	24	1.26
2	1	1	37	1.95
3	1	1	66	3.47
4	4	1	88	4.63
5	4	1	100	5.26
6	5	1	115	6.05
7	5	2	138	7.26
8	6	2	170	8.95
9	7	3	200	10.53
10	9	3	232	12.21
11	11	3	274	14.42
12	12	5	304	16.00
13	13	6	372	19.58
14	16	6	467	24.58
15	24	7	545	28.68
16	27	9	635	33.42
17	38	11	789	41.53
18	49	12	952	50.11
19	69	19	-	-
20	-	20	-	-

Table 5. Times (nearest minute) of onset of mating by successive pairs in 7 cages (A-G) each containing 30 virgin males and 30 virgin females at the start.

Cage:-	A	B	C	D	E	F	G	Total	Average
Pair									
1	3	2	1	1	1	2	1	11	1.57
2	4	2	2	2	2	2	3	17	2.43
3	4	3	3	2	2	6	4	24	3.43
4	6	4	3	3	3	8	4	31	4.43
5	7	4	4	3	3	11	7	39	5.57
6	7	5	4	3	6	15	15	55	7.86
7	9	6	6	4	8	21	17	71	10.14
8	11	7	6	4	9	24	17	78	11.14
9	19	7	8	4	10	25	17	90	12.86
10	20	8	9	4	14	25	19	99	14.14
11	20	8	13	5	15	26	19	106	15.14
12	24	11	13	7	16	27	32	130	18.57
13	24	11	14	7	16	30	32	134	19.14
14	26	14	14	7	17	31	34	143	20.43
15	27	16	18	7	17	32	36	153	21.86
16	29	20	23	11	20	32	36	171	24.43
17	30	21	27	18	20	42	37	195	27.86
18	50	31	28	20	20	43	39	231	33.00
19	79	37	43	21	23	44	41	288	41.14
20	87	37	44	21	26	45	42	302	43.14
21	93	55	48	33	27	45	43	344	49.14
22	97	62	80	36	29	47	53	404	57.71
23	159	65	91	46	29	48	54	492	70.29
24	-	81	-	55	31	55	60	-	
25	-	93	-	70	33	55	65	-	
26	-	-	-	-	37	58	66	-	
27	-	-	-	-	48	61	70	-	
28	-	-	-	-	68	66	79	-	
29	-	-	-	-	76	-	-	-	
30	-	-	-	-	-	-	-	-	

Table 6. Mating rates of residual males (first experiment). Times (nearest minute) of onset of mating by successive pairs. All cages had 40 virgin males and 20 virgin females at start. Cages A and B were run on Day 1; Cages C and D on Day 2. Cage (males were amalgamated non-mating males from cages A and B; Cage D was a control (see text).

Cage	A	B	C	D
Pair				(Control)
1	3	2	1	1
2	3	2	2	1
3	4	4	2	2
4	6	6	5	8
5	8	8	6	8
6	10	11	7	9
7	11	12	7	10
8	12	12	9	11
9	13	15	12	16
10	15	16	16	17
11	17	16	20	18
12	19	16	21	23
13	20	26	24	50
14	31	39	24	53
15	32	44	31	-
16	33	44	31	-
17	43	56	39	-
18	56	56	-	-
19	-	-	-	-
20	-	-	-	-

Table 7. Mating rates of residual males (2nd experiment).

Cage	A	B	C	D
Pair				(Control)
1	1	1	1	1
2	1	4	1	1
3	4	7	2	4
4	5	11	3	6
5	7	11	4	6
6	7	12	4	7
7	8	12	4	8
8	9	12	5	10
9	9	16	5	13
10	10	18	7	14
11	11	25	9	14
12	12	28	9	15
13	15	37	11	20
14	21	42	13	22
15	21	43	18	23
16	29	43	27	27
17	37	44	29	44
18	37	-	46	61
19	-	-	54	-
20	-	-	-	-

Table 8. Mating rates of residual females. As for Tables 6. and 7, but sex ratio reversed.

