Reproductive quality and mating strategy in stalk-eyed flies

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I, Elisabeth Mary Harley, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

Stalk-eyed flies are characterised by having eyes that project laterally from the head. Male eyespan is known to be subject to sexual selection via female mate preference for exaggerated ornaments. This thesis examines reproductive quality and mating strategy in both sexes, in laboratory and field populations, using two species that are sexually dimorphic for eyespan.

Using the African stalk-eyed fly species *Diasemopsis meigenii* I examined male sperm allocation during single and multiple matings. I demonstrate that during single matings males allocate larger quantities of sperm to highly fecund females. However, large eyespan males do not transfer more sperm than small eyespan males, despite having larger reproductive organs. All males are subject to ejaculate depletion during multiple matings, but small eyespan, and thus unattractive, males suffer the greatest degree of depletion.

I conducted novel field observations of the Malaysian stalk-eyed fly *Teleopsis dalmanni* to examine female sperm limitation and direct benefits of mating. Highly fertile females do not benefit from an additional mating, while less fertile females can dramatically increase their fertility with the sperm from a single mating. Sperm limited females show increased receptivity to additional matings by engaging in a first copulation sooner than recently mated females. Female fecundity was positively correlated with sperm storage, suggesting that the presence of sperm in the reproductive tract acts as a trigger for egg maturation.

Finally, I asked whether male and female eyespan, and the different reproductive strategies believed to be associated with the eyespan trait, resulted in differences to the trade-off between reproduction and soma. Using dietary manipulation I show that both males and females incur a cost of reproduction, as reproductive traits show reduced size under harsh conditions. Using extractable lipid content as a measure of somatic investment, I show that both males and females retain fewer lipids in their soma under harsh dietary conditions. But I find very little evidence for varying reproductive and somatic investment with eyespan.

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General introduction

1.1 Overview

In this introduction I begin by discussing various models of sexual selection, which seek to explain the mechanisms by which female preference for elaborate male sexual ornaments can evolve. Male and female reproductive quality is associated with their underlying condition. Accordingly I discuss the concept of condition within the context of sexual selection, and consider how female preference for male condition can result in male sexual trait exaggeration. I then review male reproductive quality, and in particular the costs and constraints associated with ejaculate production. I introduce the concept of male mating strategy in relation to ejaculate, and how male prudence in ejaculate allocation can result in female sperm-limitation. Finally I introduce stalk-eyed flies (Diopsidae: Diptera) as a model system in which to ask questions pertaining to reproductive quality and mating strategy, and outline the aims and content of the subsequent chapters of this thesis.

1.2 Sexual selection and exaggerated male ornaments

Sexual selection is defined as variation in individual reproductive success arising from competition over mates (Darwin, 1859, Darwin, 1871), which can take the form of scrambles, endurance rivalry, contests, sperm competition and mate preference (Andersson, 1994). These pressures have resulted in a spectacular array of male ornamentation and morphological adaptation. Male trait exaggeration, which would otherwise prove costly under naturally selective conditions, can actually increase fitness under sexual selection if females prefer to mate with elaborately ornamented males. Here I discuss various hypothetical mechanisms by which exaggerated male

traits can evolve in spite of the potential costs to male survival, with specific focus upon those models that make predictions about male ornamentation and reproductive quality.

Preference for a particular phenotypic ornament may evolve when favouring males carrying the particular trait has a direct impact upon female survival or fertility. There are numerous processes through which preference can evolve (reviewed in Kirkpatrick and Ryan, 1991). For example, females may benefit from preference for conspicuous traits because they expend less time and energy in looking for such males. Alternately, male sexual traits may correlate with the size of ejaculate received, the size of a nuptial gift given or the level of parental care provided by a male (Andersson, 1994). In these cases, signalling has to be reliable in order for the preference to persist. The simplest mechanism for maintaining signal reliability in males is physical constraint; males cannot 'fake' an elaborate trait because the ability to produce a complex ornament is directly linked to their underlying condition. For example, plumage colouration in the house finch *Carpodacus mexicanus* is directly associated with nutritional condition, and thus health or foraging ability (Hill and Montgomerie, 1994). The Handicap Principle (Zahavi, 1975) takes this hypothesis one step further by proposing that secondary sexual signals are actually costly to produce, and thus provide reliable information about the quality of the signaller. While the costs of expressing a handicap reduce male viability, the costs are greater for low quality males than for high quality males (Maynard Smith, 1987).

