# The evolution of multiple mating in the stalk-eyed fly, *Cyrtodiopsis dalmanni*

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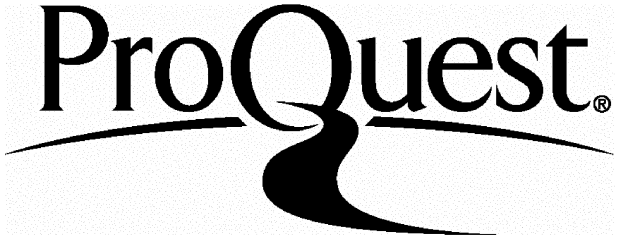

## ABSTRACT

In the stalk-eyed fly, *Cyrtodiopsis dalmanni*, male eyespan is under sexual selection. This species has an extremely high mating frequency, however, the reasons for multiple mating by females are unclear. There are a number of adaptive hypotheses proposing either direct or indirect (genetic) benefits.

I investigated three hypotheses that propose direct benefits for female multiple mating. (1) Sperm replenishment. Large females were found to have a higher number of eggs than small females and thus may require more sperm. Large females did not receive more ejaculate per mating but did mate at a higher frequency than small eyespan females. I concluded that females mate multiply to gain fertility benefits. (2) Ejaculate-derived fecundity enhancement. Mating increases female fecundity, representing a potential direct benefit of multiple mating. I established that male accessory gland proteins (components of the ejaculate) are partly or wholly responsible for the elevated fecundity caused by mating. (3) Reduction in female receptivity in response to mating. If mating is costly, then a reduction in receptivity to mating would constitute a direct benefit to females. I found no evidence that receptivity is reduced following mating.

Two non-adaptive hypotheses for multiple mating by females were also evaluated. (1) Genetic correlation in male and female mating frequency. Female mating frequency may have evolved as a correlated response to selection on male mating frequency. I found no difference in the mating frequency of females from lines of flies that had been artificially selected for increased and decreased male mating frequency. There was therefore no evidence for a genetic correlation in mating frequency between the sexes. (2) Male control of mating frequency. I made phenotypic manipulations of male eyespan (and therefore attractiveness to females) in lines artificially selected for increased and decreased male mating frequency. I found that both female preference, and male mating propensity were important determinants of mating frequency suggesting that predominant male control of mating cannot explain female multiple mating.

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# General Introduction

## 1.1 OUTLINE

Multiple mating is common in insects (Thornhill & Alcock 1983; Ridley 1988; Eberhard 1985; Andersson 1994; Choe & Crespi 1997; Arnqvist & Nilsson 2000). The primary purpose of mating, the transfer of sperm, is beneficial for both sexes. There may however be conflict between male and female interests over mating frequency (Trivers 1972; Parker 1979; Rice 1996; Arnqvist & Nilsson 2000). This conflict arises from anisogamy, the difference in male and female gamete size. Anisogamy results in differences in how the sexes maximise their fitness (Bateman 1948; Parker 1979). Sperm are small, numerous and thus available in a relatively unlimited supply. Thus male reproductive success generally increases with each mating, placing selection pressure on males to mate as frequently as possible (Bateman 1948; Trivers 1972). In contrast, eggs are large and limited in number. Females therefore maximise their reproductive success through the number of viable eggs they produce and by choosing good quality mates (Bateman 1948; Parker 1979; Andersson 1994). Females are selected to mate at a lower optimal frequency than males, determined by a trade-off between the costs and benefits of mating (Arnqvist *et al.* 2000). One or a few matings are often enough to supply females with all the sperm necessary to fertilise a lifetime's supply of eggs. Therefore, the widespread occurrence of multiple mating by females requires explanation, especially given that high rates of mating are often costly.

Adaptive hypotheses explaining multiple mating have traditionally been divided into direct and genetic benefits (Arnqvist & Nilsson 2000; Jennions & Petrie 2000). The replenishment or renewal of sperm supplies is an important direct benefit. For many insects however, mating provides more than sperm (Arnqvist & Nilsson 2000). Other direct benefits result from the transfer of nutrients (Thornhill & Alcock 1983; Gwynne 1997) and / or accessory gland proteins in the ejaculate, which increase female fecundity (Herndon & Wolfner 1995; Chen *et al.* 1988). Another direct benefit may be the reduction in female receptivity following mating, if mating imposes costs to females. In addition to direct benefits explaining multiple mating, a number of indirect, or genetic,

hypotheses have been proposed (Jennions & Petrie 2000). Females may mate multiply to obtain 'good genes' (Yasui 1997; Curtsinger 1991), to increase the genetic diversity of offspring (Philippi & Seger 1989; Yasui 1997) or to avoid inbreeding (Tregenza & Wedell 2002).

It is also possible that multiple mating in females may not have evolved as an adaptive behaviour, but could result from sexual conflict over mating frequency (Parker 1979). Strong selection for frequent male remating could result in female multiple mating if there is a genetic correlation between male and female mating frequency (Halliday & Arnold 1987). Alternatively, there could be different genes determining mating frequency between the sexes, and males and females may struggle to control mating frequency. Male control over mating could result in females being forced to mate beyond their optimum frequency (Clutton-Brock & Parker 1995; Gavrilets *et al.* 2001). To determine the reasons for female multiple mating, sexual conflict and the degree of female control over mating must be assessed in conjunction with the benefits and costs of mating for females (Rowe *et al.* 1994).

The stalk-eyed fly, *Cyrtodiopsis dalmanni*, offers an unusual and exciting opportunity to investigate female multiple mating. The species has an extremely high mating frequency, for example up to 10 times an hour in the laboratory (Wilkinson *et al.* 1998a). In addition, *C. dalmanni* is a model for sexual selection studies, enabling novel opportunities to investigate sexual conflict over mating frequency. In the first section of the thesis, three potential direct benefits of multiple mating are investigated. 1. Fertility benefits. 2. The receipt of male accessory gland proteins that boost female fecundity. 3. Mating-induced reductions in female sexual receptivity. In the second section of the thesis two non-adaptive explanations for multiple mating are investigated. First I tested for a genetic correlation between male and female mating frequency. Then I examined whether females mate multiply because males exert relatively more control over mating frequency than females.

## 1.2 MULTIPLE MATING IN STALK-EYED FLIES, *CYRTODIOPSIS DALMANNI*

The stalk-eyed fly, *Cyrtodiopsis dalmanni*, is in the Diopsid family of Diptera. Diopsids are characterised by hypercephaly, the lateral displacement of eyes and antennae on the end of stalks (Hurley *et al.* 2001; Hurley *et al.* 2002). *C. dalmanni* mate at a very high frequency each morning (Wilkinson *et al.* 1998a). During the day, individual *C. dalmanni* feed on the ground near streams in the forests of south-east Asia, but at night they form highly female-biased aggregations of between 1-10 females per male (Burkhardt & de la Motte 1983, 1987, 1988). The majority of copulations occur at dawn and dusk when the flies are aggregated (Burkhardt & de la Motte 1983). The presence of sexual dimorphism in eyespan in *C. dalmanni* and other species of Diopsid, indicates that eyespan is a secondary sexual characteristic under the influence of sexual selection. There is experimental evidence for both male-male competition to control nightly aggregations, and female preference for males with exaggerated eyespans (Wilkinson & Dodson 1997; Panhuis & Wilkinson 1999; Hingle *et al.* 2001a,b; Wilkinson 2001).

Expansion of the head capsule may have initially been naturally selected. Optical studies reveal stalk-eyed flies have a high visual resolution with binocular vision, enabling more accurate judgement of size and distance (Burkhardt & de la Motte 1983). However, there are costs associated with hypercephaly. For example, inflation of the eyestalks after eclosion takes time, and flies are subject to increased predation risk (de la Motte & Burkhardt 1983) and decreased aerial agility (Swallow *et al.* 2000). For species with sexual dimorphism males but not females have eyespans longer than their body length, and sexual selection acts in addition to natural selection to further exaggerate eyespan (Figure 1.1. Descamps 1957; Shillito 1971; Burkhardt & de la Motte 1983, 1985; Feijen 1989; Wilkinson & Dodson 1997). Sexual dimorphism has evolved multiple times within the *Diopsidae* from monomorphic species. For example, monomorphic *C. quinqueguttata* is plesiomorphic to dimorphic *C. whitei* and *C. dalmanni* (Baker *et al.* 2001b; Wilkinson & Dodson 1997).

Sexual selection in *C. dalmanni* and *C. whitei* is mediated by male-male competition and female mate choice (Burkhardt & de la Motte 1988; Wilkinson & Dodson 1997; Panhuis & Wilkinson 1999; Hingle *et al.* 2001a,b; Wilkinson 2001). Male competition occurs through ritualised fights for control of night aggregation sites (Burkhardt & de la Motte 1983, 1987; Lorch *et al.* 1993). Males fight by facing each other, rising up on their mid or hind legs with their forelegs extended and moving their body side to side (Burkhardt & de la Motte 1983, 1987). As the majority of copulations occur at dawn and dusk when the flies are aggregated, the outcome of contests between males influences male reproductive success (Burkhardt & de la Motte 1983, 1987; Burkhardt *et al.* 1994; Panhuis & Wilkinson 1999).

Males with the largest eyespan win the majority of fights in both *C. whitei* and *C. dalmanni* (Burkhardt & de la Motte 1987; Panhuis & Wilkinson 1999). An inverse relationship between the difference in eyespan between the competing males and contest duration indicates that eyespan influences competitive outcome (Panhuis & Wilkinson 1999). Despite a correlation between eyespan and body size (Burkhardt & de la Motte 1985; Wilkinson & Dodson 1997), the effect of eyespan on contest outcome is independent of body size (Panhuis & Wilkinson 1999). Artificial selection for increased and decreased eyespan relative to body length in *C. dalmanni* resulted in the production of males that differed in eyespan but not body size (Wilkinson 1993). It was found that large eyespan males won significantly more fights than small eyespan males, independently of body size (Panhuis & Wilkinson 1999).

A female preference component of sexual selection has also has also been demonstrated in *C. dalmanni* (Burkhardt & de la Motte 1988; Wilkinson & Reillo 1994; Hingle *et al.* 2001a,b). In trials with dummy males, females preferred to roost with males that had the largest eyespans. Preference is visually based, as obscuring the dummy males eliminated female roosting preference (Burkhardt & de la Motte 1988). In addition, removal of

pheromones by washing males had no effect on female choice, confirming choice is visually based (Burkhardt & de la Motte 1988). Female choice tests also demonstrate preference for large eyespan males. Choice tests were conducted by separating males of different eyespan with acetate sheets punctuated by holes large enough for females but not males to pass through. Females had increased numbers of matings with large eyespan males under this experimental set up (Wilkinson & Reillo 1994; Hingle *et al.* 2001a,b).

Female preference for large eyespan males confers a genetic advantage. Wilkinson & Reillo (1994) showed that male eyespan and female preference are genetically linked. Females that choose to mate with large eyespan males will increase their reproductive success by having daughters with a strong preference for large eyespan males, and sons with large eyespans (which win in male-male competitive bouts and thus gain more matings). Another advantage of female preference is that relative eyespan in males is a more reliable indicator of larval condition than other, non-sexual traits. Male genotypes that expressed high values of relative eyespan under good larval food conditions, also expressed high values of relative eyespan when larval food was scarce (David *et al.* 2000). A further advantage may arise from female preference because, in some populations, male eyespan can indicate resistance to X-linked meiotic drive (Wilkinson *et al.* 1998b). The sex ratio of *C. dalmanni* is female biased (Burkhardt & de la Motte 1983), due to a driving X chromosome causing Y-spermatid degeneration (Presgraves *et al.* 1997). A female biased sex ratio results in males having a higher reproductive value than females, and females consequently are under selection to produce offspring with a male-biased sex ratio. Mating with large eyespan males that have increased suppression of meiotic drive elements therefore provides a genetic benefit for females (Wilkinson *et al.* 1998b).

*C. dalmanni* is a species well suited to research questions investigating female multiple mating. The mating system of *C. dalmanni* is characterised by frequent matings by both males and females, which occur in a short time period when flies are aggregated at dawn and dusk (Wilkinson *et al.* 1998a). Copulation times are short, generally lasting less than

60 seconds (Lorch *et al.* 1993). A high number of matings of short duration within a relatively short time period enables easy estimation of mating frequency.

In addition, egg number and egg fertility are easy to determine. This permits the investigation of whether multiple mating, and ejaculate protein transfer, results in increased fecundity and egg fertility. Direct benefits of multiple mating may also include increased egg fertility through strategic ejaculate allocation, i.e. increased ejaculate transfer to highly fecund females. Sperm transfer in *C. dalmanni* occurs via a spermatophore consisting of accessory gland proteins surrounding a central sperm sac (Kotrba 1996). Ejaculate size can easily be estimated in this species by measuring the area of the spermatophore sperm sac. Other direct benefits could result from the transfer of male accessory gland proteins that reduce female sexual receptivity (Chapman 2001; Wolfner 2002). This could be beneficial for females, to reduce the cost of reproduction in female *C. dalmanni* that mate at high frequency (Reguera *et al.* submitted). Behavioural assays such as those required to assess female sexual receptivity are easy to conduct in large numbers in this species.

Bi-directional artificial selection for male mating frequency was successful in this species (Baker *et al.* in prep.). The use of these selected lines facilitates the testing of the non-adaptive hypothesis that females mate at high frequency because of a genetic correlation with male frequency. The presence of strong sexual selection makes *C. dalmanni* a particularly useful study organism, and also enables the investigation of questions in which *Drosophila melanogaster* is an inappropriate model system. For example, the strength of female preference can be varied by phenotypic manipulations of male eyespan. The presence of female preference for male eyespan (Burkhardt & de la Motte 1988; Wilkinson & Reillo 1994; Hingle *et al.* 2001a) can be used to quantify the degree of female control over mating. Predominant male control of mating may provide a non-adaptive explanation of female multiple mating in *C. dalmanni*. Comparison of male mating propensity (manipulated through artificial selection on male mating frequency) and female preference (manipulated through male eyespan) enables the degree of male and

female control of mating to be investigated in a novel experimental design. Taken together these qualities make *C. dalmanni* an ideal experimental study organism for my research questions.

## 1.3 FEMALE MULTIPLE MATING - ADAPTIVE HYPOTHESES

There are a number of hypotheses proposing adaptive advantages to multiple mating for females. These hypotheses can be divided into direct benefits (section 1.3.1) or indirect, genetic, benefits (section 1.3.2).

### 1.3.1 Direct benefits of female multiple mating.

Females may benefit from mating repeatedly through the acquisition of direct, material benefits. The principal function of mating is the transfer of sperm to the female, and thus a major potential direct benefit to multiple mating is the receipt of sufficient sperm to maximise egg fertility (Ridley 1988; Arnqvist & Nilsson 2000). However, other substances can be transferred in addition to sperm during mating which may provide direct benefits. For example, the transfer of nutrients (through the presentation of prey items or the production of spermatophores by males) may directly benefit females (Thornhill & Alcock 1983). Females may also benefit directly from mating through the acquisition of male accessory gland proteins (Chapman 2001; Wolfner 2002). Male accessory gland proteins can provide a direct benefit by increasing female fecundity (Chen *et al.* 1988; Herndon & Wolfner 1995). Mating and the transfer of accessory gland proteins also decrease female receptivity to further mating (Chapman 2001; Wolfner 2002). If mating is costly for females, a reduction in receptivity would be beneficial and constitute a direct benefit. Finally, females can gain direct benefits by mating multiply if the cost of resisting male harassment is sufficiently high (Thornhill & Alcock 1983; Rowe *et al.* 1994). Mating to avoid male harassment ('convenience polyandry') is discussed in the context of male control over mating frequency in section 1.4.2.

#### Fertility benefits of multiple mating.

Sperm are generally considered to be small, cheap to produce and numerous compared to large, relatively expensive eggs. This anisogamy is thought to fundamentally influence the way in which the sexes maximise their reproductive success. Males are selected to mate as frequently as possible whereas females are selected to mate only to obtain sufficient sperm

and / or good quality mates. This argument however depends on the assumption that males have an almost limitless supply of ejaculate.

Ejaculate production may be limiting if it represents a substantial cost to males (Dewsbury 1982). Whilst the cost of synthesising a single sperm may be insignificant, ejaculates containing vast numbers of sperm may start to incur energetic costs. The Indian meal moth *Plodia interplunctella*, reduces sperm production under conditions of dietary restriction, indicating that sperm production is costly (Gage & Cook 1994). Production costs will be increased if sperm are damaged during storage, as the male will have to produce replacement sperm. The number of viable sperm may be depleted during storage if ageing reduces the fitness of sperm (Siva-Jothy 2000), and a number of sperm can die during storage (Yamagashi *et al.* 1992). Ejaculate production may therefore not be cheap and limitless and, if males mate multiply in a short period of time, ejaculates may be allocated between females (Dewsbury 1982; Gage & Cook 1994).

A consequence of prudent male ejaculate allocation may be that females do not receive sufficient sperm to fertilise all their eggs. For example, in the turnip moth *Agrotis segetum*, female fertility is negatively correlated with the number of previous matings her partner had (Svensson *et al.* 1998). There is also evidence of male ejaculate limitation in the sandfly, *Lutzoyia longipalpis*. Females copulating with males that had copulated more than 5 times laid significantly lower proportions of fertile eggs than females copulating with males that had copulated less than 5 times, indicating male ejaculate limitation (Jones 2001).

The high mating frequency observed in the stalk-eyed fly, *C. dalmanni* (Wilkinson *et al.* 1998a), may result in females not obtaining sufficient sperm in a single ejaculate to fertilise the majority of eggs available. Baker *et al.* (2001a) showed that the number of matings a female had influenced egg fertility. Females mated once fertilised an average of 40% of their eggs, compared to 80% of eggs fertilised by multiply mated females with

continuous exposure to males (Baker *et al.* 2001a). This suggests that males may be sperm limited. However, other explanations of low fertility after mating, such as inadequate or inefficient sperm storage by females or genetic incompatibility between mates cannot be excluded. In 58% of insect species surveyed, females ran out of sperm if not allowed to remate, and in almost all species studied female fecundity and fertility was elevated through multiple mating (Ridley 1988).

Males could increase their reproductive success through the allocation of larger ejaculates to highly fecund females. In insects, female fecundity is often associated with body size (Honek 1993). Large females with increased fecundity, therefore presumably have a greater requirement for sperm. There is experimental evidence in many insect species that increased ejaculate is allocated to large females (Wedell *et al.* 2002). For example: the house cricket, *Acheta domesticus* (Gage & Barnard 1996); the stink bug, *Thyanta pallidovirens* (Wang & Millar 1997); the Indian meal moth, *Plodia interplunctella* (Gage 1998); the small white butterfly, *Pieris rapae* (Wedell & Cook 1999); the yellow dung fly, *Scatophaga stercoraria* (Parker *et al.* 1999) and the weevil, *Brentus ancorago* (Johnson & Hubbell 1984).

The cost of producing ejaculate could limit the number of matings males can achieve, and result in ejaculate partitioning between females. One consequence of male ejaculate allocation could be that females need to mate multiply in order to maximise fertility (Walker 1980; Ridley 1988; Arnqvist & Nilsson 2000). It is currently unknown whether female *C. dalmanni* mate multiply in order to receive more ejaculate (and increase fertility) or whether male ejaculate allocation decisions based on factors such as female fecundity influence female mating frequency. Fertility benefits of female multiple mating in *C. dalmanni* are investigated in Chapter 3.

**Nuptial feeding.**

The transfer of nutrients during mating provides a direct benefit for mating multiply in many orders of insects (Thornhill & Alcock 1983). Nuptial feeding can occur when a prey item is presented to the female, for example in scorpion flies, *Bittacus apicalis* and *Hylobittacus apicalis* (Thornhill 1976; 1979). In addition, salivary secretions may be produced on which the female can feed, for example in *B. apicalis* (Thornhill 1976). Spermatophores, i.e. a package of sperm surrounded by a mass of other substances such as proteins, may also provide nutrients to the female. For example the bushcricket *Requena verticalis,* produces an external spermatophore that contains, in addition to sperm, a considerable quantity of nutrients that are eaten by the female after mating. These nutrients are important to females and increase the size of the eggs that are produced (Gwynne 1997).

In insects with internal spermatophores, nutrients may be absorbed from spermatophores within the female genital tract and then incorporated in eggs. Examples of such nutrient transfer are found in butterflies, *Danaus plexippus*, *Heliconius hecale* and *H. erato* (Boggs & Gilbert 1979), the grasshopper, *Eyprepocnemis plorans* (Pardo *et al.* 1995) and the fruitfly, *Drosophila mojavensis* (Markow & Ankey 1984). The majority of species that provide ejaculate-derived nuptial gifts nuptial gifts produce large spermatophores, which can represent a substantial proportion of the male body weight (Gwynne 1984; Savalli & Fox 1998). In such species, copulation duration is usually over 30 minutes and may take hours (Butlin *et al.* 1987; Rutowski *et al.* 1987). *C. dalmanni* males produce relatively small spermatophores in comparison to other Diopsids (Kotrba 1996, Chapter 3, Figure 3.1). In addition, *C. dalmanni* has relatively short copulations, generally lasting under 60 seconds. Relatively short mating duration and small spermatophores suggest that nuptial feeding is not an important direct benefit of mating. However, the high frequency of mating may result in small-scale nutrient transfers many times a day, which cumulatively could represent a direct benefit (Baker *et al.* 2001a).

**Accessory gland proteins increase female fecundity.**

During mating males rarely transfer only sperm to females (Arnqvist & Nilsson 2000). Male insect ejaculates commonly include a large number of accessory substances. The effects of such accessory substances have been most extensively studied in *Drosophila melanogaster*. Accessory gland proteins (Acps) transferred to females during mating can have profound effects on female reproductive behaviour, and may confer direct benefits to the female such as the stimulation of fecundity or by decreasing female receptivity (Chapman 2001; Wolfner 2002).

Acps are synthesised in the paired accessory glands of the male (Wolfner 1997; Chapman 2001). The genes encoding seminal fluid proteins are expressed only in males, and are over-represented on the autosomes (Wolfner 1997; Wolfner *et al.* 1997; Swanson *et al.* 2001). There are approximately 80 Acps (Swanson *et al.* 2001), ranging from small peptides to large glycoproteins (Wolfner 1997; Wolfner *et al.* 1997; Swanson *et al.* 2001). Acps probably initially evolved to stimulate post-mating reproductive processes under natural selection. A number of Acps exhibit high rates of evolutionary change (Aguade *et al.* 1992; Tsaur & Wu 1997; Begun *et al.* 2000; Swanson *et al.* 2001), possibly due to the subsequent influence of sexual selection (Cordero 1995, 1996; Eberhard & Cordero 1995).

*D. melanogaster* Acps stimulate and regulate many reproductive processes in females following mating (Wolfner 1997; Chapman 2001; Wolfner 2002). A well-studied effect of Acps, which may represent a direct benefit to females, is the stimulation of fecundity. Mating increases female egg production (Manning 1967; Herndon & Wolfner 1995; Kubli 1996). This effect has been shown to be due to the presence of sperm and two Acps (Xue & Noll 2000; Heifetz *et al.* 2001). Persistence of increased fecundity requires the presence of sperm (Manning 1967, Kalb *et al.* 1993, Xue & Noll 2000, Heifetz *et al.* 2001). Two Acps stimulate egg production. Acp70A increases oogenesis (Chen *et al.* 1988; Aigaki *et al.* 1991; Soller *et al.* 1997, 1999) and Acp26Aa increases ovulation (Kalb

*et al.* 1993, Herndon & Wolfner 1995). Acps can also benefit females through their antibacterial actions (Lung *et al.* 2001). For example, the antibacterial protein Andropin (Samakovlis *et al.* 1991) may protect the reproductive tract of either sex, or the first egg laid, from microbial attack (Wolfner 2002).

Some Acp actions apparently benefit both males and females. For example mating, and the transfer of the Acp70A protein, decrease female sexual receptivity (Manning 1962; Chen *et al.* 1988). A reduction in female receptivity to further mating could therefore be a direct benefit if matings are costly. Males also will gain from a reduction in female receptivity if the probability of sperm competition is reduced by a delay in female remating. (For further discussion of the effects of mating on female receptivity see the following section 'Decreased female sexual receptivity').

Acp actions also appear to provide benefits for males by influencing sperm competition. The Acp PEB-me is a component of the mating plug that forms in the reproductive tract of females (Lung & Wolfner 2001). The mating plug is thought to assist the movement of sperm into storage in the female (Bairati 1968 cited in Wolfner 2002). Acps are also necessary for the efficient storage of sperm (Tram & Wolfner 1999), and a correlation has been observed between alleles at four Acp loci and a measure of sperm competition (Clark *et al.* 1995).

However, Acps have both beneficial and costly effects on females, making their net direct effect difficult to assess (Arnqvist & Nilsson 2000). The optimal female mating rate will be determined by a trade-off between the costs and benefits of mating (Arnqvist & Nilsson 2000; Gavrilets *et al.* 2001). Mating is costly for female *D. melanogaster* (Fowler & Partridge 1989) and the cost is due to the receipt of Acps (Chapman *et al.* 1995). This female cost of mating due to the receipt of Acps may indicate that females mate at a frequency beyond their optimum (Rice 1992, 1996; Holland & Rice 1998). Despite lifetime costs for females of receiving Acps, the actions of Acps on fecundity and female

sexual receptivity, and the antibacterial properties of some Acps, represent short-term direct benefits of mating for females. It is currently unknown whether male accessory gland proteins transferred in the ejaculate cause the increase in fecundity seen after mating in *C. dalmanni* (Baker *et al.* 2001a). This could represent a direct benefit of multiple mating in this species and Chapter 4 describes experiments investigating the effect of male accessory gland proteins on female fecundity in *C. dalmanni.*

**Decreased female sexual receptivity.**

Female receptivity defines the probability that a female will remate. Female resistance decreases the probability of remating, and examples of resistance behaviours in insects include walking or flying away from males, extrusion of the ovipositor, lifting the abdomen, kicking and wing movements (Connolly & Cook 1973; Bergh *et al.* 1992; Heady 1993; Fox & Hickman 1994; Ringo 1996). A reduction in receptivity through increased female resistance to remating could be beneficial to both males and females. Females will benefit from reduced receptivity if mating imposes a significant cost. Examples of mating costs include general time and energy costs (Watson *et al.* 1998), physical injury (Stutt & Siva-Jothy 2001), increased predation rates (Arnqvist 1989; Rowe 1994), parasite or pathogen infection (Hurst *et al.* 1995) or costs due to proteins transferred with the ejaculate (Chapman *et al.* 1995). A reduction in receptivity to further mating may therefore constitute a short-term benefit of mating for females.

