Sperm Displacement in
*Drosophila melanogaster*

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Abstract

This thesis presents experiments which investigated the genetics and mechanisms of sperm displacement in the fly *Drosophila melanogaster*. Sperm displacement allows males to increase their reproductive success relative to other males. Differential reproductive success of males is an important aspect of total fitness. *D. melanogaster* provides an effective system in which to investigate traits that affect reproductive success.

The experimental work presented involved both genetic studies and phenotypic manipulations. Measurements of the repeatability and heritability of sperm displacement ability (SDA) showed both parameters to be low but significantly non-zero. SDA was correlated with pre-adult viability, suggesting the heritable component of SDA and pre-adult viability may be confounded.

Since little is know of the mechanism of sperm displacement in *D. melanogaster*, experiments were carried out to investigate the effect of variation in factors which may possibly influence SDA. It was determined that males did not displace the sperm of other males in preference to their own sperm. The effect of variation in copulation duration was investigated with both spermless and normal males. The results indicated that levels of sperm displacement were determined by the amount of sperm transferred, a process which appeared to occur rapidly during copulation. The effects of variation in genetically determined male body size was also investigated. Larger males were found to be at a slight disadvantage in virgin-female matings. No significant effect of body size on SDA was observed.

Separate experiments were carried out to determine the effect of male accessory gland products on the fertility of subsequent matings. The possibility that "sperm defence" is due to accessory gland fluid physically interfering with the storage of second male sperm was shown to be unlikely.
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General Introduction

1.1 Analysing male reproductive success

1.1.1 The analysis of fitness

The object of natural selection is fitness. Accordingly, the concept of fitness is a central element of evolutionary theory and its measurement would seem to be a major goal. The measurement of total fitness, however, presents a considerable technical challenge (Prout 1971). Furthermore, its value is highly dependent on the environment in which it is measured, making the generalisation of results difficult (Mackay 1985). So rather than attempting to measure total fitness, investigators usually concentrate on measuring components of fitness which are more easily quantified. In practice, only one or a few components of fitness are measured at one time, under either natural or controlled conditions. If all relevant components could be identified, measured and given correct weightings, total fitness could, in theory, be calculated.

How then are the component traits of fitness identified? In the study of life history evolution, the most commonly used measure of fitness is $r$, the intrinsic rate of increase of a clone or gene substitution in a population growing without density limitations (Charlesworth 1984; Lande 1982). The value of $r$ is determined by the Euler-Lokta equation:

$$1 = \int_{\alpha}^{\omega} e^{-\alpha x} l_x m_x \, dx,$$

which has age-specific fecundity and mortality ($m_x$ and $l_x$ respectively) as primary factors in the calculation of $r$ ($\alpha$ and $\omega$ are ages of first and last reproduction and the $x$ subscript refers to $x$th age class). Alternatives to $r$ have been derived for populations in variable and density-dependent environments, but these still depend on fecundity and mortality as variables (Roff 1992). Empirical results also support the conclusion that fecundity and mortality are primary determinants of fitness (Mackay 1985).

Once major components of fitness have been identified, the process of breaking down of traits can be continued. Figure 1.1 illustrates this relation between fecundity and mortality to fitness at each age. Also shown are a variety of other quantifiable traits that are in turn components of fecundity. Their relative positions are determined by their
proximity to overall fitness (Arnold and Duvall 1994; Falconer 1989). The component
traits of fecundity are firstly, the success a male has in securing matings ('mating
success' in Figure 1.1) and secondly, the number of his progeny that are produced in
each mating ('per-mate fecundity' in Figure 1.1). The traits that are components of per-
mate fecundity are listed in the leftmost column; these are courtship feeding, mate-
guarding, the various peptides and proteins that enhance female fecundity and lower
receptivity ('sex-peptides') and sperm displacement. This analysis can equally be
applied to plants by substituting appropriate terms e.g. 'pollination success' for 'mating
success' (Snow 1994).

Only traits relating to per-mate fecundity are shown in Figure 1.1. Components of
mating success, such as courtship and mate searching, are not shown. Of the two
principle traits that influence overall fitness, fecundity and mortality, only fecundity will
be considered further.

1.1.2 Life history analysis

Once component traits have been identified, they can be used to predict the action
of natural selection. Two principle methods are employed in the analysis of the
evolution of traits closely related to fitness i.e. life history traits. The first is the
optimality approach which seeks to explain observed combinations of life history traits
using a proiri models. The success of a model in predicting actual trait values is taken
as a measure of the accuracy of the assumptions used in constructing the model. Those
assumptions include the choice of an appropriate measure of fitness that will be
maximised, such as 'r' or possibly other traits of more immediate interest, as well as
assumptions concerning the factors which affect the trait being optimised. The accuracy of the optimisation approach also depends on the availability of sufficient time and genetic variation for the population to reach equilibrium.

An alternate (and not inconsistent) approach is based on quantitative genetics (Lande 1982). The genetic approach also analyses life history evolution in terms of maximisation of a measure of fitness such as ‘r’. Instead of seeking to predict optimal values, evolution is modelled in terms of changing gene frequencies. As most life history characters are polygenic, the change in ‘r’ (or other fitness measure) is measured as the outcome of simultaneous changes in gene frequencies of a number of loci. The extent of the changes in gene frequencies are determined by variation in the traits, their relation to ‘r’ and the covariances between the traits. This approach has limited value in measuring long term evolutionary change because the genetic relations between traits may be unstable over time (i.e. genetic covariances change as gene frequencies change, see Charnov 1989). In the short term the genetic approach can model life history evolution accurately, especially where selection is weak and population sizes large.

Both optimality and genetic approaches analyse life history in a demographic framework, i.e. by measuring fecundities and survival probabilities (or their component traits) of the various age-classes. These traits vary over an organism’s lifespan. Central to life history theory is the idea that this variation can result from trade-offs between the various life history traits. The idea of trade-offs can be appreciated by considering a maximally fit individual. Such an individual would reproduce at a maximum rate for an indefinite period (Law 1979). Since no such individuals exist, constraints must exist which place limits on the possible levels of fecundity and lifespan. In life history studies, these constraints have been shown to enforce trade-offs between some of the traits which are components of fitness. In the absence of trade-offs, each character correlated with fitness would be maximised individually. Therefore, the persistence of many traits at values well below their maxima is indicative of the existence of trade-offs (Stearns 1989). Maximisation of one trait is expected to reduce the resources available for other traits. The equilibrium value reached by traits subject to trade-offs depends on the marginal effects of the constrained traits on ‘r’, as well as the genetic architecture of the organism. Examples of trade-offs include that between current and future reproduction, reproductive effort and lifespan and between number and size of offspring (see Stearns 1992).

1.2 Components of male reproductive success

The following sections review the major components of male reproductive success shown in Figure 1.1, i.e. mating success on the one hand (section 1.2.1) and per-mate fecundity (sections 1.2.2 to 1.2.6) on the other. The variables that have been shown to influence both these traits in different species, especially in insects, will be considered.
1.2.1 Mating success

Mating success measures the relative success of males in securing matings when in competition with other males. Evidence that mating success is a component of overall fitness has been consistently found in *D. melanogaster* (Prout 1971; Bundgaard and Christiansen 1972; Brittnacher 1981; Clark and Feldman 1981; Partridge *et al.* 1985).

As with fecundity, the effect of body size on mating success has been frequently investigated. Larger males usually have greater mating success (McLain and Marsh 1990; Fox *et al.* 1995; Zamudio *et al.* 1995), with a notable exception being Dipterans that mate on the wing (McLachlan and Allen 1987). In *D. melanogaster*, body size is heritable (Robertson 1957) and positively correlated with mating success in the field (Partridge *et al.* 1987b; Markow 1988) and in the laboratory (Ewing 1964; Partridge and Farquhar 1983; Pitnick 1991). Larger males may have proportionately more energy to dedicate to searching for mates and courtship (Partridge *et al.* 1987a; Partridge *et al.* 1987b). Searching and courtship could therefore be placed in Figure 1.1 as two traits which influence mating success.

A similar correlation between male size and mating success has been observed in various *Drosophila* species: *D. buzzatii* (Santos *et al.* 1988), *D. simulans*, *D. maojavensis* (Markow and Ricker 1992), *D. sylvestris* (Boake 1989), *D. nigrospiracula* (Markow 1988), *D. hydei* (Pitnick and Markow 1994a) and *D. testacea* (James and Jaenike 1992). In *D. buzzatii*, this correlation is only observed when individuals are grown under crowded conditions, and since resultant progeny of more successful males are not larger than average, it was suggested that there was no genetic correlation between size and mating success (Santos 1996; but see Wilkinson 1987).

Size has similar effects in the Lepidoptera. In *Jalmenus evagoras*, a Lycaenid butterfly, larger males have a greater mating success (Elgar and Pierce 1988). However, in pupal-mating *Heliconius*, mating success depends on both gaining a position on the pupae and mating once in position. Larger males are favoured in the competition for position, but smaller males are more successful at mating once in position (Dienert *et al.* 1994).

Other traits also affect mating success. Male *D. melanogaster* selected for increased territoriality achieved more matings with non-virgin females than did control lines (Hoffmann and Cacoyianni 1989). Significant amounts of additive genetic variance are maintained for this trait in natural populations (Hoffmann 1991). Similarly, territoriality and mating success may also be correlated in *D. pseudoobscura* (Partridge *et al.* 1987b) and possibly *D. nigrospiracula* (Markow 1988). Courtship song also influences mating success in *D. melanogaster*, but the heritability of this trait could not be distinguished from zero (Ritchie and Kyriacou 1994).
Trade-offs may also affect mating success. In *D. melanogaster* lines that have been selected for divergent rates of senescence show evidence of a trade-off between early-life mating success (measured as competitive mating ability) and late life mating success (competitive and non-competitive mating ability and recovery times between matings) (Service 1993). This result implies that increased early mating effort is detrimental at later ages. The effect on recovery times between matings may involve accessory gland fluid. It has been shown that the recovery time of males depends on the replenishment of accessory gland fluid rather than sperm replenishment (Hihara 1981; Lefevre and Jonsson 1962).

### 1.2.2 Per-mate fecundity

The second aspect of a male's fecundity will be the number of eggs he is able to fertilise once he has successfully mated. This will be affected by factors that influence both his potential lifetime fecundity and the number of fertilisations achieved from each mating, i.e. his per-mate fecundity.

The measurement of lifetime fecundity of males is, in principle, straightforward: simply count all offspring produced over the lifetime of a male. However, because males of most species have the potential to sire large numbers of offspring, complete offspring counts are seldom made except in where males defend a territory, harem or lek (Benjamin and Bradshaw 1993). More commonly male reproductive success is measured in terms of mating activity, including courtship and copulation (e.g. Fincke 1982; Banks and Thompson 1985; Hoffmann and Cacoyianni 1989; Oberhauser 1989), mating success and offspring sired in short periods (e.g. McLain and Marsh 1990). Few measures of lifetime offspring have been made in either the laboratory (e.g. Simmons 1988; Rowe and Scudder 1990; Benjamin and Bradshaw 1993) or the field (Fincke 1986). Since fecundity is closely related to fitness, it is expected to have a low heritability in equilibrium populations (Falconer 1989). Heritabilities of male fecundity are seldom measured directly (see for example Hughes 1995). Even fewer studies have examined the relation between male body size and lifetime fecundity. Simmons (1988) showed that larger male field crickets, *Gryllus bimaculatus*, had greater overall lifetime reproductive success. In *Drosophila hydei* progeny production over short periods is positively correlated with body size (Pitnick and Markow 1994a). In *D. melanogaster*, however, larger males have been shown to have slightly lower levels of progeny production in matings with virgin females (Pitnick 1991).

Since there must ultimately be a cost of sperm production (see below), then there will be a trade-off between energy allocation to sperm production and other functions. If so, an intraspecies correlation between sperm number and body size would be expected, as larger males will have a greater total energy budget. This relationship has been found in the flesh fly *Neobellieria bullata* (Berrigan and Locke 1991) and *D. hydei* (Pitnick...
and Markow 1994a). However, assuming larger *D. melanogaster* produce more sperm, an apparent exception to the positive relation between body size and fecundity, has been observed in *D. melanogaster* (Pitnick 1991).

It is often assumed for practical purposes that sperm production is not a limiting factor in male fecundity. Males will maximise productivity of gametes i.e. many, small, poorly provisioned sperm, while females maximise provisioning of gametes i.e. relatively few, large, well-provisioned eggs (Parker 1984; Bressac et al. 1994). Even though sperm are much less costly to produce, it has been suggested that even under optimal conditions, there will be limits on the total number of progeny a male can sire (Partridge 1988), implying that sperm production (or other components of ejaculate) eventually becomes costly to males. In *D. melanogaster*, high levels of courtship have been shown to have a high longevity cost to males (Cordts and Partridge 1996). This result suggests that it is courtship rather than ejaculate production that limits lifetime reproductive success in *D. melanogaster*, although in those experiments, males were not provided with large numbers of virgin females. However, in other organisms, sperm related costs have been demonstrated, principally in insects (e.g. Dewsbury 1982; Kirkendall 1990; Pitnick 1996; Pitnick and Markow 1994b; Smith et al. 1988), but also in birds (Birkhead 1991), fish (Nakatsuru and Kramer 1982) and a marine bryozoan (Yund and McCartney 1994). It has also been shown that in a number of insect species transfer and/or storage of sperm is insufficient to fertilise all the eggs a female may lay e.g. in *D. melanogaster* (Fowler 1973), Mediterranean fruit fly (Cunningham et al. 1971), sheep blow fly (Smith et al. 1988) and some ants (Tschinkel 1987).

The anisogamy paradigm does not apply in all cases. In certain species of *Drosophila* which produce giant sperm, each sperm represents a significant investment of resources (Bressac et al. 1994). *D. littoralis* and *D. hydei* produce sperm 7.3 and 16.9 mm long respectively. *D. littoralis* transfers only 123 sperm per mating (of which about 60% fertilise eggs) while *D. hydei* transfers an average of only 83 sperm (of which 80% fertilise eggs). In the *nannoptera* species group of *Drosophila*, species producing larger sperm are sperm limited (Pitnick and Markow 1994b). They are unable to fill female sperm storage organs and appear to allocate their limited sperm between matings, rather than attempting to maximally inseminate the first female.

If sperm are a limiting factor, males may alter their sperm usage to maximise fecundity. In species such as *D. pachea*, *D nannoptera* and *D. wassermani*, where the number of sperm transferred is usually insufficient to fill the sperm storage organs of the female, males partition their sperm among females (see also Gage and Baker 1991; Pitnick and Markow 1994b). These species appear to have adopted a 'bet-hedging' strategy (Slatkin 1974). Although transferring more sperm per mating may increase per-mate fecundity of some matings, it also increases the variance of per-mate fecundity. By
transferring fewer sperm, the variance is lowered. The particular biology of these species may make 'bet-hedging' selectively advantageous (Pitnick and Markow 1994b).

If sperm are relatively abundant, the crucial factor in determining male per-mate fecundity is the number of offspring a female will produce using the sperm of that male. There are four ways in which a male can increase this number:

i) he can attempt to increase the female's egg production or offspring fitness by providing extra nutrition at or near mating (courtship feeding or nutrient gifts);

ii) in species where females are likely to remate, males can attempt to delay female remating for as long as possible (postinsemination association or mate-guarding);

iii) as in i), he can attempt to increase egg production but by the transfer of a fecundity-enhancing substance(s) to the female (sex-peptides); and

iv) where females do remate, and the sperm of more than one male are present in the female, he can attempt to maximise the relative success of his sperm in obtaining fertilisations (sperm precedence).

These strategies are the four shown in the leftmost column of Figure 1.1.

1.2.3 Courtship feeding

Males feed females at mating in at least 10 orders of insects (Thornhill and Alcock 1983). The food offered ("nutrient gifts") can take the form of:

i) prey items, which occurs in thynnine wasps (Given 1954), empidid flies (Alcock 1973) and in scorpion flies (Thornhill 1980);

ii) male produced secretions, e.g. *D. subobscura* (Steele 1986) and Queensland fruit fly *Dacus (Bactocera) tyroni* (Fletcher 1968). Such secretions may be also be valuable as chemical defence (Eisner *et al.* 1996);

iii) spermatophores deposited externally or in the female bursa (common in grasshoppers and crickets e.g. Gwynne 1986; Boggs 1990; Wedell 1993; Neuner *et al.* 1996); and

iv) by internal absorption of nutrients contained in the male ejaculate e.g. in butterflies (Karlsson 1996) and *D. mojavensis* (Markow and Ankney 1984) or defensive chemicals which can be later incorporated in eggs, e.g. the beetle *Neopyrochroa flabellata* (Eisner *et al.* 1996). Although the transfer of accessory gland fluid is of crucial importance in male reproductive success in *D. melanogaster*, there is no evidence that the fluid functions as courtship feeding (Bownes and Partridge 1987; Chapman *et al.* 1994).
There has been considerable debate over how nutrient gifts function to affect male reproductive success. Such feeding may function in two distinct, but not exclusive ways: either as a "paternal investment" or as part of an increased mating effort (e.g. Sakaluk 1986; Simmons 1990; Wedell 1993). Paternal investments function to increase the number or fitness of the female's offspring without necessarily ensuring that the sperm of the male providing the paternal investment fertilises the extra eggs produced as a result of the investment. An example of paternal investment occurs in the bush cricket Requena verticalis, where there is a positive correlation between gift size and female fecundity (Gwynne 1984a). Where paternity cannot be assured, males would be expected to reduce any paternal investment, since they receive no assured paternity benefit. Provision of a parental investments will only be selectively adaptive where sperm precedence is low or absent, as is found in R. verticalis. In R. verticalis later males are rarely successful in displacing the sperm of the first male (Gwynne 1988).

Contrasting with paternal investment function is the view that some nutrient gifts function to directly increase per-mate fecundity ("mating effort"). The nutrient gift does not simply increase the number or fitness of eggs the female lays, but directly increases the number of eggs fertilised by the male providing the nutrient gift. In a survey of 28 bush cricket species, it was found that increased spermatophore size did not increase female fecundity, but did increase the female refractory period which in turn correlated positively with male reproductive success (Wedell 1993).

The characterisation of nutrient gifts as either paternal investments or mating effort is not always clear. Paternal investments are an indirect influence on per-mate fecundity and their effectiveness depends crucially on other behavioural characteristics, particularly the likelihood of sperm precedence. For example, although the gift of the grasshopper Decticus verrucivorus provides nutrients in the form of a spermatophore, the female usually remates before the extra nutrients are incorporated into fertilised eggs (Wedell and Arak 1989). Similarly, in the grasshopper Chorthippus brunneus, females increase their fecundity using spermatophore derived nutrients but remate many times before egg-laying (Butlin et al. 1987). Using a phylogenetic approach, it has been shown that nutrient provision in butterflies increases with increasing polyandry, presumably to increase the female refractory period, but that the relative importance of paternal investments or mating effort is not fixed among species (Karlsson 1996).

Giant sperm, which occur in a number of Drosophila species, have been suggested to function as food gift that is dependent on fertilisation. The giant sperm provide significant numbers of mitochondria, associated transcripts and energetic reserves to the zygote (Bressac et al. 1994). However in closely studied species, it appears that most of the sperm does not enter the egg and the portion that does remains intact during most of embryogenesis (Karr and Pitnick 1996). Similarly, the smaller non-fertilising sperm in
dimorphic Drosophilids do not appear to act as nutrient donations (Snook and Markow 1996).

1.2.4 Postinsemination associations (mate-guarding)

The second trait that can influence the per-mate fecundity of males is the formation of postinsemination associations (PIAs, often generalised as "mate guarding"). PIAs benefit the guarding male by denying rival males mating opportunities with the same female. The guarding male will, however, incur a cost in the form of time lost in securing matings with other females (Parker 1970; Parker 1974). PIAs may take a variety of forms:

i) prolonged copulation - e.g. in the rhopalid bug *Jadera haemotoloma* some matings may last up to 60 times as long as necessary (up to 11 days) for maximal female fertility (Carroll 1991). Prolonged copulation has been documented in seven orders of insects, particularly bugs (Heteroptera). In the bulb mite *Rizoglyphus robini*, prolonged copulations have been shown to increase male paternity by preventing female remating which otherwise occurs frequently (Radwan and Siva-Jothy 1996). Similarly, in the walnut fly, *Rhagoletis juglandis*, local male-biased sex-ratios result in prolonged copulations, consistent with a strategy of avoidance of sperm competition (Alonso-Pimentel and Papaj 1996). A sperm competition avoidance explanation requires that copulation duration be under male control. This has been shown in *D. melanogaster* (MacBean and Parsons 1967), *D. pseudoobscura* (Kaul and Parson 1965) and the yellow dungfly *Scatophaga stercoraria* (Muhlhauser *et al.* 1996).

ii) mating plugs - usually a secreted substance which interferes physically with subsequent matings, e.g. in Lepidoptera (Alcock 1994) and *Drosophila* (Alonso-Pimentel *et al.* 1994; Markow and Ankney 1988). In the case of honey bees, the whole male reproductive tract remains attached to the female;

iii) mate grasping - which is most common in Odonata (dragonflies and damselflies), where males hold females until oviposition begins (e.g. Cordero and Miller 1992). Grasping also occurs in Coleoptera (beetles), Heteroptera (bugs) and at least one Dipteran, *S. stercoraria* (references in Alcock 1994);

iv) mate monitoring - this is also common in the Odonata (Tsubaki *et al.* 1994), where males will hover close to females until oviposition begins, but less common in Coleoptera (e.g. Alcock 1991), Orthoptera (Alcock 1994) and Diptera (e.g. the cactus fly, *Odontoloxozus longicornis*, Mangan 1979).

Alternative hypotheses advanced to explain PIAs in adaptive terms have been summarised by Alcock (1994). One possibility is that prolonged copulation may allow extra nutrients or non-gametic components of the ejaculate to be transferred to the
female. For example, in the long horned grasshopper, longer copulations allow a larger spermatophore to be passed to the female (Gwynne 1986).

1.2.5 Fecundity-enhancing substances (Sex-peptides)

Fecundity-enhancing substances (FES) are peptides or proteins that are transferred to the female during copulation. The general function of these substances is to accelerate egg production by the female after mating. The result, in cases where sperm are not limiting, will be to increase per-mate fecundity of the male (except in cases where females remate before egg production begins). FES occur in five orders of insects; the Orthoptera, Hemiptera, Coleoptera, Lepidoptera and Diptera (references in Gillott 1988; and Smith et al. 1990). They are most widely studied in the Orthoptera (grasshoppers and crickets) and the Diptera (18 species, especially *Drosophila* and mosquitoes).

FES are synthesised in different parts of the male reproductive tract, depending on the species. They can be produced in the testes, ejaculatory duct, collateral gland complex (Orthoptera) or the accessory glands (common in Diptera). FES occur as either peptides e.g. the sex-peptide of *Drosophila melanogaster* (Chen and Buhler 1970; Chen et al. 1988) or PS-2 of *D. funebris* (Baumann 1974) or higher molecular weight proteins e.g. in *Melanopus sanguinipes* (Friedel and Gillott 1976). In the genus *Drosophila*, there is some cross-species activity of sex-peptides (Ohashi et al. 1991).

The effects of many FES have been studied, although in no case is the mechanism of action completely understood. Target sites may either be in the female reproductive tract or receptors in the haemolymph, probably on the brain (Kubli 1992). FES can act by stimulating oviposition (shown in all but one species studied) or egg development (Gillott 1988). In *D. melanogaster*, the stimulation of egg production by the sex-peptide occurs only once, the effect being permanent (e.g. Partridge et al. 1986). Therefore, since a female will remate many times, the influence of the sex-peptide on male reproductive fitness is reduced for subsequent males. The sex-peptide of *D. melanogaster* has been isolated, sequenced and the female response to the synthetic analogue studied (Chen et al. 1988; Schmidt et al. 1993).

A male can also increase his per-mate fecundity by increasing the length of time after the mating for which a female remains unreceptive to further mating attempts. Many of the FES also serve as receptivity-inhibiting substances (RIS). In *D. melanogaster*, the sex-peptide also inhibits receptivity for approximately one day (Chen et al. 1988; Scott 1987) and the RIS function of the sex-peptide is thought to share the same target the FES function (Schmidt et al. 1993). RIS occur in fewer species than FES, but their biochemistry is similar (Gillott 1988).
It is possible that there are additional, but less active FES and RIS in the ejaculate of many of the insects already studied. Homogenates of accessory glands and ejaculatory ducts contain a large number of proteins: 20 in *Musca domestica* (Terranova *et al.* 1972) and at least 87 in *D. melanogaster* (von Wyl 1976; Chen 1996). Possible examples of other molecules that may have an affect on fecundity or receptivity include the myotropic activity in various fractions of the secretions of locust accessory glands (Paeman *et al.* 1990) and esterase-6 in *D. melanogaster* (produced in the ejaculatory bulb, Gilbert *et al.* 1981).

1.2.6 Sperm precedence

1.2.6.1 Mechanisms, occurrence and measurement of sperm precedence

The third trait which can influence the per-mate fecundity is sperm precedence, defined as nonrandom differential fertilisation success among males (Lewis and Austad 1990). There are three types of mechanism that will result in sperm precedence. These are:

i) sperm competition, defined as the "competition within a single female between the sperm from two or more males for the fertilisation of the ova" (Parker 1970). For the purposes of this thesis "sperm competition" will be distinguished from "sperm displacement" and "sperm defence". The term sperm competition will be only used to refer to direct competition between sperm once they have entered the "fertilisation set" (Parker *et al.* 1990). The fertilisation set refers to the randomly mixed set of sperm that are used for fertilisation and which, in insects, could equate to any transiently stable mix of sperm in the sperm storage organs from which fertilising sperm are drawn. In organisms without sperm stores, the fertilisation set is a less useful concept since it is much more difficult to identify a subset of sperm that will be used for fertilisation. "Sperm displacement" and "sperm defence" will be used to refer to processes which are distinct from sperm competition, and which have the effect of altering the overall composition of the fertilisation set. This follows the similar distinction drawn by Simmons and Siva-Jothy (in press) and contrasts with the usual usage of the term sperm competition, which encompasses sperm displacement and sperm defence as well as sperm competition (Parker 1970; Waage 1984);

ii) preferential sperm use by females (Knowlton and Greenwell 1984; La Munyon and Eisner 1993; Olsson *et al.* 1996). With this mechanism, females are able to control the paternity of their offspring by favouring the sperm of one male over another, either by reducing the number of sperm passed by particular males (La Munyon and Eisner 1993; but see Simmons *et al.* 1996) or active selection of sperm after insemination. Unambiguous evidence of female choice is difficult to establish, not least because
intense selection between males for increased sperm precedence and sperm defence are usually operating concurrently; and iii) precedence caused by the morphology of the female sperm storage organs (Walker 1980; Eberhard et al. 1993; Siva-Jothy and Hooper 1996).