Exaggerated secondary male traits may well evolve *because* they are costly, according to the Handicap Principle, but an alternate hypothesis proposes that these

traits evolve *in spite* of being costly (Fisher, 1930). Fisher (1930) proposed that when genetic variance exists for both female preference and male ornamentation, discerning females will produce more grand-offspring than those that mate at random. This is because the sons of highly ornamented male will enjoy the same reproductive advantages as their fathers. The Fisher process relies upon a genetic correlation between female preference genes and male trait genes. For the male trait to evolve two selective conditions have to be met. Initially there needs to be genetic variation in a male trait that confers increased fitness, for example increased plumage length that increases flight ability. Secondly, females with a preference for this trait will pass the trait onto their sons. As a result the alleles for the male trait become genetically linked to female preference for the trait, resulting in a feedback loop in which the alleles become increasingly frequent. Over time, males exhibiting the preferred trait gain not only increased survival, but increased mating success as the female preference alleles spread through the population. This is called the 'Fisherian Runaway Process'. This process comes to equilibrium when either the male trait becomes detrimental to survival or there is no more additive genetic variance in the male trait (Fisher, 1930).

The Handicap Principle (Zahavi, 1975) is an example of a 'good genes' or 'indicator' model. In these models a heritable genetic benefit to a female's offspring is signalled by the male trait. By selecting mates upon the basis of good genes, females use phenotypic signals that indicate good genetic quality, rather than simply based upon the possibility of 'sexy sons' (Fisher, 1930). By mating with well ornamented males, females can increase their lifetime reproductive success, manifested as superior lifetime fitness returns from sons and/or daughters. Male sexual signals are life-

history traits, and as a result will be subject to the same trade-offs as other life-history traits. Support for good genes models has come from studies showing a positive correlation between male attractiveness and other life history traits (reviewed in Moller and Alatalo, 1999, Kokko et al., 2006). However, even if males invest so much in a sexual trait that it has no correlation with any other life-history trait, this does not contradict the good genes model (Kokko, 2001). The principles of good genes still apply if mating with highly ornamented males results in superior offspring performance.

These models explain the evolution of exaggerated male traits via a basis in signalling reproductive quality, which could be either increased viability and growth, or attractiveness (Greenfield and Rodriguez, 2004). These male attributes are dependent upon underlying condition, and consequently this concept has led to the evolution of condition-dependent expression of male traits.

1.3 Condition and reproductive quality

1.3.1 Theory

The term 'condition' has come to have a wide variety of different meanings in scientific literature. In this thesis condition refers to viability; "all components of fitness other than mating success" (Maynard Smith, 1987, p.12). For good genes models to operate, including the Handicap Principle, condition is required to have two fundamental properties. The first is that higher values of condition must confer higher fitness (Maynard Smith, 1987, Cotton et al., 2004b). Secondly, condition must

have an infinite source of variance that can be environmental, genetic or a combination of the two (Iwasa and Pomiankowski, 1994, Iwasa and Pomiankowski, 1999, Rowe and Houle, 1996, Cotton et al., 2004b).

Traits that are closely related to life-history are expected to exhibit a high level of environmental variance (Price and Schluter, 1991), and condition is no different. Within a species, fitness-associated traits are expected to have an underlying association with a large number of 'metric' traits (Price and Schluter, 1991). The environmental variance of condition arises from the variance of each metric trait in addition to the variance of the fitness trait itself. Consequently, the heritability of fitness-related traits is predicted to be low, an effect that has been demonstrated in the red deer *Cervus elaphus* (Kruuk et al., 2000), the pied flycatcher *Ficedula albicollis* (Merila and Sheldon, 2000) and the great tit *Parus major* (McCleery et al., 2004). These studies have also demonstrated strong positive relationships between the environmental variance of a trait and its association with fitness.

Understanding the maintenance of variance in the genetic components of condition is potentially hindered by the prediction that strong directional selection through female choice will gradually deplete the available genetic variation. As a result, females receive diminishing benefits from their choice; a phenomenon known as the 'lek paradox' (Houle, 1992, Pomiankowski and Moller, 1995, Rowe and Houle, 1996, Tomkins et al., 2004). However, the highly polygenic nature of fitness traits offers a solution to this paradox. The genetic variance of fitness-related life-history traits could be a combination of the genetic variance of the trait itself, and the variance of the numerous morphological and physiological traits upon which the focal trait

depends (Houle, 1992). Additionally, female mate preference for condition-dependent ornaments will increase the number of genes that contribute to the phenotypic variance of the male trait (Pomiankowski and Moller, 1995, Rowe and Houle, 1996), creating a large mutational target (Houle, 1992). Consequently the lek paradox is resolved because the capture of genetic variance due to mutation will balance out the loss of variation due to directional selection.

This theoretical definition of condition infers that condition is closely related to total fitness, and consequently sexual ornament expression will be associated with many different fitness components. These relationships and the associated traits are likely to be different across different species (Cotton et al., 2004b), meaning that there is no universal trait that can be used as a cross-species index of condition. However, this limitation has not prevented the study of condition-dependent traits. For example, a long-term study of wild song sparrows has demonstrated an association between sexual ornament expression and total fitness (Reid et al., 2005). Males with wide song repertoires (the sexually selected trait) had higher lifetime fitness, measured as number of offspring and grand offspring. Studies like these help to elucidate some of the precise components that make up condition. Further studies have determined that condition can be manipulated experimentally through varying environmental (reviewed in Cotton et al., 2004b), and genetic stress (e.g. van Oosterhout et al., 2003, see also section 1.5.2).

