Costs of reproduction in female *Drosophila melanogaster*

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This thesis has been composed by me and the work contained within it is my own.

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Abstract

The experiments presented in this thesis investigated the mechanisms of costs of reproduction in female *Drosophila melanogaster.*

There was no evidence to support the existence of seminal feeding, as remating was not more beneficial for females when they were nutritionally stressed. Continual exposure to males did not lead to an increase in female reproductive success, suggesting that females were not maximising their reproductive output by frequent rematings. Females that remated often suffered a cost of mating, as a decrease in lifespan and reproductive success. The results also suggested that nutrition itself did not affect remating frequency, and that either the rate of egg-laying or the number of sperm in store accounted for differences in remating probability.

Females exposed to males that could not transfer sperm also suffered a cost of mating, suggesting that sperm was not costly; so the cost of mating is probably due to mating itself or the transfer of disease, parasites or accessory fluid. One of the accessory fluid sub-components, the sex peptide (SP), has previously been shown to cause an increase in fecundity and a decrease in female sexual receptivity following transfer at mating; it was therefore a candidate for contributing to a cost of receiving accessory fluid. However, whilst the injection of male accessory gland extract into females significantly reduced female lifespan and lifetime reproductive success, there was no evidence to suggest that the SP contributed to this cost.

Several experiments explored the possibility of a correlation between the rate of sterile egg-laying and SP-usage in virgin females, as would be predicted if there were receptors for the SP on the ovary, or some other site controlling oviposition rate. There was no evidence to suggest that this was the case. The correlation between the rate of fertile egg-laying and receptivity previously reported for mated females may therefore be an effect of sperm-depletion rates or some other consequence of mating and not of egg-laying rate *per se.*

Female *dunce* mutants were used in several experiments. *dunce* females have previously been reported to be more susceptible to the cost of mating than wild-type females and *dunce* females also remate at high levels. *dunce* flies cannot regulate intracellular cAMP levels, important in peptide hormone action; they also have memory defects. There was no evidence from the *dunce* experiments to suggest that a single mating had an adverse effect on the lifespan of *dunce* females; their increased susceptibility to the cost of mating therefore seemed to be a consequence of their high

remating rate. *dunce* females did not show rejection behaviour towards courting males after SP injection; this was not due to their memory deficiency.

To study further the effects of the functions of accessory fluid components, null-mutations are required because, although injection experiments can be used to identify the functions of accessory fluid components, they do not perfectly mimic the effects of the transfer of an ejaculate at a real mating. A mutagenesis screen for SP-null mutations was performed, using X-ray and P element-mediated techniques. A number of SP-null candidate lines were produced.

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Chapter 1. General Introduction

1.1 Life Histories

A life history is defined as the combination of age-specific survival probabilities and fertilities characteristic of a population. Life histories vary enormously; lifespan and fecundity can vary over several orders of magnitude and the age of first reproduction, which is itself highly variable, may mark the first of a number of breeding episodes or may herald a single burst of offspring production. For example, many trees have high survival rates from year to year and may only start to reproduce after several years, increasing the number of seeds in every subsequent year (eg. Harper and White, 1974). Most small birds reproduce in the year after hatching and continue to reproduce every year until death with an approximate probability of survival to the following year of only 50% (eg. Perrins, 1979). Pacific salmon (eg. *Oncorhynchus gorbuscha*), on the other hand, have a single suicidal burst of reproduction in their third year of life.

Studying the evolution of life histories may help to explain their almost bewildering diversity. An evolutionary ideal organism, or 'Darwinian Demon' (Law, 1979a) would produce offspring at a maximum rate during an infinite lifespan. This is clearly not possible. The study of life histories may clarify some of the constraints that prevent organisms from achieving this optimal life history. It is of interest to discover the factors that determine the maximum reproductive rate and the constraints on length of life and growth rate during development.

The life history leading to the greatest fitness is the one that maximises the per copy rate of increase (r) for the gene that produces it. r is the intrinsic rate of increase, or Malthusian parameter. The relationship of r to the primary life history parameters, survival probability and fertility, can be described by the Euler-Lotka equation:

$$1 = \int_0^w l_x m_x e^{-rx} dx$$

where x = age, l_x = survival probability to age x, m_x = fertility at age x and w = age at last breeding (Charlesworth, 1980; Lande, 1982).

The Euler-Lotka equation predicts that all organisms should ideally evolve as 'Darwinian Demons' (Law, 1979a) and since this does not occur, there must be constraints that prevent all of the life history variables leading to high r from being simultaneously maximised. There are two reasons why only certain constrained combinations can be realised in practice: characters that alone increase r might make the organism more vulnerable to ecological hazards such as predation or parasitism, and there may also be constraints internal to the organism that put limits on what can be achieved. The second of these possibilities is the main subject of this thesis. The evidence for the importance of ecological hazards and internal constraints is reviewed in the following two sections.

1.1.1 Ecological Hazards

The risks associated with many reproductive activities may lead to a negative correlation between reproduction and survival. These risks include:

An increase in the predation risk during courtship and mating.

Risk of injury to the female by the male.

Risk of disease or parasite transmission.

In the Phasmatodea (stick insects) copulations are often very long or frequent and males often cling to females during this period. Predators may be deterred by clinging males who can release liquid or volatile substances from exocrine glands (Bedford, 1978), eject crop contents (Key, 1957) and may even use femoral spines to

fend off predators (Bedford, 1978). A recent study (Arnqvist, 1989) showed that in the water strider, *Gerris odontogaster,* matings increased both predation risk for females and the time/energy investment in carrying passive males. There were no demonstrable balancing benefits for the female in mating multiply. Female *G. odontogaster* theoretically need only remate every 10 days for maximum survival and fecundity, but are observed to mate several times every day.

An increase in the risk of predation during mating has been demonstrated in the firefly *Photinus collustrans* (Wing, 1988). Conversely, copulating pairs of the large milkweed bug *Oncopeltus fasciatus* seem to avoid predation more successfully; it was suggested that this was because mating pairs of this aposematic insect produced a greater impression on predators (Chaplin, 1973). Female damselflies (*Enamella hageni*) can 'exchange' matings for male guarding service during the periods when they are ovipositing under water (Fincke, 1984).

Activities such as nest-guarding in fishes (eg. *Gasterosteus aculeatus,* Pressley, 1980) and distraction displays in birds (eg. Armstrong, 1942) have also been found to be costly. The nuptial colouration in a cypriodont fish (*Notobranchius guentheri*) was found to carry an increased risk of predation (Haas, 1976). During the mating season, female garter snakes (*Thamnophis sirtalis*) are often swamped by males competing for matings and suffer increased predation under these circumstances (Gibson and Falls, 1975). Gravid or ovigerous females may also be more susceptible to predation eg. in the lizard species *Anotis maccoyi*, *Lampropholis guichenoti*, *Leiolopisma coventryi*, *Leiolopisma entrecasteauxii* and *Sphenomorphus tympanum* (Shine, 1980); a copepod, *Cyclops vicinus* (Winfield and Townsend, 1983); *Daphnia galeata mendotae* (Mellors, 1975) and *Daphnia pulex*; (Koufopanou and Bell, 1984). The aggressive behaviour adopted between male blackbirds (*Turdus merula*) to obtain mates often continues after a mate has been won, and is subsequently directed towards the female and may obstruct nest building (Snow, 1958).

Female dung flies (*Scatophaga stercoraria*) may be drowned in dung by competing males (Blum and Blum, 1979). Lloyd and Park (1962) postulated that at least some of the female mortality in flour beetles (*Tribolium*) could be due to injury at mating. In water striders (*Gerris* spp.) pre-copulatory encounters are often aggressive and the female may often be injured or even killed (Wilcox, 1979, 1984; Hayashi, 1985; Arnqvist, 1988). Ikeda (1974) described a strain of *Drosophila mercatorum* with an unusually high remating rate; female fitness seemed to be reduced because of the large number of encounters with males.

The transfer of parasites at mating can incur a reproductive cost in several ways: parasitised individuals are often less vigorous (eg. Freeland, 1981) and may be less able to secure a territory (Jenkins *et al.*, 1963; Borgia, 1986); parasites may also deleteriously influence both habitat selection (Rasmussen, 1959) and the expression of male secondary sex characters important in mate choice (Wulker, 1964; Baudoin, 1975). Female *Drosophila testacea* parasitised by the nematode *Howardula aoronymphium* produce very few eggs and may even be completely sterile (Jaenike, 1988). *Drosophila melanogaster* females infected with a microsporidium (*Nosema kingi*) laid significantly fewer eggs, produced fewer offspring and had significantly shorter lifespans than non-parasitised flies (Armstrong and Bass, 1989).

1.1.2 Internal Constraints

If life history traits are competing for a share of a finite resource pool, then it will be impossible to maximise them all simultaneously. An important type of internal constraint is the 'cost of reproduction' (see reviews by Reznick, 1985; Bell and Koufopanou, 1986; Partridge and Harvey, 1988), which would be expected to constrain the combinations of reproduction with other life history traits, ie. growth and somatic maintenance. Determining the nature of the costs of reproduction in female *D. melanogaster* is the main subject of this thesis.

Many organisms resorb eggs and embryos under certain conditions, as would be predicted if resources are limiting (Bell and Brehm, 1975). The diversion of resources away from somatic function as a result of reproductive activity occurs in many plants (Harper and White, 1974) and animals (Comfort, 1979). The 'jellied flesh' appearance of many flatfish following spawning has been shown to be due to the diversion of resources from somatic to reproductive function (eg. Templeman and Andrews, 1956). In starlings, (*Sturnus vulgaris*) the production of larger clutches has been shown to be more energetically costly (Biebach, 1981). Reproduction may also cause loss in parental body weight in some species of Urodelean amphibia (Tilley, 1972; Fitzpatrick, 1973). In red deer (*Cervus elaphus*), females giving birth to calves in one season subsequently had lower survival and fecundity than non-reproducing females (Clutton-Brock *et al.*, 1982).

In many perennial plants, resources are alternately allocated to growth and reproduction, but seldom to both simultaneously (Harper and Ogden, 1970; Harper, 1977; Sohn and Policansky, 1977); these findings are consistent with the idea that growth and reproduction are competing for shares in a finite resource pool. A reduction in growth rate due to the drain of resources into gamete production has been shown to reduce the future breeding potential of a terrestrial isopod, *Armadillidium vulgare* (Lawlor, 1976). If mated female *Corixa punctata* are starved, they degrade their flight muscles irrevocably to obtain resources to maintain egg-production. Starved virgin female *Corixa punctata* preserve their flight muscles and allow their gonads to shrink and, perhaps as a result, live longer than mated females (Calow, 1977).

In a wide variety of organisms, reproductive activity inhibits growth and therefore high rates of current reproduction could inhibit future reproduction through the correlation between size and fecundity. This has been demonstrated for many species, including the bivalves *Cerastoderma edule* and *Modiolus modiolus* (Seed and

Brown, 1978), an isopod, *Armadillidium vulgare*, (Brody *et al.*, 1983), a chaetognath, *Saggitta elegans*, (McLaren, 1966), two species of barnacle, *Elminius modestus*, (Crisp and Patel, 1961) and *Balanus balanoides* (Barnes, 1962), several species of *Pardosa* spiders (Kessler, 1971), a decapod, *Palaemonetes varians*, (Jeffries, 1964), a cumeacean shrimp, *Diastylis quadrispinosa*, (Corey, 1983), several species of fish, *Salmo trutta* (Bagenal, 1969), *Pungitius pungitius* (Griswold and Smith, 1973), *Pseudopleuronectes americanus* (Tyler and Dunn, 1976) and *Thalassoma bifasciatum* (Warner, 1984), a perennial herb, *Podophyllum pellatum*, (Sohn and Policansky, 1977) and several species of trees, *Pseudotsuda menziesii*, *Abies grandis* and *Pinus monticola* (Eis *et al.*, 1965) and *Betula alleghaniensis* and *Betula papyrifera* (Gross, 1972).

Several studies have investigated the negative effects of growth on reproduction. The majority of these studies have used insects polymorphic for the presence or absence of wings. Tanaka (1976) showed that micropterous (small winged) *Pteronemobious taprobanensis* females (Orthoptera) had significantly more mature oocytes in the first 10-20 days of life than macropterous (larger winged) females. Removal of the wings of macropterous females caused regression of the flight muscles, and the number of early oocytes that matured increased. Roff (1984) observed a significant increase in the number of eggs laid by *Allonemobius fasciatus* (Orthoptera) females following natural dealation.

Many experiments have compared female longevity under different mating regimens; in many species of arthropods and nematodes, virgin females have been shown to live longer than inseminated females: eg. *Ephestia kuhniella*, Norris, 1933; *Periplanata americana*, Griffiths and Tauber, 1942; *Dysdercus fasciatus*, Clarke and Sardesi, 1959; *Tribolium castaneum*, Sonleitner, 1961 and Mertz, 1975; *Agonum fugliginosum* and *Agonum thoreyi*, Murdoch, 1966; *Trogoderma parabile*, Loschiavo, 1968; *Panagrellus redivivus*, Abdulrhaman and Samoiloff, 1975; *Corixa punctata*,

Calow, 1977; *Melanopus sangiunipes*, Dean, 1981; *Artemia* (unidentified spp), Browne, 1982; *Mesocyclops frontella*, Feifarek *et al.*, 1983; *Agromyza frontella*, Quiring and McNeil, 1984; *Diplogasteritus nudicapitatus, Paroigolaimella bernensis* and *Rhabditis curvicaudata,* Woombs and Laybourn-Parry, 1984. In *Aedes aegypti,* repeated addition of young males to a group of females shortened female lifespan compared to that of virgin females, in the absence of differences in egg-production between mated and virgin females (Liles, 1965).

The evidence therefore suggests that, in many species, some consequence of mating or association of the sexes has an adverse effect on female lifespan or future fertility.

1.2 Measuring Internal Constraints

Fisher (1930) suggested that individuals of a certain age would have a 'reproductive value' defined as the mean amount of expected future reproductive success for individuals of that age and sex in a population. Natural selection will act to maximise the reproductive value of an organism at each age by appropriate allocation of resources to growth, maintenance and reproduction (Fisher, 1930; Tinkle, 1969; Gadgil and Bossert, 1970; Schaffer, 1974a, b; Pianka and Parker, 1975; Charlesworth, 1980; Caswell, 1982). For example, it may be adaptive to postpone reproductive effort, if the advantage gained by extra allocation of resources to current growth produces a significant gain in future survival or fertility, compensating for the loss of current offspring and the risk of losing future offspring by death during the period of delay.

Evaluating internal constraints is therefore essential to adaptive accounts of life histories, but how best to measure costs empirically is a contentious issue (Partridge and Harvey, 1985, 1988; Reznick, 1985; Bell and Koufopanou, 1986; Reznick *et al.*, 1986; Van Noorwijk and de Jong, 1986; Nur, 1988; Reznick, 1992; Partridge, 1992). Several different approaches have been used to measure costs, and much of the

contention has arisen as a result of the use of inappropriate methods. The methods that have been used are outlined below:

1) Phenotypic manipulations.

2) Measurement of phenotypic correlations without manipulation.

3) Genetic experiments.

(a) genetic correlations between life history variables.

(b) correlated responses to selection.

4) Species and population comparisons

Manipulating the reproductive rates of organisms assigned randomly to groups in similar environments (approach 1 above) does usually reveal costs in future survival and fertility (Partridge and Harvey, 1985; Reznick, 1985; Bell and Koufopanou, 1986; Nur, 1988). The extent of the cost revealed depended upon the environment under which it was measured (Reznick, 1985; Bell and Koufopanou, 1986). Several field studies on birds have also revealed costs of reproduction using phenotypic manipulations of brood size (Nur, 1984a, b; Slagsvold, 1984; Winkler, 1985; Finke *et al.*, 1987; Roskaft, 1985; Lessels, 1986; Reid, 1987; Gustafsson and Sutherland 1988). Phenotypic manipulations can demonstrate how costs of reproduction are manifested in real organisms and give some indication of the magnitude of the cost.

The correlational approach (2) without manipulation has generally been the least useful (Partridge and Harvey, 1985; Reznick, 1985; Bell and Koufopanou, 1986). The majority of correlational studies measured fertility and survival in organisms allowed to reproduce at their normal rate. In such studies it is very difficult to determine the relationship between the different life history variables, because any observed correlation may be caused by a common correlation with another uncontrolled factor. Partial correlation analysis has been used to try and overcome this problem, but even

then, it is not possible to be certain that the correct covariates have been chosen. Individuals that are poor competitors (as a result of their own poor phenotype) may inhabit poor habitats that might depress fecundity and survival. A correlational approach would therefore record a spurious negative cost of reproduction in this case, or underestimate a real one.

Perhaps the most contentious issue is the use of phenotypic as opposed to genetic experiments to measure costs of reproduction. It has been argued that the demonstration of genetic correlations between life history parameters (approach 3a above) gives the only valid evidence for costs (Lande, 1982; Reznick, 1985, 1992; Rose and Charlesworth, 1981a). Genetic correlations between life history variables can be derived from the resemblance between relatives, using a method similar to that used to calculate heritabilities, and can be used to predict short-term correlated responses to selection. Genetic correlations can be obtained directly from selection experiments or from breeding experiments (Falconer, 1981a). Genetic correlations between life history variables give a measure of the degree of pleiotropy, eg. a negative genetic correlation between early fecundity and longevity would imply that on average, mutations that increase fecundity also decrease longevity (Reznick, 1985). Law (1979b) estimated life history variables in meadow grass (*Poa annua*) families from stable and disturbed habitats. There was a significant negative correlation between the number of influorescences in year 1 and the number in year 2 and a significant negative relationship between the number of influorescences in year 1 and longevity. These results suggest a cost of reproduction in terms of future survival or fecundity.

Correlated responses to selection (approach 3b above) are measured in selection experiments. For example, Rose and Charlesworth (1981b) reported a decrease in the early fecundity of lines selected for late age reproduction using old adults as the parents for each successive generation. The measurement of correlated responses to selection is undoubtedly central to the study of life history evolution, however there are difficulties

with this approach. Even in the laboratory, the measurement of quantitative traits may be imprecise, and might be even more so in field situations (Falconer, 1981b). The genetic variation in the rate of reproduction is generally much lower than can be produced by phenotypic manipulations (Rose, 1984; Luckinbill, 1984; Luckinbill *et al.*, 1987; Palmer and Dingle, 1986; Dingle *et al.*, 1988). To investigate life histories in field populations, it is necessary to derive relationships relating current to future reproductive potential; the amount of genetic variation may not be of sufficient magnitude to do this (Partridge and Harvey, 1988). Another problem with selection experiments is that it is possible inadvertently to apply selection pressure for another character, eg. in selecting for young breeding adults, there may be considerable inadvertent selection pressure for a decrease in development time (Roper *et al.*, in press).

Artificial selection experiments examine the form of sustained correlated responses to selection that arise mainly from standing genetic variance. To be able legitimately to describe the form of selection responses, the genetic correlation between two characters should remain constant as selection proceeds. There is no evidence to support this view; the genetic correlation between two traits may not remain constant as new mutations arise (Partridge and Harvey, 1988). Selection experiments allow the measurement of maximal rates of evolution and genetic correlations; they also have the advantage that they can be replicated, as can breeding and phenotypic manipulation experiments.

It is not entirely clear whether the results of selection experiments can legitimately be extrapolated to natural situations. Population cages used in many *Drosophila* selection experiments are artificial environments; it is possible that behavioural adjustments of organisms constrained within population cages could alter the evolutionary outcome of the experiment. The results of selection experiments are also only valid for the environment in which they were measured (Falconer, 1981a).

For example, Service and Rose (1985) reported that the strength of the negative genetic correlation between early fecundity and starvation resistance in *D. melanogaster* was significantly reduced when measured in a novel environment. Considerable changes in the magnitude of the correlation between life history variables and even reversals in the direction of correlations at different temperatures have also been reported (Giesel *et al.*, 1982).

Selection experiments therefore do not necessarily indicate what will happen in natural situations (Huey *et al.*, 1991). Genetic experiments can however be used purely to demonstrate that costs exists (Sokal, 1970; Lints and Hoste, 1974, 1977; Lints *et al.*, 1979; Mertz, 1975; Rose and Charlesworth, 1981a, b; Rose, 1984; Luckinbill, 1984; Luckinbill and Clare, 1985; Luckinbill *et al.*, 1987; Arking, 1987). When the results of phenotypic manipulation experiments are consistent with those from selection experiments they are a very powerful experimental tool. Both phenotypic manipulations and selection experiments were used in the experiments described in this thesis.

Species comparisons (approach 4 above) have also been used to demonstrate costs of reproduction. In several groups of animals, negative correlations between high reproductive output and repeated breeding have been reported; eg. in several species of triclads, *Dendrocoelum lacteum*, *Polycelis tenuis* and *Dugesia lugubris*, (Calow and Woollhead, 1977), two species of mites, *Phytoseiulus persimilis* and *Ambyseious andersoni* (Amano and Chant, 1977), many species of lizards, eg. *Anolis* spp., *Cnemidophorus* spp. and *Sceloporus* spp. (reviewed in Tinkle, 1969) and several species of birds, including *Larus argentatus*, *Larus ridibundus*, *Larus hyperboreus*, *Tyto alba*, *Athene noctua*, *Strix aluco*, *Erithacus rubecula*, and *Turdus merula* (Ricklefs, 1977). In general, semelparous plants (those which breed only once) allocate more to reproduction than do iteroparous plants (Calow, 1979). Species comparisons have also been used to show that large males of polygynous species suffer a higher mortality rate before reaching adulthood. For example, in red deer (*Cervus elaphus*),

males achieve their large size by rapid growth at the expense of fat deposition, increasing their chances of starvation (Clutton-Brock *et al.*, 1985).

Comparisons between the reproductive rates of different species are often complicated by confounding variables that can produce spurious correlations. Another problem can be the influence of gene-environment interactions which may occur when comparing the reproductive rates of different populations or species in a common environment to which they are unequally adapted (Partridge, 1989). Another problem with this approach can be the impact of ecological variables and population dynamics. Populations in a steady state have equal birth and death rates and high adult fecundity is therefore inevitably accompanied by reduced adult or juvenile mortality (Sutherland *et al.*, 1986; Sibly and Calow, 1987; Gustaffson and Sutherland, 1988). Therefore an association between high seasonal fecundity and low adult survival rates (eg. Tinkle, 1969) does not necessarily indicate a cost of reproduction. Correlated life history traits may also be independent adaptations to different study environments and their association need not necessarily imply a functional constraint (Partridge and Sibly, 1991).

1.2.1 Internal Constraints in *Drosophila*

As discussed in the previous section, the allocation of resources to reproduction, growth and somatic maintenance at each age, which would lead to the maximisation of an organism's reproductive value, may be constrained by the cost of reproduction. Determining the nature of the cost of reproduction is therefore central to the study of life history evolution (Charlesworth, 1980; Stearns, 1989). In this thesis, the nature of costs of reproduction in female *Drosophila melanogaster* were investigated.

Several aspects of reproduction have been shown to shorten *D. melanogaster* lifespan. Egg-production appears to be costly to females to some extent (Partridge *et*

al., 1987a), but there are also considerable costs involved in mating itself (Fowler and Partridge, 1989). It is not obvious how sexual activity has this effect on lifespan; reproduction would seem to be either a risky process, or one which can accelerate the process of senescence (Partridge, 1986). Implicit in the first argument is the idea that the current reproductive status of the individual is important to current survival probability. In the latter case, the reproductive history of the individual affects current survival probability; resources diverted away from essential repair areas as a result of sexual activity could cause a decline in body condition, lessening survival probability and fertility with age. If sexual activity acts by accelerating senescence, then the effects of sexual activity will not be reversible; a previously sexually active individual would be in a worse condition, in terms of decreased life expectancy and fertility, than a previously less sexually active individual.

A study on male *D. melanogaster* (Partridge and Andrews, 1985) showed that sexual activity had an associated risk of death that was not due to an acceleration of senescence; this effect was therefore reversible by altering male sexual activity. The situation in females is more complex, and more data are needed to clarify the situation; it appears that the mechanism may not be the same as in males (Partridge *et al.*, 1986). The aspects of reproductive activity that have been shown to shorten female *D. melanogaster* lifespan are discussed in the following section.

1.2.1.1 Non-mating Effects

Part of the cost of reproduction in female *D. melanogaster* is attributable to non-mating effects. There is a non-mating cost of exposure to males (Partridge and Fowler, 1990); females that were exposed to males that could not mate (*fruitless* males, see Hall, 1978) survived less well than those exposed to no males. Part of this cost appeared to be non-sexual in nature, eg. competition, disturbance or food contamination, as females mated once and then placed with either *fruitless* males or

sterile females did not differ in lifespan. There was also a difference in the lifespan of once mated females exposed to *fruitless* or microcauterised males. Within this mating regimen at least, another aspect of pre-mating exposure (possibly differences in the amount of courtship delivered by the two types of male) seemed to have reduced female lifespan.

1.2.1.2 Cost of Egg-production

In *Drosophila subobscura*, females that lack ovaries, or in which the ovaries are inactive have an increased life-span (Maynard Smith, 1958; Lamb, 1964). This effect has also been demonstrated in the flour beetle *Tribolium confusum* (Cork, 1957). X-irradiation of ovaryless *D. subobscura* females had no effect on lifespan, suggesting that the increase in longevity in X-irradiated wild-type females had been caused by the abolition of ovarian activity (Lamb, 1964).

Virgin *Drosophila* females also live longer then inseminated females: Bilewicz, 1953; Maynard Smith, 1958, 1963; Kummer, 1960; Malick and Kidwell, 1966; Partridge *et al.*, 1986). In *D. melanogaster* at least, this is not necessarily a cost of producing eggs because, whilst it is true that inseminated females initially lay more eggs than virgins, the rate of egg-production can soon become similar, due to a subsequent increase in the egg-laying rate of the virgin females (eg. David, 1963; Bouletreau-Merle, 1978). Since *D. melanogaster* virgins also live longer than inseminated females, the lifetime egg-production of virgin and non-virgin females can be very similar (eg. Partridge *et al.*, 1986).

It has been suggested that eggs laid by virgins and non-virgins might be qualitatively different and that perhaps the eggs laid by virgins are less costly to produce. David (1963) reported a difference in the length of fertile and non-fertile eggs; this does not necessarily mean that egg volume was different. Trevitt (1989) compared

the protein content of fertile and non-fertile eggs by gel-electrophoresis and found no obvious differences, suggesting that the two types of eggs are qualitatively similar.

1.2.1.3 Cost of Mating

Elevated rates of egg-production and exposure to males cause a drop in female lifespan (Partridge *et al.*, 1987a); these effects could be a cost of egg-production or a cost of mating because females that lay a lot of eggs also remate more often (Trevitt *et al.*, 1988). However, increased exposure to males in females whose egg-production and fertility were standardised caused a large decrease in female lifespan (Partridge *et al.*, 1987a). Females continuously exposed to intact males had a higher death rate than those exposed intermittently to wild-type males and to males that delivered courtship but did not mate (Fowler and Partridge, 1989). Females kept with intact males continuously had a higher remating rate than those only intermittently exposed; the differences in lifespan between the two groups of females therefore support the existence of a cost of mating.

The reason for the decrease in female lifespan and reproductive success resulting from the cost of mating is unclear. It could arise as a result of injury of the female at mating (eg. Lloyd and Park, 1962; Ikeda, 1984), the transfer of disease or parasites (eg. Jaenike, 1988; Armstrong and Bass, 1989) or some effect of the sperm or accessory fluid. Trevitt and Partridge (1991) showed that female *D. melanogaster* kept exclusively with normal wild-type males had lower survival rates than females intermittently exposed to XO and wild-type males, in the absence of differences in egg-production, egg-hatchability or mating frequency between the two groups of females. The results suggested that receiving sperm could be costly to females, in terms of a drop in lifespan. However, alternative explanations were possible. The genetic background of the wild-type and XO flies was not the same, and the courtship activity

of the XO and XY males was not recorded, leaving open the possibility of a difference in the non-mating costs of exposure to the different types of males (see Chapter 4).

1.3 Female Multiple Mating

It has long been recognised that male and female interests in mating may be conflicting (eg. Trivers, 1972; Parker, 1979). A male can generally increase his fitness by mating with many females (Darwin, 1871; Bateman, 1948; Trivers, 1972). For a female, however, who receives sufficient sperm to fertilise her ova, but little else from a mating, there would seem to be little gain in mating repeatedly (Daly, 1978; Parker, 1979). Females of many insect species do mate several times during their lifetimes (eg. Parker, 1970a; Thornhill and Alcock, 1983; Smith, 1984; Eberhard, 1985; Ridley, 1988). In many insect species, females can store viable sperm for a much longer period than the remating interval normally seen in the laboratory, eg. in *Locusta migratoria* (Parker and Smith, 1975), *Oncopeltus fasciatus* (Economopoulos and Gordon, 1972), *Glossina morsitans* (Dean *et al.*, 1969), *Epilachna varivestis* (Webb and Smith, 1968) and *Gerris odontogaster* (Arnqvist, 1989). There are several adaptive explanations for female multiple mating, and these include:

To renew or replenish sperm supplies (Anderson, 1974; Gromko and Pyle, 1978; Gromko *et al.*, 1984a; Newport and Gromko, 1984).

Nutrient transfer at mating (Thornhill, 1976a; Boggs and Gilbert, 1979; Turner and Anderson, 1983; Gywnne, 1984; Pitnick *et al.*, 1991).

A female tactic to reduce sexual harassment and gain male protection (Alcock *et al.*, 1977; Thornhill and Alcock, 1983; Fincke, 1984; Wilcox, 1984; Svard and Wiklund, 1986; Arnqvist, 1989).

A safeguard against the possibility of a sterile male (Walker, 1980; Gibson and Jewell, 1982) and other sperm defects.

Promoting competition among spermatozoa (Parker, 1970a).

A means for genetically diversifying offspring produced (Williams, 1975; Maynard Smith, 1978; Gladstone, 1979; Walker, 1980).

Genetic superiority of the last mate (eg. Page, 1980).

Examples of the functions listed above are given in the following sections.

1.3.1 Renewing or Replenishing Sperm Supplies

In certain insects, females do not remate until a substantial number of eggs have been laid, eg. in a delphacid bug, *Nipaparvata lugens* (Oh, 1979) and some tephritid flies, eg. *Dacus tyroni* (Fletcher and Giannakakis, 1973) and *Ceratitis capitata* (Prokopy and Hendrichs, 1979). The fertility of multiply mated female *Rhagoletis pomonella* flies has also been shown to exceed that of once-mated females (Neilson and McAllan, 1965). In *Drosophila melanogaster* it has been suggested that females remate primarily to replenish their sperm supplies (Gromko and Pyle, 1978; Gromko *et al.*, 1984a; Newport and Gromko, 1984), because females given the opportunity to remate have higher productivities than those mated only once (eg. Pyle and Gromko, 1978).

1.3.2 Seminal Feeding - Nuptial Gifts

Feeding of the female by males at mating has been described in 10 insect orders (Thornhill and Alcock, 1983). Courtship feeding could increase the number or fitness of offspring produced or could increase a male's fertilization success. The adaptive significance of courtship feeding has however been the subject of some debate (Wickler, 1985; Gwynne, 1986a, b; Sakaluk, 1986; Quinn and Sakaluk, 1986; Simmons and Parker, 1989). If courtship feeding is important to the female, it should be manifested as an increase in female lifespan or reproductive success (eg. Schal and Bell, 1982; Thornhill and Alcock, 1983; Gwynne, 1984; Steele, 1986).

Nuptial feeding may take the form of -

Prey items given to the female.

Secretions produced by the male.

Spermatophores extruded by males as food.

Internal absorption of spermatophores or nutrients.

1.3.2.1 Prey Items

Female scorpionflies (*Bittacus apicalis* and *Hylobittacus apicalis*) prefer males with a large nuptial insect gift (Thornhill, 1976a, 1979, 1980a, b). Nuptial presents are also provided by males of some predatory empidid flies (Downes, 1969; Alcock, 1973) and some thynnine wasps (Given, 1954; Alcock, 1981a, b).

1.3.2.2 Secretions Produced by the Male

Panorpa scorpion flies, *Bittacus apicalis*, (Thornhill, 1976a) and some tephritid fruit flies, (Stoltzfus and Foote, 1965 for *Eutreta* spp.; Fletcher, 1968 for *Dacus tyroni*) have been shown to provide their mates with a salivary secretion. *Drosophila subobscura* males have been shown to regurgitate crop contents during courtship (Steele, 1986); starved females that took this drop had a higher fecundity than females not allowed this food. Starved females also took more of this food than those which were well fed. Female *D. subobscura* taken from the wild had similar crop sizes to females that had been starved in the laboratory; this indicates that courtship feeding is probably very important under natural conditions in this species.

In males of another species, *Drosophila nebulosa*, courtship feeding consisted of an anal drop that was deposited and subsequently eaten by the female (Steele, 1986). There is one report of male *Drosophila melanogaster* defending such faecal deposits as well as food resources (Jacobs, 1978). A possible advantage for the male in producing

such a drop for the female to eat might be a chance to gain time to initiate courtship; for the female, the advantage might be easy access to a relatively risk free food source.

1.3.2.3 Spermatophores

In many Lepidoptera and Orthoptera, males transfer a spermatophore to the females at mating. In lepidopterans, a spermatophore is placed within the female bursa (eg. Erhlich and Erhlich, 1978). Amino acids from the spermatophore are used in egg-production (Dunlap-Pianka *et al.*, 1977; Boggs and Gilbert, 1979; Boggs, 1981a, b; Boggs and Watt, 1981). In acridid grasshoppers, spermatophores are also placed internally within the female reproductive tract where they are digested (Farrow, 1963; Loher and Huber, 1966). In the grasshopper *Melanopus sanguinipes* and in *Conocephalus nigropleurum* katydids, proteins from the internally placed spermatophore are used for egg-production (Friedel and Gillot, 1977; Gwynne, 1982).

In insects where the spermatophore is externally deposited, it is often eaten by the female (eg. Leopold, 1976; Thornhill, 1976b; Gwynne, 1982). In katydids (*Tettigoniidae*), and some crickets, the spermatophore consists of a sperm-carrying ampulla and a proteinaceous spermatophylax, which seems to be a specialised food source. In the genus *Ephippiger*, the spermatophylax may represent up to 30% of the male body weight. The spermatophore produced by male bushcrickets (*Requena verticalis*) can represent a 40% weight loss for the male (Gwynne, 1984).

Large spermatophores seem to be costly to produce; male *Orchelimum* katydids require a period of a week to produce a full sized spermatophore (Thornhill and Alcock, 1983). The size of spermatophores also decreases in recently mated males (Gwynne, 1982). Gwynne (1984) demonstrated an increase in reproductive success of female bushcrickets (*Requena verticalis*) that fed on male spermatophores, as an increase in the number and size of eggs produced. The quality of the spermatophore transferred to female bushcrickets (an unnamed species of the Zaprochiline sub-family) has been

shown to affect the number and survival probability of offspring produced (Simmons, 1990).

Urates that coat the spermatophore of German cockroaches (*Blattella germanica*) are incorporated into the female's eggs (Mullins and Keil, 1980). In another cockroach (*Xestoblatta hamata*) females feed on the urate-containing tergal glands of males; this has been shown to increase female fecundity (Schal and Bell, 1982).

1.3.2.4 Internal Absorption

Absorption of nutrients into the female somatic or reproductive tissue has been demonstrated in several studies, using radio-labelling techniques (Friedel and Gillot, 1977; Boggs and Gilbert, 1979; Markow and Ankney, 1984; Bownes and Partridge, 1987). *Drosophila mojavensis* females remate daily, and material from the male ejaculate is incorporated in female somatic tissues (Markow and Ankney, 1984). *D. mojavensis* is a relatively specialist feeder eating only rotting cactus tissue. *D. mojavensis* females, living in a harsh environment where food may be a limiting resource at certain times of year, would seem to remate daily in order to obtain nutrients from the male ejaculate.

With such a high remating frequency, it is possible that nutrients from the ejaculate of a male might supply nutrients to the progeny of another mate; this has been shown in *D. mojavensis* females (Markow, 1988), where progeny sired by the first male may also receive nutrients from the ejaculate of subsequent mates. In *D. melanogaster* only a small amount of ejaculate-derived protein is detectable after mating in the female; this material probably corresponds to male accessory gland peptides (see Chen and Buhler, 1970). Bownes and Partridge (1987), in similar experiments, suggested that for *D. melanogaster* and *D. pseudoobscura*, the total amount of ejaculate material transferred to the female at copulation was probably not of nutritional

significance. The protein content of a pair of male accessory glands is equivalent to the protein content of only approximately 5 eggs.

It is possible that males are supplying a limiting nutrient, perhaps an amino acid, essential for some aspect of egg-production. Bownes and Partridge (1987) used radio-labelled methionine to follow the fate of male ejaculate material transferred to the female reproductive tract. They found that methionine was transferred to females from the male ejaculate in the form of free amino acids or as small peptides or proteins. Flies grown on methionine-deficient medium have been shown to lay fewer eggs than those on complete medium, and these eggs are also less fertile (Sang and King, 1961); so at least one amino acid, important to egg-production, is passed to the female at copulation (see Chapter 3).

Females in the wild may be in poor nutritional condition (Bouletreau-Merle, 1978; Steele, 1986) and thus absorption of limiting nutrients from the male ejaculate may be important. Turner and Anderson's (1983) results showed an elevation of egg-production in *D. pseudoobscura* females kept with males, but as Bownes and Partridge (1987) point out, this species exhibits courtship feeding (Steele, 1986) and so any effects of seminal feeding are confounded.

In some insects with haemocoelic insemination, males sometimes mate with each other and transfer large quantities of accessory fluid and sperm (Hinton, 1964, 1974). It is possible that this represents a nutritional contribution between males; the males of *Afrocimex* bug species even seem to have abdominal modifications to facilitate homosexual copulation (Hinton, 1964).

1.3.3 Reduction of Sexual Harassment and Protection

The solitary bee *Anthidium maculosum* seems to provide a good example of reluctant females accepting superfluous matings in order to avoid harassment (Alcock *et*

al., 1977). Males of this species defend territories; females try to avoid such males but are often captured and mated. The duration of copulation in this species is only 30 seconds, so perhaps this is just a small inconvenience to avoid further harassment.

In an andrenid bee, *Nomadopsis puellae*, (Rutowski and Alcock, 1980) and ovipositing female dung flies, *Scatophaga stercoraria,* (Borgia, 1981), females are continuously receptive; it was suggested that this is to attract males who will defend females against the advances of other males. Wilcox (1984) reported that female water striders (*Gerris regimis*) could forage effectively only when carrying a copulating male who could repel copulatory attempts by other males. In spanish flies (*Lytta vesicatori*), males transfer a defensive compound, cantharidin, to the female during copulation (Sierra *et al.,* 1976). This compound is not synthesised by the female and is only transmitted sexually.

1.3.4 Safeguard Against the Possibility of a Sterile Male and other Sperm Defects

In Lepidoptera, there is a high degree of sperm displacement, thought to have arisen because of the high proportion of infertile matings in these insects (Taylor, 1967; Pliske, 1973). In several species, the incidence of impotent matings has been reported as relatively high; 50% in *Rhodnius prolixus* (Khalifa, 1950), 9.3% in *Conotrachelus nenuphar* (Johnson and Hayes, 1969), 40% in *Stomoxys calcitrans* (Harris *et al.*, 1966), 34% in *Atteva punctella* (Taylor, 1967), 19% in *Euphydryas editha* (Labine, 1966) and 47% in *Spodoptera frugiperda* (Snow *et al.*, 1970). Females of some species may therefore remate as a safeguard against male sterility or sub-fertility.

In some species, females appear to be monogamous, except if the first mating was defective in some way: in the moth *Atteva punctella*, remating seems to be induced by inadequate insemination by the first mate (Taylor, 1967); female mediterranean fruit flies (*Ceratitis capitata*) have also been reported to remate if their first mating was sterile

(Nakagawa *et al.*, 1971). After mating, female *Drosophila melanogaster* normally remain refractory to further mating for several days, but will remate again after about 1 day if the mating was sterile (Manning, 1962, 1967; Gromko *et al.*, 1984a).

1.3.5 Sperm Competition

Parker (1970a) suggested sperm competition resulted in the evolution of copulation itself, because males that could place sperm within the female reproductive tract, and thus nearer to the female's eggs, would be selectively favoured. Female remating frequency might be determined by the outcome of sperm competition. In insects that mate multiply, males will often mate with females that are already inseminated, and sperm competition is almost inevitable. Selection would be expected to favour males that can inseminate already mated females, if their sperm is used preferentially to that of earlier mates. If there is a risk of sperm displacement, then selection will also favour males that can prevent females from remating whilst carrying their sperm (Parker, 1970a, b, c, 1974). Copulation does not assure a male of exclusive paternity; in some cases, the sperm of the most recent mate is removed from the female sperm storage organs by specialised structures on the penis, as in the damselfly *Calopteryx maculata* (Waage, 1979).

The evolution of multiple mating, driven by sperm competition, could have several adaptive consequences for females. Sperm supplies would be replenished and females would avoid the cost of storing and maintaining large quantities of sperm from a single donor. Females could also replace sperm from a previous mate with that of a genetically superior individual or add sperm from a different male to their sperm supply, increasing the genetic diversity of offspring produced (see sections 1.3.6 and 1.3.7).

1.3.6 Genetic Diversification of Offspring

The advantages of genetically diverse offspring are thought to have been important in the evolution of sexual reproduction and recombination (Stearns, 1987). However, compared with the process of recombination, multiple mating is thought to contribute only slightly to the genetic diversity of sexually produced offspring (Williams, 1975; but see Knowlton and Greenwell, 1984; Parker, 1984). It is unclear how much additional zygotic diversity can be obtained by multiple mating, and Williams (1975) has suggested that insect female remating frequencies probably cannot be explained by this process alone.

Sperm displacement might increase female fitness by increasing the genetic diversity of offspring. It has proven difficult to demonstrate empirically whether there is any fitness benefit to females of producing genetically diverse offspring, or if the progeny of multiply mated females are more fit than those of females mated only once. In one study Arnqvist (1989) compared progeny from single or multiple paternity groups of water striders (*Gerris odontogaster*) and found no differences in growth or survival between them.

One example where females might remate in order to diversify their offspring is the honey bee, *Apis mellifera*. Honey bees have a haplo-diploid system of sex determination, such that haploid individuals are males. Diploid males can be produced however, if individuals are homozygous for alleles at a sex determining locus. Such diploid males are subsequently eaten by worker bees. A singly mated queen would therefore have a high probability of mating with a male carrying an identical sex-determining allele and producing a large number of diploid males (Page, 1980). There is presumably high selection pressure for female multiple mating in this species to prevent the disadvantageous production of large numbers of diploid males.

1.3.7 Genetic Superiority of Last Mate

It has been suggested that mate choice would be advantageous if females could select mates with a high genetic quality, leading to the production of offspring with high fitness; the 'good genes' hypothesis (Trivers, 1972; Hamilton, 1982; Hamilton and Zuk, 1982; Thornhill and Alcock, 1983; Weatherhead, 1984). A selective advantage could only be produced if there is additive genetic variation in fitness, and a genetic correlation between the male trait on which the female choice is exerted and offspring fitness (Maynard Smith, 1978). Natural selection tends to exhaust additive genetic variation however (Fisher, 1930). A positive fitness correlation between parents and offspring would increase the mean fitness of the population from one generation to the next and therefore a population in genetic equilibrium subject to the effects of selection only cannot show such a correlation (Charlesworth, 1987).

The amount of heritable variation in net fitness is not non-existent however, due to the input of variation from mutation. It has proven difficult to estimate the heritability of total fitness empirically and many studies have concentrated on estimating the heritability of individual fitness components. Due to the frequent existence of negative genetic correlations between fitness components (eg. Reznick, 1985), the demonstration of significant heritability for these components does not necessarily prove that there is heritable variation in net fitness (Charlesworth, 1987). Charlesworth (1987) suggested that because of temporally and spatially variable selection, there may be more variance in breeding values with respect to fitness maintained in most populations than can be accounted for on the basis of mutation. He showed that, under certain conditions, there could be significant selection pressure in favour of mate choice.

To assess the quality of a mate, females would have to be able to monitor the heritable fitness of the male. She could use characteristics such as age, which has been

suggested to be a good indicator of survival ability (Halliday, 1983; Weatherhead, 1984). Many of the courtship rituals performed by males appear to involve testing athletic ability, and it is possible that this reflects the outcome of selection for mate choice (Maynard Smith, 1978). It has been suggested that sperm storage and multiple mating by females allows selection of the sperm of superior mates for fertilisation (eg. Lloyd, 1979; Walker, 1980; Sivinski, 1984). In some insect species, the female provides nutrients for sperm in store (Davey, 1965; Davey and Webster, 1967); this may allow the female to influence the survival or competitiveness of sperm from different ejaculates (Walker, 1980).

In scorpionflies, *Hylobittacus apicalis*, females that do not receive a sufficiently large nuptial meal remate again before laying a clutch of eggs (Thornhill and Alcock, 1983). It is possible that the resource gathering quality of the male could be assessed by the time required to eat the prey item. In the house fly (*Musca domestica*), females that remate twice in succession do so the second time with a larger male than the first mate (Baldwin and Bryant, 1981). The female therefore gains a larger accessory gland secretion from the second mate, and will use his sperm for fertilisation (Leopold *et al.*, 1971a). Stretch receptors in the reproductive tract may enable females to detect a large ejaculate. A male who transfers a large ejaculate may be vigorous and desirable either genetically, or because he transfers a large amount of nutritionally important material. In some katydids, failure of a male to guard the female following mating indicates that he is subordinate; females will often mate again, possibly to receive the sperm of a more dominant male, with greater skills in sexual competition and defense (Thornhill and Alcock, 1983). However, it is possible that these rematings occur because dominant males are more efficient at securing matings with unwilling mates and that female choice is not involved.

1.3.8 Non-Adaptive Explanation for Multiple Mating

The empirical evaluations of explanations for female multiple mating, reviewed in the previous sections, provide examples of insects in which multiple mating does seem to have an adaptive basis (eg. Daly, 1978; Walker, 1980; Thornhill and Alcock, 1983; Eberhard, 1985). There are many species of insects however, where there are no demonstrable benefits for females which mate multiply (eg. Arnqvist, 1989). There is no empirical evidence, for example, that females mate multiply to improve the genetic quality of their offspring, or that females manipulate or choose sperm. However, multiple mating in females does not necessarily have to evolve as an adaptive behaviour. Halliday and Arnold (1987) have suggested that female remating has evolved as a genetic corollary to sexual selection on males. It is possible that in some cases female multiple mating has an adaptive significance, but in others, perhaps for species in which there are no demonstrable benefits, it evolved due to a genetic correlation between the sexes. There are two assumptions implicit in the Halliday and Arnold argument; that there is a genetic correlation between the sexes in mating tendency and that selection on mating tendency is stronger in males than in females.

Halliday and Arnold (1987) suggested that females remate more frequently because the genetic correlation has prevented divergence from male promiscuity. There are examples of correlations between male and female homologous characters arising as a result of gene pleiotropy, for example body size (Harrison, 1953; Korkman, 1957; Cowley *et al.*, 1986; Cowley and Atchley, 1988). Behavioural traits may also show high genetic correlation; selection on the mating speed in male *D. melanogaster* also produced an effect in females, showing that the sexes are genetically correlated (Manning, 1963; Stamenkovic-Radak *et al.*, 1992)

Because of genetic coupling, sexual dimorphism should be very slow to evolve (Fisher, 1958; Lande, 1980; Lande and Arnold, 1985). Lande (1980) calculated that if

there was strong genetic correlation between the sexes, optimal sexual dimorphism might take hundreds of thousands of generations to achieve. Halliday and Arnold (1987) suggested it is likely that selection favours multiple mating by males and single or reduced mating by females, but that genetic correlation impedes divergence. Sherman and Westneat (1988) criticised two aspects of Halliday and Arnold's (1987) hypothesis. They argued that if repeated copulation imposes a cost on females, there would be selection pressure for modifier genes to evolve, to limit the expression of genes affecting female copulation frequency. The correlation between the mating behaviour of the sexes would therefore become less strong. Sherman and Westneat's second argument was that selection for male promiscuity will result in an increase in female remating frequency only if there is a common genetic basis for remating propensity in both sexes, and that this was questionable.

Arnold and Halliday (1988) replied to both of these criticisms. They argued that Sherman and Westneat did not consider the contribution of new mutations to the genetic correlation for traits between the sexes. New mutations are not likely to be sex limited in their expression and, if the mating tendencies of the sexes are homologous, the effects of new mutations will have similar effects on both sexes. The effect of the influx of new mutations would therefore oppose the effects of selection and promote the genetic correlation between the sexes (Lande, 1980). In answer to the second criticism made by Sherman and Westneat (1988), Arnold and Halliday (1988) argued that there is no evidence that mating propensity in the sexes is not homologous. In a later discussion, Arnold and Halliday (1992) argued that only three relevant selection experiments had been performed (Manning, 1963; Dunnington and Siegel, 1983; Gromko and Newport, 1988); the results of these experiments are however equivocal.

Stamenkovic-Radak *et al.* (1992) showed that selection for slow mating speed in males produced a correlated response in females and *vice versa*. In each generation, 20 selected flies were placed with 20 unselected flies of the opposite sex and allowed to

mate without restriction for a period of 5 days. Butlin (in press) criticised this design on the basis that there was some opportunity for sexual selection to influence the contribution of the unselected flies to the next generation. Butlin suggested that, for example, in the lines selected for slow female mating speed, inadvertent selection for slow male mating speed may have resulted if slow males (for whatever reason) were more likely to mate with slow females. Alternatively, he suggested that selection for fast mating males might have been relaxed.

Butlin's hypothesis therefore requires assortative mating for mating speed. It has been demonstrated however, that males from populations with fast mating speeds have a greater advantage with slow mating females than with fast mating females, ie. there is disassortative mating for mating speed (Van den Berg *et al.*, 1984; Van den Berg, 1986). Female mating speed also shows an evolutionary increase in stocks where male mating speed is reduced by the presence of *yellow* or *ebony* mutations (Heisler, 1984; Partridge, unpublished data). It is likely that Stamenkovic-Radak *et al.* (1992) did show a correlation for mating speed between the sexes, because any disassortative mating for mating speed within the selected lines would actually lead to an underestimate of the genetic correlation between the sexes (Stamenkovic-Radak *et al.*, in press).

Two experiments on *Drosophila melanogaster* have therefore demonstrated a genetic correlation between the sexes for mating speed (Manning, 1963; Stamenkovic-Radak *et al.*, 1992). The non-adaptive hypothesis for female multiple mating therefore remains an interesting alternative to the plethora of adaptive explanations. However it is clear that more experimental work is needed.

1.4 Sexual Conflict

In *Drosophila melanogaster*, the transfer of accessory fluid from the male causes an elevation in egg-production and a decrease in female receptivity, (eg.

Kummer, 1960; Garcia-Bellido, 1963; Burnet *et al.*, 1973, reviewed in section 1.5). The male therefore maximises the production of progeny fertilised by his sperm before the female remates; this could represent a sexual conflict, because the female may produce more progeny in the short term, but incur more reproductive costs.

It has been suggested that the relative parental investment in offspring underlies the evolution of differences in sexual behaviour (Bateman, 1948; Trivers, 1972; Emlen and Oring, 1977; Simmons, 1992). In general, males allocate a large proportion of their resources to mating effort, whereas females devote a large proportion of energy to parental effort. In an experiment to measure variance in reproductive success of *Drosophila melanogaster* males and females, Bateman (1948) found that male reproductive success was more variable than that of females. Male reproductive success seemed to be limited by the ability of males to obtain matings, whereas female fitness did not depend on their capacity to persuade males to mate with them.