Evidence of sperm precedence has been documented in various groups including mammals (Ginsberg and Huck 1989), birds (Birkhead and Moller 1992; Gomendio and Roldan 1993), spiders and pseudoscorpions (Eberhard et al. 1993; Zeh and Zeh 1994), crustacea (Snedden 1990) and snails (Baur 1994). Sperm precedence has been studied in at least 61 species of insects, approximately 75% of which show last male advantage. A number of reviews of sperm precedence in insects have been published (see Parker 1970; Boorman and Parker 1976; Gwynne 1984b; Ridley 1989; also Simmons and Siva-Jothy in press).

The usual measure of sperm precedence is termed P2, which is simply the proportion of the total number of progeny sired by the second male (Boorman and Parker 1976). In general, P2 values for most insects are in the range 0.7 to 1, indicating that the second male sires most of the progeny. Examples of the entire range of P2 values between 0 and 1 have been observed. A variable affecting all P2 scores is the interval between matings. Sperm may either be used for fertilisations, leak from storage or become inviable with increasing time after the first mating. Therefore, the longer the interval, the less sperm usually remain from the first mating which will increase P2 values.

1.2.6.2 Sperm displacement, sperm defence and sperm competition

Of the three mechanisms of sperm precedence outlined above, sperm precedence caused by either sperm displacement, sperm defence or sperm competition is the most widely studied. This is due both to the relative intensity of selection on males and the technical difficulties of establishing evidence for active or passive sperm selection by females. The remainder of this section will concentrate on sperm displacement ability, sperm defensive ability and sperm competitiveness. The adaptations that have evolved to enable males to increase their reproductive success by one or more of these mechanisms will be considered.

Sperm displacement ability (SDA) is the degree to which a male can remove or incapacitate some or all of the sperm of other males previously stored. The process may involve physical removal, repositioning or dilution of prior sperm with the second male's ejaculate. By removing first male sperm, sperm displacement increases sperm precedence by increasing the ratio of second male sperm to first male sperm when sperm combine in the fertilisation set. In the absence of large differences in direct sperm competition, this will result in sperm precedence. Since the term sperm displacement
implies a mechanism for the process of sperm removal, the process has also been referred to as "pre-emption" of stored sperm (Parker 1984) and "sperm offence" (Clark et al. 1995).

Sperm defensive ability (Clark et al. 1995) describes the degree to which a male can reduce the probability of sperm of later males entering the fertilisation set. This is achieved by either reducing the probability of the female subsequently remating (see sections on courtship feeding, mate guarding and receptivity inhibiting substances above), increasing the number of first male sperm actually stored or somehow decreasing the effective number of sperm transferred in later matings. These have also been termed "anti-pre-emption" adaptations (Parker 1984).

Sperm competitiveness is the quality of sperm that results in a greater fertilisation success than that expected on the basis of the proportions of sperm in the fertilisation set. Adaptations to sperm competitiveness are more difficult to identify since relatively little is known of the details of sperm utilisation, especially in insects. Traits such as motility and longevity are thought to be important.

1.2.6.3 The 'insect model' and the 'vertebrate model'

In considering the basic modes of sperm competition, displacement and defence, Parker (1970) drew the basic distinction between the "insect model" and the "vertebrate model". The insect model is one where females possess long-term sperm storage organs and remate prior to exhaustion of stored sperm. In response to these circumstances, particular adaptations are thought likely to arise. Males will be selected for increased ability to displace stored sperm and for the ability to defend sperm against displacement. If displacement and/or defence are efficient, direct sperm competition may be of less importance (to the extent that basic fertility is still normal). The vertebrate model arises where females lack obvious sperm storage organs and sperm are relatively short-lived (but there are exceptions e.g. bats, Brock Fenton 1984; reptiles, Devine 1984; and urodeles, Halliday and Verrell 1984). Since vertebrates generally lack sperm storage organs and are larger, adaptations for sperm displacement are much less likely to arise. Since sperm are short-lived, sperm defence mechanisms may also be less selectively favoured. However, more adaptation for increased sperm competitiveness is expected, particularly increases in sperm concentration.

The degree to which selection will produce various adaptations will also depend on the relative costs of the adaptations to both sexes of the particular organism (Knowlton and Greenwell 1984; Parker 1984). Intermale conflict between SDA and sperm defence ability will be decided, in part, by the relative costs of the two sets of adaptations. There may also be male-female conflict over each set of adaptations. For example, accessory gland fluid in D. melanogaster contains a receptivity inhibiting substance (Chen et al. 1988). While passing accessory gland fluid at mating benefits
males in terms of sperm defence, there is a cost to females in terms of reduced lifespan (Chapman et al. 1995). The male paternity benefit appears to outweigh the female cost of mating. Conversely, in spiders, there appears to be widespread potential for sperm competition since females store sperm for long periods before use, males can mate with more than one female and males are unable to monopolise access to a female (Austad 1984). Despite these factors, complete first male precedence is usually the rule with no adaptations for sperm displacement having arisen. It is thought that the benefits females derive by eliminating the costs of repeated mating attempts by males outweigh benefits males could gain by increasing paternity (Austad 1984). Potential male benefits are reduced by the low frequency at which males are able to locate females. Thus different species will be expected to display different types and degrees of adaptive responses to selection for sperm precedence. The following sections discuss, in turn, sperm displacement, sperm defence and sperm competition and the particular traits that are thought to have arisen to maximise paternity in different species.

1.2.6.4 Sperm displacement adaptations

Most descriptions of adaptations for sperm displacement concern insects. Although potential for sperm displacement exists in some vertebrates, only speculative mechanisms have been described (for references, see Smith 1984). Accordingly, this section concentrates on insects, where sperm displacement is common. In species where each male is able to transfer sufficient sperm to fill the female sperm storage organs (such as *D. melanogaster*), sperm displacement will be the only method of introducing new sperm to the fertilisation set, particularly where remating occurs before significant amounts of sperm have been used (Parker 1970).

The only mechanisms of sperm displacement that are understood in detail are processes involving physical removal of sperm. In the Odonates (damselflies and dragonflies), males can remove the first male's sperm using arrays of recurved spines, bristles and barbs on the penis, before depositing their own sperm (Siva-Jothy and Tsubaki 1989; Waage 1979). Considerable variability exists among individuals in the degree to which previous male sperm is removed (Cordero and Miller 1992). Similar structures occur in the longicorn beetle *Psacoothera hilaris* (Yokoi 1990) and the millipede *Orthoporus pyrhocephalus* (Barnett et al. 1993), which are also suggested to function in sperm removal. A flushing mechanism occurs in the tree cricket *Trujalia hibinonis*. It is thought that the second male deposits ejaculate anteriorly in the sperm storage organ and the previous male's sperm is flushed posteriorly where it adheres to the shaft of the penis of the second male (Ono et al. 1989). This is effective in removing almost 90% of first male sperm. In the rove beetle, *Aleochara curtula*, spermatophore tubes extend distally into the spermathecae and then expand. The process of expansion pushes stored sperm out of the spermathecae, and at full extension, the spermatophore tubes are ruptured by spermathecal "teeth", releasing second male sperm into the
emptied spermathecae (Gack and Peschke 1994). A less obvious mechanism occurs in the carrion fly, *Dryomyza anilis*. The male must tap the female abdomen after mating in order to achieve sperm displacement, but the mechanism of this process is not understood (Otronen 1990; Otronen and Siva-Jothy 1991). Sperm removal has also been observed in higher organisms. Males dunnocks, *Prunella modularis*, appear to be able to induce females to eject sperm from earlier matings by cloacal pecking (Davies 1983).

In some species, sperm is not removed from sperm storage organs, but instead moved to more unfavourable positions in the sperm storage organs. In the dragonfly *Sympetrum rubicundulum*, inflatable lobes of the penis push stored sperm to the end of the spermathecae, distal to the oviduct, presumably preventing access for those sperm to the oviduct (Waage 1984). Similar ejaculate packing may also occur in some butterflies (Walker 1980; Drummond 1984) and in the urodele, *Ambystoma tigrum*, where spermatophores of later male are placed directly on top of spermatophores of previous males (Halliday and Verrell 1984). This prevents sperm from earlier spermatophores having access to fertilisations.

In organisms where obvious adaptations cannot be identified, sperm displacement modelling has been used to infer likely mechanisms of displacement (Parker et al. 1990; Parker and Simmons 1991; Eady 1994; Sakaluk and Eggert 1996). These models can help identify the adaptations that are important in sperm displacement. The models have been developed on the basis of a variety of different ways in which ejaculates are thought to interact. The simplest models are "raffles", where all sperm transferred by both males enters the fertilisation set (Parker et al. 1990). The raffles can be unweighted ("fair raffles") or weighted ("loaded raffles") depending on whether the interval between matings affects fertilisation success. These models are most applicable to organisms without sperm stores of fixed volumes e.g. most vertebrates and some insects such as the field cricket, *Gryllus bimaculatus*, which has expandable spermathecae (Parker et al. 1990). A second type of model includes sperm storage organs of a fixed volume, which necessitates sperm displacement. Once full, sperm can only enter the fertilisation set by displacing already stored sperm (Parker et al. 1990). Sperm mixing can take place either instantaneously during displacement or only once displacement is complete. A variant of these models assumes that rather than individual sperm being displaced, volumes of sperm store fluid (which may or may not contain sperm) are displaced by similar volumes of seminal fluid (containing sperm) of the most recent male (Parker and Simmons 1991). This type of model was shown to provide a good fit to the patterns of displacement observed in the dungfly *Scatophaga stercoraria* (Parker and Simmons 1991). These models can be modified to allow for both constant rates of sperm loss from storage organs between matings and the situation where only a proportion of sperm transferred actually enters the sperm storage organs (Eady 1994). These modifications were used by Eady (1994) to investigate sperm displacement in *Callosobruchus*
It was shown that displacement data could be accounted for by both modified sperm-for-sperm displacement models or modified volumetric displacement models, but not by a model where mixing is postponed until after displacement. The displacement models have also been modified to allow for partial sperm displacement of first male sperm by second male sperm (Sakaluk and Eggert 1996). The rationale for this modification is the idea that sperm stores can be less than perfectly elastic, so that second male sperm are accommodated by a combination of expansion of the storage organ and displacement. This model provided a good fit of sperm precedence data from the decorated cricket, *Gryllodes sigillatus* (Sakaluk and Eggert 1996).

These models can therefore identify adaptations for sperm displacement that are important in particular species. From the examples above, variation in sperm numbers are likely to be significant where spermathecae are expandable (*G. bimaculatus*) while both volume of ejaculate and sperm concentration are likely to be important in *S. stercoraria*, where volumetric displacement occurs.

### 1.2.6.5 Sperm defence adaptations

For insects, passing sufficient sperm to fertilise most or all of a female's eggs or completely fill the sperm storage organs is rarely sufficient to ensure that females do not remate. Reasons favouring female remating include, in addition to maintaining sperm supply, ensuring the presence of viable sperm, avoiding sperm storage or maintenance costs, nutritional benefits, increasing genetic diversity of offspring and avoidance of costs of rejection (Alcock et al. 1978) and increase in effective population size (Griffiths et al. 1982). Males of most species will benefit in terms of increased paternity to the extent that they can either prevent or delay female remating. The major behavioural adaptations that serve to prevent female remating are courtship feeding, mate guarding and receptivity inhibiting substances (discussed in sections 1.2.3 - 1.2.5 above).

Another adaptation that may function to reduce female remating rates are "filler" sperm. Lepidoptera commonly produce apyrene (anucleate) sperm which may function simply as physical bulk in the female reproductive tract (Cook and Gage 1995; Silberglied et al. 1984). The apyrene sperm do not enter the sperm storage organs, but their bulk in the bursa copulatrix may function as a physiological stimulus preventing remating. Similar strategies may occur in Drosophilids, where both *D. pseudoobscura* and *D. subobscura* produce sperm of two lengths. It has been shown that only the longer morphs are used for fertilisations (Bressac and Hauschteck-Jungen 1996; Snook et al. 1994).

Sperm size may also serve a sperm defence role by making sperm harder to displace when sperm size matches the size of the female sperm storage organ (Birkhead and Hunter 1990). Sperm shorter than the storage organ may be subject to stratification by later sperm, while sperm of the same length as the storage organ may ensure each
sperm has an equal chance of exiting and fertilising ova. This has led to the prediction of an arms race between male interests (in maximising fertilisation probability) and female interests (control over paternity), in which sperm storage organs and sperm both increase in size (Briskie and Montgomerie 1993). There is some weak support for a relation between sperm length and the length of female sperm storage organs in Drosophilids (Pitnick and Markow 1994b).

1.2.6.6 Sperm competition adaptations

Two factors have made it difficult to identify traits that have arisen as a direct consequence of selection for increased sperm competitive ability. Firstly, the lack of detailed understanding of the mechanics of fertilisation, especially the movement of sperm from the sperm storage organs, has made it difficult to identify the functional significance of variable sperm traits and, secondly, adaptations in female physiology make it difficult to determine whether potential sperm competition adaptations preceded evolution of the female traits (Walker 1980).

(a) Sperm number

When sperm mix in the fertilisation set, numerical superiority is the simplest way to increase the probability of successful fertilisations (Parker 1982). In insects, the ability to transfer large numbers of sperm may not be subject to the same selective intensity as in vertebrates because sperm storage organs with limited capacity may reduce any benefits derived from increasing sperm number beyond that necessary to fill the sperm storage organs. However, there are four situations in which this generalisation will not hold. Firstly, expandable sperm storage organs (as in the cricket Gryllus bimaculatus), make numerical sperm dilution an important component of sperm precedence (Simmons 1987). Secondly, where the mechanism of displacement is relatively inefficient, large numbers of sperm may overcome the inefficiency. The damselfly Coenagrion scitulum lacks the penile spines to remove sperm, which are common in other Odonates (Cordero et al. 1995). Instead, males repeatedly inseminate the female, packing the sperm of up to five consecutive inseminations into the sperm storage organs. Packing down reduces the volume of the ejaculates, suggesting sperm number rather than volume is important for paternity. Thirdly, the particular biology of the species may be significant. In the fly, Dryomyza anilis, males repeatedly inseminate females since females can discharge sperm between inseminations by the same male (Otronen 1994). Finally, sperm number would be important if displacement occurred by sperm mixing (dilution) outside the volumetric limitations of the storage organs.

Where sperm and ejaculate resources are ultimately limiting, individuals can respond to the threat of sperm competition by partitioning of ejaculate to match the probability of sperm competition. Where the probability of sperm competition is low, a
strategy of inseminating many females each with the minimum number of sperm required to ensure paternity will be favoured over a strategy of larger inseminations of a few females. Partitioning can also respond to changing probabilities of mating with already inseminated females (i.e. the probability of sperm competition). In both the butterfly, *Pieris rapae*, and the bushcricket, *Requena verticalis*, the probability of mating with a virgin female declines rapidly with time after emergence. Accordingly, sperm numbers are increased in later spermatophores (Simmons et al. 1993; Cook and Wedell 1996). Variation in sperm number may also occur in response to more immediate perceptions of the probability of sperm competition. Males can respond to this local competition by increasing the number of sperm ejaculated in a variety of species: *Ceratitis capitata* (Gage 1991), *Tenebrio molitor* (Gage and Baker 1991), *Acheta domesticus* and *Gryllodes supplicans* (Gage and Barnard 1996). Sperm partitioning also occurs in *D. pachea* which has giant sperm and where sperm competition is potentially intense (Pitnick and Markow 1994b). This may, however, be due to severe sperm limitation and particular ecological conditions which may favour submaximal insemination despite the risk of sperm competition (Pitnick and Markow 1994b). Non-fertilising (apyrene) sperm produced as "cheap filler" (Gage 1994) may serve to reduce the cost of sperm production relative to gain in paternity.

In many vertebrates, the ability to increase sperm numbers appears to be of greater significance. A major determinant of fertilisation success in male muroid rodents is the relative concentrations of sperm. Males allowed to ejaculate repeatedly increase their paternity rate over males ejaculating once, regardless of mating order (Dewsbury 1984). In the zebra finch, *Taeniopygia guttata*, the relative numbers of sperm in the female reproductive tract are sufficient to account for differences in sperm precedence (Colegrave et al. 1995; see also Smith et al. 1996). Testis size (and hence sperm production) in primates is relatively greater in groups where the risk of sperm competition is higher due to female promiscuity (Harcourt et al. 1981; Möller 1991). Together, these results suggest that increasing sperm number is important where females lack sperm storage organs (Harvey and Harcourt 1984).

(b) Sperm size

In contrast to sperm number, little is known about the effect of sperm size on sperm competitiveness. In mammals and birds, there is conflicting evidence for whether larger sperm are favoured by increased sperm competition intensity. In primates and rodents, larger sperm may occur in species where females remate more often (Gomendio and Roldan 1991; but see Harcourt 1991). In birds, sperm length is correlated with morphology of female sperm storage tubules rather than intensity of sperm competition (Briskie and Montgomerie 1992). Longer sperm may be able to swim faster (Briskie and Montgomerie 1992; Gomendio and Roldan 1991) which may be advantageous where, as in vertebrates, sperm scramble for fertilisations (Harcourt 1991).
In insects, phylogenetic aspects of testis size and sperm size and number have been recently investigated in butterflies (Gage 1994) and the Drosophilids (Pitnick 1996). Although it is generally thought that sperm should remain small and numerous (Parker 1982), relatively large sperm have arisen in a number of different insects. Larger species of butterflies were found to have relatively larger testis and to produce correspondingly larger (but not more) sperm. Of the eupyrene and apyrene butterfly sperm, only the eupyrene sperm increase in length as testis size increases. The fact that only the eupyrene sperm, and not the apyrene sperm, enter the spermathecae and therefore compete directly with other sperm, suggests an advantage (possibly based on energetics) in sperm competition for longer sperm (Silberglied et al. 1984). Similarly, in both *D. pseudoobscura* and *D. subobscura*, it has been shown that only the longer morphs are used for fertilisations (Bressac and Hauschteck-Jungen 1996; Snook et al. 1994). Increased egg-provisioning potential of larger sperm does not appear adequate to explain longer sperm in insects (Karr and Pitnick 1996; Simmons and Siva-Jothy in press). Instead longer sperm may have advantages in terms of motility, survival or spermathecal filling. Motility may be important in processes such as exiting sperm storage organs, even though the sperm of many of the *Drosophilae* are much longer than the female reproductive tract. Larger sperm may have increased longevity, which may be advantageous where longer survival overcomes the effects of some displacement mechanisms (e.g. sperm repositioning). Larger sperm may be more effective in filling spermathecae, playing a role in sperm defence, rather than sperm competition (Dybas and Dybas 1981). However, while there is no firm evidence for any of the proposed advantages of longer sperm, the trait persists in species with high levels of sperm competition. The Drosophilid species with longer sperm (*D. hydei* and *D. pachea*) inseminate the females with fewer sperm than required to fill the sperm storage organs (Pitnick and Markow 1994a; Pitnick and Markow 1994b), resulting in high levels of sperm mixing and, presumably, sperm competition. Larger sperm have been shown to be more successful competitors and to achieve more fertilisations in the bulb mite, *Rhizoglyphus robini*, which has aflagellate amoebic sperm (Radwan 1996).

Finally, sperm quality can be important. In *Caenorhabditis elegans*, male sperm always achieve precedence over a hermaphrodite's own sperm. The advantage appears to lie in sperm quality, as number and differences in activation do not explain the observed patterns of precedence (La Munyon and Ward 1995).

### 1.3 Sperm precedence in *D. melanogaster*

#### 1.3.1 Reproductive biology

A great deal is known about the reproductive biology of male *D. melanogaster*. From these accumulated observations and the general patterns of adaptations arising in
other species in response to sperm precedence, some conclusions can be drawn about the likely mechanisms of sperm precedence in *D. melanogaster*.

In *D. melanogaster*, females remate before stored sperm is exhausted, as frequently as every two days (Parker 1970; Prout and Bundgaard 1977; Marks *et al.* 1988; Van Vianen and Bijlsma 1993). During the first two days after a normal insemination, sperm use is highly inefficient (i.e. many sperm used per egg laid) and up to half the stored sperm may be used (Gilbert 1981b; although less sperm is used where high quality food is not freely available, Trevitt *et al.* 1988). At around 2-3 days, efficiency of sperm use increases to the point where only one sperm per egg is used, after which increasing numbers of unfertilised eggs are laid. Since this must be highly maladaptive for the female, there is presumably strong selection for the female to remate when she is unable to fertilise all eggs laid. It seems unlikely that females in the wild would delay remating until sperm stores are as low as 10% (as suggested by Letsinger and Gromko 1985; Newport and Gromko 1984), since this would incur a considerable cost to the female in terms of ability to fertilise eggs.

Male *D. melanogaster* are sexually mature within 24 hours of eclosion, which coincides with the filling of the seminal vesicles and accessory glands. Based on progeny counts and direct sperm counts, males usually pass enough sperm at each mating to fill the female sperm storage organs. The capacity of these storage organs is thought to total between 500-700 (Fowler 1973; Lefevre and Jonsson 1962) and 1000 sperm (Gilbert 1981a). It is likely that males often pass considerably more sperm, up to 4000-5000 (Fowler 1973; Gilbert 1981a). Sperm storage is complete within one to a few hours (Gilbert 1981b), and transport to the storage organs may involve movement along grooves observed near the entrance to the seminal receptacle (Fowler 1973). This movement probably involves the associated tubules and other accessory gland proteins (Neubaum *et al.* 1996). The tubules are stored along with the sperm. Sperm are not passed continuously throughout copulation, but instead begins at a variable time after the initiation of the copulation (1-10 min). Rates of sperm transfer have not been measured. A spermatophore-like mass ("sperm sac") has been observed in the vagina after mating, containing sperm and accessory gland products, notably paragonial (accessory gland) tubular elements, which remain associated with sperm in storage (Alonso-Pimentel *et al.* 1994; Fowler 1973). It is assumed that accessory gland products are transferred continuously throughout copulation (e.g. Harshman and Prout 1994), although this has not been shown experimentally.

Accessory gland fluid is essential for male fertility. Males whose accessory glands had been exhausted by continuous matings are effectively sterile, even though still possessing mature sperm (Hihara 1981; Lefevre and Jonsson 1962). Hihara (1981) showed that this sterility was due not to lack of sperm but of accessory gland fluid. This is consistent with a vital role for accessory gland secretions in sperm transfer and/or
storage. A number of accessory gland proteins and peptides (Acps) have been cloned from *D. melanogaster* (DiBenedetto *et al.* 1987; Monsma and Wolfner 1988; Schafer 1986). The functions of the accessory gland proteins, apart from the sex-peptide, have not been determined in detail. Acp26Aa has been shown to affect egg production for one day after mating (Herndon and Wolfner 1995). Acp36DE, the largest of the Acps, has been shown to localise at the junction of the oviduct and the uterus, suggesting that it may have a role in sperm transport or storage (Neubaum *et al.* 1996). An examination of the naturally occurring variation in accessory gland proteins has shown high levels of interspecific divergence (Thomas and Singh 1992). The coding sequences of one of the Acps, (Acp26Aa, Aguadé *et al.* 1992) has shown that there is an excess of sequence variation beyond that which can be accounted for under the assumption of neutrality. High variation is often taken to indicate the action of selective forces.

A number of studies have investigated sperm precedence in *D. melanogaster*. These studies indicate that sperm precedence is due to actual displacement of stored sperm (Boorman and Parker 1976; Lefevre and Jonsson 1962; Prout and Bundgaard 1977; Scott and Williams 1993). After rematings that occur while the sperm storage organs are still relatively full, the number of progeny that a female produces is not greatly increased, but the progeny are almost largely sired by the second male (e.g. Prout and Bundgaard 1977). This indicates that the fertilisation set has changed in content without significantly increasing in size.

Male accessory gland fluid is able to induce loss or displacement of sperm in the absence of second male sperm, as shown by males which pass no sperm (XO males) (Scott and Richmond 1990). However, Scott and Richmond (1990) were unable to exclude conclusively the possibility that the displacement observed was the result of preferential remating by the females that had low initial sperm stores (these females would subsequently produce fewer first male progeny, which would erroneously indicate sperm displacement, see Scott and Williams 1993). Harshman and Prout (1994) also observed sperm displacement using a different type of spermless male (*tudor* males). In addition, Harshman and Prout were able to show in the same paper that males that do not transfer products of the main cells of the accessory glands do not induce any displacement. This strongly suggests that accessory gland fluid contains a substance active in sperm displacement. Differences in both male and female genotype also affect sperm displacement in *D. melanogaster* (various studies cited in Gromko *et al.* 1984a). Studies using marked strains usually find that P2 values vary consistently according to the particular combination of male and female strain used. However, these differences have not been demonstrated to be due to differences in the ability to displace sperm, and may instead be due to differences in number or quality of sperm transferred.
1.3.2 Possible mechanisms of sperm displacement

Mechanisms involving physical displacement of sperm (as is common in the Odonata) are unlikely in *D. melanogaster*, since no male appendages are large or long enough to enter the uterus or to approach the entrances to the seminal receptacle and spermathecae (Miller 1950). Since females usually remate still having considerable amounts of sperm in storage, sperm precedence in *D. melanogaster* is likely to occur predominantly by displacement of sperm. The details of the mechanism are unknown. The number of sperm passed by the second male is important in a number of other species, and so could also be important in *D. melanogaster*. However, the role of accessory gland fluid must also be incorporated into any mechanism.

a) Sperm numbers

Since *D. melanogaster* are not believed to have expandable sperm storage organs (Fowler 1973), variation in sperm number will affect displacement to the extent that sperm mixing determines sperm displacement. Although sperm displacement can occur in the absence of sperm (Harshman and Prout 1994; Scott and Richmond 1990), it is not known whether sperm play an additional role in the process of sperm displacement. The excess of sperm passed by males can be explained in two ways. Firstly, it is possible that sperm storage is inefficient and that large numbers of sperm are required to ensure maximal storage. Alternatively, this excess of sperm could be important for SDA if sperm displacement did involve a process of dilution of first male sperm. If sperm numbers are important in displacement, correlations would be expected between SDA and traits that might affect the number of sperm transferred such as body size and possibly copulation duration. Also, a correlation between SDA and male productivity could be apparent.