1.3.2 Condition-dependent sexual selection and reproductive quality

The primary focus of this thesis is not upon condition *per se*, but upon the association between condition, reproductive quality and secondary sexual traits. Condition determines the ability of a male to convert energetic resources into sexual signals. As a result, honest sexual signals are described as condition-dependent. Iwasa and Pomiankowski (1994, 1999) developed a quantitative genetic framework in which to examine condition-dependent signalling. Both models consider the effect of female preference upon the evolution of a costly male signal trait, which is linked to male quality. Variation in male quality can be heritable (Iwasa and Pomiankowski, 1994) or environmentally determined (Iwasa and Pomiankowski, 1999), resulting in either indirect (by producing high quality offspring) or direct (by obtaining material benefits) benefits of mating with high quality males. Females cannot directly assess male quality, but male ornament size is determined by a condition-dependent component that is positively correlated with male quality. As a result, females can indirectly infer male quality from male ornament size. However, male assessment is assumed to carry more significant survival costs for females than random mating, as seeking a desirable partner requires a greater energetic investment. At an equilibrium point, where female preference remains constant with change in time, both models predict similar outcomes, namely that the cost of male assessment is balanced by the benefits gained of increased offspring viability. Offspring viability is increased through transmission of 'viability' genes when male quality is heritable (Iwasa and Pomiankowski, 1994), and when male quality is environmentally determined (Iwasa and Pomiankowski, 1999) the receipt of material benefits by the female increases the available resources to invest in offspring.

Direct benefits of mate choice come in many forms. Females can gain energetic benefits in the form of nuptial gifts, or benefit from higher fertility as high quality males may transfer larger ejaculates containing more sperm. For the latter to be true, male fertility has to associate with male condition, and there is a large amount of experimental evidence to suggest that this is the case. Male fertility fulfils the two requirements of condition described earlier, namely a positive association with fitness and many sources of variance. First, as an important component of reproductive success, higher values of male fertility will be associated with higher fitness. Second, male fertility shows high levels of both environmental and genetic variance. Male reproductive quality is highly sensitive to environmental manipulation in a wide range of species (e.g. yellow dung fly *Scatophaga stercoraria*, Ward and Simmons, 1991; zebra finch *Taeniopygia guttata*, Birkhead et al., 1998). Additionally, male fertility is highly susceptible to genetic stress. In *Drosophila melanogaster* mutations that cause male sterility are 10-20% as frequent as lethal mutations (Wakimoto et al., 2004), indicating that male fertility is a large mutational target. Indeed, the minimum number of genes essential to male fertility (those in which mutations affect fertility but do not cause outright sterility) is estimated to be around 500 (Wakimoto et al., 2004).

1.4 Male ejaculate limitation and female fertility

Through his classic experiment with fruit flies, Bateman (1948) demonstrated a fundamental difference between males and females: male reproductive success is limited by access to partners, whereas female success is constrained by offspring

production. Consequently, males have traditionally been considered capable of producing limitless quantities of small, cheap gamete; their sperm. Dawkins wrote in *The Selfish Gene* that "excess has no meaning for a male" (Dawkins, 1976, p.164). Sperm competition, a widespread post-copulatory phenomenon that occurs when the sperm of two or more males compete for fertilisation, also selects for increased sperm number (Parker, 1970, Parker, 1982, Birkhead and Pizzari, 2002). Ejaculates that contain large numbers of sperm are more competitive than those that do not. However, recent evidence shows that spermatogenesis has finite energetic limits (e.g. Nakatusuru and Kramer, 1982), and that males have evolved mechanisms for allocating their reserves strategically in order to maximise reproductive returns (modelled in Tazzyman et al., 2009). Here I discuss the evidence for costly ejaculate production and subsequent male allocation strategies. In addition I consider how females respond to prudent males.

Males are not capable of producing limitless quantities of sperm. Trivers (1972, p. 167-168) argued that while males are selected to mate in rapid succession, their ability to do so is likely to be limited. In addition, Dewsbury (1982) noted that although individual sperm are not costly to produce, they are not transferred individually but as part of an ejaculate that can contain millions of sperm. Producing so many sperm, coupled with the need for large quantities of accessory proteins could generate non-trivial energetic costs for males. Indeed, experimental evidence suggests that males cannot produce unlimited quantities of sperm. Nakatusuru and Kramer (1982) demonstrated that male lemon tetras could only produce four times as many offspring as females, even when access to females was unlimited. The direct energetic costs of sperm production are poorly understood. Thus far evidence

of costly sperm production has come from studies that associate sperm production with aspects of fitness or environment. For example, sex has been shown to dramatically reduce lifespan in male *Caenorhabditis elegans*, as a result of increased sperm production rather than the physical activity involved in copulating (Vanvoorhies, 1992). Sperm production is constrained by reduced diet quality in Indian meal moths *Plodia interpunctella* (Gage and Cook, 1994). Furthermore male adders, *Vipera berus,* lose significant body mass during the spermatogenesis stage of their mating cycle (Olsson et al., 1997). Clearly, creating large numbers of sperm requires a heavy resource investment. Furthermore, there is now a body of evidence in the insects demonstrating that males face a significant functional trade-off between sperm characteristics and immune function (Drnevich et al., 2002, Gershman et al., 2010, Kerr et al., 2010, Dowling and Simmons, 2012). Even if sperm production does not carry significant costs for a species, male ejaculate production may well be constrained by the need to produce large quantities of ejaculate proteins (Moore et al., 2004). Severe ejaculate limitation can limit fertility in both males and females, and result in selection for adaptations in both sexes that will maximise reproductive success.