Males will benefit from a reduction in female receptivity to further mating with rival males by increasing their chances of success in sperm competition. Sperm competition occurs when the sperm of two or more males compete to fertilise an egg (Parker 1970; Birkhead & Moller 1998; Simmons 2001). Sexual selection will favour male adaptations that enhance male sperm competitive ability (Parker 1970). There is evidence that an increased probability of sperm competition results in selection on males to decrease female receptivity. Males may gain an advantage in sperm competition by increasing ejaculate size, if sperm number is a determinant of male competitive success (Parker 1998; Wedell

*et al.* 2002). Increased ejaculate size can benefit males by providing a numerical advantage in sperm competition and by causing greater decreases in female receptivity. For example, in bushcrickets (Orthoptera: Tettigoniidae), larger male spermatophore size cause increased female refractory periods and resulted in increased male fertilisation success (Wedell 1993). In the wartbiter, *Decticus verrucivorus*, larger ejaculate volume decreases female receptivity, which may provide an advantage for males in sperm competition (Wedell & Arak 1989). In addition to strategic sperm allocation, there are a number of other mechanisms by which males control female remating. Mechanisms may be behavioural, mechanical and chemical.

Mate guarding is a behavioural mechanism to prevent female remating. For example, mate guarding and associated post-copulatory behaviour reduces female receptivity in a parasitoid wasp, *Aphytis melinus* (Allen *et al.* 1994). Mechanical mechanisms to prevent female remating include mating plugs. For example, the mating plug of the dung fly *Coproica vegans* reduces remating (Lachmann 2000). Chemical control of female receptivity may be through the transfer of anti-aphrodisiacs at mating (e.g. in *D. melanogaster* Scott 1986). A decrease in female receptivity has been shown to be due to accessory gland proteins transferred with sperm in a number of species. For example, mosquitoes, *Aedes aegypti* (Craig 1967); the house fly, *Musca domestica* (Riemann & Thorson 1969); the onion fly, *Delia antiqua* (Spencer *et al.* 1992), and the planthopper, *Prokelisia dolus* (Heady 1993).

Female receptivity has been extensively investigated in *D. melanogaster*. Female rejection of male advances, through kicking and extrusion of the ovipositor (Connolly & Cook 1973), persists for up to 11 days after mating (Manning 1962). The decrease in female receptivity after mating has been shown to be due to the receipt of the sex peptide, Acp 70A (Chen *et al.* 1988). However the transfer of sperm is required to extend post-mating reductions in receptivity beyond 24-48 hours (Gromko *et al.* 1984).

The influence of mating on receptivity in female *C. dalmanni* has not previously been investigated. A reduction in female receptivity may constitute a direct benefit of mating in female *C. dalmanni,* if the costs outweigh the benefits of further mating. Although there is evidence that female *C. dalmanni* mate multiply to gain fertility benefits (Baker *et al.* 2001a), reproduction has also been shown to cause a female longevity cost (Reguera *et al.* submitted). An investigation of mating induced inhibition of female sexual receptivity could therefore provide important information in assessing the overall gains of multiple mating in *C. dalmanni.* The effect of mating on female receptivity is examined in Chapter 5.

### 1.3.2 Genetic benefits of female multiple mating.

In addition to direct benefits, several indirect, or genetic, benefits have been proposed to explain female multiple mating. A genetic advantage occurs when certain genes, or genetic combinations, raise the offspring fitness of multiply mating females above that obtained with a single mating (Jennions & Petrie 2000). Hypotheses may be divided into benefits relating to (i) the acquisition of genes that enhance offspring fitness, including the adaptive consequences of sperm competition (Curtsinger 1991; Yasui 1997); and (ii) increased genetic diversity, including the reduction of genetic incompatibility and inbreeding (Yasui 1998). Genetic benefits of female multiple mating are not explicitly tested in this thesis, but I discuss their relevance to the *C. dalmanni* mating system.

#### Fisher, 'good genes' and sperm competition.

Females will gain indirect benefits from mating if they choose males with genes that enhance the attractiveness or viability of their offspring (Jennions & Petrie 2000). The acquisition of genes enhancing offspring fitness through sexual selection and female preference may result in female multiple mating if females mate once to ensure fertility, but later 'trade-up' by remating with another male of higher genetic quality (Petrie & Kempenaers 1998).

Models of sexual selection relating to female preference fall into two categories, Fisherian models and 'good genes' models. Fisherian models propose that female preference for attractive males results in attractive sons. Genes for female preference and the attractive male trait are inherited together, and coevolve in a process known as 'runaway' (Lande 1980; Kirkpatrik 1982; Pomiankowski *et al.* 1991). 'Good genes' models of sexual selection propose that females prefer to mate with males possessing trait values that signal high genetic quality (Pomiankowski *et al.* 1991). For example, female *C. dalmanni* exercise mate choice for large eyespan males, and the male eyespan trait signals susceptibility to larval food stress. There is genetic variation in the response of male eyespan to larval food stress. Male relative eyespan, to a significantly greater extent than

other non-sexual traits, signalled 'good genes' because genotypes that expressed large relative eyespans under good larval food conditions also had large relative eyespans under restricted larval food (David *et al.* 2000). Female *C. dalmanni* could gain indirect benefits through mating multiply if they remate with large eyespan males in order to gain genetic benefits.

Females may also acquire fitness enhancing genes through the promotion of sperm competition. When females mate multiply, sperm competition between rival ejaculates will occur within the female reproductive tract (Parker 1970). Sperm competition may promote fertilisation by genetically superior males. Females may benefit from mating with sperm-competitively superior males if competitive ability reflects general high viability and the possession of 'good genes' (Yasui 1997). While sperm number is important in determining competitive outcome (Parker 1998), the sperm of different males may also vary in their ability to reach and fertilise a female's egg or in actively preventing other males' sperm from doing so (Birkhead *et al.* 1999). If male sperm-competitive ability is heritable, females that mate multiply will benefit from their sons having high fertilisation success. This is known as the 'sexy sperm' hypothesis (Curtsinger 1991; Keller & Reeve 1995). Multiple mating in *C. dalmanni* may promote sperm competition, and could result in increased fertilisation by genetically superior males (i.e. males with 'good genes' and / or competitively superior sperm).

**Genetic diversity.**

It is possible that females are unable to exercise preference due to an inability to assess male quality or if male quality is dependent on unpredictable future conditions and therefore difficult to assess. In this situation, females may 'bet hedge' against the probability of choosing a poor quality mate by mating with a several males (Slatkin 1974; Philippi & Seger 1989; Yasui 1998). Multiple mating would insure against the chance of an inferior male siring all their offspring (Schneider & Elgar 1998), and produce genetically diverse offspring of which some may be better adapted to the prevailing

environment (Slatkin 1974; Philippi & Seger 1989). It is contentious whether multiple mating and the resulting multiple paternities of offspring result in increased genetic diversity (Thornhill & Alcock 1983). Due to the large degree of genetic diversity generated by recombination, it is unknown whether females produce enough eggs to utilise the diversity of a single ejaculate. Thus multiple mating may not significantly increase diversity (Yasui 1997). However, the genetic diversity in the sperm of one male is lower than for two or more males, and half-sib families are more than twice as diverse as full-sib families (Yasui 1998). This evidence suggests that multiple mating could significantly increase genetic diversity.

Increased genetic diversity may raise the overall fitness of a female's offspring (Jennions & Petrie 2000). For example, colonies of the bumble bee *Bombus terrestris*, which had increased genetic diversity had fewer parasites and greater reproductive success (Baer & Schmid-Hempel 1999). Newcomer *et al.* (1999) showed that females of the pseudoscorpion *Cordylochernes scorpioides*, which mated with multiple males as opposed to repeatedly with the same male, had increased numbers of offspring. It is therefore possible that multiple mating increases genetic diversity in *C. dalmanni.*

**Genetic incompatibility.**

Females could also gain indirect benefits of multiple mating through the reduction or avoidance of genetic incompatibility (Jennions 1997). Different genotypes can vary in their genetic compatibility as a result of epistatic interactions between loci, or between alleles at a locus (Tregenza & Wedell 2000). Incompatibilities can arise for a number of reasons. For example, due to selfish genetic elements (Hurst 1993; Rigaud 1999), segregation distorters (e.g. meiotic drive elements, Beeman *et al.* 1992), imprinted genes (Hurst 1998) or sexually antagonistic genes (Rice 1996). It may not however be possible to signal genetic compatibility, if compatibility depends on the interaction of the sexes (Jennions 1997). Multiple mates will therefore, as long as they are genetically diverse,

reduce the probability that all offspring are sired by an incompatible male (Zeh & Zeh 1996, 1997).

Multiple mating also introduces greater opportunities for post-copulatory female choice (Zeh & Zeh 1997). It is possible that females use chemical, cellular or molecular properties of male ejaculate to assess compatibility (Tregenza & Wedell 2000). The hatching success of eggs of the field cricket (*G. bimaculatus*) increased with the degree of polyandry, possibly due to chemical properties of ejaculates of compatible males, resulting in a higher probability of egg fertilisation (Tregenza & Wedell 1998). *C. dalmanni* has a low percentage of egg fertilisation following single matings (Baker *et al.* 2001a). It is possible that genetic incompatibility contributes to this low egg fertilisation level.

Inbreeding depression is a specific example of genetic incompatibility (Zeh & Zeh 1996). Inbreeding depression is the cost associated with mating with closely related mates due to increased homozygosity of deleterious recessive alleles and decreased heterozygosity (Pusey & Wolf 1996; Jennions & Petrie 2000). There is recent evidence from *G. bimaculatus*, that female multiple mating helps avoid the costs of inbreeding. When females were allowed to mate with two brothers, a brother and an unrelated male or two unrelated males, eggs laid by females mated to two brothers were less likely to hatch than the eggs laid by females from the other two groups (Tregenza & Wedell 2002). Inbreeding could be responsible for low egg viability in *C. dalmanni*, resulting in decreased inbreeding being an important direct benefit of multiple mating.

## 1.4 FEMALE MULTIPLE MATING - NON-ADAPTIVE HYPOTHESES

Multiple mating need not be adaptive for females, and instead could result from conflict between the sexes over mating frequency. Sexual conflict is defined as differences in the evolutionary interests of males and females (Parker 1979). A difference in the 'per mating' reproductive success of males and females will lead to conflict over mating frequency (Bateman 1948; Parker 1979). Males are predicted to gain increased reproductive success with each successive mating, if the production of ejaculate is assumed to be relatively unlimiting. In contrast, females have limited numbers of eggs and generally gain lower benefits from each additional mating than do males (Bateman 1948). Each extra mating therefore has a higher potential benefit for males than for females, and as a result males are selected to mate at a higher optimal frequency than females.

If selection is stronger on males to mate frequently than on females to limit mating frequency, a genetic correlation between the sexes for mating frequency could cause females to mate at a frequency beyond their optimum (section 1.4.1). A cost of mating for females would however impose strong selection on females to limit their mating frequency. This could result in evolutionary conflict between the sexes over mating frequency. Males and females may struggle to exert control over mating. Predominant male control over mating could result in females mating beyond the adaptive optimum, and would represent a non-adaptive explanation of female multiple mating (section 1.4.2).

### 1.4.1 Genetic correlation in mating frequency.

A genetic correlation between the sexes for mating frequency may cause female multiple mating. If the same genes determine mating frequency in both sexes, but there is stronger selection on males to increase mating frequency than on females to limit mating frequency, then females may mate at a higher than optimal frequency (Halliday & Arnold 1987). A cost of mating for females may however exert strong selection on females to reduce mating frequency (Sherman & Westneat 1988). This sexual conflict over mating frequency should eventually result in the evolution of different or sex-limited genes

controlling mating frequency in each sex (Rice 1984). Despite the presence of sexual conflict, female mating frequency could still be elevated by a genetic correlation with male mating frequency (Halliday & Arnold 1987).

Most work on testing for genetic correlations in mating frequency has been done in *D. melanogaster*. Studies in this species provide no evidence in support of a genetic correlation for mating frequency between the sexes (Sgrò *et al.* 1998). Artificial selection in females for increased and decreased time until remating (i.e. the time between the first and second mating) resulted in a direct response in both directions. No correlated response was found in female time to remating, remating frequency or the time until the first mating (Sgrò *et al.* 1998). Another study in *D. melanogaster* provided evidence for a genetic correlation (Stamenkovic-Radak *et al.* 1992). Mating speed (i.e. the time until the first mating) was selected separately in both males and females. A significant direct response to selection was found in both sexes. Correlated responses in mating speed were found in males and females from the female- and male-selected lines respectively (Stamenkovic-Radak *et al.* 1992).

However, the study by Stamenkovic-Radak *et al.* (1992) can be criticised on aspects of experimental design. This selection experiment did not control for non-random mating, which could obscure the existence of a genetic correlation in mating frequency. For example, assortative mating for mating frequency would result in inadvertent selection on the 'unselected' sex, and an overestimation of a genetic correlation (Butlin 1993). The possibility of assortative or disassortative mating was excluded in the experiment of Sgrò *et al.* (1998) by imposing random pairing between males and females and ensuring that each pairing contributed equally to the next generation.

*C. dalmanni* mates at an extremely high frequency (Wilkinson *et al.* 1998a). The finding of a cost of reproduction (Reguera *et al.* submitted) suggests that females may mate at higher than optimal frequencies. Female mating frequency could be dragged up by

selection on male mating frequency if there was a genetic correlation between the sexes. It is unknown whether the genes involved in determining mating frequency are the same in both sexes, or if there is a genetic correlation in mating frequency between males and females. Chapter 6 investigates the presence of a genetic correlation for mating frequency between the sexes in *C. dalmanni.*

### 1.4.2 Male control of mating.

Females may mate multiply if there is conflict between males and females over mating frequency, and males exert control over mating to determine the outcome of the 'battle of the sexes' (Ridley 1990; Arnqvist & Nilsson 2000). Sexual conflict over mating frequency could result in the evolution of different, sexually antagonistic, genes controlling mating frequency between the sexes (Rice 1984). Sexually antagonistic genetic variation has been demonstrated in *Drosophila melanogaster.* Chippindale *et al.* (2001) found a positive genetic correlation between male and female juvenile fitness but a negative genetic correlation between adult male and female reproductive success. At the larval stage the interests of the sexes are expected to be similar, but the interests of males and females are predicted to diverge as adults.

It is imperative to assess the level of conflict and degree of control over mating in the two sexes, when assessing whether female multiple mating confers an adaptive advantage (Rowe *et al.* 1994). Even if promiscuous females obtain a direct or indirect advantage to mating multiply, the benefits will not necessarily outweigh the costs if the mating system is controlled entirely by males (Rowe 1994). Males may employ a variety of strategies to obtain control over mating frequency. Male strategies include aggressive coercion, including forced copulation and harassment (Clutton-Brock & Parker 1995), and male attraction and seduction of females (Holland & Rice 1998). Females control mating frequency through resistance of male mating attempts and female preference.

**Male aggressive coercion.**

There are three main forms of aggressive sexual coercion: (i) forced copulation; (ii) intimidation through punishment and (iii) harassment (Clutton-Brock and Parker 1995). Forced copulation occurs when males pursue females, catch or restrain them and physically force insemination (Thornhill 1980). This behaviour may be costly to the female, in terms of energetic costs, physical injury, and reduction in offspring quality if the male is genetically inferior. In contrast, males may incur some energetic costs but also substantial fertilisation benefits (Clutton-Brock & Parker 1995). For example, male scorpion flies (*Panorpa* spp.) use forced copulation when they are unsuccessful at attracting a female by securing a gift of a dead insect or salivary mass. Females try to escape males without nuptial offerings. Males may attempt to grasp females with genital claspers, secure the female with a clamp-like notal organ, and force copulation (Thornhill 1980). Forced copulation is however thought to be relatively rare in invertebrates (Thornhill 1980). *C. dalmanni* females appear to be able to successfully reject male copulation attempts (CAG pers. obs.). Forced copulation is therefore unlikely to be an important explanation of female multiple mating in this species.

Mating to avoid continual male harassment (known as 'convenience polyandry') is however common in insects (Thornhill & Alcock 1983). Male harassment of females to mate could constitute a form of male control over mating frequency. However, if male harassment imposes significant costs to females, mating to reduce harassment may be classified as a direct benefit of multiple mating. Water striders (Heteroptera: Gerridae) provide evidence of convenience polyandry (Rowe 1992, 1994; Rowe *et al.* 1994; Arnqvist & Rowe 1995; Watson *et al.* 1998). Water strider mating behaviour includes frequent harassment of females by males and vigorous pre- and post-mating struggles (Rowe 1994). Mating is energetically costly for females (Wilcox 1984), and mating females incur increased predation and decreased foraging efficiency (Rowe 1994, Rowe *et al.* 1994).

Females are therefore not passive opponents in the struggle to control mating frequency in water striders. Morphological adaptations in both sexes influence the duration and/ or outcome of struggles. Males have adaptations for grasping on their forelegs and abdomens and females have abdominal spines that help prevent male genitalic attachment. Experimental elongation of the abdominal spines lowers remating frequency and pre-mating struggle duration, and is an adaptation to increase female resistance and promote control over copulation (Arnqvist & Rowe 1995). Further analysis of morphological adaptations across 15 congeneric species showed that changes in the balance of armaments between males and females results in evolutionary change in the outcome of sexually antagonistic interactions, indicating an 'arms race' between the sexes (Arnqvist & Rowe 2002). However, females do appear to mate at a frequency beyond that required for fertilisation (Rowe *et al.* 1994). It is possible males are currently winning the antagonistic interaction and females accept superfluous matings to avoid male harassment (Rowe 1992).

Females must assess whether the cost of continued male harassment outweighs the cost of mating (Rowe 1992; Clutton-Brock & Parker 1995). Male harassment may represent a significant cost to female *C. dalmanni*, and mating to avoid harassment could therefore represent a direct benefit. The costs of male harassment (and the potential benefit of avoiding harassment) are not directly investigated in this thesis. However, male harassment could represent a mechanism by which males exert control over mating frequency. The relative importance of male and female control of mating frequency in *C. dalmanni* is explored in Chapter 7.

**Male seduction.**

It has been suggested that males can seduce or entice females to mate beyond their optimum mating frequency (Holland & Rice 1998; Arnqvist & Nilsson 2000). Holland and Rice (1998) proposed a model of sexual selection based on the antagonistic seduction of females by males, called 'chase-away sexual selection'. The model proposed that a pre-

existing sensory bias of females selects males to evolve a rudimentary display trait that is attractive to females. These particularly attractive males induce females to mate more frequently than is adaptive. As a result, females will be counter-selected for resistance to a male display trait. Female resistance in turn selects males to evolve a more extreme trait. Cyclical antagonistic coevolution will ensue, with the model predicting diminished female attraction to the male display trait and a reduction in female fitness with attraction (Holland & Rice 1998).

Holland and Rice (1998) reviewed evidence in three areas critical to their hypothesis of 'antagonistic seduction': (i) intersexual conflict, (ii) pre-existing female sensory bias and (iii) the evolution of female resistance to attractive male traits. The experimental evidence for intersexual conflict (e.g. the female cost of mating caused by male accessory gland proteins in *D. melanogaster*, Chapman *et al.* 1995), and sensory exploitation (e.g. evidence from the frog *Physalaemus pustulosus*, that female attraction to a component of the male mating call predates the evolution of the male trait) is strong. However, the critical prediction of the model, that attraction to a male display trait reduces female fitness and consequently that females evolve diminished attraction to the male display trait, lacks evidence. Female preference for large male eyespan in *C. dalmanni* does result in genetic benefits (Wilkinson *et al.* 1998b; David *et al.* 2000). However, females are subject to a cost of reproduction that could indicate that females are seduced to mate at a frequency where the costs outweigh the benefits (Reguera *et al.* submitted). It is possible that males exert control over mating frequency through the evolution of an exaggerated trait (i.e. eyespan) that seduces females to mate beyond an adaptive optimum. The presence of antagonistic seduction is not investigated in this thesis, but potentially could be a mechanism by which males control mating frequency (Chapter 7).

**Female control through mate choice.**

Female control over mating frequency could result from female choice. Female choice can be 'passive' (occurring as a consequence of female resistance to costly mating) or 'active'

(a consequence of female preference that confers benefits to the female). Female resistance to male coercion may function as mate assessment (Jennions & Petrie 1997). For example, in the dung fly *Sepsis cynipsea*, larger males more effectively overcome female resistance and gain a mating advantage (Blanckenhorn *et al.* 2000). Similarly, large males have a mating advantage in the seaweed fly, *Coelopa ursine*. There is a positive association between male size, the duration of premating struggles and mating success which indicates that large males have a superior ability to withstand female rejection (Crean & Gilburn 1998). Sexual selection on male size is occurring as a side-effect of female resistance to avoid the costs of mating (Crean & Gilburn 1998). Females can be viewed as having exerted control over the relative mating frequency of large and small males.

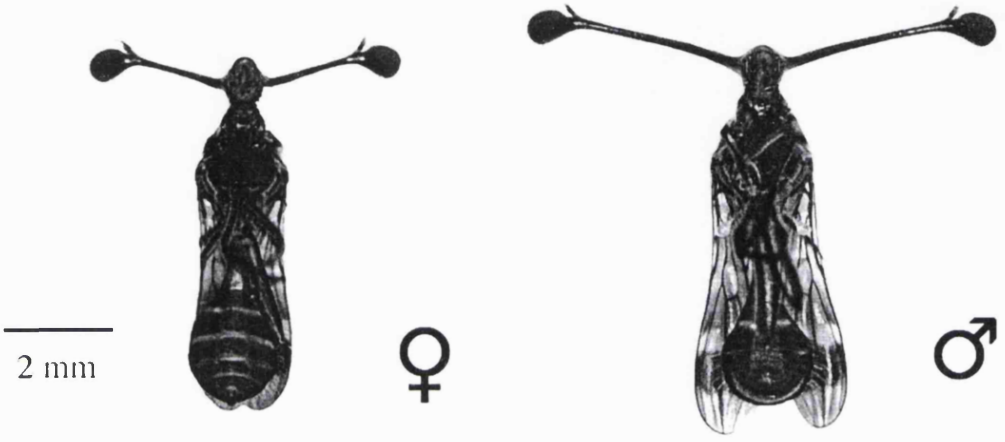
Alternatively, female control of mating could manifest as a preference that confers a benefit. Female preference can provide direct benefits to females, for example nuptial gifts or increased territory size (for a review of direct benefits of female preference see Andersson 1994). Female preference can also result in indirect, genetic benefits for females. Fisherian and 'good genes' models of sexual selection are outlined in section 1.3.2. Female preference can be interpreted as female control over the mating frequency of different males.

Stalk-eyed flies *C. dalmanni*, offer a novel opportunity to investigate the relative degree of control that males and females exert over mating (Chapter 7). This is because both female preference and male mating frequency can be experimentally manipulated to test the relative contributions of both in determining overall mating frequency. Female preference can be varied by phenotypically manipulating male eyespan. Eyespan is determined during the larval stage by the acquisition of resources (Hingle *et al.* 2001a). Manipulation of the amount of food per larva produces males with small and large eyespans, controlled for genotype. There is experimental evidence documenting female preference for the male eyespan trait (Burkhardt & de la Motte 1988; Wilkinson & Reillo 1994; Hingle *et al.* 2001a,b). Female preference can be estimated through the mating frequency of females

given a choice of mating with either large or small eyespan males (Hingle *et al.* 2001b). Strong female preference would indicate female control of mating. Male mating frequency can be manipulated by artificial selection. The cost of reproduction for female *C. dalmanni* (Reguera *et al.* submitted), suggests that females mate beyond their optimum frequency. This could indicate predominant male control of mating frequency and represent a non-adaptive explanation of female multiple mating in *C. dalmanni.*

## 1.5 FIGURES

Figure 1.1 Male and female *Cyrtodiopsis dalmanni* showing sexual dimorphism in eyespan.



## 1.6 THESIS CHAPTERS

The work was in this thesis was funded by the Natural Environment Research Council and was performed under the supervision of Kevin Fowler and Tracey Chapman. The execution of all experiments was by the author. Additional instruction and assistance was received for HPLC (R. Venn, Pfizer, Sandwich, Kent).

Chapter 2 details general materials and methods.

Chapter 3 investigates whether females mate multiply to increase fertility. Fecundity, and therefore ejaculate requirement, was shown to be related to female size. Females with large eyespan, and thus body size, have increased egg production. Male ejaculate allocation (where ejaculate size was estimated as spermatophore sperm sac area) was compared between large and small eyespan females. The effect of female eyespan on mating frequency was then examined.

Chapter 4 asks whether a male accessory gland protein transferred in the seminal fluid is responsible for increasing female fecundity after mating. Whole male accessory gland extract was injected into virgin females. The fecundity of accessory gland extract injected females was compared with females injected with a control solution, females that were mated and un-injected females. Male accessory gland proteins were then partially separated using high-pressure liquid chromatography (HPLC) into five fractions to further isolate the active protein(s). The fecundity of females injected with different HPLC fractions was compared with females injected with a control solution, females that were mated and un-injected females.

Chapter 5 reports on the effect of mating on female sexual receptivity. Receptivity was quantified as the time until the first mating, the number of rejected matings and the number of matings. The receptivity of females that had been mated and unmated was compared. In addition, the receptivity of females mated to males with large and small eyespan was

compared. Finally, male discrimination between mated and unmated females was investigated. Chapter 5 has been published recently in The Journal of Evolutionary Biology (Grant *et al.* 2002).

Chapter 6 tests whether a genetic correlation between male and female mating frequency causes female multiple mating. Lines that had been artificially selected for increased and decreased male mating frequency were used. The mating frequency of females from the selected lines was compared.

Chapter 7 is an investigation into the control that males and females exert over mating frequency. Female control of mating frequency was assessed as the extent of female preference for large male eyespan, and was manipulated by phenotypic manipulation of male eyespan. Male control of mating frequency was manipulated through the use of lines artificially selected for increased and decreased male mating frequency. The presence of female and male control was examined.

Chapter 8 summarises my key findings. It discusses potential ways in which future experiments could extend my work in order to reach a deeper understanding of the evolution of multiple mating in the stalk-eyed fly.

# 2

# General materials and methods

## 2.1 FLY STOCKS AND CULTURE METHODS

The stocks of *Cyrtodiopsis dalmanni* used were from a laboratory population founded in 1993 from individuals captured in Gombak, Malaysia. Flies were maintained since then in population cages. Population cages (height=20 cm, width=20 cm, length=30 cm) had a base of damp cotton to provide moisture and maintain cage humidity. Flies were maintained at an approximately 1:1 sex ratio, and population size has always exceeded 200 individuals, to minimise the effects of inbreeding. Flies were kept at a constant temperature of 25°C, with a 12:12 hour dark: light cycle. Artificial dawn was a half hour period of illumination from a single 60watt bulb, at the start of the light period.