Bateman also observed that the number of offspring produced by males was proportional to the number of matings they obtained whereas females that had 2 or 3 mates produced approximately the same number of progeny as females that mated once. Therefore, the reproductive success of the male was limited by the number of inseminations obtained. Female fitness seemed to be limited by the ability to produce gametes, and not necessarily by the number of matings.

A further asymmetry in the interests of the sexes at mating is provided by the fact that a male who copulates with a female is less likely to be the father of her offspring than she is to be the mother. The mechanisms by which a male ensures paternity following mating will be selectively favoured. There is no guarantee however, that the sperm donated to a female by a male will be used to fertilise all of her eggs and there is a risk that he may be assisting the progeny-production of another male (eg. Markow, 1988). There is therefore strong selection on males to ensure paternity

following mating, but no similar selection on females (Thornhill and Alcock, 1983). Selection would therefore have favoured female traits for maximising zygote survival and male traits associated with locating and fertilising female gametes. Any mechanism by which a male assures paternity after mating, for example by the effects on the female caused by the transfer of accessory fluid, could therefore represent a conflict between the sexes (see Chapter 5).

DNA-imprinting in mice seems to reflect conflict over the amount of maternal investment in embryos because of maternal costs (Haig and Graham, 1991). In mice, the genes for the insulin-like growth factor II (IGF-II) and IGF-II receptor are oppositely imprinted (DeChiara *et al.*, 1991; Barlow *et al.*, 1991). IGF-II is an insulin-related polypeptide expressed at high levels in developing embryos and at low levels in adults. Neonates that receive a defective IGF-II allele from their father produce only small amounts of IGF-II and are small, but normally proportioned. Neonates receiving a defective IGF-II allele from their mother produce normal amounts of IGF-II and are normal in size.

IGF-II has a high affinity for two unrelated receptors; the type 1 receptor is homologous to the insulin receptor and binds IGF-I and II, the type 2 receptor is the mannose-6-phosphate receptor, which as well as binding mannose-6-phosphate residues acts as a IGF-II receptor. It is unclear why the type 2 receptor binds IGF-II, as there is no evidence that IGF-II signals via the type 2 receptor. The IGF-II type 2 receptor is also imprinted: the receptor is transcribed from the maternal, but not the paternal chromosome; mice that are heterozygous for deletions at this locus die if the maternal allele is missing.

Haig and Graham (1991) argued that it is not coincidental that IGF-II and the IGF-II type 2 receptor are oppositely imprinted. They argued that the receptor may act as a sink, capturing and degrading IGF-II produced from the paternally-derived

chromosome before it can bind to the IGF-II type 1 receptor and thus influence the size of the offspring produced. The opposite imprinting may reflect the outcome of a conflict between benefits to current vs. future offspring (or other offspring of the same litter). An increase in IGF-II expression increases the size of offspring produced, with a possible metabolic cost to the mother in terms of current or future survival or reproductive success. It was suggested that the mannose-6-phosphate receptor role as a IGF-II receptor evolved as a maternal response to counter the effects of high levels of paternally-induced IGF-II.

There are also examples of physiological manipulation of females by males in higher plants (Queller, 1989) where conflict within seeds seems to have resulted in unusual fertilisation patterns and ploidy levels. If plant embryos can affect the amount of resources that are received from the mother, then the equivalent of weaning conflict is expected to arise. There are four distinct genetic entities present during the development of the seeds of angiosperms; diploid maternal sporophyte tissue, haploid gametophyte tissue (usually reduced), triploid endosperm and the diploid embryo sporophyte.

The female gametophyte contains 8 identical haploid nuclei in the embryosac, which is surrounded by diploid maternal tissue. During fertilisation, one sperm nucleus fertilises the 'egg' haploid nucleus and a second identical sperm nucleus fertilises 2 of the 'polar' haploid nuclei to produce a triploid nucleus which then divides mitotically to produce the endosperm, which acts as a nurse tissue to the embryo. The remaining haploid nuclei do not develop further.

The application of inclusive fitness theory to parent-offspring conflict in plants makes predictions about which of the genetic entities within the developing seed should fight hardest to gain extra provisioning for their own seed. The mother is equally related to all embryos and should favour the transfer of resources from one embryo to

another if the inclusive fitness benefits outweigh the costs. Aborting embryos can be favoured if the benefits to other embryos are large enough. Embryos are more related to themselves than to other offspring and should attempt to gain more resources than the mother is selected to give. The endosperm tissue is expected to favour its own embryos more than the gametophyte. The double dose of maternal alleles in the triploid endosperm tissue means that maternal and paternal pathways may depend on simple dosage effects, rather than a memory mechanism that tells a gene from which parent it came.

The endosperm nurse tissue is therefore a genetic hybrid formed from a double dose of maternal alleles and a single dose of paternal alleles. Charnov (1979) proposed that the endosperm arose as the result of a conflict of interests between the sexes. He suggested that double fertilisation is a male strategy that adaptively affects investment in the male's embryos. If this is true, then it is unclear why the maternal plant should allow double fertilisation to continue; if double fertilisation was prevented, the endosperm would revert to a less aggressive haploid form. In angiosperms, the maternal response to double fertilisation seems to have been the addition of another dose of maternal alleles to the endosperm tissue. Bulmer (1986) showed that this would be favoured if the effect of the addition of maternal alleles was to cause the endosperm to channel less resources into its embryo. It was suggested therefore that the addition of a second dose of maternal alleles was a maternal counter adaptation.

Increasing the ploidy level of the endosperm tissue is however expected to increase its vigour, which may be disadvantageous to the maternal plant. Haig and Westoby (1989) circumvented this argument by proposing that once an embryo could affect the amount of provisioning it received, there would be selection for parent-specific gene expression. Paternally-derived embryo genes would be selected to take more resources and maternally-derived genes less. If the expression of endosperm genes was limited by the availability of nucleotides, amino acids, enzymes, etc. then

doubling the dose of maternally-derived endosperm genes would increase the total number of genes expressed and reduce the proportion of resources available to paternally-derived overconsumer alleles.

1.5 Behavioural and Physiological Changes in Females after Mating

In the previous section, it was suggested that the transfer of ejaculate components at mating could represent a conflict between the sexes because of the possible manipulation of the female by the male. The effects of accessory fluid or sperm may also contribute to the cost of mating in *D. melanogaster* females (section 1.2.1.3). In this section, the changes in female behaviour brought about by the effects of the transfer of accessory fluid components are reviewed; sections 1.5.1 and 1.5.2 review the evidence for *Drosophila.*

Females of many insect species, including many *Drosophila*, mate more than once. The ultimate reasons for remating are unclear, but the physiological and behavioural changes caused by mating have been well characterised. Mating in many species of insects results in an elevation in egg-laying rate and decrease in female receptivity (reviewed by Hinton, 1974; Leopold, 1976; Gillot and Friedel, 1977; Gillot, 1988). There is a mounting body of evidence that the behavioural and physiological changes in females following mating are due primarily to the transfer of accessory gland substances from the male. Accessory glands are found throughout the insects with the exception of the Apterygota and the Palaeoptera; they have been developed from outpockets of the ejaculatory duct, or as glandular areas incorporated into the genital duct wall (Leopold, 1976). The evidence that accessory gland material affects female physiology and behaviour falls into two broad categories; accessory glands were either implanted or accessory gland extracts injected into females. Some studies assayed both receptivity and oviposition following the transfer of accessory gland material,

other studies investigated only oviposition or receptivity. The following section reviews this evidence.

(a) Studies in which both fecundity-enhancing and receptivity-inhibiting effects described:

(i) accessory glands implanted: *Drosophila melanogaster* (Garcia-Bellido, 1964; Leahy, 1966; Merle, 1968; Burnet *et al.*, 1973), *Hylema brassicae* (Swailes, 1971) and *Stomoxys calcitrans* (Venkatesh and Morrison, 1980; Morrison *et al.*, 1982).

(ii) accessory gland extract injected: *Drosophila melanogaster* (Kummer, 1960; Leahy and Lowe, 1967; Chen and Buhler, 1970; Burnet *et al.*, 1973), *Drosophila funebris* (Baumann, 1974a, b), *Aedes aegypti* (Fuchs *et al.*, 1968; Fuchs and Hiss, 1970; Hiss and Fuchs, 1972), *Hylema brassicae* (Swailes, 1971), *Stomoxys calcitrans* (Venkatesh and Morrison, 1980; Morrison *et al.*, 1982) and *Locusta migratoria* (Lange and Loughton, 1985).

(b) Studies in which only fecundity-enhancing effect described:

(i) accessory glands implanted: *Schistocerca gregaria* (Leahy, 1973), *Melanopus sanguinipes* (Pickford *et al.*, 1969), *Aedes aegypti* (Leahy and Craig, 1965; Judson, 1967), *Aedes albopictus* (Leahy and Craig, 1965), *Culex pipiens* (Leahy 1967) and *Musca domestica* (Riemann and Thorson, 1969).

(ii) accessory gland extract injected: *Melanopus sanguinipes* (Friedel and Gillot, 1976) and *Acanthoscelides obtectus* (Huignard *et al.*, 1977).

(c) Studies in which only receptivity-inhibiting effects described:

(i) accessory glands implanted: *Aedes aegypti* (Craig, 1967), *Aedes* spp., *albopictus, atropalpus, mascarensis, polynesiensis, scutellaris, sierrensis,*

togoi, triseriatus, vitattus (Craig, 1967), *Anopheles quadrimaculatus* (Craig, 1967), *Musca domestica* (Riemann *et al.*, 1967) and *Glossina morsitans* (Gillot and Langley, 1981).

(ii) accessory gland extract injected: *Aedes aegypti* (Fuchs *et al.*, 1968; Hiss and Fuchs, 1972), *Musca domestica* (Adams and Nelson, 1968; Nelson *et al.*, 1969) and *Glossina morsitans* (Gillot and Langley, 1981).

In some insect species, the accessory glands are responsible for packaging the sperm into a spermatophore (eg. Gwynne, 1984, 1988; Simmons, 1990). In mosquitos, male accessory gland substances alter female host-seeking and biting behaviour (Lavoipierre, 1958; Judson, 1967; Klowden and Lea, 1979), induce autogenous egg-development (O'Meara and Evans, 1977), increase female fertility (Adlakha and Pillai, 1975) and induce pre-oviposition behaviour in gravid females (Yeh and Klowden, 1990).

1.5.1 Behavioural and Physiological Changes in Female *D. melanogaster* after Mating

The first mating by *Drosophila melanogaster* females turns on egg-production permanently (David, 1963; Turner and Anderson, 1983; Partridge *et al.*, 1986; Partridge and Fowler, 1990). Mating also causes a decrease in female receptivity that can last 6-9 days (Manning, 1962, 1967). Chen *et al.* (1988) isolated a 36 amino acid peptide (the sex peptide, SP) that affected female egg-laying and receptivity (section 1.5.2). The increase in egg-production and decrease in receptivity following mating appears to be due both to a short-lived effect of the SP (Chen *et al.*, 1988), and a longer-term effect of receiving sperm (Manning, 1962; Gilbert *et al.*, 1981a, b; Gromko *et al.*, 1984a, b). It is not clear how either the short-term 'copulation effect' or the longer-term 'sperm effect' are achieved (Manning, 1962, 1967); both effects may carry costs to females. The maintenance of healthy sperm in the sperm storage organs

may also carry a physiological cost (Davey, 1965; Davey and Webster, 1967; Thornhill and Alcock, 1983).

Certain components transferred from male insects rapidly enter the female haemolymph and are thought to act on targets in the central nervous system (Baumann, 1974a, b; Bouletreau-Merle, 1976; Hennig *et al.*, 1973; Leopold *et al.*, 1971b; Terranova *et al.*, 1972). It is possible that the activation of the central nervous system might stimulate the endocrine system controlling sexual behaviour and egg-production (Engelmann, 1968), see Chapter 6. In *Locusta migratoria* there is evidence that there are receptors for male accessory gland substances on the ovary itself (Lafon-Cazal *et al.*, 1987; Paemen *et al.*, 1990).

The second mating by *D. melanogaster* females turns receptivity back off, but it is not clear whether this is by the same mechanism as operates after the first mating. There is evidence that the timing of remating is influenced by the number of sperm in store (Gromko and Pyle, 1978; Gromko *et al.*, 1984a; Newport and Gromko, 1984; Letsinger and Gromko, 1985), but other factors, such as nutrition and the current rate of sperm usage or egg-production may also be important (Chapter 3, this thesis).

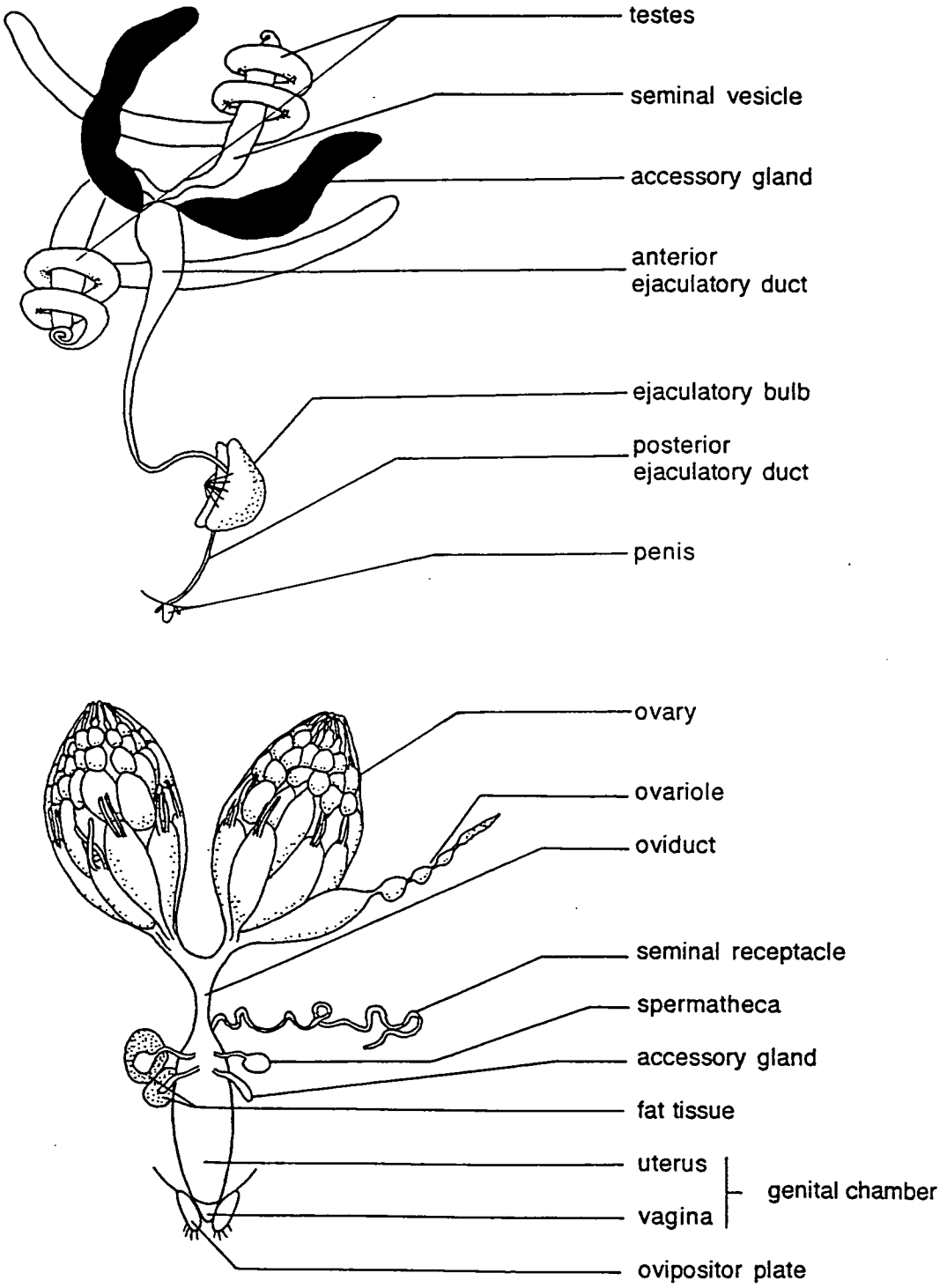
There is abundant evidence therefore, that some aspect of mating causes both physiological and behavioural changes in *D. melanogaster* females and could contribute to the cost of mating. Accessory gland sub-components are responsible for some of the changes in the behaviour of mated females and may represent a conflict between the sexes over reproductive investment (see section 1.4). The seminal fluid of *Drosophila* is a complex mixture of many sub-components, most of which are either unidentified or have unknown functions. Those components of the seminal fluid that have been characterised are reviewed in the following section.

1.5.2 Characterisation of *Drosophila* Seminal Fluid Components

The accessory glands of male *Drosophila melanogaster* (Fig.1) contain two types of secretory cells: main cells and secondary cells (Bairiati, 1968; Federer and Chen, 1982). A large variety of functions for male accessory gland components have been proposed, such as involvement in sperm transfer, storage and utilization, interaction with the pheromone system, stimulation of the *corpora allata*, the supply of nutrients to the oocyte and synthesis of non-specific proteins for general cell maintenance (Gillot, 1988). The accessory glands contain an almost bewildering diversity of secretory products including amino acids, peptides, proteins, lipids and carbohydrates; the function of by far the majority of these sub-components is still to be elucidated. The seminal fluid also contains secretions from the ejaculatory duct, eg. esterase-6 (Sheehan *et al.*, 1979; Richmond *et al.*, 1980), glucose dehydrogenase (Cavener, 1980; Cavener and MacIntyre 1983) and from the ejaculatory bulb, eg. *cis*-vaccenyl acetate (Butterworth, 1969; Brieger and Butterworth, 1970).

Baumann (1974a) isolated 2 components of *Drosophila funebris* male accessory glands that altered female reproductive behaviour. One component (PS-1), which reduced female receptivity, was composed of two types of peptide, each of 27 amino acids, differing at amino acid position 2 (either valine or leucine, Baumann *et al.*, 1975). The other component (PS-2) was a glycine carbohydrate derivative which caused an increase in oviposition (Baumann, 1974a). Schmidt *et al.* (1989) isolated a 63 amino acid peptide from the accessory glands of *D. funebris* that showed homology to serine protease inhibitors belonging to the pancreatic trypsin inhibitor (Kunitz) family. The peptide protease was found to inhibit the activity of acrosin by 95%; acrosin is a trypsin-like endopeptidase associated with the acrosome of mammalian

Fig. 1 Male and female reproductive system of *Drosophila melanogaster* (scale × 50)



sperm. Acrosomes are also present in *Drosophila* sperm (Perotti, 1969) and Schmidt *et al.* (1989) suggested that the protease was involved in fertilisation.

The transplantation of accessory glands or injection of an accessory gland extract (see section 1.5) into the abdomen of *Drosophila melanogaster* virgin females stimulates egg-laying (Kummer, 1960; Garcia-Bellido, 1964; Leahy and Lowe, 1967; Merle, 1968). Matings with XO males, which do not transfer sperm, also cause a short-term increase in the egg-laying rate of virgin females (Manning, 1967; Hihara, 1981). This finding suggested that the component causing this effect was not produced by the testes. Chen and Buhler (1970) presented biochemical and physiological evidence to suggest that peptides in the male accessory gland secretion of *Drosophila melanogaster* affected female reproductive behaviour. Chen *et al.* (1988) isolated and purified a 36 amino acid peptide (the sex peptide, SP) from male accessory glands, which induced oviposition and repressed sexual receptivity upon injection of a physiological dose of the purified peptide.

Northern-blot analysis was used to show that the SP is synthesised exclusively in the male accessory glands (Chen *et al.*, 1988). Northern analysis detects the presence of mRNA transcripts within a cell, and can be used to determine in which cells a particular gene is transcribed. RNA is extracted and fractionated by electrophoresis and the pattern of RNA bands transferred onto nitrocellulose membranes. The presence of the RNA transcript of interest is detected by hybridisation to a radioactive probe and visualised by autoradiography (Klug and Cummings, 1986).

The increase in egg-laying and decrease in receptivity lasts for about one day in females injected with SP. In mated females, receptivity returns after about 6-9 days and egg-production stays high, so the SP cannot be responsible for all of the behavioural and physiological changes seen in females after mating (see section 1.5.1). Females germ line transformed with a construct of the SP attached to the yolk protein promotor

constitutively express the SP ectopically and as a result are continually unreceptive to males and show high rates of egg-production (Aigaki *et al.*, 1991). SP-null mutants would be useful, to confirm the effects of the SP on females and to determine whether the transfer of the SP is in the reproductive interests of the female, the male or both, ie. whether the SP is the molecular manifestation of a conflict between the sexes over reproductive investment. In Chapter 7 of this thesis, a mutagenesis screen for SP-null mutations is described.

In an attempt to discover which amino acids of the SP are essential for function, the SP sequences of all the *melanogaster* species sub-group members were obtained (Kubli and Schmidt, unpublished data). If any of the *melanogaster* species sub-group SPs were inactive in *D. melanogaster*, it might be due to the substitution of one or more amino acids critical for function. All of the SPs of the *melanogaster* species sub-group were found to be active in *D. melanogaster*, including the SP of *D. suzukii*, a member of the more distantly related *suzukii* species group (Kubli and Schmidt, unpublished data). The SP of *D. melanogaster* however, is not active in *D. funebris* (Chen and Balmer, 1989), a member of the *funebris* species group, and the *D. melanogaster* SP shows no homology to the 27 amino acid peptide (PS-1) of *D. funebris*. Heterospecific injection of the accessory gland secretions of *D. busckii, D. hydei, D. nigromelanica* and *D. virilis* into *D. melanogaster* virgin females did not cause any increase in oviposition; this evidence suggests that accessory gland secretions are relatively species-specific (Chen *et al.*, 1985).

Fuyama (1983) reported that the accessory gland substances affecting oviposition in two closely related species of the *suzukii* species group, *D. suzukii* and *D. pulchrella*, were to some extent species-specific. *D. suzukii* male accessory gland extract did not cause an increase in egg-laying when injected into *D. pulchrella* females, but injection of *D. pulchrella* extract into *D. suzukii* females did cause an increase in

oviposition. Fuyama (1983) suggested that incompatibility between accessory gland secretions of different species could serve as a species isolating mechanism.

Several *D. melanogaster* male accessory gland-specific genes have been isolated and characterised by DiBenedetto *et al.* (1987), Chapman and Wolfner (1988), Monsma and Wolfner (1988) and DiBenedetto *et al.* (1990). Male-specific transcript (mst) 316 encodes a basic protein of 52 amino acids synthesised in the main cells (DiBenedetto *et al.*, 1987, 1990) and was independently isolated by Schafer (1986). The function of the male specific protein (msp) 316 is unknown. Two msts, 355a and 355b, were isolated by Monsma and Wolfner (1988). Mst 355a encodes a basic protein of 264 amino acids and mst 355b encodes an acidic protein of 90 amino acids; both of these proteins are secreted and transferred to the female at mating. The 355a protein appears to be cleaved to a smaller 90 amino acid peptide 10-15 minutes after ejaculation within the female reproductive tract (Monsma and Wolfner, 1988).

Msp 355a showed an 11 out of 17 amino acids sequence homology to the sea hare (*Aplysia californica*) egg-laying hormone (ELH). ELH works in *Aplysia* by stimulating specific ganglion cells, causing the ovotestis to contract and release the egg mass (Scheller *et al.*, 1983). It is not yet clear whether msp 355a acts as an egg-laying hormone in female *D. melanogaster*. However females germline transformed with the male-specific 355a gene showed a small (20%) but reproducible increase in egg-production; sexual receptivity was not affected. The function of msp 355b is unknown.

Apart from msp 355a, none of the proteins or peptides isolated by Schafer (1986), DiBenedetto *et al.* (1987) or Monsma and Wolfner (1988) so far tested have effects on female egg-laying or receptivity. It is possible that these peptides, unlike the SP, do not function if expressed in isolation. Combinations of two or more peptides or proteins may be have to be present at particular concentrations for any response in the female to be observed. Only effects on egg-laying or receptivity were assayed. These

accessory gland-specific proteins and peptides may have had effects on other processes that were not detectable in the assays that were used, for instance they might be involved in sperm transfer, storage or utilization.

An ovulation stimulating substance (OSS) has recently been isolated from male *D. suzukii* (Ohashi *et al.*, 1991). OSS is a peptide of at least 35 amino acids; injection of an OSS extract into virgin females stimulated ovulation and decreased receptivity. OSS was also tested in *D. melanogaster* where it caused larger responses than in *D. suzukii* or the more closely related *D. pulchrella*. However, mass spectrometry of the OSS peak showed that there were impurities in the sample, and the first 10 amino acids of one of the impurities showed complete sequence homology to the SP (Yoshiaki Fuyama and Eric Kubli, pers. comm.). Attempts to purify the sample further resulted in complete loss of activity, so it is not clear whether the major peak had anything to do with the biological activity observed; the active agent seemed to represent <5% of the major peak.

HPLC analysis of *D. suzukii* male accessory glands produced 2 peaks that affected female rejection and ovulation behaviour (Choffat, unpublished). One of these peaks reacted with the SP antibody on a Western blot and was the *D. suzukii* SP; the other peak did not react with the SP antibody and was not the *D. suzukii* OSS or SP, and therefore the sequence and the biological activity seemed to be coming from different components. The 10 amino acids at the C terminus of the *suzukii* second peak isolated by Choffat were the same as for the *D. suzukii* SP and the OSS contaminant; it seems likely therefore that there is only one SP in *D. suzukii*. There is no evidence from HPLC profiles of a second peak with biological activity in *D. melanogaster* (Choffat, unpublished), and it seems likely that there is only one SP-like peptide in *D. melanogaster*, ie. the SP itself.

Pheromones also play a role in regulating the sexual attractiveness of female *D. melanogaster*. Males appear to use *cis*-vaccenyl acetate as an anti-aphrodisiac (Jallon *et al.*, 1981) transferring it to the female at mating. It is synthesised in the male ejaculatory bulb (Butterworth, 1969; Brieger and Butterworth, 1970) and present in the cuticle. Mane *et al.* (1983) suggested that the simultaneous transfer of *cis*-vaccenyl acetate and the enzyme esterase-6, which is synthesised in the ejaculatory duct (Stein *et al.*, 1984), to the female at mating could cause the hydrolysis of *cis*-vaccenyl acetate to *cis*-vaccenol, which could be subsequently released as an anti-aphrodisiac by the female.

Van der Meer *et al.* (1986) however, found no evidence that esterase-6, *cis*-vaccenyl acetate and its hydrolysis product *cis*-vaccenol act together as an anti-aphrodisiac pheromone system. Direct chemical analyses provided no evidence that *cis*-vaccenyl acetate was converted to *cis*-vaccenol; esterase-6 also had no effect on the rate of *cis*-vaccenyl acetate loss from the reproductive tracts of mated females. *In vivo* concentrations of *cis*-vaccenyl acetate transferred to the female at copulation were found to fall below the level necessary for the inhibition of courtship within 4 hours. *cis*-vaccenyl acetate was not transferred from the reproductive tract to the abdominal cuticle which would be predicted if it were important as a pheromone (Van der Meer *et al.*, 1986). The interaction of accessory gland components with such pheromonal cues is unclear.

Esterase-6 has also been implicated in sperm usage, female remating probability and productivity (Richmond *et al.*, 1980; Gilbert, 1981; Gilbert *et al.*, 1981a, b; Gilbert and Richmond, 1982). The genetic background of different esterase-6 allele stocks however was not identical, and the conclusions of the esterase-6 studies are open to alternative interpretations. In comparisons of the rate of initial sperm use by females mated to either wild-type Oregon-R males or males with different esterase-6 alleles (Gilbert, 1981), no attempt was made to standardise the genetic background of the

males used; the results are therefore equivocal. Another enzyme present in the seminal fluid, glucose oxidase, has been suggested to act as a bactericide or fungicide (Cavener, 1980).

Filamentous structures that resemble microtubules are also synthesised by accessory glands, and are ejaculated with the sperm (Bairiati, 1966; Perotti, 1971). Immunoelectrophoresis and antigenic labelling of ultrathin sections has shown that these filamentous structures are apparently not microtubules (Federer and Chen, 1982). After mating the filamentous structures are found in the female uterus and seminal receptacle, packed between sperm. It has been suggested that they act as mechanical supports for sperm, assisting transport into the female sperm storage organs (Bairiati, 1966) or as a substrate for sperm metabolism (Perotti, 1971). Support for these explanations comes from the observation that the filaments ejaculated by spermless XO males remain in the genital chamber and do not move into the female sperm storage organs. Such filamentous structures are also found in association with the sperm of reptiles and isopods (see Perotti, 1971).

1.6 *Drosophila* as an Experimental Organism

As early as 1921, the advantages of using *Drosophila melanogaster* as an experimental organism for longevity studies were realised (Pearl and Parker, 1921). *Drosophila melanogaster* has a short generation time (approximately 10-11 days at 25°C) and is small in size; these features make it easy to raise large numbers of individuals. The genome is also small, facilitating molecular analysis; it is approximately 1/20th of the size of a typical mammalian genome (Spradling and Rubin, 1981). The genetics of *D. melanogaster* have also been well characterised; well over 3000 mutants have been described (Lindsley and Grell, 1968; Lindsley and Zimm, 1992). *D. melanogaster* has been used as an experimental tool in classical genetics, cytogenetics, biochemistry, molecular genetics, electrophysiology and cell biology

(Rubin, 1988). *D. melanogaster* is particularly suited for use in studying life histories for several reasons. There are a large number of mutations in reproductive function which can be used to determine the aspects of reproduction that are costly. Populations of outbred wild-type stocks adapted to laboratory cage culture conditions are also available. The genetics of the fruit fly are also fairly easily manipulated, to allow the production of mutant and wild-type flies with comparable genetic backgrounds.

1.7 Description of the Experiments Presented in the Thesis

This thesis investigated the mechanisms of costs of reproduction in female *Drosophila melanogaster*. Some amino acids are transferred to the female at mating and become incorporated in female tissues (Bownes and Partridge, 1987). It was reasoned that, if a limiting amino acid or other nutrient was being supplied by the male, females might remate to gain male-derived nutrients. The possibility of seminal feeding in *D. melanogaster* was investigated in Chapter 3 by measuring the lifespans, fecundities and fertilities of females kept on good and poor quality food, whose exposure to males (and therefore any male-derived benefits) were varied. Both wild-type females and females with an evolutionary history of exposure to good and poor quality food were used.

The cost of mating in female *D. melanogaster* (Fowler and Partridge, 1989) could be due to injury at mating, the transfer of disease or parasites or some effect of the sperm or accessory fluid. Chapter 4 describes work in which females were exposed to males that could not transfer sperm, to determine whether the transfer of sperm or accessory fluid was costly to females.

Several components of the accessory fluid are candidates for contributing to the cost of mating because of their effects on female behaviour and physiology (section 1.5.2). In Chapter 5 injection experiments were used to investigate the effect of receiving accessory fluid and one of its sub-components, the SP, on female lifespan and fecundity. Although the effects of the SP on receptivity and egg-laying in virgin

females have been well characterised (sections 1.5.1 and 1.5.2), virtually nothing is known about the mode of action of the SP, or where receptors are located. In Chapter 6, several experiments were performed to gain an insight into possible SP receptor sites, by investigating whether there was a correlation between the rate of egg-laying and SP-usage, as would be predicted if there were receptors for the SP on the ovary, or some other site controlling oviposition rate. *dunce* females were also used to gain an insight into the SP mode of action, because the *dunce* mutation may interfere with the SP signal pathway.

dunce females also seem to be more susceptible to the cost of mating. To investigate whether this is due to an increased susceptibility to the effects of a single mating, or a consequence of their elevated remating rate with respect to wild-type females (Bellen and Kiger, 1987), the effect of a single mating on *dunce* female lifespan was investigated (Chapter 6).

An SP-null mutant would be very useful; it would enable the effects of the SP on female receptivity and egg-laying to be confirmed and its effects on male and female reproductive success to be examined. Chapter 7 describes a mutagenesis screen for SP-null mutations.

Chapter 2. General Methods

Some of the work described in this thesis was performed during two 2-month visits to the University of Zurich, Switzerland. The remainder of the work was performed in Edinburgh, Scotland. This chapter describes general methods used in each of the two laboratories. A number of procedures were used in more than one experiment and are described here. Methods specific to each experiment are described in each chapter.

2.1 General Methods, Stocks and Cultures - Edinburgh Laboratory

2.1.1 Media

2.1.1.1 Lewis medium

Lewis medium was used for general stock maintenance; it favours larval growth as it is a relatively soft medium suitable for larvae to burrow into.

Yeast	- 17.04g
Maize meal	- 94.32g
Sugar	- 85.22g
Agar	- 6.25g
Water	- 1000ml
Nipagin	- 28ml

Nipagin solution is an anti-fungal agent and consists of 100g powdered nipagin dissolved in 950ml of absolute ethanol and 50ml water.

2.1.1.2 SY (sugar-yeast) medium (Trevitt and Partridge, 1991)

SY medium was used for general stock maintenance and in many of the experiments for storing adults. SY food favours adult survival because it is harder than Lewis medium and adults are less likely to become stuck.

Yeast	- 100g
Sugar	- 30g
Agar	- 100g
Water	- 1000ml
Nipagin	- 30ml
Propionic acid	- 3ml

Propionic acid is an anti-bacterial agent.

2.1.1.3 'High' medium (16% w/v solution)

The 'high' and 'low' food recipes were taken from David *et al.* (1971) and correspond to his 16% and 2% w/v preparations respectively. 'High' and 'Low' media were used for maintaining replicate lines of 'high' and 'low' flies (described in section 2.1.2.3) and used in two experiments where the nutrition of flies was manipulated (Chapter 3).

Flaked yeast	- 80g
Cornflour/Maize meal	- 80g
Agar	- 12g
Water	- 1000ml
Nipagin	- 30ml

2.1.1.4 'Low' medium (2% w/v solution)

Flaked yeast	- 10g
Cornflour/Maize meal	- 10g
Agar	- 12g
Water	- 1000ml
Nipagin	- 30ml

2.1.1.5 'Intermediate' medium

'Intermediate' medium is intermediate in concentration to the 'high' and 'low' media described above. Flies from the 'high' and 'low' food cages were raised on this medium in the selection experiment described in Chapter 3 to control for parental carry-over effects.

Flaked yeast	- 45g
Maize Meal	- 45g
Agar	- 12g
Water	- 1000ml
Nipagin	- 30ml

2.1.1.6 Grape juice medium (Fowler and Partridge, 1986)

Grape juice medium was used to rear flies at standardised densities (see section 2.1.4.3).

Agar	- 80g
Red grape juice	- 1000ml

Water - 600ml

The grape juice compound used was 'CWE Classic 5' (CWE Ltd. Norwich) which is a mixture of grape juice and glucose syrup.

2.1.1.7 Sugar (sucrose) food

Sugar food, which has no protein source, was used to suppress egg-production, to equalise egg-laying rates between groups of females exposed to different experimental treatments. Females kept on sugar food will survive for several weeks but lay few, if any, eggs (Bouletreau-Merle, 1971).

Agar - 20g

Sucrose - 30g

Water - 1000ml

2.1.1.8 Ringer solution (Chan and Gehring, 1971)

NaCl - 3.20g

KCl - 3.00g

$MgSO_4.7H_2O$ - 1.80g

$CaCl_2.2H_2O$ - 0.69g

Tricine - 1.79g

Sucrose - 17.10g

Glucose - 3.6g

Water - 1000ml

The pH of the Ringer solution was adjusted to 6.95 and 3ml lots dispensed into 5ml Bijou bottles, which were then autoclaved at 115lb/sq in for 15mins. The Ringer solution was then stored at +4°C until use.

2.1.2 Stocks

2.1.2.1 Dahomey wild-type

The Dahomey wild-type is an outbred stock maintained with overlapping generations in population cages at 25°C on a 12:12 hour light/dark lighting regimen. The stock was derived from individuals collected in Dahomey (now Benin), West Africa in 1970, and has been maintained at Edinburgh according to the above regimen since collection. The cages received 4 SY culture bottles per week, removed 28 days later; this was long enough for all adults to emerge from culture bottles before they were removed from the cage.

2.1.2.2 Brighton wild-type

The Brighton wild-type population was derived from individuals collected in Brighton in 1984, and has been maintained in population cages since then as described above; the cages received 4 SY culture bottles per week which were removed 28 days later.

2.1.2.3 'High' and 'Low' lines

The lines of flies selected on 'high' and 'low' quality food (hereafter called 'high' and 'low' lines) were founded in 1985, from the Brighton wild-type stock; there were three replicate cages of each line. The 'high' line flies received 3 bottles of 'high' medium each week which were taken out 28 days later. The 'low' line flies received 4 bottles of 'low' medium per week, removed 28 days later.

2.1.2.4 *fruitless*

To expose females to males that would not mate, males homozygous for the *fruitless* mutation were used, because they court females but do not mate with them (Hall, 1978). The *fruitless* stock was obtained from Dr B. Kyriacou, Leicester University.

2.1.2.5 Attached-X stock

An attached-X stock *C(1)RM, yw/0* was used to generate XO males (Cambridge Stock Centre, England). XO males were used to expose females to males that could mate but that could not transfer sperm. Balancer chromosomes marked with *Curly* and *Ultrabithorax* were used to introduce Dahomey wild-type chromosomes II and III into this stock. The resulting stock was then maintained in cage culture; 4 bottles of yeasted Lewis medium were placed in the cage every week and removed 28 days later. XO males with Dahomey X, II and III chromosomes were obtained from this stock by crossing males to Dahomey virgin females.

2.1.2.6 *transformer*

A *transformer* stock, $B^{s}Y/yw$; *mwh tra/TM3, Sb Ser* (donated by Professor R. Nothiger, University of Zurich) was used to generate XX *tra/tra* pseudomales, to expose females to males that could mate but that could not transfer sperm. The *Bar*, *yellow* and *white* mutations were removed from the stock by outcrossing to Dahomey wild-type and the resulting *mwh tra/TM3, Sb Ser* stock maintained by tossing onto new SY culture bottles every 2/3 days. The XX *tra/tra* pseudomales and XY *tra/tra* males were therefore both free of mutant markers and could be obtained from the same cultures; they were sorted apart on the basis of their size: XX *tra/tra* homozygotes are larger than XY *tra/tra* males. The XX *tra/tra* pseudomales have smaller testes than normal which were easily scored by dissection. XY *tra/tra* males were therefore a good

control for the XX *tra/tra* pseudomales because they had identical genetic backgrounds and could be obtained from the same cultures as XX *tra/tra* flies.

2.1.3 General Culturing Methods

2.1.3.1 General fly culture

For general culturing of flies and population cage maintenance, 300ml culture bottles were used, filled with 70ml medium. The experimental vials used measured 75mm*25mm and were filled with 7ml of food medium.

2.1.3.2 Fly transfer

Flies were transferred to new food vials by aspiration, cooling over ice or carbon dioxide anaesthesia on flies not less than 3 hours old. Carbon dioxide has no significant effect on either female survival or fertility (Partridge *et al.*, 1986).

2.1.4 Experimental Methods

2.1.4.1 Remating frequency

Remating frequency was scored by observing the number of copulations in experimental vials every 20 minutes for a period of 3-6 hours, starting 1-3h before lights on, when the flies were most active. Mating in *D. melanogaster* lasts approximately 20 minutes at 25°C; scoring mating every 20 minutes ensured that all matings were recorded.

2.1.4.2 Body size measurements

Thorax length was used as a measure of body size, since it correlates well with other linear measurements (Ewing 1961). Thorax length was measured by taking the distance from the tip of the scutellum to the base of the most anterior major bristle. Thorax lengths were measured in eyepiece graticule units, 25 graticule units = 1mm.

2.1.4.3 Standardised larval density

In several experiments experimental flies were raised as larvae at standardised densities to minimise differences in adult body size which could have arisen from growing larvae at uncontrolled densities. Parents of the experimental flies were placed in inverted pots, the lids of which contained Grape Juice medium smeared with a small blob of live yeast paste (Fowler and Partridge, 1986; see section 2.1.1.6). Lids containing the Grape Juice medium could easily be replaced without losing flies, by inverting the pots and tapping gently.

Flies were given 24 hours to acclimatise to the Grape Juice medium and then given new grape juice lids twice within a 4 hour ('pre-laying') period to allow plenty of oviposition space on the medium, thereby encouraging the females to lay any retained eggs. Females were then given a 2-4 hour 'laying period', the exact length of time given depending on the rate at which eggs were laid. These grape juice lids were then stored at 25°C for 26 hours from the mid-point of the laying period; first-instar larvae were then picked from the lids, using a fine brush, and placed into food vials in the required numbers (usually 100 per vial). Subsequent development continued in the food vials from which experimental adults were later collected.

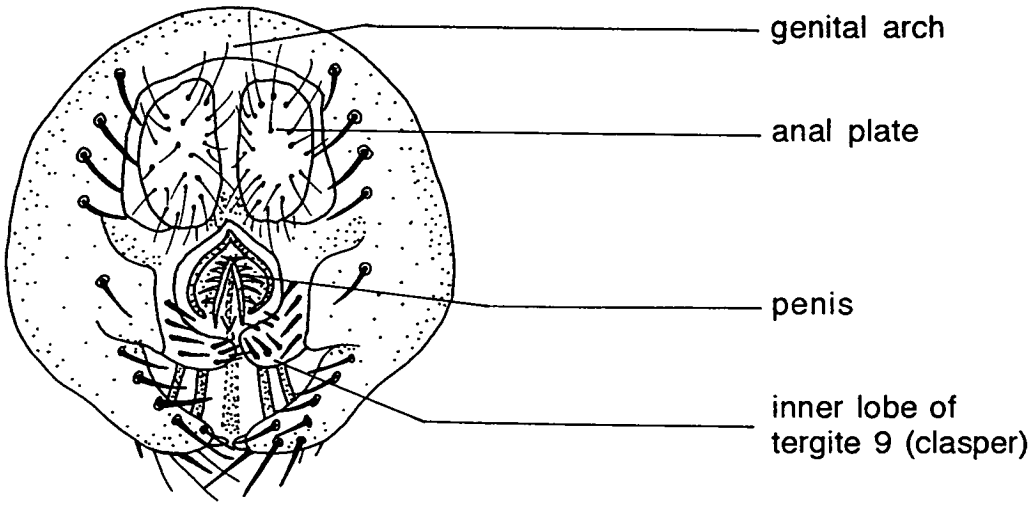
2.1.4.4 Microcautery

Microcautery was used to sterilise males by ablating their external genitalia. A current of approximately 80V was applied to the external genitalia of 2 day-old males through 2 tungsten electrodes 40μm apart, until the penis had been ablated (see Fig. 2.1). To check that the males were sterile they were screened for mating ability 2 days after the microcautery. Males were put 3 per vial together with 4, 5 day-old virgin females; any males that mated within the next 3 hours were discarded. In each screen for mating ability of the microcauterised males, control vials of intact 4 day-old wild-

type males and 5 day-old virgin females were set up to control for any environmental effects that might have affected the willingness of flies to court and mate (eg. barometric pressure, Ankney, 1984).

Fig. 2.1. Male *Drosophila melanogaster* external genitalia

(X 50)



2.1.4.5 Fly injection

The injection apparatus used consisted of a motor-driven syringe operated by a foot pedal. Depression of the pedal caused the delivery of 50nl from the end of a glass micro-needle attached to the motor-driven micro-syringe via a flexible tube. The whole system was paraffin oil-filled, making it unnecessary to calibrate the system each time a new needle was used. Females were injected in the abdomen whilst immobilised over ice; the mortality resulting from the injection procedure was minimal.

The volume of the glass micro-needles used was 2.0µl. The dose response curves for the effect of SP injection on ovulation and receptivity (Fig. 2.2) show a sharp increase in ovulation and decrease in female receptivity at about 0.6pmol SP injected per female. The standard dose injected experimentally was 3.0pmol per female in 50nl Ringer solution; this was a high enough dose to be sure of inducing a response in receptivity and ovulation. The accessory glands of a 7 day-old male contain about 6.2pmol SP, and approximately one third to one half of the contents are transferred to the female at mating (Hihara, 1981); the standard dose of SP injected, 3.0pmol, was therefore approximately a physiological dose.

2.1.4.6 The sex peptide

The amino acid sequence of the natural sex peptide is -

1 5 10

TRP-GLU-TRP-PRO-TRP-ASN-ARG-LYS-HPR-THR-LYS-PHE-HPR-ILE*-

15 20 25

HPR-SER-HPR-ASN-HPR-ARG-ASP-LYS-TRP-CYS-ARG-LEU-ASN-LEU-

30 35

GLY-PRO-ALA-TRP-GLY-GLY-ARG-CYS.

(Chen *et al.*, 1988).

Direct amino acid sequencing of the isolated peptide, and HPLC-purified proteolytic fragments, identified a substance not corresponding to any of the common amino acid derivatives at position 14. cDNA cloning clearly showed an AUU codon specifying isoleucine at this position. Analysis of the phenylthiohydantoin amino acid derivatives obtained during sequencing however, indicated that this residue was not isoleucine. Chen *et al.* (1988) concluded that the modification was probably hydroxylation, as the only modification reported for isoleucine in naturally occurring compounds is hydroxylation (eg. Shoham *et al.*, 1984). Hydroxylated isoleucine was not available as a standard, so the identity of this amino acid is still to be confirmed.

The SP injected in experiments was synthetic, and had the same biological activity as the natural peptide (Chen *et al.*, 1988). It had the hydroxyproline modifications of the natural peptide, but not the hydroxyisoleucine modification; it was otherwise identical to the natural peptide. In longevity experiments, synthetic fragments of the SP were used to control for any immunological response mounted against the synthetic sex peptide. The sequences of the two SP fragments used as controls (SP 21-36 and SP 25-36) are shown below, together with the complete SP sequence, in single letter amino acid code. At the time the SP fragments were used in experiments, both the SP 21-36 and SP 25-36 fragments were thought to have no biological activity.

SP	WEWPWNRKPTKFPIPSPNPRDKWCRLNLGPAWGGRC
SP 21-36	DKWCRLNLGPAWGGRC
SP 25-36	RLNLGPAWGGRC

The SP and SP fragments were manufactured by the Biochemistry department, University of Zurich and donated by Professor E. Kubli.

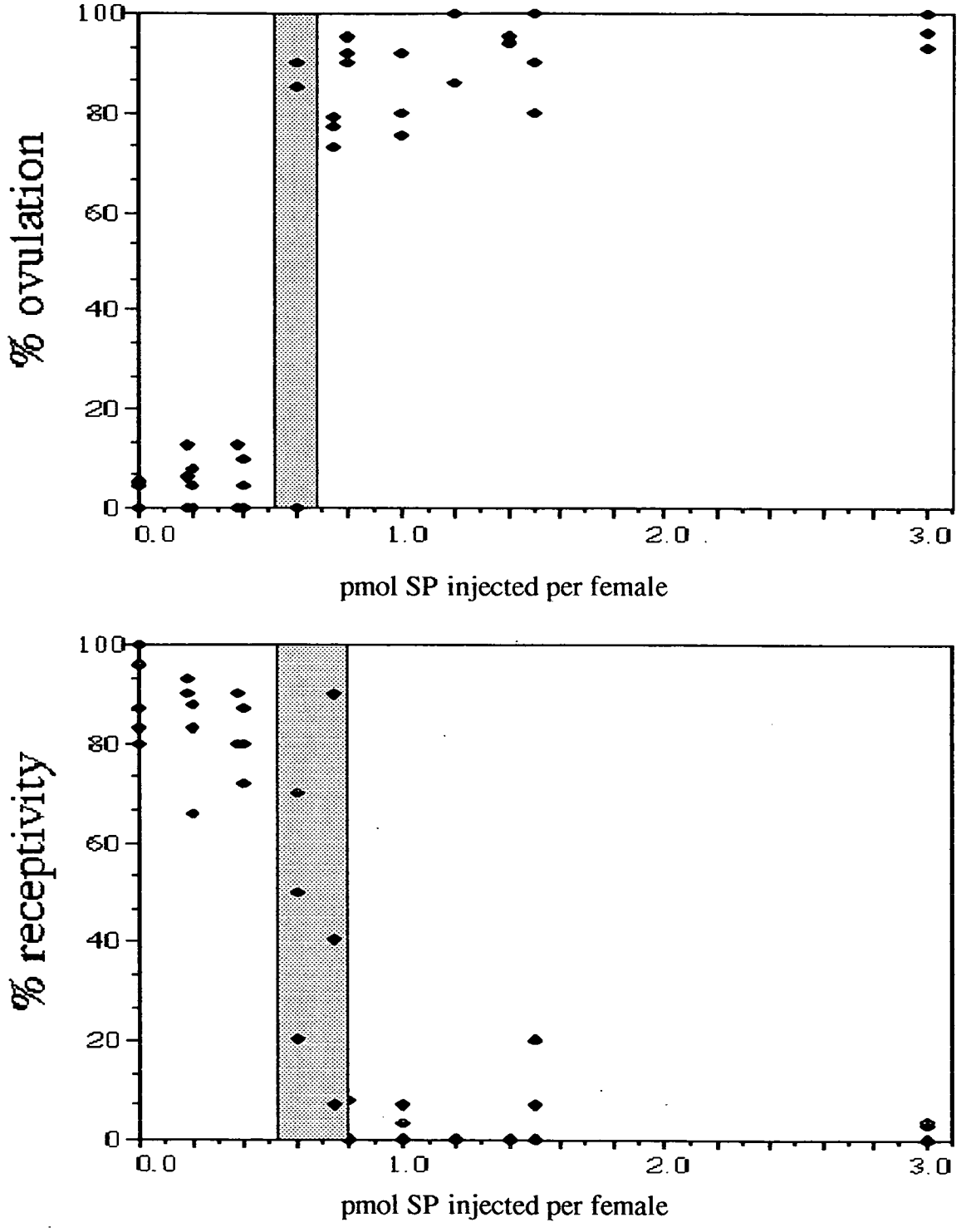


Fig. 2.2. Dose response curves for pmol SP injected against % ovulation and % receptivity. Reproduced courtesy of Yves Choffat. Grey bars indicate response threshold.

2.2 General Methods, Stocks and Cultures - Zurich Laboratory

2.2.1 Media

2.2.2.1 Zurich fly food

Agar	-	10g
Sucrose	-	75g
Fresh Yeast	-	100g
Maizemeal	-	55g
Wheatmeal	-	10g
Water	-	1000ml
Nipagin	-	15ml

Zurich food was used for general fly culturing and for rearing experimental adults.

2.2.1.2 Sugar (sucrose) food

Recipe as for Edinburgh laboratory (see section 2.1.1.7).

Zurich and sugar food were coloured black, to make counting eggs easier. One 10ml sachet of black food colouring (Werna, W Schweizer AG, CH-8832 Wollerau, Switzerland) was added per 2 litres of food.

2.2.2 Stocks

2.2.2.1 Oregon-R wild-type

The wild-type stock used was Oregon-R kept at 25°C on a 12:12 hour light:dark cycle. The stock was kept in 300ml culture bottles filled with 30ml Zurich fly food. Laying adults were tossed onto new food every day and the laying adults replaced approximately every 10-15 days.