Males from numerous species have evolved mechanisms that allow partitioning of sperm over a series of matings, suggesting possible prudence in ejaculate allocation. In many taxa, males store mature sperm in specialised regions of the reproductive tract that are adjacent to but distinct from the testes, e.g. the epididymis and vas deferentia in mammals, and the seminal vesicle in insects. When access to individual matings is high, selection will favour those males that can control their release of mature sperm, rather than releasing all their sperm at once. For example,

males of the blue head wrasse *Thalassoma bifasciatum* regulate their sperm release across successive spawnings relative to female fecundity (Rasotto and Shapiro, 1998). To facilitate such controlled ejaculate allocation, the male sperm duct is divided into numerous small chambers, the openings of which are regulated by a thin band of muscle tissue (Rasotto and Shapiro, 1998). Muscle contractions of the vas deferens control the mobilisation of sperm in rodents. A comparison of promiscuous and monogamous *Peromyscus* rodent species showed that the vas deferentia of promiscuous species have evolved high sensitivity to endogenous opioid hormones that regulate muscle action (Pound, 1999). Treatment with opium, an exogenous opioid agonist, resulted in inhibited contraction of the vas deferens. The secretion of endogenous opioid agonists depends upon social conflict cues that may well associate with the risk of sperm competition (Pound, 1999).

Ejaculate allocation is predicted to be governed by the trade-off between current and future reproduction (Parker, 1982). Budgeting energy into ejaculate production is beneficial for males if there are plenty of mating opportunities. However, this strategy reduces the energy available for looking for new females. Allocation of energy to this trade-off depends upon numerous factors. For example, if the population sex ratio is male-biased then males are predicted to budget more energy for current reproduction. This trend is reversed in female-biased populations, where selection will favour those males who allocate their ejaculates prudently. In *Drosophila pachea* males take longer on average to reach sexual maturity than females, resulting in a female-biased operational sex ratio. Consequently males partition their limited sperm carefully amongst available partners (Pitnick, 1993). Male ability to secure sexual partners will also influence the allocation of energy to ejaculate production. In

species where females select males on the basis of sexual ornamentation, attractive males are selected to be more conservative with their ejaculate allocation than unattractive males (Tazzyman et al., 2009). I discuss this phenomenon in more detail in chapters 2 and 3.

When there is ejaculate limitation, males are predicted to bias their investment towards those females that will provide the greatest fertilisation returns, and attempt to avoid engaging in costly sperm competition. There is evidence that males are capable of recognising socio-sexual cues that indicate changes to the level of sperm competition in a population, and employing ejaculate modulation as part of their avoidance strategy. Cook and Wedell (1996) showed that male small white butterflies *Pieris rapae* responded to the increased chance of sperm competition during their second mating (where the probability of encountering a virgin female is low) by transferring more sperm to the second female. Male *Tenebrio molitor* beetles allocate more sperm per ejaculate in the presence of the rival male than when unaccompanied (Gage and Baker, 1991). An alternate strategy, as opposed to varying sperm number, is for males to preferentially bias their ejaculate allocation to preferred females. Red flour beetle (Arnaud and Haubruge, 1999) and great snipe (Saether et al., 2001) males both prefer to mate with virgin females rather than mated females. Similarly, male spiny orb weaver spiders (Bukowski and Christenson, 1997) will discriminate between mated and unmated females, as first-male sperm precedence is common in this species. Males may also bias their ejaculate allocation to larger, more fecund females who will provide the greatest fertilisation returns. Kelly and Jennions (2011) demonstrated strong evidence across

multiple species that males preferentially allocate larger ejaculates to higher quality females.

Male sperm limitation and prudent allocation of sperm by males can translate into female sperm limitation. Females suffer fertility impairment as a result of mating with recently mated males (Royer and McNeil, 1993, Svensson et al., 1998), which in the case of the snow crab *Chionoecetes opilio* is caused by males using only 2.5% of their sperm reserves for insemination (Rondeau and Sainte-Marie, 2001). While dominant males often are the most frequently preferred partners, females also suffer reduced fertility as a result of mating with these males (Warner et al., 1995, Jones, 2001, Preston et al., 2001). Females may well trade-off the genetic benefits of mating with high-ranking males against direct fertility benefits. Tazzyman et al (2012) predict that female choice for male ornamentation of dominance may become more extreme in cases where females gain greater genetic benefits than fertility benefits.