Flies were fed puréed sweetcorn (maize). Food was made by blending 1.5 kg of sweetcorn with 25 ml of a mould inhibitor (10% w/v Nipagin in 100% ethanol). After the food was made, it was stored at -20°C for a minimum of three days or until use, to kill pest organisms such as mites. Before use, food was allowed to defrost thoroughly. The food in population cages was replaced twice a week. 20-25 g of food was placed on a Petri dish inside the cage. Water was added to the base of the cage to keep the cotton damp but not saturated.

Flies were cultured by collecting eggs from population cages at regular intervals (approximately every 2 months). Five 90 mm single-vent Petri dishes were placed on the base of the cage. Petri dishes were lined with cotton dampened with 5 ml of water. 15 g of puréed sweetcorn was placed on the cotton, for the larvae to feed on, and the food was covered by a circular Whatman paper (70 mm diameter). Petri dishes remained in the population cage for 4 days to allow females to lay eggs on them. The Petri dishes were then removed, and kept at a constant temperature of 25°C to allow larvae to develop and pupate. After 14 days pupae were collected and placed in a new population cage to allow them to eclose.

## 2.2 SEGREGATING FLIES BY SEX

Males reach sexual maturity on average 25 days after eclosion, females on average 22 days after eclosion (Baker *et al.* in press). To obtain virgins, flies were segregated by sex 14-21 days after eclosion. Flies were first immobilised by chilling them on ice. The sex of individuals could be determined by looking first at the eyespan (male *C. dalmanni* have an eyespan greater than their body length, Burkhardt & de la Motte 1983, 1985), and then for the presence of an ovipositor. Individuals with eyespans greater than their body length and no ovipositor, were determined male. Individuals with eyespans less than their body length, and an ovipositor, were determined female. After inspection, males and females were placed in separate population cages at 25°C.

## 2.3 MORPHOLOGICAL MEASUREMENTS

Morphological measurements were made using a video camera mounted on a monocular microscope connected to NIH image software (version 1.55). Eyespan was measured as the distance between the tips of the eye bulbs, while flies were anaesthetised over ice. Eyespan is highly correlated with other measures of body size in *C. dalmanni* and was therefore used as a measure of body size (Wilkinson & Dodson 1997).

## 2.4 ARTIFICIAL SELECTION LINES

Full details of the artificial selection protocol are given in Baker *et al.* (in prep.). In brief, two replicate lines were selected for increased male mating frequency ('high' selection regime), two replicate lines were selected for decreased male mating frequency ('low' selection regime) and two unselected 'control' lines were produced. All lines were derived from a wild-type laboratory population of *C. dalmanni* (section 2.1).

In each generation the selection protocol was a two-stage procedure. In stage one, the mating frequencies of 24 males from each line were assayed with base stock females. Mating frequency was measured as the total number of matings achieved in a one and a half hour period commencing at artificial dawn on each of two consecutive days. In stage two, the 8 males with the highest or lowest mating frequencies within each line were selected to found the next generation of the 'high' or 'low' lines respectively. Eight males were chosen at random for the control lines. Each individual male was confined with 5 females randomly chosen from their line, and 40 progeny from each of these 1-male, 5-female groups were collected as pupae to contribute to the next generation.

Measured at generation 7, there was a direct response to selection for male mating frequency in both replicates of each selection regime. High line males mated significantly more than controls, and control line males mated significantly more than males from the low lines (Baker *et al.* in prep.).

# 3

# Fertility benefits of multiple mating: the influence of ejaculate size and mating frequency

## 3.1 ABSTRACT

Female stalk-eyed flies, *Cyrtodiopsis dalmanni*, that mate only once have low egg fertility (40%) and gain high levels of fertility (80%) only after multiple matings, suggesting that females of this species may be sperm limited. I report here that females with large eyespans (and thus large body sizes) had a higher number of mature eggs in their ovaries than small eyespan females. Large eyespan females may therefore have a greater requirement for sperm than small eyespan females. Large eyespan females could receive more sperm if they mated more frequently than small eyespan females, or if males preferentially allocated ejaculates of increased size to large eyespan females. I found that there was no significant difference among females of different sizes in the amount of ejaculate received (as measured by the spermatophore sperm sac area). However, large eyespan females mated at a significantly higher frequency than small eyespan females. This suggests that large females gain increased fertility benefits through mating at high frequency and not through increased ejaculate allocation.

## 3.2 INTRODUCTION

The principal function of mating is the transfer of sperm. It has traditionally been thought that sperm are cheap to produce and therefore that male ejaculate is not a limiting factor. However, there is evidence that in some species male ejaculate production may represent a substantial cost (Dewsbury 1982). Limitation in ejaculate production could lead to strategic ejaculate allocation between females (Wedell *et al.* 2002). The amount of ejaculate allocated to each female may depend on factors such as the availability of mates and the intensity of sperm competition. Restricted ejaculate allocation can result in females receiving insufficient sperm in a single mating to fertilise all their eggs (Wedell *et al.* 2002). Indeed, it has been demonstrated that for many species of insects, an important explanation of female multiple mating is sperm replenishment to maintain high levels of fertility (Arnqvist & Nilsson 2000; Ridley 1988).

*C. dalmanni* has an extremely high mating frequency. Males mate up to 10 times per hour in the laboratory (Wilkinson *et al.* 1998a). The majority of matings occur at dawn and dusk, when females are aggregated on rootlets (Wilkinson & Reillo 1994; Wilkinson *et al.* 1998a). The number of sperm transferred at each mating is not known. However, experimental evidence suggests that it may be low. Females mated singly fertilised only 40% of their eggs. Multiply mated females given continuous exposure to males fertilised 80% of their eggs (Baker *et al.* 2001a). This suggests few sperm are transferred per mating. The spermatophore of *C. dalmanni* is also similar in size to that of it's sister species *C. whitei*, in which an average of only 90 sperm are transferred per mating (Figure 3.1. Kotrba 1996; Lorch *et al.* 1993). However, other explanations of low fertilisation rates, such as inadequate sperm storage or inefficient fertilisation cannot be excluded. Given that males mate extremely frequently, and that male ejaculate may be in limited supply, there is the potential for males to allocate sperm between females. Male ejaculate allocation could result in females receiving insufficient ejaculate from one or a few matings to efficiently fertilise their eggs, and provides an explanation of why *C. dalmanni* females mate multiply.

Males may strategically allocate increased ejaculate to females that have the potential to lay high numbers of fertile eggs. Allocation decisions could be based on female size, as fecundity is often associated with large body size in insects. Positive phenotypic correlations between body size and fecundity have been found in a number of species (Honek 1993). Specific examples are the seaweed fly, *Coelopa frigida* (Butlin & Day 1985); the cotton stainer bug, *Dysdercus fasciatus* (Kasule 1991); a species of moth, *Parapediasia teterrella* (Marshall 1990) and a parasitoid wasp, *Aphaereta minuta* (Visser 1994). Females with larger body size and higher fecundity will therefore require more sperm to fertilise all their eggs. Large females could therefore be the targets for increased ejaculate allocation by males.

Females and males will gain direct benefits through ejaculate partitioning according to female size, because large females are likely to have more mature eggs to fertilise. Female size-based ejaculate allocation may also provide indirect genetic benefits for both sexes. Body size generally shows significant heritability (Robertson 1957; Mousseau & Roff 1987; Roff & Mousseau 1987). Large females receiving increased ejaculate are able to produce increased numbers of male and female offspring with large body size (and thus increased reproductive success).

The risk of sperm competition may also affect ejaculate allocation. Sperm number is an important determinant of competitive success (Wedell *et al.* 2002; Parker 1998). Males may be predicted to increase ejaculate size and / or sperm number in situations where the risk of sperm competition is high. Theoretical models show that species with increased risk of sperm competition have greater ejaculate expenditure (Parker 1998). Sperm competition game models also predict allocation within species (Parker 1998). One situation in which the base-line level of sperm competition would be increased is if large females mate more frequently (see below). There is evidence that males of a number of species apportion increased ejaculate to larger females. For example, the house cricket,

*Acheta domesticus* (Gage & Barnard 1996); the stink bug, *Thyanta pallidovirens* (Wang & Millar 1997); the Indian meal moth, *Plodia interplunctella* (Gage 1998); the small white butterfly, *Pieris rapae* (Wedell & Cook 1999); the yellow dung fly, *Scatophaga stercoraria* (Parker *et al.* 1999) and the weevil, *Brentus ancorago* (Johnson & Hubbell 1984). Severe sperm competition may however result in decreased ejaculate size, and males have been shown to conserve sperm in intensely competitive circumstances (Parker *et al.*1997).

Regardless of whether more fecund (larger) females receive increased ejaculate through strategic male allocation, those females could also gain more sperm by mating more frequently. Thus a positive relationship between female body size, female mating frequency and fecundity is predicted. Female mating frequency would then be related to the number of eggs available for fertilisation.

In this chapter the relationship between fecundity, male ejaculate size and female mating frequency was investigated in *C. dalmanni.* I first tested whether fecundity varied with female size. I then investigated whether females with large eyespan, and thus body size, received ejaculates of increased size at mating. Finally, I tested whether females with large eyespans mated more frequently.

## 3.3 MATERIALS AND METHODS

### 3.3.1 Fly stocks and culture methods

The flies used in these experiments were from the laboratory population described in Chapter 2, section 2.1. Virgins were obtained as in Chapter 2, section 2.2. The age of experimental individuals used varied from 8 to 16 weeks.

Morphological measurements of eyespan were made as described in Chapter 3, section 2.3. Females were allocated to two groups, large eyespan (>5.9 mm) and small eyespan (<5.4 mm). In order to minimise confounding effects of variation in male eyespan, all of the males used were of intermediate eyespan (mean ± standard deviation: 8.73 ± 0.4 mm). Eyespan is highly correlated with other measures of body size in *C. dalmanni* (Wilkinson & Dodson 1997) and is used here as a measure of body size.

### 3.3.2 The effect of female size on egg production.

Egg production was measured as the total number of mature eggs in the two ovaries of each female. 24 large eyespan and 24 small eyespan females were kept individually in 400 ml containers (height=95 mm, diameter=75 mm, with damp cotton wool and 2g of puréed sweetcorn). Each virgin female was placed with a single virgin male for 24 hours. Females were then returned to their containers and held for one week before dissection. Females were immobilised over ice, dissected in PBS and their ovaries were removed and placed on a microscope slide. The number of mature eggs was then counted. Mature eggs were those at stages 12-14 of King's standard stages of oogenesis (King 1970; Ashburner 1989; Hingle *et al.* 2001a). The total number of mature eggs from the ovaries of 22 large eyespan and 24 small eyespan females was recorded.

### 3.3.3 The effect of female size on ejaculate size.

The effect of female size on male ejaculate size was investigated. Males were mated to large and small eyespan females, and the size of the ejaculate transferred was estimated by measuring the area of the spermatophore sperm sac deposited inside each female.

Experimental flies were housed from eclosion in mixed sex population cages of approximately 200 individuals with a 1:1 sex ratio. 24 hours before the first experimental matings, males were placed individually in 400 ml containers (same dimensions as those described in section 3.3.2). On the following morning a single female was added to each container. Half of the males received a large eyespan female and half received a small eyespan female. All flies were observed until a single copulation had taken place after which all the mated females were removed and frozen until dissection. 24 hours later males were allowed to mate a second time. Those that had previously mated with large eyespan females were tested with small eyespan females and *vice versa*. 'Mating order' thus assigns individual males to one of two categories according to whether they mated first to a large eyespan female (mating order L-S) or first to a small eyespan female (mating order S-L). The data were collected in blocks with equal numbers of males per mating order ($n$=10). Data were omitted from the analysis if spermatophore sperm sacs could not easily be measured or if males failed to transfer a spermatophore to both females (Table 3.1).

Spermatophore sperm sac area was used here to estimate ejaculate size. Actual counts of sperm number would have been a better and more direct measure. However, removal of the spermatophore from the female reproductive tract, and estimation of the number of sperm contained within it, proved difficult and unreliable in initial tests (CAG pers. obs). Out of 30 attempts to remove the spermatophore sperm sac from the reproductive tracts of recently mated females, only one spermatophore was retrieved intact. The small size of the spermatophore sperm sac (100-150μm, see Fig. 3.1) also made it difficult to manipulate. In the future, it will be important to develop a technique for reliably counting the number of sperm transferred.

To measure the spermatophore sperm sac area, female reproductive tracts were dissected into phosphate buffered saline (PBS), and placed on a slide with the ventral sclerite uppermost in a drop of PBS and 0.7μl of Vectashield™ mounting medium with DAPI

(which enabled the spermatophore sperm sacs to be seen more easily). Sperm sac area (excluding the tubular neck: Figure 3.1) was then measured using NIH image software on images captured from a compound microscope. Each spermatophore sperm sac area was measured twice, and the average of the two measurements taken. The repeatability of the spermatophore sperm sac area measurements was very high for both small and large eyespan females (regression of the area of the first and second measurements for large eyespan females: $r^2$=0.94, $P$<0.0001, and for small eyespan females: $r^2$=0.95, $P$<0.0001).

### 3.3.4 The effect of female size on mating frequency.

The mating frequency of 30 large eyespan and 30 small eyespan females was determined. The data were collected in 5 experimental blocks, with 6 large and 6 small eyespan females assayed in each block. Each block consisted on two assays on consecutive days. 12 virgin males were placed individually in 400 ml containers (same dimension as section 3.3.2) the evening before the first assay. At the beginning of artificial dawn, a single virgin female, with either a large or a small eyespan, was added to each container. Half the males received a large eyespan female and the other half a small eyespan female. The number of matings over and under 40 seconds was recorded for an hour after the beginning of artificial dawn. The flies remained in their containers overnight, and the number of matings was measured again for another hour at dawn on the next day. The mating frequency was calculated as the total number of matings with duration of greater than 40 seconds pooled over both days. Unpublished data shows that matings over 40 seconds almost always result in the transfer of sperm (CAG pers. obs.; R. Baker pers. comm.). In addition, evidence from sister species *C. whitei* shows that matings under 40 seconds do not transfer sperm (Lorch *et al.* 1993).

### 3.3.5 Statistical analysis.

**The effect of female size on egg production**

Shapiro-Wilk tests were used to determine whether the data were normally distributed. A one-way analysis of variance was used to compare the number of mature eggs in the ovaries of large and small eyespan females.

**The effect of female size on ejaculate size.**

I tested for differences in ejaculate size by analysing the difference in the area of spermatophore sperm sacs delivered to large and small eyespan females. The difference in spermatophore sperm sac area in large and small eyespan females mated to the same male was calculated as (area of large – area of small) irrespective of the mating order. Shapiro-Wilk tests determined that these data were normally distributed. They were then subjected to a two-way mixed model analysis of variance, with mating order (fixed effect) and experimental block (random effect) as the factors (Table 3.2). Finally I tested using a two-tailed *z*-test, whether the mean difference in sperm sac area in large and small eyespan females differed significantly from zero.

**The effect of female size on mating frequency**.

Shapiro-Wilk tests showed that the number of matings with duration of greater than 40 seconds were normally distributed. One-way analysis of variance was used to compare the number of matings over 40 seconds between large and small eyespan females. The number of matings under 40 seconds duration were not normally distributed, and the data could not be normalised through transformation. Wilcoxon rank sum tests were therefore used.

Statistical analysis was carried out using JMP statistical software (version 5, SAS Institute Inc.) for the Apple Macintosh.

## 3.4 RESULTS

### 3.4.1 The effect of female size on egg production.

Large eyespan females had an average of 21.68 ± 2.55 (mean ± standard error) mature eggs and small eyespan females an average of 12.29 ± 1.88 mature eggs in their ovaries (Figure 3.2). Large eyespan females had significantly more mature ova in their ovaries than small eyespan females ($F_{1,44}$=8.96, $P$<0.005).

### 3.4.2 The effect of female size on ejaculate size.

There was a weakly significant interaction between mating order and experimental block in determining the difference in spermatophore sperm sac area between large and small eyespan females ($F_{3,61}$=2.77, $P$<0.05). Inspection of the mean difference in spermatophore sperm sac area of mating orders across blocks (Table 3.1) shows that the difference in spermatophore sperm sac area did not show a consistent pattern between the two mating orders. In neither mating order were spermatophore sperm sacs from large eyespan females consistently larger or smaller than sperm sacs of small eyespan females. There was also no consistent increase or decrease in spermatophore sperm sac area across the experimental blocks. In addition, neither main effect was significant (block $F_{3,61}$=0.03, $P$>0.9; mating order $F_{1,3}$=0.36, $P$>0.5). Lastly, the mean difference in the spermatophore sperm sac area transferred to large and small eyespan females did not differ significantly from 0 ($z$=1.12, $df$=68, $P$>0.25). Thus whether males mated a large or small eyespan female first did not make a significant difference to the size of the spermatophore sperm sac that they transferred. There was also no evidence of strategic ejaculate allocation by males based on female body size.

### 3.4.3 The effect of female size on mating frequency.

Large eyespan females had an average of 12.36 ± 1.12 (mean ± standard error) matings over 40 seconds during the observation periods, whereas small eyespan females mated an average of 8.26 ± 0.99 times (Figure 3.3). Large eyespan females had significantly more

matings than small eyespan females ($F_{1,58}$=7.55, $P$<0.01). In contrast, large and small eyespan females did not differ significantly in the number of matings under 40 seconds (large eyespan females 1.33 ± 0.30 (mean ± standard error) matings; small eyespan 1.20 ± 0.23 matings, $n_1$=30, $n_2$=30, $P$>0.9).

## 3.5 DISCUSSION

The results of this chapter showed that female *C. dalmanni* with large eyespan, and thus large body size (Wilkinson & Dodson 1997), contained a higher number of mature ova in their ovaries than did small eyespan females. This suggests that large eyespan females have a greater requirement for sperm than small eyespan females. The problem of maintaining adequate sperm supplies may be especially acute in this species, as females need to mate multiply in order to achieve high fertility (Baker *et al.* 2001a). Large eyespan females did not however receive larger ejaculates at mating (as measured by spermatophore sperm sac area). They did mate at a higher frequency, providing evidence that large eyespan females gain fertility benefits through their increased mating frequency and not through strategic ejaculate allocation.

Female reproductive success will be limited, in general, by the number of viable eggs that can be produced and fertilised. The study shows that large eyespan females have higher rates of egg production and therefore potentially higher reproductive success than small eyespan females (Figure 3.2). However, to convert this possible reproductive advantage into a realised fitness gain, large eyespan females must have higher rates of egg production and receive or utilise more sperm, than small eyespan females. In contrast to the view that sperm are not generally limiting (Bateman 1948), females of this species must mate several times to achieve high egg fertility (Baker *et al.* 2001a). The number of sperm transferred at each mating in this species is therefore probably low, although alternative explanations for the very low single mating fertility, such as genetic incompatibility, cannot be excluded. Nevertheless, females require several matings to fertilise a high proportion of their eggs. The adaptive strategies that could be employed by more fecund females to increase their egg fertility include receiving increased ejaculates at each mating or mating more frequently.

The possibility that large eyespan females received increased ejaculate during mating was assessed by comparing the difference in the spermatophore sperm sac area delivered in

single matings to large and small eyespan females. The difference in spermatophore sperm sac area was calculated by subtracting the sperm sac area in small eyespan females from the sperm sac area in large eyespan females. Analysis of this variable takes into account the paired design of the experiment (where individual males were mated to large and small eyespan females in turn). This paired experimental design had the advantage of minimising male-derived variation in spermatophore sperm sac area.

There was a weakly significant interaction effect between mating order and experimental block in determining the difference in spermatophore sperm sac area transferred to large and small eyespan females. The direction of the differences due to mating order across blocks was not consistent. Neither was there a consistent pattern in the magnitude of differences between large and small female spermatophore sperm sac areas across experimental blocks. The biological meaning of the significant interaction between mating order and experimental block is not clear. However, there was no significant variation in the difference in spermatophore sperm sac area attributable to mating order or to experimental block. The difference between the spermatophore sperm sac area transferred to large and small eyespan females did not differ significantly from zero. Large eyespan females therefore did not receive sperm sacs with a significantly greater area than those transferred to small eyespan females.

Assuming that spermatophore sperm sac area is a legitimate estimate of ejaculate size, the results provide no evidence of strategic ejaculate allocation by males according to female size (and therefore female reproductive potential). It is important to note that the use of spermatophore sperm sac area as an estimate of ejaculate allocation is imprecise and it is not yet known whether spermatophore sperm sac area correlates with sperm number. Future work should focus on quantifying the relationship between spermatophore sperm sac area and sperm number, and / or developing a method of counting the number of sperm transferred in an ejaculate directly. Confirmation of low numbers of sperm transferred per ejaculate would argue that females do gain fertility benefits from multiple

mating through increased sperm transfer and not through reductions in genetic incompatibility.

Females and males stand to gain benefits of increased ejaculate allocation to larger females, if this results in the fertilisation of a higher proportion of eggs. It is possible that spermatophore sperm sac area is constrained by the size of the female reproductive tract, although the lack of a significant difference in sperm sac area between large and small eyespan females argues against this. Alternatively, spermatophore sperm sac area may be subject to opposing selective forces arising from sperm competition. Sperm competition is potentially higher in large females due to their increased mating frequencies. However, whilst sperm competition may result in increased ejaculate allocation (Gage & Barnard 1996; Wang & Millar 1997; Gage 1998; Wedell & Cook 1999; Parker *et al.* 1999; Johnson & Hubbell 1984), males may also adopt the opposite strategy and conserve sperm in highly competitive circumstances (Parker *et al.* 1996, Parker *et al.* 1997). Evidence supporting the latter view comes from predatory mites, *Macrocheles muscadomesticae* (Yasui 1996) and the bush cricket, *Kawanaphila nartee* (Simmons & Kvarnemo 1997).

Males can conserve sperm in highly competitive situations by stopping sperm transfer under certain circumstances. Evidence for male abstinence in situations where sperm competition is intense comes from a study on a sister species, *C. whitei.* Males mated to females that had previously mated within 6 minutes (and were therefore highly likely to contain rival male's sperm), were more likely to curtail copulation duration to less than 40 seconds and thus fail to transfer a spermatophore, than were males that mated with virgin females (Lorch *et al.* 1993). However, there was no evidence of strategic male abstinence in *C. dalmanni* in this study. Large eyespan females, in which sperm competition was likely to be intense, did not receive a higher proportion of curtailed copulations under 40 seconds than small eyespan females. There was no evidence that *C. dalmanni* females received ejaculates of increased or decreased size, or had altered likelihoods of

spermatophore transfer (i.e. the ratio of curtailed: full copulations), according to female size or the risk of sperm competition.

Large eyespan females did mate more often, and therefore had significantly more matings in which spermatophores were transferred (Figure 3.3). On average, large eyespan females had approximately a third more matings (with duration above 40 seconds) than small eyespan females. Males should prefer to mate multiple times with large females to gain fecundity benefits. However, this benefit has to be weighed against the higher probability of losing out in sperm competition in matings with large females when there are other males present. It would be interesting to address in future work whether there is an interaction between the probability of multiple matings and female size, in nightly roosting aggregations in this species.

The mating system of *C. dalmanni* may explain why females do not receive more ejaculate per mating but do remate at higher frequencies. The female biased sex ratio on roosting aggregations (Burkhardt & de la Motte 1988), and male competition to control these aggregations (Burkhardt & de la Motte 1983, 1987), may place selection pressure on the male controlling the aggregation to mate at a high frequency with all the females in his aggregation. An increase in male mating frequency could result in a male's ejaculate being increasingly partitioned and consequently leading to a decrease in ejaculate size at each mating. It has been predicted that if matings, ejaculate synthesis or transfer are limiting or costly, then males should strategically allocate their ejaculates between females of different sizes (Wedell 1992; Gage & Barnard 1996). However low mating costs may result in an optimal strategy of quicker but less discriminate ejaculate transfer. It will be important in future work to quantify the relative costs of ejaculate synthesis and transfer in this species, for example by comparing the lifespans of virgin males with males which had been allowed to mate multiply.

## 3.6 TABLES

Table 3.1. The sample size and mean ± standard error of the spermatophore sperm sac areas ($mm^2$ x $10^{-4}$) in large and small eyespan mated females, and the difference between the spermatophore sperm sac areas in large and small eyespan mated females.

| Block | Mating order | *n* | Large eyespan female | Small eyespan female | Difference |
|---|---|---|---|---|---|
| A | L-S | 8 | 47.27 ± 3.37 | 46.08 ± 3.32 | 1.19 ± 5.23 |
| A | S-L | 7 | 45.56 ± 4.94 | 44.33 ± 4.83 | 1.23 ± 6.00 |
| B | L-S | 9 | 53.86 ± 3.27 | 54.56 ±3.54 | -0.70 ± 5.42 |
| B | S-L | 9 | 53.99 ± 4.73 | 49.35 ± 3.06 | 4.63 ± 4.57 |
| C | L-S | 10 | 50.37 ± 3.64 | 43.79 ± 3.69 | 6.58 ± 5.24 |
| C | S-L | 10 | 44.31 ± 3.07 | 47.04 ± 3.37 | -2.70 ± 4.38 |
| D | L-S | 8 | 40.77 ± 3.24 | 46.98 ± 3.88 | -6.20 ± 4.44 |
| D | S-L | 8 | 49.41 ± 3.77 | 37.87 ± 2.38 | 11.54 ± 2.65 |
| Total | | 69 | | | |

Table 3.2 ANOVA on the difference between large and small eyespan female spermatophore sperm sac area ($mm^2$). The difference was calculated as the area of the small eyespan female spermatophore sperm sac subtracted from the large eyespan female spermatophore sperm sac area. Mating order (whether a male mated a large or small eyespan female first) is a fixed factor and experimental block is a random factor.

| **Source of variation** | ***df*** | ***SS*** | ***MS*** | ***F*** | ***P*** |
|---|---|---|---|---|---|
| Mating order | 1 | $2.03 \times 10^{-6}$ | $2.03 \times 10^{-6}$ | 0.36 | >0.50 |
| Block | 3 | $1.6 \times 10^{-7}$ | $5.33 \times 10^{-8}$ | 0.03 | >0.90 |
| Mating order x block[1] | 3 | $1.68 \times 10^{-5}$ | $5.61 \times 10^{-6}$ | 2.77 | <0.05 |
| Error[2] | 61 | $1.23 \times 10^{-4}$ | $2.02 \times 10^{-6}$ | | |

[1] Error term for $MS^{\text{mating order}}$

[2] Error term for $MS^{\text{mating order x block}}$, $MS^{\text{block}}$

## 3.7 FIGURES

Figure 3.1. Diopsidae spermatophores (reproduced from Kotrba 1996, Figure 2). Spermatophore size was estimated for *C. dalmanni* (c) by measuring the area of the sperm sac (grey shaded area) excluding the tubular neck.