Cage	A	B	C	D
Pair				(Control)
1	2	4	19	7
2	12	4	19	11
3	22	5	19	23
4	26	5	46	64
5	26	32	46	72
6	27	33	48	73
7	33	37	57	106
8	46	49	80	109
9	53	49	101	125
10	60	55	104	131
11	76	63	104	171
12	85	69	110	-
13	96	84	113	-
14	102	89	134	-
15	112	97	134	-
16	127	170	167	-
17	154	-	-	-
18	167	-	-	-
19	-	-	-	-
20	-	-	-	-

Table 9. Times (nearest minute) of onset of mating of successive pairs, in a cage containing 80 virgin females and 20 virgin males at t = 0; another lot of 20 virgin males were added at t = 60.

Pair	1st lot of males	2nd lot of males
1	1	1
2	1	1
3	1	1
4	1	1
5	2	1
6	3	1
7	4	2
8	4	2
9	6	3
10	11	3
11	13	6
12	14	13
13	15	16
14	15	16
15	17	17
16	18	18
17	19	36
18	20	46
19	46	-
20	-	-

Table 10. As for Table 9, but with sexes reversed

Pair	1st lot of females	2nd lot of females
1	1	1
2	1	2
3	2	3
4	2	7
5	8	9
6	9	10
7	15	10
8	17	10
9	18	11
10	21	11
11	21	12
12	28	16
13	34	22
14	39	27
15	49	28
16	51	36
17	-	51
18	-	51
19	-	-
20	-	-

Table 11. Age of flies, and ambient temperature, in the experiments in Section 1.

Experiment	Cages	Age of male in days	Age of female in days	Temp °C
1	A-C	7/8	7/8	21.5-23
2	A-C	7/8	7/8	25.5-26.5
3	A-F	7/8	6/7	27.0-28.0
4	A and B	7/8	7/8	26.0-27.0
5	A-D	5/6	5/6	27.0-28.0
6	E-G	no note made		25.0
6ai	A and B	7/8	7/8	28.0
	C and D	8/9	8/9	29.5
6aii	A and B	7/8	7/8	29.5-31
	C and D	8/9	8/9	27.5-29
6b	A and B	6/7	5/6	22.5-24.5
	C and D	7/8	6/7	22.5-26.0
7	One only	6/7	6/7	25.0

Table 12. Calculation of regression line shown in Fig. 13.

x	m-x	f-x	$\frac{m-x}{f-x}$	$\frac{(m-x)}{\log(f-x)}$	X	Y	$\hat{Y}$
0	40	20	2	.301030	.011280	24	19.56
1	39	19	2.052632	.312310	.012200	13	20.60
2	38	18	2.111111	.324510	.013243	29	21.77
3	37	17	2.176471	.337753	.014430	22	23.12
4	36	16	2.250000	.352183	.015792	12	24,65
5	35	15	2.333333	.367975	.017375	15	26.44
6	34	14	2.428571	.385350	.019218	23	28.52
7	33	13	2.538462	.404568	.021399	32	30.98
8	32	12	2.666667	.425967	.024001	30	33.92
9	31	11	2.818182	.449968	.027153	32	37.48
10	30	10	3.000000	.477121	.031034	42	41.87
11	29	9	3.222222	.508155	.035913	30	47.38
12	28	8	3.500000	.544068	.042197	68	54.47
13	27	7	3.857143	.586265	.050557	95	63.91
14	26	6	4.333333	.636822	.062148	78	77.00
15	25	5	5.000000	.698970	.079181	90	96.24
16	24	4	6.000000	.778151	.106456	154	127.04
17	23	3	7.666667	.884607	.156786	163	183.87
18	22	2	11.000000	1.041393			

Calculated regression equation $_{(Y.X)}$ $Y = 1129.26\ X + 6.82$

Analysis of variance table.

	df	ss	ms	F
Explained variance	1	30279.6	30279.6	146.2 ($p<.001$)
Unexplained variance	15	3106.4	207.1	
Total variance	16	33386.0		

APPENDIX 3.

The taxonomic position of Glossina

Introduction

With the knowledge that Bibio and Dilophus use spermatophores (see Appendix 4), the statement that spermatophores occur in the Bibionidae can be made. The genus Glossina is often placed in the Muscidae (Imms, 1954), but this family is so diverse and ill-defined, and Glossina so untypical as a member of it, that drawing the inference that certain Muscidae have spermatophores seemed unwise without some discussion of the taxonomic placing of the genus. Evaluation of published information has led to the conclusion that Glossina shows more affinities to the Hippoboscidae than to the Muscidae, and is best regarded as related to the Hippoboscidae. The evidence is summarised here.