Females have always been assumed to be the choosy sex, but these studies demonstrate the presence of substantial selection for male choice via the cost of ejaculate production. In this thesis I address various questions that arise as a result of male ejaculate limitation, using a recently established model species: the stalk-eyed fly.

1.5 Stalk-eyed flies

1.5.1 Introduction

Over the last 30 years stalk-eyed flies (Diptera: Diopsidae) have become an established model species for studying sexual selection (Wilkinson et al., 1998, Chapman et al., 2005). Here I review our existing knowledge of various species of stalk-eyed fly, in the context of the questions posed in this thesis about reproductive quality and mating strategy questions.

Approximately 150 extant species of Diopsids have thus far been characterised (Wilkinson and Dodson, 1997), distributed primarily across South East Asia and Africa. Species have also been discovered in North America (Wilkinson and Dodson, 1997) and Europe (Papp et al., 1997), and a prehistoric genus called *Prosphyracephala* has been recovered from fossilised amber dating from 22 million years ago (Schumann, 1994). All Diopsids are characterised by hypercephaly; the eyes and antennae of both sexes are located on elongated lateral projections from the head capsule (Shillito, 1940). Eyespan, defined as the distance between the outermost tips of the eyebulbs, is highly variable across the species and in some can even exceed body length (Baker and Wilkinson, 2001). Sexual dimorphism for eyespan is believed to have evolved independently at least four times (Baker and Wilkinson, 2001), although monomorphic eyespan is plesiomorphic within the family (Wilkinson and Dodson, 1997, Baker et al., 2001, Baker and Wilkinson, 2001). For example, both extant (e.g. *S. beccari*, Cotton et al. 2004b) and extinct (e.g. *Prosphyracephala*, Schuman, 1994) species exhibit monomorphism.

In stalk-eyed flies the evolution of sexual dimorphism is associated with increased male eyespan relative to that of females (Baker and Wilkinson, 2001, De la Motte and Burkhardt, 1983). The origin of hypercephaly is thought to be the result of

natural selection for increased visual capacity. The number of ommatidia (optical components of the insect compound eye) present increases with eyespan (Burkhardt and De la Motte, 1983), such that having longer eyestalks can improve near field vision by 400 to 800 times (Burkhardt and De la Motte, 1987). Increased numbers of ommatidia also increase the field of binocular vision (135°, *Teleopsis whitei*, Burkhardt and De la Motte, 1983), and reduces the distance between ommatidia resulting in increased visual resolution (1.3° divergence angle between ommatidia, *T. whitei*, Burkhardt and De la Motte, 1983). Despite the benefits of having a large eyespan, the changes in the nervous system required to support the trait are likely to result in reduced ability to rapidly resolve visual signals (Buschbeck and Hoy, 1998). As a result, while stalk-eyed flies may have visual acuity similar to that of much larger insects, e.g. the dragonfly, their ability to move at speed will be constrained by their inability to resolve visual images quickly (Buschbeck and Hoy, 1998). Reduced aerial performance is a feature of stalk-eyed flies (Swallow et al., 2000, Ribak and Swallow, 2010). Males carrying large eyespans suffer from increased risk of predation (Worthington and Swallow, 2011), and having a large eyespan may well increase the likelihood of damage.

1.5.2 Condition dependence of male eyespan

Condition has a large environmental component, and traits closely related to fitness are thought to be more susceptible to environmental variation than those that are not. As a result, change in environment should result in a change in condition. In stalk-eyed flies, male eyespan shows heightened condition-dependence relative to other non-sexual traits in both sexually dimorphic (David et al., 1998, Cotton et al.,

2004a) and monomorphic species (Cotton et al., 2004c). In response to environmental manipulation (changing the quality and quantity of larval diet) the eyespan of sexually dimorphic adult male *Teleopsis dalmanni* decreased more in response to stress than did female eyespan, male wing length or female wing length (David et al., 1998, Cotton et al., 2004a). Male eyespan is similarly sensitive to larval environmental stress in sexually monomorphic species where the eyespan trait shows minimal exaggeration (Cotton et al., 2004c), suggesting that condition-dependence of eyespan is ancestral. These findings are not simply the result of allometric scaling with body size. A study using the African species *Diasemopsis aethiopica* has further demonstrated that males reared under low dietary stress invest their additional resources into increased eyespan rather than body size (Knell et al., 1999). Female larvae raised on high quality diets had larger adult eyespans and body lengths than their low quality counterparts. In contrast, low stress males had larger eyespans but similar body lengths compared to high stress males.