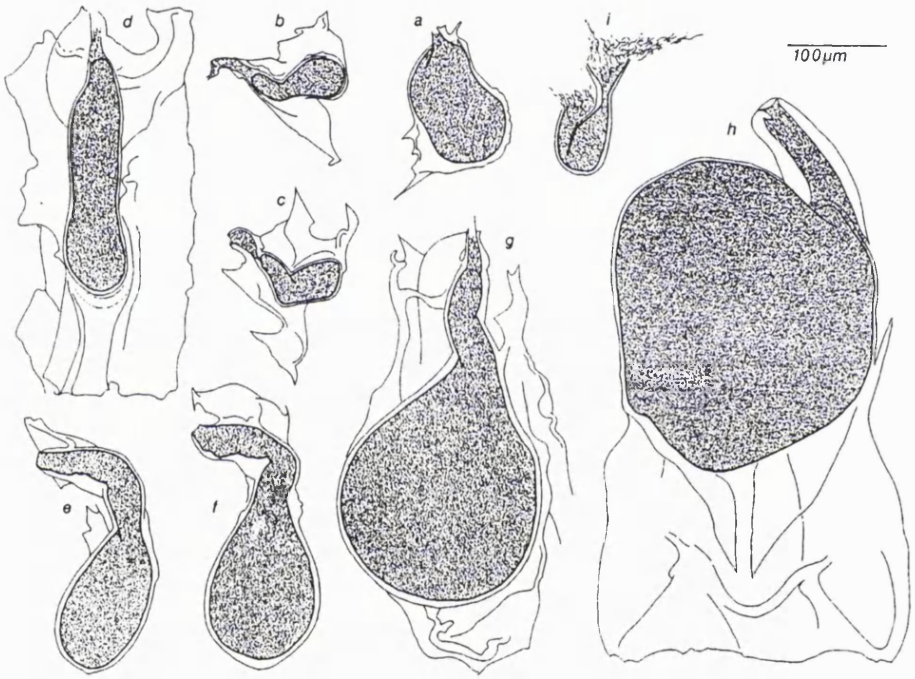



Figure 2. Spermatophores of Diopsidae. (a) *Sphyracephala brevicornis*; (b) *Cyrtodiopsis whitei*; (c) *Cyrtodiopsis dalmanni*; (d) *Cyrtodiopsis quinqueguttata*; (e) *Diasemopsis silvatica*; (f) *Diasemopsis dubia*;(g) *Teleopsis breviscopium*; (h) *Teleopsis quadriguttata*; (i) *Diopsis fumipennis*. Magnification is equal in all drawings. Scale bar = 100 µm.

Figure 3.2 The mean ± standard error number of mature eggs in the ovaries of large ($n$=22) and small ($n$=24) eyespan females.

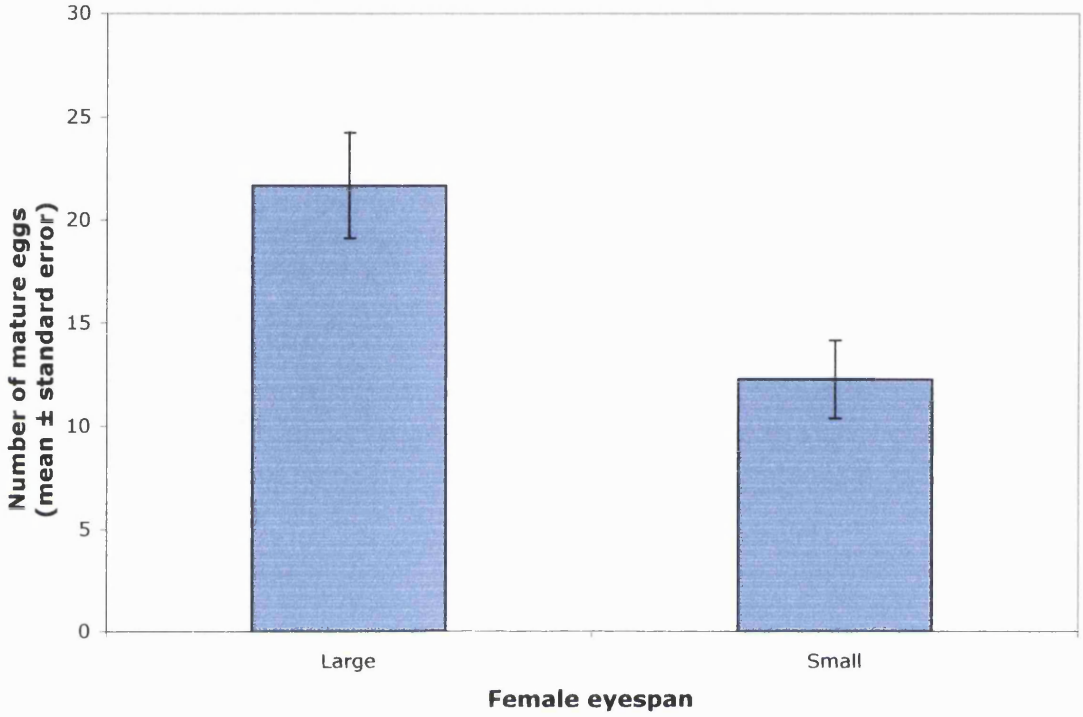
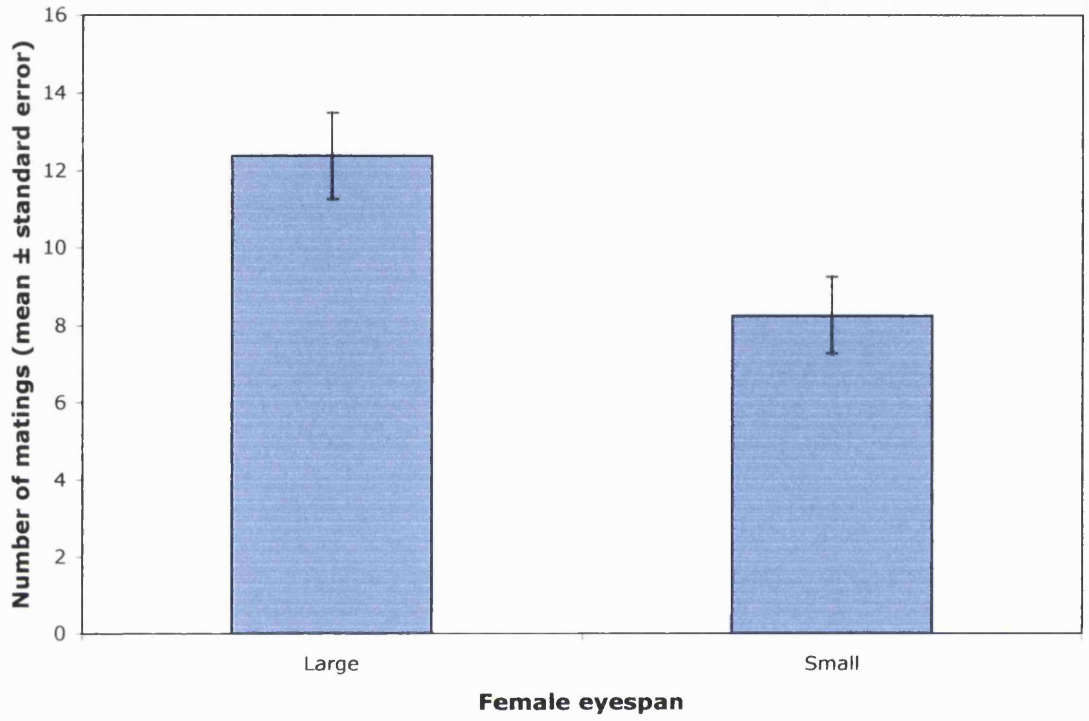


Figure 3.3 The mean ± standard error number of matings by large ($n$=30) and small ($n$=30) eyespan females. The graph shows the number of matings (of duration greater than 40 seconds) within two 1 hour assay periods on consecutive days.



# 4

# Male accessory gland proteins increase female fecundity

## 4.1 ABSTRACT

In many insects mating increases fecundity. This increase in fecundity is often due to proteins transferred in the seminal fluid, for example the accessory gland proteins of *Drosophila melanogaster*. Mating also increases fecundity in the stalk-eyed fly, *Cyrtodiopsis dalmanni* and the experiments in this chapter test whether *C. dalmanni* accessory glands are responsible. In the first two experiments, whole male accessory glands were homogenised and the extract was injected into the abdominal cavity of virgin females. Injection of male accessory gland proteins significantly stimulated fecundity in comparison to control, saline-injected virgin females, although the effect was less pronounced and occurred earlier than the increase in fecundity following mating itself. Accessory gland proteins were then separated into five fractions by reverse-phase HPLC, and injected into virgin females. The fecundity of injected females was then compared to that of control injected virgin females and mated females. The results showed that a protein or proteins isolated in one of the fractions is partially or wholly responsible for the increase in fecundity following mating.

## 4.2 INTRODUCTION

In many insects mating causes an increase in fecundity. This is often caused by the transfer of seminal fluid proteins by mating males (reviewed by Chapman 2001; Wolfner 2002). The increase in fecundity caused by mating is interesting, as it could result from either natural or sexual selection (Eberhard & Cordero 1995). Natural selection could act upon this process to coordinate mating and egg laying. This would ensure that eggs only start to be produced in large numbers once sperm have been received. There are also however opportunities for sexual selection. Males should be selected to boost egg production following transfer of their sperm, so that the number of progeny they father is maximised before the female remates. The increased fecundity caused by mating could also constitute a form of female mate choice (Eberhard 1996). Females would increase egg production to the highest level in response to matings with fit males i.e. those males capable of transferring highly effective fecundity enhancing proteins. This type of mate choice could result in direct and / or indirect benefits for females. It is therefore of interest to discover the mechanism by which fecundity is enhanced in *C. dalmanni*. This is addressed in the work described in this chapter, in which I tested whether seminal fluid proteins of male *C. dalmanni* increase female fecundity.

In *Drosophila melanogaster* and *C. dalmanni* mating increases fecundity. In *D. melanogaster* this has been shown to be due to the actions of accessory gland proteins (Acps). In *D. melanogaster* these Acps stimulate female oogenesis and ovulation. In addition, Acps reduce the probability of female remating, mediate sperm storage and protect against the deleterious actions of microbes (Wolfner 1997; Chapman 2001; Wolfner 2002). Acps act to increase male reproductive success, but receipt of high levels of Acps is costly to females, causing a decrease in longevity and reproductive success (Chapman *et al.* 1995). This 'cost of mating' is thought to be a deleterious side effect of male-beneficial Acp functions (Chapman *et al.* 1995).

The effect of mating on *D. melanogaster* female fecundity is pronounced. A young virgin female will produce and lay only a few eggs per day, after mating this increases to 40-80 eggs per day at high levels of nutrition (Manning 1967; Herndon & Wolfner 1995; Kubli 1996). Elevated oviposition lasts for about 7-10 days then decreases to virgin levels (Manning 1967). Egg production can be divided into a number of stages. Eggs develop within the ovarioles (oogenesis), they are then released into lateral oviducts (ovulation) and finally travel down the common oviduct to the uterus before being laid. One or more Acps could act at any or all stages in these processes (Wolfner 2002). Two Acps have so far been shown to influence different stages of egg production, Acp70A and Acp26Aa (Chen *et al.* 1988; Aigaki 1991; Kalb *et al.* 1993; Herndon & Wolfner 1995). In addition to these two Acps, the presence of sperm in females contributes to the initial increase in oviposition (Xue & Noll 2000; Heifetz *et al.* 2001) and storage of sperm is required for the longer-term elevation of fecundity (Manning 1967; Kalb *et al.* 1993; Xue & Noll 2000; Heifetz *et al.* 2001).

Acp26Aa ('ovulin') is a 264 amino-acid peptide precursor that, like peptide hormones, undergoes proteolytic cleavage before reaching its active form (Monsma & Wolfner 1988; Monsma *et al.* 1990; Park & Wolfner 1995). Acp26Aa is necessary to stimulate egg laying in mated females. Females that lack the Acp26Aa protein laid fewer eggs than control females on the first day post-mating (Herndon & Wolfner 1995). The amino acid sequence of the protein Acp26Aa is similar to egg-laying hormones made by the sea hare, *Aplysia californica* (Scheller *et al.* 1983; Rothman *et al.* 1986). Acp26Aa stimulates the early release of oocytes by the ovaries following mating (Heifetz *et al.* 2001) suggesting that it functions to clear mature eggs from the ovaries (Wolfner 2002). Within three minutes of mating, Acp26Aa is found in the female reproductive tract (Lung & Wolfner 2001), it then passes into the haemolymph (Monsma *et al.* 1990). Consistent with its action, Acp26Aa is localised primarily at base of the ovaries after mating (Monsma *et al.* 1990; Heifetz *et al.* 2001). Therefore, Acp26Aa might act directly on the ovaries or trigger endocrine or neural signals that stimulate ovulation (Wolfner 2002). Females mated to

males lacking Acp26Aa show some elevation of egg laying, indicating that other molecules also stimulate egg production (Herndon & Wolfner 1995; Kalb *et al.* 1993).

The second molecule acting on egg production in *D. melanogaster* is the 36 amino acid 'sex peptide' Acp70A (Chen *et al.* 1988). Chen *et al.* (1988) purified Acp70A from the male accessory glands. Male accessory gland proteins were separated using reverse-phase high pressure liquid chromatography (HPLC). Two injection experiments were then performed to isolate the biologically active protein in a single homogenous fraction. The protein was characterised using peptide sequencing, and through the construction of a cDNA library, the structure of the mRNA was found. Northern analysis was then used to demonstrate tissue specific expression in male accessory glands (Chen *et al.* 1988).

Acp70A increases egg production and decreases receptivity following its transfer at mating (Chen *et al.* 1988), as shown by ectopic expression of Acp70A and injection of synthetic Acp70A (Chen *et al.* 1988, Aigaki *et al.*1991). Soller *et al.* (1997, 1999) showed that both mating and Acp70A injection increase the transcription of yolk protein genes in the ovaries, and stimulate the development of vitellogenic oocytes. The Acp70A binding site must be accessible via the haemolymph, because injection of Acp70A into the abdomen of virgin females causes an increase in egg production (Chen *et al.* 1988). Nakayama *et al.* (1997) used ectopic expression of Acp70A in transgenic females. They found that cytoplasmic, but not membrane, bound Acp70A decreased receptivity and increased egg production, indicating that Acp70A must act outside the cells.

However, outside *D. melanogaster*, little is known about Acps. *C. dalmanni* therefore offers a novel opportunity to investigate the function and structure of male accessory gland proteins in a species with strong female mate choice. *C. dalmanni* exhibits extreme male exaggeration of eyespan and sexual dimorphism, and female preference has been demonstrated for large male eyespan (Burkhardt & de la Motte 1988; Wilkinson & Reillo 1994; Hingle *et al.* 2001a,b). The investigation of Acps in other stalk-eyed fly species will

eventually permit the study of Acp sequences and evolution. A great deal is known about the evolutionary history of eyespan exaggeration amongst stalk-eyed fly species. A molecular phylogenetic analysis of over 30 Diopsid species shows that exaggerated male eyespan evolved independently at least four times from the ancestral state of monomorphism and has also undergone repeated reductions (Baker & Wilkinson 2001; Baker *et al.* 2001b). Comparisons of Acp structure and function could be made between pairs of closely related species of stalk-eyed fly that differ in the degree of sexual dimorphism, to shed light on Acp evolution. For example, Acp function and structure in sexually dimorphic *C. dalmanni* would be compared with *C. quinqueguttata,* a closely related species that is almost monomorphic. Understanding the mechanism by which fecundity is increased after mating may also help to determine whether receipt of Acps represents a direct or indirect benefit of multiple mating.

This chapter reports two experiments in which a preparation of homogenised male accessory glands was injected into the abdominal cavities of virgin female *C. dalmanni.* The effect on fecundity was then assayed over the following 6 days and compared to control females that had been injected with saline, females that had been mated and virgin females. In the second experiment, an additional control group of sham-injected females was also included. In a third experiment, accessory gland proteins were separated using reverse-phase HPLC (as in Chen *et al.* 1988) and divided into five fractions. The five fractions were injected into virgin females. Female fecundity was assayed and compared to control females injected with saline, females that had been mated and virgin females.

## 4.3 MATERIALS AND METHODS

### 4.3.1 Fly stocks and culture methods.

Flies used were from the laboratory population described in Chapter 2, section 2.1. Virgin flies were obtained as in Chapter 2, section 2.2. The age of experimental individuals used varied from 6 to 10 weeks.

### 4.3.2 General procedures.

**Dissection of male accessory glands.**

The paired accessory glands of 100 males were dissected. Males were dissected between 6 and 8 weeks post-eclosion, and had been kept in a population cage of approximately 100 flies at a 1:1 sex ratio. Males were killed by placing them in a –20°C freezer for 10 minutes. Reproductive tracts were dissected at x10 magnification using a dissection microscope and placed on to a microscope slide in 20µl of phosphate buffered saline (PBS). The testes and ejaculatory bulb were removed and the paired accessory glands transferred into PBS and chilled on ice. For the whole gland injection experiments 1 and 2, 100 paired accessory glands were placed in100µl of PBS each. For the Acp separation by HPLC experiments, two lots of 50 paired accessory glands were stored in 50µl of PBS. The dissected accessory glands were stored at –80°C until use.

**Microinjection.**

Females were injected using a custom-made microinjection machine (kindly made by the laboratory of Prof. E. Kubli at the University of Zürich). In this machine a motor was attached to the plunger of a 5µl Hamilton syringe, allowing accurate and repeated delivery in the range of 20-100nl. Capillary needles were attached to the syringe via a paraffin-filled flexible tube. Capillary needles were made by heating the centre of a 1mm glass capillary tube using a Bunsen burner and rapidly stretching the tube by hand to narrow the bore in the centre. After the tube had cooled it was broken in two at the narrowest point, placed under a dissection microscope at approximately x10 magnification, and a piece of sharpened tungsten wire used to chip a bevel where the bore was finest. Needles were

filled with approximately 1μl of injection solution, allowing the injection of approximately 20 females with 50nl each before refilling the needle. Females were immobilised on ice for injections. They were placed dorsally on a microscope slide under x10 magnification under a dissecting microscope, and injected by Dr Tracey Chapman on the ventral side of the abdomen, approximately 1mm above the ovipositor on the right hand side.

### 4.3.3 Experiment 1. Whole male Acp extract injection.

**Preparation of male Acp extract for injection.**

Acp extract was prepared on the day of the injections. Accessory glands were defrosted and homogenised by sonication (at a frequency of 7μ for 10s, then 4μ for 20s). The extract was spun down in a centrifuge (15000 rpm for 15 minutes) and the supernatant removed. The sonication, centrifugation and removal of supernatant steps were repeated three times, to remove the cellular debris from the Acps.

**Experimental females.**

180 virgin females, aged between 6 and 8 weeks post eclosion, were placed individually into numbered containers (400ml; height=95mm, diameter=75mm). Females were placed in containers a week prior to treatment to allow them to acclimatise. The base of the containers had damp cotton wool covered by a circle of blue tissue paper, and 2g of puréed sweetcorn. Females were assigned to one of 6 experimental groups ($n$=30 per group): 1. Females left with a single male for 2 hours to mate *ad libitum*, to estimate the effect of mating on female fecundity; 2. Virgin females, to act as a non-injected control for comparison to mated females; 3. Females injected with 0.05μl of Acp extract (approximately equivalent of 1/20 of one male's accessory glands, an estimation of the quantity of accessory gland proteins transferred in a single mating); 4. Females injected with 0.05μl of PBS, i.e. controls for females injected with Acp extract.

At the start of artificial dawn on the day of treatment (i.e. mating or injection), 30 males were placed into the containers of the females in the mated group, one male per container.

Males used were between 6 and 8 weeks post-eclosion and had been kept in a cage of approximately 100 flies at a 1:1 sex ratio since eclosion. The males were placed in the containers for a two-hour period, to ensure all the females were mated. The number of copulations each female received was not counted, however, a single male and female in identical containers mate between 1 and 10 times in the period of an hour (CAG unpublished observation). After two hours the males were removed and discarded. At mid-day the injections were initiated. Females injected with Acp extract were injected first, followed by females injected with PBS. The injection needle was replaced between each group, to avoid cross-contamination of injection solutions. Virgin females were not injected.

**Fecundity assay.**

Eggs were counted by removal of the blue paper on the base of each fly container. The blue paper was replaced along with 2g of fresh sweetcorn, and the cotton moistened to provide water. Eggs laid on the blue paper were easily visible and were counted without the use of a microscope. Eggs were counted on the day of treatment, as an estimate of pre-treatment fecundity. After treatment the number of eggs each female laid were counted every two days for 6 days. Females that died after treatment were excluded from the analysis. Final sample sizes were: mated females $n$=28; virgin females $n$=28; females injected with Acp $n$=28; females injected with PBS $n$=30.

### 4.3.4 Experiment 2. Whole male Acp extract injection.

**Preparation of male Acp extract for injection.**

Preparation of the Acp extract was identical to experiment 1.

**Experimental females.**

150 virgin females, between 6 and 8 weeks post eclosion, were placed individually into the same sized containers as used in experiment 1. Females were placed in containers a week prior to treatment to allow them to acclimatise. Females were assigned to five experimental

groups ($n$ = 30 per group): 1. Females mated three times; 2. Virgin females; 3. Females injected with 0.05µl of Acp extract; 4. Females injected with 0.05µl of PBS; 5. Females sham-injected.

On the treatment day, a single male was placed in the container of each female in the mated group at the start of artificial dawn. Each container was observed until 3 matings had occurred. Males were between 6 and 8 weeks post-eclosion and had been kept in a cage of approximately 100 flies at a 1:1 sex ratio. 24 hours before the matings males were removed from their holding cage and placed in isolation, 1 male per cage, to allow replenishment of sperm and Acps. After mating, males were removed and discarded. Injection of females with Acp extract was initiated at mid-day and was followed by injection of females with PBS. Females of group 5 were treated last, and sham-injected with an empty needle. Injections were performed as described in experiment 1.

**Fecundity assay.**

Eggs laid on the blue paper of each container were counted in the same way as for experiment 1. Eggs were counted on the day of treatment as an estimate of pre-treatment fecundity. After treatment, eggs were counted every 24 hours for the first 4 days, and then on day 6 (which included eggs laid on days 5 and 6). Egg counting commenced at the same time each day in order to standardise the time between egg counts to 24 hours and reduce variation in fecundity within the female groups. Females that died after treatment were excluded from the analysis. Final sample sizes were: mated females $n$=29; virgin females $n$=28; females injected with Acp $n$=30; females injected with PBS $n$=28; sham-injected females $n$=25.

### 4.3.5 Experiment 3. Injection of separated Acp fractions.

**Preparation of Acp extracts for injection by high-pressure liquid chromatography (HPLC).**

HPLC was performed in November 2000 at Pfizer, under the supervision of Dr. Richard Venn. 100 paired male accessory glands were dissected and eluted in two samples of 50 paired glands in 50μl of PBS. The glands were extracted using 500μl of Bennett's solution per 50 glands (5% formic acid, 1% trifluoroacetic acid (TFA), 1% w/v sodium chloride, in 1 M hydrochloric acid) with 10% methanol added (Bennett *et al.* 1979 cited in Esch *et al.* 1983). The solution was homogenised using sonication (7μ for 10s, then 4μ for 20s) and centrifuged (5 mins at 12500 rpm) to remove cellular debris. The pellet was extracted twice, and the supernatants pooled, before being lyophilised in a Turbo-Vac drier. Before elution, the dried sample was re-suspended in 200 μl of the mobile phase (Bennett's solution), vortexed to dissolve the sample and centrifuged (10000 rpm for 5 mins). The supernatant was removed and loaded into a Jupiter $C_5$ column. The sample was eluted with a linear gradient of 5% - 50% of buffer (0.1% TFA in acetonitrile) within 25 minutes, 50% - 90% over 5 minutes and was held at 90% for a further 5 minutes. Peaks representing groups of proteins were recorded using a UV detector (Figure 4.1), and a fluorescence detector (Figure 4.2). Fractions from each sample of 50 paired accessory glands were pooled in tubes at two-minute intervals. The tubes were then combined in five fractions: A: 2-4 minutes; B: 4-12 minutes; C: 12-16 minutes; D: 16-22 minutes; E: 22-32 minutes. Each fraction was then lyophilized in a Turbo Vac drier and stored at -80°C, until required. Fractions were resuspended on the morning of the injection day in 100 μl of distilled water, vortexed to dissolve the sample and centrifuged (15000 rpm for 15 mins). The supernatant was then removed into an Eppendorf tube and stored on ice until injection.

**Experimental females.**

240 virgin females, between 6 and 10 weeks post eclosion, were placed individually into numbered containers as before. Females were placed in containers a week prior to treatment, allowing them to adjust to the containers, and were then assigned to one of 8 experimental groups ($n$ = 30 per group): 1. Mated females, placed with a single male each

and allowed to mate *ad libitum* for 2 hours; 2. Virgin females; 3-7. Females injected with 0.05μl of Acp fractions A–E, respectively; 8. Control females injected with 0.05μl of PBS.

On the treatment day, a single male was placed into the container of each female of the mated group at the beginning of artificial dawn, and left for two hours. Males used had been kept in a mixed sex cage at a 1:1 sex ratio, for between 6 and 10 weeks after eclosion. Virgin females were not injected. Injection of females began with PBS injected females and then proceeding through the HPLC fractions A–E. The injection needle was replaced between each group, to avoid cross-contamination of injection solutions.

**Fecundity assay.**

Eggs laid on the blue paper of each container were counted in the same way as for experiment 1. Eggs were counted on the day of treatment as an estimate of pre-treatment fecundity. After treatment, eggs were counted every 24 hours for the first 4 days, and then on day 6 (which included eggs laid on days 5 and 6). Females that died after treatment were excluded from the analysis. Final sample sizes were: mated females $n$=24; virgin females $n$=23; females injected with Acp fractions A $n$=22, B $n$=22, C $n$=22, D $n$=23, E $n$=23; females injected with PBS $n$=23.

### 4.3.6 Statistical analysis.

Fecundity was compared between different female groups before treatment and then after treatment. The pre-treatment fecundity (number of eggs laid before treatment) was not normally distributed, and could not be transformed to normality. Pre-treatment fecundity was therefore analysed using Kruskal-Wallis tests ($\chi^2$ values). The post-treatment fecundity (number of eggs laid after treatment) was the total number of eggs laid over the 6 days divided by 6, to give the average number of eggs laid per day. These values were transformed using the square root, to normalise the data. Post-treatment fecundity was then analysed using a one-way analysis of variance. Significant differences between the

groups were identified using Tukey-Kramer Honestly Significant Difference tests ($Q$ values).