2.2.2.2 *dunce*

dunce mutants were used in several of the experiments described in Chapter 6 because they appear to be susceptible to the effects of mating (Bellen and Kiger, 1987). The *dunce* and *dunce* revertant stocks used were obtained from John Kiger, University of California. The genotypes of the stocks used were as follows -

dunce stock - *y dnc* ^{M14} *ec f/FM7* (Salz *et al.*, 1982)

dunce revertant - *y dnc*$^{+M14R1}$ *ec f/FM7* (Bellen and Kiger, 1987)

y dnc^{+M14R1} *ec f/FM7* is a fertile, spontaneous revertant isolated from the *y dnc*^{M14} *ec f/FM7* stock and exhibits wild-type phosphodiesterase (PDE) activity (Bellen and Kiger, 1987).

2.2.3 General Culturing Methods

2.2.3.1 General fly culture

Stocks were cultured in 300ml culture bottles filled with 30ml Zurich fly food. Vials measured 90mm*30mm and were filled with 10ml Zurich fly food.

2.2.3.2 Tissue plate system of fly culture

A new system of keeping flies was tried in the Zurich laboratory. 24-well tissue culture 'cluster^{24}' plates (Costar, 205 Broadway, Cambridge, MA 02139, USA) were used to keep batches of 24 experimental females together. Each of the 24 wells was 16mm in diameter. Fly food was poured into the lid of the tissue culture plate and left to cool and set. Experimental females were placed into the wells of the other half of the plate (1 female per well) by cooling on ice.

The lid of the culture plate was then replaced and pushed onto the other half so that there was no gap between each well and the food-filled lid. The whole plate was then inverted food-side down and the food lid secured by rubber bands at each end. Each of the 24 wells had small ventilation holes drilled in the top. In order to change the food plate and count eggs, it was necessary to simultaneously anaesthetise all 24 females. This was achieved in 2 ways:

(i) Inverting the plate and cooling it down for 15 mins in a refrigerator, followed by cooling for 5 mins on ice, tapping the food lid several times to immobilise females on the surface of the plate next to the ice. The food plate could then be removed and replaced.

(ii) Inverting the plate and placing it on a carbon dioxide-diffuser plate for approximately 30 seconds, again tapping the food lid to ensure that all the females were immobilised and away from the food lid when it was replaced.

Once the food plate was replaced, eggs could be counted; the eggs were easily visible against the black background. In the initial experiments, food plates were changed by cooling. This led to problems with condensation inside the wells of the plates. In later experiments CO_2 was used; this proved to be quicker, more convenient and left the plates drier.

2.2.4 Experimental Methods

2.2.4.1 Female rejection behaviour assay

In several experiments a rejection test was performed to assay the rejection behaviour of females towards courting males, normally (but not always) 3 hours after injection. The day before the test, males were stored 7 per vial overnight. The exact age of the males differed between experiments but was typically about 5-7 days. After injection, females were stored 3 per vial. 3 hours later, vials of 7 males and 3 females were put together and the number of copulations in the following hour recorded. The rejection behaviour of females following SP injection can be influenced by certain weather conditions (eg. barometric pressure, Ankney, 1984). It was very important to control for this effect. Usual controls were 5-day old Oregon-R virgin females injected with Ringer or SP; 3 hours later, their rejection behaviour was tested simultaneously with the experimental females. All of the experiments used Oregon-R males for rejection tests.

Chapter 3. Remating and Male-derived Nutrients

3.1 Introduction

A large number of evolutionary explanations for remating by females have been suggested (see Chapter 1, section 1.3). Remating may renew or replenish dwindling sperm supplies (Anderson, 1974; Gromko and Pyle, 1978; Gromko *et al.*, 1984a; Newport and Gromko, 1984). It could also be an insurance against male-sterility and sub-fertility (Gibson and Jewell, 1982). Females may gain nutrients at mating (eg. Pitnick *et al.*, 1991) and there may sometimes be benefits from increased genetic diversity among offspring (Williams, 1975). Multiple mating could also promote competition among ejaculates of different males (Parker, 1970a).

Remating by females could also occur as a result of conflict with males. For instance, harassment of females by males during courtship could prevent them from feeding (Alcock *et al.*, 1977) or make them conspicuous to predators. Female remating could also evolve as a correlated response to sexual selection on males (see Chapter 1, section 1.3.8), if the remating rates of the two sexes are genetically correlated (Halliday and Arnold, 1987; but see Sherman and Westneat, 1988; Arnold and Halliday, 1988; Cheng and Siegel, 1990; Gromko, 1992; Arnold and Halliday, 1992; Stamenkovic-Radak *et al.*, 1992). In some insects, remating is costly to females, because it lowers their lifetime reproductive success by shortening their lifespan (eg. Arnqvist, 1989; Fowler and Partridge, 1989).

Among animals there is considerable variation in the extent to which females remate (Halliday and Arnold, 1987). This diversity can be seen among the members of the genus *Drosophila*, where *D. pachea* has been reported to remate several times daily (Pitnick *et al.*, 1991) while *D. subobscura* from northern populations do not remate in

the course of a lifespan that can last several weeks (Maynard Smith, 1956; Steele, 1984). High and variable rates of multiple insemination in field populations have been reported for *D. melanogaster* (47% estimated by Milkman and Zeitler, 1974; 12-65% by Marks *et al.*, 1988) and *D. pseudoobscura* (>50% estimated by Anderson, 1974; 30-60% by Cobbs, 1977; 60-100% by Levine *et al.*, 1980). Multiple insemination in the field has also been reported as >23% for *D. subobscura* (Loukas *et al.*, 1981), >35% for *D. eronotus* (Stalker, 1976) and 83% for *D. athabasca* (Gromko *et al.*, 1980). It is not clear if the intraspecific variation in remating rates is mainly genetic or environmental in origin, and ecological variables can be important (Marks *et al.*, 1988).

Remating rate is known to be heritable in *D. melanogaster* (Gromko and Newport, 1988) and would therefore be expected to be responsive to natural selection. Identifying the functions of repeated mating is of interest, because they may explain the wide variation in remating frequency that is observed both between different species and between different populations of the same species. The aim of the experiments presented in this chapter was to assess the importance of seminal feeding in *Drosophila melanogaster*. Amino acids from the seminal fluid enter the female haemolymph and yolk proteins (Bownes and Partridge, 1987, see Chapter 1 section 1.3.2.4), but it is not clear if these or any other seminal components contribute significantly to female survival, fecundity or fertility. Male-derived contributions to the female are more likely to be important for her fecundity or survival when nutrients are scarce (Turner and Anderson, 1983). The opportunity to remate would then be expected to increase female survival or reproductive output more under conditions of nutritional stress. The effects of remating frequency on fecundity and survival of *D. melanogaster* females under different feeding regimens has not been previously investigated.

Turner and Anderson (1983) varied the amount of yeast supplied to female *D. pseudoobscura*, and compared the lifetime production of adult offspring in females either exposed briefly to males at the beginning of their adult lives, or given continuous

access to males. Access to males increased productivity of both groups of females, but the proportional increase was much greater for the females kept without yeast. The results could have been explained by nutrient transfer at mating, or by higher death rates of stored sperm in the poorly fed females. Male *D. pseudoobscura* have since been found to transfer nutrients to females, both through courtship feeding (Steele, 1986) and through the ejaculate (Bownes and Partridge, 1987; Markow and Ankney, 1988).

Two approaches were used to investigate the importance of seminal feeding in *D. melanogaster*:

1) A comparison of the remating rates, survival and fertility of females that had an evolutionary history of exposure to poor and good quality food. It was reasoned that if seminal feeding is important, then females persistently exposed to poor nutrition might be expected to evolve higher remating rates, leading to either higher fecundity or higher survival rates under these conditions.

2) Measurement of the remating rates, survival, fecundity and fertility of wild-type females with low or high opportunities for remating when they were exposed to poor or good quality food. It was reasoned that, if the male nutritional contribution is important, females in poor nutritional condition would benefit more in their fecundity or survival from extra matings.

These experiments involved measurement of female remating rates. There is some controversy about the best way to measure these in the laboratory; different experimental designs (eg. continuous versus intermittent confinement of the sexes) can have a large effect on remating frequency (Newport and Gromko, 1984). The evolutionary history of all the stocks used in this study was one of continuous exposure to males in cage culture. Continuous exposure of females to males was therefore used as the highest opportunity for remating in the experiments. Remating rates tend to be

higher using this type of experimental design than in ones involving intermittent exposure (Newport and Gromko, 1984; Fowler and Partridge, 1989). Continuous exposure of the sexes and high rates of remating may be representative of the natural situation, where adults can occur on food resources at continuously high local densities (Johnston and Heed, 1975, 1976); remating rates under these conditions can be very high (Partridge *et al.*, 1987b). The reports of high multiple insemination rates in field populations of *D. melanogaster* (eg. Milkman and Zeitler, 1974; Gromko *et al.*, 1980; Marks *et al.*, 1988) also support the idea that remating may be frequent in nature.

This work has contributed to a manuscript which has been submitted for publication (Chapman *et al.*, submitted, see Appendix A for manuscript).

3.2 Effects of Natural Selection on High and Low Quality Food

3.2.1 Method

The evolutionary responses of remating, egg-production, development time and larval and adult survival to level of nutrition were investigated by long-term culture of the same base stock on medium of two different concentrations. The aims were to examine whether remating rate was higher in lines with a history of low nutrition, and whether any increase in remating rate was associated with increased female survival or fecundity, as would be predicted if seminal feeding was important.

The 'high' and 'low' selection regimens were set up in 1985 using flies from the Brighton base stock, and 3 replicate population cage cultures were kept in each selection regimen using sugar-free food media described by David *et al.* (1971), see Chapter 2, sections 2.1.1 and 2.1.2.3. These lines had been in existence for 5 years at the time the experiments on them were carried out.

To control for any maternal effects, the parents of the experimental flies were all reared at standard larval density on 'intermediate' food (see Chapter 2, section 2.1.1.5), which was intermediate in concentration to the 'high' and 'low' media. To eliminate the problem of inbreeding depression through homozygosity of deleterious recessive alleles in the selected lines, hybrids between the lines within a selection regimen were used. Additive genetic effects in the selected lines would still have been present in the hybrids. Parental flies from replicate lines of the same selection regimen were crossed, males of replicate 1 with females of replicate 2, males of 2 with females of 3 and males of 3 with females of 1, and eggs from the crosses were collected on grape juice medium (Fowler and Partridge, 1986; see Chapter 2, section 2.1.1.6). Newly hatched F_1 first-instar larvae from all crosses were then collected and placed at a density of 40/vial on 'low' food and 150/vial on 'high' food; larvae from each regimen were reared on both types of food. Virgin females were then collected under CO_2 anaesthesia and aged in single-sex groups of 20/vial for 2 days.

Development time and larval survival were recorded by counting the number of adults eclosing every 8 hours from the 'high' and 'low' food vials. To measure remating rate, reproductive performance and survival of adult females, 20 F_1 females from each cross were collected as virgins, and placed individually in food vials of the same type as that on which they had been reared, together with 2, 3 day-old wild-type Brighton males. Remating frequency was sampled 4 times per week by scanning along vials every 20mins for 3h starting 1h before lights on. Female deaths were recorded daily. Females were transferred to fresh food vials every 3 days and the males were renewed then. The vacated vials were retained to count adult progeny emerging. The 'low' food vials were seeded with active yeast, to provide a sufficient food supply to ensure high (>90%) and constant survival rates for the larvae. In a separate experiment, the thorax lengths of 5 adult females from each replicate hybrid cross reared on 'low' and 'high' food were measured (see Chapter 2, section 2.1.4.2).

3.2.2 Results

The proportion (p) of first-instar larvae surviving to adults was calculated and then arc sin transformed for analysis of variance (ANOVA); Table 3.1 shows the mean proportion of survivors +/- 95% confidence intervals. Growth food had a significant effect on larval survival; more larvae survived to become adults on 'high' food than on 'low' food ($F_{1,4}df = 11.07$, $p<0.05$). There were no significant differences in larval survival between selection regimens.

Development times +/- 95% confidence intervals for males and females are shown in Tables 3.2 and 3.3. Growth food had a large effect on development time in both males and females, $F_{1,4}df = 369.89$ ($p<0.001$) and $F_{1,4}df = 342.65$ ($p<0.001$) respectively; flies on 'low' growth food had a much longer development time than flies on 'high' food, irrespective of selection regimen. Females from 'high' hybrid lines had a longer development time than 'low' hybrid line females (on both types of food) $F_{1,4}df = 9.37$ ($p<0.05$); this effect was not significant in males. For males and females there was a borderline significant growth food-selection food effect, $F_{4,24}df = 2.79$ and $F_{4,24}df = 2.80$ respectively; the extent of the difference in development time between 'low' and 'high' line hybrids ('high' hybrids had a longer development time) was larger on 'low' growth food. There was significant variation in development times between male ($F_{4,24}df = 8.05$, $p<0.001$) and female ($F_{4,24}df = 5.08$, $p<0.01$) replicate hybrid lines.

To calculate remating rates, each day of observation was taken as one opportunity for mating to occur, and for each female each sampling day was scored as an opportunity 'taken' or 'not taken'. Because mating was infrequently observed, and because there were no significant differences in mating frequency between them, the data from the 3 replicate lines from each selection regimen were combined for the first 4 remating samples, when most of the females were still alive (Table 3.4). Each group

had the potential for producing 240 sampling points (20 females*3 replicates*4 samples); some of the females died during this period, so the number of sampling points was slightly less than 240. Females from both selection regimens kept on 'high' food remated more frequently than those kept on 'low' food. Chi-squared analysis showed that on 'high' food the 'high' line females remated significantly (χ^2 = 4.29 1df, $p<0.05$) more often, whereas on 'low' food the 'low' line females remated significantly more frequently (χ^2 = 9.87 1df, $p<0.01$).

There were no significant differences in lifespan between replicates ($p>0.05$). The survival data from the replicate lines were combined and survival curves plotted (Fig. 3.1). For statistical analysis a non-parametric distribution-free method was used (Mantel-Cox test, BMDP software manual Vol. 2, Ed. W. J. Dixon, 1988). This test generalizes the Log Rank test (Miller, 1981). It cumulates, for each successive sampling interval (day), both the observed and expected number of deaths for each group. The expected number of deaths is calculated by allocating the number of deaths in each group in each sampling interval in proportion to the number of individuals entering that sampling interval. The total observed and expected deaths are used to generate a chi-square value. Females from the 'high' selection regimen kept on 'high' food (median lifespan=18d) lived significantly ($p\leq0.012$) longer than females from the other 3 groups, between which there were no significant differences, (median lifespan of 'low' lines on 'low' food=16d, 'low' lines on 'high' food=14d and 'high' lines on 'low' food=17d).

The results for age-specific progeny-production (Fig. 3.2) showed a marked tendency for females kept on 'low' food to produce fewer progeny than those on 'high' food, with no overlap in the egg counts from the two environments. Within an environment, females from the two selection regimens were compared using a nested analysis of variance, with replicate lines nested within selection regimen. There was a tendency throughout for the 'high' line females to produce more progeny on 'high'

food, significant ($p<0.05$) in intervals 1, 3 and 6 and marginally non-significant ($p=0.057$) in interval 2, and there was also a tendency towards the opposite effect on 'low' food, with 'low' females producing significantly more progeny in intervals 2 and 3.

The progeny produced in each sampling interval by each female were cumulated to give a measure of lifetime progeny-production (Table 3.5). Nested analysis of variance on the data from 'high' food showed that females from the 'high' lines produced significantly more progeny ($F_{1,4}$ df = 10.85, $p<0.05$), while on the 'low' food the opposite effect occurred ($F_{1,4}$ df = 13.68, $p<0.05$).

The thorax lengths of females measured in the separate experiment were subjected to analysis of variance (Table 3.6). Females were larger if reared on the same type of food on which they had been selected ($F_{1,4}$df = 8.05, $p<0.05$).

Table 3.1 Mean proportion (and 95% confidence intervals) of 1st-instar larvae surviving to adulthood from the 'low' and 'high' selection regimens kept on 'low' and 'high' food - the data are arc-sin transformed.

		Food	
		'Low'	'High'
Selection Regimen	Replicates		
'Low'	1	0.791 (0.485-1.098)	0.893 (0.696-1.089)
	2	0.758 (0.478-1.038)	0.897 (0.628-1.167)
	3	0.883 (0.847-0.919)	0.886 (0.599-1.173)
'High'	1	0.875 (0.659-1.090)	0.899 (0.774-1.024)
	2	0.833 (0.643-1.023)	0.904 (0.793-1.014)
	3	0.891 (0.855-0.927)	0.928 (0.844-1.012)

Results of nested analysis of variance on the larval survival scores in Table 3.1. Analysis was a 2-way cross-classification of selection regimen and growth food, with replicate lines nested within selection regimen.

Source	ss	ms	df	F
Growth Food[B]	308.52	308.52	1	11.07*
Selection Food[A]	49.02	49.02	1	2.40
Lines Within Selection[C]	81.43	20.35	4	0.38
Growth*Selection Food[B]	30.89	30.89	1	1.10
Growth*Lines Within Selection[C]	111.42	27.85	4	0.53
Error	1261.12	52.54	24	

Error Terms:

A - Lines Within Selection is the correct error term for Selection Food.

B - Growth*Lines Within Selection is the correct error term for both Growth Food and Growth Food*Selection Food.

C - Error is the correct error term for Lines Within Selection and Growth Food*Lines Within Selection.

*$p<0.05$

Table 3.2. Mean male development times (and 95% confidence intervals) in hours of males from the 'high' and 'low' selection regimens kept on 'high' and 'low' food.

		Food	
		'Low'	'High'
Selection Regimen	Replicates		
'Low'	1	292.51 (289.16-295.85)	236.90 (235.78-238.01)
	2	322.59 (315.98-329.19)	245.08 (244.03-246.12)
	3	294.80 (291.73-297.86)	237.23 (236.20-238.25)
'High'	1	323.98 (318.07-329.88)	247.53 (245.97-249.08)
	2	319.38 (313.92-324.83)	246.00 (244.75-247.24)
	3	324.75 (318.43-331.06)	245.95 (245.05-246.84)

Results of nested analysis of variance on male development time scores in Table 3.2 Analysis was a 2-way cross-classification of selection regimen and growth food, with replicate lines nested within selection regimen.

Source	ss	ms	df	F
Growth Food[B]	44598.41	44598.41	1	369.89***
Selection Food[A]	1577.41	1577.41	1	4.53
Lines Within Selection[C]	1391.41	347.85	4	8.05*
Growth*Selection Food[B]	336.72	336.72	1	2.79
Growth*Lines Within Selection[C]	482.28	120.57	4	2.79 ns/*
Error	1036.08	43.17	24	

A, B and C as in Table 3.1
*p<0.05, ***p<0.001

Table 3.3. Mean female development times (and 95% confidence intervals) in hours of females from the 'high' and 'low' selection regimens kept on 'high' and 'low' food.

		Food	
		'Low'	'High'
Selection Regimen	Replicates		
'Low'	1	282.13 (278.51-285.74)	228.21 (235.78-238.01)
	2	310.00 (305.62-314.37)	232.16 (231.14-246.12)
	3	287.57 (284.35-290.78)	228.92 (229.10-229.73)
'High'	1	319.21 (313.79-324.62)	238.58 (237.49-239.66)
	2	310.59 (306.19-314.98)	236.30 (235.28-237.31)
	3	319.01 (312.71-325.30)	239.41 (238.32-240.49)

Results of nested analysis of variance on female development time scores in Table 3.3. Analysis was a 2-way cross-classification of selection regimen and growth food, with replicate lines nested within selection regimen.

Source	ss	ms	df	F
Growth Food[B]	45319.32	45319.32	1	342.65***
Selection Food[A]	2248.34	2248.34	1	9.37*
Lines Within Selection[C]	959.03	239.75	4	5.08**
Growth*Selection Food[B]	504.00	504.00	1	3.81
Growth*Lines Within Selection[C]	529.04	132.26	4	2.80 */ns
Error	1131.90	47.16	24	

A, B and C as in Table 3.1
*p<0.05, **p<0.01, ***p<0.001

Table 3.4. Remating frequencies for the first 4 remating samples of the experiment for females from the 'high' and 'low' selection regimens on 'high' and 'low' food.

Selection Regimen	Food	Remating Opportunities		
		Taken	Not Taken	% Taken
'High'	'High'	68	162	30.0
	'Low'	10	229	4.2
'Low'	'High'	44	166	21.0
	'Low'	28	204	12.1

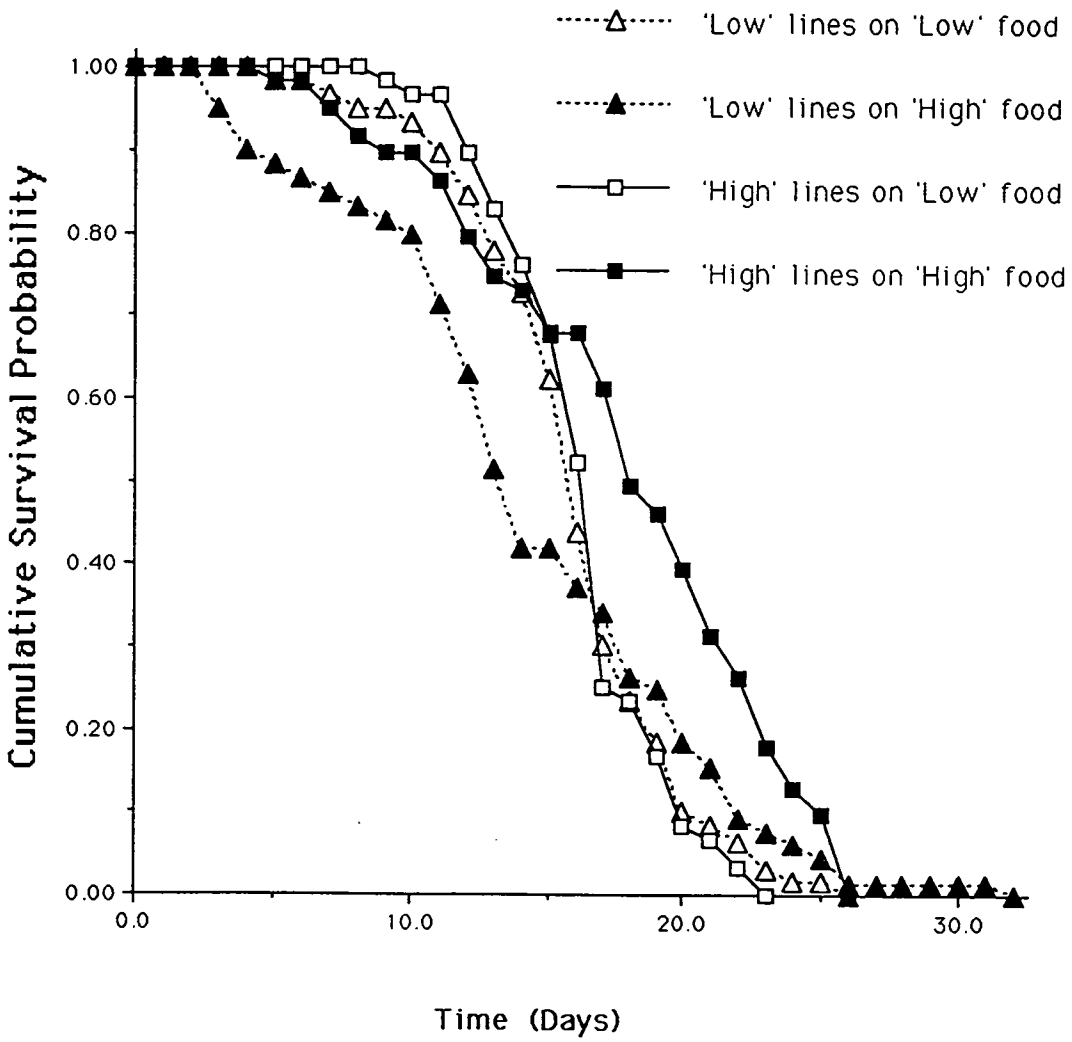


Fig. 3.1. Cumulative probability of survival against time (days) for pooled replicates of 'high' and 'low' line females kept on 'high or 'low' food.

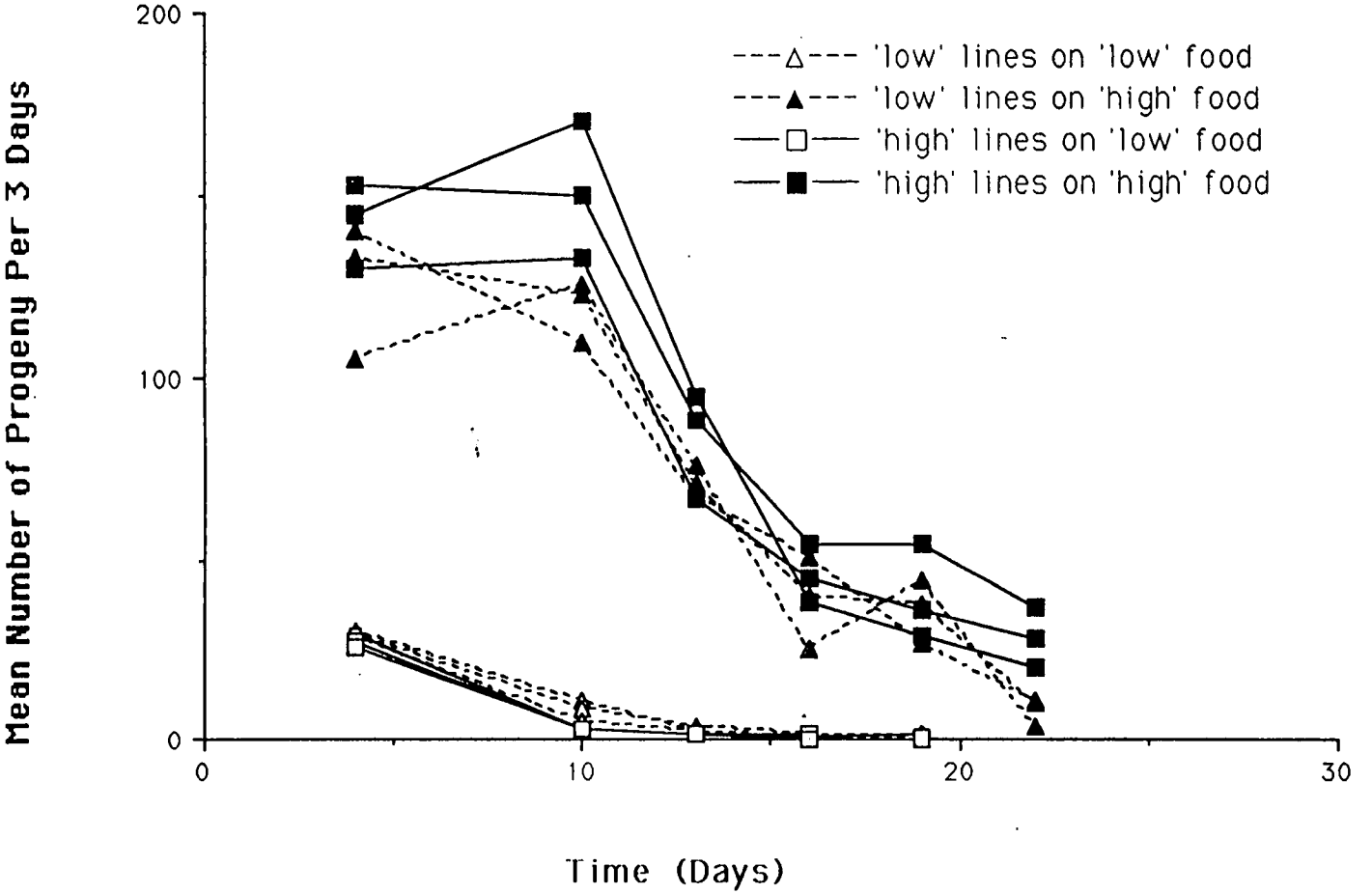


Fig. 3.2. Mean number of progeny produced per 3 day sampling interval for 3 replicate lines of 'high' and 'low' females kept on 'high' and 'low' food.

Table 3.5. Means (and standard errors) of number of progeny produced in their lifetimes by females from the 'high' and 'low' selections regimens kept on 'high' and 'low' food.

		Food	
		'Low'	'High'
Selection Regimen	Replicate		
'Low'	1	44 (3.2)	361 (38.8)
	2	44 (2.7)	350 (44.3)
	3	37 (2.2)	328 (30.5)
	mean	42	346
'High'	1	35 (1.8)	396 (30.0)
	2	33 (1.9)	468 (36.3)
	3	31 (2.5)	469 (26.6)
	mean	33	444

Results of nested analysis of variance on the lifetime progeny-production scores in Table 3.5.

(a) On 'high' food

	df	ss	ms	F
Selection regimen	1	2095	2095	10.85*
Replicate lines	4	773	193	1.75
Error	107	11761	110	

(b) On 'Low' food

	df	ss	ms	F
Selection regimen	1	255978	255978	13.68*
Replicate lines	4	74838	18709	0.86
Error	101	2186107	21645	

*$p<0.05$

Table 3.6 Mean female thorax length (and 95% confidence intervals) of females from the 'high' and 'low' selection regimens kept on 'high' and 'low' food. Measurements in graticule units; 25 units = 1mm.

		Food	
		'Low'	'High'
Selection Regimen	Replicates		
'Low'	1	25.08 (24.60-25.55)	24.50 (24.15-24.84)
	2	24.24 (23.73-24.74)	23.88 (23.28-24.48)
	3	24.96 (24.60-25.31)	24.10 (23.59-24.72)
'High'	1	25.26 (24.65-25.86)	25.22 (24.74-25.69)
	2	24.44 (23.81-25.06)	25.44 (25.04-25.83)
	3	23.96 (23.16-24.75)	25.72 (25.32-26.12)

Results of nested analysis of variance on female thorax length scores in Table 3.6. Analysis was a 2-way cross-classification of selection regimen and growth food, with replicate lines nested within selection regimen.

Source	ss	ms	df	F
Growth Food[B]	1.176	1.176	1	0.866
Selection Food[A]	2.645	2.645	1	2.104
Lines Within Selection[C]	5.029	1.257	4	1.277
Growth *Selection Food[B]	10.922	10.922	1	8.048*
Growth*Lines Within Selection[C]	5.429	1.357	4	1.517
Error	42.92	0.894	48	

A, B and C as in Table 3.1
*$p<0.05$

3.3 Effect of Males on Lifetime Reproductive Success of Well and Poorly Fed Females

3.3.1 Method

The aim of this experiment was to investigate how level of nutrition interacted with the availability of males in determining female remating rate, longevity, fecundity and fertility. Dahomey wild-type flies were reared on Lewis medium (see Chapter 2, section 2.1.1.1) under relaxed larval competition, collected under CO_2 anaesthesia and aged for 3 days in mixed-sex groups of 5 males and 5 females per vial. Adult females in this experiment then had their nutrition and exposure to males manipulated; females were divided into 4 experimental groups of 40 females, and exposed to 1 of the 4 possible combinations of high and low nutrition and high and low availability of males. Nutrition was manipulated by keeping 2 groups each on the 16% w/v ('high') and 2% w/v ('low') media of David *et al.* (1971) as used in the previous experiment.

Remating opportunity was varied by keeping 2 groups each exposed continuously to 2 wild-type males, or to 2 wild-type males for 1 day in 3 and to 2 *fruitless* males for the other 2 days. The *fruitless* males were present as a control for non-mating effects of exposure to males on female survival or fertility (Partridge and Fowler, 1990), as they court females but do not mate with them (Hall, 1978). New 2-3 day-old wild-type and *fruitless* males were provided every 3 days. Fresh vials were provided every 1- and 2-days alternately, coinciding with the introduction and removal of the *fruitless* males in the 2 experimental groups that were exposed to them. All fly transfers were by mouth aspiration.

Remating frequency was recorded on the days when all females were exposed to wild-type males by scanning the vials every 30min for 2-3h after lights on and recording the number of matings that were seen. Female deaths were recorded daily,

and egg-counts were made every third day in the vials in which all females had been exposed to wild-type males. These vials were retained to count the emerging progeny. 'Low' food vials were seeded with active yeast after the egg-counts, to provide a sufficient food supply to ensure high survival rates for the larvae.

Since the number of rematings observed was very low, a short repeat experiment was performed in order to increase the sample size of the remating data. 3 day-old inseminated Dahomey females were exposed to one of the 4 combinations of 'high' and 'low' food and high and low availability of males as before. Remating frequency was scored every day for 5 days by scanning vials every 20mins for 6h, starting 3h before lights on.

3.3.2 Results

The number of matings observed in the main experiment was low. The data on remating rates up to day 9 (Table 3.7a), when female deaths began to occur at high rates, were analysed by calculating Fisher exact probabilities. There were no significant differences within a food regimen in remating rates on days when all females were exposed to intact wild-type males ('high' food $p=0.33$, 'low' food $p=0.09$). There were more rematings on 'high' food than on 'low' (19 versus 6) but this difference was not significant. Since females continuously exposed to wild-type males mated as often as intermittently exposed females on the days when both groups had wild-type males, by extrapolation it follows that they probably mated more often in total. Table 3.7b shows the remating frequencies from the repeat experiment on the 2 days out of 5 when all females were exposed to wild-type males. There were no significant differences in remating frequency within a food regimen ('high' food, Fisher exact $p=0.46$, 'low' food $p=0.055$). Females as a whole on 'high' food remated significantly more than females on 'low' food, ($p<0.000$). Matings were observed in continuously exposed females on the 3 days out of 5 when the other two groups of females were with

fruitless males (34 matings in the the 'high' food continuously exposed group and 5 matings in the 'low' food continuous group during this period). Therefore females continuously exposed to males did remate more often in total than those which were only intermittently exposed.

The survival curves for the 4 groups of females (Fig. 3.3) were subjected to a survival analysis as described previously. Females exposed to 'high' food survived for significantly ($p<0.001$) longer than females exposed to 'low' food, and on 'high' but not on 'low' food continuous exposure to wild-type males significantly ($p<0.001$) reduced female lifespan. The data on egg-production (Fig. 3.4) were compared using a Kruskal-Wallis test, which showed that females kept on 'high' food produced significantly ($p<0.05$) more eggs than those kept on 'low' food in all sampling intervals, and that continuous exposure to wild-type males had no significant effect on egg-production under either feeding regimen in any sampling interval.

The progeny-counts were used to calculate egg-hatchability, which was compared for the 4 groups using Kruskal-Wallis tests (BMDP software, 1988). There were significant differences only on days 16 and 19, with intermittently exposed females on 'low' food having first significantly ($p<0.05$) lower and then higher hatchabilities than both continuously exposed females on 'low' food and intermittently exposed females on 'high' food; these differences would be expected by chance. The data on egg- and progeny-production were used to generate lifetime scores for each female, and these were compared using t-tests for the 2 groups of females kept on each type of food. For the females on 'high' food, continuously exposed females produced significantly fewer eggs (means continuous 299.5, intermittent 361.9, $t=2.177$, df 71, $p<0.05$) and progeny (means continuous 217.5, intermittent 268.1, $t=2.474$, $p<0.05$) in their lifetimes than did intermittently exposed females. These differences were not significant in females on 'low' food for either egg-counts (means continuous 34.5, intermittent 39.1) or progeny-counts (means continuous 28.8, intermittent 32.6).

Table 3.7

(a). Remating frequency of females kept on 'low' or 'high' food and intermittently or continuously exposed to wild-type males. Data presented for days when all females exposed to wild-type males. For details of sampling frequency see text.

Food	Male Exposure	Remating Opportunities		
		Taken	Not Taken	% Taken
'High'	Intermittent	8	137	5.8
	Continuous	11	107	10.2
'Low'	Intermittent	1	99	1.0
	Continuous	5	78	6.4

(b). Extra remating frequency data .

Food	Male exposure	Remating Opportunities		
		Taken	Not taken	% Taken
'High'	Intermittent	14	47	29.7
	Continuous	27	68	39.7
'Low'	Intermittent	6	54	11.1
	Continuous	2	93	2.1

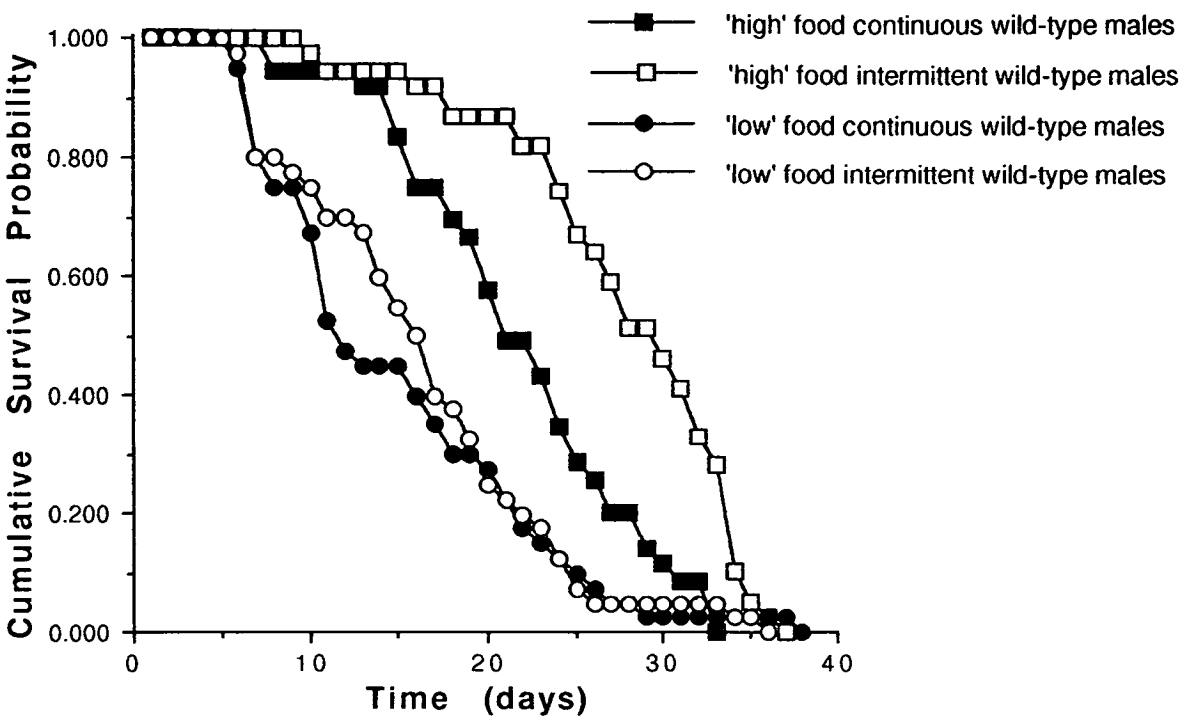


Fig. 3.3. Cumulative probability of survival against time (days) for females kept on 'high' or 'low' food and intermittently or continuously exposed to wild-type males.

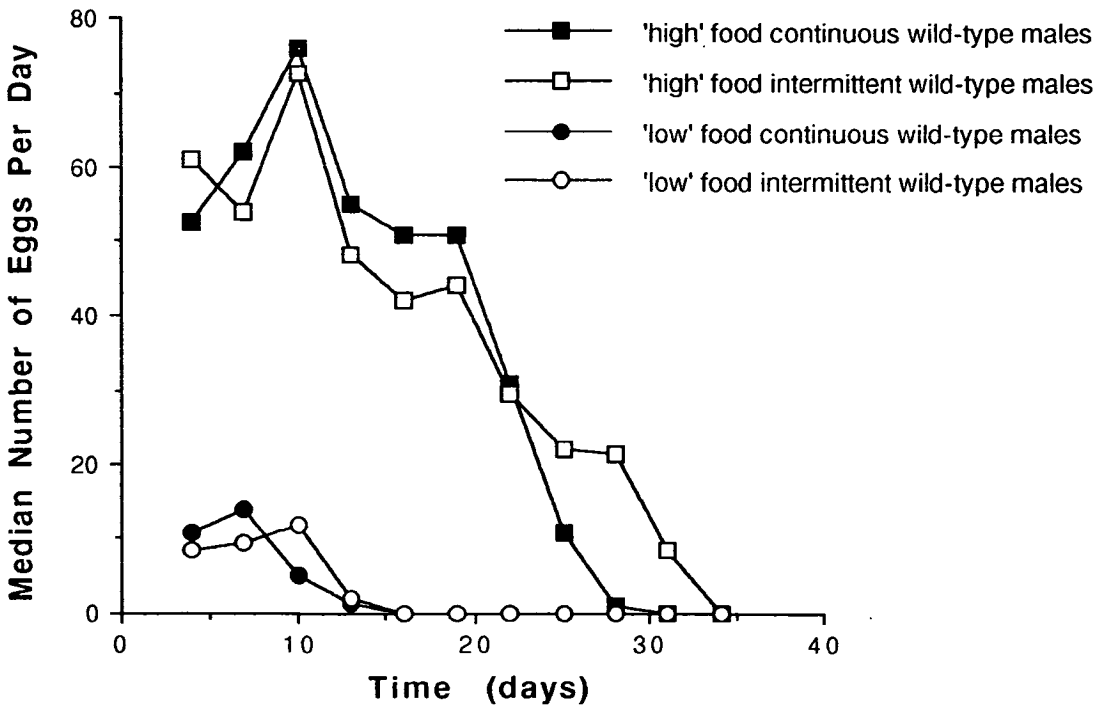


Fig. 3.4. Median number of eggs produced per 1 day sampling interval for the same groups of females as in Fig. 3.3.

3.4 Discussion

Neither of the experiments provided any evidence that remating by females was more beneficial for their fecundity or survival when they were nutritionally stressed. When the nutrition and access to males of wild-type females was manipulated, any nutritional benefits from mating should have been more apparent on 'low' food, as an increase in the fecundity or survival of continuously exposed females, because they remated approximately 3 times as frequently as intermittently exposed females. The lifespans, egg-production, egg-hatchabilities and lifetime reproductive success of the two groups of females on 'low' food did not differ significantly, so that increased remating was without benefit to females under these circumstances. In the females kept on 'high' food, increased remating was detrimental in that it decreased the lifespan and lifetime reproductive success of the continuously exposed females, as has been reported previously (Fowler and Partridge, 1989). The rate of mating was higher in these females than in those kept on 'low' food, which may explain why only they showed the cost of mating.

The females with an evolutionary history of poor nutrition also provided no support for the importance of male-derived nutrients, because their willingness to remate was no greater than that of the females from the 'high' lines, and instead there was evidence of gene-environment interaction; females from each regimen remated most frequently when they were kept on the food on which they had been selected. The data therefore suggested that females from the two selection regimens differed in the factors controlling remating rate, either because they had gained different benefits from remating in their evolutionary past, or because their remating rates were associated with some other evolved difference between them. One such difference was that females from each selection regimen produced more progeny when kept on the food on which they had been selected. Remating rate could then have been a response to fecundity *per*

se or to sperm depletion, which is more rapid in females that lay more eggs (Trevitt *et al.*, 1988).

The evolutionary adaptation to the two different culture regimens was also apparent in lifetime progeny-production. On 'high' food both the longevity and age-specific fertility of the 'high' line females contributed to their advantage, whereas on 'low' food only the age-specific fertility of the 'low' line females was significantly higher. It was not clear how the adaptation to diet was achieved; there were no significant differences in the number of ovarioles of the females from the two selection regimens when they were reared on intermediate food (Chapman, unpublished data). The analysis of the female thorax length data showed that female flies were larger if reared on the same type of food on which they had been selected. This suggests that there is a phenotypic correlation in the 'high' and 'low' selected lines between large adult body size, longevity ('high' line females only) and fertility (both 'high and 'low' line females).

Flies on 'low' growth food seemed able to compensate for the lack of food by having a longer development time. This prolonged the period of larval feeding, giving larvae longer to assimilate the necessary food reserves for the development of adult structures during pupation. It would be useful to know the pupariation times (from egg to eversion of the anterior spiracles of the pupa) to determine whether the larval feeding period, pupation period or both is prolonged in flies reared on 'low' food. Larval survival was also greater on 'high' food where food supply was not limiting. Flies grown up on 'low' food spent longer in the food medium and were exposed to toxic waste products within the culture bottles for a longer period of time than flies that developed on 'high' food; this may have been costly in terms of larval survival. Larval competition was relatively relaxed in the selection experiment; it is possible that clearer effects in terms of differences in larval survival might be observed by increasing the severity of competition by putting more larvae into the experimental vials initially.

'Low' food selected lines had evolved faster development times (although the effect was not significant in males). Although development time was much slower when nutrients were scarce, flies from the 'low' food selection regimen had evolved mechanisms to develop faster on this type of food than flies that had not had an evolutionary history of exposure to it ('high' lines). When flies were given access to much higher levels of nutrients ('high' growth food), the 'low' line flies still developed faster. The difference in development time between 'low' and 'high' selected flies was significantly larger on 'low' growth food, when resources were scarce, than on 'high' growth food, which may simply have reflected the extended development times of flies from both selection regimens on 'low' food.

Flies that develop faster and eclose first gain access to resources that may be ephemeral and in short supply, both in populations cages and in nature. This might be particularly important when nutrients are scarce (eg. the 'low' food cage situation). Selection pressures for changes in development time may not be equal under different nutritional regimens; this might have been the reason for the difference in the response in the 'low' and 'high' food lines. It would be useful to know how development time has shifted in relation to the base stock from which these lines were derived.

In both experiments, females remated more frequently when they were exposed to high quality food, as has been reported previously (Gromko and Gerhart, 1984; Harshman *et al.*, 1988; Trevitt *et al.*, 1988). This finding does not support the importance of seminal feeding. In a separate experiment performed by Sara Trevitt (Chapman *et al.*, submitted), 2 groups of females were exposed to poor ('dilute') quality food for 2 days followed by good ('rich') quality food for 2 days. The 2 groups of females ('rich-dilute' and 'dilute-rich') experienced this nutritional cycling in antiphase. Every 4 days a simultaneous remating test was performed, ie. after one group of females had been exposed to 'dilute' food for 2 days ('rich-dilute') and the

other to 'rich' food for 2 days ('dilute-rich'). Females remated significantly more after exposure to 'rich' food and both groups of females laid significantly more eggs when they were on 'rich' food. 'Rich-dilute' females laid significantly more eggs during their lifespans than 'dilute-rich' females; this was attributable to a significantly higher score on 'rich' but not 'dilute' food.

The 'dilute' food contained no source of protein, and a pilot experiment had shown that females kept solely on this food were all dead by day 13 from first mating. The 'rich-dilute' females had been kept on this food for two days prior to their remating test, yet, despite being under nutritional stress, they remated significantly less often than the 'dilute-rich' females. The data suggested that females remated primarily to replenish their sperm supplies (Gromko and Pyle, 1978; Gromko *et al.*, 1984a; Newport and Gromko, 1984), which are used up more rapidly under conditions of good nutrition and high rates of egg-production (Trevitt *et al.*, 1988).

The results of the experiments on laboratory natural selection and nutritional cycling described above, throw some light on the proximate mechanisms by which females regulate their remating rate. In the selection experiment, the females from the two selection regimens were compared on both types of food. Under identical nutritional conditions, the females that laid more eggs remated more frequently. This result suggests that nutrition itself did not affect remating frequency, and that either the rate of egg-laying or the number of sperm in store accounted for differences in remating probability. Sperm stores have been previously implicated in this regard (Gromko and Pyle, 1978; Gromko *et al.*, 1984a; Newport and Gromko, 1984; Letsinger and Gromko, 1985). However, the results of the nutritional cycling experiment suggest that other factors may also have been important. These females regulated their rate of egg-deposition closely in accordance with their current nutritional status, as has been found previously (Sang and King, 1961; Fairbanks and Burch, 1974; Partridge *et al.*, 1987a), and shown to be mediated by changes both in the rate of transcription of the yolk-

protein genes and in the rate of uptake of the yolk proteins by the ovary (Bownes, 1989).

The 'rich-dilute' females also had higher overall fecundity. Their entry into the nutritional cycle on 'high' food may have resulted in fuller ovarian development (Manning, 1967), conferring a greater capacity for egg-production which was expressed when the females were on 'rich' food. Since high nutrition and fecundity result in higher rates of sperm use (Trevitt *et al.*, 1988), these females presumably depleted their sperm stores more rapidly than did the 'dilute-rich' females. At the time of the remating test therefore, the 'rich-dilute' females had lower current nutrition, fecundity and sperm stores, and higher cumulative fecundity and they remated less frequently than the 'dilute-rich' females. The differences in remating rate between the groups could therefore not be accounted for by differences in cumulative fecundity or the amount of sperm in store, and must instead have been related to differences in current nutrition, rate of egg-laying or rate of sperm use.

The results of the experiments presented in this chapter provide no support for the importance to female *D. melanogaster* of male-derived nutrients. It is suggested that factors other than sperm currently in store may also affect the probability of remating by females (Chapman *et al.*, submitted).

Chapter 4. Costs of Mating with Males that do not Transfer Sperm

4.1 Introduction

Both elevated rates of egg-production and increased exposure to males cause a drop in the lifespan of female *D. melanogaster* (Partridge *et al.*, 1987a), and part of the survival cost of exposure to males is some consequence of mating itself (Fowler and Partridge, 1989), see Chapter 1, section 1.2.1.3. The cost of mating was also demonstrated in Chapter 3 of this thesis, where well fed females continuously exposed to wild-type males remated to the extent that their lifetime reproductive success was impaired. The nature of this cost of mating is unclear, and there seem to be at least 3 possibilities: mechanical injury at copulation (Lloyd and Park, 1962; Ikeda, 1974), the transfer of disease or parasites to the female, or some effect of sperm or accessory fluid. This chapter investigated the last of these possibilities, that is whether all or part of the cost of mating is caused by effects of the sperm or accessory fluid.

In the General Introduction (Chapter 1, section 1.5.1), the effects of the first and subsequent matings on female egg-production and receptivity were described. The mechanisms by which the short-lived SP effect and the longer term 'sperm effect' operate to reduce female receptivity are unclear, but both could carry costs to females. Receptivity after remating is also reduced, it is not clear whether by the same mechanism as in the first mating. The number of sperm in store can affect the probability of female remating (Gromko and Pyle, 1978; Gromko *et al.*, 1984a; Newport and Gromko, 1984), but the current rate of sperm-depletion and egg-laying can also be important (Chapter 3, this thesis). Several other constituents of the male

seminal fluid have also been reported to affect female reproductive behaviour, see Chapter 1, section 1.5.2.

Costs incurred as a result of the transfer of sperm or accessory fluid could occur as a result of females maximising their own reproductive success, for instance through lifetime egg-production. However, the evidence from the previous chapter showed that under conditions of good nutrition, the lifetime reproductive success of females continuously exposed to wild-type males was significantly lower than that of females only intermittently exposed to wild-type males (see also Fowler and Partridge, 1989). Any cost of receiving seminal fluid was therefore not accompanied by an increase in female reproductive success. Costs could also be a manifestation of sexual conflict with the male over future costs of reproduction (see Chapter 1, section 1.4). Sexual conflict resulting from the transfer of accessory fluid and/or sperm could therefore be responsible for, or contribute to, the cost of mating.

The experiments described in this chapter aimed to determine whether the cost of mating was due to the transfer of accessory fluid or sperm. A potential way to test for a cost of receiving sperm is to make use of males that are incapable of transferring it. Two experiments were done with two different types of males that could not transfer sperm, XO males and *transformer* (*tra*) pseudomales (see Chapter 2, sections 2.1.2.5 and 2.1.2.6).