Keys

Buxton (1955) regarded a discussion of the taxonomic position of the genus Glossina in relation to other flies as outside the scope of his survey, referring the reader to Imms (1944) and Smart (1948), and simply accepted that the genus falls within the Muscidae in a wide sense.

Imms (ninth edition, 1957) gives a key for the Calypteratae which does not work for Glossina, since the Muscidae (under which Glossina is treated) is reached by a couplet giving the choice between:-

(a) "Pteropleural and hypopleural bristles present."

and (b) "Pteropleural bristles absent; hypopleural bristles present only in the genus Eginia ..... Muscidae."

Glossina has pteropleural bristles, but no hypopleurals (Austen, 1911).

Smart (1948) confines his attention to flies of medical importance and his key does not therefore contribute to this discussion.

The key in Brues et al. (1932, revised 1954), copes poorly with Glossina. For example, some of the dichotomies which have to be passed before the genus is finally separated from the Muscidae, as the Glossinidae are as follows:-

"Coxae close together ..... ; adults not ectoparasitic upon mammals, birds or bees; rarely viviparous, in which case the new born young are very immature."

"..... post humeral and intra-alor bristles usually both present; .. .... front of male usually narrow or the eyes meeting .....; abdominal spiracles of at least segments two to five located in side margins of tergites, very rarely in the membrane."

The structure of Glossina either differs from these conditions, or formed the unusual instance mentioned in the "escape" clause.

The above remarks clearly indicate that the taxonomic position of Glossina is a neglected field of study.

Viviparous features

Glossina and all the hippoboscids reproduce viviparously, and larvae remain within the enlarged uterus feeding on female secretions until growth is complete. The larva pupates almost immediately after deposition by the female. The female reproductive system is modified by having only two ovarioles per ovary, instead of many (Saunders, 1960; Bequaert, 1953). Ova are shed one at a time from the ovaries, in a strict sequence R^1, L^1, R^2, L^2, ..., where R and L represent right and left ovaries respectively, and the suffixes denote the two ovarioles per ovary (Pratt, 1899; Saunders, 1960, 1962, 1964). There is a specialised region in the uterus, the chorioth&te, which in Glossina is thought to aid the removal of the egg membrane and larval integuments, at hatching and

moulting; in Hippobosca it is present but its function is less certain (Bursell and Jackson, 1957).

In the uterus the larva feeds on secretions from modified female accessory glands (Roubaud, 1909; Hardenberg, 1929). The larvae of both Glossina and at least some Hippoboscidae are polypneustic, i.e. there are "very numerous supernumary stigmata on the surface of the respiratory lobes of third instar ..... larvae" (Bursell, 1955); the similarities between these larvae led Newstead (1918) to comment that "the larvae of the two groups, respectively, are so alike in form and structure that, if studied apart from the adults, one would not hesitate to group them together as members of the same family."

The indirect wing muscles in the thorax of Glossina grow considerably after eclosion (Bursell, 1961). The growth is due to increase in size of already existing musle tissue, not the development of new muscle from previously undifferentiated tissue (Glasgow and Glasgow, 1962). In Lipoptena capreoli, Theodor (1928) showed that the mid-gut of 14-20 day old keds was 4-5 times the length of the same organ in the newly-emerged insect; the growth was by increase in cell size, not cell number. This type of imaginal growth has been regarded as an adaptation to viviparity, as it keeps to a minimum the final weight of the larva, and hence of the load that a pregnant female has to carry (Bursell, 1961).

Blood-feeding and ectoparasitic features

The blood-feeding and ectoparasitic habits are obviously linked, and will be considered here together. In Glossina and hippoboscids both sexes are exclusively blood-feeders, like Stomoxys, but unlike tabanids and the biting Nematocera. The mouthparts have been studied by Jobling (1933) and are similar in the two groups, except that those of Glossina are non-retractile. Bequaert (1953) remarked that this similarity "raises the possibility that both groups are rather closely

related and that the ancestral Hippobossidae may have acquired the present perfected mouth parts, as well as the exclusive blood diet, before they became permanent ectoparasites."

In both groups the cuticle is toughened; spiracles are set in the membranous sides of the abdomen, rather than in the lateral areas of the tergites as in normal for calypterate flies. The mid-coxae of Glossina are separated by the forward growth of the meta-sternum; the coxae of hippoboscida are well separated. Both groups are vectors of trypanosomes (see review for Hippoboscidae by Baker, 1967), and both use warm-blooded vertebrates as hosts, principally.