The post-treatment fecundity of females from different experimental groups was compared every two days in experiment 1, and every day for experiment 2. This enabled differences in the timing of effects on fecundity to be compared. Kruskal-Wallis tests were used to compare fecundity, as the data were not normally distributed, and could not be normalised. Multiple comparisons between groups were performed using Dunn's nonparametric tests ($q$ values). For experiment 3 (Acp fraction injection), the analysis of post-treatment fecundity on a per day basis showed no consistent pattern of differences between female groups (data not shown). Therefore results are only reported for fecundity post-treatment averaged over the 6-day period.

Statistical analysis was carried out using JMP statistical software (version 5, SAS Institute Inc.) for the Apple Macintosh.

## 4.4 RESULTS

### 4.4.1 Experiment 1. Whole male accessory gland extract injection.

There was no significant difference in female fecundity between the experimental groups before treatment (Kruskal-Wallis: $\chi^2$=1.07, *df*=3, $p$>0.75). After treatment there was a significant difference between female experimental groups, when fecundity was estimated as the mean number of eggs laid per day over 6 days (Figure 4.3: mated, 6.10 ± 0.60 (mean ± standard error); virgin, 3.79 ± 0.47; Acp extract injected, 4.79 ± 0.62; PBS injected, 3.22 ± 0.42. $F_{3,110}$=4.73, $p$<0.005). Multiple comparisons show that mated females laid significantly more eggs per day than virgin females and females injected with PBS (mated *v* virgin, $q$=5.008, $p$<0.005; mated *v* PBS injected, $q$=3.874, $p$<0.05). No other groups differed significantly.

Alterations in female fecundity were then investigated as the mean number of eggs laid every two days for the 6 days post-treatment (Figure 4.6). On day 2 a significant difference in fecundity was found between the experimental groups (Kruskal-Wallis: $\chi^2$=14.08, *df*=3, $p$<0.005). Multiple comparisons show that mated females and females injected with Acp extract laid significantly more eggs than females injected with PBS (mated *v* PBS injected, $Q$=3.422, $p$<0.005; Acp extract injected *v* PBS injected, $Q$=2.777, $p$<0.05). On day 4 the difference between experimental groups was more pronounced (Kruskal-Wallis: $\chi^2$=28.90, *df*=3, $p$<0.0001), mated females laid significantly more eggs than all other groups (mated *v* virgin, $Q$=3.040, $p$<0.02; mated *v* Acp extract injected, $Q$=3.529, $p$<0.005; mated *v* PBS injected, $Q$=5.264, $p$<0.001). By day 6 there were no significant differences in fecundity between any of the experimental groups (Kruskal-Wallis: $\chi^2$=1.04, *df*=3, $p$>0.075).

### 4.4.2 Experiment 2. Whole male Acp extract injection.

There was no significant difference in female fecundity between the experimental groups before treatment (Kruskal-Wallis: $\chi^2$=6.67, *df*=4, $p$>0.1). Analysis of post-treatment fecundity (mean number of eggs laid per day over 6 days) shows that treatment

significantly affected female fecundity (Figure 4.4: mated, 5.02 ± 0.45 (mean ± standard error); virgin, 2.39 ± 0.24; Acp extract injected 3.96 ± 0.37; PBS injected, 1.75 ± 0.23; sham-injected, 1.81 ± 0.23. $F_{4,135}$=19.86, $p$<0.0001). Multiple comparisons show that both mated females and females injected with Acp extract laid significantly more eggs per day than virgin females, females injected with PBS and sham-injected females (mated *v* virgin, $q$=55.735, $p$<0.001; mated *v* PBS injected, $q$=77.348, $p$<0.001; mated *v* sham-injected, $q$=9.477, $p$ <0.001; Acp injected *v* PBS injected, $q$=7.545, $p$ <0.001; Acp extract injected *v* sham-injected, $q$=6.893, $p$ <0.025; Acp extract injected *v* virgin, $q$=4.672, $p$<0.05). No other groups differed significantly.

Fecundity of the different experimental groups was then compared every day after treatment (Figure 4.7). On days 1, 2 and 3 there was a significant difference in fecundity between experimental groups (Kruskal-Wallis: day 1, $\chi^2$=21.45, $df$=4, $p$<0.0005; day 2, $\chi^2$=19.72, $df$=4, $p$<0.001; day 3, $\chi^2$=37.49, $df$=4, $p$<0.0001), but by day 4 and on day 6 there were no significant differences in fecundity (day 4, $\chi^2$=2.98, $df$=4, $p$>0.5; day 6, $\chi^2$=5.39 $df$=4, $p$>0.2). On day 1 and 2, females injected with Acp extract laid significantly more eggs than females injected with PBS, and sham-injected females (day 1: Acp injected *v* PBS injected, $Q$=3.581, $p$<0.005; Acp extract injected *v* sham-injected, $Q$=3.596, $p$<0.005; day 2: Acp injected *v* PBS injected, $Q$=3.075, $p$<0.05; Acp extract injected *v* sham-injected, $Q$=3.147, $p$<0.02). On day 3 mated females laid more eggs than all other experimental groups, including Acp extract injected females (mated *v* virgin, $Q$=4.895, $p$<0.01; mated *v* Acp extract injected, $Q$=3.224, $p$<0.02; mated *v* PBS injected, $Q$=4.938, $p$<0.001; mated *v* sham-injected, $Q$=4.414, $p$<0.001). No other groups differed significantly.

### 4.4.3 Experiment 3. HPLC fraction injection.

There was no significant difference in fecundity between the experimental groups before treatment (Kruskal-Wallis: $\chi^2$=3.76, $df$=7, $p$>0.8). There was a significant difference in fecundity (mean number of eggs laid per day over 6 days) between experimental groups

after treatment (Figure 4.5: mated, 10.42 ± 0.77 (mean ± standard error); virgin, 4.20 ± 0.81; fraction A, 4.18 ± 0.81; fraction B, 7.20 ± 0.89; fraction C, 5.64 ± 0.63; fraction D, 4.88 ± 0.68; fraction E 2.67 ± 0.53; PBS injected females, 3.38 ± 0.66. $F_{7,174}$=10.52, $p$<0.0001). Multiple comparisons show that mated females laid significantly more eggs per day than virgin females, females injected with fractions A, C, D and E and females injected with PBS (mated *v* virgin, $q$=7.618, $p$<0.001; mated *v* fraction A, $q$=7.486, $p$<0.001; mated *v* fraction C, $q$=6.370, $p$<0.001; mated *v* fraction D, $q$=6.444, $p$<0.001; mated *v* fraction E, $q$=10.140, $p$<0.001; mated *v* PBS injected, $q$=8.870, $p$<0.001). Females injected with fraction B laid significantly more eggs than females injected with PBS and females injected with fraction E (fraction B *v* PBS injected, $q$=8.676, $p$<0.001; fraction B *v* fraction E, $q$=6.532, $p$<0.001). Females injected with fraction C laid significantly more eggs than females injected with fraction E (fraction C *v* fraction E, $q$=5.075, $p$<0.001). No other groups differed significantly.

## 4.5 DISCUSSION

The results of these experiments show that accessory gland proteins (Acps) transferred in male seminal fluid cause, at least in part, the increase in fecundity seen as a result of mating in *C. dalmanni*. The results of experiments 1 and 2, where extracts of whole male accessory gland were injected into virgin females, showed that one or more Acps caused a significant increase in fecundity. In experiment 2, females injected with Acps laid significantly more eggs than females injected with the same volume of PBS, virgin females, and females that had been sham-injected. Fecundity of Acp injected females did not differ significantly from that of mated females. The results of experiment 1 are less clear, as there was no overall significant difference between females injected with Acps and females injected with PBS or virgin females, although mated females did not lay significantly more eggs than Acp injected females. However, analysis of day by day fecundity following treatment shows that injection of Acps did have a similar affect on female fecundity in experiment 1 as in experiment 2, although the timing of the effect differed.

In experiment 1, the fecundity of females injected with Acps peaked on day 2 (Figure 4.6), and they laid significantly more eggs than females injected with the same volume of PBS. In experiment 2, Acp-injected female fecundity peaked on day 1 (Figure 4.7), when they laid significantly more eggs than females injected with PBS and females that were sham-injected. Fecundity in accessory gland injected females remained significantly higher than control groups on day 2. As eggs were only counted every two days in experiment 1, it is possible that Acp-injected female fecundity did peak on day 1, as in experiment 2 where the eggs were counted daily. In both experiments the peak in fecundity in females injected with Acps preceded the peak in fecundity seen in mated females. In experiment 1, mated female fecundity peaked on day 4; in experiment 2, it peaked on day 3. This discrepancy could also be explained by the fact that eggs in experiment 1 were counted every two days and in experiment 2 every day.

The difference in the timing of fecundity increases between mated females and Acp-injected females could result from the treatment of the females. Injected females were cooled on ice and injected, unlike mated females. Another explanation might be the location of the target receptor for the fecundity increasing protein in relation to the mode of delivery of the Acps. In *D. melanogaster*, two accessory gland proteins increase female fecundity, acting at different stages of egg production (reviewed by Wolfner 1997; Chapman 2001; Wolfner 2002). Acp26Aa stimulates the release of mature eggs from the ovaries (Heifetz *et al.* 2001), and although Acp26Aa is primarily localised at the base of the ovary after mating (Monsma *et al.* 1990; Heifetz *et al.* 2001) it also passes into the haemolymph (Monsma *et al.* 1990) to possibly trigger endocrine or neural signals. Acp70A stimulates oogenesis (Soller *et al.* 1997, Soller *et al.* 1999), and previous injection experiments in *D. melanogaster* show that the protein still elicits a response when injected into the haemolymph. *D. melanogaster* Acps have been shown to enter the haemolymph through a permeable area in the female reproductive tract, the vaginal intima, which is permeable to all but the largest Acps (Lung & Wolfner 2001). Acps in *D. melanogaster* and *C. dalmanni* reach at least some of their target receptors via the haemolymph. Any effect of Acps on egg production in *C. dalmanni* might be quicker if the proteins were delivered into the haemolymph without passing through the reproductive tract, if the transfer of Acps from the reproductive tract into the haemolymph takes time in this species.

Male accessory gland proteins were separated into 5 fractions using reverse-phase HPLC. The fecundity of females injected with the fractions was then compared to that of females injected with PBS, mated and virgin females. After treatment, mated females laid significantly more eggs per day than PBS injected females, virgin females and females injected with all fractions except for fraction B. Females injected with fraction B laid significantly more eggs than females injected with PBS and females injected with fraction E, but did not lay significantly more eggs than females injected with any other fraction or than virgin females (Figure 4.8). This experiment provides preliminary evidence that one

or more proteins in fraction B may contribute to the increase in fecundity seen after mating.

The increase in fecundity caused by injecting fraction B was less pronounced than the effect seen when whole accessory gland extract was injected into females. Females injected with fraction B did not lay significantly more eggs than virgin females, or females injected with most other fractions. One explanation is that the increase in egg production resulting from mating is only in part caused by the protein(s) in fraction B. In *D. melanogaster* two proteins are responsible for the increase in egg production (Acp26Aa and Acp70A, Chapman 2001; Wolfner 2002). If two or more proteins are responsible for the increase in fecundity in *C. dalmanni* they could have been isolated in different fractions when separated by HPLC. It is also possible that the proteins work in a complementary way, and that the full response is dependent on the presence of both proteins. In addition, the presence and storage of sperm has been shown to contribute to the initial increase in oviposition in *D. melanogaster*, and a prolonged effect of mating on fecundity is dependent on the storage of sperm (Xue & Noll 2000; Heifetz *et al.* 2001). It is possible that this is also true in *C. dalmanni*, and may partially explain why the effect of injecting whole accessory glands and injecting fraction B does not elevate fecundity to the same degree as mating.

The experiments presented here show that male Acps partly or wholly cause the increase in female fecundity seen as a result of mating. The receipt of Acps could represent a direct or indirect benefit of mating for females. A direct benefit could result from the coordination of mating, boosting fecundity only after the receipt of sperm. Females may gain indirectly through producing sons that transfer highly effective fecundity-enhancing Acps. Sexual selection will act on males to increase female egg production before the female remates, in order to fertilise the maximum number of the female's eggs. Indirect benefits for females could also result from the production of daughters who exercise mate

choice by increasing egg production in response to Acps from fit males (i.e. males that transfer Acps that are most effective in increasing egg production).

Separation of the accessory gland proteins, and the increase in fecundity caused by one of the fractions, represents the first step towards isolating the protein(s) responsible for the increase in egg production. In future work it would be beneficial to repeat the HPLC, and replicate the fraction injection experiment to test whether the results are repeatable. Further steps would purify male accessory gland proteins through more HPLC stages, until one or more fractions that elicit the effect on fecundity have only a single homogenous peak. The peptide could then be characterised through amino acid sequencing, or by the construction of a cDNA library to find the mRNA structure. Northern analysis would identify where the gene was expressed. It would be interesting to compare the function and structure of the protein, and the sequence of the gene, between other species of stalk-eyed fly from the same genus. The most informative comparisons would be between closely related pairs of species that differ in the degree of sexual dimorphism. For example, the comparison between *C. dalmanni* and *C. quinqueguttata*, a close relative that is almost monomorphic. This would allow novel areas of research into the evolution of male accessory gland proteins.

## 4.6 FIGURES

Figure 4.1. HPLC UV spectrophotometer reading (set at 210 nm). Elution of 50 paired accessory glands extracted in Bennett's solution (Chen *et al.* 1988) on a Jupiter $C_5$ column. The sample was eluted with a linear gradient of 5% - 50% of buffer (0.1% TFA in acetonitrile) within 25 minutes, then from 50% - 90% for 5 minutes and then held at 90% for 5 minutes.

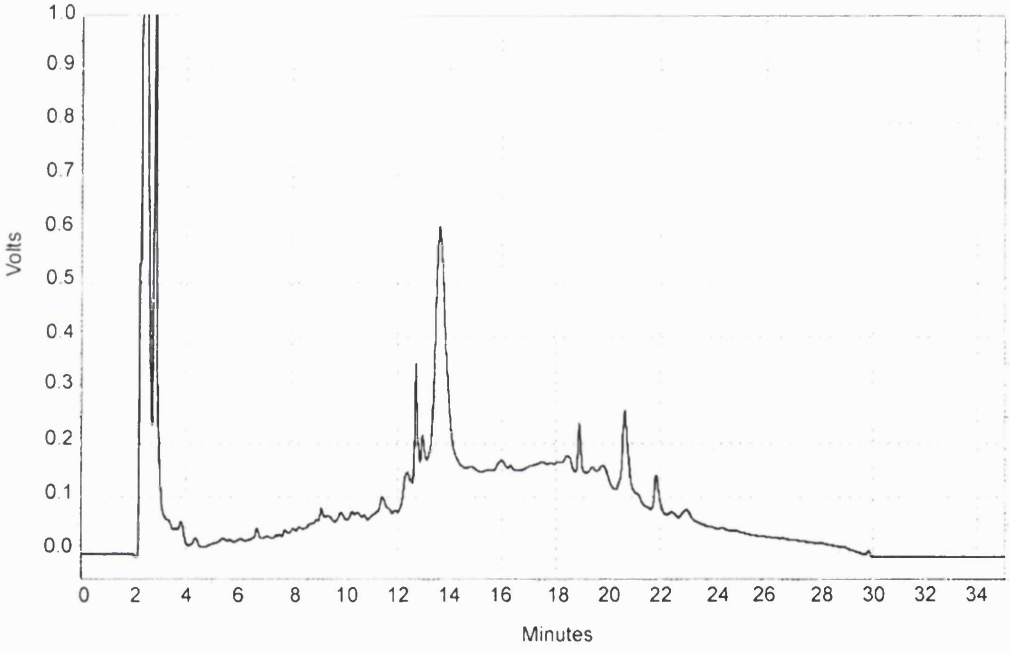


Figure 4.2. HPLC fluorescence detector reading (set at an excitation of 215 and an emission of 283 of tyrosine). Elution of 50 paired accessory glands extracted in Bennett's solution (Chen *et al.* 1988) on a Jupiter $C_5$ column. The sample was eluted with a linear gradient of 5% - 50% of buffer (0.1% TFA in acetonitrile) within 25 minutes, then from 50% - 90% for 5 minutes and then held at 90% for 5 minutes.

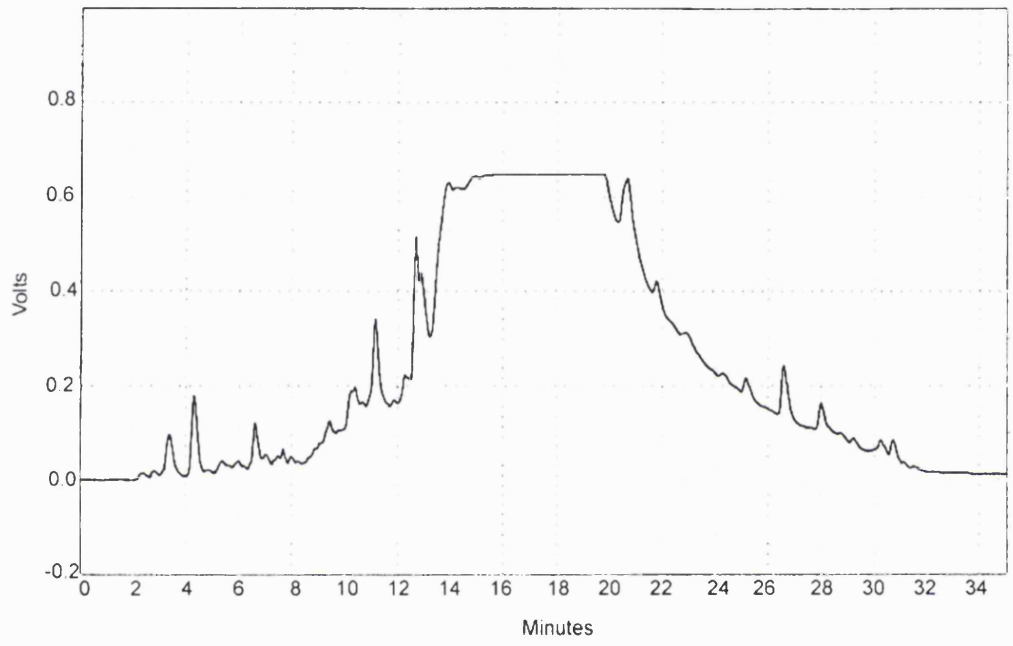


Figure 4.3. Fecundity of females from the Acp extract injection (experiment 1). The mean ± standard error number of eggs laid per day over the 6 days post-treatment, for females that had been mated, virgin females, females injected with 0.05μl of Acp extract and females injected with 0.05μl of PBS.

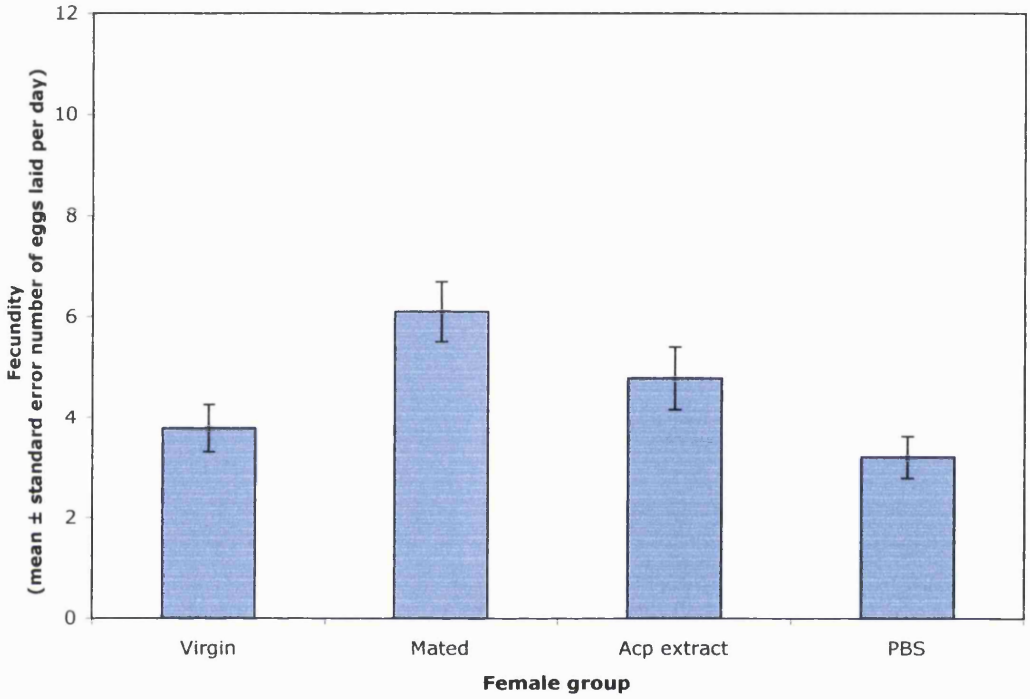


Figure 4.4. Fecundity of females from the Acp extract injection (experiment 2). The mean ± standard error number of eggs laid per day over the 6 days post-treatment, for females that had been mated, virgin females, females injected with 0.05μl of Acp extract and females injected with 0.05μl of PBS.

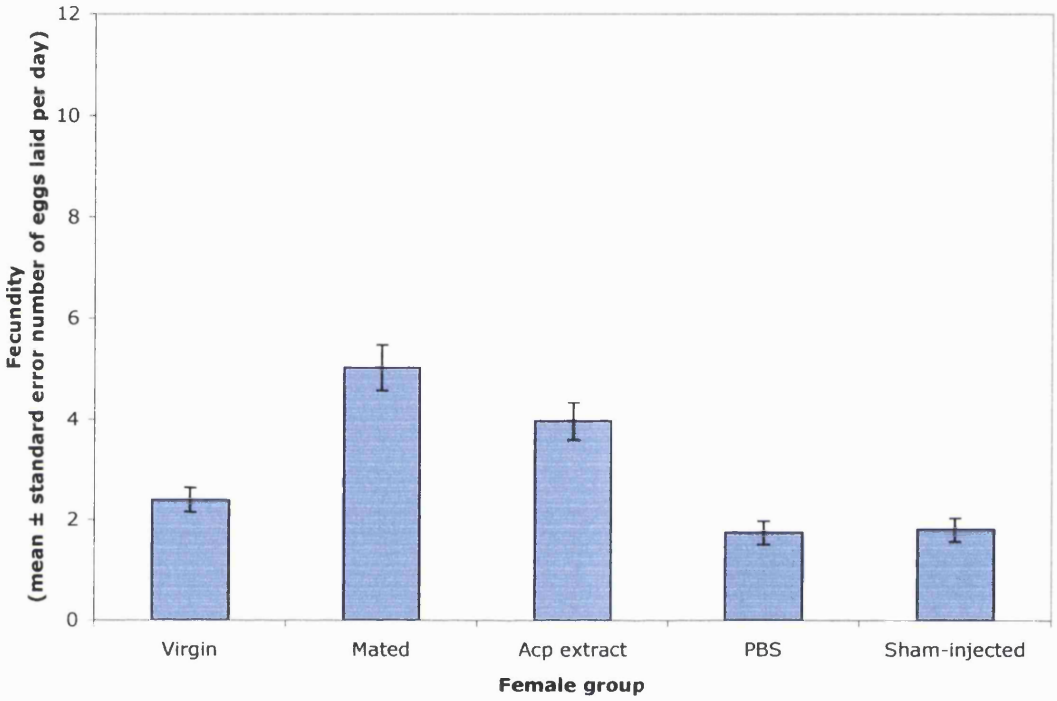


Figure 4.5. Fecundity of females from the HPLC fraction injection (experiment 3). The mean ± standard error number of eggs laid per day over the 6 days post-treatment, for females that had been mated, virgin females, females injected with 0.05µl of HPLC separated fractions A, B, C, D and E and females injected with 0.05µl of PBS.

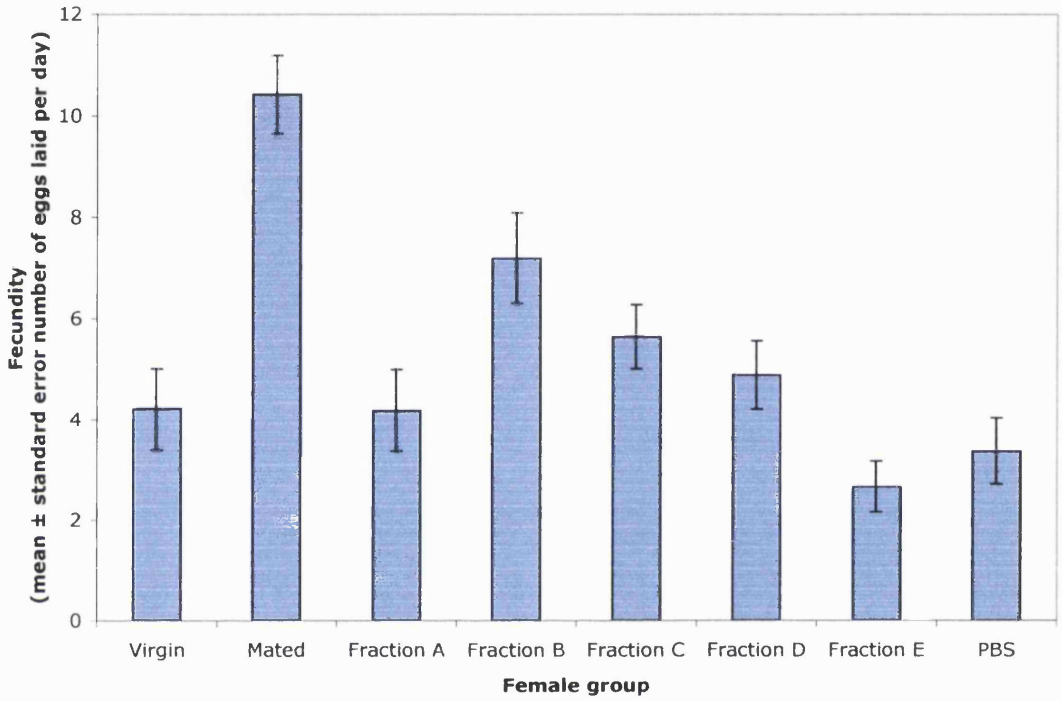


Figure 4.6. Fecundity of females from Acp extract injection (experiment 1). Fecundity (mean ± standard error number of eggs laid per two days) of mated females, virgin females, females injected with 0.05µl of Acp extract and females injected with 0.05µl of PBS.