In other species, the transfer of greater sperm numbers is especially important where females have expandable sperm storage organs, can otherwise manipulate sperm in storage or where males "know" the likelihood of mating a virgin or already mated female. None of these would appear to apply to *D. melanogaster*. It is not known whether male *D. melanogaster* respond to higher numbers of immediate rivals by changing the numbers of sperm transferred.

b) Accessory gland fluid

Given that accessory gland fluid is necessary for sperm displacement, there are three possible ways in which it may influence sperm displacement.

Firstly, displacement may occur as an integral part of sperm storage. The process of storing second male sperm may incidentally cause first male sperm to be released or repositioned in storage and P2 would be independent of first male effects, since the
process would be dominated by the second male storage ability. Since there is little additive genetic variation for male productivity (Hughes 1995), there may also be little additive genetic variance for displacement in this case.

Secondly, the degree of displacement may be determined by the volume of accessory gland fluid passed. If accessory gland fluid is passed at a constant rate during copulation, SDA would covary with copulation duration. There may also be a relation between male size and displacement if the rate of accessory gland fluid transfer was size-dependent. Alternatively, larger males, which may pass more accessory gland fluid may have higher SDA.

Finally, particular proteins in the accessory gland fluid may mediate sperm displacement in addition to any role they may have in sperm storage. These proteins would presumably be subject to selective forces proportional to their ability to improve SDA. There may or may not be additive genetic variance for these loci. This mechanism may be independent of body size and/or copulation duration, unless size determines the quantity transferred and the response to the active component is concentration dependent.

c) Modelling sperm displacement

Modelling has been used in a number of other species to study mechanisms of sperm displacement. The fitting of models requires that certain basic information be available, including estimates of $P_2$, estimates of the number of sperm transferred by each male and estimates of the sperm storage capacity of the female (Parker et al. 1990). Rates of sperm transfer have been estimated in other species from copulation duration, spermatophore attachment times and direct sperm counts. However, in *D. melanogaster* measurement of sperm transfer is complicated by the fact that sperm transfer does not appear to vary linearly with copulation duration. Accordingly, data has not been applied to the available models. For this reason it is important to determine if any relationship can be found between variables such as size or copulation duration and $P_2$.

1.3.3 Possible mechanisms of sperm defence

As there is no appreciable mate-guarding or seminal feeding in *D. melanogaster* (Bownes and Partridge 1987), sperm defence must rely mainly on physiological and biochemical mechanisms.

The receptivity inhibiting function of the *D. melanogaster* sex-peptide has a role in sperm defence via its effect on female remating (Chen et al. 1988). In addition, remating rates are also affected by the "sperm effect" (Manning 1962; Scott 1987). The "sperm effect" describes the reduction in female remating rates observed when those females have greater numbers of sperm in storage. Variation in either the receptivity
inhibiting function of the sex-peptide, other receptivity inhibiting components of accessory gland fluid or male ability to manipulate the female perception of the "sperm effect" could contribute to sperm defence. While direct selection for male ability to affect female remating rates has failed (Newport and Gromko 1988), a genetic basis for male ability to influence female remating rates was found by Service and Fales (1993) and Service and Vossbrink (1996).

Apart from effects on female remating rates, genetic variation also appears to exist for other aspects of sperm defence. In experiments where females were remated after a fixed period, four accessory gland protein alleles were found to correlate with sperm defence (Clark et al. 1995), one of which, Acp26Aa, had previously been shown to have a high level of sequence variability (Aguadé et al. 1992). In the experiments of Service and Fales (1993), intraspecific variation was found in the ability of the sperm of different first males to "survive" rematings. These findings suggest that there may be appreciable additive genetic variance in sperm defence ability.

While the few studies of sperm defence have had success in identifying factors associated with the variation in sperm defence, the mechanism remains unclear. Proposed mechanisms have to be able to account for the following results:

i) first males can vary in their effect on female remating rates and the number of their sperm surviving displacement (Service and Fales 1993);

ii) variation in defensive ability is associated with variation in accessory gland alleles (Clark et al. 1995); and

iii) females mated to poorly defending males lay more eggs initially and have less sperm in storage at the time of remating (Service and Vossbrink 1996, but this result may be dependent on the particular experimental conditions used).

These results indicate that sperm defence in *D. melanogaster* is not only dependent on a male's ability to delay female remating. Sperm defence may be due only to differences in male fertility (i.e. number of sperm passed), but this seems unlikely given the correlation with Acp alleles and the suggestion of additive genetic variance for defence. It is probable, therefore, that sperm defence also involves accessory gland fluid, possibly interacting with sperm. Accessory gland fluid may defend sperm directly through physical associations with either the stored sperm or the sperm storage organs, hindering displacement. Alternatively, the effect of accessory gland components could be indirect, with variation in accessory gland fluid quantitatively affecting the numbers of sperm stored which could, in turn, affect SDA.

In *D. melanogaster*, the study of defence is complicated by variation in experimental designs, particularly remating protocols, which complicate the comparison of results. Therefore, further investigations of sperm defence will require methods which avoid or control for these compounding effects.
1.4 Thesis outline

In this thesis, an analysis was undertaken of SDA, a component trait of total fitness in male *D. melanogaster*. Little is known of the genetics or mechanism of sperm displacement in *D. melanogaster*. Therefore, a variety of basic approaches were used in the present work to investigate SDA. Some of the experimental work investigated aspects of sperm defence. The experiments undertaken are presented in the following order:

Chapter 3: The heritability of SDA in an outbred cage population was examined. This gives an indication of the selective intensity acting on SDA. Correlations with other traits were also investigated, as they may have confounding effects on the measurement of SDA.

Chapter 4: Since the degree of sperm displacement may also depend on the genotypes of both the female and the first male, a method was investigated that allows detection of the role of variation in those two genotypes on sperm displacement and related traits. The method involved using crosses between different chromosome extraction lines to provide large numbers of individuals with identical second and third chromosomes.

Chapter 5: The possibility exists that males may be able to preferentially displace the sperm of other males, in preference to their own. This possibility was investigated using fertile males to inseminate females, after which the males could be sterilised by irradiation and remated to the same females.

Chapter 6: The effect of variation in copulation duration on SDA was investigated using both interrupted and uninterrupted matings. Both spermless males and normal males were used as second males.

Chapter 7: The effects of variation in body size on male productivity, remating speed and SDA was investigated. Males derived from lines selected for both increased and decreased size were used, as well as males from hybrid crosses.

Chapter 8: Since accessory gland fluid may play a role in sperm defence, the effect of the presence or absence of accessory gland fluid on the productivity of subsequent matings was studied.
2

General Methods

2.1 Drosophila melanogaster stocks

2.1.1 Wild type Dahomey

All wild type flies used were from an outbred wild type stock collected in Dahomey, West Africa (now Benin) in 1970. The stock has since been maintained in mass culture in population cages at 25°C on a 12/12 hour dark/light cycle. The cages receive three bottles containing 80ml SY food (see section 2.2 below) each week on a four week rotation.

2.1.2 sepia stock: (Chapters 3, 4, 6, 7, and 8)

A sepia (se) stock was constructed by backcrossing a se strain from the Umea stock centre (stock number 71400) to the Dahomey stock en masse for four generations. After each backcross generation, F2 homozygotes were selected for the next backcross. The stock was maintained as duplicate cage populations, in an identical manner to the Dahomey cage stocks, for six months before being used in any experiments.

2.1.3 Irradiated males: (Chapters 5 and 6)

Male flies were irradiated in a paper grid in a plastic petri dish with a 10 kR X-ray dose (250 kV tube voltage, unfiltered) which is sufficient to sterilise male D. melanogaster permanently (Welshons and Russel 1957). Sterility is not immediate because irradiation does not incapacitate all mature sperm and sperm derived from mature spermatocytes (Ashburner 1989). To exhaust these surviving viable sperm, irradiated males were 'brooded' with 3 successive groups of 3 virgin females in the days following irradiation.

2.1.4 son-of-tudor males: (Chapters 6 and 8)

tudor is a maternal-effect gene which results in the embryos of homozygous females lacking polar granules and consequently developing without germ cells (Boswell and Mahowald 1985). In these experiments, homozygous tudor females were taken from a tud1 bw sp/SM5 stock and crossed to wild type Dahomey males to produce male offspring that are phenotypically normal, but lacking testes, and are therefore sterile. Since the accessory glands are derived from somatic tissue, they develop
normally in these son-of-tudor males (referred to simply as tudor males). tudor males mate normally and pass normal amounts of accessory gland fluid but no sperm.

2.1.5 XO males: (Chapters 6 and 8)

XO males were produced from crosses between Dahomey virgin females and males from a C(1)RM, yw/O stock (described in Lindsley and Zimm 1992). Males from the C(1)RM, yw/O stock are X^YO, and their sons XO. A Dahomey chromosomal background had previously been introduced into the parental C(1)RM, yw/O stock as described in Chapman (1992). XO males pass accessory gland fluid but no functional sperm. Sperm maturation is affected at a late stage so that bundles of sperm are formed in the testis, but these sperm never become motile (Safir 1930).

2.2 Maintenance of stocks

2.2.1 Media

The standard fly medium used in all experiments was based on the traditional cornmeal/agar/treacle recipe used in many Drosophila labs (Roberts 1986; Ashburner 1989). The particular recipe used for "standard" food was:

- 1.0% (w/v) agar
- 8.5% sugar
- 6.0% maize meal
- 2.0% dried yeast extract
- 2.5% (v/v) of a 10% Nipagin solution in ethanol.

SY (Sugar-Yeast) food used to feed cage stocks was:

- 2.0% (w/v) agar
- 10.0% sugar
- 10.0% dried yeast extract
- 3.0% (v/v) of a 10% Nipagin solution in ethanol
- 0.3% (v/v) propionic acid.

Low Food used to suppress egg laying was:

- 1.3% (w/v) agar
- 1.0% maize meal
- 1.0% dried yeast extract
- 3.0% (v/v) of a 10% Nipagin solution in ethanol.

Grape juice medium used for egg-laying and subsequent collection of larvae was:

- 30% grape juice
- 4.0% agar.

Apart from population cages, flies were cultured in either 75 x 25 mm glass vials with 7ml of food or 1/3 pint milk bottles containing 80ml of food.
2.2.2 Collection of stocks

Unless otherwise stated, all experimental flies were reared at a standard density of 60 larvae/vial. Eggs were collected either directly from cage stocks (by placing a petri dish containing grape juice medium in the cage for a few hours) or from smaller "laying pots" containing parental flies and a small amount of grape juice medium. First instar larvae hatching from the collected eggs were picked with a brush and transferred to vials containing 7ml of standard cornmeal/yeast/agar medium. All flies were reared at 25°C and collected by immobilisation over ice within 8 hours of eclosion. Sexes were stored separately, up to 20 flies per yeasted vial. Virginity was checked by checking storage vials for larvae. All transfers of flies were performed by aspiration or by chilling briefly over ice.

2.2.3 Production of mated females

Almost all the experiments presented involved the mating of females with at least two males. Since, in practice, it is impossible to control the number of sperm a female stores from the first mating, all that could be done in the attempt to produce uniformly inseminated females was to standardise the conditions of the first mating as far as possible. A system was developed in which large numbers of uniformly mated females could be produced, the majority of which would remate two days after the first insemination if paired with a male for a sufficient length of time. This was felt to be a biologically realistic remating interval (e.g. Van Vianen and Bijlsma 1993) and was also a stage at which the females still had large numbers of sperm in storage, important if variation in sperm displacement ability was to be detected.

The final system involved pairing 25 3-4 day old virgin females with a slight excess of males (27-30) in 1/3 pint bottles with 50ml of yeasted standard food soon after lights on. The bottles were left undisturbed for 6-7 hours and the males removed. The females could then lay freely before being used in remating experiments on the second morning after the initial matings (i.e. 38-40 hours later). It was not necessary to ensure that first matings were single copulations (unlike rematings), and it was actually preferable that during the 6-7 hour period any poorly inseminated females had the opportunity to remate. This ensured a higher percentage of females were maximally inseminated at the end of the 7 hours.

Ideally, these females should all be equally well inseminated, so that subsequent measures of sperm displacement are not biased, since having females with low numbers of sperm in storage will inflate apparent displacement and such females may also remate faster (Manning 1962; Fukui and Gromko 1989). However, there is no reliable indicator of the level of insemination of a mated female. A number of papers have used the female productivity (i.e. the number of adult progeny produced by a female) as an indicator of the relative numbers of sperm in storage and concluded that female remating is largely dependent on the numbers of sperm in storage (Gromko et al. 1984;
Newport and Gromko 1984; Letsinger and Gromko 1985). However, the results in those experiments were obtained when a short remating period was used, typically 2 hours per day. As noted by Newport and Gromko (1984), with longer pairings of males and females (24 hours), this sperm dependence breaks down.

The degree of sperm dependent remating in the present experiments (i.e. at least 8 hour confinements of the remating pairs) was tested using the criteria of Gromko et al. (1984). The correlations between female productivity and remating speed were measured in experiments using the experimental design outlined above are shown in Table 2.1. The lack of any significant correlations indicates that, at least by the criteria of female productivity, there is no overall sperm dependence in the remating design used in the present experiments. However, in the first two hours after pairing there was evidence of a non-significant negative correlation, consistent with some of the first females remating because of lower sperm stores (see discussion in Chapter 3).

<table>
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<tr>
<th></th>
<th>n</th>
<th>correlation</th>
<th>P</th>
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<tbody>
<tr>
<td>Day 1</td>
<td>405</td>
<td>0.055</td>
<td>0.27</td>
</tr>
<tr>
<td>Day 2</td>
<td>429</td>
<td>0.026</td>
<td>0.59</td>
</tr>
<tr>
<td>Day 3</td>
<td>419</td>
<td>0.055</td>
<td>0.26</td>
</tr>
</tbody>
</table>

Table 2.1. The correlations between the total number of progeny produced by a female before remating and the time taken to remate after pairing with the second male. Data collected over three days of an experiment reported in Chapter 3 (n is the number of females tested).

It could be argued that female productivity is not a useful indicator of sperm numbers, since the relation between variation in the number of sperm transferred and female productivity is not clear. It is known that the numbers of sperm used are often in large excess over the numbers of progeny produced (Gilbert 1981b). Whether the degree of this excess influences female productivity is unknown. Nevertheless, samples of the sizes shown in Table 2.1 should have been large enough to detect any systematic effect of under-insemination of females on remating time.

2.2.4 Statistical analysis

In most published studies since Boorman & Parker (1976), sperm displacement has been measured in terms of the P2 statistic, which is simply the proportion of the post-remating progeny sired by the last male. Since in D. melanogaster, P2 and angular transformed P2 are rarely, if ever, normally distributed (see Figures 2.1 and 2.2), another transformation of the raw data was sought. The adjusted ratio of second to first male progeny, $a/(b+1)$ was used, where $a$ is the number of second male progeny and $b$ the number of first male progeny (Haldane 1956; Hughes 1997). In these experiments, this
statistic was transformed by taking the fifth root. This gave a normally distributed measure of sperm displacement, as shown in Figure 2.3 (checked using a Shapiro-Wilk W test, P < 0.05). A third root transformation had been previously used by Hughes (1997) and produced very similar results in all parametric tests, but was found to be non-normal in the present study (using a Shapiro-Wilk W test).

For highly skewed data, such as the number of first male progeny produced after rematings or measures of daily egg and progeny production, non-parametric Kruskal-Wallis one-way tests were used. Where significant heterogeneity was indicated, multiple comparisons were calculated according to the method of Siegel and Castellan (1988) using a Basic program written for the Macintosh by me.

Figure 2.1 Distribution of mean P2 values (each calculated from up to three rematings) in the two generations used in the heritability experiments in Chapter 3.
Figure 2.2 Distribution of the mean arcsin transformed P2 values calculated from the same data as shown in Figure 2.1.

Figure 2.3 Distribution of mean sperm displacement ability (SDA) values calculated from the same data shown in Figure 2.1. This transformation of the data, described in section 2.2.4, was applied to all sperm displacement measures in this thesis.
3

Heritability of Sperm Displacement Ability

3.1 Summary

Variation in reproductive success can result from differences in the ability of males to displace the sperm of previous males from female sperm storage organs, i.e. variation in sperm displacement ability (SDA). Since the trait may be subject to strong directional selection, it is of considerable interest to measure the additive genetic variance of SDA. Using conventional methods based on counts of adult offspring, the heritability of SDA in Drosophila melanogaster was measured as 0.10 ± 0.08. However, since a number of factors could produce variation between sires in the relative numbers of adult progeny, correlations between SDA and a variety of other traits were also measured. A significant positive genetic correlation between SDA and pre-adult viability of offspring was found. Since both traits affect the relative numbers of adult progeny sired and both are heritable, apparent heritable variation in SDA may be largely due to heritable variation in pre-adult viability.

3.2. Introduction

The opportunity for sperm competition arises in many insect species, including Drosophila melanogaster, because females remate before stored sperm is exhausted (Parker 1970; Prout and Bundgaard 1977; Marks et al. 1988; Van Vianen and Bijlsma 1993). In D. melanogaster, the mechanism of sperm competition involves later males displacing the sperm of earlier males from the female sperm storage organs (Lefevre and Jonsson 1962; Boorman and Parker 1976; Prout and Bundgaard 1977). This trait, termed sperm displacement ability (SDA), is mediated, at least in part, by products of the second male's accessory glands (Scott and Williams 1993; Harshman and Prout 1994; Chapman, et al. 1995).

While SDA is a widely studied trait, little is known of its genetic basis. Variation in SDA between different strains of D. melanogaster has been well documented (Lefevre and Jonsson 1962; Prout and Bundgaard 1977; Gromko et al. 1984a). Genetic variation in SDA between lines homozygous for the second and/or third chromosome recently sampled from the wild has been shown, but this may have been non-additive in
Hughes (1997) did not find significant amounts of additive genetic variance for SDA on the third chromosome of *D. melanogaster*. A useful first step in the estimation of additive genetic variance of SDA is to measure its heritability. Quantifying the additive genetic variance is useful as it indicates both the ability of the trait to respond to selection (evolvability) and the intensity of forces acting on the variation (Houle 1992). However, misleading conclusions could be drawn regarding the heritability of SDA if the trait is not accurately measured. It should, ideally, be calculated directly from either counts of the number of first male sperm in storage before remating and the numbers of sperm subsequently stored from each male after remating or from zygotic paternity. This would give the "true" SDA of each male, assuming that differences between sperm in the ability to achieve fertilizations after storage are negligible.

Instead, investigators routinely use adult progeny counts to estimate SDA, assuming that the ratio of adult progeny is a sufficient indicator of the ratio of sperm stored from different males (see Peacock and Erickson 1965 for an example of the relation between sperm counts and progeny in *D. melanogaster*). The disadvantage of adult progeny counts is that they are affected not only by SDA, but by any other traits or variables that influence relative numbers of emergent adults. The effects of these confounding variables can be minimised by either appropriate randomisation (e.g. of genotypes of females and first males) or experimental design (e.g. control of remating interval, avoidance of multiple rematings). However, some traits are difficult to control, such as variation in pre-adult viability of offspring, variation in male fertility (including differences between stored sperm in the ability to achieve fertilisations, i.e. direct sperm competitive ability) and preferential use of sperm by females (Parker 1992). In order to identify confounding variables, it is essential to measure potential correlations between SDA and a number of other traits.

In these experiments, the repeatability and heritability of SDA were measured using conventional methods based on progeny counts. Repeatability is a combined measure of all genetic and within individual variance (as opposed to general environmental variance, Falconer 1989), and provides an upper limit for heritability estimates. Correlations between SDA and pre-adult viability, male productivity, mating success, mating speed, male size and female productivity were also measured. The results indicate that the use of adult progeny as a measure of SDA can be seriously misleading if the aim is to demonstrate genetic variation for SDA.

### 3.3. Materials and Methods

All wild type flies used in this experiment were from the outbred 'Dahomey' stock, (Chapter 2). A stock carrying the recessive *sepia (se)* eye colour marker in a Dahomey
genetic background was constructed as described in Chapter 2. Both stocks were maintained as cage stocks for one year before the experiments. Unless otherwise stated, all experimental flies were reared at a standard density and virgins collected within 8 hours of eclosion. Virginit y was checked by inspecting storage vials for larvae. All transfers of flies were performed by aspiration.

The procedure used for rematings in both repeatability and heritability experiments was that described in detail in Chapter 2. Groups of 25 virgin se females were placed with 27 virgin se males in bottles for 7 hours, after which the males were removed and the females allowed to oviposit for a further 42 hours (62 hours in the first repeatability experiment). The mated se females were then paired with single 3-day-old wild type males in yeasted vials and the vials scanned for rematings every 10-14 minutes for up to 10 hours. Times of all rematings were recorded and males removed. Remated females were placed in fresh yeasted vials. Females that remated twice were discarded and any females producing fewer than 10 offspring were excluded a priori from the analysis in order to reduce the sampling variance.

To estimate the repeatability of SDA, each experimental wild type male was given remating opportunities on three consecutive days, with new, mated se females being used each day. Post-remating progeny were scored for paternity and SDA calculated (as described below). The repeatability of SDA for each male was then calculated (Becker 1992). In the first repeatability experiment, females were tipped over into new vials after three days and left in the second vial for a further three days. Progeny from both vials were scored for paternity. In all other experiments, progeny from only one vial per female were scored. In the two repeatability experiments, a total of 2619 rematings were observed and 255322 progeny scored.

The heritability of SDA was estimated from a parent-offspring regression analysis. Males in each generation were again given remating opportunities on three consecutive days. SDA was calculated for each remating and averaged over all measurements for each male. The second generation of males were raised by mating parental males to randomly chosen Dahomey virgin females. Mated females were allowed to lay in a fresh vial for 18 hours, resulting in offspring generation males being raised at a low but variable density. In the heritability experiment, a total of 2863 rematings were observed and 183365 progeny scored.

Sperm displacement was measured as the adjusted ratio of second to first male progeny, $a/(b+1)$, where $a$ is the second male progeny and $b$ the first male progeny (Haldane 1956; Hughes 1997), as described in Chapter 2. The fifth root of the ratio was used since it gives a normally distributed measure of sperm displacement (checked using a Shapiro-Wilk W test). Calculations of heritability and confidence intervals followed Becker (1992).
Pre-adult viability was measured in separate experiments. Two days after measuring the SDA of wild type males (as in the heritability experiment), each male was placed with five virgin se females. From the offspring of each male, 50 first instar larvae (all se/+) were collected and placed in a fresh unyeasted vial of standard food along with 50 first instar larvae reared from eggs collected directly from the se stock cage (i.e. se/se larvae). All emerging adults were scored for genotype. The relative pre-adult viability was calculated as \( a/(a+b+1) \) where \( a \) is the number of se/+ adults and \( b \) is the number of se/se adults. The genotypes present in each vial (se/se and se/+), and the total density (100 larvae per vial) were similar to those of the vials scored in the repeatability and heritability experiments. However, the pre-adult viability vials contained a random sample of se/se genotypes, whereas in the repeatability and heritability experiments the se/se larvae were full sibs. Repeatability of the pre-adult viability of each male's offspring was calculated from up to 3 independent measurements to determine whether the variation observed in this trait might simply be due to sampling differences between the groups of females.

Male productivity was measured for males of the parental generation of the heritability experiment. Two days after the last rematings, males were consecutively mated to three Dahomey wild type virgin females over two days. Two matings were made on the first day, at least 3-4 hours apart and the final mating on the next day. All matings were observed to ensure only single copulations. After mating, females were placed in fresh yeasted vials and removed three days later. The 3-day progeny counts were averaged across females to estimate the productivity of each male.

Female productivity was estimated for the females used in the first repeatability experiment. The number of progeny produced before remating was used as an index of female productivity. This quantity was chosen since it is independent of the effects of the second male. There is a high correlation between the total number of progeny produced by females before and after remating (\( n = 1255, r = 0.44, P < 0.0001 \)). During the 50 hour period before remating, almost all eggs laid are fertile (Chapter 5).

Male size was estimated by measuring the wing area for each experimental male in the first repeatability experiment. Wings were removed from the males with tweezers and placed in a drop of propanol on a glass slide. A drop of permanent mounting medium, Aquamount, was smeared on a coverslip which was then placed on the wing. Gentle weight was applied until the mountant was dry. The area of the wing was measured using a camera lucida attached to a dissecting microscope projecting onto a graphics tablet linked to a computer. Each wing was measured between three and five times.

All analyses were performed using JMP statistical package version 3.1 for the Macintosh (SAS 1994).
3.4 Results

Low but significant repeatability of SDA was found in both experiments (Table 3.1). Despite the differing intervals before remating, the estimates from both experiments were similar, demonstrating that SDA is a repeatable trait of individual males.