D. melanogaster XO individuals, which lack a Y-chromosome, are phenotypically normal males; they transfer accessory fluid but no sperm at mating and are therefore sterile (Bridges, 1916; Stern, 1929). The testes of XO and XY males appear to be similar in both size and shape, but those of XO males contain non-motile sperm (Safir, 1920). Spermiogenesis does occur in XO males, and spermatids elongate but degenerate before maturation (Keifer, 1966; Hess and Meyer, 1968; Hardy *et al.*, 1981) and are not transferred to the female at mating. The protein synthesis and

constitution of XO male accessory glands has been reported to be normal (Von Wyl, 1976; Ingman-Baker and Candido, 1980; Chen, 1984), but the courtship and mating success of XO males have been reported to be deficient (Aslund *et al.*, 1978; Gromko *et al.*, 1984a). To compare the reproductive behaviour of XO and XY males directly, the genetic backgrounds of the 2 types of fly should ideally be the same so that any differences are due to the presence or absence of a Y chromosome and not to differences in the genetic background of the flies. The relative fitnesses of XO and XY genotype flies may also be unequal in different genetic backgrounds (Voelker and Kojima, 1971).

The *transformer* gene is part of the regulatory hierarchy controlling sex determination (Belote, 1989). XX *tra/tra* homozygotes are transformed into sterile males with fully developed sex combs, a male coloured abdomen and normal male external and internal genitalia (see Fig. 1, Chapter 1 and Fig. 2.1, Chapter 2), although testis size is reduced. Dosage compensation is not affected by the *tra* mutation. XX *tra/tra* pseudomales are female in size and larger than XY males, presumably as a result of the effects of non-dosage-compensated size-determining genes on the X chromosome. The *transformer* gene is not essential in males; XY *tra/tra* flies are normal, fertile males.

Trevitt and Partridge (1991) showed that female *D. melanogaster* kept exclusively with normal wild-type males had lower survival rates than females intermittently exposed to XO and wild-type males, in the absence of differences in egg-production, egg-hatchability or mating frequency between the two groups of females. The results suggested that receiving sperm could be costly to females, in terms of a drop in lifespan. However, alternative explanations were possible. The genetic background of the wild-type and XO flies was not the same, and the courtship activity of the XO and XY males was not recorded, leaving open the possibility of a difference in the non-mating costs of exposure to the different types of males.

The two experiments presented in this chapter tested directly for effects of receiving sperm and accessory fluid on female survival. To control female egg-production and egg-hatchability, all females were exposed to intact wild-type males for 1 day in 3. On the other two days, females could receive both sperm and accessory fluid (by exposure to intact wild-type or *tra/tra* XY males), only accessory fluid (by exposure to intact XO males or *tra/tra* XX pseudomales) or neither sperm nor accessory fluid (by exposure to wild-type, XO, XY tra/*tra*, or XX *tra/tra* males with microcauterised external genitalia). The separate effects of sperm and accessory fluid on female lifespan could therefore be deduced, provided that the non-mating effects of XO and wild-type males or XY *tra/tra* and XX *tra/tra* males did not differ, and that female fecundity and fertility were unaffected by the experimental treatments.

The XO experiment described in this Chapter has given rise to a publication (Chapman, 1992), see Appendix B.

4.2 Effect of Exposure to Wild-Type and XO Males on Female Lifespan and Reproductive Success

4.2.1 Method

The method used to obtain XO males was described in Chapter 2, section 2.1.2.5. The XO males used in this experiment had the same genetic background as the Dahomey wild-type males; the two types of males therefore differed only by the presence or absence of a Y chromosome.

XY and XO males were microcauterised by ablating their external genitalia with tungsten electrodes (see Chapter 2, section 2.1.4.4). Microcauterised males were screened for mating ability 2 days after microcautery; males were placed 3 per vial with

4, 4 day-old wild-type virgin females and any males that mated during the 3 hours of the test were discarded. Environmental effects such as barometric pressure can significantly affect remating propensity (Ankney, 1984). To control for these effects in the screen for mating ability of the microcauterised males, at least 10 control vials of 3 intact males (wild-type or XO) of the same age and 4, 4 day-old wild-type virgin females were always set up. In each screen, all of the control intact males mated within 1 hour.

Experimental females were from the Dahomey stock and were reared at a density of 100 larvae per vial. 160 adult virgin females were collected at eclosion by cooling on ice, and were then aged in groups of 20/vial for 3 days; females were then randomly assigned to 4 experimental groups, each of 40 females:

1) Females kept with 2 wild-type males continuously, 1 set of wild-type males for 1 day in 3 and another set for the other 2 days = WT+WT INTACT group.

2) Females kept with 2 wild-type males for 1 day in 3 and 2 XO males for the other 2 days = WT+XO INTACT group.

3) Females kept with 2 wild-type males for 1 day in 3 and 2 wild-type microcauterised males for the other 2 days = WT+WT MICROCAUT group.

4) Females kept with 2 wild-type males for 1 day in 3 and 2 XO microcauterised males for the other 2 days = WT+XO MICROCAUT group.

Each female was placed with 2 males in a freshly-yeasted food vial and females were given new food every time that the males were changed. Males were initially 5 days old when introduced into the experiment, and were renewed from fresh cultures every 9 days. Males were stored 20 per vial when not kept with females. Female deaths were recorded daily. Eggs were counted in the vials laid up on the days when all

females were with wild-type males, and these vials were retained to count emerging adult progeny.

Remating frequency was recorded every 3 days on the days when all females were with wild-type males, by scanning vials every 20 minutes for 3 hours starting 1h before lights-on and counting the number of matings. Female behaviour was assayed every 3 days on one of the 2 days out of 3 when females were exposed to experimental males, to assess whether the 4 types of males differed in activity or courtship behaviour. Vials were scanned every 20 minutes for 3 hours starting 1h before lights on and the behaviour of the female recorded as moving or not moving, and as courted (male within 5mm of female, following or orientated towards her, licking, performing wing vibration or attempted copulation) or not courted. Matings by XY and XO intact males on these days were also recorded.

4.2.2 Results

Female survival curves (Fig. 4.1) were analysed using a distribution-free Mantel-Cox test (BMDP Software, 1988). WT+WT INTACT females (median lifespan = 12 days) had significantly shorter lifespans than WT+WT MICROCAUT females (median lifespan = 17 days, Mantel-Cox (MC) test statistic=13.26, p=0.0003) and WT+XO INTACT females (median lifespan = 21 days) had significantly shorter lifespans than WT+XO MICROCAUT females (median lifespan = 28 days, MC=24.74, p<0.0000). In the 2 groups where females were exposed continuously to intact males, WT+XO INTACT females lived significantly longer than WT+WT INTACT females (MC=4.85, p=0.027). WT+WT MICROCAUT females lived significantly less long than WT+XO MICROCAUT females (MC=6.65, p=0.010). Females in the WT+WT MICROCAUT group lived significantly longer than females in the WT+XO INTACT group (MC=4.28, p=0.038).

Egg-production is shown in Fig. 4.2; medians were plotted because preliminary investigation of the egg-production data indicated that they were not normally distributed. They were analysed using Kruskal-Wallis tests including multiple comparisons to show between which groups any significant differences lay. In general, egg-production did not differ significantly between the groups. The WT+WT INTACT group tended to lay fewer eggs than the other groups (day 1 $p=0.0003$, day 4 $p=0.0333$ and day 10 $p=0.0176$). There were no significant differences in egg-hatchability between the 4 groups (Fig. 4.3), except on day 10 when the hatchability of the WT+XO INTACT group was significantly ($p<0.05$) lower than the WT+XO MICROCAUT group and on day 19, when the hatchability of the WT+XO INTACT group was significantly ($p<0.05$) lower than either the WT+WT MICROCAUT or WT+XO MICROCAUT groups. Because of the large number of comparisons for each sampling interval, this number of significant results would be expected by chance (eg. 1 in 20 at the $p<0.05$ significance level).

The total number of progeny produced per female was analysed using a Kruskal-Wallis test. WT+XO MICROCAUT females produced significantly ($p<0.05$) more progeny in their lifetimes (median = 288.5) than any of the other groups (median lifetime progeny WT+WT INTACT=137, WT+XO INTACT=179 and WT+WT MICROCAUT=204). WT+WT MICROCAUT females also produced significantly more lifetime progeny than WT+WT INTACT females ($p<0.05$).

Mating frequency was recorded on the days when all females were kept with wild-type males. Each morning of mating observations was treated as 1 mating opportunity 'offered' for each female and, if a mating was scored, this was a mating opportunity that was 'taken'. The number of matings recorded in each sampling interval was small (maximum percentage of opportunities taken was 22, minimum 1), so the remating frequencies of 2 successive sampling intervals were summed throughout the

experiment to give a larger sample size. The data for the 4 groups were compared using a χ^2 test, and none of the comparisons revealed significant differences ($p>0.05$). Since the mating frequencies did not differ significantly on days when intact XY males were present, and since XY and XO matings were seen on the other days, those females exposed to intact males on the other 2 days must have had higher overall mating frequencies than females exposed to microcauterised males.

The data on behaviour were used to calculate, for each female in each sampling interval, the proportion of observations in which she was either courted or not courted and either moving or still (Figs. 4.4 and 4.5). The data were then subjected to the angular transformation and a one-way analysis of variance. For courtship, microcautery had no significant effect on wild-type males, while intact XO males delivered more courtship than microcauterised XO males in interval 6. The chromosomal constitution of the male had a large effect, with wild-type males delivering significantly more courtship than XO males in sampling intervals 1-3, and at least 1 significant comparison in intervals 6 and 7. For female movement, microcautery had little effect: females exposed to microcauterised and intact wild-type males showed no significant differences throughout the experiment, and females exposed to microcauterised XO males moved significantly less than those exposed to intact XO males only in the first sample. Chromosomal constitution of the male did have an effect: females exposed to both types of XY males moved more than those exposed to XO males in samples 1-3, and females exposed to microcauterised wild-type males moved more than those exposed to XO males in sample 7.

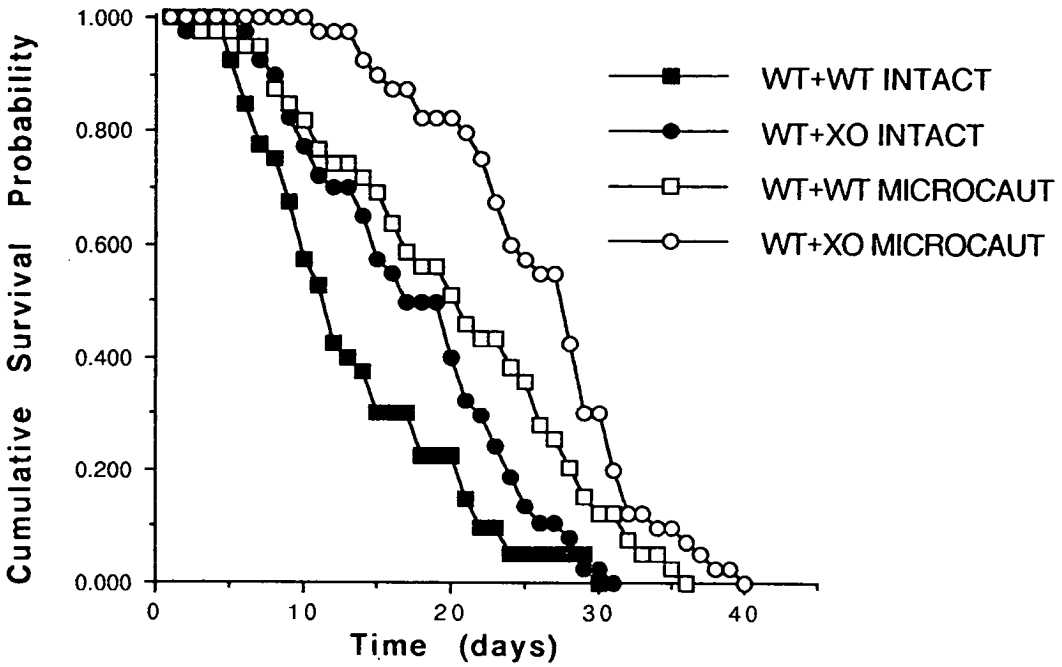


Fig. 4.1. Cumulative probability of survival against time (days) for females exposed to wild-type males continuously, to wild-type males intermittent with XO males or to wild-type males intermittent with either wild-type or XO microcauterised males.

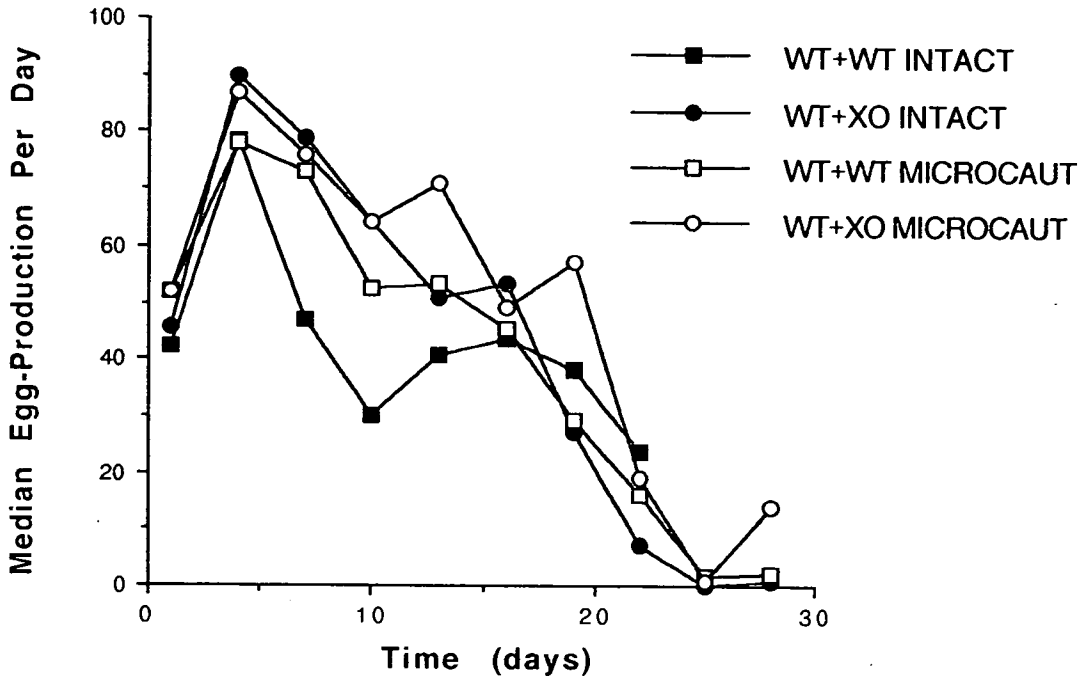


Fig. 4.2. Median number of eggs produced per 1 day sampling interval for the same groups of females as in Fig. 4.1.

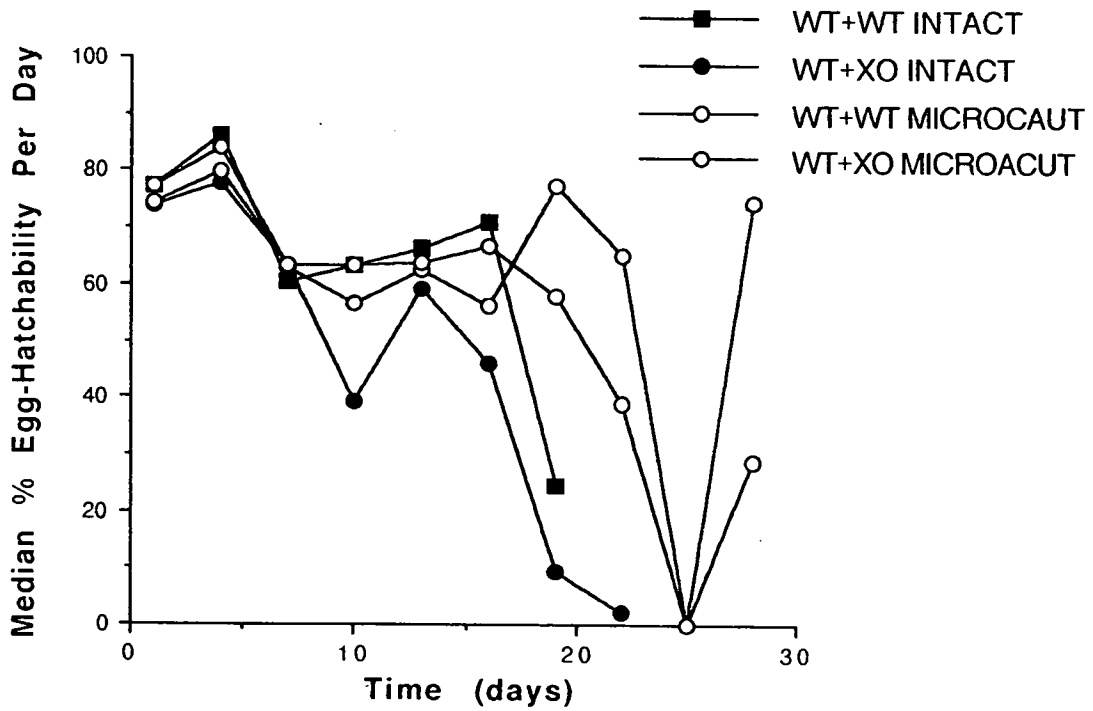


Fig. 4.3. Median egg-hatchability from 1 day egg samples for the same groups of females as in Fig. 4.1.

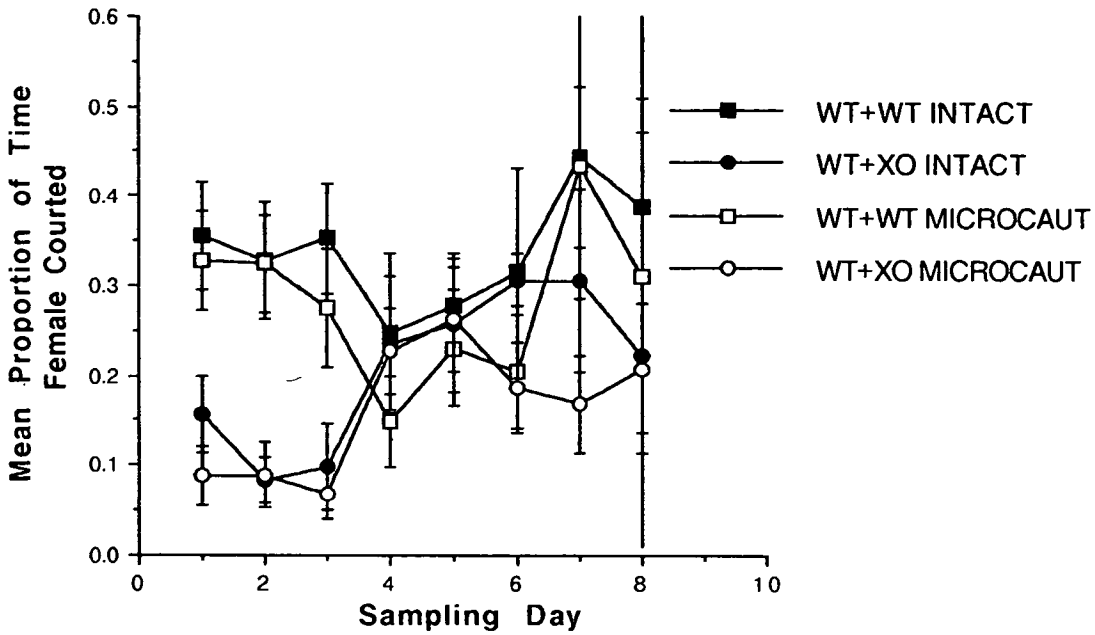


Fig. 4.4. Mean and 95% confidence intervals for the proportion of time females courted against time (sampling day) on one of the 2 days out of 3 when females were kept with wild-type or XO, intact or microcauterised males.

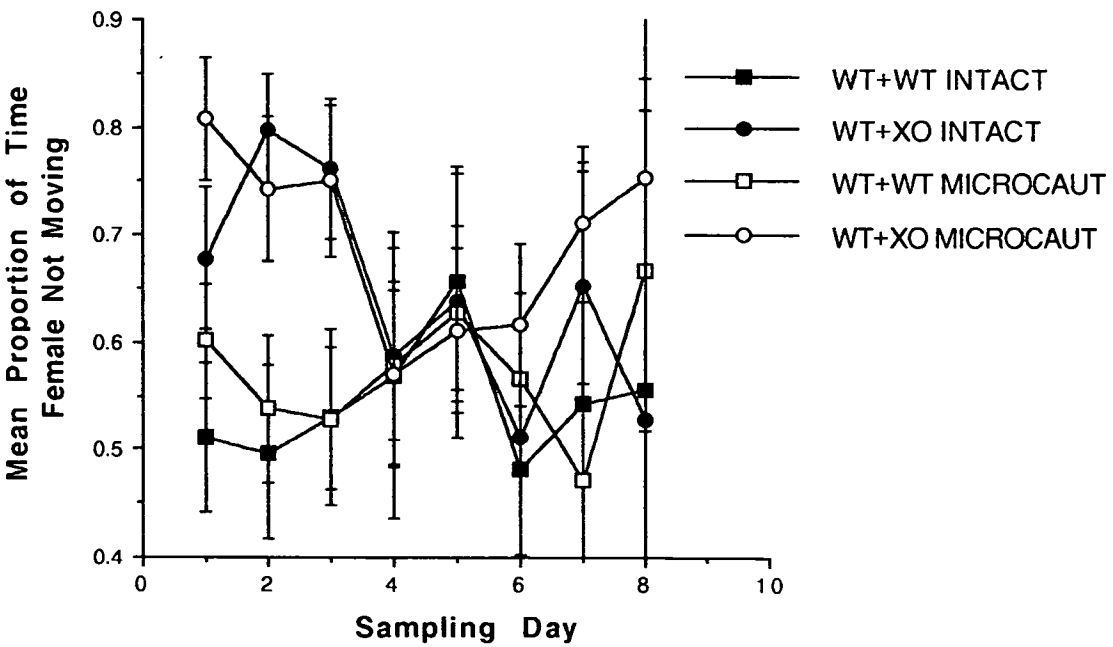


Fig. 4.5. Mean and 95% confidence intervals for the proportion of time females not moving against time (sampling day) on one of the 2 days out of 3 when females were kept with wild-type or XO, intact or microcauterised males.

4.3 Effect of Exposure to XY *tra/tra* and XX *tra/tra* Males on Female Lifespan and Reproductive Success

4.3.1 Method

The *transformer* stock used was described in Chapter 2, section 2.1.2.6. XX *tra/tra* and XY *tra/tra* males were sorted apart on the basis of their size: XX *tra/tra* males are larger than XY *tra/tra* males. Samples of XX *tra/tra* (N=98) and XY *tra/tra* males (N=101) were later dissected to calculate the efficiency of sorting them by eye. The XX *tra/tra* males have smaller testes than normal which were easily scored by dissection.

In the XO experiment, there were differences in non-mating costs of exposure to spermless (XO) and wild-type males. To ensure that this was not the case in the *transformer* experiment, a short pilot experiment was performed to determine whether there were differences in the amount of courtship delivered by XY *tra/tra*, XX *tra/tra* and Dahomey wild-type males. Females were exposed to wild-type (N=30), XY *tra/tra* (N=35) or XX *tra/tra* males (N=35), and placed 1 per vial with 2 males each. Courtship activity was sampled over a period of 8 days by scanning the vials every 20 mins for 3 hours, starting 1h before lights on. Female behaviour was recorded as courted or not courted (male within 5mm of female and performing wing vibration, licking genitalia, following the female or attempting copulation) and moving or not moving. The proportion of time that females in the 3 groups were courted and not moving are shown in Figs. 4.6 and 4.7. The data were then subjected to the angular transformation and one-way analysis of variance.

The amount of courtship delivered by XY *tra/tra* and XX *tra/tra* males was significantly less than that delivered by wild-type males on all occasions ($p<0.05$). The drop in courtship was associated with lowered levels of female movement. The level of

courtship delivered by the spermless XX *tra/tra* males was not comparable with that of wild-type males. The amount of courtship delivered by XY *tra/tra* males however (which transfer sperm and accessory fluid), was generally comparable to that of XX *tra/tra* males (significantly higher on days 4 and 6 only, $p<0.05$); the non-mating costs of exposure to XY *tra/tra* and XX *tra/tra* males were therefore probably similar. The XY *tra/tra* and XX *tra/tra* males were therefore suitable to use in this experiment because their genetic background was identical and their courtship behaviour comparable.

The aim of the experiment was to investigate the costs of receiving accessory fluid and sperm, by comparing the survival of females exposed to microcauterised males, which could not mate, with that of females exposed either to intact XX *tra/tra* males which transfer only accessory fluid, or to XY *tra/tra* males which transfer both sperm and accessory fluid, while controlling female fecundity and fertility (exposing all females to wild-type Dahomey males for 1 day out of every 3). XY *tra/tra* and XX *tra/tra* flies were microcauterised by ablating their external genitalia with tungsten electrodes (Chapter 2, section 2.1.4.4). Microcauterised males were screened for mating ability 2 days after microcautery as in the previous experiment. Experimental females were from the Dahomey stock and were reared at a density of 100 larvae per vial. 160 adult virgin females were collected at eclosion using CO_2 anaesthesia, and were then aged in groups of 20/vial for 3 days; females were then randomly assigned to 4 experimental groups, each of 40 females:

1) Females kept with 2 wild-type Dahomey males for 1 day in 3 and 2 XY *tra/tra* intact males for the other 2 days = WT+XY *tra* INTACT group.

2) Females kept with 2 wild-type Dahomey males for 1 day in 3 and 2 XX *tra/tra* intact males for the other 2 days = WT+XX *tra* INTACT group.

3) Females kept with 2 wild-type Dahomey males for 1 day in 3 and 2 XY *tra/tra* microcauterised males for the other 2 days = WT+XY *tra* MICROCAUT group.

4) Females kept with 2 wild-type Dahomey males for 1 day in 3 and 2 XX *tra/tra* microcauterised males for the other 2 days = WT+XX *tra* MICROCAUT group.

Each female was placed with 2 males in a freshly-yeasted SY food vial and was given new food every time that the males were changed. Males were initially 5 days old when introduced into the experiment, and were renewed from fresh cultures every 9 days. Males were stored 20 per vial when not kept with females. Female deaths were recorded daily. Eggs were counted in the vials laid up on the days when all females had wild-type males, and these vials were retained to count emerging adult progeny.

Remating frequency was recorded every 3 days on the days when all females were with wild-type males, by scanning vials every 20mins for a period of 3-5 hours starting 1-3h before lights-on, and counting the number of matings. Female behaviour was assayed every 3 days on one of the 2 days out of 3 when females were exposed to the 4 types of experimental males, to assess whether the males differed in their activity or courtship behaviour. Vials were scanned every 20mins for 3 hours starting 1h before lights-on and the behaviour of the female recorded as moving or not moving and as courted or not courted, as in the previous experiment.

4.3.2 Results

The efficiency of sorting XY *tra/tra* males (N=101) was 100% and was 75% for XX *tra/tra* males (N=98). Therefore, the WT+XX *tra* INTACT and WT+XX *tra* MICROCAUT groups had reduced exposure to XX *tra/tra* males. However, the WT+XX *tra* INTACT group of females would still have been exposed to lowered

sperm levels overall (kept for 2 days out of every 3 with males 75% of whom could not transfer sperm).

Female survival curves (Fig. 4.8) were analysed using a distribution-free Mantel-Cox test (BMDP Software, 1988). Intermittent exposure to microcauterised males had a significant effect on female lifespan: WT+XY *tra* MICROCAUT females (median lifespan = 20d) lived significantly longer than WT+XY *tra* INTACT females (median lifespan = 17d, Mantel-Cox (MC) test statistic = 5.21, p=0.02) and WT+XX *tra* MICROCAUT females (median lifespan = 19d) lived significantly longer than the WT+XX *tra* INTACT group (median lifespan = 15d, MC=5.87, p=0.01). Chromosomal constitution did not have a significant effect; there were no differences between the lifespans of the WT+ XY *tra* INTACT and WT+ XX *tra* INTACT groups, or between the WT+XY *tra* MICROCAUT and WT+XX *tra* MICROCAUT groups.

Egg-production (Fig. 4.9) was analysed using multiple comparison Kruskal-Wallis tests as previously. Egg-production did not differ significantly between the groups except in the initial sampling interval where the WT+XY *tra* INTACT group laid significantly fewer eggs than the WT+ XY *tra* MICROCAUT and WT+XX *tra* MICROCAUT groups ($p<0.05$). Median egg-hatchability was calculated and plotted against female age (Fig. 4.10) and analysed using Kruskal-Wallis tests; egg-hatchability did not differ between the 4 groups except in the first sampling interval where the WT+XX *tra* MICROCAUT group had significantly higher egg-hatchability than the WT+XX *tra* INTACT and WT+XY *tra* INTACT groups ($p<0.05$). Both of these results would be expected by chance with so many comparisons (eg. 1 in 20 at the $p<0.05$ level).

The total number of progeny produced from egg samples was calculated and compared between groups using a Kruskal-Wallis test. There were no significant differences between any of the groups, although the lifetime progeny-production of the

WT+XY *tra* MICROCAUT and WT+XX *tra* MICROCAUT groups (medians 201 and 205 respectively) were higher than those of the WT+XY *tra* INTACT and WT+XX *tra* INTACT groups (medians 162 and 179.5 respectively).

Mating frequency was recorded on the days when all females were kept with wild-type males. Each morning of mating observations was treated as 1 mating opportunity 'offered' for each female and, if a mating was scored, this was a mating opportunity that was 'taken'. The number of matings recorded in each sampling interval was small (maximum percentage of opportunities taken was 10, minimum 0), so the remating frequencies were summed throughout the experiment to give a larger sample size. The data for the 4 groups were compared using a χ^2 test, and there were no significant differences in remating frequency, $\chi^2=3.39$ 3df. Since the mating frequencies did not differ significantly on days when intact wild-type males were present, and since matings with XY *tra/tra* and XX *tra/tra* males on the other days were seen, those females continuously exposed to intact males must have had higher overall mating frequencies than females intermittently exposed to microcauterised males.

The data on behaviour were used to calculate, for each female in each sampling interval, the proportion of observations in which she was either courted or not courted and either moving or still (Figs. 4.11 and 4.12). The data were then subjected to the angular transformation and one-way analysis of variance. In the first 2 sampling intervals, females exposed to XX *tra/tra* intact males were courted significantly more ($p<0.05$), and moved around significantly more ($p<0.001$) than the other groups of females. After this point, the 4 groups of females did not differ significantly in the amount of courtship they received, or the proportion of time that they spent moving around.

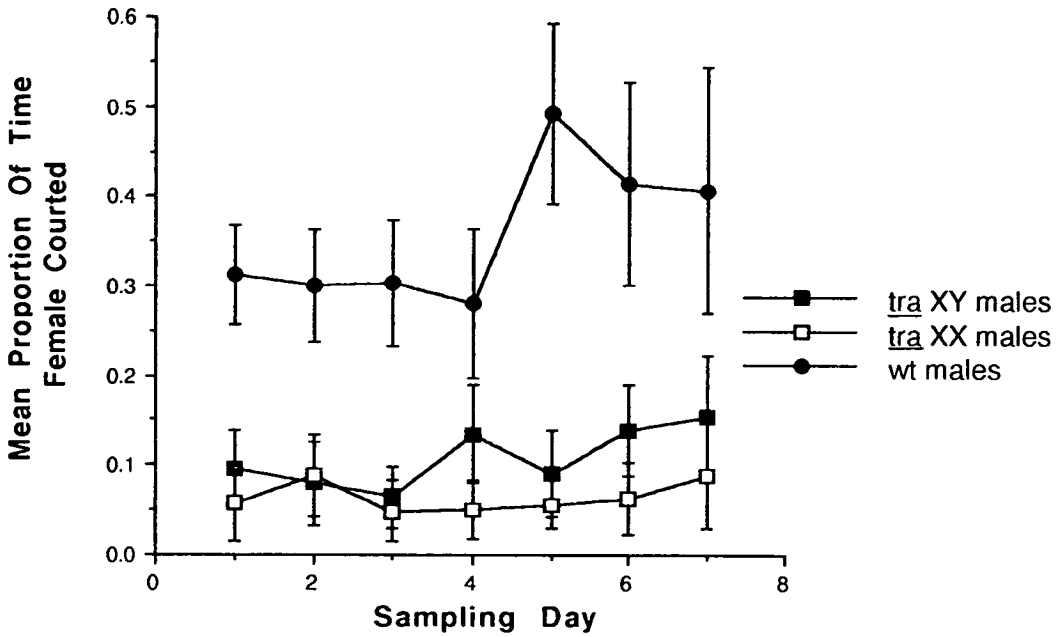


Fig. 4.6. Courtship pilot experiment. Mean and 95% confidence intervals for the proportion of time females courted against time (sampling day) for females exposed to XY tra/tra, XX tra/tra or wild-type males.

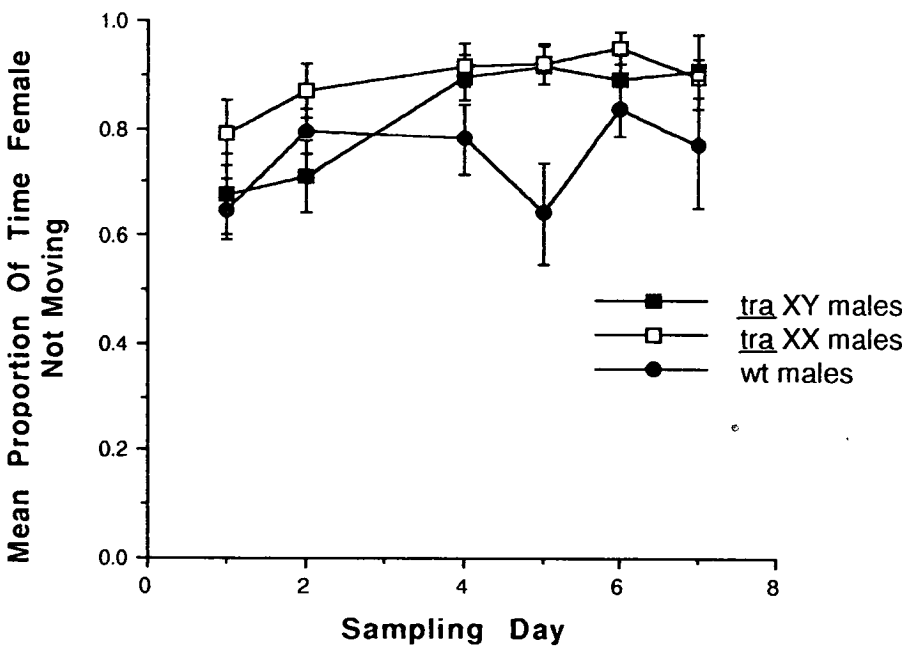


Fig. 4.7. Courtship pilot experiment. Mean and 95% confidence intervals for the proportion of time females not moving against time (sampling day) for females exposed to XY tra/tra, XX tra/tra or wild-type males.

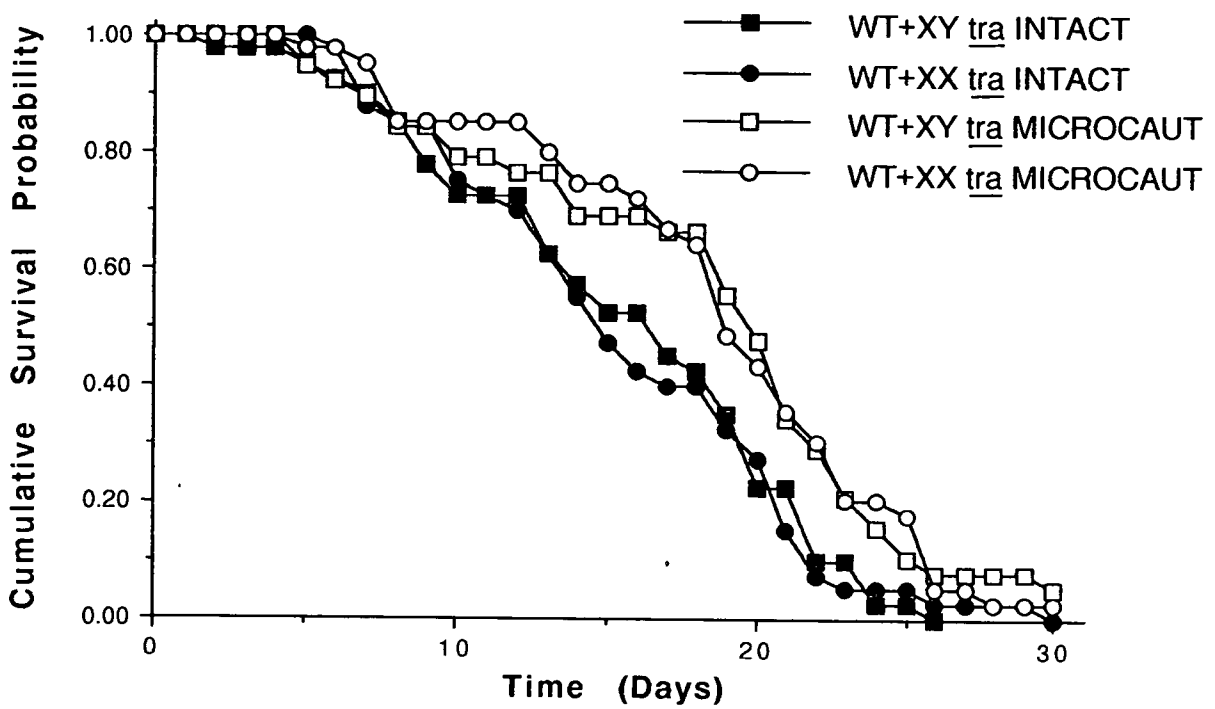


Fig. 4.8. Cumulative probability of survival against time (days) for females exposed to wild-type males intermittent with XY tra/tra or XX tra/tra, intact or microcauterised males.

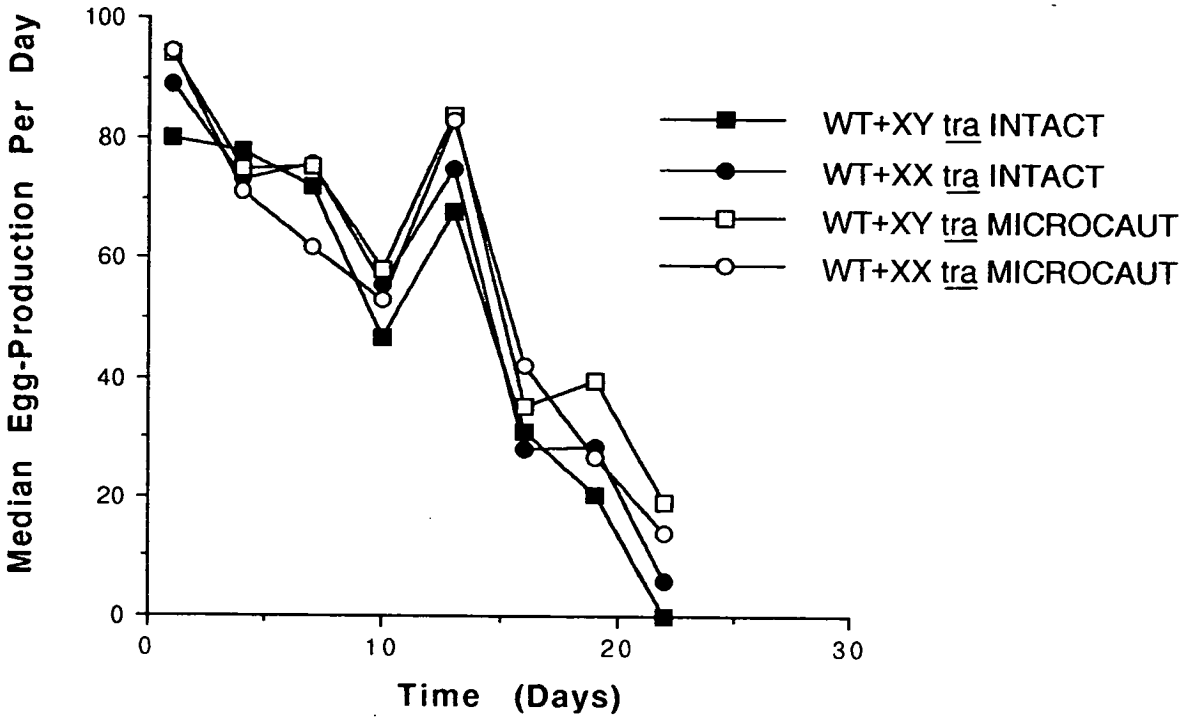


Fig. 4.9. Median number of eggs produced against time (days) per 1 day sampling interval for the same groups of females as in Fig. 4.8.

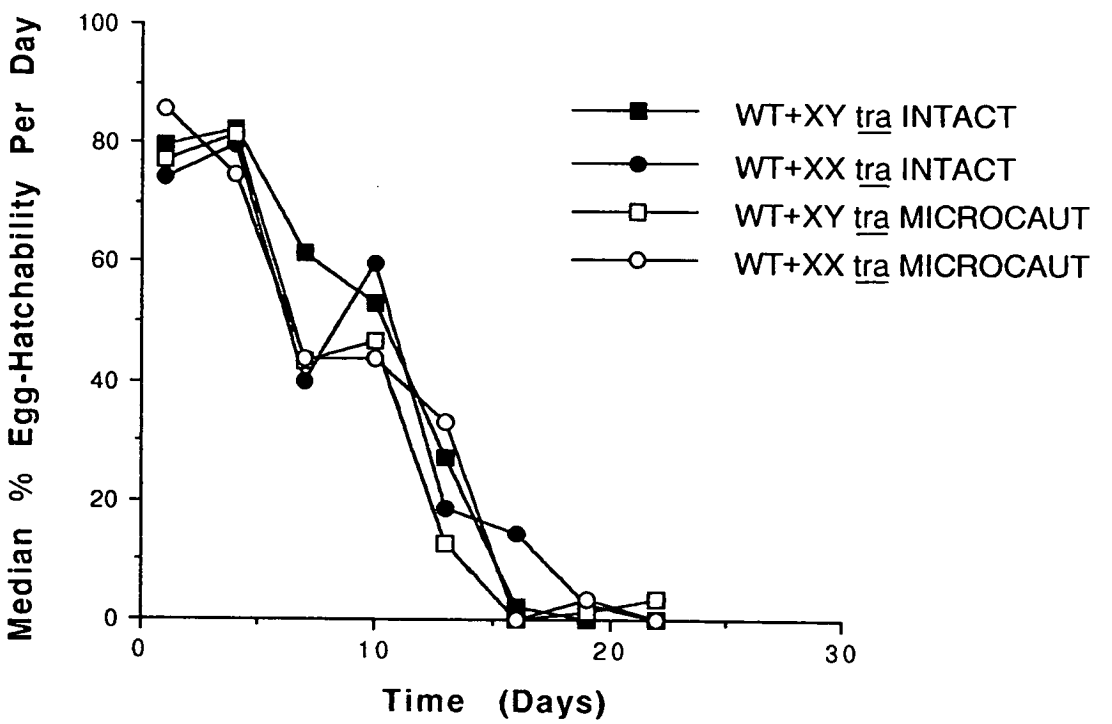


Fig. 4.10. Median egg-hatchability against time (days) for 1 day egg samples for the same groups of females as in Fig. 4.8.

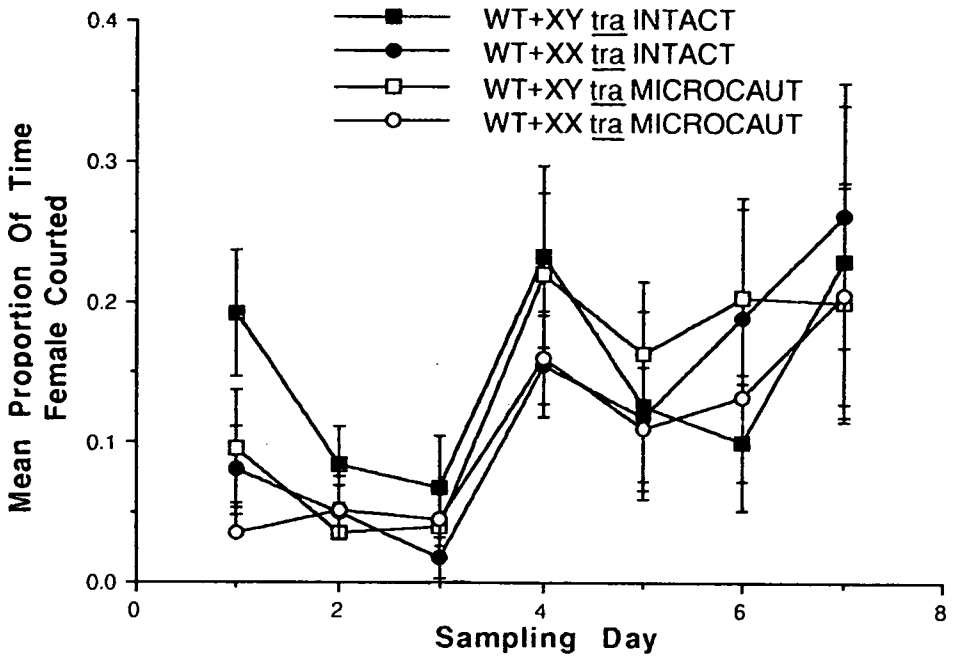


Fig. 4.11. Mean and 95% confidence intervals for the proportion of time females courted against time (sampling day) on one of the 2 days out of 3 when females were kept with XY tra/tra or XX tra/tra, intact or microcauterised males.

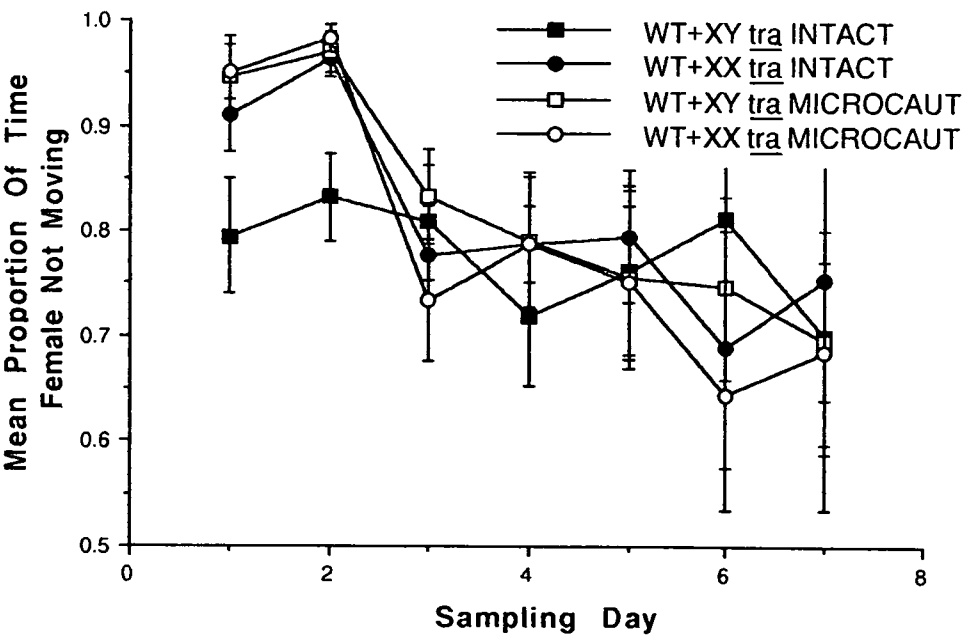


Fig. 4.12. Mean and 95% confidence intervals for the proportion of time females not moving against time (sampling day) on one of the 2 days out of 3 when females kept with XY tra/tra or XX tra/tra, intact or microcauterised males.

4.4 Discussion

The most important finding in the XO experiment was that mating with males that could not transfer sperm significantly reduced female lifespan. This conclusion was evident from comparison of the groups of females exposed intermittently to either intact or microcauterised XO males. Females from the former group survived less long than females from the latter, in the absence of significant differences between them in egg-production, egg-hatchability and frequency of mating with XY males. Females exposed to microcauterised XO males did receive less courtship and moved less than females exposed to intact XO males in one sampling interval each, but this would be expected by chance with so many comparisons between groups. The decreased survival rate in the WT+XO INTACT females is therefore likely to have been caused by their matings with XO males. It is not possible to deduce whether the cost of mating with XO males is a cost of receiving accessory fluid or some other consequence of mating. Despite the lack of replication in the present experiment the results seem robust in that where they overlap previous findings (Fowler and Partridge, 1989; Trevitt and Partridge, 1991) they replicate them. The differences in the effects on female lifespan of intermittent exposure to either microcauterised XO or microcauterised XY males and to either intact XO or microcauterised XY males have not been previously recorded.

The results confirmed the cost of mating with XY males (Fowler and Partridge, 1989); females exposed continuously to intact wild-type males had significantly shorter lifespans than females intermittently exposed to microcauterised XY males, in the absence of significant differences in egg-hatchability, movement and amount of courtship received. The WT+WT INTACT females also tended to lay fewer eggs than the WT+WT MICROCAUT females. However this difference was in the opposite direction to that needed to explain the difference in lifespan between the groups. The WT+WT INTACT females probably laid fewer eggs because they were in a poorer

physiological condition than the females in the other groups, as a result of their extra matings with intact XY males.

The results also confirmed another earlier finding, that intermittent exposure to intact XY males leads to lower female survival than intermittent exposure to intact XO males, in the absence of differences in egg-production and egg-hatchability (Trevitt and Partridge, 1991). In the present experiment the genetic background of the XO and XY males was otherwise similar, ruling out any explanation in terms of other genetic differences between the XO and XY males. A possible explanation for the difference in survival is a cost of receiving sperm, but the present data suggest an alternative or additional explanation. The XO males were less likely to court than were the XY males and, perhaps in consequence, the females exposed to XO males were less like to be moving than were the females exposed to XY males. The non-mating costs of exposure to XY males may therefore have been higher. Both extra expenditure of energy by the females and prevention of feeding may be consequences of extra male courtship and female movement. It is unclear why there should be a survival cost to receiving sperm; it is possible that the maintenance of healthy sperm in the female sperm storage organs (the seminal receptacle and spermathecae, see Fig. 1, Chapter 1) has a physiological cost (Thornhill and Alcock, 1983). It is also possible that the XY males mated more frequently than the XO males. However, this cannot have been the whole explanation of the differing effects on female survival, because it was also apparent in the 2 groups of females intermittently exposed to microcauterised males, indicating that the difference could not have been entirely caused by a consequence of mating, and must have been in part attributable to non-mating effects.

The genetic background of the XY and XO males used was identical; the flies differed only in the presence or absence of a Y chromosome. The difference in the amount of courtship delivered by the different males suggests that the Y chromosome may control certain aspects of mating behaviour, as in *Anopheles* mosquitos (Fraccaro

et al., 1977). In the XO experiment it proved impossible to test satisfactorily for costs of receiving sperm and accessory fluid, because any such effects were confounded with differences in behaviour between XO and XY males.