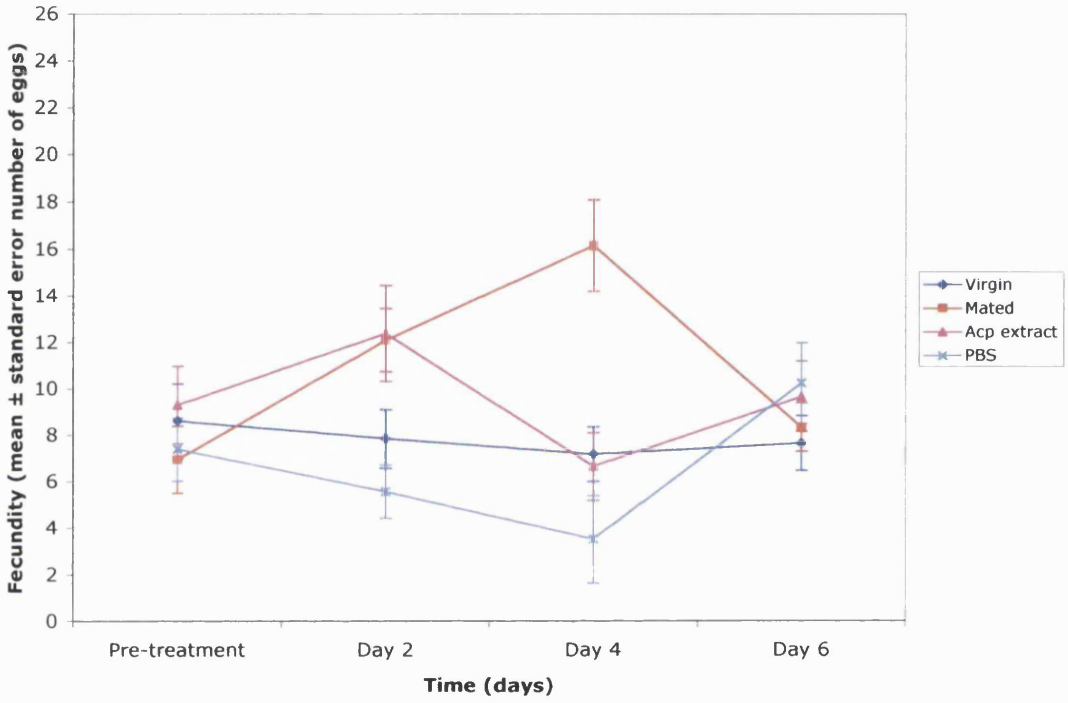


Figure 4.7. Fecundity of females from the Acp extract injection (experiment 2). Fecundity (mean ± standard error number of eggs laid per day) of mated females, virgin females, females injected with 0.05µl of Acp extract, females injected with 0.05µl of PBS and sham-injected females.

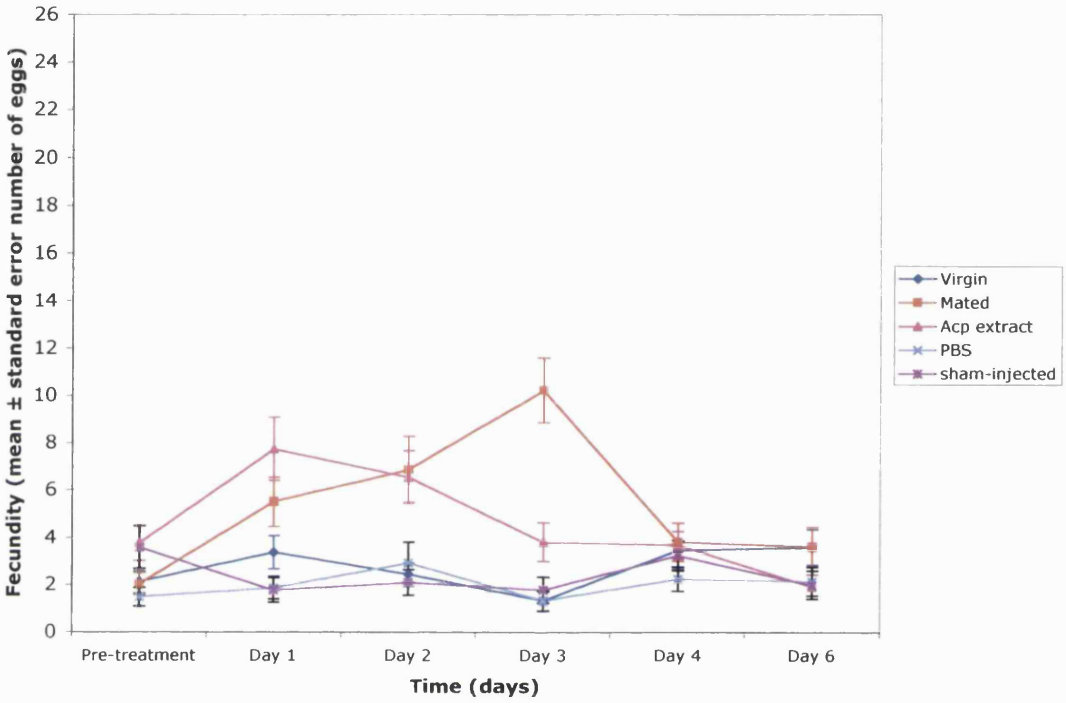
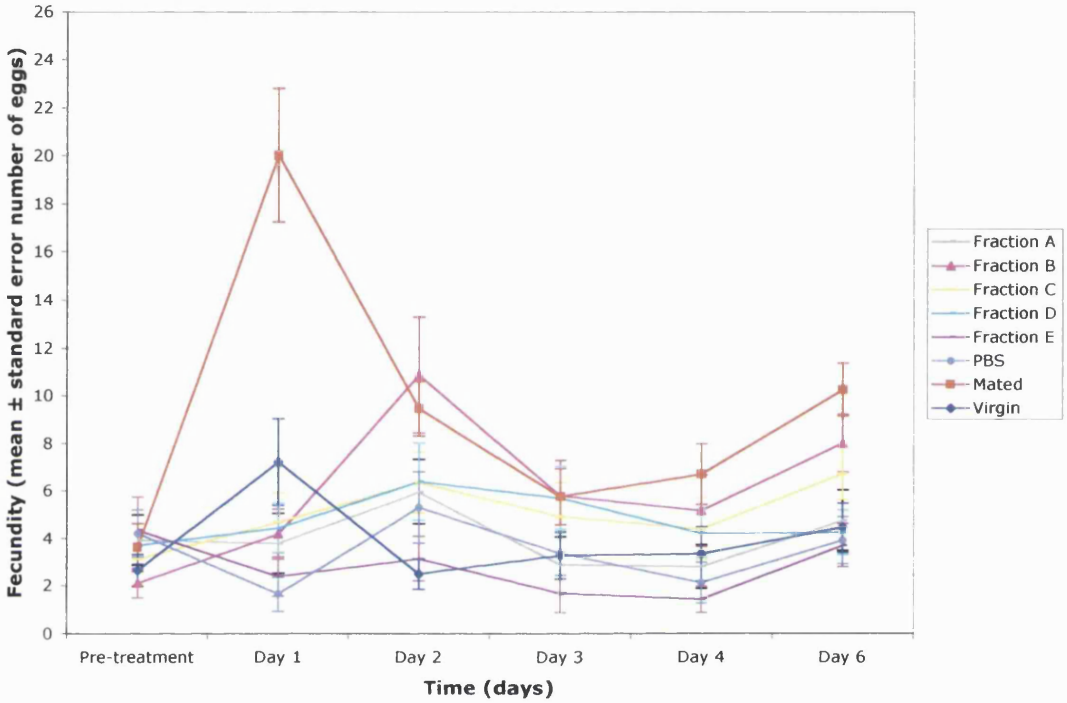


Figure 4.8. Acp HPLC separated fraction injection (experiment 3). Fecundity (mean ± standard error number of eggs laid per day) of mated females, virgin females, females injected with 0.05µl of HPLC separated fractions A-E and females injected with 0.05µl of PBS.



# 5

No reduction of female sexual receptivity following mating

## 5.1 ABSTRACT

The level of female sexual receptivity is an important component of male and female reproductive success. In many insects, mating itself causes a sharp decline in female receptivity. This can be a direct result of the physical act of mating, or due to actions of sperm or seminal fluid proteins. The degree to which males can decrease female receptivity will directly affect their reproductive success, by altering the chance that their sperm will be used in fertilisations in the interval before the female mates again. In this chapter, the effect of mating on female receptivity in the sexually dimorphic stalk-eyed fly, *Cyrtodiopsis dalmanni*, was investigated. The results showed no evidence for mating-induced reductions in female receptivity. In addition, matings with males that differed in eyespan did not cause differences in the level of female receptivity. There was also no evidence that females remated sooner when presented with large eyespan males. These results are surprising, given the indirect benefits that females gain from matings with large eyespan males. Finally I demonstrated that males do not appear to discriminate between females on the basis of female mating status.

## 5.2 INTRODUCTION

Female sexual receptivity is an important component of both male and female fitness. The level of sexual receptivity defines the probability that a female will mate, and may be affected by the male she previously mated with, and the identity of the new mating partner as well as by environmental factors. The level of female receptivity is expected to be associated with sperm supply. Female fitness depends on receiving sufficient sperm to maximise fertility, indeed, sperm replenishment was found to be the predominant explanation of multiple mating in a variety of insects (Ridley 1988; Arnqvist & Nilsson 2000). Females are generally predicted to have an optimum mating rate that is lower than that for males, because given a sufficient sperm supply, females gain less of an increase in reproductive success than do males with each additional mating. Thus, on average, females are expected to be less willing to mate than males. If forced copulation is assumed to be uncommon in insects (Thornhill 1980), it follows that male fitness is also dependent on female acceptance of copulation.

Female behaviour is known to influence the acceptance of copulation in a number of species. Physical rejection of advances by males may be achieved by walking or flying away, extrusion of the ovipositor or lifting the abdomen, as well as kicking and wing movements (Connolly & Cook 1973; Bergh *et al.* 1992; Heady 1993; Fox & Hickman 1994; Ringo 1996). If females are able to successfully reject male mating advances then fertilisation may be, at least in part, controlled by female acceptance of copulation.

Female receptivity can be influenced by environmental or physiological factors in insects. Female *Drosophila melanogaster* given access to food were shown to remate at a higher level than those denied food (Harshman *et al.* 1988). Another environmental influence is the availability of oviposition sites. In *D. melanogaster* (Trevitt *et al.* 1988) and the bruchid beetle, *Callosobruchus maculates* (Fox & Hickman 1994), females able to oviposit regained receptivity to courting males more readily. In addition to these environmental influences, mating itself causes a sharp decline in female receptivity in many

insects.

The effects of mating on female receptivity fall into three categories: behavioural, mechanical and chemical. The post-copulatory behaviour of the anthophorid bee, *Centris pallida*, involves tactile and acoustic displays that suppress female receptivity (Alcock & Buchmann 1985). Allen *et al.* (1994) found that guarding and associated post-copulatory behaviour helped "switch off" female receptivity in a parasitoid wasp, *Aphytis melinus*. The presence of male ejaculate in the female reproductive tract may act as a mechanical signal or physical barrier to decrease receptivity. For example, in two species of cockroach, *Supella longipalpa* and *Blattella germanica*, receptivity is suppressed temporarily by a spermatophore in the bursa copulatrix, and completely by the presence of sperm in the spermathecae (Smith & Schal 1990; Liang & Schal 1994). In the tsetse fly, *Glossina mositans*, receptivity can be suppressed by implanting glass beads into the uteri of virgin females (Gillott & Langley 1981). The presence of mating plugs can also prevent remating, for example in the dung fly, *Coproica vagans* (Lachmann 2000). Seminal fluid proteins secreted from male accessory glands have been shown to decease female receptivity in a number of species, including mosquitoes, *Aedes aegypti* (Craig 1967); the house fly, *Musca domestica* (Riemann & Thorson 1969); the onion fly, *Delia antiqua* (Spencer *et al.* 1992) and the planthopper, *Prokelisia dolus* (Heady 1993).

Several studies have shown that a combination of behavioural, mechanical and chemical factors influence female receptivity. Bergh *et al.* (1992) found that the mechanical stimuli associated with the act of mating where important for triggering a refractory period in the Hessian fly, *Mayetiola destructor*. However, the transfer of a chemical component of the male reproductive tract in the ejaculate was necessary to prolong the suppression of sexual receptivity. In *Drosophila melanogaster,* a male accessory gland protein (Acp) known as the 'sex peptide' (or Acp70A), which is transferred to females during mating, can decrease female receptivity for 1-2 days (Chen *et al.* 1988). The presence of sperm is necessary for a persistent reduction in female receptivity for up to 12 days (Kalb *et al.* 1993). In the

grasshopper *Gomphocerus rufus* (Hartmann & Loher 1999), female receptivity can be decreased by behavioural factors (copulation duration is over 2 hours while insemination occurs in only 3-4 minutes), mechanical factors (the empty spermatophore acts as a mating plug) and chemical factors (secretions from the male accessory glands).

In species in which both males and females mate multiply, sexual selection will favour male adaptations that enhance the success of their own versus rival sperm (Parker 1970). Males may be selected to decrease female receptivity in order to increase their chances in sperm competition. Females of the stalk-eyed fly, *Cyrtodiopsis dalmanni*, show high sexual receptivity and frequent multiple mating by both sexes. Recent work by Baker *et al.* (2001a) demonstrates direct benefits of multiple mating for female *C. dalmanni*. High egg fertility (80%) is only achieved after multiple mating. Matings occur predominately in the dawn and dusk period when flies are aggregated on root hairs (Wilkinson & Reillo 1994; Wilkinson *et al.* 1998a).

*C. dalmanni* exhibit sexual dimorphism for eyespan width, with males having exaggerated eyespans. Selection for the extreme exaggeration in males is presumed to have been driven by sexual selection. Male eyespan is an important fitness trait, influencing competitive success in contests with other males (Burkhardt *et al.* 1994; Panhuis & Wilkinson 1999). In addition, female choice for males with large eyespans has been demonstrated (Burkhardt & de la Motte 1988; Hingle *et al.* 2001a,b), and shown to have indirect genetic benefits (Wilkinson *et al.* 1998b; David *et al.* 2000).

In this chapter, the effect of mating on female receptivity was investigated. I tested whether stalk-eyed fly females mated with 'initial' males had reduced sexual receptivity to subsequent matings with 'assay' males, as compared to females that had no 'initial' matings. Given the strong female preference for matings with large eyespan males (Burkhardt & de la Motte 1988; Hingle *et al.* 2001a,b), I tested the predictions that females would (a) show decreased receptivity after matings with large eyespan 'initial'

males and (b) show increased probability of remating with large eyespan 'assay' males. Finally, I investigated whether there was any bias among 'assay' males for matings with either unmated or mated females.

## 5.3 MATERIALS AND METHODS

### 5.3.1 Fly stocks and culture methods

The flies used were from a laboratory population of *Cyrtodiopsis dalmanni* described in Chapter 2, section 2.1. Virgins were obtained using the method described in Chapter 2, section 2.2. The age of experimental individuals used varied from 8 to 10 weeks. Eyespan was measured as described in Chapter 2, section 2.3. Males were assigned to groups as follows: large eyespan males had an eyespan >8.5 mm, small eyespan males had an eyespan of <7.5 mm and intermediate eyespan males had an eyespan between 7.5-8.5 mm. Females used were selected randomly with respect to eyespan.

### 5.3.2 Receptivity tests

A series of three experiments were performed. In each experiment I compared females that had been mated to 'initial' males with females that were unmated, by quantifying their subsequent receptivity with 'assay' males. Three days before the receptivity assays, females were placed in single sex groups. On the morning of the assay, one half of the females were placed with 'initial' males in a container at a 1:1 sex ratio for 1hr following lights on, at the artificial dawn (hereafter, 'mated' females). The other half of the females were similarly placed in a container at dawn, but in single sex groups, without males ('unmated' females).

In each experiment, the subsequent receptivity of 'mated' and 'unmated' females was quantified by placing them together with 'assay' males at various times, depending on the experiment, after the artificial dawn. Receptivity was then scored over the next 1-1.5hr (depending on the experiment) as (i) the time until first mating, (ii) number of female rejections, and (iii) number of matings over 40 seconds. Rejection behaviour was defined as kicking or wing movement, when a male attempted to mount the female. Matings shorter than 40 seconds are unlikely to result in the transfer of ejaculate (CAG pers. obs.; R. Baker pers. comm.), and were omitted from the analysis.

**The effect of mating on female receptivity.**

'Mated' and 'unmated' females were placed individually in 400ml containers (height = 95mm, diameter = 75 mm) with one intermediate eyespan 'assay' male each. Receptivity was recorded for 1hr, and three assays were performed: 1hr after dawn ($n$=55 for 'mated' and 'unmated' groups); 2.5hr after dawn ($n$=56, each group) and 6hr after dawn ($n$=44, each group).

**The effect of large and small eyespan males on female receptivity.**

We investigated whether the eyespan of the 'initial' male altered the magnitude of any mating-induced reductions in receptivity. In addition, the receptivity of females mated to 'assay' males with both large and small eyespans, was compared. Females were placed into one of three experimental groups: (i) 'unmated' females ($n$=40), (ii) females mated with small eyespan 'initial' males ($n$=40) (iii) females mated with large eyespan 'initial' males ($n$=40). Females from each group were placed individually in containers (dimensions specified above) with a single assay male each. For each assay, half of each group of females was assayed using a large eyespan 'assay' male, the other half were assayed using a small eyespan 'assay' male. Receptivity was scored for 1.5hr beginning 1hr after dawn.

**Are matings more likely with unmated or recently-mated females?**

Males were placed together with groups of 'mated' and 'unmated' females, to determine the effect of female mating status. For each assay, intermediate eyespan 'assay' males ($n$=63) were placed individually in containers with 3 'mated' and 3 'unmated' females each. This sex ratio was chosen to allow males a choice of 'mated' and 'unmated' females, and to allow females some opportunity of rejecting undesired matings. Females from each group were distinguished by marking them with a small dot of orange or green non-toxic paint on the thorax. The colours used for 'mated' and 'unmated' females were switched

half way through the experiment. The mating status of the first females to mate ('mated' or 'unmated') was then recorded for each assay. Female receptivity was assayed for 1hr, starting 1hr after dawn.

### 5.3.3. Statistical analysis.

In all experiments, time until the first mating (in seconds) was normalised using a log transformation and means were compared using *t*-tests. The number of matings and number of female rejection behaviours were analysed using Wilcoxon rank sum tests as the data were not normally distributed (as determined using Shapiro-Wilk tests).

Statistical analysis was carried out using JMP statistical software (version 5, SAS Institute Inc.) for the Apple Macintosh.

## 5.4 RESULTS

### 5.4.1 The effect of mating on female receptivity

There were no significant differences in the mean time until first mating, or the mean number of matings between 'mated' and 'unmated' females, for any of the three assay times (Table 5.1). There were no significant differences in the number of female rejections for two out of the three assay times (1hr after dawn and 6hr after dawn). However, unmated females performed significantly more rejections than mated females 2.5hr after dawn ($P<0.05$).

### 5.4.2 The effect of large and small eyespan males on female receptivity.

The eyespan of neither 'initial' nor 'assay' males influenced female receptivity (Table 5.2). There were no significant differences between 'mated' females mated to small eyespan 'initial' males and tested with small eyespan 'assay' males, and 'unmated' females tested with small eyespan 'assay' males, in the time until first mating ($df$=34; $P>0.75$), number of matings ($n_1$=18, $n_2$=20; $P>0.50$) or the number of rejections ($n_1$=19, $n_2$=20; $P>0.05$). There were no significant differences between 'mated' females mated to large eyespan 'initial' males and tested with large eyespan 'assay' males, and 'unmated' females tested with large eyespan 'assay' males, in the time until first mating ($df$=38; $P>0.25$), number of matings ($n_1$=20, $n_2$=20; $P>0.80$) or the number of rejections ($n_1$=20, $n_2$=20; $P>0.10$).

We also tested whether the eyespan size of the 'initial' male had any influence on subsequent female receptivity with 'assay' males. Females that mated a small eyespan 'initial' male and were tested with a small eyespan 'assay' male were compared to females that mated with a large eyespan 'initial' male and were tested with a large eyespan 'assay' male. There were no significant differences in the time until the first mating ($df$=35; $P>0.50$), the number of matings ($n_1$=20, $n_2$=18; $P>0.75$) or the number of rejections ($n_1$=20, $n_2$=19; $P>0.10$).

Finally, the influence of the eyespan size of the 'assay' male used for the assay was

investigated. 'Unmated' females assayed with small eyespan 'assay' males were compared with 'unmated' females tested with large eyespan 'assay' males. There was no significant difference in the time until the first mating ($df$=37; $P$>0.50), the number of matings ($n_1$=20, $n_2$=20; $P$>0.25) or the number of rejections ($n_1$=20, $n_2$=20; $P$>0.10).

### 5.4.3 Are matings more likely with unmated or recently mated females?

63 males were tested and 33/63 males mated an 'unmated' female first, and 30/63 males mated a 'mated' female first. There was no significant difference between 'mated' and 'unmated' females in the mean number of matings recieved ($n_1$=62, $n_2$=62; $P$>0.10) or the number of rejections they performed ($n_1$=62, $n_2$=62; $P$>0.10).

## 5.5 DISCUSSION

The results of all three experiments showed no significant effect of mating on subsequent female receptivity, for a period of up to 6 hours after the initial matings. No significant differences in female receptivity were found between females that had not mated for three days ('unmated' females) and females that had been recently mated, in all but one of the assays. Unmated females performed significantly more rejections than mated females 2.5 hours after dawn. This may indicate that mated females have increased acceptance of further mating, or that males made more attempts to mate with females which had not copulated that morning. However, this significant difference was markedly isolated. Coupled with the fact that females relatively rarely reject a mating (an average of 0.6 and 1.1 rejections per hour for 'mated' and 'unmated' females respectively), this suggests that this result may represent a type I error, rather than a true indication of altered female receptivity.

The second experiment investigated whether the eyespan of the 'initial' male or the eyespan of the 'assay' male, had any influence on female receptivity. Comparisons of unmated and mated females were consistent with the findings of the first experiment, that mating did not reduce female receptivity. The results showed that the eyespan of the 'initial' male had no effect on subsequent female receptivity with 'assay' males. The eyespan of the 'assay' male also had no significant effect on receptivity. These results are surprising, because female *C. dalmanni* have a preference for matings with large eyespan males (Burkhardt & de la Motte 1988; Hingle *et al.* 2001a,b). The results show no evidence that this preference is expressed in terms of female receptivity to mating or rejection behaviour.

The finding of no effect of mating upon subsequent receptivity in the first two experiments was supported by the results of the third experiment, in which single males were assayed with 3 'mated' and 3 'unmated' females each. There was no significant difference in the

number of matings or rejections performed by 'mated' or 'unmated' females. Thus, even when females had a greater opportunity to avoid copulation through the presence of other females in the container, no mating-induced reduction in receptivity was evident. In this experiment, males also had the opportunity to mate with 'mated' or 'unmated' females. However, there was no evidence of a bias in favour of matings between males and 'unmated' females, suggesting that males cannot discriminate on the basis of female mating status.

The lack of a reduction in female receptivity following mating could be due to: (i) an absence of factors that change female mating propensity as a result of mating, (ii) a lack of capacity to detect changes in female receptivity in the experimental design, (iii) sexually antagonistic factors that alter female receptivity but whose effects are masked by counter-adaptation.

The results support the first of these possibilities. Male and female *C. dalmanni* both mate multiply, which led me to the *a priori* prediction that males may be selected to decrease female receptivity, in order to delay remating with other males, and therefore enhance the success of their own versus rival sperm (Parker 1970). However, the assumption that females immediately remate with other males may not be met in this species. Females of this species choose roosting aggregations controlled by large eyespan males, and the majority of matings occur at dawn within the aggregation (Burkhardt & de la Motte 1988), meaning that females may mate multiply with the same male on a given morning. Ejaculate limitation (see Chapter 3) could increase the probability that females remate repeatedly with one male controlling each aggregation, which would not lead to selection on males to decrease female receptivity. An additional factor that would decrease selection pressure on males to prevent female remating is strong first male sperm precedence. A sister species, *Cyrtodiopsis whitei*, shows sperm mixing and first male sperm precedence (Lorch *et al.*1993), although the pattern of sperm precedence in *C. dalmanni* is currently unknown. While female multiple mating led me to predict male mediated initiation of a refractory

period, the lack of a significant decrease in female receptivity as a consequence of mating in *C. dalmanni* may be explained in the context of the ecology, behaviour and physiology of this species.

The second possibility is that mating does decrease female receptivity but that the design of the three experiments minimised the detection of any effect. Previous experiments (e.g. Hingle 2001b) have found that females are able to discriminate between males with a certain degree of difference in eyespan in pairwise choice tests. It is possible that in the experimental design, where females were not given the opportunity to compare large and small eyespan males simultaneously, females are less discriminating in their rejection behaviour. Additionally, these experiments do not make a distinction between male persistence and female resistance. However, the third experiment provides some evidence that male persistence is not influenced by female mating status, as there is no difference in the distribution of matings between, or in the number of matings rejected by, 'mated' and 'unmated' females. The experiment also increases the likelihood that females could avoid male mating attempts by increasing the female sex ratio bias, although if male mating propensity was sufficiently high then any attempts by females to reject male copulation attempts in a limited space might have been ineffective. However, sexually immature *C. dalmanni* females frequently successfully reject male copulation attempts, by leg and wing movements as well as through running and flying away (CAG, pers. obs.) in conditions similar to those in these experiments. This suggests that females do have the ability to reject matings but do not exercise this as a result of mating, or under these experimental conditions.

Finally, it is possible that males are selected to decrease female receptivity but that 'Red Queen' style sexual antagonistic coevolution may have masked any effects attributable to mating (Rice 1996; Holland & Rice 1998). A decrease in receptivity upon mating, in females that are already sperm limited, would have strong fitness consequences, and would select for adaptation in females to counter the effects of mating on receptivity. It has been

suggested that sexually selected traits, such as the male eyespan of *C. dalmanni*, may have evolved partly to seduce females with low receptivity into remating (Sakaluk *et al.* 1995). The results do not support this hypothesis, however, as females assayed with large eyespan males were not more willing to mate. As female preference for males with large eyespan has been previously demonstrated under experimental conditions where females are presented with a binary mate choice (Hingle *et al.* 2001b), future work should be based around experiments designed to incorporate female preference.