<table>
<thead>
<tr>
<th>Estimate</th>
<th>N₀</th>
<th>F ratio (df)</th>
<th>Repeatability (95% confidence limits)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.39</td>
<td>1.37*** (476,686)</td>
<td>0.13 (0.063 - 0.200)</td>
</tr>
<tr>
<td>2</td>
<td>2.10</td>
<td>1.35*** (535,431)</td>
<td>0.14 (0.058 - 0.227)</td>
</tr>
</tbody>
</table>

Table 3.1. The results for the two estimates of repeatability of SDA. (N₀ is group size for an unbalanced design and *** indicates significance at the 0.001 level).

In the heritability experiment, the mean SDA of the parental and offspring generations differed (transformed values of 1.73 and 1.65 respectively, t = 3.60 P < 0.001) but the variances of the two generations were not significantly different (0.134 (parental) and 0.127 (offspring), F[592,571] = 1.047 ns). The average number of rematings per male was 2.49 (parental) and 2.48 (offspring).

The parent-offspring regression for SDA of 556 father-son pairs is shown in Figure 3.1. The narrow sense heritability, h², calculated as twice the regression coefficient, was 0.21 ± 0.08 (s.e.). This represents the heritability of approximately 2.5 measurements of the SDA of individual males. Since heritabilities based on multiple records are over-estimates, a correction is necessary (Becker 1992). The correction factor is \( \frac{m}{1 + (m - 1)R} \) where m is the number of records and R the repeatability. For m = 2.5 and R = 0.13, the correction factor is 2.1. Thus the heritability of a single measurement of SDA can be summarised as 0.10 ± 0.08.

The point in the lower left of Figure 3.1 can be excluded from the regression, without affecting the significance of the result (b = 0.09, t = 2.13, P = 0.033).
Figure 3.1. The transformed parental sperm displacement ability (SDA) plotted against transformed offspring SDA. The solid regression line shown indicates significant heritable variation \((n = 556, b = 0.106, t = 2.56, P = 0.011)\). The dashed line indicates the regression obtained when the point in the lower left-hand corner is omitted \((n = 555, b = 0.09, t = 2.13, P = 0.033)\).
The coefficients of additive genetic variation ($CV_A$) and residual variation ($CV_R$) were also calculated (Charlesworth 1984; Houle 1992). Since the untransformed data were extremely skewed, the coefficients were calculated on transformed data. The coefficients were $CV_A = 6.7\%$, $CV_R = 20.2\%$ (parental generation) and $CV_A = 6.8\%$, $CV_R = 20.4\%$ (offspring generation).

The mean value for relative pre-adult viability was 1.14, indicating that the $sel/+ \,$ larvae had a higher mean viability than the $sel/se \,$ larvae. The correlation with SDA was significant (Spearman's coefficient, $r_S = 0.14$, $P = 0.03$; Figure 3.2), indicating that males that had a higher SDA also produced offspring with a higher viability in a mildly competitive environment. The repeatability of pre-adult viability of offspring was calculated for each wild type male with two or more measurements (Donner and Koval, 1980) and was significantly greater than zero: $R = 0.16 \pm 0.08$. This figure represents an upper limit for the heritability of the sire component of pre-adult viability as measured in this experiment.

![Figure 3.2](image-url)
Male productivity was estimated for 526 individuals. Each male was mated to an average of 2.7 virgin females. The correlation between male productivity and SDA was marginally non-significant ($r = 0.085, P > 0.06$; Figure 3.3).

The distribution of mating success (in terms of one, two or three matings over the three days) in both the repeatability and heritability experiments did not differ significantly from the binomial expectation (first repeatability group: $\chi^2_{[3]} = 10.24, P < 0.05$). Allowing for multiple comparisons, the distribution of matings appeared to be essentially random (Rice 1989). Mean SDA did not vary between males according to their mating success. Average remating speed was also calculated for each male from records of the time between pairing and copulation. There was no correlation with SDA in any generation (largest $r_S = -0.052, P > 0.23$). It should be noted that all rematings occurred under non-competitive conditions and that correlations measured in competitive situations may differ. No significant correlation between wing size and SDA was found for 428 males ($r = -0.021, P = 0.67$), although the size range of these flies was relatively small as they were grown at standard densities under uncrowded conditions.
Table 3.2. Summary of the correlations between sperm displacement ability (SDA) and the traits indicated. Number of individuals indicates the sample size on which each correlation is based. For mating success, speed and female productivity the number of individuals was summed over more than one test. Parenthesis indicate marginal significance of P = 0.06.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Number of individuals</th>
<th>Correlation with SDA</th>
</tr>
</thead>
<tbody>
<tr>
<td>larval viability</td>
<td>300</td>
<td>+</td>
</tr>
<tr>
<td>male productivity</td>
<td>526</td>
<td>(+)</td>
</tr>
<tr>
<td>remating success</td>
<td>&gt; 6000</td>
<td>0</td>
</tr>
<tr>
<td>remating speed</td>
<td>4586</td>
<td>0</td>
</tr>
<tr>
<td>size</td>
<td>428</td>
<td>0</td>
</tr>
<tr>
<td>female productivity</td>
<td>1163</td>
<td>-</td>
</tr>
</tbody>
</table>

The correlations between female productivity (i.e. number of pre-remating progeny) and the subsequent degree of sperm displacement was calculated separately for females remated on different days. Two of the three correlations were highly significant (Day 1, $r_s = -0.15$, $P < 0.005$; Day 2, $r_s = 0.005$, $P > 0.92$; Day 3, $r_s = -0.13$, $P < 0.007$). Combining probabilities (Sokal and Rohlf 1995) gives $\chi^2 = 21.7$, $P < 0.015$, indicating a significant negative correlation between female productivity and SDA.

3.5. Discussion

3.5.1 Heritable variation in SDA

These results show that SDA, as measured by conventional methods, is a repeatable trait in individual males and that it has a low but significant heritability. The two traits that were found to correlate with SDA were pre-adult viability of offspring, which showed a positive correlation, and female productivity, which was negatively correlated with SDA.

An important feature of the results was the correlation observed between SDA and pre-adult viability. This correlation suggests that the apparent heritability of SDA, calculated from progeny counts, may actually be caused by heritable differences in pre-adult viability, rather than by heritable differences in true SDA. Variation in both traits affects the relative numbers of adult offspring of each male. Both traits have been shown to be heritable (for heritability of larval viability see Garcia et al. 1994) and the sire component of pre-adult viability was shown to be a repeatable trait in these experiments. Thirdly, the correlation between the traits must have consisted of additive genetic effects because the genetic variance among sires for both traits must have been
largely additive. For SDA, the small difference between the heritability and repeatability measures indicate the presence of little non-additive variance. For pre-adult viability, differences would also have been mainly additive because the trait was measured across a number of females. Furthermore, there was no environmental correlation because the traits were measured separately. Finally, since additive genetic correlations between traits suggest a common genetic basis, and since SDA and pre-adult viability seem unlikely to share many additive genes, I suggest instead that they are in fact the same trait and that heritability of SDA may simply be due to heritable differences in pre-adult viability. If the two traits are confounded, then the additive genetic correlation between them should approach 1. This is consistent with (but not proved by) the low phenotypic correlation observed, as shown by the following expression for the phenotypic correlation, $r_p$:

$$r_p = h_{SDA}h_{V}r_a + e_{SDA}e_{V}e$$

where $r_a$ is the additive genetic correlation, $e$ the environmental correlation (including non-additive effects), $h = \sqrt{h^2}$ of the indicated trait ($V =$ pre-adult viability) and $e = \sqrt{(1-h^2)}$. Assuming the $h^2$ for both traits to be 0.01, $r_a = 1$ and $e = 0$, then the expected phenotypic correlation would be 0.1. This is comparable to the correlation observed in these experiments. However, an additive genetic correlation of 1 is unlikely since SDA and pre-adult viability were measured under slightly different conditions. The direct estimation of $r_a$ would require extremely large experiments due to the large standard errors associated with its estimation. If heritable variation of SDA calculated from progeny counts is due to heritable differences in pre-adult viability, then the heritability of true SDA is probably very low. This is consistent with earlier genetic investigations of SDA. Clark et al. (1995) found no association between accessory gland protein variation and SDA, which would be expected if heritable variation were largely due to viability rather than SDA differences. Low additive genetic variance for true SDA would indicate that natural selection for this trait is intense and there is little capacity to respond to selection.

An alternative explanation for the correlation between SDA and pre-adult viability is that females preferentially store or use for fertilisation the sperm of males with higher pre-adult viability. It has been suggested that some reptiles preferentially use the sperm of less related males (e.g. Olsson et al. 1996), while in the moth Utetheisa ornatrix, females prevent migration of sperm from the spermatophores of smaller males to the spermathecae (LaMunyon and Eisner 1993). This explanation would require female D. melanogaster to be able to assess pre-adult viability from traits of the male or his sperm. Females may assess sperm quality before mating via correlations between pre-adult viability and other traits such as mating success or mating ability. In the absence of any relevant correlations in the present data and no reported evidence of post-copulatory
sperm selection in *D. melanogaster*, preferential sperm use by females seems a less likely explanation of the present results.

Other sources of heritable variation in SDA are possible, but these do not explain the correlation between SDA and pre-adult viability. Firstly, second males may vary in sperm production, which could vary their ability to dilute or displace first male sperm with their own sperm. While the marginally non-significant correlation between male productivity suggests a possible role of sperm number in SDA, the additive genetic variance for male productivity would be expected to be very low, as confirmed by Hughes (1995). A second possible explanation for heritable variation in SDA are the non-sperm components of the male ejaculate. Although Clark *et al.* (1995) found no correlations between SDA and seven accessory gland protein alleles, it remains possible that heritable variation in SDA is due to allelic variation of other accessory gland products, as there are at least 85 accessory gland proteins in total (Chen 1996).

The negative correlation between female productivity and the degree of sperm displacement was unexpected. *A priori*, females with higher fecundity might be expected to use stored sperm faster, leaving less sperm to be displaced by the second males. This would result in a positive correlation. A possible explanation of the negative correlation is that larger females, although they produce more progeny (Robertson 1957), may initially store more first male sperm, or their remaining sperm may be more difficult to displace.

These experimental results indicate that the accurate measurement of the genetic parameters of true SDA requires direct estimates of sperm numbers or zygotic paternity. Without control of variation in pre-adult viability, progeny counts may be a biased representation of relative numbers of stored sperm. The confounding effect of heritable differences in pre-adult viability may also be important in the measurement of other traits which utilise adult progeny counts, such as studies of direct sperm competition and female sperm selection.

### 3.5.2 Mechanisms of SDA

The most significant aspect of these results concerning the mechanism of sperm displacement was the weak correlation between male productivity and SDA. The productivity measure used here (i.e. three day progeny counts) was necessarily simple for practical reasons. Three day progeny counts correspond to the period when a freely laying female on abundant food uses sperm least efficiently (Gilbert 1981b). During this time, quantitative differences in the number of sperm stored are likely to be obscured. A better measure of productivity involves scoring progeny well past the point where the female efficiency of sperm use has fallen below one and sperm are effectively limiting. A repeat of the type of experiment performed here, using a single generation
and a more comprehensive male productivity measure, may find a significant correlation.

Only one previous study has purported to demonstrate a relation between sperm number and SDA (Letsinger and Gromko 1985). Using crosses between various marked strains, they found a high correlation between the "single mating productivity" of the cross and mean P2 values for the same cross. However, their remating design used relatively short confinement times (a maximum of 2 hours) for the remating pairs. Females were exposed to males daily for 2 hours until they remated. Therefore, remating could take place many days after the first matings, such that the remating females were those with the least sperm in storage. The result of this pattern of remating is that little sperm displacement occurs because females that remate have relatively fewer sperm that can be displaced (Letsinger and Gromko 1985).

In the present experiments, where females were exposed to males for up to 11 hours, female remating did not appear to be determined by sperm numbers. If female remating speed was determined by the numbers of sperm in storage, then there should be some evidence of a negative correlation between remating speed and SDA values. An indication of the degree of sperm dependence of remating in the present experiments is shown in Figure 3.4. Although there is a suggestion of higher SDA values in the first two hours after pairing, there is no significant overall correlation. Therefore, SDA, as measured in these experiments, is not determined by the numbers of first male sperm in storage. The sperm dependence of remating is also absent when females are exposed to males for 24 hours (Newport and Gromko 1984). This excludes the possibility that the near-significant relation between male productivity and SDA observed in the present experiments was merely due to females delaying remating until they have only relatively low amounts of sperm in storage. Accordingly, a single generation experiment, using this remating design and with a more extensive measure of male productivity, may provide experimental evidence that the number of sperm passed by the second male is important in determining SDA in D. melanogaster. This assumes that male productivity accurately reflects the numbers of sperm transferred.

The importance of second male sperm number has been shown in a number of other species which have different modes of sperm displacement: e.g. the bushcricket Decticus verrucivorus (Wedell 1991), where sperm mixing occurs; the beetle Necrophorus orbicollis (Muller and Eggert 1989) where males mate repeatedly; the yellow dung fly Scatophaga stercoraria, (Simmons and Parker 1992), where larger males transfer more sperm; and the arctiid moth, Utetheisa ornatrix (LaMunyon and Eisner 1994) where larger males pass larger spermatophores.
Figure 3.4. Mean SDA of rematings as a function of remating speed for three consecutive days of rematings in the offspring generation of the heritability experiment. Day 1, upper panel; Day 2, middle panel; Day 3, lower panel. Error bars are 95% confidence limits.
Sources of Variation in Sperm Precedence

4.1 Summary

In any measurement of sperm precedence, there are a number of potential sources of variance. In addition to the variance intrinsic to the second male, the first male and the female may also contribute significantly to the variance in precedence. In order to perform an analysis of variance, repeated measures of the same genotypes are necessary. In *Drosophila melanogaster*, balancer chromosomes can be used to simultaneously extract sets of chromosomes, which can then be used to produce large numbers of chromosomally identical individuals. This method was investigated as a means of determining the contributions of females and first males to variation in remating speed, productivity and sperm precedence in *D. melanogaster*. With only a small sample of genotypes, both females and first males were shown to significantly affect the characters assayed. Thus, the method presented may provide a useful tool in the analysis of sources of variation in sperm displacement in *D. melanogaster*.

4.2 Introduction

The high variance of sperm displacement ability (SDA) within species was first noted by Lewis and Austad (1990). In most species for which they were able to collect data, values spanned at least half the maximum range from 0 to 1. It is likely that this variance has multiple causes, rather than solely differences in the ability of second males to displace sperm. Other potential sources of variation include preferential use of sperm by females and variation in the sperm defence ability of males. The relative contribution of different sources to variation in SDA has been quantified only in three studies.

In the first study, Lewis and Austad (1990) measured sources of variation in sperm precedence in the red flour beetle, *Tribolium castaneum*. They used 11 pairs of males and mated each pair, in the same order, to 8 replicate females. Analysis of variance showed significant variation among male pairs and among females. These results indicated that while sperm competition, defence or displacement were significant (the male component), a larger proportion of the variance was due to differences among females. They speculated that the large female component of variance indicated a role for female sperm selection in determining sperm precedence.
Simmons et al. (1996) used a similar approach in an investigation of sperm precedence in the yellow dung fly, *Scatophaga stercoraria*. Variation was measured between matings involving males of different sizes. They found no evidence of a female effect on SDA. Importantly, they were also able to estimate numbers of sperm transferred by males of different sizes and their characteristic sperm displacement rates. Correcting observed SDA for these purely size-related differences among the males, they were able to show that sperm were drawn randomly from the sperm storage organs (the fertilisation set). This refuted earlier suggestions that female *S. stercoraria* were able to use preferentially sperm of larger males (Ward 1993). This study also demonstrated the necessity of estimating actual sperm numbers transferred if sources of variance are to be analysed accurately.

Recently, Hughes (1997) used a quantitative genetic approach to analyse genetic variance in both sperm defence and SDA between lines of *D. melanogaster* carrying different third chromosomes. She found significant variation between lines, and because of the large number of lines tested, was able to test for significant additive and dominance genetic variance and for environmental variance. No significant additive variance was detected and small amounts of dominance variance were found for both traits but the bulk of the variance was environmental.

In *D. melanogaster*, variation in sperm precedence is high, with values of P2 commonly falling between 0.5 and 1 (Lefevre and Jonsson 1962; Gromko et al. 1984a). Studies have shown that second males either from different mutant strains, from different populations or with different homozygous second and/or third chromosomes often differ in mean SDA (Gromko et al. 1984a; Clark et al. 1995). However, it is reasonable to assume that variance of SDA originates not only with the second male, but may also include variation originating from female and first male. It has been argued that females are important in determining levels of SDA, since they may often not remate until numbers of first male sperm are reduced below a threshold which, if as low as suggested, would mean very little sperm needs to be displaced to explain observed SDA values (Gromko et al. 1984b; Letsinger and Gromko 1985). Therefore, females may affect sperm precedence via determination of remating threshold. Alternatively, females may be able to preferentially use the sperm of different second males or females may have differences in sperm storage organ morphology that select between sperm of different males (e.g. as in some spiders, Eberhard et al. 1993).

A role for variation caused by differences among first males is suggested by the findings of (Clark et al. 1995). They found significant variation in sperm defence ability among recently sampled homozygous second and/or third chromosome extraction lines. This variation was found to be associated with variation at particular accessory gland protein loci. Service and Fales (1993), measuring sperm defence in lines artificially selected for divergent rates of senescence, found some lines were both
better able to delay female remating after a first mating and to have larger numbers of sperm 'surviving' displacement by other males.

Ideally, the degree and effect of these various sources of genetic variation for SDA would be assessed using replicated measures of a number of genotypes drawn at random from a population of interest. Lewis and Austad (1990) used repeated measures on the same males only after showing that those males could remate repeatedly without exhaustion. The same assumption could not be made with male *D. melanogaster*, as males are easily exhausted by rapid rematings (Lefevre and Jonsson 1962) and exposure to virgins and high amounts of courtship affect longevity (Partridge and Farquhar 1981; Cordts and Partridge 1996). Highly inbred strains of *D. melanogaster* could be used instead, but they have disadvantages. Inbreeding depresses fertility (Partridge et al. 1985), which would adversely affect investigations of a trait such as SDA which is closely related to fertility. Also, inbred strains are time consuming to construct and considerable selection may occur during their construction. Hybrids between inbred strains can be used to circumvent the problem of inbreeding, but still represent a considerable amount of work to produce each line.

In the present experiments, a more rapid method of bringing together different haploid sets of *D. melanogaster* second and third chromosomes was tested as a means of investigating sources of variance in SDA. The pairs of second and third chromosomes were extracted using balancer chromosomes (Figure 4.1.A). Similar methods were used by Clark et al. (1995) and Hughes (1997). Balancers are non-recombining chromosomes (due to multiple overlapping inversions) containing dominant markers. The balancers allow the extraction of entire unmarked chromosomes from outbred stocks without the danger of recombination altering the extracted chromosome. Subsequently, different sets of extracted chromosomes can then be brought together by a simple cross to form identically heterozygous individuals (Figure 4.1.B).

In this experiment, a small set of randomly selected outbred genotypes was created using the balancer extraction technique. Individuals of each genotype were used as both females and first males in an experiment measuring the SDA of randomly selected wild type males. The effects of the females and first males on the second male's remating speed and his productivity were also measured. Variation in remating speed, female and male productivity and SDA were each analysed with respect to variation in both the female and first male genotype. The results obtained are strictly applicable only to the restricted set of extracted chromosomes that were assayed.

### 4.3 Materials and Methods

Chromosome extraction lines were drawn from the *sepia* cage population described in Chapter 2. Pairs of second and third chromosomes were extracted simultaneously using a double balancer stock *SM5(Cy)/Pm; TM6B (Ser)/Sb* with first
and fourth chromosomes derived from the Dahomey stock. The double balancer stock was constructed in this laboratory. The extraction procedure is shown in Figure 4.1.A.

The females and first males with replicated, heterozygous second and third chromosomes that were used in the experiments were generated by crossing different balanced stocks and selecting the non-balancer *sepia* offspring (Figure 4.1.B). The three different heterozygote combinations were used as both females and first males in a 3x3 design, with group sizes of 40 for each of the 9 possible crosses (total = 360 females). Each mated female was then allowed to lay for 40 hours, before being given the opportunity to remate with a wild type Dahomey male. The experiment was repeated on the following day with another 360 females and first males.

One extracted line (number 5) was used as a common parent for all three heterozygote combinations used in the experiments. In addition, two of the crosses shown in Figure 4.1.B were reciprocal, combining the same two extracted sets to produce the "5/7" and "7/5" heterozygotes (parental female genotype number shown first). The X chromosomes were all derived from the double balancer stock and should be randomly distributed among the genotypes. However, the reciprocal cross offspring differed in the origin of the Y chromosome (i.e. either from the 5 or 7 extraction line).

The factorial crossing design meant that some females were crossed to chromosomally identical first males (i.e. 5/7 x 7/5), giving equal numbers of offspring homozygous and heterozygous for chromosomes II and III. In comparison, females crossed to chromosomally different males (5/7 x 8/5, 8/5 x 7/5) only gave 25% homozygous offspring. Previously, pairs of chromosomes with one or other of the second or third chromosomes homozygous lethal (or nearly so) were discarded to minimise the effects of this inbreeding. Remaining effects of inbreeding were tested by comparing the total numbers of progeny produced between the 25% and 50% inbreed groups. No significant effect was found ($t_{263} = 0.27, P = 0.79$).

Results were analysed by standard analysis of variance, treating both female and first male genotypes as random effects. Except for remating time, results were pooled over the two days of the experiment. Since no interaction terms were significant, all mean squares were tested over the interaction mean square. Although some interaction mean squares were relatively small, all significant results remained significant if tested over the error (second males within crosses) mean square. Where progeny counts were highly skewed (notably the small but highly variable numbers of first male progeny produced after remating), non-parametric one-way Kruskal-Wallis tests were used.
Figure 4.1 The crossing scheme used in these experiments to extract pairs of second and third chromosomes (bars represent major chromosomes of *D. melanogaster*, females on the left). The upper section (A) shows the extraction of pairs of second and third chromosomes from the *sepia* stock (shaded) using balancer chromosomes (hatched). Unmarked chromosomes are derived from the balancer stock. The lower section (B) shows the crossing of two different extraction lines (denoted by subscripts) to produce the individuals used as females and first males. *Cy*, *Pm*, *Sb* and *Ser* are dominant markers (described in Lindsley and Zimm 1992).
4.4 Results

4.4.1 Second male remating time

Remating time was analysed separately for each day of the experiment. On Day 1, female genotype had a significant effect on second male remating speed (Table 4.1.A), however on the Day 2 no significant effect was apparent (Table 4.1.B). The first male did not affect second male remating speed on either day.

4.4.2 Pre-remating progeny

The number of *sepia* progeny produced before the second mating was influenced by differences between female genotypes (Table 4.1.C). There was no significant effect of first male genotype, suggesting that there was little variation between the three male genotypes in the number of progeny sired ("male productivity").

4.4.3 Post-remating progeny

After remating, three related aspects of the absolute numbers of progeny produced were measured. These were the absolute total number of progeny produced and the separate numbers of first and second male offspring which comprise that total. As with pre-remating progeny, the total number of offspring varied significantly with female genotype (Table 4.1.D), as did second male productivity (Table 4.1.E). No effect of the first male was apparent. The numbers of first male progeny produced by each female after remating was much smaller than the number of second male progeny (mean/female = 8.5 first male progeny and 43 second male progeny). Non-parametric analysis of the numbers of first male progeny produced after remating showed that it was influenced by both female and first male genotypes (female, KW = 18.19, P < 0.0001; male, KW = 32.41, P < 0.0001). This result was altered if rematings after which no first male progeny were produced were removed from the calculations. The effect of female genotype then became marginally non-significant (KW = 5.19, P < 0.07), while the first male effect remained significant (KW = 15.60, P < 0.0004).

4.4.4 Sperm precedence

Highly significant effects of both female genotype and first male genotype on the SDA of the second male were observed (Table 4.1.F). The variance components (expressed as percentages) showed that the female and first male effects were similar and relatively small compared to the error (among second males) variance.
### A. Second male remating speed (Day 1)

<table>
<thead>
<tr>
<th>Source of variance</th>
<th>df</th>
<th>MS</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>2</td>
<td>1.2723</td>
<td>8.08 *</td>
</tr>
<tr>
<td>Male</td>
<td>2</td>
<td>0.0048</td>
<td>0.03 ns</td>
</tr>
<tr>
<td>F*M</td>
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</tr>
<tr>
<td>Error</td>
<td>267</td>
<td>0.2434</td>
<td></td>
</tr>
</tbody>
</table>

### B. Second male remating speed (Day 2)

<table>
<thead>
<tr>
<th>Source of variance</th>
<th>df</th>
<th>MS</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>2</td>
<td>1.4997</td>
<td>4.19 ns</td>
</tr>
<tr>
<td>Male</td>
<td>2</td>
<td>0.6383</td>
<td>1.78 ns</td>
</tr>
<tr>
<td>F*M</td>
<td>4</td>
<td>0.3587</td>
<td>1.47 ns</td>
</tr>
<tr>
<td>Error</td>
<td>283</td>
<td>0.2438</td>
<td></td>
</tr>
</tbody>
</table>

### C. Total pre-remating progeny

<table>
<thead>
<tr>
<th>Source of variance</th>
<th>df</th>
<th>MS</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>2</td>
<td>65454.00</td>
<td>191.89***</td>
</tr>
<tr>
<td>Male</td>
<td>2</td>
<td>389.46</td>
<td>1.14 ns</td>
</tr>
<tr>
<td>F*M</td>
<td>4</td>
<td>340.88</td>
<td>0.41 ns</td>
</tr>
<tr>
<td>Error</td>
<td>663</td>
<td>828.60</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.1. Analysis of variance of the effects of female and first male genotype on the indicated variable. F*M is the interaction term between female and first male.
### D. Total post-remating progeny

<table>
<thead>
<tr>
<th>Source of variance</th>
<th>df</th>
<th>MS</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>2</td>
<td>88358.00</td>
<td>22.71**</td>
</tr>
<tr>
<td>Male</td>
<td>2</td>
<td>1566.99</td>
<td>0.40 ns</td>
</tr>
<tr>
<td>F *M</td>
<td>4</td>
<td>3893.69</td>
<td>2.05 ns</td>
</tr>
<tr>
<td>Error</td>
<td>556</td>
<td>1901.00</td>
<td></td>
</tr>
</tbody>
</table>

### E. Total second male progeny

<table>
<thead>
<tr>
<th>Source of variance</th>
<th>df</th>
<th>MS</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>2</td>
<td>65469.60</td>
<td>51.33**</td>
</tr>
<tr>
<td>Male</td>
<td>2</td>
<td>243.33</td>
<td>0.19 ns</td>
</tr>
<tr>
<td>F *M</td>
<td>4</td>
<td>1275.04</td>
<td>0.80 ns</td>
</tr>
<tr>
<td>Error</td>
<td>556</td>
<td>1592.20</td>
<td></td>
</tr>
</tbody>
</table>

### F. Second male SDA

<table>
<thead>
<tr>
<th>Source of variance</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>Variance Component (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>2</td>
<td>2.1138</td>
<td>38.12**</td>
<td>8.0</td>
</tr>
<tr>
<td>Male</td>
<td>2</td>
<td>2.8753</td>
<td>51.28**</td>
<td>11.4</td>
</tr>
<tr>
<td>F *M</td>
<td>4</td>
<td>0.0553</td>
<td>0.39 ns</td>
<td>0.6</td>
</tr>
<tr>
<td>Error</td>
<td>415</td>
<td>0.1432</td>
<td>80.0</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.1. continued.
4.5 Discussion

The results of these experiments must be interpreted with caution since the number of chromosome pairs tested was small. However, a number of the results were highly significant, indicating that significant effects might also be obtained in a more extensive analysis. In the present experiment, lack of an effect implies either females or first males did not significantly affect the trait analysed, or that there was relatively little variation for the trait in question between the sampled genotypes.