In addition to environmental variance, further studies have shown that male eyespan exhibits a high level of genetic variance in response to dietary stress. David et al. (2000) used full- and half-sib families of the South East Asian species *T. dalmanni* to demonstrate a strong genotype-by-environment interaction upon the development of male eyespan. Certain genotypes proved less stress-susceptible than others, producing large absolute male eyespans consistently across both high and low stress larval environments. Contrastingly, other genotypes produced smaller absolute eyespan with increasing stress. Furthermore, the male genotypes that produced the largest eyespans under high stress also produced the largest eyespans under low stress. Non-ornamental traits including female eyespan, male

wing length and female wing length showed genetic variation in condition-dependent expression, but this genetic response can be accounted for by body size scaling. This study (David et al., 2000) attempted to account for the effects of allometric scaling by dividing the trait value by thorax length. This ratio method is flawed, as it does not remove the effect of body size unless the trait allometries pass directly through the origin (Packard and Boardman, 1999). To overcome the deficiencies of this approach, Cotton (2004b) included thorax length as a covariate in their analyses, and confirmed the findings of the earlier study (David et al., 2000). In inbred lines of *T. dalmanni* (Cotton et al., 2004b) male relative eyespan showed far higher sensitivity to environmental stress than female relative eyespan. Both studies demonstrate that the genetic variation of male eyespan increases when subject to dietary stress.

Recent studies have examined the condition-dependent nature of male eyespan through its responses to genetic stress. Prokop et al. (2010) and Bellamy et al. (in review) induced genetic stress in *T. dalmanni* and *Diasemopsis meigenii* respectively through inbreeding. The Handicap Principle predicts that sexual traits will be more susceptible to inbreeding than non-sexual traits. After one generation of inbreeding, *T. dalmanni* showed significantly higher inbreeding depression in male eyespan than the homologous trait in females. However, these effects could be entirely explained by an associated decrease in body size, suggesting that inbreeding depression upon eyespan is low. By contrast, the *D. meigenii* study applied greater genetic stress through eleven generations of inbreeding, resulting in a significant decrease in male eyespan relative to other non-sexual traits (including female eyespan) that could not be explained by changes in body size. By crossing the inbred lines to generate

outbred offspring, Bellamy et al (in review) showed distinct heterosis in male eyespan relative to non-sexual traits and female homologs. This provides evidence of heightened genetic condition-dependence of male eyespan.

Finally, consistent with the assumptions of condition-dependent sexual selection, male eyespan is characterised by high levels of additive genetic variance. In both *T. dalmanni* and *Teleopsis whitei* the additive genetic variance in male eyespan is over twenty times greater compared to the variance of a non-sexual trait (thorax width) (Meier and Baker, 2002). Additionally, the additive genetic variance observed in a monomorphic congener *Teleopsis quinqueguttata* was three times less than that of its sexually dimorphic relatives (Meier and Baker, 2002). When compared to female eyespan, the eyespan of male *T. dalmanni* and *T. whitei* had treble the additive genetic variance (Wilkinson and Taper, 1999). While the genes that contribute to the genetic variance in eyespan have not been fully identified, there is evidence to suggest that the X chromosome has significant influence upon male relative eyespan. In *T. dalmanni* artificial selection for large and small male eyespan ratios resulted in strong bidirectional trait changes (Wilkinson, 1993). The X chromosome has subsequently been shown to account for 25% of the difference between the selected lines (Wolfenbarger and Wilkinson, 2001, Johns et al., 2005).

All these studies demonstrate that hypercephaly is a complex polygenic trait with a strong association to male condition. This link may have arisen prior to the evolution of female preference for hypercephaly, demonstrated by the presence of heightened condition-dependence of eyespan relative to wing length in the monomorphic species *S. beccari* (Cotton et al., 2004c). This observation suggests that female

preference for exaggerated male eyespan may have evolved so frequently because hypercephaly directly reflects male quality.

1.5.3 Female preference for male eyespan

An initial selective advantage associated with hypercephaly would provide a basis for female choice, leading to the eventual exaggeration of male eyespan beyond that which is naturally selected for. Thus far the majority of the experimental work in stalk-eyed flies has used the Asian species *T. dalmanni* and *T. whitei*. In the wild these flies form nocturnal aggregations on exposed root hairs overhanging rainforest streams (Burkhardt and De la Motte, 1985, Wilkinson and Reillo, 1994, Wilkinson and Dodson, 1997). Males arrive shortly before dusk and compete to control the best sites; these contests are frequently won by the male with the largest eyespan (Panhuis and Wilkinson, 1999, Hingle et al., 2001a, Hingle et al., 2001b, Small et al., 2009). Females arrive shortly afterwards, and will preferentially roost and mate with the largest eyespan males (Wilkinson and Dodson, 1997, Wilkinson and Reillo, 1994, Hingle et al., 2001b, Cotton et al., 2010). More than 90% of copulations occur at dawn, and individual males have been reported to mate up to 40 times per day (Burkhardt et al., 1994). Mixed-sex aggregations are highly variable in size; the largest can contain 1-4 males and up to 24 females (Lorch et al., 1993). Males will typically mates with all the females on their lek, so the number of females present is a good indicator of male reproductive success (Lorch et al., 1993, Burkhardt et al., 1994).