## 5.6 TABLES

Table 5.1. Mean ± standard error of the time until first mating (seconds), the number of matings and the number of rejections, for 'unmated' or 'mated' females (all matings were with males of 'intermediate' eyespan width). Females were assayed with males (1 male, 1 female) in three 1hr assay periods, commencing 1, 2.5 and 6hr after dawn. Probability values are for comparisons between the mated and unmated females. Two-tailed *t*-tests were used to compare means for the logarithmic transformation of the time until first mating, the degrees of freedom and probability values are given. Wilcoxon rank sum tests were used to compare the number of matings and rejections, the sample size of the mated ($n_1$) and unmated ($n_2$) group and the probability values are given.

| | **Mating status of female** | | |
|---|---|---|---|
| | **Unmated** | **Mated** | ***P* values and *df* or sample sizes** |
| **Mean time until first mating (s)** | | | |
| 1hr after dawn | 611±890 | 683±960 | $df$=95; $P$>0.25 |
| 2.5hr after dawn | 1018±118 | 1008±120 | $df$=94; $P$>0.75 |
| 6hr after dawn | 1414±157 | 1068±146 | $df$=58; $P$>0.10 |
| **Mean number of matings** | | | |
| 1hr after dawn | 3.89±0.39 | 3.75±0.36 | $n_1$=55, $n_2$=55; $P$>0.75 |
| 2.5hr after dawn | 2.27±0.19 | 2.16±0.23 | $n_1$=55, $n_2$=55; $P$>0.50 |
| 6hr after dawn | 1.80±0.23 | 1.47±0.21 | $n_1$=43, $n_2$=40; $P$>0.25 |
| **Mean number of rejections** | | | |
| 1hr after dawn | 1.1±0.21 | 1.10±0.26 | $n_1$=55, $n_2$=55; $P$>0.50 |
| 2.5hr after dawn | 1.1±0.16 | 0.64±0.13 | $n_1$=56, $n_2$=55; $P$<0.05 |
| 6hr after dawn | 0.50±0.12 | 0.77±0.15 | $n_1$=43, $n_2$=40; $P$>0.25 |

Table 5.2. Mean ± standard error of the time until first mating (seconds), the number of matings and the number of rejections, for 'unmated' females, or females mated with large or small eyespan 'initial' males and subsequently assayed with either large or small eyespan 'assay' males. All relevant pairwise comparisons were non-significant, see the results section for probability values.

| | Mating status of female | | |
|---|---|---|---|
| **Size of 'assay' male** | **Unmated** | **Mated: small 'initial' male** | **Mated: large 'initial' male** |
| **Mean time until mating (s)** | | | |
| Small eyespan | 469±106 | 514±125 | 426±93 |
| Large eyespan | 425±76 | 699±133 | 542±91 |
| **Mean number of matings** | | | |
| Small eyespan | 6.05±0.61 | 7.11±1.04 | 7.50±0.62 |
| Large eyespan | 6.95±0.65 | 6.45±0.80 | 6.65±0.83 |
| **Mean number of rejections** | | | |
| Small eyespan | 0.50±0.20 | 1.16±0.31 | 1.00±0.31 |
| Large eyespan | 0.75±0.14 | 0.50±0.15 | 0.45±0.14 |

# 6

# No genetic correlation between male and female mating frequency

## 6.1 ABSTRACT

One explanation of the high mating frequency of female stalk-eyed flies, *C. dalmanni*, is that female mating frequency has evolved as a correlated response to selection on male mating frequency. The mating frequency of females from lines that had been artificially selected for 8 generations for increased or decreased male mating frequency was measured. Despite a direct response to selection in males, the mating frequency of females from lines selected for high male mating frequency did not differ significantly from that of females from lines selected for low male mating frequency. This provides evidence against a genetic correlation between male and female mating frequency.

## 6.2 INTRODUCTION

Multiple mating is common in insects (Ridley 1988). Males are predicted to have a higher optimal mating frequency than females (Arnqvist & Nilsson 2000; Gavrilets *et al.* 2001). This is because males are assumed to be able to produce sperm in unlimited quantity and thus can increase their reproductive success with each successive mating (Bateman 1948). In contrast, female reproductive success may be limited by egg production; therefore females do not necessarily increase their reproductive success with each extra mating (Bateman 1948; Parker 1979). Selection on males to increase mating frequency is predicted to be stronger than selection on females to limit mating frequency (Parker 1979). Female optimal mating frequency is determined by a trade-off between the benefits and costs of mating (Arnqvist & Nilsson 2000; Gavrilets *et al.* 2001).

There are many hypotheses proposing benefits for female multiple mating (e.g. Arnqvist & Nilsson 2000; Ridley 1988; Jennions & Petrie 2000). Adaptive explanations include direct benefits. For example, the receipt of sufficient sperm to maximise fertility (Arnqvist & Nilsson 2000; Ridley 1988), incorporation of male derived nutrients into egg or somatic tissue (Boggs & Gilbert 1979; Butlin *et al.* 1987) or the receipt of male accessory gland proteins with sperm that increase fecundity (Chapman 2001; Wolfner 2002). Adaptive hypotheses also include indirect, genetic benefits. For example, an increase in the genetic diversity of offspring (Tregenza & Wedell 2002), and the 'good sperm hypothesis' (Yasui 1997) [for full discussion of benefits of female multiple mating see Chapter 1 section 1.3].

An alternative to these adaptive hypotheses, is that a genetic correlation between the sexes for mating frequency causes female multiple mating (Halliday & Arnold 1987). If mating frequency is controlled by the same genes in males and females, then selection on males to increase their reproductive success by mating more frequently may result in females mating at a higher than optimal frequency. The argument assumes that selection on males

to mate frequently is stronger than on females to mate at their lower optimal mating frequency (Halliday & Arnold 1987).

A cost of mating will result in selection on females to limit their mating frequency (Sherman & Westneat 1988). Female mating costs can be caused by time and energy expenditure (Watson *et al.* 1998), physical injury (Stutt & Siva-Jothy 2001), increased predation risk whilst mating (Arnqvist 1989; Rowe 1994), parasite infection (Hurst *et al.* 1995) or chemicals transferred in male seminal fluid (Chapman *et al.* 1995). If there is a common genetic basis for mating frequency in both sexes, selection on females to lower their mating frequency should result in the evolution of modifier genes that limit the expression of genes for increased female mating frequency. This would eventually lead to different genes controlling mating frequency in males and females (Sherman & Westneat 1988). New mutations however are unlikely to be sex-limited, and a mutation with the same effect in both males and females would tend to strengthen a genetic correlation between the sexes in mating frequency (Arnold & Halliday 1988).

The presence of a genetic correlation between the sexes for mating frequency has been investigated in *Drosophila melanogaster* through the use of artificial selection experiments (Manning 1963; Gromko & Newport 1988; Stamenkovic-Radak *et al.* 1992; Sgrò *et al.* 1998). Taken together, these experiments provide equivocal results and have been criticised on aspects of experimental design (Arnold & Halliday 1992; Butlin 1993). For example, remating speed (time between the first and second mating) was selected in females in each of two populations of *D. melanogaster* (Gromko & Newport 1988). A significant direct response to selection was seen in one population only. A correlated response in male remating speed was found in the population lacking a significant direct response to selection (Gromko & Newport 1988). The study provides inconclusive evidence for a genetic correlation between the sexes because the correlated response was only seen in one out of two populations. Furthermore, the design of the selection experiment did not incorporate replication within populations, or proper control lines to

which the selection lines could be compared. The results could therefore be attributable to genetic drift (Arnold & Halliday 1992; Sgrò *et al.* 1998).

The time until the first mating ('mating speed') has been used in *D. melanogaster* as an estimate of mating frequency (Manning 1963). Selection on two replicate lines for 'fast' male mating speed (i.e. selection for males with decreased time until the first mating) was unsuccessful. A direct response to selection was seen in one of two lines selected for 'slow' male mating speed (i.e. selection for males with increased time until the first mating). A significant correlated response in female mating speed was seen in the line that had a significant direct response to selection for 'slow' male mating speed. There was no significant correlated response in female mating speed in the other line selected for slow male mating speed (in which there was no direct response to selection) (Manning 1963). Correlated responses were not tested in females from the two lines selected for 'fast' male mating speed. The lack of a direct response to selection in the two lines selected for 'fast' male mating speed, and one of the lines selected for 'slow' male mating speed, weakens this study as evidence for a genetic correlation in mating frequency between the sexes (Sherman & Westneat 1988).

In one study, artificial selection for mating speed (measured as the time until the first mating) in *D. melanogaster*, was applied independently to males and females (Stamenkovic-Radak *et al.* 1992). A significant direct response to selection was found in both sexes, although the response was largely attributable to the response of the 'slow' lines. Significant correlated responses were found in both sexes. Males from the 'fast' female-selected lines, and females from the 'fast' male-selected lines, both mated significantly faster than their 'slow' line counterparts (Stamenkovic-Radak *et al.* 1992). However, the experiment by Stamenkovic-Radak *et al.* (1992) does not provide strong evidence for a genetic correlation in *D. melanogaster*. This is because the design of the selection experiment did not enforce random mating between the selected individuals and

their unselected mates during production of the next generation for selection (Butlin 1993).

Non-random mating could take the form of assortative mating between males and females for mating speed, e.g. males from lines selected for fast male mating speed could preferentially mate with fast mating females. One consequence of such assortative mating would be unintended selection on the 'unselected' sex. This would lead to an overestimate of any genetic correlation (Butlin 1993). There is however some evidence for disassortative mating for mating speed in *D. melanogaster*. Males from populations that have fast mating speeds have a greater advantage with females from slow mating populations, and *vice versa* (Van de Berg *et al.* 1984; Van de Berg 1986). Disassortative mating for mating speed would lead to an underestimate of any genetic correlation (Stamenkovic-Radak *et al.* 1993).

Sgrò *et al.* (1998) provide the most convincing test for a genetic correlation in remating speed between the sexes in *D. melanogaster*. They selected females for increased and decreased time until remating (i.e. the time between the female's first and second mating). The possibility of assortative or disassortative mating was excluded by imposing random pairing between selected females and unselected males within selection regimes. In addition, each pair contributed equally to the next generation, which ensured that the strength of selection was consistent across all selection lines and replicates. Equal family size also minimised variation between lines due to assortative or disassortative mating (Sgrò *et al.* 1998). No correlated response was found in males in any of three traits used to estimate mating frequency: time to remating, time to first mating for virgins or remating frequency. The results provide strong evidence against a genetic correlation between males and females for remating speed in *D. melanogaster*.

However, none of the previous studies practised selection on mating frequency *per se*. Instead they focussed selection on initial matings, e.g. first virgin matings (e.g. Manning

1963), or the time from the first until the second matings (e.g. Sgrò *et al.* 1998). To investigate fully whether there is a genetic correlation between the sexes for mating frequency, it is necessary to select directly on mating frequency itself, rather than related traits such as time until mating or remating.

The sexually dimorphic stalk-eyed fly, *Cyrtodiopsis dalmanni*, is an ideal study organism in which to practice artificial selection for mating frequency because of its extremely high mating frequency (Wilkinson *et al.* 1998a). The number of matings that can easily be observed during relatively short assay periods of just a few hours is considerable, facilitating selection on this trait. Females gain direct fecundity and fertility benefits from multiple mating (Baker *et al.* 2001a). However, there is a cost of reproduction for female *C. dalmanni*. Females housed with males had significantly shorter life spans than virgin females housed with other females (Reguera *et al.* submitted). A cost of reproduction may indicate that female *C. dalmanni* mate at frequencies beyond their optimum.

Here the hypothesis that the high level of female multiple mating in *C. dalmanni* is a consequence of a genetic correlation between the sexes is evaluated. I used lines that had undergone 8 generations of artificial selection for increased ('high' lines) and decreased ('low' lines) male mating frequency (Baker *et al.* in prep.). Male mating frequency was manipulated by selecting up or down for the number of matings and there was a significant direct response to selection. Males from the high selected lines mated significantly more frequently than males from the low selected lines (Baker *et al.* in prep.). I asked whether there was a genetic correlation in mating frequency between males and females, by testing for a correlated response in the mating frequency of females from the lines artificially selected for high and low male mating frequency. I used two estimates of female mating frequency; a direct measure, the number of matings and an indirect measure, the time until the first mating.

## 6.3 MATERIALS AND METHODS

### 6.3.1 Fly stocks and culture methods.

Flies used were from the laboratory population described in Chapter 2, section 2.1. Virgins were collecting using the method in Chapter 2, section 2.2. The age of experimental individuals used varied from 6 to 8 weeks.

### 6.3.2 Artificial selection lines.

Details of the production of the artificially selected lines can be found in Chapter 2, section 2.4. The present study used samples taken from the selected lines after 8 generations of selection.

### 6.3.3 Correlated response in female mating frequency.

Females from the high and low male-selected lines and the control line were observed simultaneously. For each assay, five females from each selection regime (high, low or control) were placed in a container (400ml: height=95 mm, diameter=75 mm) that had damp cotton wool and 2g of puréed sweetcorn on the base. There were four containers of females from each of the two replicate lines of each selection regime, a total of 24 containers. Females were placed in the containers two days before observation to acclimatise. On the morning of observation, a single base stock male was added to each container of females. Observation began at the start of artificial dawn and lasted an hour and a half. The number of matings over 40 seconds by all five females ('number of matings') and the number of seconds until the first female in a container mated ('time until the first mating') were recorded.

Females were observed as described above on three consecutive days, i.e. one experimental 'block'. Males were assigned to either replicate line 1 or replicate line 2. To control for variation in male mating frequency, each male was then placed with females from each selection regime in turn; one day with females from the high selection regime, one day with females from the low selection regime and one day with control females. The day (1,

2 or 3) that a male encountered females from each selection regime (high, control or low) was randomly assigned. After each experimental block was completed, all females and males were discarded. 8 experimental blocks were conducted in total, and each block comprised an independent sample of females from each replicate line of each selection regime and base stock males.

### 6.3.4 Statistical analysis.

#### Number of matings.

The number of matings was defined as the total number of matings by all 5 females in a container in each experimental block, resulting in 32 data points (Table 6.1). Shapiro-Wilk tests showed that the number of matings was normally distributed. A one-way analysis of variance showed that block did not have a significant effect on the number of matings ($F_{7,184}$=0.87, $P$>0.5). The data were therefore pooled across blocks for the remainder of the analysis. The data for number of matings were analysed using ANOVA with selection regime as a fixed effect and replicate line nested within selection regime as a random effect (Table 6.2).

#### Time until the first mating.

The time until the first mating was defined as the mean time (in seconds) until the first female in a container mated in each block, resulting in 32 data points (Table 6.1). Shapiro-Wilk tests showed that the time until the first mating was not normally distributed. A logarithmic transformation was used to normalise the data. A one-way analysis of variance showed that block did not have a significant effect on the $\log_e$(time until the first mating) ($F_{7,164}$=1.15, $P$>0.3). Data were therefore pooled across blocks for the remainder of the analysis. The data for $\log_e$(time until the first mating) were analysed using ANOVA with selection regime as a fixed effect and replicate line nested within selection regime as a random effect (Table 6.2).

The experimental design was fully balanced. Statistical analysis was carried out using JMP statistical software (version 5, SAS Institute Inc.) for the Apple Macintosh.

## 6.4 RESULTS

### 6.4.1 Number of matings.

There was no significant effect of replicate line on the number of matings ($F_{3,186}$=1.05, $P$>0.25, Table 6.2(a)). There was no significant difference in the mean number of matings by females from the high, low or control selection regimes ($F_{2,3}$=0.24, $P$>0.75, Table 6.2(a)).

### 6.4.2 Time until first mating.

There was no significant effect of replicate line on the $\log_e$(time until the first mating) ($F_{3,166}$=2.30, $P$>0.05, Table 6.2(b)). There was no significant difference in the $\log_e$(time until the first mating) by females from the high, low or control selection regimes ($F_{2,3}$=0.07, $P$>0.75, Table 6.2(b)).

## 6.5 DISCUSSION

There was no evidence for a genetic correlation between male and female mating frequency, in the stalk-eyed fly *Cyrtodiopsis dalmanni*. Artificial selection for increased and decreased male mating frequency (number of matings) produced a direct response in both directions (Baker *et al.* in prep.). However, here I found no significant differences in the mating frequency of females from these high, low or control selection regimes, when mating frequency was estimated either directly (number of matings) or indirectly (time until the first mating).

If mating frequency was controlled by the same genes in males and females, females might mate beyond their optimal frequency if there was stronger selection on males to mate at a high frequency than on females to mate just once or a few times (Halliday & Arnold 1987). A cost of reproduction in female *C. dalmanni* could impose selection pressure on females to limit their mating frequency (Reguera *et al.* submitted). Strong selection on females to reduce mating frequency could lead to the evolution of modifying elements to limit the expression of genes which increase mating frequency (Sherman & Westneat 1988). This would lead to sex limitation in the genes determining mating frequency.

The lack of genetic correlation between the sexes for mating frequency in *C. dalmanni* suggests that mating frequency is under the control of different or sex-limited genes in males and females. Little is known regarding the determinants of male and female remating in this species. Experimental evidence suggests females are stimulated to remate by visual cues. Given a pairwise choice, females mate more frequently with males with large eyespan than with small eyespan males. The strength of female preference is influenced by the difference in eyespan between males (Hingle *et al.* 2001b). Female mating frequency is also related to female eyespan, large eyespan females mate more frequently than small females (see Chapter 3, section 3.4.3). Large eyespan females have significantly more mature eggs in their ovaries and mate at a higher frequency than small eyespan females, perhaps to maximise their fertility (see Chapter 3, section 3.4.1). The

difference in mating frequency between large and small eyespan females could also be due to males preferring to mate with large, more fecund females. This could indicate that visual cues are also important in stimulating male mating. There are many other factors that could determine remating in both male and female *C. dalmanni*. These include environmental influences such as availability of oviposition sites, or physiological limitations such as ejaculate size or sperm storage capacity, or chemical cues such as male accessory gland proteins (although see Chapter 5).

The lack of a genetic correlation between the sexes for mating frequency in *C. dalmanni* is consistent with evidence from *D. melanogaster* (Sgrò *et al.* 1998). The factors involved in determining remating in *D. melanogaster* are well characterised, in comparison with *C. dalmanni*, and differ between the sexes. Female *D. melanogaster* are stimulated to remate by nutritional status (Harshman *et al.* 1988), availability of oviposition sites (Trevitt *et al.* 1988), sperm storage (Letsinger & Gromko 1985), and accessory gland proteins transferred by males with sperm (Chen *et al.* 1988). Males are stimulated to remate by visual stimuli (Willmund & Ewing 1982), female epicuticular hydrocarbons (Scott 1986) and the number of recent matings obtained (Markow *et al.* 1978). If different factors determine mating in males and females, it is unlikely that selection on the mating frequency in one sex will significantly affect the mating frequency of the unselected sex.

A genetic correlation between the sexes in *C. dalmanni* could be underestimated if a particular form of non-random mating occurred in the selection line (Butlin 1993). Disassortative mating for mating frequency would occur if male *C. dalmanni* from lines selected to mate at a high frequency preferentially mated with females with low mating frequency, or males from lines selected to mate at a low frequency mated preferentially with fast mating females. Disassortative mating would then inadvertently result in selection on female mating frequency. This would obscure the appearance of any genetic correlation present.

The problem of non-random mating within selected lines can be addressed by enforcing random pairing between males and females (e.g. Sgrò *et al.* 1998). The selection protocol used for the lines evaluated here involved partially enforced random mating (Baker *et al.* in prep.). In each generation, selected males were placed with 5 selection line females chosen at random. The imposition of equal family size can also reduce variation between lines due to disassortative mating (Sgrò *et al.* 1998). During selection of the lines tested here, family size variation was minimised, but not eliminated. The contribution of different males to future generations was equalised across all regimes by taking an equal number of progeny from each selected male every generation. However, as each male was placed together with 5 females, it is possible that the contribution by females varied across regimes. Rigorously enforced random mating and equalisation of family sizes would require equal numbers of offspring to have been taken from individual males paired with single, randomly-chosen females. It would be interesting in future experiments to determine the extent of non-random mating by testing for disassortative and assortative mating in laboratory and field populations of *C. dalmanni*. The present study however provides no evidence for a genetic correlation between males and females for mating frequency in the stalk-eyed fly, *C. dalmanni*. The hypothesis that female mating frequency has evolved as a correlated response to selection on male mating frequency is not supported.

## 6.5 TABLES

Table 6.1. Mean ± standard error for the number of matings, and the time until the first mating, for females from the high, low and control lines artificially selected for male mating frequency.

| Selection regime | Replicate line | No. of matings | Time until first mating (s) |
|---|---|---|---|
| High | 1 | 13.22 ± 0.78 | 2159.58 ± 274.47 |
| | 2 | 11.97 ± 0.74 | 1543.62 ± 235.44 |
| Control | 1 | 12.50 ± 0.60 | 1726.51 ± 183.13 |
| | 2 | 13.62 ± 0.68 | 1593.53 ± 166.17 |
| Low | 1 | 12.78 ± 0.72 | 1746.90 ± 153.19 |
| | 2 | 13.09 ± 0.55 | 1690.83 ± 185.92 |

Table 6.2. ANOVA of a) number of matings and b) $\log_e$(time until the first mating) of females from the high, low and control lines artificially selected for male mating frequency, with selection regime (fixed effect) and replicate line nested within selection regime (random effect).

| Source of variation | *SS* | *df* | *MS* | *F* | *P* |
|---|---|---|---|---|---|
| **(a) Number of matings** | | | | | |
| Selection regime | 7.54 | 2 | 3.77 | 0.24 | >0.75 |
| Replicate line within selection regime[1] | 46.81 | 3 | 15.60 | 1.05 | >0.25 |
| Error[2] | 2758.13 | 186 | 14.83 | | |
| **(b) $\log_e$(time to the first mating)** | | | | | |
| Selection regime | 0.11 | 2 | 0.05 | 0.07 | >0.75 |
| Replicate line within selection regime[1] | 2.39 | 3 | 0.80 | 2.30 | >0.05 |
| Error[2] | 57.59 | 166 | 0.35 | | |

[1] Error term for $MS^{\text{selection regime}}$

[2] Error term for $MS^{\text{replicate line within selection regime}}$

# 7

# Male and female control of mating frequency

## 7.1 ABSTRACT

Female multiple mating may be non-adaptive and result from predominant male control over mating frequency. This chapter reports an experiment to investigate the relative degree of control of males versus females over mating frequency in the stalk eyed fly, *Cyrtodiopsis dalmanni*. Two variables were manipulated: male mating frequency and female preference. Male mating frequency was manipulated by using males from lines artificially selected for increased and decreased male mating frequency. Female preference was altered by a phenotypic manipulation of male eyespan within each selected line, to produce large and small eyespan males. The mating frequency of large and small eyespan males from both of the high and low male mating frequency lines was then measured. I predicted that if there was total female control, then female preference for large male eyespan would predominate and there would be no significant effect of male genotype (high or low selection line) on mating frequency. I predicted that if there was total male control, then there would be significant differences in the mating frequency of high and low selection line males, but no significant effect of male eyespan (i.e. female preference). The results showed significant effects of male genotype and of male eyespan, suggesting that neither sex has total control over mating frequency in this species. This view is further supported by the finding that there was no significant difference in the number of matings between small eyespan males from the high selection regime and large eyespan males from the low selection regime. The lack of evidence for predominant male control over mating frequency suggests that female multiple mating is not a non-adaptive consequence of selection on male mating frequency.

## 7.2 INTRODUCTION

It has been suggested that female multiple mating could be non-adaptive, and result from selection on male mating frequency. A genetic correlation between male and female mating frequency could cause females to mate at higher than optimal mating frequencies (see Chapter 6). Another non-adaptive explanation of female multiple mating is that males exert stronger control over mating frequency than females. Therefore females might mate at a higher than optimal frequency because they have little control over mating decisions. Males are predicted to under strong selection to mate as frequently as possible; whereas females are selected to mate at a lower optimal frequency that is determined by a trade-off of the costs and benefits of mating (see Chapter 1).

Males may employ a variety of strategies to control mating frequency (Chapter 1, section 1.4.2), e.g. aggressive strategies such as forced copulation and harassment (Clutton-Brock & Parker 1995). Forced copulation, where a male physically restrains and inseminates a female, is thought to relatively uncommon in insects (Thornhill 1980). In contrast, male harassment of females is common in insects, and can result in selection on females to mate to reduce harassment. This strategy is known as 'convenience polyandry' (Clutton-Brock & Parker 1995; Thornhill & Alcock 1983). For example, in water striders (Heteroptera: Gerridae) mating behaviour involves vigorous pre- and post-mating struggles, which represent a significant cost to females. Females accept superfluous matings to avoid further harassment (Rowe 1992, 1994; Rowe *et al.* 1994; Arnqvist & Rowe 1995; Watson *et al.* 1998).

Another male strategy to control mating is the coercion of females through 'antagonistic seduction' (Holland & Rice 1998). Males may evolve traits to entice females to mate at a frequency beyond the female optimum. This results in mating costs to females, and therefore selection on females to resist the male trait. It has been suggested that this can lead to cyclical antagonistic coevolution between the male trait and female resistance, or 'chase-away sexual selection' (Holland & Rice 1998). Unlike traditional models of sexual

selection (e.g. Fisherian or 'good genes' models) that predict a benefit to female mate choice, the 'chase-away' model predicts that female mate choice will be costly.

Female may control mating decisions through mate choice (Chapter 1, section 1.4.2). Female mate choice can result from resistance to male attempts to solicit copulation (Jennions & Petrie 1997). Females can control the relative mating frequency of males with different phenotypes, if those males differ in their capacity to overcome female resistance. For example, larger males of the dung fly (*Sepsis cynipsea*) and the seaweed fly (*Coelopa ursine*), are both able to more effectively overcome female resistance to mating than are small males (Blanckenhorn *et al.* 2000; Crean & Gilburn 1998). Females appear to be controlling the mating opportunities of males according to male size. Sexual selection therefore occurs as a side effect of female resistance to mating (Crean & Gilburn 1998). Female mate choice and control of mating can also be a result of female preference. Female preference has been demonstrated to provide both direct (Andersson 1994) and indirect, genetic benefits for females (Chapter 1, section 1.3.2).

Female *C. dalmanni* prefer males with large eyespan, and female preference has been shown to confer an indirect benefit (Burkhardt & de la Motte 1988; Wilkinson & Reillo 1994; Hingle *et al.* 2001a,b; Wilkinson *et al.* 1998b). Large relative eyespan signals male susceptibility to larval food stress. Male genotypes that expressed large relative eyespan under plentiful larval food conditions, also expressed large relative eyespan when larval food was scarce (David *et al.* 2000). Large male eyespan also indicates increased suppression of meiotic drive elements, which is beneficial to females in populations with a female-biased sex ratio (Wilkinson *et al.* 1998b).