Testicular form

Of all the organ systems in the body, the male reproductive system is the least likely to be modified as a result of either the viviparous method of reproduction of the blood-feeding/ectoparasitic habit. Consequently the anatomy of this system is of particular interest for the comparison being made here.

The testes of calypterate flies are normally simple in shape, varying from ovoids to somewhat more elongate forms (fusiform, banana-shaped etc.). Glossina, however, has very long, but coiled, tubular testes, in common with the Hippoboscidae. The distinction between the two forms of testes (i.e. simple and coiled tubular) is well displayed by employing the "T-index" of Hori (1960). This is $\frac{\text{greatest width of testis}}{\text{length of testis}} \times 100$. Hori has measured the T-index of 83 species (1125 individuals) of Japanese calypterate flies, selected on the basis of local availability. All his specimens have a T-index of between 10 and 100; but Glossina morsitans, Stenepteryx hirundinis, and Ornithomyia avicularia, measured in the present work, were all found to have a T-index of between 1 and 10.

Concluding remarks

It would seem that the main reason for placing Glossina in the Muscidae is the similarity in the mouth-parts of Glossina and Stomoxys, an otherwise fairly conventional muscid. The similarity of the hippoboscid mouth-parts to those of Glossina is dismissed as a convergence, by this argument.

If it is accepted that the hippoboscids and Stomoxys must be classified apart in different families, as surely they must, it is clear that the two (hippoboscids and Stomoxys) have seen a convergent evolution of piercing, blood-sucking mouthparts, regardless of the taxonomic placing of Glossina. If this feature is consequently left out of consideration, one has to decide which of the following is more important for taxonomic purposes.

(a) the relatively unmodified external features of Glossina and Stomoxys, or

(b) the considerable similarities between Glossina and hippoboscids in adenotrophic viviparity (including all the other matters linked with this, such as larval structure), and in testis shape.

The evidence in (a) above is of a negative nature (i.e. not much external specialisation has taken place). The evidence in (b) is positive and detailed. One should also remember that blood feeding is rare in the Muscidae, but universal in the Hippoboscidae. The ancestors of the present day Hippoboscidae must have passed through a stage in which the external features were less modified for the ectoparasitic life than they are now; such a fly would resemble a tsetse fly even more than do the present day forms. One may conclude therefore that Glossina (as Glossinidae) is better placed near the Hippoboscidae than in the Muscidae.

Spermatophores have yet to be recorded from either the Hippoboscidae or the Muscidae. This may be due simply to lack of information, though

it is certain that Musca domestica does not have spermatophores (Rivàsecchi, 1958). Roberts (1927) makes a passing reference to spermatophores in his description of the male reproductive system of Hippobosca, but it does not seem that he ever saw one, nor did he apparently appreciate that at that date spermatophores had never been observed in Diptera. Nevertheless, one may expect that spermatophores will eventually be found in the Hippoboscidae. The secretion within the male accessory glands of Ornithomyia avicularia coagulates to a firm mass when released into water, following the puncture of a gland (personal observation).

APPENDIX 4.

Spermatophores in Bibionidae

Introduction

Downes's (1968) review of sperm transfer processes in the lower Diptera did not deal with the Bibionidae and it seemed worthwhile to investigate whether or not spermatophores were used by this group.

Spermatophores: occurrence and description

A colleague caught on request some copulating bibionids in the fields and lanes near the Marlborough Downs, and stored them in clean glass tubes till the writer could inspect them. On examination the day after capture, two of the tubes were found to contain expelled spermatophores. Two different species were involved, Bibio marci (L.) and Dilophus humeralis Zett.; identifications were made with the aid of Edwards' (1925) Key.

Curious pocket-like structures found near the orifice of the male reproductive system raised the suspicion that spermatophores might be held there, and that possibly two spermatophores at a time might be formed. A Bibio marci copulating pair caught at Silwood Park, Sunninghill, Berks, parted naturally about fifty minutes after capture. The sexes were placed at once into separate clean tubes and kept for observation. The tube used for housing the copulating pair was examined, but no spermatophore was present. When the other tubes were inspected just over three hours later, the male tube contained a single spermatophore. The next morning no other spermatophore had appeared in either tube. The female was then dissected and all three spermathecae found to contain sperm, but no spermatophore was seen; neither did the male show signs of another one.