Second male remating speed varied principally between female genotypes, with no first male effect. The lack of a first male effect on second male remating speed indicates little variation between the extracted male genotypes in their ability to delay remating. The significant effect of female genotype could have been caused by variation in a number of underlying traits. Remating in females is thought to be dependent on the short term response to the sex peptide and possibly other accessory gland products ("copulation effect") and a longer term response to the amount of sperm stored ("sperm effect"). At the remating interval (2 days) used in these experiments, it is more likely that the major factor delaying female remating is the sperm effect (Scott 1987). For the genotypes tested, females may vary in either their response to the sperm effect (assuming equal insemination), the number of sperm stored (assuming equal response to sperm effect) or the efficiency with which they use stored sperm (which would result in variation of the time at which any sperm effect threshold was crossed). Alternatively, females may vary in their ability to discriminate between males (i.e. their "choosiness"), some inter-strain variation for which has been shown to reside on chromosome III (Scott 1994).

In measures of absolute numbers of progeny, females were shown to have a large effect on variation, while first males had little effect. Both the number of pre-remating progeny (i.e. all progeny produced between the first and second matings) and the numbers of post-remating progeny varied significantly between females. This effect is consistent with the highly significant correlation observed between pre-remating progeny and post-remating, both in this experiment (Day 1, \( r = 0.86, P < 0.001 \); Day 2, \( r = 0.88, P < 0.001 \)) and in the previous chapter. While there is little additive genetic variance for female egg production, the amount of non-additive genetic variance is substantial (Robertson 1957). Therefore high variation between lines in female egg production may effectively mask differences in male fertility (i.e. number of sperm transferred and stored). As long as the first male is able to transfer and store a reasonable number of sperm, differences in egg production and sperm wastage may have overshadowed any variation in fertility among the first males tested.

The only significant effect of the first male on absolute numbers of progeny was on the numbers of his progeny produced after remating. These numbers were small
(typically 0-10) and were affected significantly by both first males and females. Between the three types of male tested, no effect on female remating speed was observed (Table 4.1.A), suggesting that the first male effect on the numbers of his sperm surviving the second mating were not due to delayed remating. The first male effect could have been due to variation in pre-adult viability between the first males, although the viability differences would have to have been large to account for this (the mean numbers of progeny for the three first male genotypes were approximately 12, 10 and 5). In a larger experiment, differences in pre-adult viability could be measured directly.

A further indication that sperm defence could be an important component of male reproductive success was seen in the ability of first males to affect second male SDA (Table 4.1 E and F). First males accounted for about 12% of the variance in second male sperm precedence. Females had an equally significant effect, accounting for 8-9% of the variance in the SDA of the second male. However, the bulk of the variation in SDA lay with the second males, which accounted for 80% of the variance in SDA. Nevertheless, these limited results indicated that significant amounts of variance in SDA can be attributed to both first males and females. This result shows that a more extensive survey of genotypes may be able to establish definite roles for the first male and female in determining SDA.

The significant effect of first male genotype is consistent with earlier indications of variation in sperm defence in *D. melanogaster* (Service and Fales 1993; Clark et al. 1995; Service and Vossbrink 1996). The basis of sperm defence is unknown, although it could involve variation in quantity or quality of either sperm transferred or stored or of accessory gland fluid components. In this experiment, it appeared that first males were able to significantly affect SDA without significantly affecting female remating rates. This suggests sperm defence may not rely on simply manipulating female remating rates via the "sperm effect". Nor do differences in pre-adult viability seem a sufficient explanation of the first male effect. This last possibility could be easily tested by comparing the relative pre-adult viability of male genotypes in a larger experiment.

The significant influence of the female on SDA observed in this experiment also warrants further investigation. While a female effect is consistent with preferential use of sperm by females, there are alternative explanations that have to be eliminated before such a mechanism can be confidently proposed (Simmons et al. 1996). In the heritability study presented in Chapter 3, a negative correlation was found between female productivity and SDA, and the possibility of an effect of female body size suggested. The significant female effect observed in this experiment may be based on similar differences between females. Alternatively, the female effect observed could also be due to pre-adult viability differences.

This experimental design could also be useful in addressing other problems faced in sperm precedence studies. Firstly, the ability to accurately estimate the numbers of
sperm transferred by each male in a sequence of matings is ultimately essential for determining mechanisms of sperm precedence (Simmons et al. 1996; Simmons and Siva-Jothy in press). In the absence of convenient indicators of the numbers of sperm transferred in *D. melanogaster*, such as copulation duration or spermatophore size, it may be that direct sperm counts, obtained by dissecting females, are necessary. However, sperm counts in *D. melanogaster* are subject to large amounts of variation (Gilbert 1981b). One method of overcoming this problem may be to use the experimental design used in the present experiment. Firstly, outbred combinations of lines from which males performed well or poorly in sperm defence could be identified. Using the same combinations, direct sperm counts could be made after first matings involving replicated males. The use of divergent, replicated genotypes may reduce some of the variability in sperm counts. Any significant effects of first males sperm numbers on subsequent SDA are then more likely to be detected.

The most obvious extension of this type of experiment is to simply increase the number of genotypes sampled so that a quantitative genetic analysis of variance components can be performed (Kearsey and Pooni 1996). Of particular interest is the additive genetic variance which indicates the amount of evolutionarily significant variance present in a population. The recent work of Hughes (1997) demonstrated this type of experiment, but failed to find additive variance for either sperm defence or sperm displacement. However, her experiment only examined differences between third chromosomes (approximately 40% of the genome) with an average of 8 rematings for each extracted chromosome and did not control for multiple rematings (remating pairs were left together for 24 hours without observation). In comparison, the present experiment tested pairs of second and third chromosomes with an average of over 60 observed rematings per extraction. Hughes (1997) examined 40 different third chromosomes. The present experimental design could be expanded to cover a similar number of chromosomes.

While the small number of genotypes tested makes the present conclusions tentative at best, use of the method has shown that both the first male and the female may have significant effects on the SDA of the second male. Measurement of correlated characters, such as remating speed, can be used to investigate how these effects may be mediated. The results demonstrate the utility of this experimental design in dissecting sperm precedence mechanisms. It is a relatively efficient method of detecting and analysing sources of variation in SDA in *D. melanogaster*, limited only by the number of genotypes that can be tested.
5

Preferential Displacement of Sperm

(Gilchrist, A.S., and L. Partridge 1995 J. Insect Physiol. 41, 1087-1092)

5.1 Summary

In *D. melanogaster*, the exact mechanism of sperm displacement is unknown. If a sperm incapacitation process is involved, a male's accessory gland fluid would have to be able to distinguish between self and nonself sperm, otherwise a male would incapacitate his own sperm as well as the sperm already stored in the female. This experiment investigated whether males differentially displaced stored sperm when it was their own as opposed to that of another male. Females were remated to the same or a different male that had been sterilised between matings, allowing the degree of displacement of first male sperm to be determined unambiguously. Although no differential displacement was observed, the results suggest that sperm displacement is directed primarily against sperm stored in the seminal vesicle rather than the spermathecae.

5.2 Introduction

In natural populations of *Drosophila melanogaster* females may remate as often as every second day (Prout and Bundgaard 1977; Marks *et al.* 1988; Van Vianen and Bijlsma 1993), which is well before the sperm stored from the previous mating is exhausted. In these circumstances, rematings will usually result in a disproportionately large number (80-90%) of subsequent progeny being sired by the last male (Prout and Bundgaard 1977; Gromko *et al.* 1984a; Scott and Williams 1993). This increase in relative reproductive success by the last male has been termed sperm displacement (Parker 1970) and must affect male reproductive fitness.

Sperm displacement involves the last male actively displacing, incapacitating or otherwise reducing the effective number of the previous males' sperm in storage. Although the exact mechanism of sperm displacement in *D melanogaster* is unknown, recent studies have shown that the male's accessory gland fluid may be responsible for the loss or displacement of sperm. Scott and Richmond (1990) observed that female fertility was reduced when remated to males that passed accessory gland fluid but no
sperm (XO males). However, they were unable to exclude conclusively the possibility that the displacement observed was the result of females with low initial sperm stores preferentially remating with the spermless males (see also Scott and Williams 1993). Harshman and Prout (1994) obtained similar results with another strain of spermless males (tudor males). In addition, they were able to show in the same experiment that males that do not transfer products of the main cells of the accessory glands do not induce any displacement.

It is unclear how a male's accessory gland fluid displaces previously stored sperm without apparently affecting his own sperm transferred in the same mating (Harshman and Prout 1994). A male's sperm is present in the uterus of the female together with his own accessory gland fluid for all but the first few minutes of the mating (Fowler 1973). It is possible that the accessory gland fluid can somehow distinguish between the sperm already in storage and the sperm being transferred.

This chapter describes an attempt to detect such a 'self recognition' mechanism. If a male can recognise his own sperm during a mating, it is possible that whatever identifies the sperm will remain intact until the next mating. If so, a male should be less able to displace his own sperm from a previous mating if those sperm are his own. In the present study, wild type males were sterilised by X-irradiation before remating them to the same or different females. Any self recognition system should be apparent as differences in the progeny production of females remated to the same or different males.

Central to the interpretation of this experiment is the effect of the irradiation on the accessory glands of the males. Adult Drosophila are very radioresistant (Ashburner 1989) but as X-rays primarily damage dividing cells, it is possible to disrupt spermatogenesis at doses that have minimal effect on non-dividing tissues such as the accessory glands. Preliminary experiments (described below) revealed that irradiation, at the doses used to sterilise the males, had no discernible effect on male ability to reduce female receptivity after matings. This is consistent with normal accessory gland function.

5.3 Materials and Methods

All flies used in this experiment were from the outbred stock 'Dahomey' stock as described in Chapter 2. Experimental flies were reared at a standard density and collected within 8 hours of eclosion. Flies were stored for 2-3 days in groups of 5 in yeasted vials before use in the experiments. General culture conditions are described in Chapter 2.

Three days after eclosion (i.e. Day 1 of the experiment) the initial matings were performed. Single pairs of flies were aspirated into yeasted vials and observed until mating took place. Each mated pair was separated within 40 minutes of the completion of copulation and the flies assigned identifying numbers. Females were immediately
placed in individual unyeasted vials of Low food. This medium was used in order to suppress egg laying rates and hence sperm usage in the 5 days before the rematings (Trevitt et al. 1988). Females were transferred to new unyeasted Low food vials on Day 3. On Day 5, the evening before the rematings, the females were transferred to yeasted standard medium.

Male flies were irradiated in a numbered grid with a 10 kR X-ray dose (250 kV tube voltage) which is sufficient to sterilise male *D. melanogaster* permanently (Welshons and Russel 1957). Sterility is not immediate because irradiation does not incapacitate all mature sperm and sperm derived from mature spermatocytes (Ashburner 1989). To exhaust these surviving viable sperm, irradiated males were 'brooded' with 3 successive groups of 3 virgin females in the days following irradiation. As a 10 kR dose of X-rays kills all cells at earlier stages of spermatogenesis (Welshons and Russel 1957), little if any replacement sperm should be produced after the brooding. In the 36-40 hours immediately prior to the rematings, the males were stored without any females to allow recovery of accessory glands (Lefevre and Jonsson 1962). Preliminary experiments confirmed that such males were almost completely sterile at the time of remating (average number of offspring produced per male was 0.3 (n = 92) with no individual male producing more than 2 offspring).

Preliminary experiments gauged the ability of the irradiated, brooded males to reduce the receptivity of females after mating. The rapid decline in receptivity after mating is apparent for 1-2 days following mating (Manning 1962; Scott 1987). This effect is mediated, at least in part, by the sex-peptide which is produced in the male accessory glands (Chen et al. 1988; Schmidt et al. 1993). During the brooding process, irradiated males have the opportunity to mate with at least 9 virgin females in 3 days. As it takes approximately 4 matings to exhaust the contents of the accessory glands (Hihara 1981), most sex-peptide present 5 days after irradiation should be newly synthesised. The remating rate of virgin females mated to irradiated and brooded males was tested 3 and 24 hours after the matings by placing them individually with wild type males for 1 hour. In the initial matings, 68/72 virgin females mated with irradiated males. At 3 hours, only 2/65 females remated and at 24 hours, 10/56 of the singly mated females remated (Fisher exact test P < 0.001). Thus irradiation does not appear to affect the ability of brooded males to reduce female receptivity.

On Day 6 females were remated to the same or different male from the initial matings on Day 1. Rematings were carried out in the yeasted vials in which the females had been stored overnight. Females that remated with the same male from the initial matings comprised the 'Same' group, while females that remated with males that had previously been mated to a different female comprised the 'Different' group. Prior to the rematings, a group of females were randomly selected to serve as the 'Control' group. These females were not given the opportunity to remate. A random selection of females that had declined the opportunity to remate comprised the 'Non-remating' group.
Remating pairs were separated within 1 hour of the completion of the copulation. Females from all four experimental groups were aspirated into freshly yeasted vials and transferred daily to new food until Day 16 and again on Day 18. On Days 21 and 23, only the few females still producing fertile eggs were transferred to fresh vials to allow lifetime progeny counts to be completed.

All eggs laid after the initial matings were counted until no further fertile eggs were laid. Vials were kept at 25°C during progeny development and all progeny were counted within 2-3 days of eclosion.

Results were analysed using a Kruskal-Wallis one-way analysis of variance. Multiple comparisons between treatments were performed by the method described in Seigal and Castellan (1988).

5.4 Results

In the period before the rematings (Days 1-5), there were no significant differences in egg deposition rates (KW statistic = 6.88; P = 0.076), percentage of eggs producing adults (KW statistic = 3.64; P = 0.30) or the number of progeny produced per day (KW statistic = 4.42; P = 0.22) between any of the groups. In this period, the mean number of progeny produced per female was 29 which accounted for 12.3% of the mean lifetime progeny production for the Control and Non-remating groups.

Total egg production after the second mating and until loss of fertility did not differ significantly between any of the groups (KW statistic = 4.81; P = 0.19). Daily egg production (Figure 5.1) differed significantly between the groups only on Days 7, 10, 11, 15 and 16 (P < 0.05) mainly due to slightly higher production of the Different group.

The fertility data for each group are shown in Figure 5.2. After the second mating the fertility of the eggs laid by both the Same and Different groups fell by approximately 80%. The fertility of both the Same and Different groups continued to be significantly lower than the two once-remated groups until Day 12. There were no significant differences between the Control and Non-remating groups until Day 14 (P > 0.05). The Same and Different groups did not significantly differ at any time (P > 0.05).

The total progeny production for all groups before and after the rematings is shown in Table 5.1. The total progeny production after the rematings differed significantly between the groups (KW statistic = 54.82, P < 0.001), with both the Same and Different females producing fewer progeny than the Control and Non-remating females (P < 0.01). There was no significant difference between the Same and Different groups, or between the Control and Non-remating groups. The daily production of offspring is shown in Figure 5.3. The once-mated Control and Non-remating females produced significantly more offspring than remated Same and Different females for the 5 days following the second matings. The once-mated groups did not differ
5.1. Median number of eggs laid per day plotted against time (days) for each of the four experimental groups. Filled symbols represent remated females and open symbols represent once-mated females.

Figure 5.2. Median percentage of eggs giving rise to adult progeny for the same experimental groups as in Figure 5.1.
Figure 5.3. Median number of adult progeny produced per day plotted against time (days) for the same experimental groups as in Figure 5.1.

Table 5.1. Mean progeny production (±SEM) of each experimental group before and after the rematings were performed on the Same and Different groups on Day 6.
significantly in progeny production until Day 14. The remated groups showed no significant differences in progeny production.

Thus no significant differences were found in any aspect of the productivity of females remated to the same or different males. However, during a period of nearly equal egg production, both groups of remated females suffered greatly reduced fertility and progeny production in comparison to once-mated females.

5.5 Discussion

Numerous studies have investigated whether insects are capable of nonself recognition. While transplant experiments usually give negative results, experiments using suspended mixed cell cultures have shown reactions indicative of nonself recognition within insect species (reviewed in Ratcliffe et al. 1984). All these experiments, however, have been concerned with haemocyte reactivity, and do not necessarily bear on the existence of a separate nonself recognition mechanism that may be mediated by accessory gland secretions in the female genital tract.

In this study, no evidence has been found to indicate that D. melanogaster is able to recognise nonself sperm. The degree of sperm displacement induced by the second male is not altered if the first male's sperm are his own. The patterns of egg-laying, fertility and progeny production were virtually indistinguishable regardless of the identity of the second male. This result does not exclude the possibility that a self recognition system does exist. This method relies on whatever may identify or mark the sperm remaining intact until the rematings. It is possible that these markers may have deteriorated during the 5 day period before the rematings. It is not strictly necessary that any marker should persist longer than the time needed to store the second male's sperm. This is the time during which the markers would have to 'defend' the second male's sperm from the incapacitating effects of his own accessory gland fluid. As storage of sperm takes only a few hours (Fowler 1973), the markers may need to be effective only in the few hours before storage is completed.

There are two alternative experimental methods that could reduce the period before the rematings. Both eliminate the need to irradiate and brood the experimental males. The first would be to interrupt the second matings before sperm transfer when small amounts of accessory gland fluid have already been transferred (Fowler 1973). This would allow the rematings to be performed within hours of the initial matings (Scott and Williams 1993). However, the method is complicated by the variability of the timing of the beginning of sperm transfer (Harshman and Prout 1994; Fowler 1973; Gromko, et al. 1984b) and the difficulty in achieving appreciable levels of displacement with smaller amounts of seminal fluid and large numbers of sperm in storage (Gromko et al. 1984b; Fukui and Gromko 1989).
The second, preferable alternative is to use males from highly inbred lines. Females could then be remated within hours to an already sterilised full-sib of the first male. With sufficiently inbred lines, the full-sib males could be assumed to be almost genetically identical.

These results provide further evidence that accessory gland fluid is involved in the process of sperm displacement. Of previous studies investigating sperm displacement using spermless males (either XO males or tudor males), two have observed displacement (Scott and Richmond 1990; Harshman and Prout 1994) and one has not (Gromko et al. 1984b). In this experiment, sperm transfer by irradiated males was checked by examination of the sperm storage organs of females that had been mated to irradiated males, as described in Welshons and Russel (1957). Examination of those females showed that only 8/38 (21%) of those males transferred sperm. However, this sperm is largely inviable. If sperm were necessary for sperm displacement, and only 21% of irradiated males transfer sperm, it is impossible that these males could have induced the levels of displacement seen across the remated groups.

Fowler (1973) suggested that the primary target of sperm displacement is the seminal receptacle. In this study, the contrasting patterns of progeny production provide further evidence that the primary target is the seminal receptacle. To investigate these patterns, the progeny production of females from all four experimental groups with similar levels of progeny production were analysed. All the remated females (n = 125, mean = 133 progeny) were compared to the lower end of the distribution of once-mated females i.e. those that produced fewer than 230 progeny in total (n = 35, mean = 147 progeny). These once-mated females are presumably those that stored a lower than average number of sperm from their mating.

Figures 5.4 and 5.5 show the contrasting rates of egg and progeny production of the two groups. Despite both groups having similar numbers of sperm in storage at Day 6, the twice-mated females displayed a much lower and more consistent rate of progeny production than the once-mated females. Although there were significant differences in egg production on Days 9-12, this was not reflected in progeny production due to decreasing egg fertility.

Previous studies have shown that sperm stored in the seminal receptacle is used rapidly during the first 3 or 4 days following mating when a high proportion of fertile eggs are produced (Gromko et al. 1984a). As the seminal receptacle is exhausted, the rate of fertile egg production becomes lower as a result of the lower rates of sperm utilisation from the spermathecae, which act as longer-term sperm storage organs (Gromko et al. 1984a). The spermathecae of a once-mated female with a total of 200 or more sperm in storage typically contain 40-100 sperm (Fowler 1973). However, when a female stores fewer than 200 sperm, the number stored in the spermathecae is typically 30 or less (Fowler 1973).
These reports suggest the following interpretation of the results of this study. The high initial rate of progeny production by the once-mated females is due to the rapid utilisation of sperm stored in the seminal receptacle. As the once-mated females presumably stored fewer sperm after their only mating (evidenced by their low progeny production), the absolute number of sperm stored in the spermathecae should also be relatively low. This would explain the rapid decline in fertility after exhaustion of the seminal receptacle. In contrast, the twice-mated females would have had more sperm stored after their first mating and correspondingly greater absolute number of sperm stored in their spermathecae. If the remating displaces most of the sperm in the seminal receptacle only, no initial peak of progeny production should be apparent. Instead these females should produce offspring at the lower rate characteristic of sperm utilised largely from the spermathecae. This is in fact what is observed in Figure 5.5. This inference is, however, only tentative and needs to be reconciled with the observation by Harshman and Prout (1994) of sperm displacement in females 10-12 days after the first mating, when most remaining sperm would be stored in the spermathecae.

A second feature of these results is the lack of significant differences between the Control and Non-remated females. It has previously been observed that remating is at least partially dependent on depletion of stored sperm (Gromko and Pyle 1978; Fukui and Gromko 1989). However this experiment used longer confinement times for remating pairs than the earlier studies. As has been observed previously (Scott and Williams 1993) females confined for periods much longer than 2 hours tend to remate irrespective of their level of stored sperm.

Although this study has found no evidence of differential sperm displacement, it is possible that any incapacitation mechanism may only be detectable for a few hours. It is probable that the most efficient method of further investigation of this process is to use males that do not express one of more of the many peptides included in the accessory gland fluid of *D. melanogaster*.
Figure 5.4. Median number of eggs laid per day plotted against time (days) for all remated females (filled symbols) and all once-mated females with total progeny production of less than 230 progeny.

Figure 5.5. Median number of adult progeny produced per day plotted against time (days) for the same experimental groups as in Figure 5.4.
Copulation Duration and Sperm Displacement in *Drosophila melanogaster*

6.1 Summary

The experiments presented in this Chapter investigated the effects of variation in copulation duration on sperm displacement in *Drosophila melanogaster*. Both spermless and normal males were used as second males in the experiments. Displacement induced by accessory gland fluid alone was shown to be a relatively inefficient process, producing continuous variation in levels of displacement. In contrast, displacement in the presence of both sperm and accessory gland fluid was found to be bimodal and to begin earlier during copulation. This indicated a process in which the level of displacement was determined by the amount of sperm transferred. The results indicated that sperm transfer occurred during a period of a few minutes, which began at a variable time after the initiation of copulation. The results are consistent with sperm displacement by dilution in rematings with spermless males and sperm mixing in rematings with normal males.

6.2 Introduction

In species where detailed genetic analysis is difficult, if not impossible, some general inferences about mechanisms of sperm displacement can be made from some easily observed mating parameters. For example, where $P_2$ is affected linearly by the ratio of the copulation duration of the first and second male, a simple sperm mixing process is indicated, since (in most species) sperm numbers are the most likely component of the ejaculate to vary with copulation duration. $P_2$ values observed in such cases are correspondingly low, averaging 0.57 in 8 species (summarised by Simmons and Siva-Jothy in press). Alternatively, if $P_2$ is observed to increase asymptotically with increasing second male copulation duration alone, a flushing mechanism is more likely. As displacement proceeds, the rising concentration of second male ejaculate in the sperm storage organ results in more second male and less first male ejaculate being displaced. Sperm storage organs are not likely to be elastic. In species where this asymptotic relationship has been observed, $P_2$ values are higher, averaging 0.86 (summarised by Simmons and Siva-Jothy in press).
More detailed modelling of mechanisms of sperm precedence requires estimates of rates of sperm transfer by first and second males. From these estimates, inferences about the mechanisms of sperm displacement can be made (e.g. Parker 1990; Parker and Simmons 1991; Sakaluk and Eggert 1996). Models of sperm precedence use the estimates of the relative numbers of sperm transferred to test the linear relations predicted to exist between the observed degree of sperm displacement and some function of the number of sperm transferred. These models have been applied to data from a variety of species, and have shown that different aspects of displacement are important in different species (Parker 1990; Parker and Simmons 1991; Eady 1994). For example, it was recently shown in the cricket, *Gryllodes sigillatus*, using spermatophore attachment time as an indicator of numbers of sperm transferred, that second male sperm do not displace equal numbers of first male sperm, suggesting a degree of expansion is probably occurring in the sperm storage organs simultaneously with displacement (Sakaluk and Eggert 1996).