In the *transformer* experiment, the amount of courtship delivered by XY *tra/tra* and XX *tra/tra* males was not significantly different. However, the efficiency of sorting XX *tra/tra* males was only 75% and this would have reduced the differences between the XX *tra/tra* and XY *tra/tra* treatments. Females intermittently exposed to intact XX *tra/tra* males would still have received substantially lowered sperm levels overall. The most important result from the *transformer* experiment was that there seemed to be no cost of receiving sperm. This was evident because the lifespans, egg-production, egg-hatchability and remating rates of females intermittently exposed to males that could (XY *tra/tra* males) and could not (XX *tra/tra* males) transfer sperm were not significantly different. The cost of mating with males that could not transfer sperm was again evident; females intermittently exposed to microcauterised males lived significantly longer than females intermittently exposed to XX *tra/tra* males. This difference in longevity could be explained in terms of a cost of receiving accessory fluid, but it is not possible to distinguish this possibility from other explanations, eg. a cost of receiving other ejaculatory duct components, injury at mating or the transfer of disease or parasites.

The results of the *transformer* experiment confirmed that the difference in lifespan of females exposed to wild-type and spermless (XO) males in the XO experiment was due to differences in the pre-mating costs of exposure to the 2 types of male. In the *transformer* experiment there were no significant differences in the amount of courtship delivered by spermless or XY males, and no significant differences in the lifespans of females intermittently exposed to microcauterised XX *tra/tra* or microcauterised XY *tra/tra* males. This suggests that there were no significant differences in the premating costs of exposure to XY *tra/tra* and XX *tra/tra* males.

If the accessory gland secretion does contribute to the cost of mating, it might act through manipulating female reproductive behaviour by ensuring the maximum production of progeny fathered by the male before the female remates; this might cause the female to incur extra reproductive costs. In both experiments, females continuously exposed to intact males had significantly shorter lifespans and lowered lifetime reproductive success compared to females only intermittently exposed to intact males. The lifetime reproductive success of females exposed to intact males was significantly reduced in the XO experiment, and reduced, but not significantly so, in the *transformer* experiment. Both the amount of courtship and remating rates of *transformer* males were lower than those of XO males. The differences in lifetime reproductive success of females in the *transformer* experiment might therefore have been larger if the courtship intensity and remating rates of the males had been increased (for example by keeping 3 males with each female), therefore exposing females to more reproductive costs.

The reduction in lifetime reproductive success in females continuously exposed to intact XY males suggests that males can persuade females to remate more than is in their reproductive interests (Fowler and Partridge, 1989) and supports the idea of a sexual conflict over the amount of parental investment in offspring. Peculiar patterns of fertilization and ploidy levels within the seeds of higher plants are thought to have arisen out of a similar conflict (Queller, 1989). DNA-imprinting in mice also seems to reflect a conflict over the amount of maternal investment in embryos because of maternal reproductive costs (Haig and Graham, 1991), see Chapter 1, section 1.4.

It would be possible to investigate directly whether accessory fluid or mating itself is costly to females, if males that transfer sperm but not accessory fluid and males that transfer nothing at mating could be obtained. Sperm probably cannot be transferred without accessory fluid, although even very small amounts of fluid allow some transfer (Hihara, 1981). Males could be made to transfer small amounts of accessory fluid by

mating them serially with several females before use. Males which transfer nothing at mating could be obtained by serially mating males and then injecting them with cycloheximide to inhibit protein synthesis. Cycloheximide injection can greatly inhibit protein synthesis in males for a few days without other adverse effects (Mary Bownes, pers. comm.) and the technique has been successfully used in females (Bownes *et al.*, 1987). To confirm that such males transfer nothing, they could be radio-labelled and mated to females; the females would then be assayed for progeny-production and radioactivity.

Another approach would be to examine the effects of accessory fluid components by using males that do not transfer products of particular accessory gland cell types because those cells have been ablated, eg. flies without accessory gland main cells are available (Mariana Wolfner, pers. comm.). Another approach, in the absence of truly accessory-glandless males, would be to use injection experiments. Accessory gland extracts or particular gland components could be injected into females with appropriate controls and their effect on female lifespan and reproductive success investigated. Experiments of this type are described in Chapter 5 of this thesis. Accessory glands and their components are focussed upon because there is evidence that they can alter the behaviour and physiology of the female (Chapter 1, section 1.5.2) and as such are candidates for contributing to the cost of mating.

Chapter 5. Effect of Male Accessory Gland Extract and one of its Sub-Components, the Sex Peptide, on Female Lifespan and Egg-production

5.1 Introduction

The cost of mating (Fowler and Partridge, 1989) was demonstrated in Chapters 3 and 4. In Chapter 3, wild-type females maintained on good quality food and continuously exposed to males, remated more and had significantly shorter lifespans and lower lifetime reproductive successes than females only intermittently exposed to males. In Chapter 4, females that were continuously exposed to intact males lived significantly less long than females only intermittently exposed to intact males, in the absence of differences in age-specific egg-production. The lifetime reproductive success of females continuously exposed to intact males was lower than that of females intermittently exposed to intact males. The lifetime reproductive success of females exposed to intact males was significantly reduced in the XO experiment, and reduced, but not significantly so, in the *transformer* experiment (see Chapter 4).

As suggested in the previous chapter, the cost of mating could be due to the transfer of disease or parasites, injury at mating or some effect of the accessory fluid. The results of the *transformer* experiment in Chapter 4 suggested that receiving sperm is not costly to the female and therefore any components of the seminal fluid that adhere to the sperm are probably also not costly. Females intermittently exposed to males which could only transfer accessory fluid (XX *tra/tra* males) had significantly shorter lifespans and lower lifetime reproductive successes than females intermittently exposed to males that did not mate, and hence transferred nothing: this suggested that females that had received elevated levels of accessory gland components had incurred more

reproductive costs. The results however could not exclude the possibility of injury or transfer of disease or parasites at mating.

The experiments presented in this chapter investigated directly whether receiving accessory fluid and one of its sub-components, the sex peptide (SP) by injection had any effect on female survival or fecundity. If injection of the accessory gland extract turned out to be costly, attempts could then be made to investigate which of the sub-components of the accessory fluid were responsible. The *D. melanogaster* SP was used for several reasons: it was a candidate for contributing to the cost of mating because of its effects on female egg-laying and receptivity, which have been well characterised (Chen *et al.*, 1988; Aigaki *et al.*, 1991). The SP is probably not the only accessory gland component involved in female post-mating responses, but was available in a pure synthetic form, making the injection of a specific and controllable dose possible. Injected extracts could have immunological as well as reproductive effects, therefore a salivary gland extract was used as a control for the accessory gland extract and inactive fragments of the SP as controls for the SP. A Ringer-injected group was included to test for any biological activity and non-sexual effects (eg. immunological) of the SP fragments used.

Any cost of exposure to the injected extracts might also depend on elevation of egg-production rates, which vary with nutrition; female nutrition was therefore varied by supplying a varying level of protein. Sucrose food with no source of protein (see Chapter 2, section 2.2.1.2) was used to suppress egg-laying. The first experiment (section 5.2) investigated the effect of accessory gland injection on female lifespan and egg-production. The next three experiments (5.3a, 5.3b and 5.4) investigated the effect of injection of one of the accessory fluid components, the SP, on female lifespan and egg-production.

The first two SP-injection experiments (sections 5.3a and 5.3b) were performed in the Zurich laboratory. In the first experiment, the response to SP-injection in females kept on unyeasted Zurich medium was investigated. The second experiment used sucrose food to equalise the egg-laying rates of all females, to investigate any non-egg-mediated effect of SP-injection on female lifespan. The sucrose food was also drier than the Zurich fly food and led to less problems with condensation in the culture plates used. The third experiment, conducted in Edinburgh, (section 5.4) combined these 2 approaches using 3 nutritional regimens.

5.2 Effect of Accessory Gland Injection on Female Longevity and Egg-production

5.2.1 Preparation of Accessory and Salivary Gland Extracts

Wild-type Dahomey males were separated from females at eclosion and stored 20 per vial until they were 3-4 days old. The accessory glands of these males were then dissected into Ringer solution; only whole pairs of glands were used. 50 pairs of glands were dissected into 20µl of Ringer, spun down in a microcentrifuge and 15µl of the supernatant then removed, leaving 50 pairs of glands in 5µl Ringer. A total of 500 accessory glands were dissected. 500 pairs of salivary glands from wild-type Dahomey third-instar larvae were similarly prepared; after dissection, accessory and salivary glands were stored at -70°C.

After all the glands had been dissected, they were defrosted and homogenised using an Eppendorf homogeniser. The homogenised extracts were pooled and diluted with Ringer solution to give a concentration of one third of a pair of accessory or salivary glands per 50nl; a female receives about one third of the contents of the male

accessory glands at mating (Hihara, 1981). The prepared extracts were then refrozen at -4°C until they were used in the experiment.

5.2.2 Method

Dahomey flies were reared in vials of SY food at a standardised density of 100 larvae per vial (Chapter 2, section 2.1.4.3). Virgin females and males were collected by cooling on ice not less than 3 hours after eclosion. Virgin females were randomly assigned to 1 of 3 nutritional regimens (120 females in each group): SY food with added live yeast granules (yeasted food), SY food with no added live yeast (unyeasted food) and food with no protein source (sucrose food), see Chapter 2, section 2.1.1. The females were then aged for 2 days in single sex groups of 5 per vial on the 3 types of food. Males were aged for 2 days in yeasted SY food vials in single sex groups of 20 per vial. All females were mated on day 3 after eclosion to turn on egg-production (see Chapter 1, section 1.5.1); 1 female and 2 males were placed in each vial (on yeasted, unyeasted or sucrose food) and observed until mating took place; the males were then removed and discarded. On day 4 after eclosion, the mated females on each type of food were randomly assigned to 1 of 3 experimental groups, each of 40 females, and injected with accessory gland extract, salivary gland extract or Ringer solution (see Chapter 2, section 2.1.4.5); females were stored individually in food vials. The 9 experimental groups were therefore as follows -

yeasted food - (i) accessory gland extract-injected
(ii) salivary gland extract-injected
(iii) Ringer-injected

unyeasted food - (i) accessory gland extract-injected
(ii) salivary gland extract-injected
(iii) Ringer-injected

sucrose food - (i) accessory gland extract-injected

(ii) salivary gland extract-injected

(iii) Ringer-injected

All females were immobilised over ice for injections; each female was injected with 50nl of accessory gland extract, salivary gland extract or Ringer. This dose represented one third of a pair of accessory glands or one third of a pair of salivary glands. Females were injected every 4 days throughout their lifespan; deaths were recorded every day and eggs counted every 2 days when the females were transferred to new food vials by aspiration.

5.2.3 Results

Female survival was analysed as previously using a distribution-free Mantel-Cox (MC) test (BMDP software, 1988); the median lifespans of all the experimental groups are given in Table 5.1. There were highly significant differences between the lifespans of the 9 experimental groups (Mantel-Cox test statistic = 373.39, $p<0.001$). Within the 3 food regimens, the differences in lifespan were all in the same direction: Ringer-injected females lived significantly longer than accessory gland extract-injected females (yeasted food, MC=49.02, $p<0.001$; unyeasted food, MC=8.89, $p=0.002$; sucrose food, MC=18.19, $p<0.001$) which in turn lived significantly longer than salivary gland-injected females (yeasted food MC=4.32, $p=0.037$; unyeasted food MC=6.77, $p=0.009$; sucrose food MC=5.40, $p=0.02$).

There were significant differences in female lifespan between different food regimens irrespective of injection treatment, ie. yeasted groups combined vs unyeasted groups combined vs sucrose groups combined (MC=268.03, $p<0.001$). Yeasted groups and unyeasted groups combined did not differ significantly in lifespan, but both lived significantly longer than the groups kept on sucrose food. Within the Ringer-injected treatment, yeasted and unyeasted groups did not differ significantly in lifespan;

for the other 2 treatments, females kept on unyeasted food lived significantly longer than those kept on yeasted food (salivary gland-injected MC=5.44, p=0.019; accessory gland-injected MC=4.36, p=0.036).

Median egg-production is shown for all groups in Fig. 5.1 and for the separate food regimens in Figs. 5.2a, 5.2b and 5.2c. Egg-production was compared between groups using Kruskal-Wallis tests as described previously (BMDP software, 1988). Females kept on yeasted food laid significantly more eggs than those kept on unyeasted food, which in turn laid significantly more eggs than sucrose females.

Within the yeasted food regimen, there were no significant differences in the number of eggs laid between Ringer, salivary and accessory gland-injected females on days 2-6 ($p>0.05$). Thereafter, Ringer-injected females laid significantly more eggs than salivary gland-injected females and significantly more eggs than accessory gland-injected females on days 8-18 ($p<0.05$). Accessory gland-injected females laid significantly more eggs than salivary gland-injected females on 2 occasions (days 10 and 12).

Within the unyeasted food regimen, Ringer-injected females laid significantly more eggs than salivary gland-injected females until day 18, and significantly more eggs than accessory gland-injected females until day 8 ($p<0.05$), after which egg-production between these 2 groups was not significantly different. Accessory gland-injected females also laid significantly more eggs than salivary gland-injected females on days 6-14 ($p<0.05$). There were no significant differences in egg-production between any of the 3 groups kept on sucrose food (most females laid no eggs).

Table 5.1. Median lifespan (and interquartile range) of females kept on yeasted, unyeasted or sucrose food and injected with Ringer solution, salivary gland or accessory gland extract.

	Food		
Injection	Yeasted	Unyeasted	Sucrose
Ringer	20 (17-24)	21 (18-25)	9 (6-9)
Salivary Gland	13 (12-15)	14 (7-18)	5 (1-6)
Accessory Gland	13 (11-17)	17 (7-21)	5 (3-7)

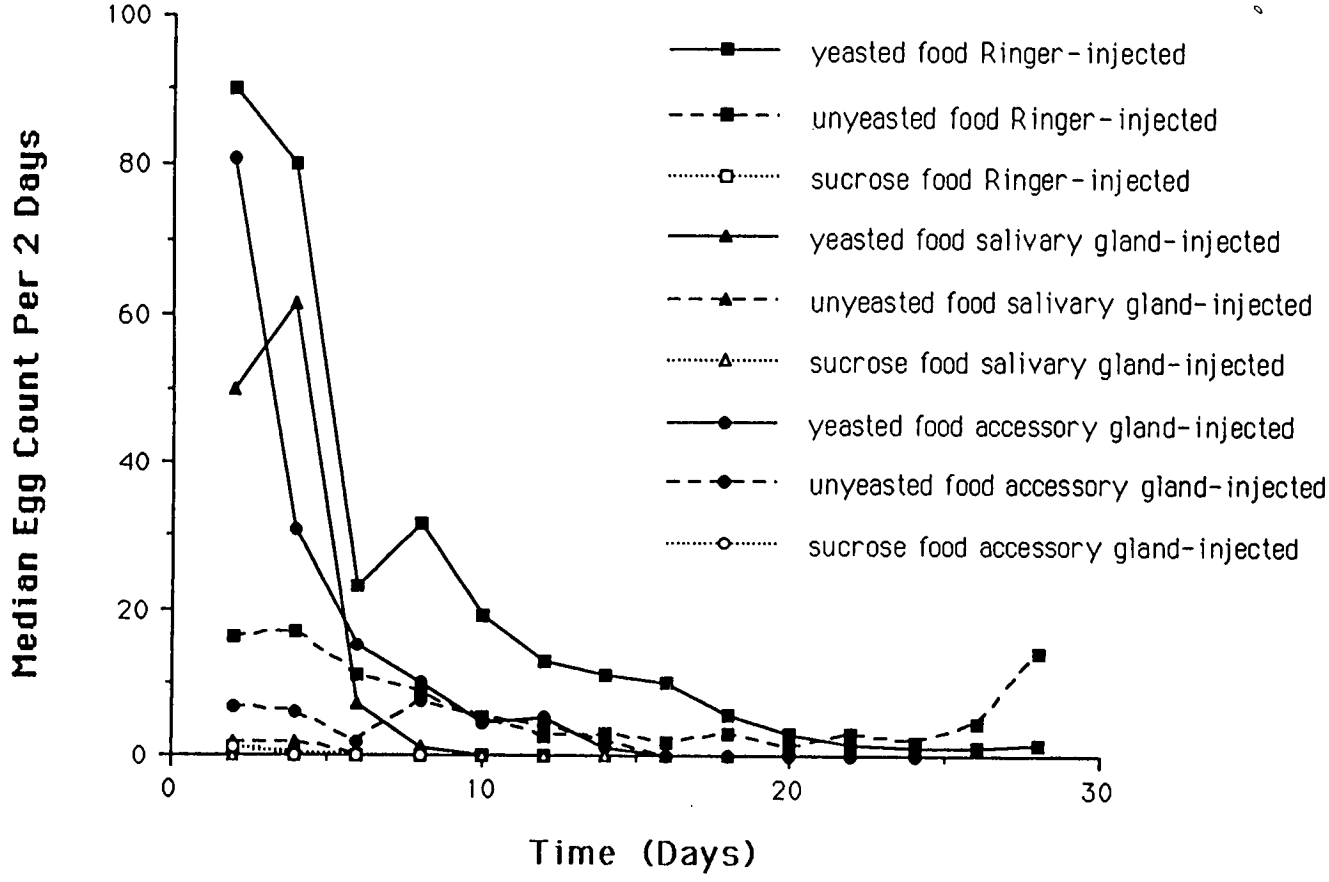


Fig. 5.1. Median egg-production per 2 days against time (days) for females kept on yeasted, unyeasted or sucrose food and injected with Ringer, salivary gland or accessory gland extract.

Fig. 5.2a

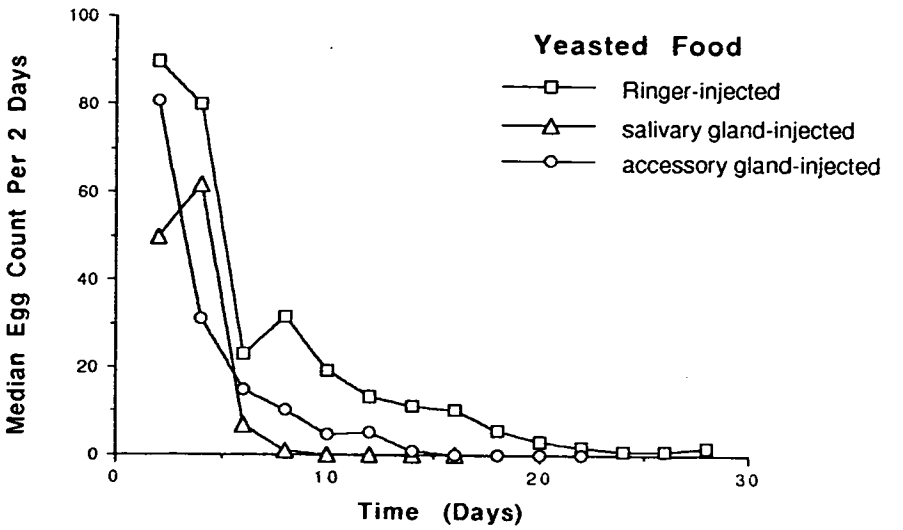


Fig. 5.2b

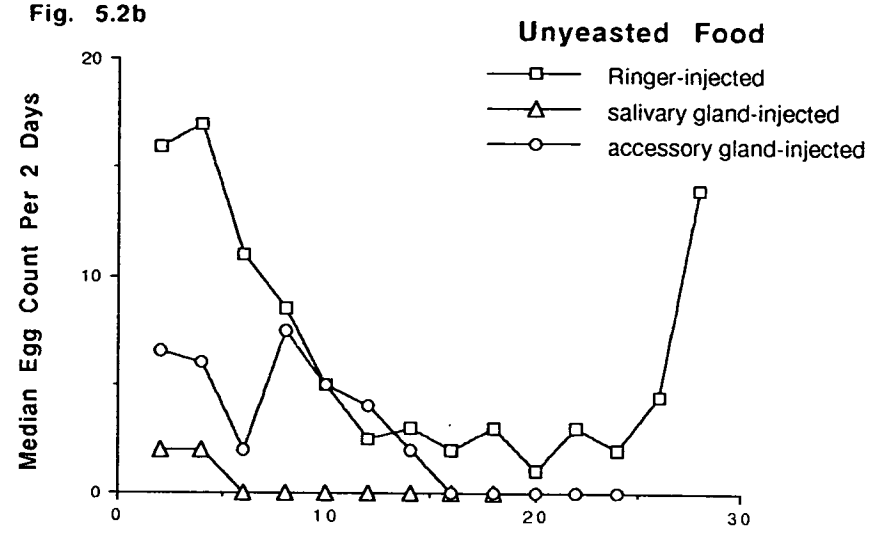
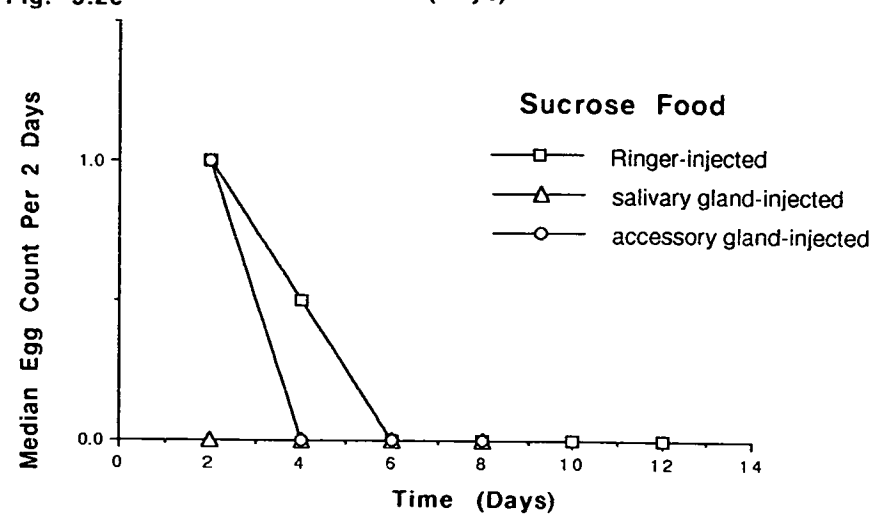


Fig. 5.2c



Figs. 5.2a, 5.2b and 5.2c. Median egg-production per 2 days against time (days) for females kept on yeasted, unyeasted or sucrose food and injected with Ringer, salivary gland or accessory gland extract.

5.3 (a) Effect of Sex Peptide Injection on Female Longevity and Egg-production

5.3.1 Method

Approximately 280 virgin females and 500 wild-type Oregon-R males were collected and sorted by cooling over ice. Virgin females were stored 50 per culture bottle and tossed onto new food every 2 days; males were stored 100 per culture bottle. Four days after collection, females were mated to turn on egg-production by putting them together with males in vials and removing the males after copulation; 250 females mated and were subsequently stored 5 per vial and tossed onto new food every 2 days. 7 days after mating, females were randomly assigned to 3 experimental groups of 72 females each and injected with Ringer, 21-36 SP fragment or SP (see Chapter 2, section 2.1.4.5). The SP fragment was injected to control for any immunological response mounted by females to the SP.

The dose of SP and 21-36 SP fragment injected was 3.0pmol per female in 50nl Ringer. Females were injected after immobilisation by cooling on ice and injected every 3rd and 4th day alternately throughout their lives. The tissue culture plate system was used (see Chapter 2, section 2.2.3.2); there were 3 plates per experimental group (3*24=72 females per group). The food plates were changed every day by cooling females over ice; female deaths were scored and eggs counted daily.

5.3.2 Results

The survival curves are shown in Fig. 5.3. Female lifespans were compared using a distribution free Mantel-Cox (MC) test (BMDP software, 1988) as described earlier. There were no differences in longevity between any of the 3 groups, MC test statistic = 2.01 (p=0.36). Median longevities of the experimental females were very

low (Ringer-injected = 9d, SP fragment-injected = 10d, SP-injected = 10d); whilst it is true that twice weekly injections were probably stressful, the reason for the rapid rate of mortality in this experiment was the death of many females by drowning and sticking in the condensation produced by cooling the plates in order to change the food.

Fig. 5.4 shows median egg-production by the 3 groups of females. The data were analysed as before using Kruskal-Wallis tests. SP-injected females laid significantly more eggs than either SP 21-36-fragment or Ringer-injected females ($p<0.05$). The rate of egg-laying of the SP injected females increased after every injection and then dropped the following day. The rate of egg-laying of SP 21-36 fragment-injected females, although consistently higher, was mostly not significantly different from that of the Ringer-injected females, except on the days following injection, when the rate of egg-laying of the 21-36 SP fragment-injected females was significantly higher than that of the Ringer-injected controls ($p<0.05$). The 21-36 SP fragment therefore seemed to have significant biological activity and was an unsuitable control. This experiment was therefore repeated (next section) using a shorter 25-36 SP fragment as a control for SP injection. Sucrose food was used to suppress and therefore equalise the egg-laying rates of all females.

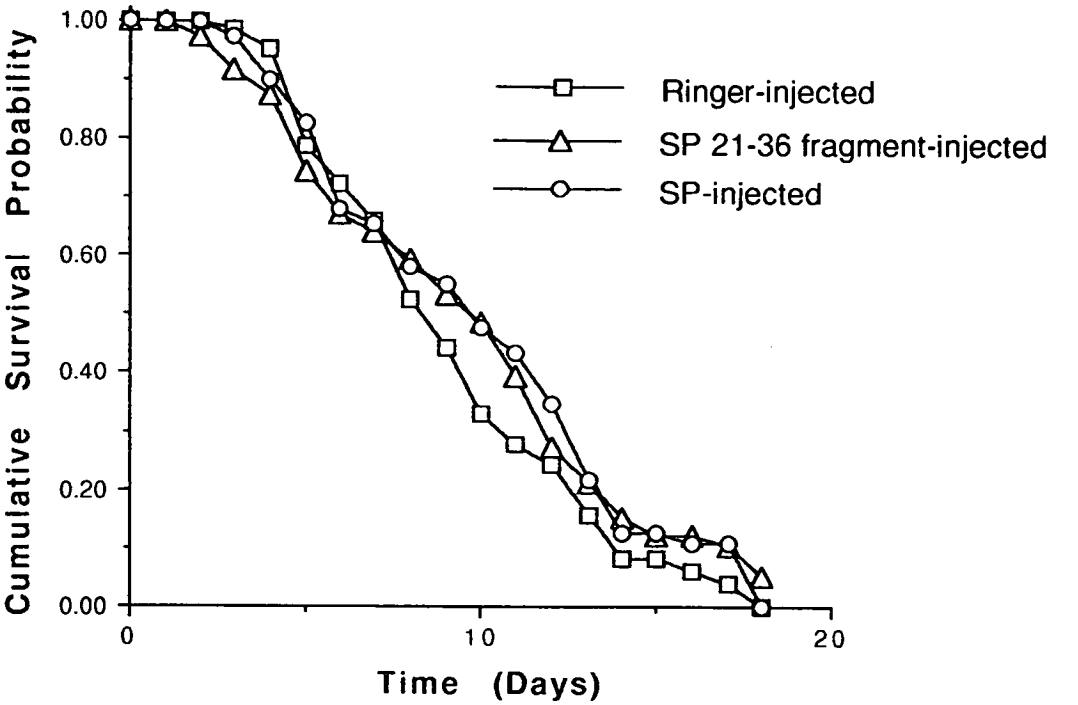


Fig. 5.3. Cumulative probability of survival against time (days) for females kept on unyeasted Zurich medium and injected with Ringer, SP 21-36 fragment, or SP.

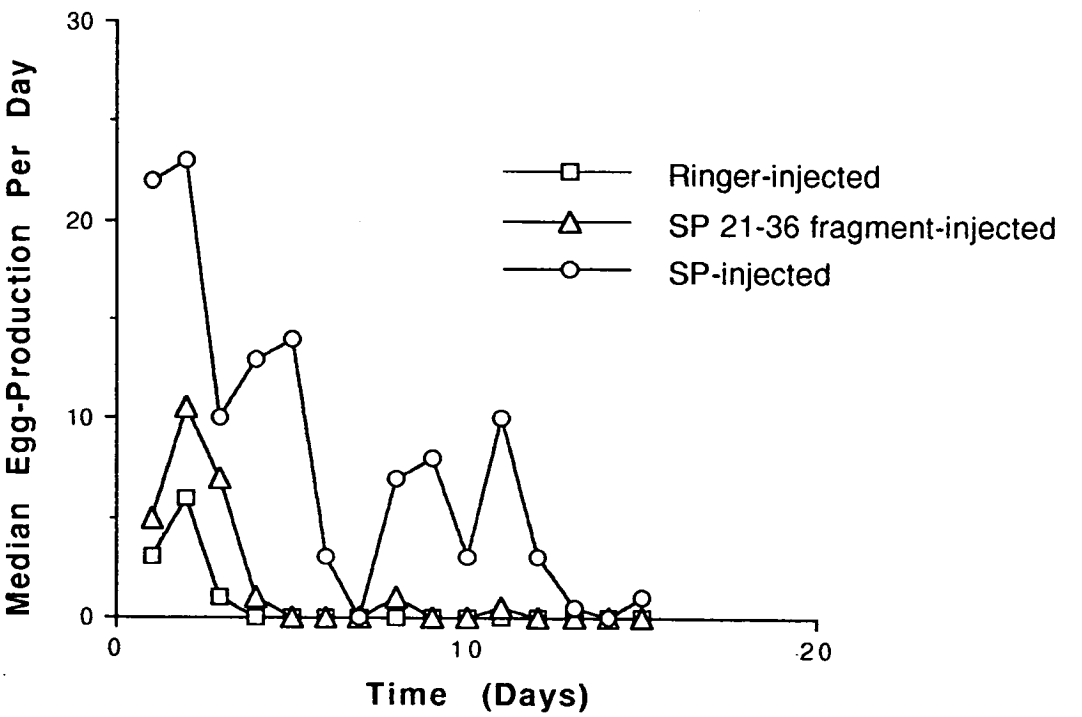


Fig. 5.4. Median egg-production per day against time (days) for the same groups of females as in Fig. 5.3.

5.3 (b) Effect of Sex Peptide Injection on Egg-laying Rate and Female Longevity - Repeat Experiment with Sucrose Food

5.3.3 Method

The method used was the same as for the previous experiment, except that a shorter (25-36) amino acid SP fragment was used as a control for the SP; the 21-36 SP fragment used in the previous experiment had turned out to have significant biological activity. Sucrose food (Chapter 2, section 2.2.1.2) was used throughout to equalise the egg-laying rates of all females, so that any effect of the SP on female lifespan could be seen directly. Culture plates were used as in the previous experiment. Females were stored in culture bottles (100 per bottle) prior to the first injections, instead of in vials as in the previous experiment.

5.3.4 Results

Female survival curves are shown in Fig. 5.5 and were analysed as before (BMDP software, 1988). There were no significant differences in the lifespans of females in the 3 experimental groups (Mantel-Cox test statistic = 3.12, p=0.20). The median lifespans of the 3 groups were: Ringer-injected = 15d, SP fragment-injected = 15d and SP-injected =15d. Median egg-production in shown in Fig. 5.6. The data were analysed by using Kruskal-Wallis tests as before. After day 6, egg counts of the groups were not significantly different; egg counts of the 2 control groups were not significantly different from one another throughout the experiment. The egg-laying rate of the SP-injected group was significantly ($p<0.05$) higher on days 2-5 than in the other 2 groups.

Females in this experiment were stored on Zurich medium prior to the first injections. There was therefore an opportunity for females to assimilate food reserves before being exposed to sucrose food, which has no protein source. This was probably responsible for the initial and undesirable disparity in the egg-laying rates between the 3 groups of females, because energy reserves were diverted into producing eggs in the SP-injected group. In the experiment described in the next section, females were randomly assigned to 1 of 3 nutritional regimens (yeasted, unyeasted and sucrose) and stored in the 3 types of food vials directly after eclosion, to prevent assimilation of food reserves in females kept on sucrose food.

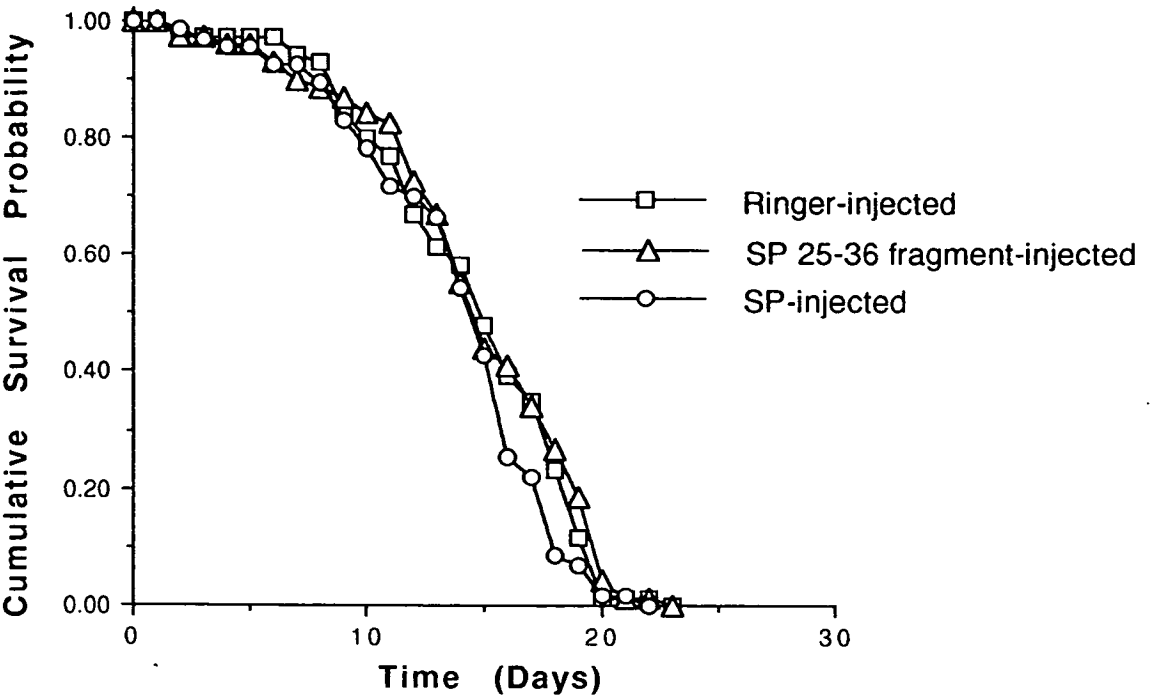


Fig. 5.5. Cumulative probability of survival against time (days) for females kept on sucrose food and injected with Ringer, SP 25-36 fragment or SP.

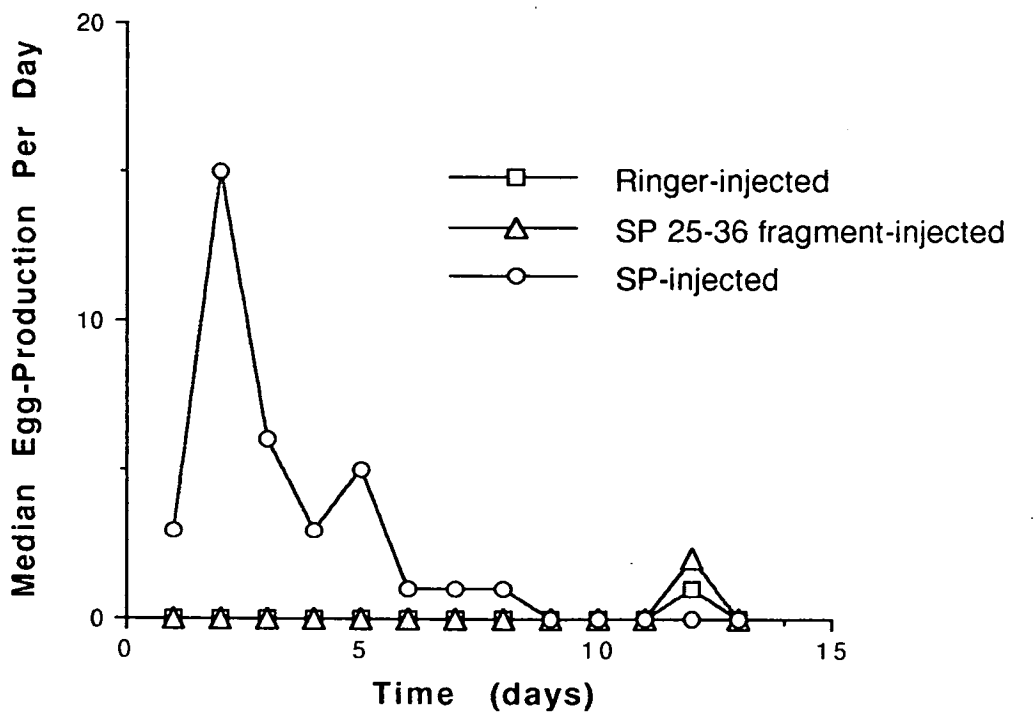


Fig. 5.6. Median egg-production per day against time (days) for the same groups of females as in Fig. 5.5.

5.4 Effect of Sex Peptide Injection on Female Longevity and Egg-production under three Nutritional Regimens

5.4.1 Method

Dahomey wild-type flies were reared at a standardised density of 100 larvae per vial. Virgin females and males were collected at eclosion by cooling on ice; females were randomly assigned to 1 of 3 nutritional regimens (120 females in each group): SY food with added live yeast granules (yeasted food), SY food with no added yeast (unyeasted food) and sucrose food (no protein source), see Chapter 2, section 2.1.1.

Virgin females were aged for 2 days in single sex groups of 5 per vial on the 3 types of food. Males were aged for 2 days in groups of 20 in SY yeasted food vials. All females were mated on day 3 after eclosion to turn on egg-production; 1 female and 2 males were placed in each vial (yeasted, unyeasted or sucrose) and observed until mating took place. Males were then removed and discarded. On day 4 after eclosion the mated females on each type of food regimen were randomly assigned to 1 of 3 experimental groups, each of 40 females, and injected with SP, 25-36 SP fragment or not injected; females were stored individually in food vials. The uninjected group was present to determine the effect of injections on female lifespan and egg-production. The 9 experimental groups were as follows:

Yeasted food - (i) uninjected
(ii) SP-injected
(iii) SP 25-36 fragment-injected

Unyeasted food - (i) uninjected
(ii) SP-injected
(iii) SP 25-36 fragment-injected

Sucrose food - (i) uninjected

(ii) SP-injected

(iii) SP 25-36 fragment-injected

All females were immobilised over ice for injection: SP-injected females each received 3pmol of SP in 50nl Ringer; SP fragment-injected females each received 3pmol of 25-36 SP fragment in 50nl Ringer and uninjected females were cooled over ice but not injected. Injections were administered every 4 days until day 34. Female deaths were recorded every day and eggs counted every 2 days when the females were transferred to new food vials by aspiration.

5.4.2 Results

Female survival (Table 5.2) was analysed as previously, using a distribution-free Mantel-Cox (MC) test (BMDP software, 1988). There were highly significant differences in the lifespans of the 9 groups (MC=362.17, $p<0.001$). On yeasted food, the uninjected group lived significantly longer (MC=69.17, $p<0.001$) than either of the injected groups, between which there were no significant differences. Within the unyeasted food regimen, the uninjected group had a significantly longer lifespan then the SP fragment-injected group (MC=6.41, p=0.011), but not the SP-injected group; there were no differences in longevity between the 2 injected groups. There were no significant differences in the lifespans of the 3 groups of females kept on sucrose food.

Unyeasted food groups as a whole lived significantly longer than groups kept on yeasted food (MC=4.05, p=0.044) which in turn lived significantly longer than groups kept on sucrose food ($p<0.001$). Females kept on unyeasted food lived significantly longer than yeasted groups within the SP-injected (MC=6.98, p=0.008) and SP fragment-injected treatments (MC=11.60, p=0.0007); there were no significant differences in lifespan between yeasted and unyeasted uninjected females.

Median egg-production for the 9 groups is shown in Fig 5.7, and separated out into the 3 nutritional regimens in Figs. 5.8a, 5.8b and 5.8c. Egg-counts were analysed using Kruskal-Wallis tests as described previously (BMDP software, 1988). Females kept on yeasted food had significantly higher egg-production than females kept on unyeasted food, which in turn laid significantly more eggs than females kept on sucrose food. On yeasted food, egg-production between the 3 groups was not significantly different until day 16 after which uninjected females laid significantly more eggs ($p<0.05$) than either of the 2 injected groups which did not differ significantly in egg-production. After day 20, the yeasted uninjected group of females had a significantly higher rate of egg-production than any of the other groups, except on 2 occasions (day 36 and day 56). There were no significant differences in egg-production at any time between any of the groups kept on unyeasted or sucrose food; most females in the latter group laid no eggs.

Table 5.2. Median lifespan (and interquartile range) of females kept on yeasted, unyeasted or sucrose food and injected with SP 25-36 fragment, SP or not injected.

Injection	Food		
	Yeasted	Unyeasted	Sucrose
Uninjected	43 (37-48)	39 (33-46)	13 (12-15)
SP 25-36	27 (23-30)	34 (24-39)	14 (12-16)
SP	25 (22-28)	27 (23-34)	15 (12-17)

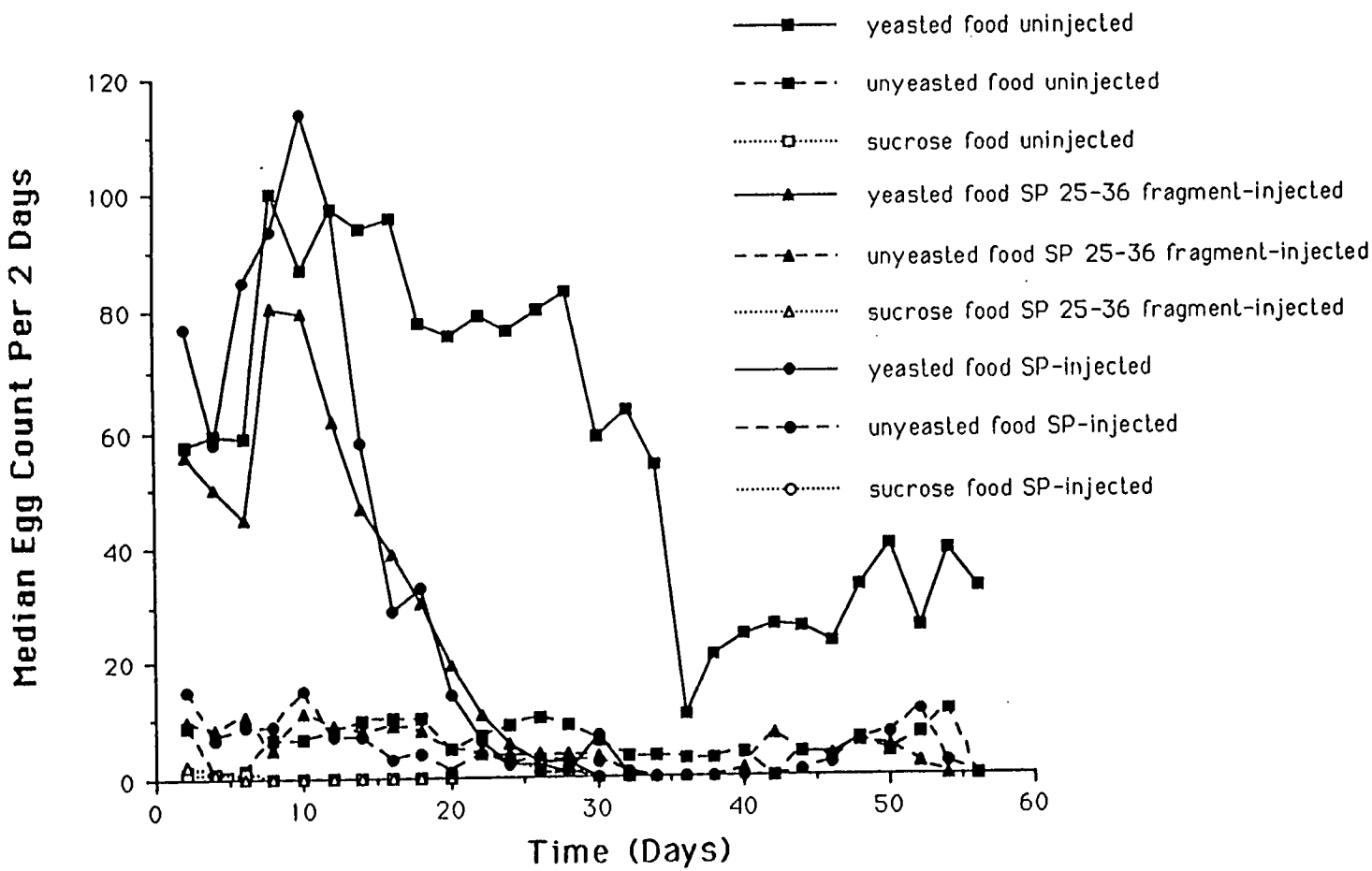


Fig. 5.7. Median egg-production per 2 days against time (days) for females kept on yeasted, unyeasted or sucrose food and injected with SP 25-36 fragment, SP or not injected.

Fig. 5.8a

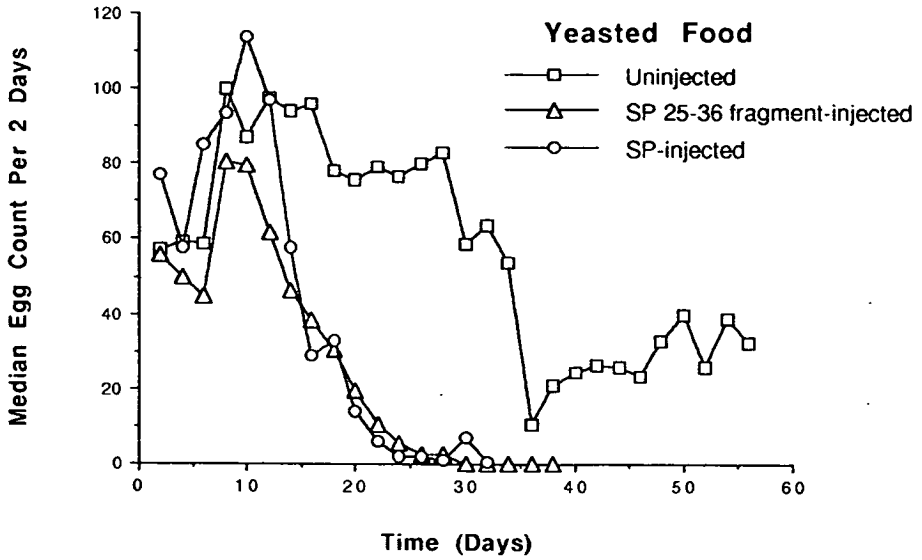


Fig. 5.8b

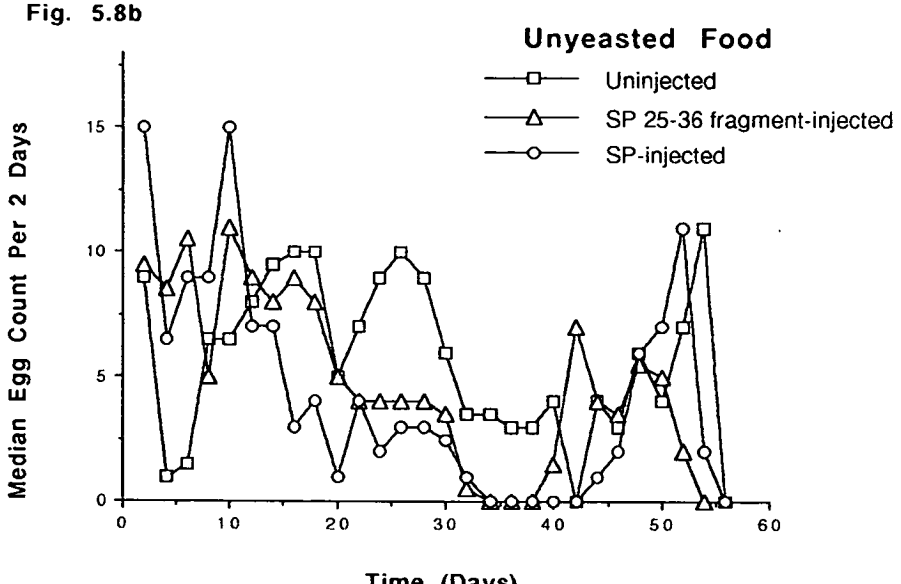
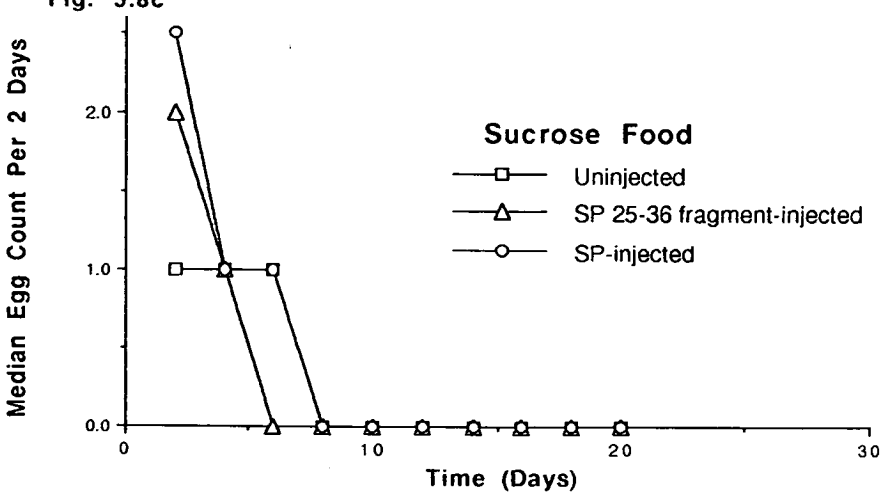


Fig. 5.8c



Figs. 5.8a, 5.8b and 5.8c. Median egg-production against time (days) for females kept on yeasted, unyeasted or sucrose food and injected with SP 25-36 fragment, SP or not injected.

5.5 Discussion

The most important finding from the first experiment (5.2) was that injection of accessory gland extract significantly shortened female lifespan and reduced age-specific egg-production. On each type of food, accessory gland-injected females had significantly shorter lifespans than Ringer-injected females. There were significant differences in egg-production between these 2 groups (Ringer-injected females tended to lay significantly more eggs than accessory gland-injected females), but the differences in lifespan were in the wrong direction to be explained by a cost of producing eggs (Partridge *et al.*, 1987a). Salivary gland-injected females had significantly shorter lifespans than Ringer or accessory gland-injected females on all types of food and also significantly lower egg-production (except on sucrose food where there were no significant differences in egg-production between any groups). It seems likely that enzymes in the salivary gland extract were digesting female abdomens; the salivary gland extract was therefore not suitable as a control for accessory gland-injection. A more suitable control for future experiments might be an ovary extract; this is less likely to contain enzymes that would digest female abdomens upon injection.

Injection of accessory gland extract did not cause the expected short-term increase in egg-production (Garcia-Bellido 1964; Leahy 1966; Merle, 1968). Garcia-Bellido (1964) observed an increase in egg-production after injection of one accessory gland equivalent or implantation of 1 pair of accessory glands per virgin female. Leahy (1966) implanted a single pair of glands into virgin females and observed an increase in egg-production to the level of mated females. Merle (1968) observed an increase in egg-production and decrease in sexual receptivity of virgin females implanted with 1 pair of accessory glands; a small transitory increase in the egg-production of females implanted with 1 ejaculatory bulb was also evident.

In the accessory gland experiment, each of the injections was a physiological dose of one third of a pair of accessory glands into once mated females. This dose represented what a female would receive at mating, but administered by an abnormal route. It is possible that this dose was not high enough to cause an increase in egg-production after injection and it would be possible to increase the dose injected or inject more frequently. It is also possible that the increase in egg-production and decrease in receptivity following accessory fluid transfer is less important in the once-mated females used in this experiment. The increases in egg-production observed previously used whole accessory gland implants or injections into virgin females (Garcia-Bellido, 1964; Leahy, 1966; Merle, 1968).