Despite the potential importance of models of evolutionary change such as the 'chase-away' (Holland & Rice 1998) in determining whether female multiple mating is adaptive, the relative roles of the sexes in determining overall mating frequency have rarely been investigated. This chapter addresses this issue, and tests whether males or females have

strong control over mating frequency in *C. dalmanni*. I produced males with large or small eyespan from lines that had been artificially selected for increased ('high' selection regime) and decreased ('low' selection regime) male mating frequency. The variation in male eyespan phenotype was achieved by a simple manipulation of larval nutrition. I tested for male control over mating frequency by comparing the mating frequency of males that differed in selection regime history but were matched for eyespan phenotype. Female control over mating frequency was tested by comparing the mating frequency of males that were matched for selection regime history but differed in eyespan.

## 7.3 MATERIALS AND METHODS

### 7.3.1 Fly stocks and culture methods.

Females used were from the laboratory stocks described in Chapter 2, section 2.1. Males used were from lines that had been artificially selected for increased and decreased male mating frequency, outlined in Chapter 2, section 2.4. This study used flies taken from generation 10 of artificial selection.

Males with large (>8.6 mm) or small (<6.4 mm) eyespan were generated from both replicates of the 'high' and 'low' selection regimes. Males with large and small eyespans were produced by manipulating larval condition. Larval condition is determined by the amount of resources accrued during the larval stage, and determines adult body size and eyespan (Hingle *et al.* 2001a). Larvae were therefore supplied with either excess food (to generate large eyespan males) or restricted food (to generate small eyespan males). The food quantities used were previously determined in pilot experiments (CAG unpublished data).

Eggs were collected from each selection line every 24 hours. Eggs were collected from population cages (cage specification in Chapter 2, section 2.1) containing 200 flies at a 1:1 sex ratio. Paper on which the females laid eggs was placed on the base of the cage to collect eggs. Batches of 30 eggs were placed on Petri dishes containing damp cotton and supplied with either 0.19g of puréed sweetcorn (to generate small eyespan males) or 1.17g of puréed sweetcorn (to generate large eyespan males). Larvae were allowed to pupate in the Petri dishes and were then placed in population cages in which they eclosed. The flies were sexed as described in Chapter 2, section 2.2. Male eyespan was measured as described in Chapter 2, section 2.3.

### 7.3.2 Experimental design.

30 males with a large eyespan and 30 males with a small eyespan were randomly chosen from each replicate of both selection regimes and placed separately in 'large eyespan' and

'small eyespan' holding cages (20cm x 20cm x 30cm). 10 males with a large eyespan and 10 males with a small eyespan from each replicate of both selection regimes were held as reserves to replace any of the 30 chosen males that died (see Table 7.1 for final sample sizes). The mating frequency of males with large and small eyespan from 2 replicates of each of the selection regimes (2 replicate lines from the high selection regime, and 2 replicate lines from the low selection regime) was assayed (Table 7.1).

On every day of the observations, mating frequency was scored in containers (400ml: height=95mm, diameter=75 mm, with damp cotton and 2 g of food in the base). There were 48 containers, 24 containers with a large eyespan male and 24 containers with a small eyespan male. An equal number of males with large and small eyespan were observed simultaneously from both replicate of each selection regime (i.e. there were 6 containers with a large eyespan male, and 6 containers with a small eyespan male, from each replicate line of both the high and low selection regime). Males were randomly sampled from the holding cages on the day of observation. Mating frequency was scored each day as the number of matings over 40 seconds occurring in an hour and a half, beginning at the start of artificial dawn. After observation on each day, males were removed from the containers and returned to the holding cages. Observations were made over four consecutive days, and this procedure was repeated 4 times (i.e. 4 experimental blocks). Two observers scored mating frequency each day, and each person observed an equal number of mating containers with large and small eyespan males from each replicate of the 2 selection lines.

Uncontrolled variation in mating frequency due to females was minimised in three ways. (i) Females were placed at random in groups of 6 in each mating container, to even out variation due to individual females. (ii) Each group of 6 females was rotated so that within each replicate (i.e. replicate 1 of the high selection regime and replicate 1 of the low selection regime), groups of females were tested with males with both large and small eyespan from the high and low selection regimes. (iii) The order in which groups of

females encountered males with large or small eyespan from the high or low selection regimes over the four observation days of a block was rotated.

### 7.3.3 Statistical analysis.

#### Male eyespan.

It was important to confirm that the absolute values of each category of eyespan phenotype were similar in each selection regime. For the large eyespan phenotype, the values of male eyespan from the two replicate lines of each selection regime were compared using a one-way analysis of variance. Similarly, values for the small eyespan phenotype were compared using a one-way analysis of variance. A further one-way analysis of variance was used to test whether the difference between large and small male eyespan classes, when pooled across replicate lines and selection regimes, was significant.

#### Male mating frequency.

The mating frequency of males with large or small eyespan from each selection line was compared. Mating frequency was defined as the total number of matings over the four observation days in a block. Shapiro-Wilk tests showed that the data were normally distributed. A one-way analysis of variance showed that block did not have a significant effect on the number of matings ($F_{3,764}$=0.51, $P$>0.6). Therefore the data were pooled across blocks for the remainder of the analysis. The data were analysed using a two-way mixed model analysis of variance. Male eyespan was a fixed effect, and replicate line nested with selection regime was a random effect (Table 7.2). The interaction between male eyespan and selection regime was investigated by using a *t*-test to compare the mean number of matings of small eyespan males from the high selection regime with mean number of matings of large eyespan males from the low selection regime.

Statistical analysis was carried out using JMP statistical software (version 5, SAS Institute Inc.) for the Apple Macintosh.

## 7.4 RESULTS

### 7.4.1 Male eyespan.

The absolute values of each male eyespan class (large or small) did not differ between selection regimes. There was no significant difference in the mean eyespan of large eyespan males from the pooled data of the two replicates from each of the high and low selection regimes ($F_{3,106}$=1.03, $P$>0.3). There was also no significant difference in the mean eyespan of small eyespan males from the pooled data across selection lines ($F_{3,116}$=1.04, $P$>0.3). Pooled across both replicates of both selection regimes, the difference between large eyespan males (8.71 ± 0.04 mm, mean ± standard error) and small eyespan males (5.17 ± 0.04 mm) was significant ($F_{1,228}$=3372.52, $P$<0.0001).

### 7.4.2 Male mating frequency.

There was a significant effect of male eyespan on the number of matings ($F_{1,2}$=27.30, $P$<0.05) with female preference for large male eyespan (that is, large eyespan males had significantly more matings than small eyespan males). There was also a significant effect of selection regime on the number of matings ($F_{1,2}$=125.47, $P$<0.01). Males from the high selection regime had significantly more matings than males from the low selection regime. There were no significant differences in mating frequency between replicates of each selection regime ($F_{2,760}$=1.56, $P$>0.2).

There was a significant interaction between eyespan and replicate line nested within selection regime ($F_{2,760}$=7.99, $P$<0.001). However, inspection of the mean number of matings shows that large eyespan males had a consistently higher number of matings than small eyespan males (Table 7.1). The significant interaction between eyespan and replicate line nested within selection line indicated that the magnitude of the difference in the number of matings between large and small eyespan males differed between replicate lines within each selection regime.

The interaction between eyespan and selection regime was not significant ($F_{1,2}$=6.18, $P$>0.1). Further analysis of this interaction showed that there was no significant difference between the number of matings by small eyespan males from the high selection regime and the number obtained by large eyespan males from the low selection regime ($t$=0.2, $df$=382, $P$>0.5).

## 7.5 DISCUSSION

This chapter provides evidence for both male and female control over mating frequency in the stalk-eyed fly, *C. dalmanni*. It has been suggested that total or predominant male control over mating frequency may provide a non-adaptive explanation of female multiple mating. Selection on males to mate frequently is predicted to be stronger than selection on females to limit mating frequency. This is because males can increase their reproductive success with each mating. If male ejaculate production is assumed to be relatively unlimiting, then males will be selected to mate as frequently as possible. Female optimal mating frequency, in contrast, is predicted by a trade-off between the costs and benefits the female receives from each mating. Females are generally assumed to be limited by the production of eggs, and therefore do not necessarily increase their reproductive success with each mating. Female optimal mating frequency is therefore generally predicted to be lower than that of males. If male control over mating frequency was sufficiently greater than female control, females could be forced to mate at a higher than optimal frequency.

Evidence that both males and females exert control over mating frequency in *C. dalmanni* provides evidence against predominant male control as a non-adaptive explanation of female multiple mating. Male control over mating frequency was manipulated by using males artificially selected for increased (high selection regime) and decreased (low selection regime) mating frequency. Males from the high selection regime had significantly more matings than males from the low selection regime, providing evidence for some male control over mating frequency, regardless of female preference.

Female control over mating frequency was manipulated by using males with different eyespans. There is strong experimental evidence in *C.dalmanni* that females prefer to mate with large eyespan males (Burkhardt & de la Motte 1988; Wilkinson & Reillo 1994; Hingle *et al.* 2001a,b). Female preference was measured in a previous study as the difference in the number of matings by males with large and small eyespan (Hingle *et al.* 2001a). The present study used a similar protocol to demonstrate female preference. Large

eyespan males had significantly more matings than small eyespan males regardless of selection regime, providing evidence for some female control over mating frequency.

The degree to which the sexes control mating frequency was further examined by comparing the mating frequency of males that differ in both selection regime and eyespan i.e. small eyespan males from the high selection regime and large eyespan males from the low selection regime. Predominant male control predicts that males selected to mate at a high frequency will have significantly more matings than males selected to mate at low frequencies, even when phenotypically small and discriminated against by females. Conversely, predominant female control predicts that large eyespan males will have significantly more matings than small eyespan males, even when small eyespan males have been selected to mate at a higher frequency. I found no significant difference in the number of matings by small eyespan males from the high selection regime and large eyespan males from the low selection regime. The results of this experiment, with the given parameters of the difference in male eyespan and the difference in male mating frequency between selection regimes, show that there is no evidence for total male control of mating frequency.

There is therefore evidence for simultaneous male and female control over mating frequency. It is important to note however that this experiment used a specific set of parameters to estimate male and female control. It is possible that other outcomes would be observed with other combinations of parameters. For example, the number of generations of artificial selection on male mating frequency will influence the size of the difference in the number of matings between males from the high and low selection regime. Similarly, the size of the difference between large and small male eyespan will influence the strength of female preference, i.e. the difference in the number of matings by males with large and small eyespan. Hingle *et al.* (2001b) found strong female preference for males with a mean eyespan difference of 3.17 mm. The large and small eyespan males

in this study had a mean eyespan difference of 3.53 mm, enabling the clear detection of female preference.

However, the combination of values that I used is biologically realistic. After the experimental manipulation, the average values of both high and low mating frequency and large and small male eyespan, remain within the range of mating frequencies and eyespan seen typically in laboratory and field populations (Burkhardt & de la Motte 1983; Wilkinson *et al.* 1998a). However, the relative importance of male versus female control must be interpreted with caution. In the future it would be interesting to extend these findings. One possibility would be to use a similar design but to use males selected for 'high' and 'low' mating frequency and with a continuous distribution of male eyespan (rather than a pair of discrete phenotypic categories).

Despite caution in assessing the relative importance of male and female control, this experiment provides evidence of male and female control over mating frequency acting simultaneously. Given that female *C. dalmanni* are able to exert at least some control over mating frequency, the hypothesis that male control of mating is causing non-adaptive elevation of female mating frequency is weakened. Male *C. dalmanni* may attempt to control mating frequency through harassing females to mate, leading to 'convenience polyandry' (i.e. females mating multiply to avoid male harassment) (Thornhill & Alcock 1983; Clutton-Brock & Parker 1995). Another male strategy employed by male *C. dalmanni* may be to seduce females, using the sexually selected eyespan trait, to mate at non-adaptively high frequencies (Holland & Rice 1998). Whilst neither hypothesis of male harassment or antagonistic male seduction is tested by this experiment, the demonstration of some female control of mating indicates that even if males pursue these strategies, it does not result in complete male control over mating frequency. The mating frequency of *C. dalmanni* appears therefore to be determined by both males and females. The evidence against predominant male control of mating frequency suggests that multiple mating may be an adaptive female trait.

## 7.5 TABLES

Table 7.1. The sample size (denoted *n*) and mean ± standard error for the total number of mating by large and small eyespan males from each of the high and low lines artificially selected for male mating frequency.

| Selection Regime | Replicate Line | Male Eyespan | *n* | Total number of matings |
|---|---|---|---|---|
| High | 1 | Large | 27 | 6.38 ± 0.36 |
| High | 1 | Small | 30 | 2.13 ± 0.16 |
| High | 2 | Large | 27 | 5.35 ± 0.23 |
| High | 2 | Small | 30 | 2.80 ± 0.29 |
| Low | 1 | Large | 30 | 2.88 ± 0.18 |
| Low | 1 | Small | 30 | 1.44 ± 0.14 |
| Low | 2 | Large | 26 | 2.30 ± 0.16 |
| Low | 2 | Small | 30 | 1.33 ± 0.12 |

Table 7.2. ANOVA of the total number of matings of large and small eyespan males from lines artificially selected for increased and decreased male mating frequency. Analysis was a two-way cross-classification of selection regime and eyespan, with replicate line nested within selection regime.

| Source of variation | *SS* | *df* | *MS* | *F* | *P* |
|---|---|---|---|---|---|
| Selection regime | 912.19 | 1 | 912.20 | 125.47 | <0.01 |
| Replicate line within selection regime[1] | 14.53 | 2 | 7.27 | 1.56 | >0.20 |
| Eyespan | 1019.82 | 1 | 1019.82 | 27.30 | <0.05 |
| Selection regime x eyespan | 230.78 | 1 | 230.78 | 6.18 | >0.10 |
| Eyespan x replicate line within selection regime[2] | 74.70 | 2 | 37.35 | 7.99 | <0.001 |
| Error[3] | 3550.11 | 760 | 4.67 | | |

[1] Error term for $MS^{\text{selection regime}}$

[2] Error term for $MS^{\text{eyespan}}$, $MS^{\textit{selection regime x}\ \text{eyespan}}$

[3] Error term for $MS^{\text{replicate line within selection regime}}$, $MS^{\text{eyespan x replicate line within selection regime}}$

# 8

# General Discussion

## 8.1 SUMMARY OF FINDINGS

Females of many insect species mate multiple times (Ridley 1988; Arnqvist & Nilsson 2000). Female mating frequency may exceed that required to maintain full fertility. In addition, high frequencies of mating are often found to be costly. Frequently mating females of many insect species often suffer reduced longevity and reproductive success in comparison to females mating at lower rates or to virgin females (reviewed in Chapman *et al.* 1998). The widespread occurrence of female multiple mating is therefore one of the great puzzles in evolutionary biology.

In this thesis I investigated some of the possible reasons for female multiple mating. I chose to do this in the stalk eyed fly *C. dalmanni*, which was ideally suited to my research questions because this species is easy to manipulate both phenotypically and genetically, has a high mating frequency and exhibits strong sexual selection. I investigated a number of adaptive, direct benefits of multiple mating. I asked whether females mate multiply to replenish sperm supplies (Chapter 3), or to gain fecundity benefits from the receipt of male accessory gland proteins (Chapter 4). I then tested whether females showed any mating-induced reductions in receptivity, and whether this varied with the eyespan of the mating male (Chapter 5). I also tested whether multiple mating arises for non-adaptive reasons. If there is a genetic correlation for mating frequency between the sexes, the generally stronger selection on males to mate frequently could increase female mating frequency. I tested this in females from lines artificially selected for male mating frequency (Chapter 6). In addition, I examined whether females mate multiply because of a conflict of interests between the sexes over mating frequency, with males having overall control over mating decisions (Chapter 7). In the following sections I summarise briefly the findings of each chapter.

Chapter 3. Fertility benefits of multiple mating: the influence of ejaculate size and mating frequency.

I found that females with large eyespan, and therefore large body size (Wilkinson & Dodson 1997), had more mature eggs in their ovaries than did small eyespan females. Large females may therefore have a greater requirement for sperm than small females. There was no evidence that males could exploit this phenomenon via strategic ejaculate allocation. Males did not allocate spermatophores of increased size (as measured by spermatophore sperm sac area) to large eyespan females. It is possible therefore that the high mating frequency of *C. dalmanni* results in indiscriminate ejaculate transfer. However, large eyespan females had significantly more matings than small eyespan females. This suggests that large females can meet their demand for increased sperm supply by mating at a higher frequency than small eyespan females.

Chapter 4. Male accessory gland proteins increase female fecundity.
Substances other than sperm that are transferred during mating can directly benefit females. I showed that male accessory gland proteins are partly or wholly responsible for the increase in fecundity seen as a result of mating. Females injected with accessory gland protein extract had significantly greater fecundity in comparison to control females injected with saline. The increase in fecundity was similar to that seen as a result of mating. A protein or proteins isolated in one of five fractions separated by HPLC appeared to be responsible for the increase in fecundity.

Chapter 5. No reduction of female sexual receptivity following mating.
Females could benefit from mating-induced reduction in receptivity if it decreased the frequency of further costly male mating attempts. Males will be selected to decrease the sexual receptivity of the female they have mated in order to increase their chances in sperm competition. However, I found no evidence that mating with large or small males decreases female sexual receptivity. Females that had been recently mated did not differ significantly in their receptivity in comparison to unmated females, when receptivity was quantified by the number of times a female rejected mating, the number of matings and the time until the first mating occurred.

Chapter 6. No genetic correlation between male and female mating frequency.
A positive genetic correlation between the sexes for mating frequency could result in females mating at higher than optimal frequencies (Halliday & Arnold 1987). There is evidence that *C. dalmanni* females incur a longevity cost of reproduction (Reguera *et al.* submitted), suggesting that females might be mating at a frequency above their optimum. However, I found no evidence for a genetic correlation in mating frequency between the sexes and therefore no evidence that female multiple mating in *C. dalmanni* is non-adaptive. I conclude that mating frequency in male and female *C. dalmanni* is determined by different, or sex-limited, genes.

Chapter 7. Male and female control of mating frequency.
Another non-adaptive explanation of female multiple mating is that males exert predominant control over mating frequency. Male control of mating could be through forced copulation (Thornhill 1980; Clutton-Brock & Parker 1995), continual harassment (Thornhill & Alcock 1983), or antagonistic seduction (Holland & Rice 1998). However, there was no evidence for total male control of mating, and mating frequency appeared to be under the control of both sexes.

Taken overall, my results provide evidence for adaptive direct benefits of multiple mating in some contexts. I failed to find support for two non-adaptive explanations. I did not make any explicit tests of indirect benefits but I note that there is increasing evidence that "good genes" sexual selection may play an important role in some circumstances (e.g. David *et al.* 2000).

I have added to a body of research that has investigated the reasons for multiple mating in females. My choice of experimental organism however, has allowed me investigate multiple mating in a novel context, i.e. in a system in which the males possess an ornamental sexually selected trait. In addition, I have been able to utilise the experimental

rigour that comes from using a well-developed laboratory experimental system. To understand the generality and significance of explanations for multiple mating, it is important to expand the range of species subject to experimental investigation, and not just to rely upon the findings from model systems such as *Drosophila melanogaster*.

## 8.2 FUTURE DIRECTIONS

### Direct benefits of multiple mating.

A direct benefit of mating multiply identified from the work in this thesis is to maximise egg fertility (Chapter 3; Ridley 1988; Arnqvist & Nilsson 2000). It would therefore be interesting to investigate further why one or a few matings result in low egg fertility. One explanation is that few sperm are transferred at mating, as in *C. whitei*, in which there are only 90 sperm per mating transferred (Lorch *et al.* 1993). In *C. dalmanni* the number of sperm transferred could be counted by dissecting out the female sperm storage organs (spermathecae) and counting the number of sperm inside using a haemocytometer (as in Lorch *et al.* 1993). The rate of sperm storage could also be important for egg fertility, and could be measured by counting the number of sperm reaching the females' sperm storage organs at specific times following mating.

Low egg fertility following mating could also result from ineffective sperm transfer due to variation in spermatophore formation and / or orientation. This could be assessed by measuring spermatophore shape and recording the proportion of singly mated females that have incorrectly aligned spermatophore sperm sacs, i.e. in which the tubular neck does not open into the base of the spermathecae. It is also possible that high numbers of sperm are transferred at each mating, but that egg fertility following single matings is low because there are defects in egg fertilisation. This could be investigated by using anti-sperm-tail antibodies to examine the proportion of recently laid eggs that contain a sperm in the correct orientation (as in Chapman 2001). This would allow an investigation of whether defects occur early, before fertilisation, or whether fertilised eggs fail to successfully complete embryogenesis. If a high proportion of eggs were fertilised, but died as embryos following single matings, it would suggest that genetic incompatibility might be an important explanation of low egg fertility. If this were the case, female multiple mating would then confer additional genetic benefits (see indirect genetic benefits below).

Future work could also further investigate fecundity benefits of multiple mating. The function and structure of the male accessory gland protein(s) responsible for increasing fecundity as a result of mating could be determined (as discussed in Chapter 4). Once the sequence of the fecundity-enhancing protein and the gene that encodes it were known, it would be possible to search for homologous sequences in other species of Diopsids. The well-characterised phylogeny of the Diopsid family (Baker & Wilkinson 2001; Baker *et al.* 2001b) would allow informative comparisons within and between species of stalk-eyed fly. Pairs of closely related species, such as sexually dimorphic *C. dalmanni* and monomorphic *C. quinqueguttata*, could enable accessory gland protein evolution to be investigated in the context of species with and without female mate choice. It would be interesting to test whether there was an association between the rate of evolutionary change in fecundity-enhancing genes and the presence of eyespan dimorphism. Such differences might represent the molecular signatures of sexual selection and / or sexual conflict.

Fecundity benefits of mating in *C. dalmanni* may also result from nutrient transfer. Male *C. dalmanni* transfer sperm in a spermatophore formed internally in the female. Compared to other Diopsid species, the spermatophore is small, implying few nutrients are transferred (Kotrba 1996). It may be however that the high frequency of mating results cumulatively in a significant transfer of nutrients. The increase in fecundity seen after mating reported in Chapter 4 could be a result of nuptial feeding in addition to the effect of accessory gland proteins. Other insect studies have demonstrated, using radiolabelling, that nutrients transferred at mating are incorporated into eggs (Boggs & Gilbert 1979). Similar techniques could be employed in *C. dalmanni* to see if nutrient transfer represents a direct benefit of mating multiply. If significant spermatophore-derived material was found to be incorporated in eggs, it would be interesting to test whether receipt of high numbers of spermatophores can result in increased egg size, which could potentially lead to higher egg viability.

**Indirect genetic benefits of multiple mating.**

The subject of indirect (genetic) benefits, was not investigated experimentally in my thesis but should be examined. Female *C. dalmanni* could obtain genetic benefits as a result of multiple mating through (i) the acquisition of genes that enhance the fitness of offspring; or (ii) the increased genetic diversity of mates reducing genetic incompatibility and raising the genetic diversity of offspring (Yasui 1997).

Female *C. dalmanni* could benefit by mating multiply if they initially mate indiscriminately to safeguard the receipt of sperm, but subsequently remate and exercise female preference in order to obtain fitness-enhancing genes (Jennions & Petrie 2000; Petrie & Kempenaers 1998). It would be possible to test this by exploiting female preference for large male eyespan, which is known to provide genetic benefits to females (Wilkinson *et al.*1998b; David *et al.* 2000). One suitable experimental design would be to restrict initial mating opportunities (e.g. by providing intermittent exposure to males at a level which keeps egg fertility low), and then to allow a period of more abundant mating opportunities. The strength of female preference for male eyespan under each of these conditions could then be tested.

The potential benefit of multiple mating on sperm competitive ability could also be investigated. Mating with more than one male can result in sperm competition between rival male ejaculates (Parker 1970). Multiple mating affords females the opportunity to have their eggs fertilised by sperm with superior competitive ability (Curtsinger 1991). To test whether this is an important direct benefit in *C. dalmanni*, the sperm competitive ability of the offspring of multiply mated and singly mated females could be compared. Molecular markers, such as microsatellites, are being developed to enable the fertilisation success of different males to be determined.

The increased genetic diversity of mates that results from multiple mating may also constitute a direct benefit for female *C. dalmanni*. Increased genetic diversity of mates

may reduce the probability of genetic incompatibility. Genetic incompatibility may be a contributing factor in the low percentage of egg fertilisation seen as a result of single matings (Baker *et al.* 2001a). It would be possible to test for genetic incompatibility by examining the proportion of fertilised eggs that subsequently fail to develop (as described above) in singly and multiply mated females. Molecular markers (e.g. microsatellites) would also be useful to compare whether the offspring of females mated multiply were significantly more genetically diverse than the offspring of singly mated females.

Inbreeding depression could also be important and contribute to low egg fertility. It can also be viewed as a form of genetic incompatibility. The degree of inbreeding in the flies used is thought to be low as large population sizes were always maintained in stock cages. However, it would be possible to estimate the genetic diversity of my study population by genotyping a sample of individuals at several microsatellite loci. Any potential benefits of multiple mating in reducing inbreeding depression could then be revealed in experiments where the reproductive success (e.g. the percentage of eggs hatching) is compared between females mated to two brothers, a brother and an unrelated male, or two unrelated males (as in Tregenza & Wedell 2002).

The evolutionary reasons for female multiple mating may be determined by a complex interaction between the benefits (both direct and genetic) and costs of mating. Future work should also focus on elucidating the nature of reproductive costs in *C. dalmanni* and other Diopsid species. Previous work has identified a cost of reproduction to female stalk-eyed flies (Reguera *et al.* submitted). However, the mechanism has not yet been identified. It would be of interest to determine the relative contribution of pre- and post-mating costs to the total cost of reproduction. Pre-mating costs could result from the deleterious effects of receiving male courtship. Mating costs could result from energetic costs of receiving and maintaining sperm supplies, parasite or pathogen transmission, physical damage or from accessory substances transferred in the seminal fluid. Elucidation of the nature of mating costs would then permit an investigation of whether females can evolve resistance to them.

Ultimately, it is important to consider the importance of the findings of laboratory experiments in a natural context. It would therefore be extremely interesting to conduct fieldwork to establish the relative importance of the costs and benefits of mating in determining overall female mating frequency in wild populations of *C. dalmanni*. The measurement of life-history variables such as fecundity, fertility and longevity requires following individuals throughout their lives and, although ambitious, this could be achieved in a natural setting in field cage experiments. Mating frequency could then be varied by confining females together with different numbers of males. Some traits, such as fecundity, egg fertility, and the strength of female preference could be measured on wild-caught females.

In conclusion it is clear that identifying the relative importance of the costs and benefits of multiple mating is a complex task. In the future it will be important to verify the generality of my findings by examining a diverse array of other species. The Diopsid clade is especially well suited to this task because of the well-characterised phylogeny which will allow rigorous comparative analyses. The potential rewards of this research are significant and will ultimately solve the evolutionary riddle of multiple mating in females.

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