In order to apply the models of sperm displacement to *D. melanogaster*, a quantitative indicator of the numbers of sperm transferred is required. The number of adult offspring produced by a female has been used to estimate sperm numbers in storage (Letsinger and Gromko 1985). However, males pass many more sperm than are stored, so female progeny production is unlikely to accurately reflect variation in numbers of sperm transferred. The alternative of making direct counts of the sperm transferred is difficult in *D. melanogaster* (Gilbert 1981b), and precludes the possibility of measuring displacement in the same female.

Studies using external indicators of sperm transfer obviously depend on the important variable in sperm displacement, i.e. either number of sperm transferred or volume of ejaculate, being first demonstrated to be proportional to copulation duration. Unfortunately, in *D. melanogaster*, it is unlikely that sperm transfer is a constant process. It has been reported that sperm are not passed continuously during copulation and that the initiation of sperm transfer occurs at a highly variable time after the initiation of copulation (MacBean and Parsons 1967, Fowler 1973). No correlation between copulation duration and sperm transfer has been observed (unpublished studies by Yanders, reported in Fowler 1973), despite earlier suggestions to the contrary (MacBean and Parsons 1967). Also, spermless males mate for periods comparable to normal males. Therefore, it seems unlikely that a simple relationship exists between copulation duration and sperm transfer. Furthermore, sperm may not be the major displacing agent in the *D. melanogaster* ejaculate. The role of second male sperm in determining levels of displacement is not clear, since displacement can be induced by spermless males (Scott and Richmond 1990; Harshman and Prout 1994). Harshman and Prout assumed that accessory gland fluid is transferred constantly throughout copulation, although apart from their demonstration that some displacement can occur with the amount of accessory gland fluid passed prior to sperm transfer (i.e. at about 4
minutes in their experiments), little is known of the rate of transfer or of the quantitative effects of variation in the amount of accessory gland fluid passed. It is possible, therefore, that accessory gland fluid, unlike sperm, is passed at a constant rate during copulation.

If the rates of transfer of sperm and accessory gland fluid are qualitatively different, they could provide a basis for assessing the relative roles of sperm and accessory gland fluid in sperm displacement. Assuming that accessory gland fluid is transferred continuously during copulation while sperm are not, then a relation between copulation duration and sperm displacement ability (SDA) would indicate that the level of displacement was determined by the amount of accessory gland fluid passed. If SDA was determined instead by relative numbers of sperm, then a relation with copulation duration would be less likely, since sperm transfer may be aggregated at one point during copulation rather than bearing any simple linear relationship to total copulation duration.

These simple predictions could, however, be misleading if sperm transfer, although starting well after the start of copulation, still occupied a consistent proportion of the duration of the copulation. This could also produce a relation between SDA and copulation duration if displacement depends on relative sperm numbers. However, if the timing of the beginning of sperm transfer was highly variable between individuals, then any relation between SDA and copulation would be extremely difficult to detect.

A third possibility is that SDA occurs in a threshold manner, whether due to the effects of accessory gland fluid or sperm. A particular threshold amount of either sperm or of a particular accessory gland component may be necessary to trigger displacement. The sex-peptide provides an example of a component of accessory gland fluid which acts in a concentration dependent manner (Chen et al. 1988). In this situation a simple "all-or-nothing" response to variation in copulation duration could be observed. Therefore, distributions of SDA as a function of copulation duration could appear bimodal.

Since no quantitative information on any relation between sperm displacement and copulation duration in D. melanogaster is available, the present experiments were undertaken to investigate the effects of variation in copulation duration on sperm displacement. Displacement was measured in both uninterrupted and interrupted rematings and with both spermless and normal males as second males. Both uninterrupted and interrupted rematings were used since little is known about rates of transfer of the factors determining sperm displacement. If most males transfer similar total amounts of fluid or sperm, then interrupted matings are necessary to induce variation. Alternatively, if the total amount transferred varies between individuals, then differences between uninterrupted copulations may be significant. Both these possibilities were investigated. While spermless males allow quantification of the
effects of accessory gland fluid in the absence of sperm, the transfer of sperm introduces an extra variable into the determination of SDA. The relation between copulation duration and sperm displacement in normal male rematings was investigated using wild type second males with a genetically marked strain as first male. This allowed detection of second male paternity among the progeny of both uninterrupted and interrupted rematings.

6.3 Materials and Methods

Two types of experiments are reported in the chapter. The first involves spermless males and the second, normal males which transfer normal amounts of sperm. In the spermless male experiments, displacement can only be measured as a reduction in the number of subsequent progeny produced by a female after the remating. This was assumed to reflect the loss of sperm from female sperm storage organs. With the normal male rematings, SDA can be calculated from the ratio of progeny sired by each male, as described in Chapter 2.

Two types of spermless male were used as second males (irradiated and tudor) and rematings were both interrupted and uninterrupted. In the second set of experiments, wild type males were used and, again, displacement in both interrupted and uninterrupted rematings were measured.

6.3.1 Spermless males

a) Irradiated males

Since the experiments presented in Chapter 4 had successfully demonstrated an experimental design in which irradiated males could cause significant levels of sperm displacement, that same design was used to investigate interruptions in rematings with spermless males. The irradiated males used in these experiments were prepared as described in Chapter 2. Mated females were prepared by individually mating 3-day-old virgin wild type females to similarly aged wild type males. All matings were observed, and the females removed to individual unyeasted vials of Low food for 5 days. This medium suppressed egg laying rates and hence sperm usage in the 5 days before the rematings (Trevitt et al. 1988). On the evening before the rematings, the females were transferred to yeasted vials of standard medium.

For rematings, irradiated males were introduced to the vials containing the females and the vials continuously monitored. The time of the initiation of any copulation was noted. Matings were interrupted at either 0, 2, 4 or 10 minutes or left uninterrupted. Pairs of flies were assigned sequentially to groups to avoid any bias caused by time of day. This produced 5 experimental groups (0 min, 2 min, 4 min, 10 min, and Uninterrupted). A random selection of once-mated females were set aside before the rematings to serve as one control group (No remating). The other control group
comprised a random selection of the females that had declined the opportunity to remate (Remating refused). This experiment was repeated (as Experiment 2), omitting the 10 min and Remating refused groups.

After remating, females were transferred daily to new food to allow lifetime progeny counts to be completed.

b) tudor males

*tudor* males were used as an alternative strain of spermless male. The *tudor* males and the mated wild type females were prepared as described in the Chapter 2. Single *tudor* males were paired with single mated females and the vials scanned continuously. Rematings were either interrupted at 10 or 15 minutes or left undisturbed. All progeny produced after rematings were counted.

6.3.2 Normal male rematings

Wild type males from the Dahomey stock were used as second males that transferred normal amounts of sperm. Females and first males were from the Dahomey stock carrying the recessive eye-colour mutant *sepia* (*se*) stock described in Chapter 2. Mated *se* females were prepared as described in Chapter 2 and paired with the wild type males for remating 42 hours after the first mating. The vials were scanned continuously for the initiation of copulation, and rematings were interrupted after either 5 or 10 minutes or left undisturbed. The experiments of Harshman and Prout (1994) suggested that sperm transfer may begin around 4 minutes. Accordingly, the 5 minute group was intended to assay the effect of relatively low numbers of transferred sperm, while the 10 minute group should include a greater number of matings with more complete sperm transfer. Egg counts were not made in this experiment.

Where results were not normally distributed or were significantly heteroscedastic, results were analysed using the Kruskal-Wallis one-way analysis of variance. Multiple comparisons between treatments were performed only when a Kruskal-Wallis test indicated significant heterogeneity among groups.
6.4 Results

6.4.1 Spermless males

a) Interrupted rematings

In total, three interrupted mating experiments were carried out with the spermless males. Experiments 1 and 2 used irradiated males, and Experiment 3 used tudor males.

Experiment 1

The total number of eggs laid did not differ significantly between any of the groups (KW statistic = 6.212, P = 0.4). The median number of eggs laid per day is shown in Figure 6.1. At no time did the groups differ significantly in the number of eggs laid (maximum KW statistic = 11.72, P = 0.06). The effect of Low food on egg production is evident on Days 1-5. The %hatchability of those eggs is shown in Figure 6.2. On Days 6-9, the 10 min and Uninterrupted groups had significantly lower hatchabilities than the remaining groups.

The median numbers of progeny produced per day are shown in Figure 6.3. Significant differences were only apparent on Days 6, 7 and 8. These differences were due primarily to the lower medians for the 10 min and Uninterrupted groups. On Day 8 the 2 min group also dropped significantly compared to the higher groups.

Total progeny production did vary significantly between the groups (KW statistic = 18.15, P = 0.006). Figure 6.4 shows a histogram of the total number of progeny produced by each group. Multiple comparisons of the totals show that the groups fall into two broad classes. The class with higher totals are the 4 min, the No remating and Remating refused groups. The class with the lower totals include the 2 min, 10 min and Uninterrupted groups. The 0 min group fell between these classes.

While sperm displacement was evident, as expected, in the 10 min and Uninterrupted groups, the main query arising from these results concerns the possibility of displacement in the 2 min group, particularly as compared to the 4 min group. An indication that this result might not represent real differences comes from Figure 6.3. Here the peak progeny production of all the shorter copulation groups (0, 2 and 4 min) was very close to the No remating group, as opposed to the longer copulations (10 min and Uninterrupted), which were noticeably lower. In Chapter 5, it was argued that a relative deficit at the peak of early progeny production is indicative of sperm displacement from the seminal receptacle. As the 2 min group did not show this deficit, it is unlikely that the reduction in progeny is due to displacement. Nevertheless, this suggestion of displacement was investigated more closely in Experiment 2.
Figure 6.1. The median number of eggs laid per day by groups of females remated to irradiated males for the time indicated (Experiment 1).

Figure 6.2. The median % hatchability of eggs laid by the groups of females remated to irradiated males for the time indicated (Experiment 1).

Figure 6.3. The median number of progeny produced per day by groups of females mated to irradiated males for the time indicated (Experiment 1).
Experiment 2

In this experiment, the 10 min and non-remating groups were omitted to allow an increase in the sample sizes of the remaining groups. As in Experiment 1, the total number of eggs laid did not differ significantly between any of the groups (KW statistic = 3.59, P = 0.46). The number of eggs laid per day after the rematings is shown in Figure 6.5. Figure 6.6 shows the %hatchability of those eggs. The only significant differences involved the Uninterrupted group which had lower %hatchability on Days 7-9.

Figure 6.7 shows the progeny produced on each day after the rematings. It can be seen that only the Uninterrupted group showed a relative deficit in progeny production at the peak on Day 7. Multiple comparisons showed these differences to be significant on Days 6 and 7. Total progeny production varied between the groups (KW statistic = 19.20, P = 0.001). Figure 6.8 shows a histogram of the total number of progeny produced by each group. There were no significant differences between the groups except that the Uninterrupted group produced significantly fewer progeny. Thus, with larger sample sizes, the differences in progeny production between the 0, 2 and 4 min groups were no longer apparent, confirming the absence of detectable displacement in shorter copulations with spermless males.
Figure 6.5. The median number of eggs laid per day by groups of females remated to irradiated males for the time indicated (Experiment 2).

Figure 6.6. The median %hatchability of eggs laid by the groups of females remated to irradiated males for the time indicated (Experiment 2).

Figure 6.7. The median number of progeny produced per day by groups of females mated to irradiated males for the time indicated (Experiment 2).
Figure 6.8 The mean total number of progeny produced by females mated to irradiated males for each group (Experiment 2). Ordinates indicate the numbers of minutes after which the remating was interrupted and the Uninterrupted (Unint’d) and No remating (No rem.) groups. Error bars are 95% confidence intervals.

Experiment 3

In this experiment, the effect of interruptions later in the course of spermless male copulations was investigated. Rematings were interrupted at 10 minutes (as in Experiment 1) and at 15 minutes. Egg and progeny counts were recorded for 55 rematings, and the results are shown in Figure 6.9. Neither egg nor progeny production differed significantly between the two groups. Thus, as observed in Experiment 1, displacement induced by spermless males does not seem to increase appreciably after 10 minutes.

Figure 6.9. The total number of eggs (open bars) and progeny (shaded bars) produced by females after rematings with irradiated males that were interrupted at the indicated time (Experiment 3). Error bars are 95% confidence intervals.
b) Uninterrupted spermless male rematings

Two separate experiments were carried out to gauge the effect of variation in uninterrupted copulation duration on the number of eggs laid and progeny produced after the remating. In the first smaller experiment using irradiated males, data was collected for 50 rematings carried out 2 days after the first insemination. Figure 6.10 shows the total progeny production plotted against the uninterrupted copulation duration. The correlation between copulation duration and the total number of progeny was not significant ($r = 0.06, P = 0.7$). In the second larger experiment, data were collected for 155 rematings with *tudor* males as the second male. Figure 6.11 shows the total progeny production plotted against uninterrupted copulation duration. There was no significant correlation with copulation duration ($r = 0.12, P = 0.11$; for the correlation between egg production and copulation duration, $r = 0.01, P = 0.88$; data not shown).

![Figure 6.10](image)

*Figure 6.10. Total numbers of progeny produced by individual females in uninterrupted rematings with irradiated males plotted against the copulation duration of the remating.*

![Figure 6.11](image)

*Figure 6.11. Total numbers of progeny produced by individual females in uninterrupted rematings with *tudor* males plotted against the copulation duration of the remating.*
6.4.2 Normal male matings

When normal males are used as second males, significant displacement is observed when matings are interrupted at both 5 and 10 minutes (Figure 6.12). There was significant heterogeneity among the groups (KW = 20.35, P < 0.001). Multiple comparisons indicated that the 5 minute group SDA was significantly lower than either the uninterrupted (control) group or the 10 minute group. The 10 minute and uninterrupted groups did not significantly differ (Figure 6.12).

Figure 6.13 shows the distribution of individual SDA values for the rematings interrupted at either 5 or 10 minutes. It can be seen that in the 5 minute group, the number of matings in which no sperm displacement (zero SDA) has occurred is much greater than in the 10 minute group. Also, a number of the 5 minute group show intermediate SDA values near 1.2, indicating that interruption at 5 minutes is more likely to interrupt the process determining the level of SDA.

The distribution of non-zero SDA values in the 10 minute group was similar to that of complete matings, as can be seen from a comparison with the distribution of SDA shown in Chapter 2, Figure 2.3. The data in Figure 2.3 were collected from over 1000 uninterrupted rematings carried out under a protocol identical to that used in the present experiment. The uninterrupted SDA values shown in Figure 2.3 are normally distributed with a mean of approximately 1.7 (the same as the uninterrupted control group, Figure 6.12). The SDA values in Figure 6.13 are similarly distributed, with the exception of the individuals at zero SDA. This indicates that, by 10 minutes, displacement was complete in most females, but a small subset remained in which displacement had not begun.

![Figure 6.12](image_url)

Figure 6.12 The mean SDA for rematings with normal males interrupted at the time indicated. The Control group rematings were uninterrupted. Sample sizes were: 5 minutes n = 62, 10 minutes n = 57, control n = 34. The error bars are 95% confidence intervals.
Figure 6.13. The distribution of individual SDA values observed after rematings with normal males had been interrupted at either 5 or 10 minutes. The distribution of uninterrupted matings, not shown, had no individuals in the zero SDA class.

Figure 6.14. The distribution of progeny counts observed after interrupted rematings with spermless males (fewer progeny indicates greater displacement). The data for 10 and 15 minute interruptions were combined since they were not significantly heterogeneous. The data for the two groups come from different experiments, so mean values are not comparable (4 min, n = 53; 10-15 min, n = 59).
6.5 Discussion

a) Spermless males

The results of the experiments with the spermless males showed that essentially no displacement is observed in short matings (i.e. 4 minutes or less). However, after 10 minutes, significant levels of displacement were consistently observed. (Note that Figure 6.14 is misleading in this respect. Since the data came from different experiments, mean levels of displacement should not be compared).

Although appearing to be at odds with the results of Harshman and Prout (1994) who measured displacement after 2 minutes, there were crucial differences between their experiments and the experiments presented here. Harshman and Prout used females 10 - 12 days after the first matings, by which time their sperm stores would be expected to be very low. Their justification for using such a long remating interval was that they had to maximise any effect of the small amounts of accessory gland fluid passed within 2 to 4 minutes. They were successful in detecting displacement with the 2 and 4 minute rematings. In contrast, the present results showed that against much larger amounts of stored sperm (i.e. the amount present at 2 days after the first mating), the amount of accessory gland fluid passed after 4 minutes did not induce detectable levels of displacement. This lack of sensitivity in these experiments may have been partly due to the fact that the levels of displacement achieved were not as high as those observed in the experiments presented in Chapter 5.

The results did show that significant displacing activity of accessory gland fluid alone on larger amounts of sperm only became apparent after 10 minutes, and did not appear to increase markedly thereafter. No evidence for a bimodal distribution of sperm displacement values was seen in the interrupted copulations with spermless males (Figure 6.14). Instead, the data were consistent with a process in which the degree of displacement by spermless males was continuously variable.

The present results also stand in contrast to the results of Gromko et al. (1984b) who found no displacement with uninterrupted matings with XO males. However, the experimental design used in their experiments also differed from the present design. They used a shorter period of mating (stated as "2-6 hours") and retested females on successive days until they mated. In contrast, these experiments used a longer remating period on a single day (10 hours), two days after the initial mating during which time 70-80% of females will remate (Chapter 2).

b) Normal males

The results of the interrupted rematings with normal males differed qualitatively from the spermless male rematings, as can be seen in the comparison of Figures 6.13
and 6.14. The results are highly suggestive that the level of sperm displacement in mating with normal males is determined by the number of sperm transferred.

Appreciable SDA was observed at both 5 and 10 minutes, and a significant increase in SDA was observed between 5 and 10 minutes. SDA values showed a bimodal distribution (Figure 6.13). Since no displacement was observed before 10 minutes in spermless matings, the factor determining the degree of displacement (but not necessarily causing displacement) in the 5 minute normal male rematings must have been the second male sperm. The upward shift in mean SDA between 5 and 10 minutes, coupled with a corresponding drop in the numbers of individuals showing zero SDA, suggests a simple model of how the degree of SDA is determined.

In this model, the degree of sperm displacement is closely associated with sperm transfer. Once sperm transfer begins (some variable number of minutes after the start of copulation), the degree of sperm displacement is determined rapidly, and is essentially fixed within a few minutes. This is indicated by the observation that in most interrupted rematings, SDA was either zero or complete, with few intermediates. The mode at zero in each distribution was taken to represent the number of matings interrupted before sperm transfer had begun. The higher frequency of intermediate SDA values in the 5 minute group was attributed to the higher probability of interrupting sperm transfer. By 10 minutes, most males had completed sperm transfer (explaining the mode at a SDA of 1.6 and the drop in zero SDA scores), so relatively fewer rematings (than the 5 minute group) will remain that can be interrupted during sperm transfer, and fewer individuals will remain in the zero SDA group.

Under the model proposed, SDA is determined by the number of sperm transferred. Such a result which would be consistent with findings in a number of other species (see introduction to this Chapter) in which P2 has been shown to vary with numbers of sperm transferred. The major difference between \textit{D. melanogaster} and the other species is that sperm transfer in \textit{D. melanogaster} appears to occur rapidly in comparison to the total duration of copulation. Accordingly, unlike other species, total copulation duration of individual males will provide no information as to the number of sperm transferred. In other species, e.g. \textit{G. sigillatus} (Sakaluk and Eggert 1996) or \textit{Scatophaga stercoraria} (Simmons and Parker 1992), investigators have been able to use individual estimates of sperm transfer to test models of sperm displacement. While individual SDA values in \textit{D. melanogaster} may not be useful, mean SDA values for different groups of males that have been interrupted at various times, could provide data that could be used for model fitting.

If this proposed model is correct, one immediate prediction is that sperm transfer should be completed rapidly (i.e. within minutes of initiation). Dissections of \textit{D. melanogaster} have shown that sperm transfer begins some minutes after the start of copulation (Fowler 1973), but the timing of the end of sperm transfer has not been
examined. The idea of rapid transfer of sperm in *D. melanogaster* is supported indirectly by observations that sperm are deposited in the female reproductive tract in a "sperm sac" (Fowler 1973; Alonso-Pimentel et al. 1994), by observations of a sperm mass (Gilbert 1981b) and by the observations of Garcia-Bellido (1964) who reported a large increase in sperm numbers in the female reproductive tract between 4 and 8 minutes.

A second prediction would be that levels of sperm displacement should be low in spermless matings. Presumably, first male sperm that have had no opportunity to mix with second male sperm either remain in storage or are restored. However, in the results of other studies (Scott and Williams 1993; Harshman and Prout 1994) and in the experiments presented in this Chapter, significant amounts of displacement were observed in the absence of sperm. In the present experiments, the level of sperm displacement in spermless matings was lower than the normal male rematings and did not appear to be bimodal. An explanation for this could be that displacement in spermless matings occurs not as a result of sperm mixing, but is instead due to sperm dilution with the second male's accessory gland fluid. This dilution should also occur in normal matings, but it would be hard to detect against the background of high levels of displacement induced by sperm, and it may also affect first and second male sperm equally, leaving SDA unaffected. An experiment that could test this possibility would be to use large and small spermless males. Assuming larger males pass greater volumes of accessory gland fluid, the level of displacement observed for a fixed period of copulation should be greater in the large spermless males.

If sperm transfer does occupy only a small proportion of total copulation time, then the question arises as to why copulation can last for 25 minutes or more. Some of the extra time must be necessary for the transfer of sufficient accessory gland products to ensure sufficient storage and/or displacement of sperm. Copulation duration in *D. melanogaster* is mainly under male control (MacBean and Parsons 1967) as is also the case in *D. pseudoobscura* (Kaul and Parson 1965). In both *D. melanogaster* and *S. stercoraria*, copulation duration has been shown to be a heritable male trait (MacBean and Parsons 1967; Gromko et al. 1991; Muhlhauser et al. 1996). Copulation duration in *S. stercoraria* has been shown to be optimised with respect to male body size to maximise male paternity gains (Parker and Simmons 1994). It is possible that copulation duration is similarly optimised in *D. melanogaster*. The results presented in this Chapter show that the transfer of sperm and determination of the level of SDA in *D. melanogaster* may occupy only a short period of the entire copulation. Elucidation of any functional significance of the remainder of the copulation duration may provide further insights into important aspects of male reproductive success in *D. melanogaster*. 

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Male Size, Reproductive Success and Sperm Displacement

7.1 Summary

The experiments presented in this chapter investigated the effects of genetically determined body size on male productivity, copulation duration and sperm displacement ability (SDA). Larger males were found to have low early productivity, while Control males were found to copulate for significantly longer periods. Only marginal variation was found in SDA among the size classes. Examination of the trends apparent in this data suggest different mechanisms may be responsible for determining male reproductive success in matings with virgin and non-virgin females.

7.2 Introduction

Phenotypic studies of the effect of body size have often found positive correlations between body size and male reproductive success (Roff 1992, p128). Traits such as mating success and fecundity usually increase with body size (e.g. Partridge and Farquhar 1983; Partridge et al. 1987b; Boake 1989; Berrigan and Locke 1991; Fox et al. 1995; but for exceptions see McLachlan and Allen 1987; Zamudio et al. 1995).

Analysis of reproductive performance in terms of its components (i.e. mating success and per-mate fecundity; Figure 1.1, Chapter 1), shows that the effects of size on the individual traits comprising male reproductive performance in D. melanogaster are not uniformly positive. Larger males have a higher mating success and live longer (Partridge and Farquhar 1983), deliver more courtship (Partridge et al. 1987a) and are more aggressive in male-male encounters (Dow and Schilcher 1975). Larger size has also been shown to positively influence mating times with virgin females (Partridge and Farquhar 1983) and remating times, with females remating faster with larger males, regardless of first male size (Pitnick 1991). However, in the same study, larger males were also shown to copulate for shorter times and produce slightly fewer early progeny, suggesting adverse effects of larger size on per-mate fecundity.
The value of the sort of phenotypic correlations outlined above in describing evolutionary change depends on the degree to which those phenotypic correlations reflect the underlying genetic correlations. The subject of the correspondence between phenotypic and genetic correlations has been reviewed by Roff and Mousseau (1987) and Cheverud (1988). A recent survey by Roff (1996) has suggested that, as a general rule, phenotypic correlations between two metric characters or between metric and life history characters provide "adequate" estimates of underlying genetic correlations. Therefore, the phenotypic correlations listed above between larger body size (a metric trait) and components of reproductive success (a life history trait) are probably useful indicators of underlying genetic correlations. If genetically larger body size were correlated with increased reproductive success, this would provide a constant selective pressure for increased body size. However, body size is thought to be under stabilising selection. Local optima for body size are produced by the patterns of genetic correlations between body size and various fitness components. The phenotypic correlations outlined above suggest that not all genetic correlations between the components of male reproductive success and body size are positive. Therefore, it is of interest to examine genetic correlations between components of male reproductive success for two reasons. Firstly, some of the correlations may contribute to local size optima. Also, of more specific interest in terms of sperm displacement ability (SDA), is that genetic correlations can provide indications of the mechanism of SDA.