Within the Ringer injected groups, there was no significant difference between the lifespans of yeasted and unyeasted females; for accessory gland-injected females, those kept on unyeasted food lived significantly longer than those on yeasted food. This difference can be attributed to a cost of producing eggs (Partridge *et al.*, 1987a); females on yeasted food laid considerably more eggs than those kept on unyeasted food and, perhaps as a consequence, had significantly shorter lifespans.

There were three experiments that investigated the effect of SP injection on female lifespan and egg-production. The first two experiments (5.3a and 5.3b) did not provide any evidence that the SP had any effect on female lifespan; the lifespans of SP-injected, SP fragment-injected and Ringer-injected females did not significantly differ in either experiment. However there were problems with the methods employed in each: in the first experiment (5.3a) females drowned or became stuck in the condensation produced by cooling the culture plates, any effects of the SP may therefore have been masked by extrinsic effects on mortality. The 21-36 aa SP fragment used as a control was not a good choice as it turned out to have significant biological activity.

In the second SP injection experiment (5.3b), food with no protein source was used (sucrose food) to equalise the egg-laying rates of all the groups by suppressing it; any direct effect of the SP on female lifespan could then have been seen. However, females were given an opportunity to assimilate food reserves before being put onto sucrose food and this caused an initial disparity in the egg-laying rates of the experimental groups. The third SP injection experiment (5.4), performed in Edinburgh, corrected these problems: 3 nutritional regimens were used, females were stored on the 3 types of food directly after eclosion and the shorter 25-36 amino acid SP fragment was used as a control; only this experiment will be discussed in detail.

There was no evidence to suggest that SP injection had any adverse effect on female lifespan or egg-production: the lifespans and egg-production of SP-injected females were not significantly different from SP 25-36 fragment-injected controls on any of the 3 nutritional regimens studied. The uninjected group lived significantly longer and laid more eggs than the other groups on yeasted food, and also lived significantly longer than the other groups on unyeasted food (where egg-production was not significantly different from the other groups). There were no significant differences in lifespan or egg-production between any groups on sucrose food. Thus injecting the females every 4 days was harmful, but the effect was smaller when egg-laying was reduced (on unyeasted and sucrose food).

Females kept on unyeasted food lived significantly longer than those on yeasted food for both groups of injected females; the corresponding difference for uninjected females was not significant. The rates of egg-production for females kept on yeasted food were considerably higher than for females on unyeasted food; the females on yeasted food might therefore have incurred more of the cost of producing eggs (Partridge *et al.*, 1987a) and suffered a decrease in lifespan as a direct result. There was some evidence of a short-term increase in egg-production following SP injection, but

only under intermediate levels of egg-laying on unyeasted food; this effect was also evident in the first of the SP injection experiments (5.3a) at similar levels of egg-production. Egg-production in the SP-injected group on unyeasted food did go up and down alternately every 2 days until day 25 or so; however, the SP fragment-injected females also showed a similar pattern of egg-production. It is not possible to be certain that the SP had no effect on female lifespan or fecundity if the 25-36 SP control fragment also had biological activity; it would have been useful to have had a Ringer-injected control group to clarify this.

The SP 25-36 fragment seemed to have biological activity, because females injected with this fragment did not differ in terms of egg-production from those injected with the full length SP. The small increases that were observed in egg-production following SP-injection in females kept on unyeasted food were also seen in the SP 25-36-injected females. The SP 25-36 fragment does not cause an elevation in ovulation or decrease in receptivity in virgin females upon injection of a single 3pmol dose (Yves Choffat and Thomas Schmidt, pers. comm.); it seems that repeated injection of the 25-36 SP fragment is therefore necessary before any biological activity is seen. A more suitable SP control without biological activity could be obtained by synthesising a peptide with a scrambled full length SP sequence.

The SP seemed to affect egg-laying only at intermediate levels of egg-production. No effects of the SP on egg-laying were seen on either sucrose or yeasted food; the reason for this is unclear. The SP effect on unyeasted food did not persist beyond about day 20 or so; this suggests that it is important for young or virgin females. It would be possible to investigate this by injecting SP into young and old virgin and inseminated females and assaying ovulation and receptivity.

The results of these experiments indicate that injection of male accessory gland material does cause a drop in female lifespan; however, the SP does not appear

to contribute to this cost. Future clarification of the exact components responsible for the effect awaits further characterisation of accessory gland and seminal fluid components (Chapter 1, section 1.5.2). In particular, it is important to discover whether there are other proteins or peptides apart from the SP that directly affect female receptivity and fecundity. Injection experiments of the type described in this chapter require a significant quantity of a purified preparation of the component of interest to inject. This may soon be possible for some of the male-specific accessory gland proteins isolated by DiBenedetto *et al.*, (1987) and Monsma and Wolfner (1988). Alternatively, females could be mated to males that lack certain accessory gland products and assayed for effects on longevity and egg-production. Null mutations for particular accessory gland components would therefore be very useful and Chapter 7 of this thesis describes a mutagenesis screen for SP-null mutants. Several of the accessory gland-specific genes isolated by Monsma and Wolfner (1988) have P element insertions nearby; these will be used to obtain null mutants by secondary P element mutagenesis (Mariana Wolfner, pers. comm.), see Chapter 7, section 7.1.2.

Males lacking whole sets of accessory gland components have been obtained by chemically ablating particular cell types within their accessory glands. Main cell protein synthesis was chemically ablated by fusing the diptheria toxin subunit A to a main cell specific promotor (Mariana Wolfner, pers. comm.). The main cells of these flies do not produce secretory products and secondary cell expression is unaffected. These males could be used to look at the importance of secondary cell products on female lifespan and egg-production. Accessory gland extracts could also be depleted of specific components by antibody precipitation; this would require specific antibodies to the components of interest, several of which are already available. The effects of many of the components of the accessory fluid and their possible contribution to the cost of mating remain to be elucidated.

Chapter 6. Sex Peptide Mode of Action

6.1 Introduction

The effects of the sex peptide (SP) on egg-laying and receptivity in wild-type virgin females have been well characterised (Chen *et al.*, 1988), see Chapter 1, section 1.5.2, but nothing is known about the SP mode of action, or the location of receptors for the SP. The SP induces both rejection and an increase in ovulation in virgin females, but it is not clear whether the two effects are linked or induced separately (Chen *et al.*, 1988). The target of the SP is unknown; there may be a single target or independent targets in different tissues, eg. peptide receptors in neuronal as well as ovarian cells. The SP does not show any homology to other small peptides that are involved in information transfer in neuronal cells (Hokfelt *et al.*, 1987).

Three approaches were used to identify the location of the SP receptor: affinity chromatography, expression library screening with a radioactive ligand and incubation of labelled SP on female head sections (Eric Kubli and Thomas Schmidt, pers. comm.); none of these approaches were conclusive and the SP target is still unknown. The dose response curves for the SP (see Chapter 2, Fig. 2.2) show a sharp threshold of the ovulation and receptivity responses at 0.6pmol SP injected per female. The sharp threshold of the dose-response curve may indicate that 0.6pmol is the concentration at which a nerve is fired or perhaps that the sex peptide acts as a dimer, with two molecules coming together only after a certain threshold concentration has been exceeded.

The SP may have receptors in the brain, themselves causing the release of a brain peptide or peptides which could then act upon other receptors, perhaps in the ovary. Brain peptides could be involved in mediating the ovulation reaction and there is

some evidence that certain concentrated head peptide fractions do cause an increase in ovulation (Saudan, unpublished data). The SP causes an increase in egg-production and decrease in receptivity (and therefore must find its target) when expressed ectopically in females transformed with a construct of the SP gene attached to a heat shock promotor (Aigaki *et al.*, 1991); however, the SP is not detectable in the haemolymph of these females.

In the first part of this chapter, three experiments are described which aimed to investigate whether there are receptors for the SP on the ovary, or some other site controlling ovulation rate (experiments 6.2, 6.3 and 6.4). It was reasoned that, if the processes controlling ovulation rate were directly influenced by the SP, then the rate of SP usage could be changed by altering the rate of egg-laying. There could then be differences in the rejection behaviour of females that had had different rates of egg-production following SP injection, ie. females that laid a lot of eggs after SP injection might be expected to be more receptive than females that laid fewer eggs following SP injection.

Zurich fly food and sucrose food, which has no source of protein, (see Chapter 2, section 2.2.1) were used to cause a disparity in the egg-laying rates of 2 groups of females, to investigate whether the magnitude of the rejection response to the SP (normally lasting about 1 day in abdominally-injected females) could be altered by suppressing the rate of egg-production after SP injection (experiment 6.2). The second two experiments of the three (6.3 and 6.4) had an additional mated female group to confirm that females that lay more eggs remate more often (Trevitt *et al.*, 1988). Mated females that had laid lots of eggs (kept on Zurich food) were therefore expected to be more receptive to males than mated females that had laid fewer eggs (those kept on sucrose food).

To gain a further insight into the mode of action of the SP, several experiments with *dunce* (*dnc*) females were performed and are reported in the second part of the chapter (experiments 6.5, 6.6 and 6.7). The defect in *dunce* flies is the failure to regulate cyclic adenosine monophosphate (cAMP) because of altered or abolished cAMP-specific phosphodiesterase II (PDE II) activity (Salz *et al.*, 1982). Bellen and Kiger (1987) reported that *dunce* females homozygous for null enzyme alleles exposed to wild-type males had significantly shorter lifespans than wild-type females exposed to wild-type males. It was suggested that the transfer of male seminal fluid components activated adenylate cyclase, resulting in elevated cAMP levels, which could not be regulated in the *dunce* females due to the defect in the PDE II enzyme. This could have disrupted basic physiological functions leading to a decrease in longevity.

dunce females are also reported to remate almost twice as often as wild-type females (Bellen and Kiger, 1987) and would therefore be expected to show increased costs of mating (Fowler and Partridge, 1989). Bellen and Kiger (1987) also reported that a single mating was enough to reduce the lifespan of *dunce* females; this implies that as well as suffering from an increased cost of mating due to their elevated remating rate, they are also more susceptible to the effects of a single mating. The first of the *dunce* experiments (6.5) repeated the Bellen and Kiger (1987) experiment to investigate whether a single mating could cause a drop in lifespan of *dunce* females. Sucrose food was used to equalise the egg-production of all groups so that any effect of a single mating on lifespan could be seen directly and would not be confounded with differences in egg-production between *dnc* and dnc^+ females.

The last of the *dunce* experiments (6.6 and 6.7) investigated the rejection responses of *dunce* females to SP injection. Peptide hormones (of which the SP may be one) act via a second messenger system, binding to a specific receptor on the cell membrane. This triggers the release of a 'second messenger', often cAMP, within the

cell which then activates specific kinase enzymes causing physiological changes, specific to the type of cell (Lamb *et al.*, 1980). The effect of the SP injection in *dunce* females was therefore investigated because it was reasoned that the elevated levels of cAMP in *dunce* females might interfere with the SP signal, causing abnormal or even absent rejection behaviour following SP injection.

dunce was originally isolated as a memory mutant (Quinn and Greenspan, 1984); it has defects in both associative and non-associative learning. It was reasoned that if the rejection behaviour of *dunce* females following SP injection turned out to be abnormal, it would then be monitored at shorter time intervals after SP injection than in the standard test (3 hours) to investigate whether rejection behaviour had been prematurely lost as a result of the *dunce* memory defect.

All the experiments presented in this chapter were performed in the Zurich laboratory. The *dunce* stocks used are described in Chapter 2, section 2.2.2.2. A fertile spontaneous *dunce*$^+$ (*dnc*$^+$) revertant, isolated from the *dunce* stock, exhibiting wild-type phosphodiesterase (PDE) activity was used as a control for *dunce*. The *dnc* and *dnc*$^+$ flies therefore had the same genetic background.

6.2 Effect of Egg-laying Rate on SP-usage

6.2.1 Method

200 Oregon-R virgin females and 600 males were collected by sorting flies over ice. Females were then stored 100 per culture bottle, and males 150 per bottle; flies were tossed onto new food every 2 days. 5 days later females were randomly assigned to 4 experimental groups (98 females per group); and injected with Ringer or SP (3pmol per female), see Chapter 2, section 2.1.4.5. Half of the females in each group

were then placed on normal Zurich fly food culture plates and half on sugar food culture plates (see Chapter 2, section 2.2.1).

Food plates were changed every day, deaths scored and eggs counted; food plates were changed by cooling females. 3 days after injection, females were collected from the culture plates, stored 3 per vial and a rejection test performed (see Chapter 2, section 2.2.4.1).

6.2.2 Results

Egg-production was analysed using Kruskal-Wallis tests as described previously (BMDP software, 1988). Fig. 6.1 shows the egg-laying rates of all 4 groups in the 3 day period after injection and before the rejection test. On the unyeasted Zurich food, SP-injected females laid significantly more eggs ($p<0.05$) than Ringer-injected females except on the third day after injection when the egg-laying rates of these 2 groups were not significantly different. Within the sucrose food regime, SP-injected females laid significantly more eggs than Ringer-injected females on all 3 days ($p<0.05$). Females injected with SP laid significantly more eggs when kept on Zurich food than when kept on sucrose food. There were no differences in egg-production between the 2 Ringer-injected groups on the 2 food regimens, except on day 3, when significantly more eggs were laid by the Ringer-injected group on Zurich food ($p<0.05$).

The results of the rejection test are shown in Table 6.1; a G test was used to test for differences in the frequency of rejection behaviour between the different groups (Sokal and Rohlf, 1969). There were no significant differences in the frequency of rejection behaviour between the 4 experimental groups of females ($G=4.07$, 3df).

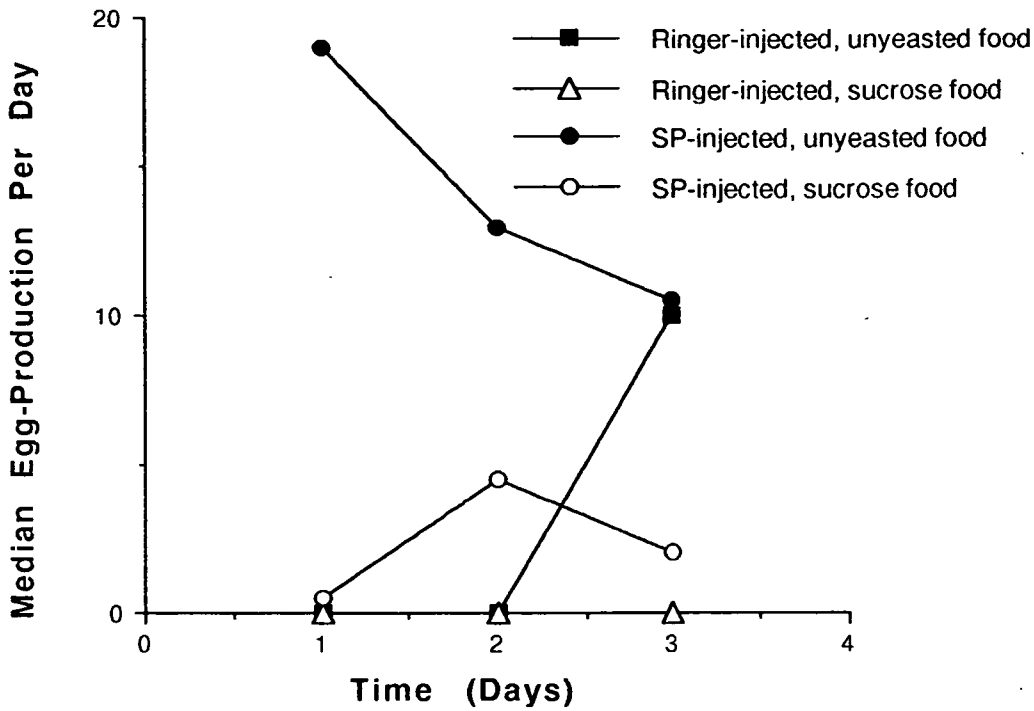


Fig. 6.1. Median egg-production per day, for the 3 day period after injection and before the rejection test, for females kept on unyeasted or sugar food and injected with Ringer or SP.

Table 6.1. Rejection responses of wild-type females kept on unyeasted Zurich food or sugar food for 3 days after injection with Ringer or SP.

Genotype of female	Injection	pmol /50nl	Number of females injected	Number of Copulations			
				0-30min	30-60 min	Total in 60 min	Total %
Oregon-R sugar food	Ringer	-	30	27	1	28	93
Oregon-R sugar food	SP	3.0	30	20	6	26	87
Oregon-R unyeasted Zurich food	Ringer	-	24	19	3	22	92
Oregon-R unyeasted Zurich food	SP	3.0	30	20	2	22	73

6.3. Effect of Egg-production on SP-usage (with Addition of Mated Female Group)

6.3.1 Method

To check that the culture plate system used produced a large enough disparity in egg-laying rates for any differences in rejection behaviour to be observed, the previous experiment was repeated with an additional mated female group. Mated females that lay a lot of eggs are known to remate more often (Trevitt *et al.*, 1988). Therefore if the differences in egg-production produced by the culture plate system were large enough, mated females kept on Zurich food would have been expected to be more receptive in the rejection test than mated females kept on sucrose food.

300 wild-type Oregon-R virgin females and 600 males were collected and stored as in the previous experiment. 3 days later, females were randomly assigned to 3 experimental groups of 98 females and injected with Ringer or SP, or mated. Half of the females in each group were placed on Zurich food culture plates and half on sugar food culture plates. Food plates were changed and eggs counted every day. In the previous experiment, females were immobilised by cooling in order to change food plates, but this led to problems with unwanted condensation. Food plates in this experiment were changed using CO_2, as this was quicker, more convenient and left the food plates drier. 4 days after injection or mating, the rejection responses of all groups were tested, with 7 day-old virgin females (injected with Ringer or SP 3 hours before the rejection test) as controls for any environmental effects that might have affected female remating probability.

6.3.2 Results

Fig. 6.2 shows the egg-production rates for the 4 day period after injection and before the rejection test. Egg-production was analysed using Kruskal-Wallis tests. On Zurich food, the egg-production of the mated and SP-injected groups was significantly higher than the Ringer-injected group ($p<0.05$), except on day 3 after injection when the rates of egg-production of the SP and Ringer-injected groups were not significantly different. The rate of egg-production on sucrose food was mostly significantly lower than on Zurich food ($p<0.05$). Within the sucrose food regime, mated females laid significantly more eggs than Ringer-injected females on all days ($p<0.05$); SP-injected females laid significantly more eggs than Ringer-injected females on two occasions (days 1-2, $p<0.05$). There were no significant differences in egg-production between the Ringer-injected groups on the two foods, except on day 3 when significantly more eggs were laid by the Ringer-injected group on Zurich food ($p<0.05$).

The results of the rejection test are shown in Table 6.2; the data were analysed using a G test, as previously. The overall G statistic was highly significant ($G=27.48$ 5df, $p<0.001$). Partitioning of the G test statistic showed that this was due to significant differences in rejection behaviour between the different treatments (mating, Ringer or SP injection), irrespective of food regime ($G=22.85$ 2df, $p<0.001$). Mated females kept on sucrose food were significantly less receptive than any of the other groups. The receptivity of SP-injected females kept on sugar food was 13% lower than the receptivity of SP-injected females kept on Zurich food and 14% lower than that of Ringer-injected females on sugar food, but these effects were not significant.

The control flies were not included in the analysis because they were included only to monitor atmospheric conditions that might have affected remating probability, and had not been subjected to any of the experimental treatments. Under ideal conditions, 100% of the Ringer-injected and 0% of the SP-injected controls would have

been expected to copulate in the rejection assay. The actual results observed were 97% copulation in the Ringer-injected control group and 3% in the SP-injected control group. There was no evidence therefore to suggest that female remating propensity was affected by external factors such as barometric pressure on the day of the rejection test.

6.4 Effect of Egg-production on SP-usage (Repeat Experiment with Mated Female Group)

6.4.1 Method

The results for the previous experiment suggested that there was a small decrease (13%) in the receptivity of SP-injected females kept on sugar food compared to SP-injected females kept on Zurich food. The previous experiment was therefore repeated with larger sample sizes to clarify whether this was a reproducible effect. The method used was exactly as in the previous experiment.

6.4.2 Results - Repeat Experiment

Fig. 6.3 shows the egg-production for the 6 groups in the 4 day period after injection and before the rejection test. Kruskal-Wallis tests were used to analyse egg-counts as before. On Zurich food, mated and SP-injected females laid significantly more eggs than Ringer-injected females ($p<0.05$), except on day 4 when the egg-production of the SP and Ringer-injected females was not significantly different. Ringer and SP-injected females on sucrose food laid significantly fewer eggs than the groups of females on Zurich food ($p<0.05$). On days 3-4, the egg-production of mated females on sucrose food and mated and SP-injected females on Zurich food was not significantly different. Within the sucrose food regime, mated females laid significantly more eggs than the other groups on days 2-4 ($p<0.05$). The rate of egg-production of

the SP and Ringer-injected females on sucrose food was not significantly different at any point.

The results of the rejection test are given in Table 6.3. The frequency of rejection was analysed as previously using a G partitioned test. The overall G test statistic was highly significant ($G=33.65$ 5df, $p<0.001$). Partitioning of the G statistic showed that this was due to a significant effect of food medium ($G=5.28$ 1df, $p<0.05$), experimental treatment ($G=13.95$ 2df, $p<0.001$) and the interaction between experimental treatment and food medium ($G=14.42$ 2df, $p<0.001$). As in the previous experiment (section 6.3), the receptivity of the mated females kept on sucrose food was significantly lower than that of the other groups.

There was however a problem with the controls for the rejection test in this experiment. 64% of Ringer-injected and 11% of SP-injected virgin control females copulated in the test. 100% of Ringer-injected females and 0% of SP-injected virgin females would normally be expected to copulate under these conditions; environmental effects may therefore have affected female remating probability on the day of the rejection test.

Fig. 6.2

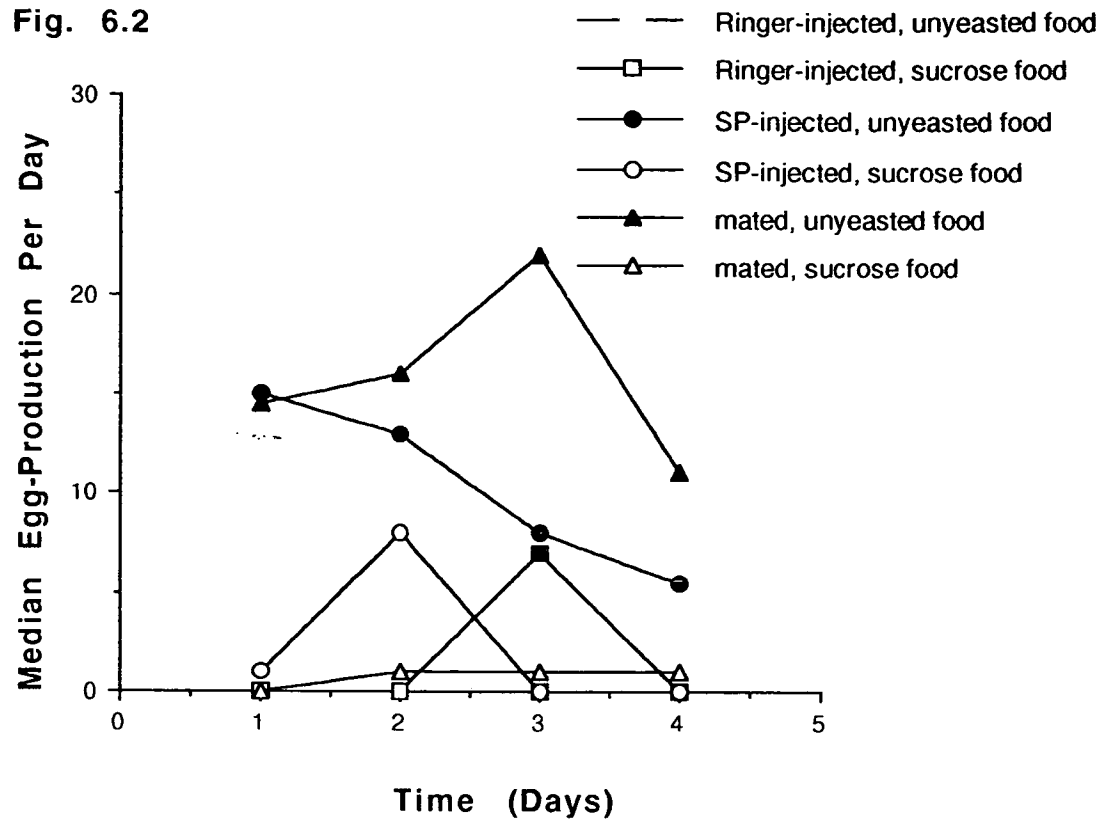
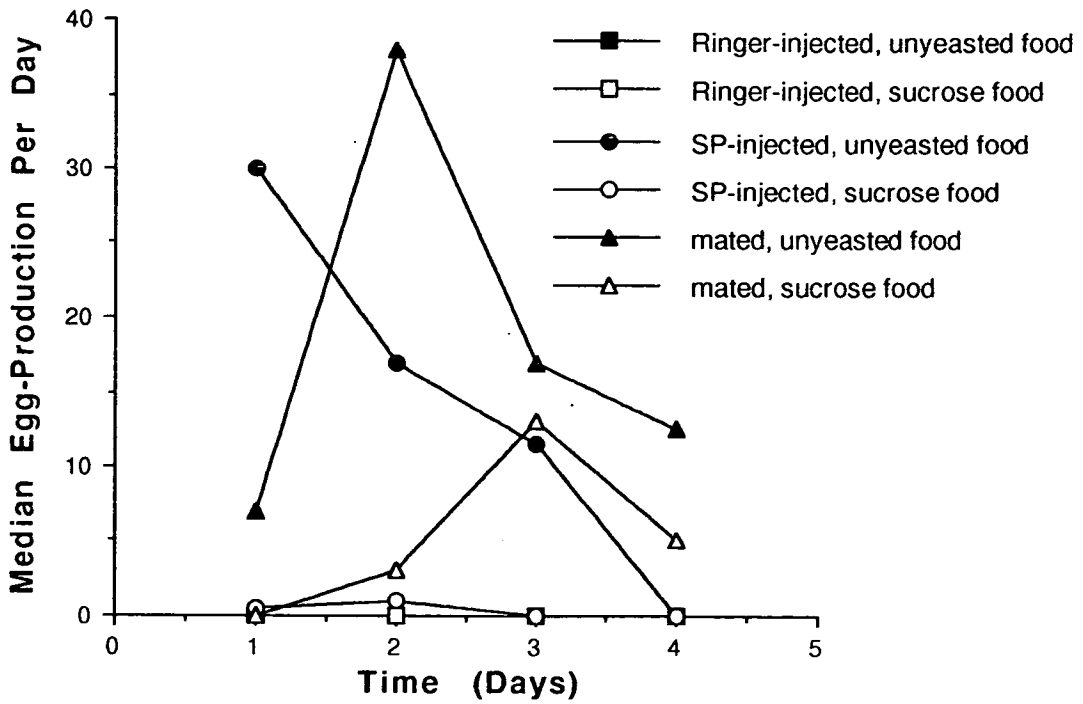


Fig. 6.3



Figs. 6.2 and 6.3. Median egg-production per day for the 4 day period after injection and before the rejection test, for females kept on unyeasted or sucrose food and injected with Ringer or SP, or mated.

Table 6.2. Rejection responses of wild-type females kept on unyeasted Zurich food or sugar food for 4 days after mating or injection with Ringer or SP.

Genotype of female	Injection	pmol /50nl	Number of females injected	Number of Copulations			
				0-30min	30-60 min	Total in 60 min	Total %
Oregon-R unyeasted Zurich food	Ringer	-	24	22	1	23	96
Oregon-R unyeasted Zurich food	SP	3.0	26	23	1	24	92
Oregon-R unyeasted Zurich food	Mated	-	30	16	5	21	70
Oregon-R sugar food	Ringer	-	30	25	3	28	93
Oregon-R sugar food	SP	3.0	29	21	2	23	79
Oregon-R sugar food	Mated	-	30	9	6	15	50
Oregon-R virgin female control	Ringer	-	30	25	4	29	97
Oregon-R virgin female control	SP	3.0	30	1	0	1	3

Table 6.3. Rejection responses of wild-type females kept on unyeasted Zurich food or sugar food for 4 days after mating or injection with Ringer or SP - repeat experiment.

Genotype of female	Injection	pmol /50nl	Number of females injected	Number of Copulations			
				0-30min	30-60 min	Total in 60 min	Total %
Oregon-R unyeasted Zurich food	Ringer	-	19	11	3	14	74
Oregon-R unyeasted Zurich food	SP	3.0	26	11	4	15	58
Oregon-R unyeasted Zurich food	Mated	-	26	12	4	16	62
Oregon-R sugar food	Ringer	-	48	20	7	27	56
Oregon-R sugar food	SP	3.0	44	16	12	28	64
Oregon-R sugar food	Mated	-	35	3	1	4	11
Oregon-R virgin female control	Ringer	-	42	21	6	27	64
Oregon-R virgin female control	SP	3.0	42	2	6	8	19

6.5 Effect of a Single Mating on the Longevity of *dunce* and *dunce*$^+$ Females

6.5.1 Method

100 *dnc* and 100 *dnc*$^+$ virgin females were collected over a 2 day period and stored in culture bottles. The following day, 98 *dnc* and 98 *dnc*$^+$ virgin females were placed on sugar food culture plates. Food plates were changed using CO_2, deaths scored and eggs counted every day; after 3 days, half of each group of virgins were mated with Oregon-R males and then returned to culture plates. Food plates were replaced every day, deaths scored and eggs counted until day 18 when the experiment was terminated.

6.5.2 Results

Fig. 6.4 shows the survival curves for the 4 groups. Lifespans were analysed using a distribution free Mantel-Cox test (BMDP software, 1988) as used previously. *dnc*$^+$ females lived significantly longer than the *dnc* females (MC=5.13, p=0.02); there were no significant differences between any of the other experimental groups. Median lifespans were as follows: *dnc* virgins=7d, *dnc* mated=8d, *dnc*$^+$ virgins=11d and *dnc*$^+$ mated=10d. Fig. 6.5 shows the median egg-production for the 4 groups. The data were analysed using Kruskal-Wallis tests as before. Egg-production was not significantly different between any of the groups, except on days 4-8 when the egg-production of the mated *dnc*$^+$ females following mating (on day 3) was significantly higher ($p<0.05$) than in any of the other groups.

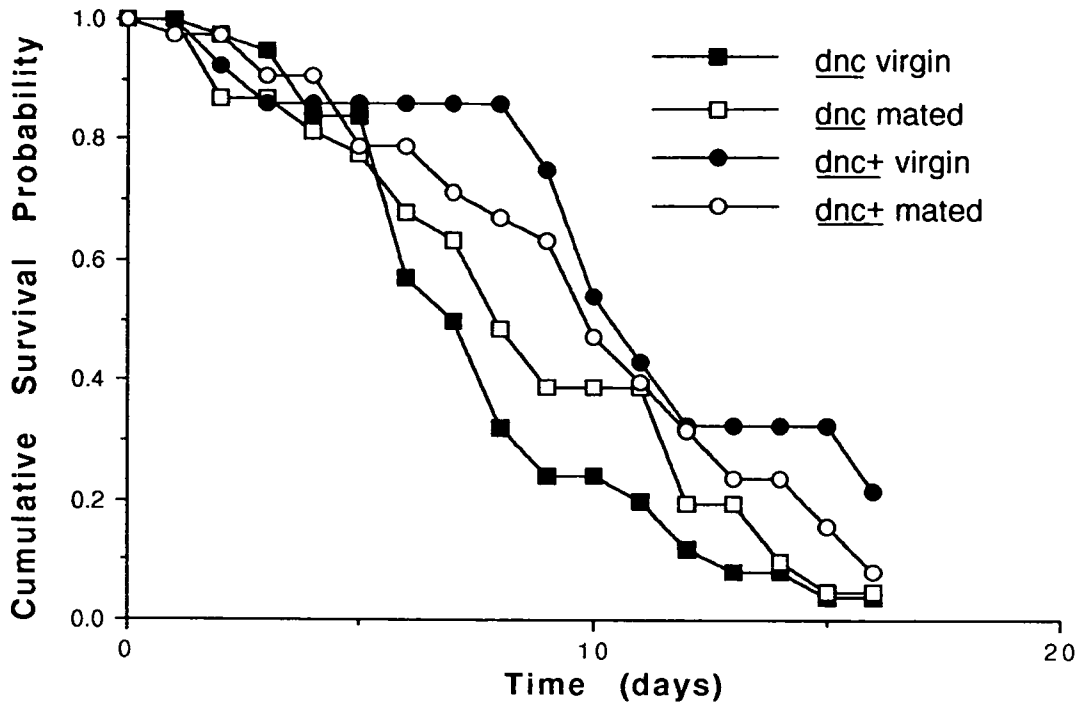


Fig. 6.4. Cumulative probability of survival against time (days) for virgin or once mated dnc or dnc+ females kept on sucrose food.
The 2 mated groups were mated once on day 3 of the experiment.

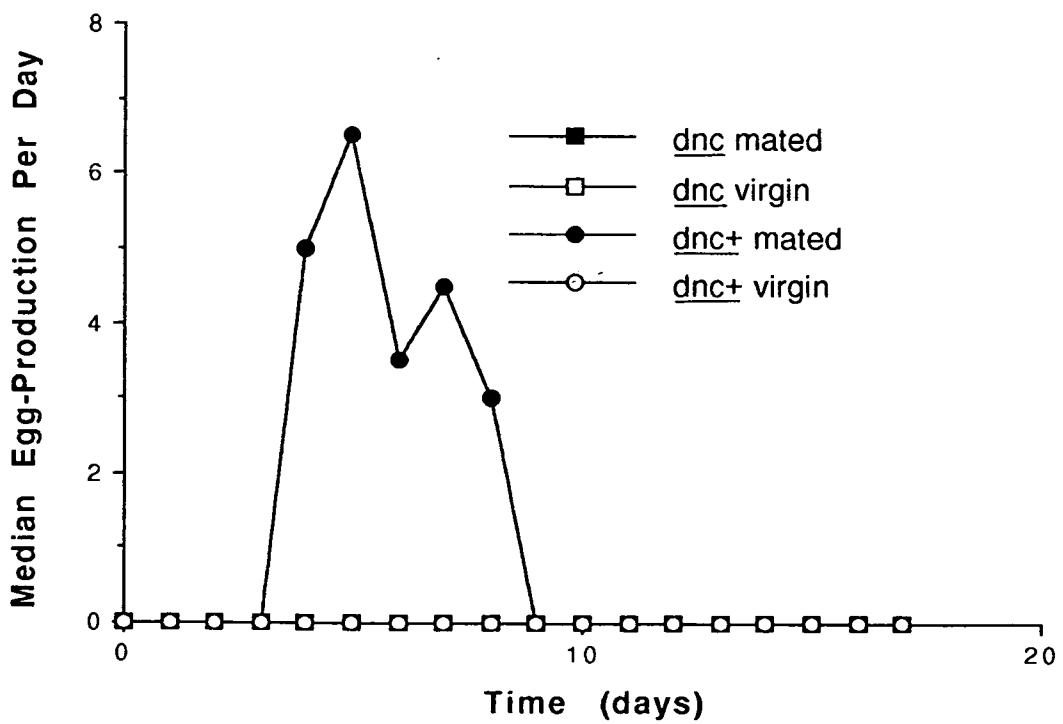


Fig. 6.5. Median egg-production per day against time (days) for the same groups of females as in Fig. 6.4.

6.6 Effect of SP on Rejection Behaviour in *dunce* Females

6.6.1 Method

60 each of *dnc* and *dnc*$^+$ virgin females were collected by sorting on ice, stored 10-15 per culture bottle and tossed onto new food every 2 days. Males for the rejection test were collected and stored approximately 100 per culture bottle and tossed onto new food every 2 days. 5 days later, half of each group of females was injected with Ringer or SP. Three hours after injection, the rejection behaviour of all females was assayed (Chapter 2, section 2.2.4.1).

6.6.2. Results

The results of the rejection test are shown in Table 6.4, the data were analysed as previously, using a partitioned G test. The overall G statistic was highly significant ($G=46.83$ 3df, $p<0.001$). Partitioning of the G statistic showed that this was due to a significant effect of female genotype ($G=6.46$ 1df, $p<0.05$), injection treatment ($G=18.60$ 1df, $p<0.001$) and the interaction between female genotype and injection treatment ($G=34.69$ 1df, $p<0.001$). *dnc* virgin females did not show rejection behaviour after SP injection whereas 100% of *dnc*$^+$ virgin females rejected males following SP injection.

6.7. Effect of SP on Rejection Behaviour in *dunce* females 30 and 90 minutes after SP Injection

6.7.1 Method

120 each of *dnc*, *dnc*$^+$ and wild-type Oregon-R virgin females were collected, stored 10-20 per culture bottle and tossed onto new food every 2 days; the wild-type virgins were included as additional controls. Oregon-R males for the rejection tests were collected and stored 150 per bottle. 5 days later, 2 rejection tests were performed. The first recorded rejection behaviour 30 minutes after half of each group was injected with either Ringer or SP, and the second 90 minutes after injection with Ringer or SP.

6.7.2 Results

The results of the rejection tests are shown in Tables 6.5a and 6.5b. The frequency of rejection behaviour of all females was analysed using partitioned G tests as previously. The overall G statistics for both experiments were highly significant (G=122.89 and G=160.44 5df, $p<0.001$). Partitioning of the G statistics showed that this was due to significant effects of female genotype (G=28.78 and G=18.33 2df, $p<0.001$), injection treatment (G=55.20 and G=102.67 1df, $p<0.001$) and the genotype-injection treatment interaction (G=38.90 and G=39.42 2df, $p<0.001$). SP injection had no effect on *dnc* females either 30 or 90 minutes after injection. The rejection response of the *dnc*$^+$ females took time to develop; only 50% of the *dnc*$^+$ females rejected copulations 30mins after injection, compared with 83% rejection 90 mins after injection.

Table 6.4. Rejection responses of *dnc* and *dnc+* females 3 hours after injection with Ringer or SP .

Genotype of female	Injection	pmol /50nl	Number of females injected	Number of Copulations			
				0-30min	30-60 min	Total in 60 min	Total %
dnc+	Ringer	-	30	23	5	29	97
dnc+	SP	3.0	10	0	0	0	0
dnc	Ringer	-	30	27	2	29	97
dnc	SP	3.0	30	21	5	26	87

Table 6.5a. Rejection responses of wild-type, *dnc*+ and *dnc* females 30 mins after Ringer or SP injection.

Genotype of female	Injection	pmol /50nl	Number of females injected	Number of Copulations			
				0-30min	30-60 min	Total in 60 min	Total %
Oregon-R	Ringer	-	30	18	10	28	93
Oregon-R	SP	3.0	30	0	0	0	0
dnc+	Ringer	-	30	28	2	30	100
dnc+	SP	3.0	30	9	6	15	50
dnc	Ringer	-	30	24	3	27	90
dnc	SP	3.0	30	23	4	27	90

Table 6.5b. Rejection responses of wild-type, *dnc*+ and *dnc* females 90 mins after Ringer or SP injection.

Genotype of female	Injection	pmol /50nl	Number of females injected	Number of Copulations			
				0-30min	30-60min	Total in 60 min	Total %
Oregon-R	Ringer	-	30	29	1	30	100
Oregon-R	SP	3.0	30	0	0	0	0
dnc+	Ringer	-	30	30	0	30	100
dnc+	SP	3.0	30	5	0	5	17
dnc	Ringer	-	29	25	2	27	93
dnc	SP	3.0	30	18	5	23	77

6.8 Discussion

In the first 3 experiments (6.2, 6.3 and 6.4) females kept on Zurich food had significantly higher rates of egg-production than females kept on sucrose food. Mated females that lay a lot of eggs have been shown to remate more often (Trevitt *et al.*, 1988) and this was supported by the finding in experiments 6.3 and 6.4 that mated females kept on sucrose food, that laid few eggs, were significantly less receptive in the rejection tests than all the other groups of females. However, there was no evidence from any of the experiments of a correlation between the rate of egg-laying and the magnitude of the SP response in virgin females. Virgin females injected with SP and which then experienced different rates of egg-production for 3 to 4 days did not differ in their rejection behaviour towards wild-type males. Therefore, in the absence of sperm (ie. in the unmated groups of females), elevating the rate of sterile egg-production did not alter female receptivity. The correlation between the rate of egg-laying and receptivity seen in mated females was therefore presumably an effect of sperm depletion rates or some other consequence of mating itself and not of the rate of egg-laying *per se.* The magnitude of the differences in egg-production in virgin females obtained by using unyeasted Zurich food and sugar food was only of the order of 10-20 eggs/day for 3 or 4 days however, and it is possible that the disparity in egg-laying rates between the groups of females was not large enough or did not persist for long enough to see any significant differences in rejection behaviour. The differences in egg-production between mated females kept on Zurich and sucrose food were larger than for the injected virgin females; this might explain why only mated females showed differences in receptivity.

There was no evidence from the first of the *dunce* experiments (6.5) that a single mating caused a drop in *dnc* female lifespan; there was no significant difference in the lifespans of once-mated *dnc* females and *dnc* virgins; this finding is in disagreement with the result obtained by Bellen and Kiger (1987). Sucrose food

suppressed the rate of egg-production of all groups so that any effect of the single mating on female lifespan could be seen directly. The lifespans of the females in the *dunce* experiment however were not very long and it is possible that females starved before any differences in lifespan could be manifested. There were no differences in egg-production between any of the groups except for a small increase in the mated dnc^+ group following mating. The results of the *dnc* longevity experiment suggested that *dnc* females are more susceptible to costs of mating because of their high remating rate and not because they are more susceptible to the effects of a single mating.

Bellen and Kiger (1987) reported that *dnc* females had significantly higher remating rates than wild-type females, and therefore the drop in lifespan they observed in *dnc* females kept with wild-type males was probably a direct consequence of the high cost of mating they incurred (Fowler and Partridge, 1989). The longevity of dnc^+ once mated females was shorter, but not significantly different, from dnc^+ virgins. It is possible that the early burst of egg-production by the dnc^+ mated females was costly (Partridge *et al.*, 1987a) producing this minor effect.

The results of the first *dunce* SP injection experiment (6.6) showed that *dunce* virgin females did not exhibit rejection behaviour 3 hours after injection with the SP. If the loss of the SP response was due to the *dunce* memory defect, then the SP response might only have been apparent shortly after injection; the evidence does not support this hypothesis however, because *dnc* virgin females did not reject males either 30 or 90 minutes after SP injection (experiment 6.7). It is unclear why *dunce* virgin females did not respond to the SP. If the SP acts as a peptide hormone, it may be unable to trigger the 'second messenger' system of cells (see Introduction to this chapter) because of the already elevated levels of intracellular cAMP in *dunce* females. Any signal produced by the binding of the SP to cell membrane receptors may therefore be insufficient to cause rejection. Alternatively, *dnc* females may have been unable to reject for another reason, normal rejection behaviour such as ovipositor extrusion may be impossible for females

with such a grossly disturbed cAMP system; *dunce* females may simply be unable effectively to reject males under any circumstances. The results also showed that rejection behaviour in the SP-injected dnc^+ females took time to develop; only 50% of dnc^+ females rejected males 30 minutes after SP injection as compared to 83% and 100% rejection shown by dnc^+ females 90mins and 3 hours after SP injection. The reason for this is unclear; it may reflect an effect of the genetic background of the dnc^+ flies used.

There are many details of the SP mode of action that remain unclear. Further experiments should address the exact mechanism by which the SP operates, and the location of SP receptors. The nature of the interaction of the male ejaculate, the female sperm storage organs, ovary and central nervous system (CNS) is also unknown. Feedback information from the ovary or sperm storage organs (the seminal receptacle and the spermathecae, see Fig. 1, Chapter 1) concerning current rates of egg-production and sperm usage could affect female remating probability. Such a feedback loop could operate via neural or secondary biochemical cues (Scott, 1987). The nature of the feedback loop between ovary and CNS could be investigated by implanting ovaries; the neural network between ovary and brain would be disrupted in implanted females, but any humoral factors produced in response to the SP, or other accessory gland component, could still be released by the implanted ovary. Implantation of full and empty seminal receptacles between females could provide information about whether the feedback loop between female sperm storage organs and CNS operates via neural or humoral cues.

To investigate if there are receptors for the SP on the ovary, it would be possible to dissect ovaries and bathe them in a solution of the SP and look for myogenic egg-laying activity. Alternatively, it would be possible to use females from a stock transformed with a heat shock promotor-SP gene construct (Aigaki *et al.*, 1991). The expression of the SP gene is turned on by heat shock in these flies. Isolated female

abdomens could be heat shocked to investigate whether there is an elevation in egg-production which would be predicted if there are SP receptors on the ovary itself. There is evidence that there are receptors for male accessory gland substances on the ovary of at least one other insect, *Locusta migratoria* (Lafon-Cazal *et al.*, 1987; Paemen *et al.*, 1990).

Investigation of the SP response in germlineless (eg. ovo^{D}) females could provide an insight into the SP mode of action. If the ovary has receptors for the SP, germlineless females might have elevated levels of free, unbound SP in the haemolymph and might therefore be more unreceptive to males.

Chapter 7. Mutagenesis Screen for Sex Peptide-null Mutants

7.1 Introduction

The aim of the experiments presented in this chapter was to produce mutant lines of flies carrying a deletion in the SP gene. An SP-null mutant would be extremely useful; it would enable the effects of the SP on female behaviour and physiology to be confirmed and extended. The advantage of assessing the importance of the SP using SP-null males, as opposed to injecting females with SP or SP-depleted accessory gland extracts, is that the accessory gland material is transferred via the normal route, and the female also experiences the effects of mating itself. Accessory gland injection is useful to identify the effects of accessory gland components, but does not perfectly mimic the effects of the transfer of an ejaculate at mating (see Chapter 1 section 1.5.1). It would also be possible to use SP-null males to examine whether the SP acts in the reproductive interests of the male, the female or both, and thus to determine whether the SP represents the molecular manifestation of a conflict between the sexes over investment in offspring (see Chapter 1, section 1.4).

The accessory glands of male *D. melanogaster* contain many different proteins and peptides. One-dimensional electrophoretic separation of male accessory gland proteins yielded about 12 fractions on 7.5% native starch gels and at least 40 fractions on 10% sodium dodecyl sulphate (SDS) gels, with molecular weights ranging from 15 to 175 kilo-Daltons. Two dimensional separation yielded at least 85 fractions (Stumm-Zollinger and Chen, 1985). The differences in the estimates of the number of accessory gland proteins are due to differences in degree of resolution and separation in the methods used. Autoradiography of two-dimensional gels by Ingman-Baker *et al.* (1980) demonstrated the presence of approximately 1200 accessory gland proteins in

male *D. melanogaster*. Ingman-Baker *et al.* did not separate out the glandular epithelium from the accessory gland secretion before electrophoresis, and the number of accessory gland proteins they reported is an over-estimate of the number of secreted proteins contained in the accessory gland lumen.

Some of the small proteins and peptides of the accessory fluid have been characterised, and these include the sex peptide, SP, (Chen *et al.*, 1988) and male specific proteins (msps) 316, 355a and 355b (DiBenedetto *et al.*, 1987; Chapman and Wolfner, 1988; Monsma and Wolfner, 1988; DiBenedetto *et al.*, 1990), see Chapter 1, section 1.5.2. The effects of by far the majority of the sub-components of the accessory fluid, and their possible contribution to the cost of mating are unclear. The experiments presented in Chapter 5 suggested that receiving accessory fluid was costly to females but the SP did not appear to contribute to this cost. Components of the accessory fluid other than the SP were probably responsible for the drop in lifespan of accessory gland-injected females.

Injection experiments such as those described in Chapter 5 require a significant quantity of a purified preparation of the component of interest to inject. Purified preparations of some of the msps isolated by DiBenedetto *et al.* (1987) and Monsma and Wolfner (1988) will soon be available. Additionally, accessory gland extracts could be depleted of particular components by antibody precipitation, and then injected into females. This would require specific antibodies to the components of interest, several of which are already available. Another approach would be to mate females to males that lack particular accessory gland products. Two approaches could be used to produce males lacking particular accessory gland components: chemical ablation of particular cell types within the accessory glands, which would produce males that lack whole sets of accessory gland products, and the production of null mutations in specific accessory gland genes.

There are two types of secretory cells within the accessory glands; there are about 1000 main cells and about 45 secondary cells at the distal tip of each lobe (Mariana Wolfner, pers. comm.). Two different methods have been used to obtain males with chemically ablated accessory gland main cells (Mariana Wolfner, Dominik Styger and Eric Kubli, pers. comm.). Main cell protein synthesis was chemically ablated by transforming flies with a diptheria toxin subunit A (DTA) main cell-specific promotor construct (Mariana Wolfner, pers. comm.). The main cells of the DTA flies do not produce secretory products, while secondary cell expression is unaffected. DTA males are also sterile. This is thought to be due to leakiness of the main cell promotor allowing a small amount of expression of the diptheria toxin in the testis, causing sterility (one molecule of the DTA toxin is enough to block transcription in a cell). Spermatogenesis is not dependent on the presence of accessory gland products because it does occur in *tuh-3/Df C4* males which do not have accessory glands (Mariana Wolfner, pers. comm.). Therefore the sterility of the DTA males is probably not due to the absence of accessory gland main cell products. Spermatogenesis does occur in flies transformed with a less strong diptheria toxin (0.01 DTA) construct, but females mated to 0.01 DTA males produce fewer progeny than controls (Mariana Wolfner, pers. comm.).

DTA males could be used to look at the importance of secondary cell products for female lifespan and egg-production. DTA males have low fertility and to directly compare their effects on females with those of control males, both types of males could be made sterile. This could be achieved by making all males XO, or using the male offspring of DTA and control males crossed to homozygous *tudor* mothers. XO males lack a Y chromosome and do not transfer sperm at mating (Bridges, 1916; Stern, 1929). The *tudor* (*tud*) mutation has a maternal effect that blocks the formation of pole cells in all progeny of *tud/tud* mothers (Boswell and Mahowald, 1985). The offspring have no germline and male offspring therefore do not produce sperm.

Ablation of the main cells of accessory glands was also attempted by transforming flies with a main cell promotor-mouse hair follicle keratin gene construct (Dominik Styger and Eric Kubli, pers. comm.). The mouse gene contains a very high proportion of cysteine residues (McNab *et al.*, 1989). Expression of this gene within a cell in which it is not normally expressed should result in the formation of a large number of disulphide bridges between the cysteine residues, cross-linking proteins and causing cell death (Eric Kubli and Gabriel Vogeli, pers. comm.). The main cells of males transformed with the high sulphur (HS) mouse gene-main cell promotor construct should therefore be ablated.

The HS protein was expressed in the main cells but seemed to be released into the gland lumen, cross-linking the lumen contents and leaving the cells intact. Nuclear (DAPI) staining of the accessory gland nuclei confirmed that the main cells were still present (Dominik Styger, pers. comm.). Large clumps were visible in the accessory gland lumen of the HS transformed flies; these could be dissolved by injecting the glands with a reducing agent (DTP). The reducing activity of the DTP presumably broke apart the disulphide bridges, dissolving the clumps. Examination of the accessory glands of the flies transformed with the HS construct by electron microscopy showed that golgi apparatus function was affected in the main cells and that both main and secondary cells were still present (Perotti, unpublished data). Males transformed with the HS construct were also sterile, possibly due to leakiness of the promotor allowing some expression of the HS protein in the testes. It is unclear at present whether the HS construct transformed males are able to transfer accessory gland components at mating (Dominik Styger, pers. comm.).