Five copulating pairs of Bibio species (probably B. leucopterus) were captured at Silwood Park, and placed in tubes. On returning to the laboratory about half an hour later, all but one pair had parted,

and each disengaged pair had dropped a single spermatophore. The fifth pair was allowed to separate naturally and the two flies at once put into separate tubes. A few minutes later the male discarded a spermatophore.

The spermatophores of all three species are pear shaped, with apparently a single sperm mass within (Fig 56). The spermatophore of Bibio marci was 612 µm along the main axis; that of Dilophus humeralis, slightly damaged in mounting, was approximately 400 µm. In both, the exit is at the narrower end, from which sperm could be seen issuing. The gelatinous capsule is completely transparent, and took up borax camine faintly.

Discussion

From these preliminary observations, which are to be extended next season, the following conclusions can be drawn.

1. Spermatophores are used for sperm transfer in the Bibionidae.
2. One spermatophore is used for any one mating, which is normal in insects.
3. The spermatophore can be expelled a few minutes after the end of mating, at least in one species, and is dropped by the male, apparently.

This is the first record of spermatophores from Diptera not having the blood sucking habit, the Chironomidae being here regarded as a fundamentally blood-sucking group (Downes, 1967). This fact stands in the way of any attempt to conclude that spermatophore use is a by-product of the blood feeding habit; on the other hand it is a strong argument supporting the view that spermatophores are a primitive feature of dipteran organisation. This argument is elaborated in Section 9. Anisopus fuscipennis (Macq.), regarded by Downes (1968) as a Bibionomorph, was inferred by him to use a closed sperm transfer system.

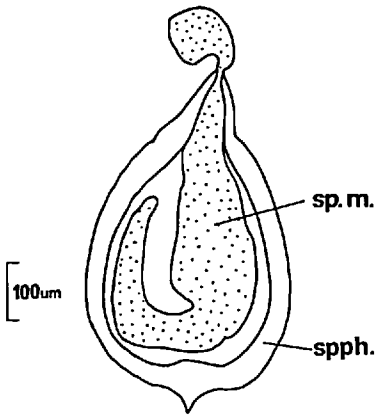


Fig. 56

The possibility that spermatophore is retained by the male is particularly interesting. Such a retention would be previously unknown in Diptera, and is very rare in insects as a whole. Males of some Acridiidae retain part of the spermatophore for a time after sperm transfer. If confirmed (more observations are certainly required) it may well represent the earliest stage in the evolution of spermatophore transfer, coming before the first of Gerber's (1970) four stages of evolution of spermatophore formation.

ACKNOWLEDGEMENTS

The writer is much indebted to all those who have assisted in the completion of the present work.

Mr. J. Donnelly and Mr. D. Tarry of the Central Veterinary Laboratory, Weybridge, Surrey, contributed the original supply and periodic replenishment of Lucilia sericata stocks.

Dr. T.A.M. Nash, C.M.G., O.B.E., sent stocks of Glossina austeni and G. morsitans.

Dr. P.F.L. Boreham and the Staff of the Immunology Laboratory, Imperial College Field Station, Sunninghill, Ascot, Berks, gave useful advice and much practical assistance in the carrying out of precipitin tests.

Mr. R.H. Williams took the photographs which appear in Fig. 40 and Fig. 41 of this thesis; he also photographed all the line figures.

Mr. J. Donnelly very kindly made available to the writer certain unpublished observations on mating in L. sericata; these have been referred to in the text.

Professor J.S. Kennedy, F.R.S. made valuable criticisms of an earlier draft of the mating rate work; Professor M.S. Bartlett, F.R.S., very kindly explained a statistical technique set out in Appendix 1.

I am most grateful to my supervisor Dr. C.T. Lewis, whose encouragement and shrewd advice throughout the work have been much appreciated; he also suggested that my general interest in mating in insects should take the form of a study of mating in Lucilia sericata, in particular.

I thank Professor T.R.E. Southwood for providing laboratory facilities at Imperial College Field Station, Silwood Park, Sunninghill, Ascot, Berks.