Phenotypic correlations between body size and sperm precedence have been studied in a number of other insects, but not *D. melanogaster*. In each case, size related variation in P2 was thought to depend on variation in the number of sperm transferred. P2 was positively correlated with body size in red flour beetles, *Tribolium castaneum*, (Lewis and Austad 1990; Lewis and Austad 1994), the yellow dungfly, *Scatophaga stercoraria*, in which P2 varies with both relative body size of the first and second males and their relative copulation times (Simmons and Parker 1992), the Diptera, *Dryomyza anils* (Otronen 1994) and a coccinellid beetle (ladybird) *Harmonia axyridis* (Ueno 1994). Although these studies were best explained by assuming larger males transferred more sperm per unit time, it is also possible that larger males transfer non-sperm components, which are active in sperm displacement, at the same rate as sperm. This second possibility is suggested by the observation that the accessory gland fluid transferred by *D. melanogaster* males has been shown to cause displacement (Harshman and Prout 1994). The effects of larger body size on SDA are not always mediated by fertility differences. In the damselfly *Ischnura graellsii*, the positive correlation with body size is due to larger males having larger (though highly variable) penile hooks to scrape out first male sperm (Cordero and Miller 1992).

The phenotypic correlations observed in other species suggests that variation in sperm number may be important in determining SDA. If so, then assuming larger males pass more sperm (Berrigan and Locke 1991) a genetic correlations between body size
and male productivity or SDA in *D. melanogaster* would be expected. Alternatively, non-sperm components of ejaculate may be important in determining SDA. In an attempt to clarify some of these relationships in *D. melanogaster*, the effect of genetically determined body size variation on male productivity, copulation duration and sperm displacement ability (SDA) of males was investigated. The effect of body size on SDA in *D. melanogaster* has not previously been studied.

### 7.3 Materials and Methods

The Large, Small and Control males used in these experiments originated from unreplicated lines selected in this laboratory at 25°C for large and small wing size by Dr. Bas Zwaan. Both these lines and the controls were derived from 48 isofemale lines collected in North Carolina in 1994. The lines were first used at generation 18, by which time significant size differences were apparent. These changes have occurred as a result of changes in both cell size and cell number. The selection lines were not replicated.

All wild type females were from the Dahomey stock, and *sepia* (*se*) flies were from the *sepia*-marked Dahomey stock. All experimental flies were raised at a standard density. Both the stocks and the general culture conditions are described in Chapter 2.

Male productivity was measured for males of generation 18 from each of the three selection lines. Males were individually mated to 4 day old virgin wild type females. All pairings were observed to ensure single insemination, after which females were placed in fresh yeasted vials. Female were placed in new vials after three days and left in the second vial for a further six days, producing vials containing Days 1-4 progeny and Days 4-9 progeny. All progeny emerging from both vials were counted.

For the experiments measuring SDA, five types of second male were used. These consisted of males from the three size selection lines (Large, Small and Control) and males raised from the reciprocal crosses of the Large and Small lines (referred to as L*S and S*L). The males from the reciprocal crosses were intended to allow detection of any gross inbreeding or maternal effects in comparison to males from the selected lines. The size of the males from the two reciprocal crosses was not measured.

The mated females to which the selected males were mated were produced by single matings of 3-day-old virgin *se* females to similarly aged *se* males. All first matings were observed and females removed to new vials in which they were stored in groups of three for approximately 45 hours. Of a random sample of singly mated females not subsequently remated, 100% (n=72) were found to be fertile and of these all but one produced at least 50 *se* offspring.

For the rematings, individual mated *se* females were paired with individual selected males and all vials scanned continuously for rematings. The start and finish of
copulations were noted. Even with continuous scanning, it is impossible to see the initiation of every copulation. However, for a short period (perhaps 30 seconds) after pair formation, the male is not properly positioned on the female. This was used as an indication of whether mating had recently begun. After mating, females were placed in fresh vials and progeny counts made sixteen days later.

SDA was measured on two consecutive days. Total experimental size on each day was limited by the number of vials that could be scanned continuously for initiation of rematings. On each day, 50 males of each of the five groups of males was given the opportunity to mate with non-virgin females. SDA was calculated using adjusted progeny ratios described in Chapter 2.

Where results were not normally distributed or heteroscedastic, results were analysed using the Kruskal-Wallis one-way analysis of variance. Multiple comparisons between treatments were performed only when a Kruskal-Wallis test indicated significant heterogeneity among groups. The method used was that described in Siegel and Castellan (1988). Normally distributed data were analysed using standard anova techniques, and multiple comparisons performed using the Tukey-Kramer method. All analyses were performed using JMP statistical package version 3.1 for the Macintosh (SAS 1994).

7.4 Results

7.4.1 Male productivity

After single matings to males from the selection lines, females were found to produce significantly different numbers of offspring in the first three days following mating (Table 7.1; KW statistic = 7.47, df = 2, P = 0.024). Multiple comparisons showed that Large males sired significantly fewer offspring than both the Small and Control males on Days 1-3. On Days 4-9, the productivity of the females did not differ significantly (KW statistic = 0.64, df = 2, P = 0.73). Also the total productivity did not differ between the groups (KW statistic = 0.57, df = 2, P = 0.75).

<table>
<thead>
<tr>
<th>Male</th>
<th>n</th>
<th>Median progeny Days 1-3</th>
<th>Median progeny Days 4-9</th>
<th>Median progeny Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large</td>
<td>146</td>
<td>149.0 a</td>
<td>68 a</td>
<td>223.5 a</td>
</tr>
<tr>
<td>Small</td>
<td>136</td>
<td>159.5 b</td>
<td>48 a</td>
<td>206.0 a</td>
</tr>
<tr>
<td>Control</td>
<td>124</td>
<td>154.0 b</td>
<td>66 a</td>
<td>210.5 a</td>
</tr>
</tbody>
</table>

Table 7.1. The number of progeny produced by females mated once to males from the different selection lines. Letters denote statistically significant groupings.
The mating success of the males from the different groups did not differ significantly from that expected from a random distribution of matings among groups (Table 7.2; Day 1, $\chi^2_{[4]} = 1.22, P > 0.75$; Day 2, $\chi^2_{[4]} = 0.36, P > 0.98$). Similarly, mating speed did not differ significantly between any of the groups on either day (Table 7.2; larger KW statistic = 5.86, $P = 0.21$).

### 7.4.2 Copulation duration

Control males had significantly longer copulation duration on both days (Figure 7.1; Day 1, KW statistic = 13.02, $P = 0.011$; Day 2, KW statistic = 36.43, $P < 0.0001$). Multiple comparisons showed that none of the other four groups differed in their copulation duration.

### 7.4.3 SDA

The patterns of SDA were not consistent over the two days. Mean SDA over both days was high, corresponding to a mean P2 of 0.87, but differed significantly between the days (Day 1 mean = 2.53; Day 2 mean = 3.25; KW statistic = 16.9, $P < 0.001$). Therefore SDA was analysed separately for each day. On Day 1 significant differences were apparent ($F_{[4,199]} = 3.43, P = 0.01$). However, on Day 2, there were no significant differences between the groups ($F_{[4,199]} = 1.047, P = 0.71$). Multiple comparisons indicated that the SDA of the Control males was significantly lower than the S*L group on Day 1 (Figure 7.2). However, after correction for multiple comparisons, this comparison was not significant (Rice 1989).

Correlations between copulation duration and SDA were non-significant within groups, with the exception of the Large males on Day 2, where a large negative correlation was observed ($r = -0.54, P < 0.0005, n = 34$). To determine whether this result indicated a real relation or occurred as an anomalous effect related to the

<table>
<thead>
<tr>
<th>male</th>
<th>Mating success (%)</th>
<th>Mating speed (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
</tr>
<tr>
<td>Large</td>
<td>68</td>
<td>76</td>
</tr>
<tr>
<td>Small</td>
<td>62</td>
<td>80</td>
</tr>
<tr>
<td>Control</td>
<td>58</td>
<td>84</td>
</tr>
<tr>
<td>L*S</td>
<td>74</td>
<td>82</td>
</tr>
<tr>
<td>S*L</td>
<td>70</td>
<td>84</td>
</tr>
</tbody>
</table>

Table 7.2 The mating success and mating speed for males of each of the five size groups used in the SDA experiments.
relatively small sample size, the relation between copulation duration and SDA in Large males was tested in a larger trial (n = 110). No significant correlation between copulation duration and SDA was found (r = 0.02, P = 0.80; Figure 7.3).

Figure 7.1. The copulation duration of males of differing size plotted for both experimental days. Combined sample sizes were: Large, n = 72; Small, n = 71; Control, n = 73; L*S, n = 80; S*L, n = 79. Error bars are 95% confidence intervals.

Figure 7.2. The SDA of males of differing size plotted for both experimental days for the same individuals shown in Table 7.2. Error bars are 95% confidence intervals.
7.5 Discussion

7.5.1 Male productivity

Since the selection lines used in these experiments were not replicated, any correlations observed cannot automatically be assumed to have a genetic basis. However, the lines did not appear to be suffering from any appreciable degree of inbreeding, so it is likely that the results are at least qualitatively informative. These results show the unpredictability of the effects of body size variation on male reproductive success. Larger males had the lowest productivity of the three lines and the smallest males were not significantly different from the Controls. While this appears contrary to expectations based on studies of other species (which usually involve a small relative size range), it agrees with the finding of Pitnick (1991) that large *D. melanogaster* males have low early productivity. His and the present experiments differ in that Pitnick used environmentally induced size variation, whereas this experiment utilised genetically determined size variation. Pitnick observed that the drop in productivity was due partly to differences in early female egg production rather than egg fertility. This, Pitnick reasoned, was more likely to be due to differences in accessory gland product quality or quantity (rather than variation in sperm number) since a component of accessory gland fluid (the sex peptide) has been shown to have analogous short term effects on egg laying (Chen et al. 1988). Pitnick further speculated that, since larger males will secure more rematings, they may pass less accessory gland fluid at each mating as a means of ejaculate partitioning. Such partitioning has been since reported in a coral reef fish, *Thalassoma bifasciatum* (Warner et al. 1995). The problem with this hypothesis in *D. melanogaster* is that the
period of the deficit in male productivity occurs during the period of highly inefficient sperm use by the female (i.e. days 1-3 after mating; Gilbert 1981). During this period many sperm are released per egg which, while ensuring maximal fertility of the female's eggs, would also serve temporarily to mask quantitative differences in numbers of sperm stored. These differences would only become apparent at the point when the sperm to egg ratio dropped below one (i.e. Day 2 or 3 after remating). Therefore differences in numbers of sperm stored would be expected to be least apparent at the only time at which those differences were actually observed in both experiments. Furthermore, progeny totals were similar. If ejaculate and sperm were partitioned, overall totals would be expected also to vary.

Alternate explanations are suggested by considering the basis of size differences. Size variation in *D. melanogaster* is caused by both variation in cell size and cell number (Robertson 1959). Different selective regimes, such as laboratory versus field, have been shown to produce similar body size changes by different mechanisms, i.e. cell size changes in one environment, cell number in the other (Partridge *et al.* 1994; James *et al.* 1995). Since accessory glands are somatic tissue, they may be affected by the same differences that affect overall body size, although whether accessory glands increase in size by increasing cell size or number has never been investigated. If, however, the accessory glands of larger males had larger cells, rather than more cells, then during copulation, these males will pass larger quantities of accessory gland fluid, but this fluid may be relatively dilute. This could have the effect of a slight reduction in the stimulation of egg laying induced by accessory gland fluid. This would, in turn, produce a transient reduction in male productivity. Evidence of such effects being mediated by accessory gland fluid comes from the finding that females which mate with males lacking the Acp26Aa protein have been shown to suffer a similar slight reduction in egg laying on the day following mating (Herndon and Wolfner 1995). Furthermore, Service and Vossbrink (1996) found that selected males which had lower sperm defence ability also caused females to increase egg production soon after mating. They attributed this effect on egg laying to variation in accessory gland fluid components. An explanation based on variation in accessory gland fluid also predicts that overall progeny totals should not vary greatly, since no variation in numbers of sperm stored is postulated. Thus dilution of accessory gland fluid provides a better explanation of the slight reduction in early productivity of females mated to larger males, than does ejaculate partitioning.

A related explanation is that "dilute" accessory gland fluid may not be as efficient at storing sperm in the female. The effects of such a mechanism would be open to the same objections as the ejaculate partitioning explanation, and it therefore it seems less likely explanation. Alternatively, larger males may pass greater volumes of accessory gland fluid which may reduce the concentration of sperm passed, which may in turn, reduce the numbers of sperm stored.
7.5.2 Copulation duration

The pattern observed on both experimental days, where Control males mate for significantly longer, was verified in the same selected lines after a further two generations of selection (W. Lucas unpublished). Thus the observed pattern of variation in copulation duration does reflect real differences between the selected lines. Furthermore, the reciprocal crosses, which mated for similar times to the Large and Small lines, indicated that the selection lines were not affected by inbreeding depression for genes controlling copulation duration. This contrasts with the finding of Pitnick (1991) of a weak negative phenotypic correlation between body size and copulation duration.

The lack of any concordance between the patterns of copulation duration and male productivity suggest that they are not determined by the same variable(s). While the early drop in Large male productivity implicated accessory gland fluid quality as an important variable in productivity, no similar explanations are apparent for variation in copulation duration. The Control males, although having copulated for significantly longer, were intermediate in each productivity measure, which is evidence against any appreciable differences in quantities of accessory gland fluid or sperm passed despite the longer copulations. Earlier selection experiments on male copulation duration have demonstrated that there is extant additive genetic variance for the copulation duration, and that males that copulate for longer begin sperm transfer later (MacBean and Parsons 1967). If Control males did transfer sperm later, it would not necessarily affect productivity. Thus variation in copulation duration may be uninformative regarding amounts of sperm or accessory gland fluid transferred, being instead a product of drift between the selected population for the trait. Unfortunately, these selected lines are unreplicated. Comparison between replicates would indicate whether the variation was truly size related. Males from the reciprocal crosses, which would have produced flies of approximately intermediate size, did not copulate for longer, supporting the idea that the longer copulation duration is not size related.

7.5.3 SDA

Despite the significant differences in SDA observed on Day 1, the large differences in size appear to have only marginal effects on the ability to displace sperm. The lack of a significant improvement in SDA of the reciprocal crosses suggests little effect of inbreeding on the SDA of the selection lines.

However, the pattern of decreasing SDA among the Large, Small and Control lines, apparent in Figure 7.2, although not significant, is repeated on both days and is different to that expected on the basis of the male productivity rankings. Above, it was argued that variation in male productivity could be better understood in terms of variation in the quality of accessory gland fluid affecting female productivity, rather than differences in the number of sperm a male transfers or stores. Moderate variation
in sperm number will be expected to make little difference to early productivity, since sperm are initially released or leave the sperm storage organs in excess of the number required for fertilising all eggs laid. However, in matings with non-virgin females, the first male sperm (and possibly accessory gland fluid products) will already be present, and will interact with the second male ejaculate. In this situation, as has been inferred in a number of other insect species, SDA in D. melanogaster may be influenced by the numbers of sperm passed. The results obtained in the study of heritability of sperm displacement, presented in chapter 3, also provide indirect evidence for this hypothesis. If larger D. melanogaster pass more sperm then, as a result of sperm mixing and dilution, the larger male should achieve high levels of sperm displacement. While Figure 7.2 does show a higher mean SDA for the Large males, the ordering of the Small and Controls was not qualitatively consistent with this explanation. The generally high performance of the reciprocal crosses may have been caused by fertility-related benefits of outbreeding.

The other notable qualitative aspect of the data shown in Figure 7.2 was the relatively low SDA of the Control line observed on both days of the experiment, which raises the possibility of a relation between copulation duration (for which the Controls were the longest) and SDA. If sperm number is the more important variable in determining SDA, then the longer copulation duration of the Controls may imply the transfer of less sperm. The productivity data (Table 7.1) does not support this idea. Alternatively, longer copulation may imply a delay in the relative time of sperm transfer for the Control males (MacBean and Parsons 1967). Although highly speculative this suggests that the delay in sperm transfer may reduce SDA, possibly because some restorage of displaced first male sperm may occur before appreciable numbers of second male sperm are present to dilute the displaced first male sperm. This possibility would be difficult to test except by a detailed time course examination of physical sperm location in newly remated females.

7.5.4 Effects on male reproductive success

The results do provide circumstantial evidence of the relative importance of accessory gland fluid and sperm number in different mating situations. In virgin matings, accessory gland fluid may be the major variable, via its effect on female egg laying rate, while in non-virgin matings, the relative numbers of sperm may be more important. Variation in copulation duration may only reflect differences in the timing of initiation of sperm transfer, as suggested by (MacBean and Parsons 1967). A summary of these ideas and how they might impact on male fecundity is presented in Figure 7.4. This speculation about the relative importance of accessory gland fluid and sperm number in virgin and non-virgin matings does not explain all the trends (non-significant) in the data, for example, why Small males appeared to have intermediate SDA. It is suggestive, however, that a trade-off may exist between mating success and fertility (Warner et al. 1995).
In order to clarify the relations between body size and reproductive success, the most useful technique would be a method of regulating or measuring the numbers of sperm passed. Unfortunately, coefficients of variation for traits closely related to fertility (and therefore fitness) are often very high, so isogenic lines may not provide sufficient control over sperm numbers. Direct estimation of sperm numbers would allow differences in sperm transfer to be directly related to productivity, copulation duration and SDA. Additionally, the relation between body size and accessory gland cell size and cell number could be investigated in flies of different sizes. The accessory glands are easily dissected out and cell number easily counted using fluorescent nuclear stains to visualise the characteristic binucleate cells. The relation between cell size (presumably affecting accessory gland fluid concentration) and productivity and SDA could then be quantified.

Figure 7.4. A representation of the qualitative effects of large male size on overall fecundity, based on the ideas presented in the text.
8

The Role of Accessory Gland Fluid in Sperm Defence

8.1 Summary

It is possible that accessory gland fluid has a direct effect on sperm defence by physically interfering with the storage of second male sperm. This possibility was investigated using spermless males that differed in the amount of accessory gland fluid transferred at mating. Females were mated to these males and then subsequently mated to normal males. The effect of variation in the amount of accessory gland fluid received in the first mating on the productivity of the second male was measured. No defensive role for accessory gland fluid was detected. The result is discussed in the context of other possible mechanisms of sperm defence.

8.2 Introduction

Sperm defence has the potential to be an important aspect of male reproductive success. In many species, males defend their sperm from displacement and/or numerical competition by providing either courtship feeding, mate guarding or transferring specific receptivity inhibiting substances. None of these affect sperm in storage directly, although courtship feeding can result in greater numbers of sperm being stored (Sakaluk 1984; Simmons 1986). Rather, they "impose" on the female a longer period during which she must use the sperm of the defending male. Females would not be expected to be strongly selected to resist this imposition, so long as the remating delay did not impair their overall fertility (but see Warner 1995). Since female productivity is an extremely important component of overall fitness (Mackay 1985), the cost to a female of altering her pattern of progeny production so as to allow the use of more sperm of different males would probably not be compensated by the small benefits of earlier remating. Functional differences between sperm storage organs within individuals may allow some sperm diversity to be maintained despite sperm precedence (Siva-Jothy and Hooper 1996).
In *D. melanogaster*, courtship feeding and mate guarding have never been reported and seminal feeding is known not to occur due to the small amounts of nutrient passed at mating (Bownes and Partridge 1987; courtship feeding does occur in at least one species of *Drosophila, D. subobscura*, Steele 1986). The sex peptide of *D. melanogaster* has, however, been shown to function as a receptivity inhibiting substance (Chen *et al.* 1988). Two recent investigations of the genetic aspects of sperm defence have been reported. In the first, the effect of selection for delayed senescence in *D. melanogaster* was shown to improve a male's ability to "defend" his sperm against displacement by subsequent males (Service and Fales 1993). The ability of males to defend sperm was affected by both selection regime and the age of the male. If the first male was from the selected "old" line, the proportion of females that remated was lower and the number of his sperm that survived subsequent matings was greater. Thus defence, as measured, occurred both by delayed remating and greater sperm "survival". No effect was found of selection or age on the ability to displace sperm. The second study also identified variation in sperm defence, in this case between lines homozygous for different second and/or third chromosomes (Clark *et al.* 1995). Significant correlations between the presence of particular alleles of four accessory gland proteins (Acps) and the ability of the inbred lines that contained those alleles to resist displacement of sperm were found. Their results suggest that some accessory gland components may have a role in sperm defence.

The results presented in Chapter 4, from an analysis of the effects of first males and females on remating speed, productivity and SDA, also indicated that first males can significantly influence the numbers of sperm "surviving" displacement. However, among the small number of genotypes tested, first males did not appear to influence remating speed, suggesting that variation in the first males ability to delay remating was not the only component of sperm defence.

These findings strongly suggest that accessory gland fluid could be involved in sperm defence. A number of mechanisms by which *D. melanogaster* may defend their sperm are possible. The first is sperm defence mediated directly by accessory gland fluid. Variation in the receptivity inhibiting function may be appreciable (as implied by the finding of a remating delay by Service and Fales 1993), either as functional variation among alleles of the receptivity inhibiting substances, or indirectly by effects on female perception of the "sperm effect". This second possibility could involve a role for bulking agents in accessory gland fluid.

Alternatively, accessory gland fluid could directly affect the resistance of the first male's sperm to displacement. Certain proteins may delay or hinder the displacing activity of second male ejaculate (suggested by the association in the storage organs between sperm and paragonial tubules, Fowler 1973). An indirect effect of accessory gland fluid could arise where sperm precedence was determined ultimately by sperm mixing. In this case, accessory gland fluid could affect either the number of first male
sperm stored after the initial mating or it may affect the rate of usage of first male sperm by the female. Both could affect the numbers of first male sperm present at remating, with consequent effects on precedence.

There are, therefore, a variety of means by which accessory gland fluid could affect sperm defence. The possibility that accessory gland fluid has a direct effect on defence by physically interfering with sperm displacement can be tested experimentally. I therefore decided to attempt to assay any effect of accessory gland fluid on the fertility of subsequent matings. Various strains of *D. melanogaster* are available that transfer all or only a fraction of accessory gland fluid at a mating. Furthermore, strains are available that deliver normal amounts of accessory gland fluid and no sperm. Using these strains as first males, it was possible to produce females who have, in their first mating, received only small, or normal, amounts of accessory gland fluid and no sperm. The females could then be remated to wild type males whose productivity could be measured in relation to the amount of accessory gland fluid passed in the earlier mating.

### 8.3 Materials and methods

Seven strains of flies were used in these experiments:

i) 20B. A strain constructed by transformation of an *Adh*£6cn *ry502* stock with a Carnegie 20 vector carrying *ry* and part of the diphtheria toxin subunit A (DTA). The transformation vector was constructed by replacing the *lacZ* fragment of an *Acp95EF-lacZ* construct (described in DiBenedetto et al. 1990) with a 700bp fragment containing the DTA gene and placing the fusion in the Carnegie 20 vector (Kalb et al. 1993). This produced a strain which expresses the DTA gene under the control of the accessory gland specific promoter of Acp95EF. The construct causes the main cells of the accessory glands to completely degenerate shortly after eclosion after which the expression of other Acps is undetectable. There is no apparent effect on either the expression of Esterase-6 which is produced in the ejaculatory bulb or on viability or behaviour. Homozygous 20B flies are sterile because they produce no sperm past the primary spermatocyte stage, thought to be due to 'leakage' of the Acp95EF promoter in the testis (Kalb et al. 1993).

Phenotypes of the 20B males were expected to result in significantly shorter remating since they pass no sex peptide. Analysis of the remating speed (see Table 8.3) showed females mated to 20B males were faster to remate (KW statistic = 14.45, P < 0.001).

ii) PPF4. This stock was constructed in a similar manner to 20B, but uses a fragment of the DTA gene which encodes a less toxic product. The vector used was the *w*-marked vector pW8, transformed into a *w;ry Sb P[ry* D2-3]*/TM6 background. This strain expresses another Acp (Acp26Aa) at 1% of normal levels. These males
therefore have some accessory gland function remaining and also transfer sub-normal amounts of sperm.

iii) tudor. These males were the progeny of homozygous Tudor females. They are phenotypically normal, but lack testis, and are therefore sterile. Accessory glands develop and mating ability and transfer of accessory gland fluid are normal. The stock is described in Chapter 2.

iv) XO males produced by the method described in Chapter 2. These males, like Tudor males, pass accessory gland fluid but no sperm.

v) Wild type. These flies were from the Dahomey stock described in Chapter 2.

vi) scarlet. This stock, homozygous for the recessive eye colour marker scarlet (st), was derived by introducing st into a Dahomey background with repeated backcrossing to produce an outbred st-marked stock.

vii) sepia flies were collected from a stock homozygous for the eye colour marker sepia in a Dahomey background, described in Chapter 2.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Accessory gland main cell products (% of normal)</th>
<th>Sperm</th>
</tr>
</thead>
<tbody>
<tr>
<td>20B</td>
<td>0</td>
<td>none</td>
</tr>
<tr>
<td>PPF4</td>
<td>1</td>
<td>few</td>
</tr>
<tr>
<td>Tudor XO</td>
<td>100</td>
<td>none</td>
</tr>
<tr>
<td>Dahomey</td>
<td>100</td>
<td>normal</td>
</tr>
</tbody>
</table>

Table 8.1. Summary of the amounts of accessory gland fluid and sperm passed to females during a mating with a male of the indicated genotype.

The underlying design of all three experiments was to first mate females to one of the strains listed above. These females and their controls were then mated to normally fertile males, allowing comparisons of the effects of different first males on second male fertility. Experiments 1 and 2 used initially virgin females, while Experiment 3 used non-virgin females. In Experiments 1 and 2, different intervals between rematings were employed (either 24 hours or immediate). In each experiment, groups of females were identified by the type of male used in the first mating.

All flies used were grown under uncrowded conditions of 60 larvae per vial and collected on ice within 8 hours of eclosion. Flies were aged for 3-4 days in single-sex groups of 5 per yeasted vial.
8.3.1 Experiment 1: 24 hour rematings

*st* females were mated individually to 20B, PPF4, *tudor* or Dahomey males on Day 1 and the males removed within one hour of the completion of copulation. Females were placed in fresh yeasted vials overnight and rematings to *st* males carried out in the same vials on Day 2. Each female was provided with a virgin *st* male and all vials were scanned every 10 minutes until females remated. Experimental females remated readily (i.e. within 1-2 hours) with the exception of the females mated to wild type males. This was expected due to the 'sperm effect', in which females that have relatively full sperm storage organs usually take two or more hours to remate (Manning 1962; Scott, 1987).