An alternative approach for studying the function of specific accessory gland products is to produce null mutations. Several of the accessory gland specific genes isolated by Monsma and Wolfner (1988) have P element insertions nearby; these could

be used to obtain null mutants; imprecise excision of a P element during mobilisation could delete part of a nearby gene, creating a null mutation. When these flies are available, they could provide data on the effect of mating females to males that lack specific accessory gland proteins. This chapter describes experiments designed to produce a null mutation in one accessory gland-specific gene, namely the SP gene.

The SP gene has been sequenced and localized in the cytological region 70A of chromosome III (see Fig 7.1); it is a short gene (266bp from transcription start site to the polyA signal), single copy and contains an intron of 65bp at about two-thirds of its length (Styger *et al.*, unpublished data). A previous approach used to obtain flies with reduced or absent SP was to produce an SP-null mutation by expressing SP antisense RNA under the control of the endogenous SP promotor in transgenic flies (Styger, unpublished data). Antisense RNA is an RNA molecule with a nucleotide sequence that is completely complementary to a specific RNA sense (normal) transcript. The expression of antisense SP RNA therefore blocks the translation of sense SP RNA by binding to it. If the amount of antisense RNA expressed is high enough, the amount of SP produced would be substantially reduced. Females do not respond to the SP at low concentrations (see dose response curve, Chapter 2, Fig 2.1) and therefore if the amount of SP could be reduced below this level (about 0.6pmol for abdominally-injected females) the males would be effectively null for the SP.

The antisense method for making SP-null males was partly successful; a stock where a double antisense SP construct was present on chromosomes II and III showed reduced levels of SP expression as detected by Western blots. Females mated to the double antisense construct males were significantly more receptive to males after mating than were females mated to wild type males, as would be predicted if they had received reduced SP at mating (Dominik Styger, pers. comm.). In the absence of an SP-null mutant, these flies could be used to investigate the effect of receiving reduced levels of SP on female lifespan and fecundity.

All of the approaches used to obtain flies with reduced or absent SP have been partly successful, but a true SP-null mutant is still not available. This chapter describes a mutagenesis project designed to produce such a mutation; both P element mutagenesis and classical mutagenesis techniques were used; all experiments were performed in the Zurich laboratory.

7.1.1 X-ray Mutagenesis

Radiation has been widely used as a mutagen; exposures of 3000-4000 rads (r) are typically used, as this dose produces a high frequency of mutations with minimum sterility (5-10%, Grigliatti, 1986). The penetration of X-rays into cells causes the ejection of electrons from the atoms of molecules in the path of the X-ray. Thus stable atoms and molecules are transformed into free radicals and reactive ions which may then undergo chemical changes. Purines and pyrimidines within the DNA may be altered, producing point mutations; breaks in DNA strands may also occur, resulting in chromosomal aberrations. X-ray mutagenesis experiments are generally more prone to deleterious secondary effects of the mutagenesis procedure than P element techniques. The proportion of X-ray induced homozygous viable mutations depends upon the radiation dose used, partly because at high doses the probability of multiple hits increases. In addition to effects on the germline, radiation may also cause somatic damage. Many of the F_1 progeny of irradiated males are also sterile (Demerec, 1933; Moore, 1934).

7.1.2 P Element Mutagenesis

P element mutagenesis falls into two broad categories:

(i) primary P element mutagenesis refers to the induction of new mutation, usually by P element insertion, into a locus previously unoccupied by a P element.

(ii) secondary P element mutagenesis results from the excision of a P element already resident in a locus.

Secondary P element mutagenesis was used in these experiments. Mobilisation of a resident P element may result in precise or imprecise excisions, deletions or chromosomal rearrangements at, or in the vicinity of, the location of the termini of the existing P element insertion. P elements can be excised by X-irradiation, or by using the activity of the transposase enzyme, which is encoded by the P element itself. A stock containing a defective P element, which cannot itself be excised but which produces high levels of the transposase enzyme, is usually crossed to a stock containing a resident P element which is subsequently excised.

In mutagenesis experiments, stable deletions are generally produced by inducing chromosome breakage and then selecting for pseudodominance of a recessive allele, or reversion of a dominant mutation. Pseudodominance is the expression of a recessive gene when present in a single dose, eg. when it is opposite a deletion in the homologous chromosomal strand (Klug and Cummings, 1986). The deletion of a dominant gain of function mutation (such as *Glued*, used in these experiments) will revert the phenotype from mutant to wild-type (eg. Hanson, 1928). The applicability of dominant reversion screens has been increased by the use of transformed stocks carrying the wild-type alleles of recessive loci at new locations within the genome. For example, an integrated P element carrying the *rosy*$^+$ allele can be considered a dominant mutation when in a *rosy* background. A stock of this type could therefore be mutagenised and screened for the appearance of the *rosy* phenotype.

Deletions are usually detected among the F_1 progeny of parents subjected to X-ray or P element mutagenesis procedures. There is no recombination in male *D. melanogaster* and therefore, if the putative deletion is recovered in a male, the individual male is mated to several virgin females from a balancer stock. A stock is then set up

from virgin female and male offspring from this cross which carry the putative deletion over the balancer chromosome. The stock can then be screened for the mutant phenotype.

If the putative deletion is recovered in a female, recombination makes it a little more difficult to follow the deletion-bearing chromosome in subsequent generations, because a crossing-over event could separate the deletion from visible markers. To overcome this problem, several lines are usually set up from each putative deletion and each line subsequently tested for the presence of the deletion. Females with the deletion are mated to balancer males; the male offspring of this cross carrying the putative deletion over the balancer are mated separately to balancer females to establish balanced lines which can then be screened separately for the mutant phenotype. Deletions may also be detected by their failure to complement another deletion or deficiency.

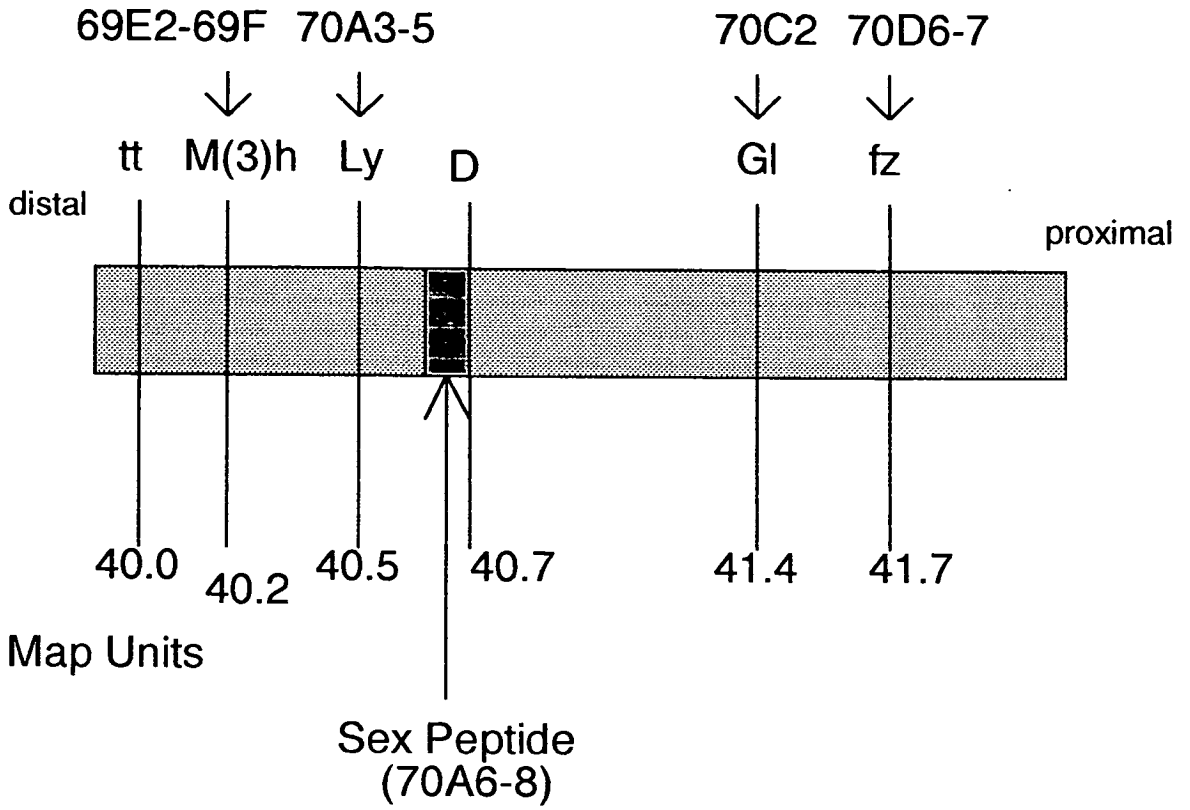
A previous screen for SP-null mutations using primary P element mutagenesis proved unsuccessful (Dominik Styger and Eric Kubli, pers. comm.). Two stocks were available (P389 and P1052) however, with a P element in the 70AB region of chromosome III. Imprecise excision of the P element from these stocks, using X-rays or jumping it out using transposase activity, could cause a deletion in the nearby SP gene. Stocks P389 and P1052 were homozygous for *rosy* (*ry*); the P elements were marked with *rosy*$^+$ (*ry*$^+$). The transposase method excised the P element from stocks P389 and P1052 by crossing them to a stock carrying a defective P element. The defective P element, which has a Δ2-3 deletion, cannot itself be excised, but produces high levels of the transposase enzyme causing the excision of normal P elements in flies to which it is crossed. Loss of the P element from these stocks was visible by the appearance of the *ry* phenotype. The second approach used a classical X-ray technique to induce a deletion in the 70AB region, visible by the reversion of a dominant phenotype (*Glued*, *Gl*) in the nearby region 70C.

For simplicity, a biological test was chosen to screen the large number of lines generated by the mutagenesis experiments; the alternative was to perform Western blots on hundreds of lines. The test described below was used as an initial step to separate out SP-null candidate lines from a large number of lines. Accessory glands from candidate lines were then dissected and Western blots performed to detect lines in which the SP was not produced.

Transfer of the SP from males to females at mating causes females to increase their rate of egg laying and become unreceptive to courting males for 1-2 days. A mated female will not normally mate again for a period of about 5-7 days; prolonged unreceptivity of the female seems to be due to the transfer of sperm, the 'sperm effect' (Manning, 1967), or some other consequence of mating. Females mated to SP-null mutant males that produce no SP might be expected to remate much sooner than normally mated females because they would not show behaviour associated with the action of the SP, ie. rejection of males. The rejection behaviour of females mated to SP^- null males would therefore be expected to be higher than that of females mated to SP^+ males on the second day after the mating took place.

Fig. 7. Sex Peptide Chromosomal Location

Cytological Region:



Visible mutations in the cytological region from 69C (eyg) to 70D6-7 (fz):

Mutation	Map Unit	Name	Description
eyg	37.5	eye gone	eyes and head small
pyd	39	polychaetoid	extra bristles.
tt	40	tilt	wings spread, warped, L3 interrupted.
Ly	40.5	Lyra	wing margins exised, homozygous lethal
D	40.7	Dichaete	wings spread, alulae missing.
Gl	41.4	Glued	eyes small and oblong, shiny.
fz	41.7	frizzled	hairs and bristles directed irregularily, eyes rough.

7.2 General Methods

Aims: To produce a deletion in the 70AB region of chromosome III. Two approaches:

1) Deletion of P(ry^+) elements in the 70AB region of chromosome III, two approaches:

   (a) Imprecise excision of the P element in 2 stocks (P389 and P1052) carrying P (ry^+) in the 70AB region of chromosome III using X-rays.

   (b) Excision of the P element from the same 2 stocks using the transposase activity of a defective Δ 2-3 P element, to generate an imprecise excision deleting all or part of the SP gene.

2) Deficiencies produced by X-ray treatment to revert a dominant mutation (*Glued*) from stock 61400 (*Gl Sb/In(3L)P*) in region 70C, with associated deletion in the SP gene.

7.2.1 Stocks

Name	Genotype	Used for
Or-R	wild-type	copulation assays, biological screen
E42	$\frac{ftz^{6H3h}\ ry^{506}\ e^{5}}{TM3,\ Sb\ ry^{rk}}$	jump out experiment
E65	$\frac{ry^{506}(ry^{+}\Delta 2\text{-}3)}{ry^{506}(ry^{+}\Delta 2\text{-}3)}$	transposase activity of defective Δ2-3 P element used to jump out P (ry^{+}) constructs from 70AB in jump out experiment
P1052	$\frac{P(ry^{+}),\ ry^{506}}{P(ry^{+}),\ ry^{506}}$	stock has P element in 70AB region, used in jump out and X-ray experiments to produce a deletion by loss of ry^{+}
P389	$\frac{P(ry^{+}),\ ry^{506}}{P(ry^{+}),\ ry^{506}}$	second of the stocks with a P element in 70AB. Used in X-ray and jump out experiments to produce a deletion in this region by loss of ry^{+}
3-7	$\frac{Ki\ ry^{506}}{Ki\ ry^{506}}$	excision of ry^{+} by X-ray experiment
Df Ly	$\frac{Df(3L)Ly}{TM3,\ Ser\ or\ TM3,\ Sb\ Ser}$	analysis of generated deficiencies, establishing stocks.

fz/TM1	$\frac{fz}{TM1}$	analysis of generated deficiencies
pyd	*pyd/pyd*	analysis of generated deficiencies
61400	$\frac{Gl\ Sb}{In(3L)P}$	reversion of *Gl* phenotype and analysis of deficiencies
X4	$\frac{X4}{TM3,\ Ser}$	X4 is a dominant purple eye colour mutation. Used in *Gl* reversion experiment as a dominant marker
fz^{Glb3b}/ *TM3a, Sb*	$\frac{fz^{Glb3b}}{TM3a,\ Sb}$	has a large deletion from cytological region 69D-70D, used for analysis of deficiencies.

7.2.2 Biological Screen for SP-null Candidates

The biological test that was used to screen large numbers of SP-null candidate lines was as follows: 4 day-old virgin Oregon-R (Or-R) females were mated to SP-null candidate males, control base stock males (the stock from which the candidates were generated, either P1052, P389 or 61400 males) or Or-R control males. To mate flies, 1 male and 1 female were placed together in a vial and observed until mating took place; males were then removed. Mated females were then collected and stored 10 per vial until the copulation assay 2 days later. It was not always possible to collect 10 males from each candidate line, or to get 10 males to mate with Or-R virgin females; in these cases, smaller sample sizes were tested and more males collected from the line to retest.

Two days after mating, all females were challenged with Or-R males in a copulation assay: Or-R males were placed 6 per vial the morning or day before the test. At the start of the assay, 3 mated females (where possible) were introduced into the vials by aspiration. The number of copulations within the following hour was then recorded. Lines in which more than 75% of the females remated were investigated further by Western blotting, to determine the presence or absence of SP in the male accessory glands.

7.2.3 Detection of SP-null Males by Western Blot

Up to 8 pairs of accessory glands (if available) were dissected from SP-null candidates (as determined by the biological screen) directly into SDS-PAGE gel (sodium dodecyl sulphate polyacrylimide gel electrophoresis) loading buffer. Glands were heated at 80°C for 10 min and then centrifuged. Extracts were loaded onto a 17% SDS-gel and run for half an hour. The proteins separated by the SDS-PAGE were then transferred onto nitrocellulose membranes, and blocked for 1 hour with non specific serum. The membranes were washed 5 times with 1% phosphate buffered saline (PBS) and incubated in SP primary antibody solution (obtained from rabbits) overnight at 4°C. The next day, the membranes were washed 5 times in 1% PBS, and incubated for 2 hours at room temperature with goat anti-rabbit secondary antibody (coupled to alkaline phosphatase enzyme). The membranes were then washed 5 times in 1% PBS and phosphate substrate added to visualise the location of the SP antibody binding. The membranes took approximately half an hour to develop at room temperature in the dark.

7.3 Mutagenesis Methods

In each of the crosses described below, females are always listed first.

7.3.1 Deletion of P(ry^+) Constructs in the 70AB Region of Chromosome III

7.3.1.1 Excision of *ry+* by X-ray - Crossing Program

Stocks with P(ry^+) in cytological region 70AB of chromosome III = P1052 and P389

Males from these 2 stocks were separated from females after eclosion and irradiated (4000 rads, r) at approximately 3 days old. Irradiated males were replaced in the crosses every 3 days to ensure a high proportion of irradiated sperm in matings with the 3-7 virgin females. Offspring were screened for loss of ry^+, and the number of flies screened was recorded.

Ki ry^{506} / *Ki* ry^{506} X *P(ry+),* ry^{506} / *P(ry+),* ry^{506}

(3-7 stock virgin females) 4000r

(P-), ry^{506}*/Ki* ry^{506} males and females from this cross were collected and crossed according to the scheme shown below, (P-) = loss of ry^+ from P element-carrying stock.

(i) If *(P-), ry506/Ki ry506* was a male:

Df (3L)Ly / TM3, Ser X *(P-),* ry^{506} / *Ki* ry^{506}

(P-),ry^{506}*/TM3, Ser* virgin females and male offspring from the above cross were collected and crossed *inter se* to set up stocks; there is no recombination in male *Drosophila* and therefore males which had lost the P element from the 70AB region of chromosome III were candidates for having an SP gene deletion. Males from

homozygous viable stocks (*(P-), ry^{506}/(P-), ry^{506}*) were tested using the biological assay. Males from lines that were not homozygous viable were crossed to *fz^{GLb3b}/TM3a* virgin females; this stock has a large deletion from 69D-70D which should include the region of the SP, if the mapping is correct. Therefore, if the deletion generated by the X rays did knock out the SP, *(P-)/fz^{GLb3b}* males should be SP-null. Lines that were not homozygous viable could also be crossed together to obtain overlapping deletions. Homozygous males from such lines could also be tested using the biological assay.

(ii) If *(P-), ry506/Ki ry506* was a virgin female:

(P-), ry^{506} / Ki ry^{506} X *Df (3L)Ly / TM3, Ser*

There is recombination in female *D. melanogaster* and therefore, to circumvent the problem of a recombination event separating markers, *(P-), ry^{506}/Ki ry^{506}* females were crossed to males carrying a 3rd chromosome balancer, as shown above, and separate lines established from 10 of the male progeny carrying the putative deletion. 10 *(P-), ry^{506}/TM3, Ser* males (if possible) were collected from this cross and mated separately to *Df Ly/TM3, Ser* virgin females; each of these lines was an SP-null candidate (in some of these male lines recombination would not have occurred between the putative deletion and the marker). The lines that were lethal over *Df Ly* were kept because they were better candidates than those which were viable, since the putative deletion extended distally from the 70 AB region and overlapped with *Df Ly* (see Fig. 7). This deletion would therefore probably include the SP gene. Homozygous male flies from these stocks were candidates to be tested. Homozygous lethal males could also be crossed to *fz^{Glb3b}/TM3a, Sb* virgin females (this stock contains a large deletion from 69D to 70D which should include the SP). *(P-), ry^{506}/fz^{Glb3b}* males are SP-null candidates and could be tested using the biological assay.

(iii) If *(P-), ry506/Ki ry506* was a non-virgin female:

(P-), ry506 / Ki ry506 X *ftz ry506 e / TM3, Sb ryrk* (E42)

If the putative deletion was recovered in a non-virgin female, isolating the deletion-bearing chromosome opposite a balancer was complicated by the fact that, not only is there recombination in female *D. melanogaster*, but also the female might already be carrying sperm from another male. This problem was overcome by crossing females to E42 males, as shown above. This allowed male progeny carrying the putative deletion opposite the *ry506 ftz e* chromosome to be distinguished from all the other possible types of progeny, even bearing in mind all the genotypes of the sperm that the female might possibly be carrying. 10 *(P-), ry506/ry506 ftz e* males (if possible) were collected from this cross and mated separately to *Df Ly/TM3, Sb Ser* virgin females, to set up SP-null candidate lines:

Df Ly / TM3, Sb Ser X *(P-), ry506 / ry506 ftz e*

(P-), ry506/TM3, Sb Ser virgin females and males were selected from the progeny and crossed *inter se*. Lines were also tested for viability over *Df Ly*; those which were not viable opposite *Df Ly* were better SP-null candidates than those which were viable, for the reason explained in the previous section. Homozygous male flies from these stocks were candidates to be tested.

7.3.1.2 Jump out P(*ry*+) by Transposase Activity - Crossing Program

The P1052 and P389 stocks with P(ry^+) in the 70 AB region of chromosome III were crossed to the stock carrying a defective P element (Δ2-3 deletion), to excise the P element:

P(ry+), ry506 / P(ry+), ry506 X *ry506(ry+ Δ2-3) / ry506(ry+ Δ2-3)* (E65)

Male offspring were collected and crossed to E42 virgin females, to balance the putative deletion on the 3rd chromosome:

ftz ry^{506} *e / TM3, Sb* ry^{rk} X *P(*ry^{+}*),* ry^{506} */ (*ry^{+} *Δ2-3),* ry^{506}

(E 42 virgin females)

Because there is no crossing over in male *D. melanogaster*, both types of *ry* male offspring (those with the putative deletion opposite the balancer *TM3, Sb* ry^{rk} and those with the putative deletion opposite *ftz* ry^{506} *e*) from the above cross could be used to make SP-null candidate stocks. The two types of *ry* male offspring generated from the above cross were:

(P⁻), ry^{506} */ TM3, Sb* ry^{rk} or *(P⁻),* ry^{506} */ ftz* ry^{506} *e*

Single males of both of the above types were used to set up a total of 1000 SP-null candidate lines by crossing to E42 virgin females to balance the putative deletions opposite the *TM3, Sb* ry^{rk} chromosome:

ftz ry^{506} *e / TM3, Sb* ry^{rk} X *(P⁻),* ry^{506} */ TM3,Sb* ry^{rk}

(E 42 virgin females)

and

E42 virgin females X *(P⁻),* ry^{506} */ ftz* ry^{506} *e*

Sb ry non *e* virgin female and male offspring of these lines (ie. males and females with the putative deletion balanced opposite the *TM3, Sb* ry^{rk} chromosome) were crossed *inter se* to make SP-null candidate stocks: 500 lines were set up (250 from *(P⁻),*ry^{506}*/TM3, Sb* ry^{rk} males, *250* from *(P⁻),* ry^{506}*/ftz* ry^{506} *e* males) from each of P1052 and P389 lines.

(P1052⁻), ry^{506}/TM3 Sb ry^{rk} 500 lines

(P389⁻), ry^{506}/TM3 Sb ry^{rk} 500 lines

Homozygous males from these stocks, *(P⁻), ry^{506}/(P⁻), ry^{506}* were tested as SP-null candidates. Stocks that were not homozygous viable could be crossed together to get overlapping deletions; homozygous males from these crosses could also be tested as SP-null candidates.

7.3.2 Reversion of *Glued* Phenotype by X-ray - Crossing Program

Males were separated at eclosion, kept in single sex groups and irradiated (4000r) at approximately 3 days old. Irradiated males were crossed to virgin *X4/TM3, Ser* females to recover putative deletions in the progeny opposite the *TM3, Ser* balancer, or the *X4* dominant marker. Irradiated males were replaced every 3 days to ensure a high proportion of irradiated sperm in the matings with the *X4/TM3, Ser* females:

X4 / TM3, Ser X *Gl Sb / In(3L)P* (61400 stock)

(virgin females) 4000r

Offspring of this cross were screened for reversion of the *Glued* phenotype, ie. for male and female *Sb* flies that were not *Glued* (*Gl**= *Gl* revertant). The number of flies that were screened was recorded. Revertants could be of 2 genotypes, *Gl* Sb/TM3, Ser* or *Gl* Sb/X4.* Male progeny (in which there was no recombination) were crossed to balancer stocks to stabilise the putative deletion. Female progeny underwent an additional generation of crossing to circumvent the problem of possible recombination of the deletion-bearing chromosome.

(i) If male and *Gl* Sb/TM3*, Ser:

Male offspring of the above cross carrying the putative deletion were mated to *Df Ly/TM3, Ser* virgin females to make SP-null candidate stocks and stabilise the deletion opposite the *TM3, Ser* chromosome:

Df Ly/TM3, Ser X *Gl* Sb/TM3, Ser*

Gl Sb/TM3, Ser* offspring were selected and crossed *inter se* to make SP-null candidate stocks; homozygous males were then tested using the biological assay.

(ii) If male and *Gl* Sb/X4*:

Procedure same as in (i) above.

(iii) If virgin female and *Gl* Sb/X4* (no *Gl* Sb/TM3, Ser* flies were obtained):

Gl Sb/X4* X *TM1/TM3, Ser*

No non-virgin females were obtained, obviating the need for consideration of all the possible genotypes of males with which the females might previously have mated. Because of the possibility of recombination of the deletion-bearing chromosome, it was stabilised by crossing females carrying the *Gl*-reverted chromosome (*Gl**) to males carrying 3rd chromosome balancers (*TM1/TM3, Ser*). 10 non-*Glued*, *Stubble* and *Serrate* male offspring (ie. *Gl* Sb/TM3, Ser*) were selected, and crossed separately to *Df Ly/TM3, Ser* virgin females to obtain SP-null candidate lines. *Gl* Sb/TM3, Ser* virgin females and male progeny from this cross were selected and crossed *inter se* to make stocks. If the resulting stocks were homozygous viable they were discarded, because the deletion was unlikely to have been large enough to extend distally to overlap with the SP gene (see Fig. 7). Homozygous non-viable

stocks could be crossed to other stocks (eg. *fz*^{Glb3b}*/TM3a, Sb*) to obtain overlapping deletions. Males with overlapping deletions could then be tested as SP-null candidates.

7.4 Results

7.4.1 Deletion of P(*ry*+) Constructs from the 70AB Region of Chromosome III

7.4.1.1 Excision of *ry*+ by X-ray

Excision of *ry*$^+$ from P1052 stock:

A total of c.8500 flies were screened for the loss of *ry*$^+$; a total of 9 excisions were obtained. The following lines were set up:

Excision of *ry*$^+$, male lines:

P1052 1M - male was sterile. No stock.

P1052 2M - male fertile, stock established which was homozygous lethal. Males from this line were crossed to *fz*^{Glb3b}*/TM3a* virgin females and males of the genotype *(P-)/fz*^{Glb3b} selected for testing with the copulation assay. Oregon-R (Or-R) females mated to these males did not remate in the copulation assay (0 out of 8 in first test, 0 out of 9 in the second). Not SP-null.

P1052 3M - not a complete loss of *ry*$^+$, eyes looked half *ry*$^+$ and half *ry*, and male was sterile. No stock obtained.

P1052 4M - male fertile, stock established; seemed to be homozygous lethal. Stock not otherwise characterised.

P1052 5M - not a complete loss of ry^+, eyes looked half ry^+ and half ry; stock established but not otherwise characterised.

P1052 6M - male fertile, stock established; seemed to be homozygous lethal, not otherwise characterised.

All these male lines were viable over *Df Ly*, the deletions produced by X-rays therefore did not extend distally from 70AB to overlap with *Df Ly*. The deletions were therefore unlikely to have overlapped with the SP gene and were poor SP-null candidates.

Excision of ry^+, female lines:

P1052 1F - Female died, no stock.

P1052 2F - Female fertile but not virgin. 3 lines were established from single male *(P-)*, ry^{506}/ry^{506} *ftz e* progeny. All lines were viable over *Df Ly* and homozygous lethal, not tested further yet.

P1052 3F - Female fertile and virgin, 3 lines from single *(P-)/TM3, Ser* males were established, 1 male was infertile. Other 2 lines seemed to be homozygous lethal and were viable over *Df Ly*, not tested further yet.

Excision of ry^+ from P389 stock:

A total of c. 6300 flies were screened for loss of ry^+ and a total of 8 excisions were obtained. The following lines were set up:

Excision of ry^+, male lines:

P389 1M - male fertile, stock was established which was homozygous viable and fertile. *(P-)*, ry^{506}*/(P-)*, ry^{506} males tested in the biological assay; Or-R

females mated to these males did not remate in the copulation assay (0 out of 5, 1 out of 2 and 0 out of 4 in the 3 tests performed). Males from this stock were also crossed to *fz^{Glb3b}/TM3a* virgin females to obtain *(P^{-})/fz^{Glb3b}* males; Or-R females mated to these males did not remate in the copulation assay (n=4), the stock was not SP-null.

Excision of *ry*$^{+}$, female lines:

P389 1F - female fertile but only 3 progeny emerged, not of correct phenotype to proceed. No stock.

P389 2F - female died before initial cross. No stock.

P389 3F - virgin female, fertile but not enough progeny of correct phenotype emerged to proceed. No stock.

P389 4F - female fertile but not enough progeny of correct phenotype to proceed. No stock.

P389 5F - female died before initial cross. No stock.

P389 6F - female fertile but not enough progeny of correct phenotype to proceed. No stock.

P389 7F - female fertile but not enough progeny to proceed. No stock.

7.4.1.2 Jump out of P(*ry*+) from 70AB

The frequency of P element jump out was 40.2% for the P389 stock and 29.7% for the P1052 stock. The following lines were set up:

P1052 jump outs:

P1052- 1 to 250 (from *P1052^{-}/ftz ry e* males)

P1052- 1Sb to 250Sb (from *P1052⁻/TM3, Sb ry^{rk}* males)

P389 jump outs:

P389- 1 to 270 (from *P389⁻/ftz ry e* males)

P389- 1Sb to 230Sb (from *P389⁻/TM3, Sb ry^{rk}* males)

Considering all 1000 lines together, approximately 640 were fertile and approx. 480 were estimated to be homozygous viable (although not all have been characterised yet). Homozygous males, ie *(P⁻)/(P⁻)* were collected and tested using the biological screen. Out of the first 100 homozygous viable stocks tested, 14 gave a positive result in the copulation assay, ie. 75% or more of Or-R females mated to males from these stocks remated in the copulation assay. Western blots were performed on the following stocks which gave favourable results in the copulation assays with Or-R, P389 and P1052 males as controls:

57 P1052, 46 P1052, 23 P389, 46Sb P1052, 41Sb P1052, 19Sb P1052, 55Sb P1052, 65 P1052, 6 P1052, 3Sb P1052, 52Sb P1052, 63Sb P1052, 32 P389

There was no detectable SP band for 3 stocks:

46 P1052, 46Sb P1052 and 19Sb P1052

7.4.2 Reversion of *Gl* Phenotype by X-ray

Approximately 24 400 flies were screened and a total of 9 reversions obtained. The following lines were set up:

***Gl* reversion male lines:**

Gl rev 1M - line from *Gl* Sb/TM3, Ser* male, but sterile. No stock.

Gl rev 2M - line generated from *Gl* Sb/X4* male. Progeny collected but not enough of the correct phenotype to proceed. No stock.

Gl rev 3M - line from *Gl* Sb/X4* male but sterile. No stock.

Gl rev 4M - line generated from *Gl* Sb/X4* male. Progeny collected and stock set up. *Gl*Sb/TM3, Ser* stock seemed to be homozygous viable, stock not characterised further.

_Gl_ reversion female lines:

(all lines generated from *Gl* Sb/X4* females)

Gl rev 1F - female fertile but not enough male progeny of correct phenotype to proceed. No stock.

Gl rev 2F - female fertile and 9 lines set up from male progeny, lines not characterised further.

Gl rev 3F - female sterile. No stock.

Gl rev 4F - female sterile. No stock.

Gl rev 5F - female fertile but not enough progeny of the correct type to proceed. No stock.

7.5 Discussion

Three stocks generated from the jump out mutagenesis experiment (46 P1052, 46Sb P1052 and 19Sb P1052) had no detectable SP in their accessory glands by Western analysis. The 46 P1052 and 46Sb P1052 lines were homozygous sterile and the 19Sb P1052 line fertile. Further analysis of the 3 SP-null candidate lines by Southern analysis showed that the SP gene was still present (Eric Kubli and Irene Fleischmann,

pers. comm.). In Southern analysis, DNA restriction fragments are separated on a gel and transferred onto a nitrocellulose membrane. A radioactive probe is then used to visualise the location of the gene of interest by autoradiography (Klug and Cummings, 1986). The SP-null candidate lines were crossed to obtain overlapping deletions; the resulting transheterozygotes did not seem to be SP-null on the basis of a biological assay (females mated to the transheterozygotes showed effective rejection responses for at least 2 days, Helbling, Heller, Tassini and Rohaly, unpublished data). The lines were not expected to have been able to complement each other to produce a functional SP product when crossed. For both reasons there is some doubt as to whether the 3 lines were truly SP-null.

Overlapping deletions could have produced a functional SP product through intragenic complementation; this arises when different parts of a functional gene product are produced by different chromosomal strands, and are able to complement each other in a way that restores the activity of the gene product. However, it seems unlikely that intragenic complementation could have produced a functional SP in all three of the transheterozygote lines unless the SP acts as a dimer (see Introduction to Chapter 6); this possibility merits further investigation. Another possibility is that the overlapping deletions allowed small, biologically active fragments of the SP to be produced which were able to bind to different types of SP receptors and thus induce the response. However, the sharpness of the SP dose response curve (Chapter 2, Fig. 2.2) suggests that there is only 1 SP target and if small, biologically active fragments had been produced by the original SP-null candidate lines, they would not have turned up as candidates in the first place. Since the SP gene is still present in all 3 SP-null candidate lines it seems unlikely that they are truly SP-null, although Southern analysis is unlikely to have been able to resolve a very small, or point deletion. Further characterisation of these lines is needed.

Western blots could be repeated on the SP-null candidate lines to check whether the SP is really absent from accessory glands. Females mated to males from the 3 SP-null candidate lines did not show normal post-mating rejection responses towards males. If the lines turn out to be SP^+, it would suggest that some other aspect of mating with males from these stocks was not sufficient to induce female rejection responses of normal duration. The absence of the receptivity-inhibiting 'sperm effect' (Manning, 1962, 1967) cannot be the only explanation, as one of the SP-null candidate lines (19Sb P1052) was fertile and therefore did produce sperm. There were also other stocks in which females remated within 2 days but were not SP-null (ie. candidates which did give a SP signal on the Westerns): it would be interesting to investigate further these contradictory results. There are a large number of other stocks still to be analysed from the jump-out experiment and it is possible that SP-null mutants could be found within these lines.

The results of the X-ray experiments were less successful. Of the few candidate lines which were tested, none were SP-null; many of the progeny of the irradiated males were sterile, possibly as a result of secondary effects of the X-ray procedure. It was therefore impossible to establish stocks from all of the progeny which might have carried a deletion. The P element mutagenesis method had the advantage that it did not cause deleterious secondary effects, as seemed to occur with the X-ray mutagenesis procedure.

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Appendix A

Chapman, T., S. Trevitt and L. Partridge, submitted

REMATING AND MALE-DERIVED NUTRIENTS IN *DROSOPHILA MELANOGASTER*

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Running head: REMATING IN *DROSOPHILA*

Abstract.-Laboratory natural selection and environmental manipulations were used to investigate the importance of male-derived nutrients to female *Drosophila melanogaster.* No evidence for the importance of such nutrients was found. Females from the same wild type base stock exposed as adults to low quality food did not show elevated fecundity or survival when they remated more frequently, and on high quality food the females showed a 'cost of mating' in reduced survival. Laboratory evolution on low quality food did not lead to elevated rates of remating by females; females from each selection regimen remated more frequently when kept on the food type to which they had been exposed for the previous 5 years, on which they also showed higher fecundity. Even under conditions of extreme nutritional stress, when females were exposed to a short term (4-day) cycle of exposure to very low and high quality food, they remated more frequently immediately after exposure to high quality food. The results of this last experiment suggested that, under these circumstances, current nutrition, fecundity or rate of sperm usage was more important than number of sperm in store or cumulative fecundity in determining the probability that a female would remate.

A large number of evolutionary explanations for remating by females has been suggested. Remating may renew or replenish dwindling sperm supplies (Anderson, 1974; Gromko and Pyle, 1978; Gromko *et al.*, 1984; Newport and Gromko, 1984). It could also be an insurance against male-sterility and sub-fertility (Gibson and Jewell, 1982). Females may gain nutrients at mating (eg. Pitnick *et al.*, 1991) and there may sometimes be benefits from increased genetic diversity among offspring (Williams, 1975). Multiple mating could also promote competition among ejaculates of different males (Parker, 1970).

Remating by females could also occur as a result of conflict with males. For instance, harassment of females by males during courtship could prevent them from feeding (Alcock *et al.*, 1977) or make them conspicuous to predators. Female remating could also evolve as a correlated response to sexual selection on males, if the remating rates of the two sexes are genetically correlated (Halliday and Arnold, 1987; but see Sherman and Westneat, 1988; Arnold and Halliday, 1988; Cheng and Siegel, 1990; Gromko, 1992; Arnold and Halliday, 1992; Stamenkovic-Radak *et al.*, 1992). In some insects, remating is costly to females, because it lowers their lifetime reproductive success by shortening their lifespan (eg. Arnqvist, 1989; Fowler and Partridge, 1989).

Among animals there is considerable variation in the extent to which females remate (Halliday and Arnold, 1987). This diversity can be seen among the members of the Genus *Drosophila*, where *D. pachea* has been reported to remate several times daily (Pitnick *et al.*, 1991) while *D. subobscura* from northern populations do not do so in the course of a lifespan that can last several weeks (Maynard Smith, 1956; Steele, 1984). High and variable rates of multiple insemination in field populations have been reported for *D. melanogaster* (47% estimated by Milkman and Zeitler, 1974; 12-65% by Marks *et al.*, 1988) and *Drosophila pseudoobscura* (>50% estimated by Anderson, 1974; 30-60% by Cobbs, 1977; 60-100% by Levine *et al.*, 1980). Multiple insemination in the field has also been reported as >23% for *D. subobscura* (Loukas *et*

al.,1981), >35% for *D. eronotus* (Stalker, 1976,) and 83% for *D. athabasca* (Gromko *et al.*, 1980). It is not clear if the intraspecific variation in remating rates is mainly genetic or environmental in origin; ecological variables can be important (Marks *et al.*, 1988).

Remating rate is known to be heritable in *D. melanogaster* (Gromko and Newport, 1988) and would therefore be expected to be responsive to natural selection. Identifying the functions of repeated mating is of interest, because it may explain the wide variation in remating frequency that is observed both between different species and between different populations of the same species. The aim of this study was to assess the importance of seminal feeding in *Drosophila melanogaster*. Amino acids from the seminal fluid enter the female haemolymph and yolk proteins (Bownes and Partridge, 1987), but it is not clear if these or any other seminal components contribute significantly to female survival, fecundity or fertility. Male-derived contributions to the female are more likely to be important for her fecundity or survival when nutrients are scarce (Turner and Anderson, 1983). The opportunity to remate would then be expected to increase female survival or reproductive output more under conditions of nutritional stress. The effects of remating frequency on fecundity and survival of *D. melanogaster* females under different feeding regimens has not been investigated. Turner and Anderson (1983) varied the amount of yeast supplied to female *D. pseudoobscura*, and compared the lifetime production of adult offspring in females either exposed briefly to males at the beginning of their adult lives, or given continuous access to males. Access to males increased productivity of both group of females, but the proportional increase was much greater for the females kept without yeast. The results could have been explained by nutrient transfer at mating, or by higher death rates of stored sperm in the poorly fed females. Male *D. pseudoobscura* have since been found to transfer nutrients to females, both through courtship feeding (Steele,

1986) and through the ejaculate (Bownes and Partridge, 1987; Markow and Ankney, 1988).

We used three approaches to investigate the importance of seminal feeding in *D. melanogaster*:

1) We compared the remating rates, survival and fertility of females that had an evolutionary history of exposure to poor and good quality food. We reasoned that if seminal feeding is important, then females persistently exposed to poor nutrition might be expected to evolve higher remating rates, leading to either higher fecundity or higher survival rates under these conditions.

2) We measured the remating rates, survival, fecundity and fertility of wild-type females with low or high opportunities for remating when they were exposed to poor or good quality food. We reasoned that, if the male nutritional contribution is important, females in poor nutritional condition would benefit more in their fecundity or survival from extra matings.

3) We examined the effects of short-term variation in nutritional level of females (exposing them to a 4 day cycle of exposure to very poor and good quality food) on rates of re-mating, egg-production and survival. We reasoned that females under short-term extreme nutritional stress might be expected to increase their remating probability to gain male-derived nutrients.

GENERAL METHODS

The Dahomey wild-type stock was collected in Dahomey, West Africa (now Benin) in 1970; the Brighton wild-type stock was collected in Brighton, UK in 1984. Both have been maintained since collection in mass culture in population cages. A stock homozygous for the mutant *fruitless* was used to provide males that courted but would not mate with females (Hall, 1978). Cultures and experimental flies were kept at 25^{o}C

on a 12h light-12h dark cycle. All anaesthesia was under carbon dioxide administered not less than 3h after eclosion. All experimental females were housed individually in vials (75mm depth, 25mm diameter) containing 7ml of food medium.

These experiments involved measurement of female remating rates. There is some controversy about the best way to measure these in the laboratory; different experimental designs (eg. continuous versus intermittent confinement of the sexes) can have a large effect on remating frequency (Newport and Gromko, 1984). The evolutionary history of all the stocks used in this study was one of continuous exposure to males in cage culture. We therefore used continuous exposure of females to males as the highest opportunity for remating in the experiments. Remating rates tend to be higher using this type of experimental design than one involving intermittent exposure (Newport and Gromko, 1984, Fowler and Partridge, 1989). Continuous exposure of the sexes and high rates of remating may be representative of the natural situation, where adults can occur on food resources at continuously high local densities (Johnston and Heed 1975, 1976); remating rates under these conditions can be very high (Partridge *et al.*, 1987). The reports of high multiple insemination rates in field populations of *D. melanogaster* (eg. Milkman and Zeitler, 1974; Gromko *et al.*, 1980; Marks *et al.*, 1988) also support the idea that remating may be frequent in nature.

EFFECTS OF LABORATORY NATURAL SELECTION ON HIGH AND LOW QUALITY FOOD.

Methods.-The evolutionary responses of remating, egg-production, and survival to level of nutrition were investigated by long-term culture of the same base stock on medium of two different concentrations. The aims were to examine whether remating

rate was higher in lines with a history of low nutrition, and whether any increase in remating rate was associated with increased female survival or fecundity, as would be predicted if seminal feeding were important.

The 'high' and 'low' selection regimens were set up in 1985 using flies from the Brighton base stock, and three replicate population cage cultures were kept in each selection regimen using sugar-free food media described by David *et al.*, (1971). Each 'high' line cage was supplied with 3 bottles per week of 'high' food medium (16% w/v flaked yeast and maize meal) and each 'low' line cage with 4 bottles per week of 'low' food medium (2% w/v flaked yeast and maize meal); the bottles were left in the cages for 4 weeks which was sufficient for all adults to emerge before the bottles were removed. These lines had been in existence for 5 years at the time the experiments on them were carried out.

To control for any maternal effects, the parents of the experimental flies were all reared at standard larval density on food intermediate in concentration to the high and low media. To eliminate the problem of inbreeding depression through homozygosity of deleterious recessive alleles in the selected lines, hybrids between the lines within a selection regimen were used. Additive genetic effects in the selected lines would still have been present in the hybrids. Parental flies from replicate lines of the same selection regimen were crossed, males of replicate 1 with females of replicate 2, males of 2 with females of 3 and males of 3 with females of 1, and eggs from the crosses were collected on grape juice medium (Fowler and Partridge, 1986). Newly hatched F_1 first instar larvae from all crosses were then collected and placed at a density of 40/vial on low food and 150/vial on high food; larvae from each regimen were grown up on both types of food. Virgin females were then collected under anaesthesia and aged in single-sex groups of 20/vial for 2 days.

To measure remating rate, reproductive performance and survival of females, 20 F_1 females from each cross were collected as virgins, and placed individually in vials of food of the same type as that on which they had been reared, together with 2 3-day-old wild type Brighton males. Remating frequency was sampled 4 times per week by scanning along vials every 20 mins for 3 hours starting 1h before lights on. Female deaths were recorded daily. Females were transferred to fresh food vials every 3 days and the males were renewed then. The vacated vials were retained to count adult progeny emerging. The 'low' food vials were seeded with active yeast, to provide a sufficient food supply to ensure high (>90%) and constant survival rates for the larvae.

Results.- To calculate remating rates, each day of observation was taken as one opportunity for mating to occur, and for each female each sampling day was scored as an opportunity 'taken' or 'not taken'. Because mating was infrequently observed, and because there were no significant differences in mating frequency between them, the data from the 3 replicate lines from each selection regimen were combined for the first 4 remating samples, when most of the females were still alive (Table 1). Each group had the potential for producing 240 sampling points (20 females*3 replicates*4 samples); some of the females died during this period, so the the number of sampling points was slightly less than 240. Females from both selection regimens kept on 'high' food remated more frequently than those kept on 'low' food. Chi-squared analysis showed that on 'high' food the 'high' line females remated significantly (χ^2 = 4.29, df 1, $p<0.05$) more often, whereas on 'low' food the 'low' line females remated significantly more frequently (χ^2 = 9.87, df 1, $p<0.01$).

There were no significant differences in lifespan between replicates ($p>0.05$). The survival data from the replicate lines were combined and survival curves plotted (Fig. 1). For statistical analysis a non-parametric distribution-free method (Mantel-Cox

test) was used. This test generalizes the Log Rank Test (Miller, 1981). It cumulates, for each successive sampling interval (day), both the observed and expected number of deaths for each group. The expected number of deaths is calculated by allocating the number of deaths in each group in each sampling interval in proportion to the number of individuals entering that sampling interval. The total observed and expected deaths are used to generate a chi-square value. Females from the 'high' selection regimen kept on 'high' food (median lifespan=18d) lived significantly ($p<0.012$) longer than females from the other 3 groups, between which there were no significant differences, (median lifespan of 'low' lines on 'low' food=16d, 'low' lines on 'high' food=14d and 'high' lines on 'low' food=17d).

The results for age-specific progeny production (Fig. 2) showed a marked tendency for females kept on 'low' food to produce fewer progeny than those on 'high' food, with no overlap in the egg counts from the two environments. Within an environment, females from the two selection regimens were compared using a nested analysis of variance, with replicate lines nested within selection regimen. There was a tendency throughout for the 'high' line females to produce more progeny on 'high' food, significant ($p<0.05$) in intervals 1, 3 and 6 and marginally non-significant ($p=0.057$) in interval 2, and there was also a tendency towards the opposite effect on low food, with 'low' females producing significantly more progeny in intervals 2 and 3.

The progeny produced in each sampling interval by each female were cumulated to give a measure of lifetime progeny production (Table 2). Nested analysis of variance on the data from 'high' food (Table 3a) showed that females from the 'high' lines produced significantly more progeny, while on the 'low' food (Table 3b) the opposite effect occurred.

EFFECT OF MALES ON LIFETIME REPRODUCTIVE SUCCESS OF WELL AND POORLY FED FEMALES

Methods.-The aim of this experiment was to investigate how level of nutrition interacted with the availability of males in determining female remating rate, longevity, fecundity and fertility.

Dahomey wild-type flies were reared on Lewis medium (6.2g agar, 85.2g sugar, 94.2g maize and 17g flaked yeast per 1000ml water) under relaxed larval competition, collected under anaesthesia and aged for 3 days in mixed-sex groups of 5 males and 5 females per vial. Adult females in this experiment then had their nutrition and exposure to males manipulated; females were divided into 4 experimental groups of 40 females, and exposed to 1 of the 4 possible combinations of high and low nutrition and high and low availability of males. Nutrition was manipulated by keeping 2 groups each on the 16% w/v ('high') and 2% w/v ('low') media of David (1971) as used in the previous experiment. Remating opportunity was varied by keeping 2 groups each exposed continuously to 2 wild type males, or to 2 wild type males for 1 day in 3 and to 2 *fruitless* males for the other 2 days. The *fruitless* males were present as a control for non-mating effects of exposure to males on female survival or fertility (Partridge and Fowler, 1990). New 2-3 day old wild type and *fruitless* males were provided every 3 days. Fresh vials were provided every 1- and 2-days alternately, coinciding with the introduction and removal of the *fruitless* males in the 2 experimental groups that were exposed to them. All fly transfers were by mouth aspiration.

Remating frequency was recorded on the days when all females were exposed to wild-type males by scanning the vials every 30 min for 2-3h after lights on and recording the number of matings that were seen. Female deaths were recorded daily,

and egg-counts were made every third day in the vials in which all females had been exposed to wild-type males. These vials were retained to count the emerging progeny. Low food vials were seeded with active yeast after the egg-counts.

Since the number of rematings observed was very low, a short repeat experiment was performed in order to increase the sample size of the remating data. 3-day old inseminated Dahomey females were exposed to one of the 4 combinations of high and low food and high and low availability of males as before. Remating frequency was scored every day for 5 days by scanning vials every 20mins for 6h, starting 3h before lights on.

Results.- The number of matings observed in the main experiment was low. The data on remating rates up to day 9 (Table 4a), when female deaths began to occur at high rates, were analysed by calculating Fisher exact probabilities. There were no significant differences within a food regime in remating rates on days when all females were exposed to intact wild type males ('high' food p=0.33, 'low' food p=0.09). There were more rematings on 'high' food than on 'low' (19 versus 6) but this difference was not significant. Since females continuously exposed to wild type males mated as often as intermittently exposed females on the days when both groups had wild type males, by extrapolation it follows that they probably mated more often in total. Table 4b shows the remating frequencies from the repeat experiment on the 2 days out of 5 when all females were exposed to wild-type males. There were no significant differences in remating frequency within a food regimen ('high' food, Fisher exact p=0.46, 'low' food p=0.055) Females as a whole on 'high' food remated significantly more than females on 'low' food, (p=0.000). Matings were observed in continuously exposed females on the 3 days out of 5 when the other two groups of females were with *fruitless* males (34 matings in the the 'high' food continuously exposed group and 5

matings in the 'low' food continuous group during this period). Therefore females continuously exposed to males did remate more often in total than those which were only intermittently exposed.

The survival curves for the 4 groups of females (Fig. 3) were subjected to a survival analysis. Females exposed to 'high' food survived for significantly ($p<0.001$) longer than females exposed to 'low' food , and on 'high' but not on 'low' food continuous exposure to wild type males significantly ($p<0.001$) reduced female lifespan. The data on egg-production (Fig. 4) were compared using a Kruskal-Wallis test, which showed that females kept on high food produced significantly ($p<0.05$) more eggs than those kept on low food in all sampling intervals, and that continuous exposure to wild-type males had no significant effect on egg-production under either feeding regimen in any sampling interval.

The progeny counts were used to calculate egg hatchability, which was compared for the 4 groups using Kruskal-Wallis tests. There were significant differences only on days 16 and 19, with intermittently exposed females on 'low' food having first significantly ($p<0.05$) lower and then significantly higher hatchabilities than both continuously exposed females on low food and intermittently exposed females on high food; these differences would be expected by chance. The data on egg- and progeny-production were used to generate lifetime scores for each female, and these were compared using t-tests for the 2 groups of females kept on each type of food. For the females on 'high' food, continuously exposed females produced significantly fewer eggs (means continuous 299.5, intermittent 361.9, $t=2.177$, df 71, $p<0.05$) and progeny (means continuous 217.5, intermittent 268.1, $t=2.474$, $p<0.05$) in their lifetimes than did intermittently exposed females. These differences were not significant in females on 'low' food for either egg counts (means continuous 34.5, intermittent 39.1) or progeny counts (means continuous 28.8, intermittent 32.6).