Laboratory studies have extensively demonstrated female preference for large eyespan males (Wilkinson et al., 1998, Wilkinson and Reillo, 1994, Hingle et al., 2001b, Cotton et al., 2006). The majority of early studies have used a choice-based experimental design, where females are given two different male options to choose between. Female *T. whitei* presented with large and small 'dummy' males (with artificially lengthened eyestalks) chose to roost with the larger males (Burkhardt and De la Motte, 1988). Wilkinson and Reillo (1994) demonstrated a genetic association between female choice and male eyespan by subjecting flies to thirteen generations of selection for large or small eyespan to body length ratios. When presented with a choice between either a large or small male, females from the large lines and a non-selected control population chose to roost with large eyespan males. Contrastingly, small line females chose to roost with small males. Selection was restricted to male eyespan by matching males for their body size, allowing female choice to be attributed to a genetic association with male eyespan (Wilkinson and Reillo, 1994).

Further simultaneous choice tests have demonstrated that female preference for male eyespan results in an increased frequency of mating with preferred males. In sexually dimorphic species (*T. dalmanni* and *T. whitei*) large eyespan males obtain a higher proportion of copulations than small eyespan males (Wilkinson et al., 1998, Hingle et al., 2001b). This trend was not observed in the related monomorphic species *T. quingueguttata* (Wilkinson et al., 1998). However, female ability to accurately discriminate between similarly sized males is limited (Hingle et al., 2001b). Based on the proportion of copulations received by large and small eyespan males, *T. dalmanni* females were able to distinguish between males when the difference in male eyespan was large (mean difference = 3.17mm), but not when the

difference was intermediate (2.40mm) or small (1.45mm). In the intermediate difference group, large females showed stronger preference for male eyespan than small females, suggesting that female preference varies with female condition. Further studies confirmed this hypothesis by showing that adult females raised on a high quality diet (sweetcorn) mated more frequently with large eyespan males than small (Hingle et al., 2001a). This pattern was shown to be reversible: when female diet was switched from high quality to low (sweetcorn to sucrose) females switched from strong preference for male eyespan to random mating irrespective of male eyespan.

T. dalmanni do not exhibit any obvious rejection response, and as a result discerning male effects and female effects upon copulation number can be difficult. For example, male *T. dalmanni* may be able to force copulations. More recent laboratory investigations of female preference have focussed upon the South African stalk-eyed fly *D. meigenii*. While little is known about the natural history of this species, females exhibit an unambiguous rejection response in the presence of unwanted males characterised by vigorous abdominal shaking and ovipositor extrusion (Cotton et al., 2006, Small, 2009). In contrast to the earlier studies, which are complicated by a lack of obvious female acceptance or rejection, studies using this species are able to quantify individual female preference independent from male phenotypic effects or an artificial choice situation. New studies have adopted a different method in which females are sequentially presented with a range of male phenotypes (as opposed to two: large and small) and scoring the acceptance or rejection response to each mating opportunity (Cotton et al., 2006, Small, 2009).

Investigations with *D. meigenii* have added support to the hypothesis that female preference is condition-dependent. Cotton et al (2006) showed that female eyespan, a trait that is partially dependent upon larval stress (Cotton et al., 2004a), is correlated with strength of preference. Large eyespan females tended to mate more frequently with large eyespan males, and would reject the mating attempts of small eyespan males. Rejection rate was random for small eyespan females. As in *T. dalmanni*, female preference in *D. meigenii* shows evidence of being condition-dependent (Small, 2009). Females maintained on a healthy, sweetcorn diet mated more frequently with large eyespan males and were more likely to reject small males than females reared on a low quality, sucrose, diet. These investigations in both *T. dalmanni* and *D. meigenii* demonstrate that the strength of sexual selection on male eyespan is dependent upon the condition of females.

1.5.4 Sperm limitation in stalk-eyed flies

As discussed earlier, male sperm prudence or limitation can result in female fertility impairment, and this is likely to be a key factor in determining female preference in stalk-eyed flies. In field studies of *T. dalmanni* only 55% of eggs were fertilised (Cotton et al., 2010), and laboratory studies have shown that females need to mate multiply in order to maintain their fertility in both *T. dalmanni* (Baker et al., 2001) and *D. meigenii* (Small, 2009). When isolated from mating opportunities, female fertility declines significantly in *T. dalmanni* (Cotton et al., 2010) and *D. meigenii* (Small, 2009) as females use up their limited supplies of stored sperm.