SUMMARY

Mating rates

When virgin male and female flies (Lucilia sericata Mg.) are put together in cages and mating pairs tubed out as they form, mating is at first brisk, but the rate declines later in a regular fashion. A model of the mating cage situation has been formulated, based on the hypothesis that the probability of a mating event occurring within a given time interval is proportional to the density of the remaining males and to the density of the remaining females, both factors operating independently. The mating rate data are shown to be consistent with such a model. Also, it is predictable from the model that reversing the sex ratio used in mating cages should make no difference to the overall mating rate, and experimentally this has been shown to be the case. Experimental results have also been analysed using stochastic methods.

Functional morphology of genitalia

The alignment of male and female parts during copulation in Lucilia sericata has been described. Lateral phallosome ducts and exits have been fully described and illustrated. They conduct the granular male accessory secretion to the appropriate site in the female. These ducts and exits are here regarded as specialised elaborations of the original gonopore which now consists of three openings, the median one being for sperm only, not for conducting accessory secretion. This is the first insect for which separate exits for sperm and accessory secretion have been described. Sperm probably reach the end of the ejaculatory duct by the work of the ejaculatory pump; from this point the individual sperm perhaps assist their own passage up the spermathecal ducts. Lateral phallosome ducts in other species and genera of Calliphorinae

have been figured; they are probably universally present in the genus Lucilia, and at least widespread throughout the subfamily.

Mated status of male

The persistence of granular male accessory secretion in the lateral phallosome ducts in mated males has been observed and made the criterion of a test for the mated status of male L. sericata; virgin males very seldom have such adhering secretion. The test is shown to have about 95% accuracy up to 8 days after mating. It is the first test of its kind not only for the Diptera, but for any insect of medical and veterinary importance.

Sterility and the mated status test

The sterility of tepa-injected males was clearly dose dependent, but on the contrary the mating ability of the treated flies was not altered over the range of doses used. Injection of 4.32 µg tepa per male fly induced 95-97% sterility. The mated status test was valid for sterilised flies, and in principle therefore the test could be used on sterile, released and recaptured males to see if they were capable of mating with wild females under field conditions.

Male accessory secretion

The male accessory secretion was found to contain protein, at least in the non-granular fraction. An antiserum against the secretion was prepared, and used to detect the presence of secretion in females up to a week after mating. Implantation of male accessory glands into virgin females, and injection of male accessory secretion alone into virgin females, reduced female receptivity towards mating attempts by males but did not eliminate it altogether. The general damaging effect of this implantation and injection of secretion into females could not be clearly distinguished from a possible specific action against receptivity. It was concluded that any male accessory secretion circulating in the female

haemocoel as a result of mating could not be the sole cause of the degree of monogamy seen in this species.

Comparative work on other flies

The phallosome of Sarcophaga carnaria (L.) group spp. is shown to have two symmetrical apertures, one on each of the lateral filaments, through which both sperm and (probably) accessory secretions pass, though apparently at different times. No median aperture could be found and is almost certainly absent. This is the first report of twin spermatic apertures in calypterate flies. The position of the phallosome during copulation is illustrated.

The spermatophore of Glossina austeni Newstead is described for the first time. It is composed mainly or entirely of male secretions, and is expelled from the female after sperm have entered the spermatheca; this expulsion is usually within 24 hours of the onset of mating. The presence of an expelled spermatophore within the tube housing a female tsetse fly can be taken as evidence of the successful insemination of that female, a finding which should be of value in studies involving sterile males. It is argued that Glossina should be regarded as being closely related to Hippoboscidae, and that therefore spermatophores or their simple derivatives eventually will be found in that family. Spermatophores have not previously been reported from outside the Nematocera.

The phallosome of Merodon equestris F. (Syrphidae) was found to have twin orifices which are co-adapted to the openings of the spermathecal ducts, implying a closed system of sperm transfer. One male orifice serves a single large spermatheca; the other serves two smaller spermathecae, the combined volumes of which are approximately the same as that of the larger one. Insertion of multiple spermatic ducts into the spermathecal duct has not previously been reported from any cyclorrhaphous fly.

The discovery of spermatophores in Bibionidae is recorded.

Evolution of sperm transfer mechanism in the Diptera

Various observations on sperm transfer in Diptera made in the present work, and others that have appeared in the literature, have been collated. A tentative scheme delineating the evolution of sperm transfer mechanisms in Diptera has been proposed, based on the premises that spermatophores were used by the ancestral flies, and that multiple phallosome exits is a derived, not a primitive, condition.

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