Methods.-The aim of this experiment was to investigate the effects of short-term variation in nutritional level of females on their rates of remating and egg-production. If seminal feeding is important, females should elevate their rates of remating after a short period of exposure to poor quality food to gain male nutrients. The results of this experiment should also provide information on whether remating is related to current or cumulative nutrition, fecundity or sperm usage.

Virgin Dahomey wild type females were collected under anaesthesia and aged for 3 days in single-sex groups of 5. The females were then transferred to individual food vials and combined with 2 males each, by aspiration. During the next 2 hours, all the females were seen to mate, and the males were discarded. The females were then allocated randomly to 2 experimental groups. Both groups underwent a repeating nutritional cycle of 2 days on 'rich' food medium followed by 2 days on 'dilute' food medium, but since they entered the cycle at different points, they experienced it in antiphase. In the first group, 41 females entered the nutritional cycle with 2 days on 'rich' food medium while in the second 41 females entered the cycle with 2 days on 'dilute' food medium. The 'rich' food was a sugar-yeast medium (Trevitt and Partridge, 1991), while the 'dilute' food contained 2/3 the amount of sucrose and no yeast, and hence no source of protein. The females were transferred to fresh food vials every 2 days.

Every 4 days, after all the females had undergone 1 complete cycle, a synchronous mating test was carried out. Two 3-day old wild type males, aged over yeasted 'rich' food, were introduced to each individually-housed female by aspiration, and matings were scored by direct observation during a 90 minute period, after which

all the males were removed. Thus, mating opportunities were always received after 2 days on 'dilute' food in one group and after 2 days on 'rich' food in the other.

Results.- The cumulative percentage of opportunities taken to remate against time from first mating (Fig. 5) suggested that 'rich-dilute' females had a lower remating rate. Females from the two experimental groups were paired randomly and, within each pair, data were taken only up to the time at which the shortest-lived individual of the pair died. This procedure controlled for age- and time-specific changes in remating frequency. Analysis using a 2-tailed sign test showed that the 'dilute-rich' females remated significantly more frequently than the 'rich-dilute' females ($p=0.016$).

There was no significant difference in the survival rates of the two groups of females (Log Rank Test $\chi^2=0.015$, $p>0.5$). The egg-laying rate of the two groups of females (Fig. 6), in which the means and 95% confidence limits for each 2-day period are plotted against time from first mating, showed a large effect of food, with both groups of females laying significantly more eggs when they were on 'rich' food ('rich-dilute' females $t=12.181$, $p<0.001$, df 64, 'dilute-rich' females $t=6.103$, $p<0.001$, df 61). 'Rich-dilute' females laid significantly more eggs during their lifespans than 'dilute-rich' females ($t=4.760$, $p<0.001$, df 79), attributable to significantly higher score on 'rich' food ($t=4.841$, $p<0.001$, df 79) but not on 'dilute' food ($t=0.434$, $p>0.5$, df 79).

DISCUSSION

None of the experiments provided any evidence that remating by females was more beneficial for their fecundity or survival when they were nutritionally stressed. When the nutrition and access to males of wild-type females was manipulated, any

nutritional benefits from mating should have been more apparent on 'low' food, as an increase in the fecundity or survival of continuously exposed females, because they remated approximately 3 times as frequently as intermittently exposed females. The lifespans, egg-production, egg-hatchabilities and lifetime reproductive success of the two groups of females on 'low' food did not differ significantly, so that increased remating was without benefit to females under these circumstances. In the females kept on 'high' food, increased remating was detrimental in that it decreased the lifespan and lifetime reproductive success of the continuously exposed females, as has been reported previously (Fowler and Partridge, 1989; Partridge and Fowler, 1990). The rate of mating was higher in these females than in those kept on 'low' food, which may explain why only they showed the cost of mating.

The females with an evolutionary history of poor nutrition also provided no support for the importance of male-derived nutrients, because their willingness to remate was no greater than that of the females from the 'high' lines, and instead there was evidence of gene-environment interaction; females from each regimen remated most frequently when they were kept on the food on which they had been selected. The data therefore suggested that females from the two selection regimens differed in the factors controlling remating rate, either because they had gained different benefits from remating in their evolutionary past, or because their remating rates were associated with some other evolved difference between them. One such difference was that females from each selection regimen produced more progeny when kept on the food on which they had been selected. Remating rate could then have been a response to fecundity *per se* or to sperm depletion, which is more rapid in females that lay more eggs (Trevitt *et al.*, 1988). The evolutionary adaptation to the two different culture regimes was also apparent in lifetime progeny-production. On 'high' food both the longevity and age-specific fertility of the 'high' line females contributed to their advantage, whereas on 'low' food only the age-specific fertility of the 'low' lines females was significantly

higher. It was not clear how the adaptation to diet was achieved; there were no significant differences in the number of ovarioles of the females from the two selection regimes when they were reared on intermediate food (Chapman, unpublished data).

In all three experiments, females remated more frequently when they were exposed to high quality food, as has been reported previously (Gromko and Gerhart, 1984; Harshman *et al.*, 1988; Trevitt *et al.*, 1988). This finding does not support the importance of seminal feeding. In the experiment on the effects of short-term nutritional cycling, the 'dilute' food contained no source of protein, and a pilot experiment had shown that females kept solely on this food were all dead by day 13 from first mating. The 'rich-dilute' females had been kept on this food for two days prior to their remating test yet, despite being under nutritional stress, they remated significantly less often than the 'dilute-rich' females. The data suggest that females remated primarily to replenish their sperm supplies (Gromko and Pyle, 1978; Gromko *et al*, 1984; Newport and Gromko, 1984), which are used up more rapidly under conditions of good nutrition and high rates of egg-production (Trevitt *et al.*, 1988). The nutrition of the males could also have been a factor, but they were renewed every 3 days and were well fed before entering the experiments, making this explanation unlikely.

The results of the experiments on laboratory natural selection and nutritional cycling throw some light on the proximate mechanisms by which females regulate their remating rate. In the selection experiments, the females from the two selection regimes were compared on both types of food. Under identical nutritional conditions, the females that laid more eggs remated more frequently. This result suggests that nutrition itself did not affect remating frequency, and that either the rate of egg-laying or the number of sperm in store accounted for differences in remating probability. Sperm stores have been previously implicated in this regard (Gromko and Pyle, 1978; Gromko *et al*, 1984; Newport and Gromko, 1984; Letsinger and Gromko, 1985). However, the results of the nutritional cycling experiment suggested that other factors

may also have been important. These females regulated their rate of egg-deposition closely in accordance with their current nutritional status, as has been found previously (Sang and King, 1961; Fairbanks and Burch, 1974; Partridge *et al.*, 1987), and shown to be mediated by changes both in the rate of transcription of the yolk-protein genes and in the rate of uptake of the yolk proteins by the ovary (Bownes, 1989). The 'rich-dilute' females also had higher overall fecundity. Their entry into the nutritional cycle on 'high' food may have resulted in fuller ovarian development (Manning, 1967), conferring a greater capacity for egg-production which was expressed when the females were on 'rich' food. Since high nutrition and fecundity result in higher rates of sperm use (Trevitt *et al.*, 1988), these females presumably depleted their sperm stores more rapidly than did the 'dilute-rich' females. At the time of the remating test, therefore, the 'rich-dilute' females had lower current nutrition, fecundity and sperm stores, and higher cumulative fecundity and they remated less frequently than the 'dilute-rich' females. The differences in remating rate between the groups could therefore not be accounted for by differences in cumulative fecundity or amount of sperm in store, and must instead have been related to differences in current nutrition, rate of egg-laying or rate of sperm use.

The results of these experiments therefore provide no support for the importance to female *D. melanogaster* of male-derived nutrients, and suggest that factors other than sperm currently in store can also affect probability of remating by females.

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Figure Legends

Fig. 1. Cumulative probability of survival against time (days) for pooled replicate lines of 'high' and 'low' females kept on 'high' and 'low' food.

Fig. 2. The mean number of progeny produced per 3-day sampling interval for 3 replicate lines of 'high' and 'low' females kept on 'high' and 'low' food.

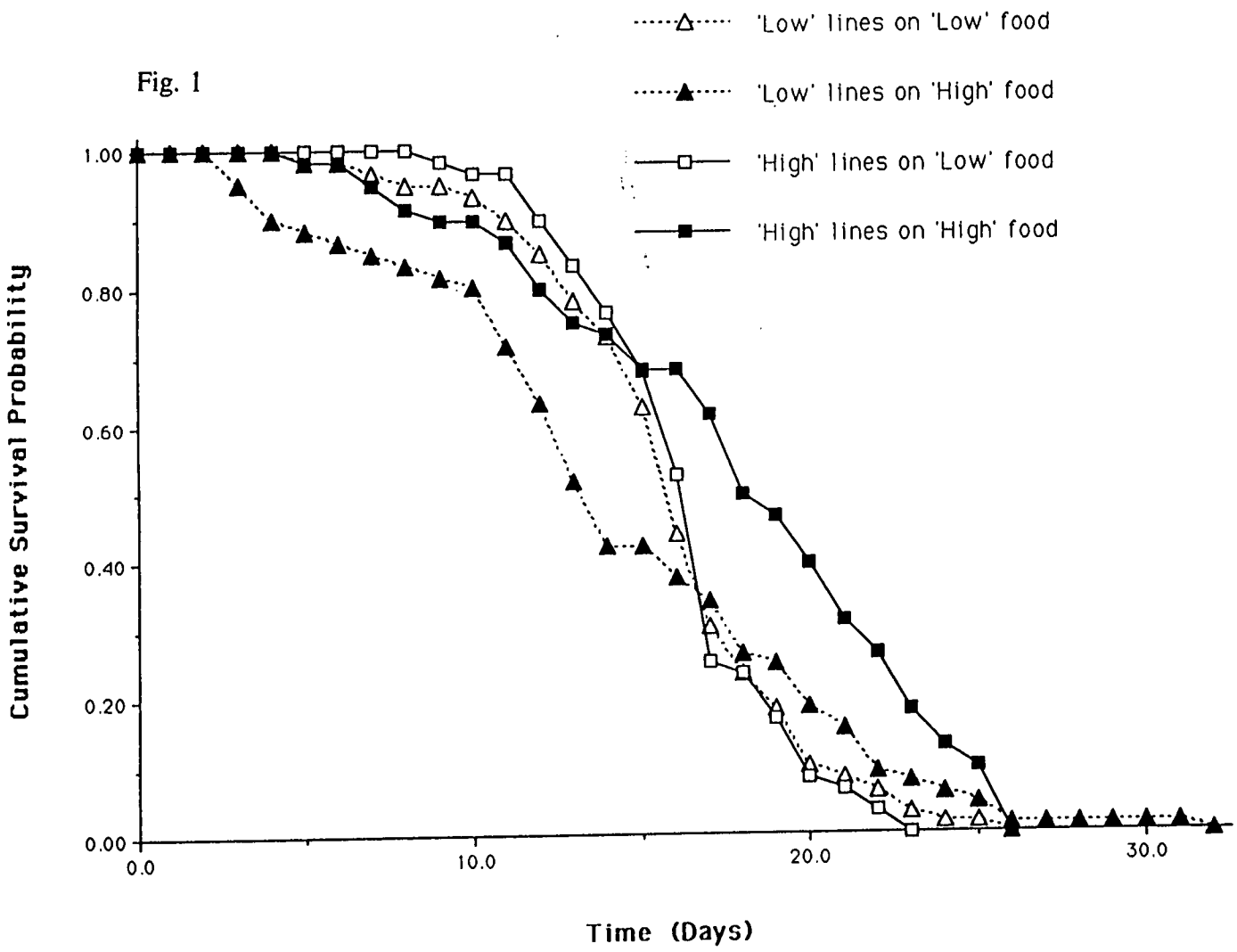
Fig. 3. Cumulative probability of survival against time (days) for females kept on 'high' or 'low' food and intermittently or continuously exposed to wild-type males.

Fig. 4. Median number of eggs produced per 1-day sampling interval for the same 4 groups of females as in Fig. 3.

Fig. 5. The cumulative percentage of mating opportunities offered which were taken against days since first mating for 'rich-dilute' and 'dilute-rich' females.

Fig. 6. The mean and 95% confidence limits of the number of eggs laid per 2 day sampling period from the time of first mating for 'rich-dilute' and 'dilute-rich' females.

Fig. 1


'Low' lines on 'Low' food
'Low' lines on 'High' food
'High' lines on 'Low' food
'High' lines on 'High' food
Cumulative Survival Probability
1.00
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0.60
0.40
0.20
0.00
0.0
10.0
20.0
30.0
Time (Days)

Fig. 2

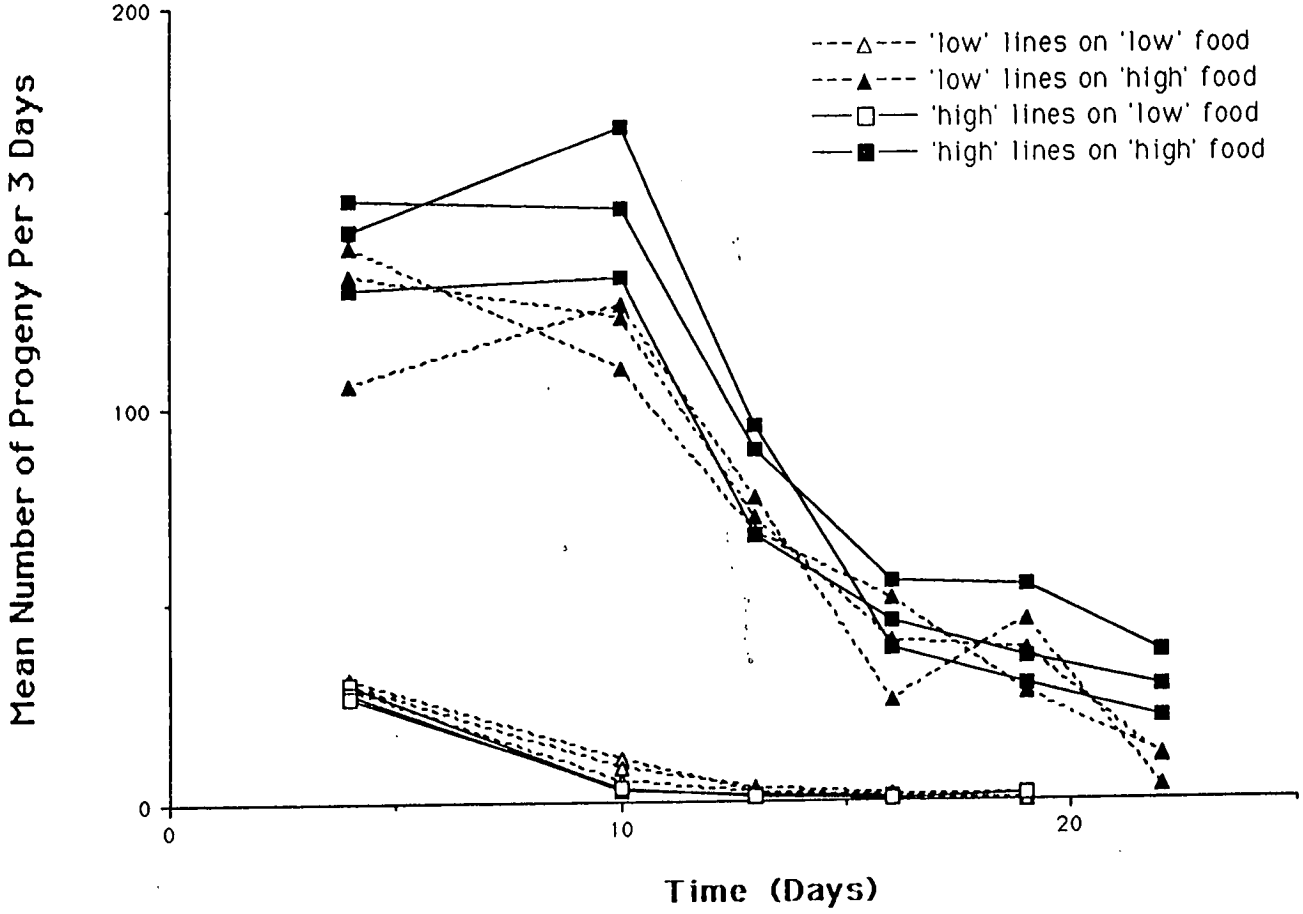
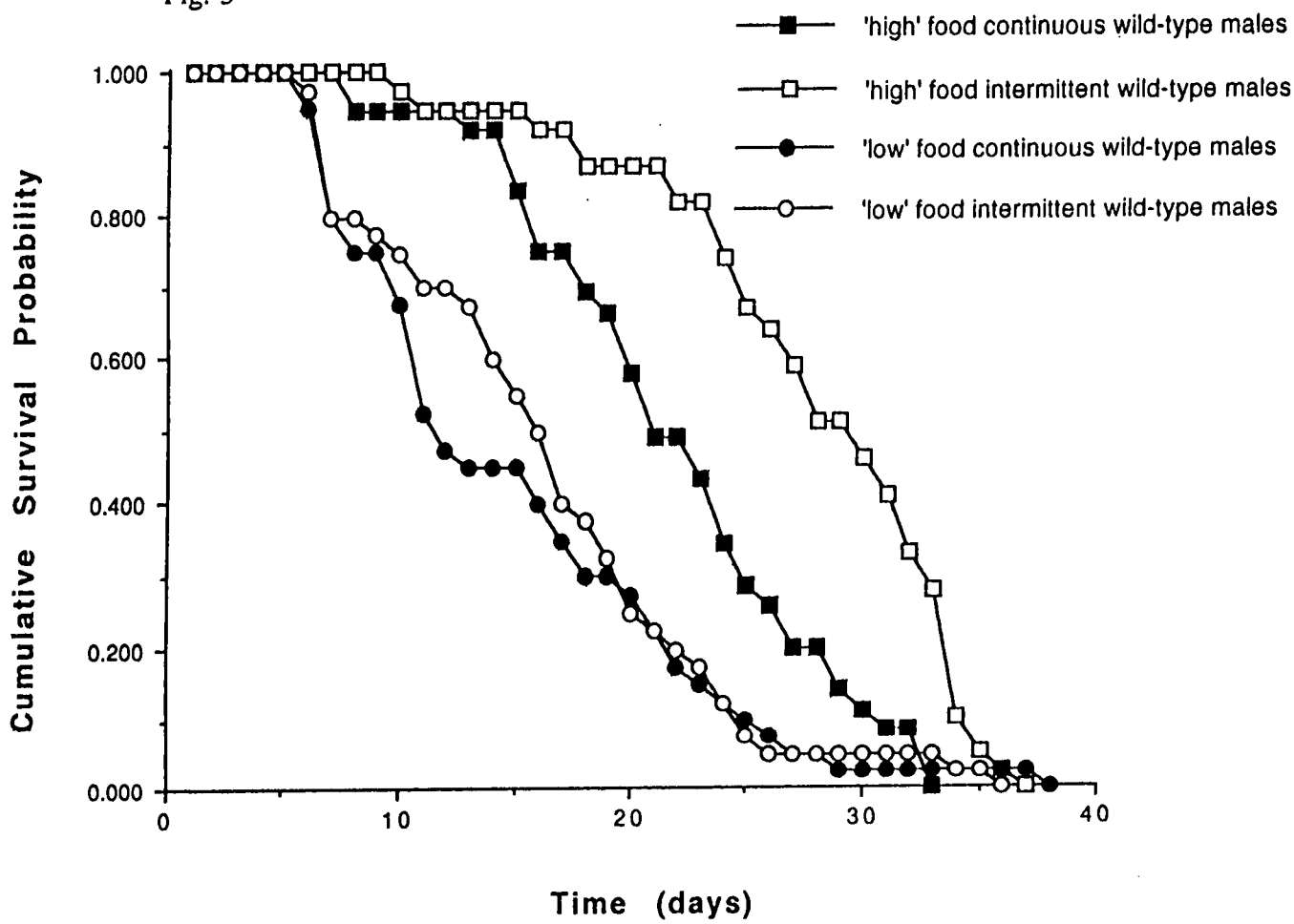

Mean Number of Progeny Per 3 Days
200
100
0
0
10
20
Time (Days)
---△--- 'low' lines on 'low' food
---▲--- 'low' lines on 'high' food
—□— 'high' lines on 'low' food
—■— 'high' lines on 'high' food

Fig. 3


'high' food continuous wild-type males
'high' food intermittent wild-type males
'low' food continuous wild-type males
'low' food intermittent wild-type males
Cumulative Survival Probability
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0.600
0.400
0.200
0.000
0
10
20
30
40
Time (days)

Fig. 4

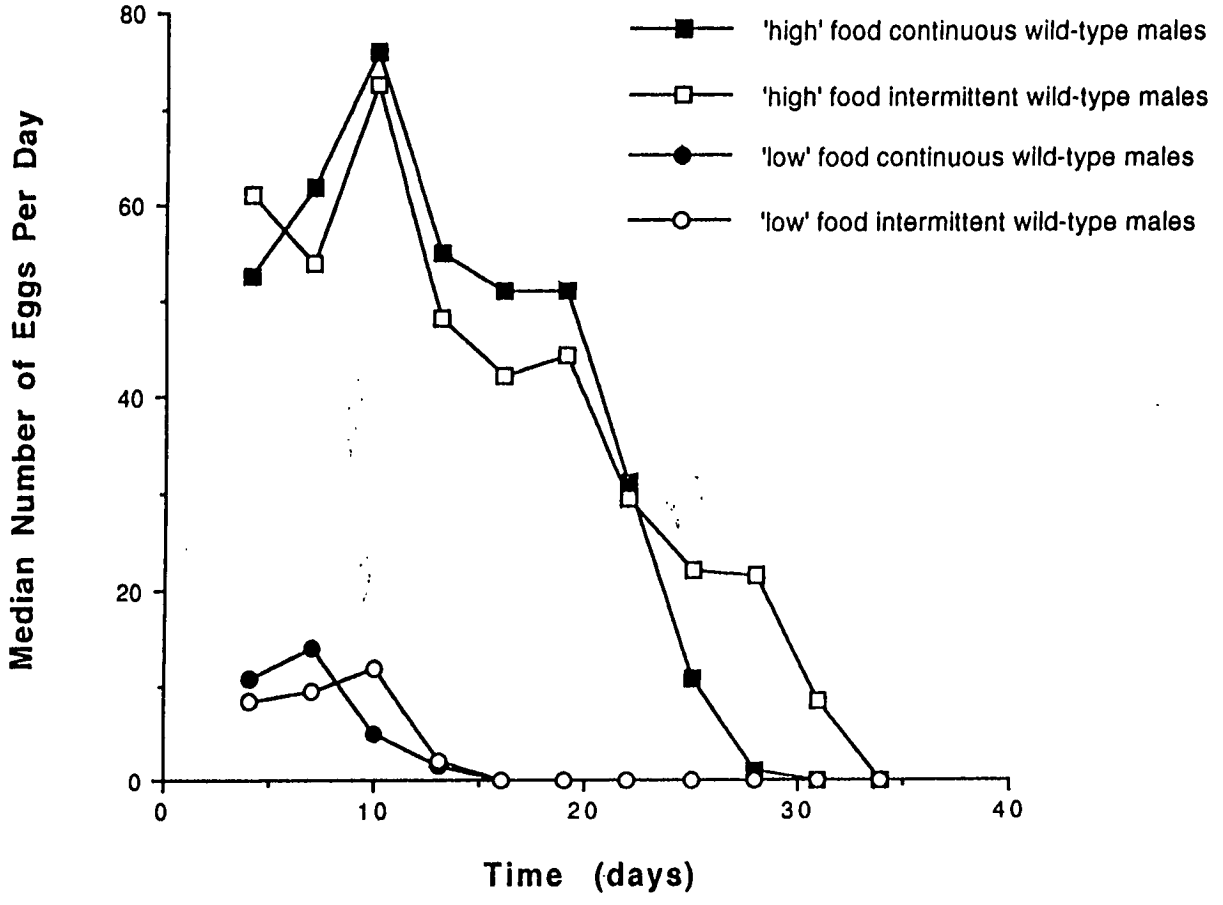
'high' food continuous wild-type males
'high' food intermittent wild-type males
'low' food continuous wild-type males
'low' food intermittent wild-type males
Median Number of Eggs Per Day
Time (days)
0
20
40
60
80
0
10
20
30
40

Fig. 5

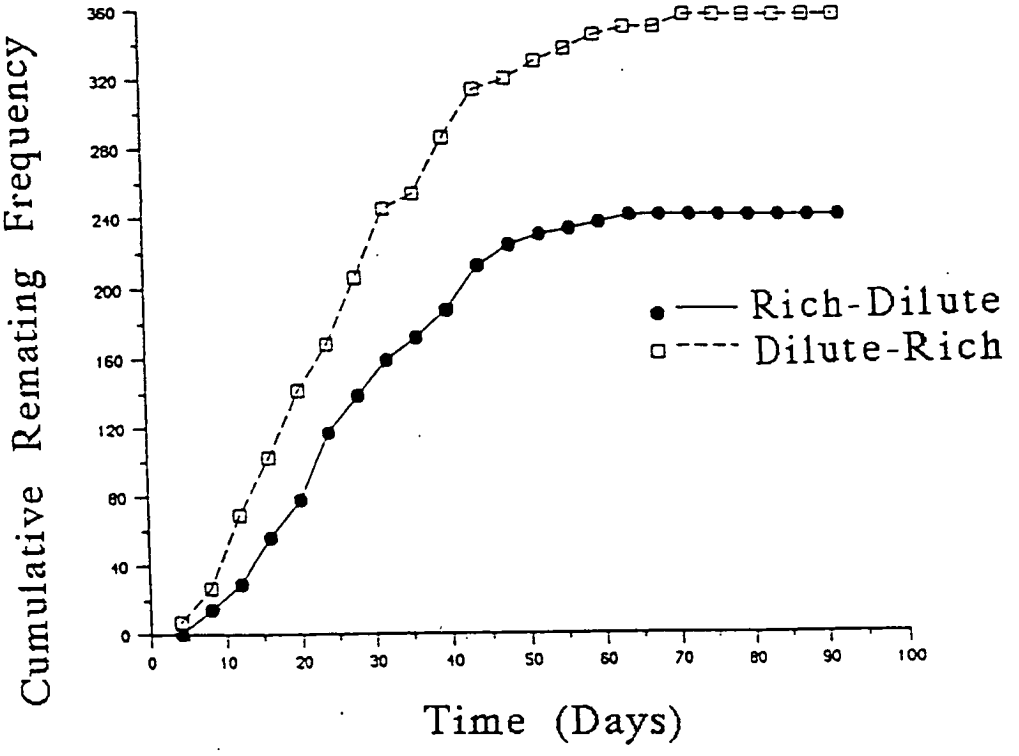
Cumulative Remating Frequency
360
320
280
240
200
160
120
80
40
0
0
10
20
30
40
50
60
70
80
90
100
Time (Days)
Rich-Dilute
Dilute-Rich

Fig. 6

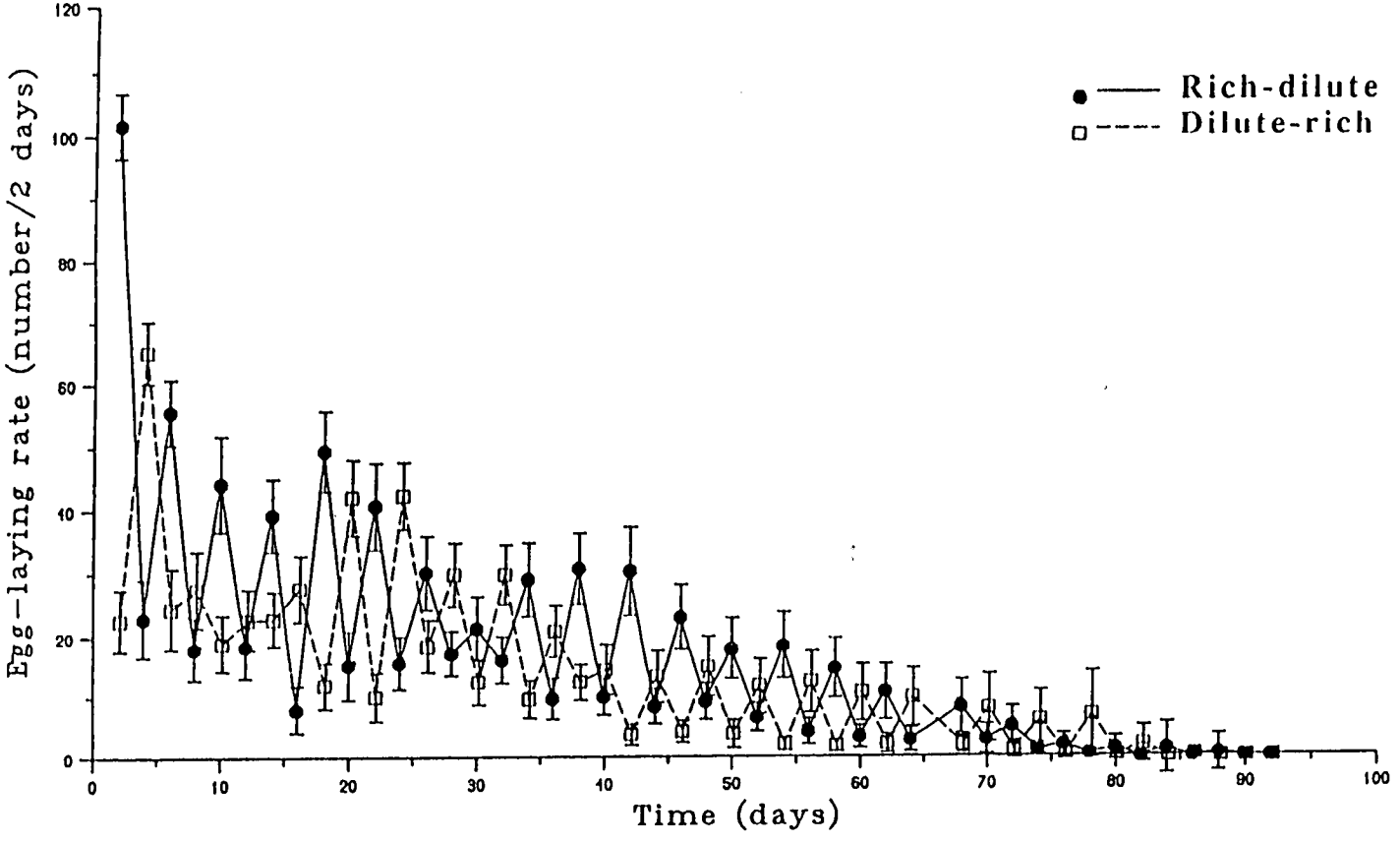
Egg-laying rate (number/2 days)
Time (days)
Rich-dilute
Dilute-rich
0
20
40
60
80
100
120
0
10
20
30
40
50
60
70
80
90
100

Table 1. Remating frequencies during the first 4 sampling intervals of the experiment for females from the 'high' and 'low' selection regimens on 'high' and 'low' food.

Selection Regimen	Food	Remating Opportunities		
		Taken	Not Taken	% Taken
'High'	'High'	68	162	30.0
	'Low'	10	229	4.2
'Low'	'High'	44	166	21.0
	'Low'	28	204	12.1

Table 2. Means (and standard errors) of number of progeny produced in their lifetimes by females from the 'high' and 'low' selection regimens kept on 'high' and 'low' food.

		Food	
		'High'	'Low'
Selection Regimen	Replicates		
'High'	1	396 (30.0)	35 (1.8)
	2	468 (36.3)	33 (1.9)
	3	469 (26.6)	31 (2.5)
	mean	444	33
'Low'	1	361 (38.8)	44 (3.2)
	2	350 (44.3)	44 (2.7)
	3	328 (30.5)	37 (2.2)
	mean	346	42

Table 3. Results of nested analysis of variance on the lifetime progeny-production scores in Table 2.

(a) On 'high' food

	df	ss	ms	F
Selection regimen	1	2095	2095	10.85*
Replicate lines	4	773	193	1.75
Error	107	11761	110	

(b) On 'low' food

	df	ss	ms	F
Selection regimen	1	255978	255978	13.68*
Replicate lines	4	74838	18709	0.86
Error	101	2186107	21645	

*$p<0.05$

Table 4. Remating rates for females kept on 'low' or 'high' food and intermittently or continuously exposed to wild-type males. Data presented for days when all females exposed to wild-type males. For details of sampling frequency see text.

(a) main experiment

Food	Male Exposure	Remating Opportunities		
		Taken	Not Taken	% Taken
'High'	Intermittent	8	137	5.8
	Continuous	11	107	10.2
'Low'	Intermittent	1	99	1.0
	Continuous	5	78	6.4

(b) extra remating frequency data.

Food	Male exposure	Remating Opportunities		
		Taken	Not taken	% Taken
'High'	Intermittent	14	47	29.7
	Continuous	27	68	39.7
'Low'	Intermittent	6	54	11.1
	Continuous	2	93	2.1

Appendix B

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A COST OF MATING WITH MALES THAT DO NOT TRANSFER SPERM IN FEMALE *DROSOPHILA MELANOGASTER*

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Abstract—The aim was to test for effects of accessory fluid and sperm on female survival, by comparing females that received both (exposed continuously to wild-type males), lowered sperm (exposed intermittently to XO males, which transfer no sperm at mating) or lowered sperm and accessory fluid (exposed intermittently to wild-type or XO males with their external genitalia microcauterized and which therefore transfer neither sperm nor accessory fluid). Within females exposed to XO males, male microcautery improved female survival, indicating a cost of mating with XO males. This cost cannot be attributed to an effect of sperm-transfer. For both microcauterized and intact males, females exposed to XO males survived longer than females exposed to wild-type males. There must therefore have been a difference in the non-mating costs of exposure to XY and XO males, and the lower levels of courtship by XO males, associated with lower levels of female movement, may have been responsible. Because of this difference in non-mating costs, it was not possible to test for costs of receiving sperm or accessory fluid.

Key Word Index: Accessory fluid; *Drosophila melanogaster*; fecundity; fertility; lifespan; mating; sperm; survival

INTRODUCTION

Costs of reproduction occur when reproductive activity adversely affects future survival or fertility (Williams, 1966). Both elevated rates of egg-production and increased exposure to males have been shown to cause a drop in female *Drosophila melanogaster* lifespan (Partridge *et al.*, 1987), and part of the survival cost of exposure to males has been shown to be some consequence of mating itself (Fowler and Partridge, 1989). Non-mating costs of exposure to males can also be important (Partridge and Fowler, 1990).

The nature of the cost of mating is unclear, and there seem to be at least three possibilities: mechanical injury at copulation (Lloyd and Park, 1962; Ikeda, 1974), the transfer of disease or parasites to the female and some effect of sperm or accessory fluid. A potential way to test for a cost of receiving sperm is to make use of males that are incapable of transferring it. *D. melanogaster* XO individuals, which lack a Y-chromosome, are phenotypically normal males, and transfer accessory fluid but no sperm at mating, and are therefore sterile (Bridges, 1916; Stern, 1929). The testes of XO and XY males appear to be similar in both size and shape, but the testes of XO males contain non-motile sperm (Safir, 1920). Spermiogenesis does occur in XO males, and spermatids elongate but degenerate before maturation (Keifer, 1966; Hess and Meyer, 1968; Hardy *et al.*, 1981) and are not transferred to the female at mating.

The protein synthesis and constitution of XO male accessory glands are reported to be normal (Von Wyl, 1976; Ingman-Baker and Candido, 1980; Chen, 1984), but courtship and mating success have been reported to be deficient (Aslund *et al.*, 1978; Gromko *et al.*, 1984). To compare the reproductive behaviour of XO and XY males directly, the genetic backgrounds of the two types of fly should ideally be the same, because the relative fitnesses of XO and XY genotype flies may be different on different genetic backgrounds (Voelker and Kojima, 1970).

Trevitt and Partridge (1991) showed that female *D. melanogaster* kept with normal wild-type males had lower survival rates than females intermittently exposed to XO and wild-type males, in the absence

of differences in egg-production, egg-hatchability or mating frequency between the two groups of females. The results suggested that receiving sperm could be costly to females, in terms of a drop in lifespan. However, alternative explanations were possible. The genetic background of the wild-type and XO flies was not the same, and the courtship activity of the XO and XY males was not recorded, leaving open the possibility of a difference in the non-mating costs of exposure to the types of males.

The aim of the present study was to examine these two possibilities, and also to test directly for effects of receiving sperm and accessory fluid on female survival. To control female egg-production and egg-hatchability, all females were exposed to intact wild-type males for one day in three. On the other two days, females could receive both sperm and accessory fluid (by exposure to intact wild-type males), only accessory fluid (by exposure to intact XO males) or neither sperm nor accessory fluid (by exposure to XO or XY males with microcauterized external genitalia). The effects of sperm and accessory fluid separately could therefore be deduced, provided that the non-mating effects of XO and XY males did not differ.

MATERIALS AND METHODS

The wild-type flies used were from a stock collected in Dahomey (now Benin), West Africa in 1970 and maintained since then in population cages, for the last 6 yr on sugar–yeast medium (30 g agar, 100 g flaked yeast, 100 g sucrose, 30 ml nipagin and 3 ml propionic acid per 1000 ml water). XO males were generated by crossing Dahomey virgin females to males from a C(1)RM, *yw*/O stock. The males in this stock had the genotype $\hat{X}Y$O and the females $\hat{X}\hat{X}$O. Dahomey wild-type genetic background was introduced into this attached-X stock using balancer chromosomes to substitute in Dahomey chromosomes 2 and 3. The stock was then maintained in population cages identical to those in which the Dahomey stock was kept. The XO males generated therefore had the same genetic background as the Dahomey wild-type males and differed only in the absence of a Y chromosome. All flies were cultured at 25°C on a 12 h light–12 h dark cycle.

XY and XO males were microcauterized by ablating their external genitalia with tungsten electrodes. The tips of these electrodes were 40 μm apart and delivered a current of approx. 80 V. Microcauterized males were screened for mating ability 2 days after microcautery; males were placed three per vial with four, 4-day-old wild-type virgin females and any males that mated during the 3 h of the test were discarded. To control for any environmental effects that might have affected the willingness of the microcauterized males to court and mate, at least 10 control vials of three intact males (wild-type or XO) of the same age and four, 4-day-old wild-type virgin females were always set up. In each screen, all of the control intact males mated within an hour.

The aim of the experiment was to investigate the costs of receiving accessory fluid and sperm, by comparing the survival of females exposed to microcauterized males, which could not mate, with that of females exposed either to intact XO males which transfer only accessory fluid, or to XY males which transfer both sperm and accessory fluid, while controlling female fecundity and fertility. Experimental females were from the Dahomey stock and were grown up at a density of 100 larvae per vial (75*25 mm containing 7 ml sugar–yeast medium). One-hundred and sixty adult virgin females were collected at eclosion by cooling on ice, and were then aged in groups of 20 per vial for 3 days; females were then randomly assigned to four experimental groups, each of 40 females:

1. Females kept with two wild-type males continuously, one set of wild-type males for one day in three and another set for the other 2 days = *WT + WT INTACT group.*
2. Females kept with two wild-type males for one day in three and 2 XO males for the other 2 days = *WT + XO INTACT group.*
3. Females kept with two wild-type males for one day in three and two wild-type microcauterized males for the other 2 days = *WT + WT MICROCAUT group.*
4. Females kept with two wild-type males for one day in three and 2 XO microcauterized males for the other 2 days = *WT + XO MICROCAUT group.*

Previous work (Fowler and Partridge, 1989) had shown that exposure to intact wild-type males on one day in three was sufficient to maintain full female fecundity and fertility.

Each female was placed with two males in a freshly-yeasted food vial and females were given new food every time that the males were changed. Males were initially 5 days old when introduced into the experiment, and were renewed from fresh cultures every 9 days. Males were stored 20 per vial when not kept with females. Female deaths were recorded daily. Eggs were counted in the vials laid up on the days when all females had wild-type males, and these vials were retained to count emerging adult progeny.

Remating frequency was recorded every 3 days on the days when all females were with wild-type males, by scanning vials every 20 min for a period of 3 h around the time of lights-on and counting the number

of matings. To assess whether the four types of experimental male differed in their activity or courtship behaviour, their behaviour was assayed every 3 days on one of the two days out of three when females were exposed to the four types of experimental males. Vials were scanned every 20 min for 3 h around the time of lights on and the behaviour of the female recorded as moving or not moving and as courted (male within 5 mm of female, following or orientated towards her, performing wing vibration) or not courted.

RESULTS

Female survival curves (Fig. 1) were analysed using a distribution-free Mantel–Cox [BMDP Life Tables and Survival Functions, BMDP Software Ltd (Dixon, 1988)]. The WT + WT INTACT group (median lifespan = 12 days) lived significantly less long than the WT + WT MICROCAUT group (median lifespan = 17 days, Mantel–Cox test statistic = 13.26, $P = 0.0003$) and the WT + XO INTACT group (median lifespan = 21 days) lived significantly less long than the WT + XO MICROCAUT group (median lifespan = 28 days, test statistic = 24.74, $P = 0.0000$). In the two groups where females were exposed continuously to intact males, WT + XO INTACT females lived significantly longer than WT + WT INTACT females (Mantel–Cox test statistic = 4.85, $P = 0.027$). WT + WT MICROCAUT females lived significantly less long than WT + XO MICROCAUT female (Mantel–Cox = 6.65, $P = 0.010$). Females in the WT + WT MICROCAUT group lived significantly longer than females in the WT + XO INTACT group (Mantel–Cox = 4.28, $P = 0.038$).

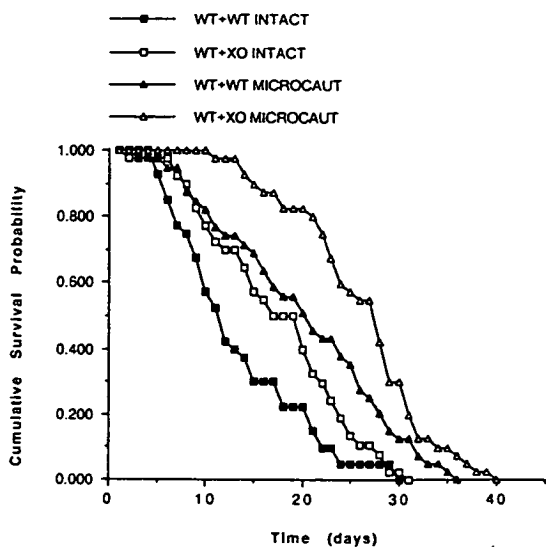

Fig. 1. Cumulative survival probability of survival plotted against time (days).

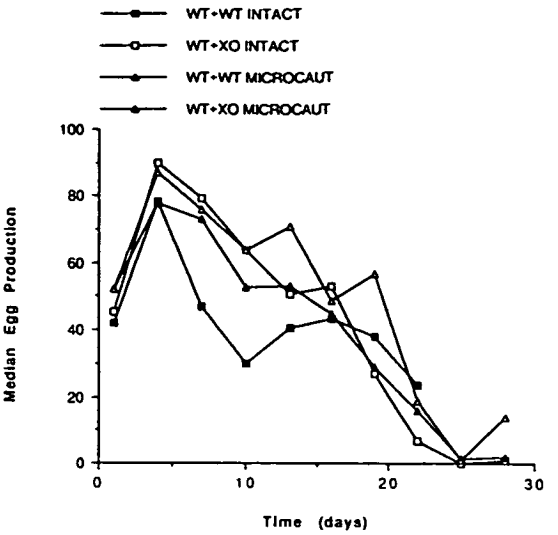

Fig. 2. Median values for egg-production (sampled every third day) plotted against time (days).

Egg-production is plotted against female age in Fig. 2. Medians are plotted because preliminary investigation of the egg-production data indicated that they were not normally distributed. They were analysed using Kruskal–Wallis tests including multiple comparisons to show between which groups any significant differences lay. In general, egg-production did not differ significantly between the groups. The WT + WT INTACT group tended to lay fewer eggs than the other groups (day 1 $P = 0.0003$, day 4 $P = 0.0333$ and day 10 $P = 0.0176$). There were no significant differences in egg-hatchability between the four groups (data not shown), except on day 10 when the hatchability of the WT + XO INTACT group was significantly ($P < 0.05$) lower than the WT + XO MICROCAUT group and on day 19, when the hatchability of the WT + XO INTACT group was significantly ($P < 0.05$) lower than either the WT + WT MICROCAUT or WT + XO MICROCAUT groups. Because of the large number of comparisons for each sampling interval (one comparison between all four groups, and six multiple comparisons) this number of significant results would be expected by chance (one in 20 at the $P < 0.05$ significance level).

Mating frequency was recorded on the days when all females were kept with wild-type males. Each morning of mating observations was treated as one mating opportunity "offered" for each female and, if a mating was scored, this was a mating opportunity that was "taken". The number of matings recorded in each sampling interval was small (maximal percentage of opportunities taken was 22, minimal one), so the remating frequencies of two successive sampling intervals were summed throughout the

experiment to give a larger sample size. The data for the four groups were compared using a χ^2-test, and none of the comparisons were significant at $P < 0.05$. Since the mating frequencies did not differ significantly on days when intact XY males were present, and since XY and XO matings were seen on the other days, those females exposed to intact males on the other two days must have had higher overall mating frequencies than the females exposed to microcauterized males.

The data on behaviour were used to calculate, for each female in each sampling interval, the proportion of observations in which she was either courted or not courted and either moving or still (Figs 3 and 4). These figures were then subjected to the angular transformation and a one-way analysis of variance. For courtship, microcautery had no significant effect on wild-type males, while intact XO males delivered more courtship than microcauterized XO males in interval 6. The chromosomal constitution of the male had a large effect, with wild-type males delivering significantly more courtship in intervals 1–3, and at least one significant comparison in intervals 6 and 7. For female movement, microcautery had little effect: females exposed to microcauterized and intact wild-type males showed no significant differences throughout the experiment, and females exposed to microcauterized XO males moved significantly less than those exposed to intact XO males only in the first sample. Chromosomal constitution of the male did have an effect: females exposed to both types of XY males moved more than those exposed to XO males in samples 1–3, and females exposed to microcauterized wild-type males moved more than those exposed to XO males in sample 7.

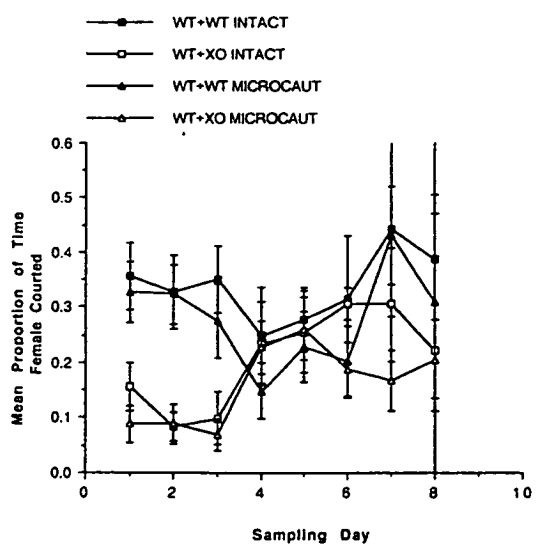

Fig. 3. The mean and 95% confidence limits of the proportion of time for which females were courted in each sampling interval.

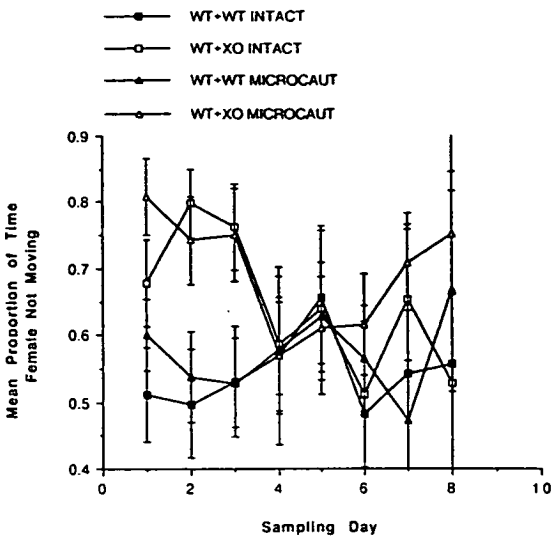

Fig. 4. The mean and 95% confidence limits of the proportion of time for which females were not moving in each sampling interval.

DISCUSSION

The most important finding in the present study was that mating with males that could not transfer sperm significantly reduced female lifespan. This conclusion is evident from a comparison of the groups of females exposed intermittently to either intact or microcauterized XO males. Females from the former group survived less well than females from the latter, in the absence of significant differences between them in egg-production, egg-hatchability and frequency of mating with XY males. Females exposed to microcauterized XO males did receive less courtship and move less in one sampling interval each, but this would be expected by chance with so many comparisons between groups. The decreased survival rate in the WT + XO INTACT females is therefore likely to have been caused by their matings with XO males. It is not possible to deduce whether the cost of mating with XO males is a cost of receiving accessory fluid or some other consequence of mating. Despite the lack of replication in the present experiment the results seem robust in that where they overlap previous findings (Fowler and Partridge, 1989; Trevitt and Partridge, 1991) they replicate them. The differences in the effects on female lifespan of intermittent exposure to either microcauterized XO or microcauterized XY males and to either intact XO or microcauterized XY males have not been previously recorded.

The results confirmed the cost of mating with XY males (Fowler and Partridge, 1989); females exposed continuously to intact wild-type males had significantly shorter lifespans than females intermittently exposed to microcauterized XY males, in the absence

of significant differences in egg-hatchability, movement and amount of courtship received. In this comparison, the WT + WT INTACT females also tended to lay fewer eggs than the WT + WT MICROCAUT females, but this difference was in the opposite direction to that needed to explain the difference in lifespan between the groups. The WT + WT INTACT females probably laid fewer eggs because they were in a poorer physiological condition than the females in the other groups, as a result of their extra matings with intact XY males.

The results also confirmed another earlier finding, that intermittent exposure to intact XY males leads to lower female survival than intermittent exposure to intact XO males, in the absence of differences in egg-production and egg-hatchability (Trevitt and Partridge, 1991). In the present experiment the genetic background of the XO and XY males was otherwise similar, ruling out any explanation in terms of other genetic differences between the XO and XY males. A possible explanation for the difference in survival is a cost of receiving sperm, but the present data suggest an alternative or additional explanation. The XO males were less likely to court than were the XY males and, perhaps in consequence, the females exposed to XO males were less likely to be moving than were the females exposed to XY males. The non-mating costs of exposure to XY males may therefore have been higher. Both extra expenditure of energy by the females and prevention of feeding may be consequences of extra male courtship and female movement. It is unclear why there should be a survival cost to receiving sperm; it is possible that the maintenance of healthy sperm in the female storage organs has a physiological cost (Thornhill and Alcock, 1983). It is also possible that the XY males mated more frequently than the XO males. However, this cannot have been the whole explanation of the differing effects on female survival, because it was also apparent in the two groups of females intermittently exposed to microcauterized males, indicating that the difference could not be entirely caused by a consequence of mating, and must be in part attributable to non-mating effects.

It therefore proved impossible to test satisfactorily for costs of receiving sperm and accessory fluid, because any such effects were confounded with differences in behaviour between XO and XY males. It is possible that the differences in behaviour would not be apparent in other flies that do not transfer sperm, such as *transformer*, and this possibility needs investigation.

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