Within an hour of remating, females were placed in fresh yeasted vials, and the remating vials retained for egg and progeny counts. Females were transferred daily thereafter into fresh yeasted vials until Day 10. Thereafter, only females that were still producing progeny were transferred in order to complete progeny counts. Vials were kept at 25°C during progeny development and all progeny were counted within 2-3 days of eclosion.

8.3.2 Experiment 2: Same day rematings

This experiment followed the same procedure as the previous experiment, except that all matings and rematings were performed on the same day. After the first mating, males were removed and replaced with single *st* males. The remating intervals were timed from the beginning of the first mating to the beginning of the second mating. Vials were observed continuously for periods up to six hours. The first matings were carried out in batches of 20 according to the strain of first male, in order to avoid any bias that may have arisen, for example, had all of one experimental group been set up in the morning and another in the afternoon. Subsequent transfers of females to new vials followed the same schedule as in Experiment 1.

8.3.3 Experiment 3: Non-virgin females

It was important that the non-virgins used in these experiments had relatively little sperm in storage when the effects of the XO males were tested, otherwise variation in female productivity could obscure any effects. The first male (*se*) sperm were exhausted by transferring *se* females to new bottles every second day for 8 days. Half the females were then mated to XO males in single pairs in new vials. All matings were observed to ensure a single insemination only. Males were removed soon after finishing copulation. On the next day, all females were paired with *se* males in new vials. After remating, females were transferred to new vials, and the progeny scored 16 days later. No wild type progeny were observed in any cross, indicating that the XO males were all sterile.

Results were analysed using the Kruskal-Wallis one-way analysis of variance. Multiple comparisons between treatments were performed only when a Kruskal-Wallis
test indicated significant heterogeneity among groups. The method used was that described in Siegel and Castellan (1988).

8.4 Results

8.4.1 Experiment 1: 24 hour rematings

Four experimental groups were used in this experiment, identified by the strain of the first male. The groups were:

i) '20B' (no main cell products, no sperm);
ii) 'PPF4' (little main cell products, few sperm);
iii) 'tudor' (full accessory gland products, no sperm); and
iv) 'wild type' (Dahomey males, full accessory gland products and sperm).

The total number of eggs laid by the females after the first matings and until Day 10 did not differ significantly between any of the groups (Table 8.2). The median number of eggs laid per day is shown in Figure 8.1. The generally lower productivity of the 20B and PPF4 groups is reflected in Figure 8.2, which shows the percentage of eggs giving rise to adults (%hatchability) plotted against time. This is taken to be indicative of a relative shortage of sperm in the 20B and PPF4 females as after Day 4 the efficiency of sperm use is high (Gilbert et al. 1981).

The total number of progeny produced by the experimental groups did significantly differ (KW statistic = 20.39, p < 0.001, Table 8.2). Multiple comparisons showed the following significant differences:

i) the 20B and PPF4 groups both produced fewer progeny than the wild type group; and

ii) the 20B group produced fewer progeny than the tudor group.

Figure 8.3 shows the median number of progeny produced plotted against time. Analysis showed that the significant differences were due principally to the lower fertility of the 20B and PPF4 groups on Days 4-7.

<table>
<thead>
<tr>
<th>First male</th>
<th>Median total number of eggs</th>
<th>Median total number of progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>20B</td>
<td>417</td>
<td>202</td>
</tr>
<tr>
<td>PPF4</td>
<td>448</td>
<td>173</td>
</tr>
<tr>
<td>tudor</td>
<td>453</td>
<td>309.5</td>
</tr>
<tr>
<td>wild type</td>
<td>496.5</td>
<td>346.5</td>
</tr>
</tbody>
</table>

Table 8.2. The median total number of eggs and progeny produced by each of the experiment groups.
Figure 8.1. The median number of eggs laid per day by groups mated to the first male indicated in Experiment 1.

Figure 8.2. Median %hatchability of eggs laid by the groups mated to the first male indicated in Experiment 1.

Figure 8.3. The median number of progeny produced per day by groups mated to the first male indicated in Experiment 1.
8.4.2 Experiment 2: Same day rematings

The four experimental groups that were used in this experiment differed from Experiment 1. The PPF4 group was omitted and replaced by the "virgin" experimental group, where no first male is used and the (virgin) females were mated to st males on the following day (along with the four other groups). The groups were:

i) '20B' (no main cell products, no sperm);
ii) 'tudor' (full accessory gland products, no sperm);
iii) 'wild type' (Dahomey males, full accessory gland products and sperm); and
iv) 'virgin' (no "first mating", just a single mating to a st male).

Significant differences were apparent in the median total number of eggs laid by each of the four experimental groups (KW statistic = 12.54, P = 0.006; Table 8.3).

<table>
<thead>
<tr>
<th>First male</th>
<th>n</th>
<th>Median total number of eggs</th>
<th>Median total number of progeny</th>
<th>Remating interval (mins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20B</td>
<td>36</td>
<td>487.5</td>
<td>333</td>
<td>119</td>
</tr>
<tr>
<td>tudor</td>
<td>52</td>
<td>447</td>
<td>306</td>
<td>178</td>
</tr>
<tr>
<td>Wild type</td>
<td>46</td>
<td>516</td>
<td>344.5</td>
<td>174</td>
</tr>
<tr>
<td>Virgin</td>
<td>49</td>
<td>419</td>
<td>268</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 8.3. The median total number of eggs and progeny produced by each of the experimental groups and the remating speed of the second males.

Multiple comparisons show that the number of eggs laid by the wild type group was significantly greater than for tudor and virgin groups. The median number of eggs laid per day by each group is plotted against time in Figure 8.4.

The %hatchabilities, shown plotted against time in Figure 8.5. The only significant differences in %hatchabilities occurred on Days 4 and 5, where the virgin group had a %hatchability lower than the highest group only.

Total numbers of progeny differed significantly between the groups (KW statistic = 9.17, P = 0.027; Table 8.3). Multiple comparisons showed that both wild type and 20B females produced significantly more progeny than the 'virgin' females. The median number of progeny produced per day is plotted against time in Figure 8.6.
Figure 8.4. The median number of eggs laid per day by groups mated to the first male indicated in Experiment 2.

Figure 8.5. Median %hatchability of eggs laid by the groups mated to the first male indicated in Experiment 2.

Figure 8.6. The median number of progeny produced per day by groups mated to the first male indicated in Experiment 2.
8.4.3 Experiment 3: Non-virgin females

The presence of only small amounts of sperm 8 days after the first mating was confirmed by the small numbers of *sepia* progeny produced by the females in the 24 hours prior to remating (non XO-mated females: 5.8 progeny/female, n = 171). Females that had been mated to XO males did not produce significantly different numbers of progeny during that 24 hours ($t_{198} = 0.49$, $P > 0.63$). However, since relatively few females were producing progeny during the 24 hours prior to the last remating, little can be inferred from this similarity.

The fertility of the final males was not affected by the earlier mating to XO males ($t_{304} = -0.81$, $P > 0.42$). The relatively small numbers of progeny produced is likely to be due to the age of the females. Thus mating with XO males appears to have no effect on later male fertility in non-virgin females.

<table>
<thead>
<tr>
<th>XO mated</th>
<th>n</th>
<th>Mean pre-remating progeny (24 hour) (95% confidence limits)</th>
<th>Mean post-remating progeny (95% confidence limits)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>153</td>
<td>6.5 (4.7 - 8.3)</td>
<td>44.5 (39.1 - 49.9)</td>
</tr>
<tr>
<td>No</td>
<td>153</td>
<td>5.8 (4.0 - 7.6)</td>
<td>47.8 (41.9 - 53.7)</td>
</tr>
</tbody>
</table>

Table 8.4. The numbers of progeny produced from rematings with wild type males where females differed in whether they had previously mated to an XO male.

8.5 Discussion

The results of all three experiments indicated that accessory gland fluid has little direct effect on the fertility of subsequent males, either in virgin or non-virgin females.

The main comparison is between the 20B group (along with the PPF4 to a lesser extent) and the *tudor* group. Any effect of the presence of the main cell products of accessory gland fluid on the productivity of the second male would be apparent between these two groups. With the same day rematings, no significant differences were observed. However, when the second male mated 24 hours later, the *tudor* group produced more progeny. This would not have been surprising had the effect been due to differences in progeny produced soon after the remating. The difference could then have been attributed to the "head-start", in terms of egg production, given to the *tudor* group females by the *tudor* male's accessory gland fluid, missing in the 20B matings (Chen *et al.* 1988). These extra eggs would have allowed the *tudor* group to make more efficient use of the excess of sperm that is released during the first few days after mating (Gilbert *et al.* 1981). However, the extra progeny were apparent throughout the duration
of the experiment, suggesting that females in the tudor group either stored more sperm initially or used whatever sperm was stored more efficiently. Whichever is the case, the results indicate that rather than acting in a sperm defence role, the extra accessory gland fluid appeared to act to increase the productivity of the second male. The positive effect of the accessory gland fluid can not be taken as evidence of a positive role in the fertility of subsequent males. Instead, the positive influence observed is likely to be a result of testing accessory gland fluid "out of context", i.e. with no first male sperm present. It would be possible to test accessory gland fluid "in context". An already inseminated female would be mated to a tudor (or similar) spermless male and then secondly, to a wild type male. The results of this would be difficult to interpret due to the confounding effects of displacement and sperm defence, but if either could be controlled, some useful results might be obtained.

One comparison of interest in Experiment 2 (same day rematings) was that the group that received no main cell products in the first mating (20B) did not differ significantly in subsequent fertility from the wild type group which received both full accessory gland products and sperm. In addition, both the 20B and wild type groups produced significantly more progeny than the virgin group. While in the comparison between wild type and 20B the effects of accessory gland fluid and sperm are confounded, the comparison between 20B and virgin groups should only be due to the secondary cell products, which are probably still produced in the 20B males (Kalb et al. 1993). The basis of the effect of the secondary cell products is unclear and it would be of interest to determine if this effect of 20B matings is repeatable.

The failure of these experiments to find evidence of a role for accessory gland secretions in sperm defence does not exclude the possibility that they do affect sperm defence. Some function for accessory gland fluid is strongly suggested by the association between defence ability and Acp alleles (Clark et al. 1995). Of the possible defence mechanisms listed in the introduction, the present results rule out only one, i.e. that any defensive effect of accessory gland fluid is due to direct bulking or blocking effects of accessory gland proteins or other male secretions. However, the other mechanisms, in which accessory gland fluid influences displacement via an effect on sperm, remain possible.

Of these other mechanisms, only one is consistent with the results of previous investigations of sperm defence. This mechanism is that in which defence occurs as an indirect effect of accessory gland fluid on the number of first male sperm initially stored. The results of both Clark et al. (1995) and Service and Fales (1993) are consistent with the idea that numbers of sperm that are stored are important in defence. This hypothesis requires that there were differences between the various strains of first male tested either in the number of sperm they transfer or the number they cause to be stored. While ordinarily there will be little genetic variation for male fertility, differences in the number of sperm transferred by the first male could, however, have
been induced by selection (Service and Fales 1993) or inbreeding (Clark et al. 1995), and at a level that would not necessarily be apparent from progeny counts (since the level of productivity is determined mainly by the female, see Chapter 4). But variation in the number of sperm transferred does not explain the correlation between certain Acp alleles and variation in sperm defence found by Clark et al. (1995). No correlation would be expected between genes controlling spermatogenesis and Acp alleles. A better rationale is that the reduction in stored sperm numbers, leading to poorer sperm defence, was caused by inbreeding at the Acp loci rather than at spermatogenic loci. This would explain the correlation of defence and the four Acp alleles. A caveat on the argument that accessory gland fluid plays a role in sperm defence is raised by the results of Herndon and Wolfner (1995). They found that null variants of one of the alleles (Acp26Aa) identified by Clark et al. (1995) as varying with sperm defence, had no effect on sperm defence.

The tentative conclusion from this reasoning is that sperm defence may depend on having more sperm in storage, but that the number stored is determined, at least in part, by the quality of the accessory gland fluid rather than, or as well as, the number of first male sperm transferred. The present experiment would not be expected to detect any effect of the presence or absence of accessory gland fluid in this scenario.

If there is significant variation at Acp alleles that affects sperm defence, then it should be discernible in a heritability or selection experiment. It would be possible to correlate the results of such experiments with Acp alleles to test their involvement in first male productivity and sperm defence.
9

General Discussion

9.1 Summary

The following discussion considers the four major aspects of sperm precedence in *Drosophila melanogaster* that were addressed by the experiments presented in this thesis. The aspects discussed are the evolutionary significance, the genetics and the mechanisms of sperm displacement ability (SDA), and the importance of sperm defence. Some areas of uncertainty in the understanding of each aspect of sperm precedence are identified, and experimental approaches to these problems suggested.

9.2 The evolutionary significance of sperm displacement

The evolutionary importance of sperm precedence depends on its effect on male reproductive success, as indicated by the relationships shown in Figure 1.1, Chapter 1. Laboratory measures of second male sperm displacement ability (SDA) have consistently demonstrated that second males are highly successful in displacing first male sperm from storage (Lefevre and Jonsson 1962; Boorman and Parker 1976; Prout and Bundgaard 1977; Gromko *et al.* 1984a), although there is considerable variance of SDA within and between strains. SDA has potentially large effects on male reproductive success. Female *D. melanogaster* have the capacity to produce over a hundred offspring within two days of a single mating (the minimum period before which remating is likely to occur, see below). The gain in paternity to a male which can displace, for example, 95% rather than 70% of first male sperm, will be of the order of 30 or more progeny.

However, laboratory measures do not necessarily reflect the importance of a character under natural conditions. Of the large number of variables that may alter under artificial conditions, one is the female remating rate. On the basis that laboratory remating rates are artificially high, Gromko and co-workers have argued that remating rates in nature are considerably lower (e.g. Pyle and Gromko 1981). Under their laboratory approximation of natural conditions (in which remating opportunities are
limited to 2 hours per day), female remating rate declines markedly, to the point that females that do remate usually have only low levels of sperm remaining in storage. Under these conditions, only small amounts of sperm displacement are observed (Newport and Gromko 1984; Letsinger and Gromko 1985) and variation in SDA would contribute relatively little to male reproductive success. Natural selection on SDA would be correspondingly weaker. This raises the question of whether variation in SDA in nature would realise the large potential paternity gains indicated as possible under laboratory conditions.

In the experiments presented in this thesis, an alternative design was used where females effectively all remate two days after the first mating. This fixed interval was chosen because earlier measurements made on the same Dahomey stock had indicated that females remate at least every two days (N. Grey, unpublished results). Van Vianen and Bijlsma (1993) have observed similar rates of remating in recently caught wild type populations. Thus, the assumption underlying the present experiments was that females remate at least every other day in the cage populations, and possibly as frequently in natural populations. Under these conditions, the experiments presented in Chapter 3 showed that SDA had little, if any, heritable variation, suggesting that selection on SDA, or correlated characters, in the Dahomey cage populations is intense.

These alternative experimental approaches to the measurement of SDA highlight a gap in the understanding of an important parameter affecting SDA. For insects, remating intervals are difficult to measure in natural populations. One parameter that has been measured repeatedly in wild populations of *D. melanogaster* (and other *Drosophila* species) is the frequency of detectable multiple insemination (concurrent multiple paternity). The values found have been as low as 23% (Griffiths et al. 1982), but are more commonly well over 50% (47%, Milkman and Zeither 1974; up to 64%, Marks et al. 1988; 52-56%, Ochando et al. 1996). While these values are suggestive of generally high female remating rates, they cannot be used to estimate remating rates because they are consistent with both sperm displacement and with females remating when sperm stores are low. Therefore, it would be of considerable value to studies of SDA to be able to measure and compare remating rates of flies from wild and cage populations.

**9.3 The genetics of sperm displacement**

The results presented in Chapter 3, which showed that the heritability of SDA is extremely low and probably compounded with heritable variation in pre-adult viability, represent the only measurement of heritability of SDA. The result of Hughes (1997) describes only the additive and dominance variance for SDA associated with the third chromosome of *D. melanogaster*. 117
A finding of very low heritability for a trait usually implies that the trait is under strong directional selection (Falconer 1989). Although the heritabilities of life history traits are generally lower than heritabilities for either physiological, behavioural or metric traits, heritabilities of life history traits still average 0.26 (Roff and Mousseau 1987). Thus even in comparison only to life history traits, the heritability of SDA, measured as 0.10 ± 0.08 in Chapter 3, is still quite low. This poses the question of whether low heritability is, itself, informative for SDA, particularly whether it implies that there is an unusually low level of additive genetic variance for the trait.

A first possible explanation for the low heritability is that the high intrinsic variability of SDA makes any measure of the trait necessarily imprecise. Measures of male SDA in *D. melanogaster* involve a large amount of environmental variance (shown in Chapter 3, by the low repeatability of SDA) as well contributions of variance from females and first males (Chapter 4). These combined sources of variance increase the total phenotypic variance, proportionally reducing the second male additive component (and therefore the heritability) to levels where it may be difficult to distinguish from zero. An alternative measure of heritability is the coefficient of additive genetic variance, CVA, which as shown in Chapter 3, is very high for SDA. A high CVA is consistent with the interpretation that the heritability is low, not because of an absence of additive genetic variance, but because the phenotypic variance is comparatively large (Houle 1992). If this interpretation is correct, then there may be considerable amounts of additive genetic variance for SDA. This is the most parsimonious explanation of the low heritability of SDA, and indicates that there may not be unusually low levels of additive genetic variance for the trait.

A second possibility is that SDA is actually subject to unusually strong selection. This possibility is suggested by two recent studies of sperm displacement. The first is that of Rice (1996) who found evidence that when females are prevented from evolving in response to the costly effects of male ejaculate, the cost to females of receiving ejaculate increases. This implies that males continually evolve more toxic ejaculates (presumably of shorter term benefit to male fecundity) and females continually evolve in parallel to minimise those toxic effects. The expectation from this is that at least some components of accessory gland fluid will be under very strong and continual selection. A second study by Price (submitted) is consistent with the ideas presented by Rice (1996). Price measured sperm precedence in females that were sequentially remated with different combinations of con- and heterospecific males. In contrast to the strong second male precedence observed when both males are conspecifics, her results showed that conspecific males always had a high level of sperm precedence over heterospecific males, whether mating as the first or second male. Furthermore, using XO males, she showed that accessory gland fluid of conspecific XO males significantly reduced the number of hybrid progeny, irrespective of mating order.
The results of Price (submitted) are similar to those of Rice (1996) in that they suggest that male accessory gland fluid components are highly adapted to the reproductive physiology of females in the same population. Differentiation among populations in accessory proteins (Acp) variants may explain some of the excess sequence variation observed in Acp26Aa (Aguadé et al. 1992). Rapid evolution of Acps may have been a contributory factor to the failure of Clark et al. (1995) to find associations between Acp alleles and SDA. They tested homozygous lines from two localities (North Carolina and Maryland) against females from a cinnabar-marked laboratory stock. Populations from these two localities may have diverged sufficiently to confound the results of Clark et al. (1995).

The relevance of the work of Rice and Price to the present study is that they provide evidence that selection on components of accessory gland fluid is intense. This may explain why the heritability of SDA is so low. If variation among Acp alleles is the major determinant of variation in SDA, and this variation is subject to strong selection, then a mutation/selection balance will be the most likely mechanism maintaining genetic variation for the trait. Since there are about 87 Acp loci (Chen 1996), not all of which would be expected to contribute to additive genetic variance for SDA, the amount of variation maintained by a mutation/selection balance may be low.

However, an objection to this explanation is that variation in sperm production and transfer would still be expected to contribute to additive variance for SDA. The results presented in Chapters 3 and 6 suggested a role for variation in sperm number in SDA. Thus, although likely to be under intense selection (e.g. Falconer 1989), some additive genetic variance for sperm number should be maintained by a mutation/selection balance, since hundreds of genes are known to be involved in spermatogenesis (Castrillon et al. 1993). While in D. melanogaster, significant additive genetic variance has been found for female egg production (heritabilities ranging from 0.04 to 0.18 Robertson 1957; Tantawy and Rakha 1964; Tantawy and El-Helw 1966), there is only one known measurement of additive variance for male productivity (numbers of progeny), which found a value not significantly different from zero (Hughes 1995). However, selection on sperm numbers may not be under intense selection in virgin matings, since males transfer more than enough sperm to fill the female sperm storage organs. This may contribute to the level of additive variance. In contrast, in non-virgin resamplings, high levels of displacement probably depend critically on the transfer of large numbers of second male sperm (Chapter 6), suggesting that, where remating rates are high, selection on sperm number may be intense. Thus, although selection on Acps and sperm numbers may be intense, this explanation seems less likely to account for any unusually low levels of additive variance for SDA.

The relative contributions of variation in accessory gland function and sperm number to genetic variation in SDA cannot be easily distinguished. However, one strategy for trying to disentangle their effects might be to exploit their different
developmental origins. Testes are germ tissue derived, while accessory glands are somatic tissue. Any regime that could be shown to induce a trade-off between the amounts of the two types of tissue (i.e. between reproduction and growth) could be examined for effects on both male productivity and SDA. Courtship has been shown to be a highly costly aspect of male reproduction (Cordts and Partridge 1996), and larger males have been shown to be capable of delivering more courtship to females (Partridge et al. 1987), possibly because they have a greater total energy budget. Consequently males selected for divergent courtship rates (a costly trait) without any change in body size, may vary the relative amounts of energy devoted to spermatogenesis. Such males may vary in the relative mass of germ line and somatic tissues and correlations between this variation and measures of reproductive performance (including productivity and SDA) could be measured.

9.4 The mechanism of sperm displacement

One of the major problems facing investigations of SDA in *D. melanogaster* is the difficulty of estimating the numbers of sperm transferred in both virgin and non-virgin female matings. Experiments presented in this thesis were successful in identifying two methods by which estimates of sperm numbers stored and transferred might be made. In Chapter 3, a crude measure of male productivity (number of adult progeny) was found to have a near-significant relation to SDA. In Chapter 6, the results of interrupted matings suggested strongly that the level of SDA in normal matings was determined by the numbers of sperm transferred. Both these results were interpreted as reflecting differences in sperm numbers. However, in neither case could the possibility that the observed effects were in fact due to accessory gland fluid be excluded.

The continual presence of confounding factors when interpreting such results reflects a basic problem presented by the reproductive biology of *D. melanogaster*. While it is possible to study the effects of accessory gland fluid in the absence of sperm (using XO males for example), it is not possible to study variation in sperm number in the absence of accessory gland fluid (such matings are sterile, Hihara 1981). Since both traits are subject to strong selection (Chapter 3, see section 9.3), most genetic variants (of either trait) would be expected to have similar, detrimental effects on male reproductive success. This prevents clear attribution of causes of variation in SDA to either trait.

One method by which this problem could be approached could be to minimise variation in one trait, while measuring the effect of variation in the other trait. This could be achieved using identically heterozygous males (created by the methods presented in Chapter 4) as either first or second males. The SDA and the male productivity of these males could be measured and correlations between them estimated. Although these males would be genetically identical for both accessory gland genes and
genes determining sperm number, it is likely that the coefficient of phenotypic variance is greater for sperm number than for the effects of accessory gland products. One reason for thinking this may be the case is that spermatogenesis involves many more loci than are known for accessory gland products (DiBenedetto et al. 1987; Hackstein 1991; Castrillon et al. 1993). If the number of sperm transferred was a repeatable trait of individual males, then variation in numbers of sperm transferred may be observed among males within genetically uniform groups. This may allow the effects of variation in sperm number on male productivity and SDA to be detected.

9.5 Sperm defence

Of the experiments presented in this thesis, only two investigated sperm defence. In Chapter 8, it was shown that accessory gland products do not appear to play a role in physically interfering with second male sperm transfer or storage. In Chapter 4, variation in sperm defence ability between different first males was shown to occur in the absence of significant effects on female remating speed. This result was consistent with variation between males in the effect of ejaculate on either sperm storage or ability to avoid displacement.

A major difference between sperm displacement and sperm defence in D. melanogaster is that there appears to be appreciable additive genetic variance for sperm defence. Increased sperm displacement ability has been indirectly selected (Service and Fales 1993) and shown to correlate with particular Acp alleles (Clark et al. 1995), although null alleles of one of the loci identified by Clark et al. (1995) has been shown to have no effect on SDA (Acp26Aa, Herndon and Wolfner 1995). It would be informative to measure any additive genetic variance for defence. If significant additive variance was found then it would imply that defence is not solely due to variation in sperm numbers (although it could still be involved) since large amounts of additive genetic variance is not expected for sperm number. Large amounts of additive variance would indicate that defence was not subject to the same intensity of selection as SDA. One reason for this may be that the numerical paternity gain for increased defensive ability is much smaller than for increased SDA. In experiments presented in Chapter 4, the difference in the number of first male progeny produced after remating between the best and worst defensive males was only 7 progeny. Under natural conditions, 7 extra progeny produced over a number of days may be the object of much weaker selection than the paternity benefit of increased SDA (at least 30 progeny over the first 1-2 days).

Mechanisms of defence are also difficult to investigate because of the confounded effects of sperm number and accessory gland fluid. Although the presence of additive genetic variance could make selection experiments an option for investigating the basis of the trait, it may be more profitable to investigate more thoroughly the factors affecting sperm transfer and storage of first male sperm. Various identically
heterozygous lines of first males could be initially screened for repeatable differences in male productivity (i.e. by progeny counts). In those combinations showing large differences, direct counts of stored sperm could then be made and correlations between the direct counts and a number of other factors then measured. The other factors could include effects on female remating rates, sperm defence and the presence of particular Acps. This could also provide information pertinent to many of the assumptions made in studies of male reproductive success, particularly the assumption that number of offspring of males (male productivity) are proportional to the numbers of sperm stored.
References


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