In *T. dalmanni*, female sperm-limitation could be due to the small size of spermatophore transferred (Kotrba, 1996), and the number of sperm stored in the spermathecae of females following a single mating is very small (~35, Wilkinson et al., 2005; ~142, Rogers et al., 2006). Female *T. whitei* store ~34 sperm following a single mating, and produce 20 offspring on average (Fry and Wilkinson, 2004). These observations suggest that sperm numbers per ejaculate are small in stalk-eyed flies, possibly due to prudent male ejaculate allocation, as described earlier. Stalk-eyed fly ejaculates appear to be orders of magnitude smaller than those of other Diptera. For example, *D. melanogaster* females store up to 1000 sperm per insemination and use 400 to fertilise eggs (Qazi et al., 2003), and *Drosophila pseudoobscura* males can pass up to 25,000 sperm in a single ejaculate (Snook et al., 1994). Coupled with low sperm numbers, it appears that 25% of copulations in *T. dalmanni* do not result in successful sperm transfer (Baker et al., 2001). In wild populations the issue of sperm-limitation is further complicated by the presence of an X-linked meiotic drive element, carried by up to 25% of *T. dalmanni* males (Presgraves et al., 1997). The 'drive' allele stunts the elongation of Y-carrying sperm thus impairing their ability to fertilise (Wilkinson and Sanchez, 2001, Wilkinson et al., 2006, Johns and Wilkinson, 2007). Consequently females mated to meiotic drive-carrying males suffer from decreased fertility (Wilkinson and Fry, 2001, Wilkinson and Sanchez, 2001, Wilkinson et al., 2006).

There is evidence to suggest that male reproductive quality is associated with male reproductive organ size, which in turn is determined by male condition (Rogers et al., 2008). The size of male accessory glands in *T. dalmanni* is genetically (Rogers et al., 2005a) and phenotypically (Baker et al., 2003, Rogers et al., 2005b) associated with

male mating frequency. Following mating, females will store more sperm from males with large testes than those with small (Fry, 2006) suggesting that testis size has a direct influence upon female fertility. As male eyespan is a key predictor of male reproductive organ length (Rogers et al., 2008), sperm-limited females may well select large eyespan males based upon their fertilisation potential. In support of this hypothesis is the finding that female *T. dalmanni* housed with large eyespan males had higher fertility than those housed with small eyespan males (Rogers et al., 2008). Interestingly there is currently little evidence of sperm precedence in stalk-eyed flies. Given female multiple mating for fertility assurance, males may well be expected to invest heavily in sperm competition. There is some evidence to suggest that when female *T. whitei* mate in rapid succession (20 minutes) the spermatophore of the first male acts as a mating plug to prevent the uptake of sperm from subsequent males (Lorch et al., 1993). However, studies in *T. dalmanni* (Corley et al., 2006) and *D. meigenii* (Bellamy, 2012) have failed to find any evidence of consistent first male sperm precedence.

1.6 Thesis structure

The previously unanticipated (Bateman, 1948) costs associated with both gamete and ejaculate production in males form the basis for the questions asked in this thesis. There are three categories of experimental chapters, grouped by my choice of model system and methodological approach to address questions about male ejaculate allocation strategy, female promiscuity, and trade-offs associated with the cost of reproduction. In the first two chapters I examine male ejaculate allocation strategy during single and multiple matings using the African stalk-eyed fly *D.*

meigenii. For chapters 4 and 5 I use wild populations of the South-East Asian stalk-eyed fly *T. dalmanni* to ask questions about multiple mating and female sperm storage. Finally in chapter 6 I use laboratory-reared *T. dalmanni* to ask whether variation in reproductive strategy in both males and females is reflected in the investment of adult-derived nutrients into reproduction and somatic renewal.

Chapter 2

I report the results of an empirical test of male ejaculate allocation during single matings using *D. meigenii*. Female size is a significant predictor of fecundity in these species, and males accordingly mated for longer with and allocated more sperm to large females. However, while male eyespan was a strong predictor of reproductive organ length, I found no variation in ejaculate allocation relative to eyespan. This is in contrast to the predictions of the phenotype-linked fertility hypothesis, which implies that large eyespan males should transfer more sperm during mating, resulting in increased female fertility. This finding does provide partial support for a recent model of sperm competition, which predicts that male ejaculate allocation is negatively correlated with attractiveness. I consider how female mate preference for large male eyespan can continue to be adaptive despite the lack of obvious direct benefits during a single mating. All the experiments reported in this chapter were conducted by me, with the exception of the male testes and accessory gland length data, which were collected by Jen Small, and the mathematical model of ejaculate allocation, which was written by Sam Tazzyman.

This chapter has been accepted for publication in *Ecology and Evolution.*

Chapter 3

Stalk-eyed flies are highly promiscuous, and as a result differences in ejaculate strategy between different eyespan males may only become apparent during multiple matings. Furthermore, in a recent model of sperm allocation strategy during multiple matings, Tazzyman predicts that attractive males will become depleted at a much slower rate than unattractive males, as a result of differential investment of resources in future versus current matings (model presented in Appendix 3). In order to test these theoretical predictions I examine the ejaculate allocation of large and small eyespan males over the course of three sequential matings, using *D. meigenii*. My findings support Tazzyman's theoretical predictions: I found that both large and small eyespan males show different ejaculate depletion patterns during multiple matings. Both sizes of male show evidence of ejaculate depletion, measured as reduced spermatophore size and sperm contents, between matings one and three. For small eyespan males the rate of ejaculate depletion is much more rapid than that of large males. These findings have interesting consequences for our current understanding of sperm competition outcomes in this species, as unattractive males may well be able to bias fertilisation success away from attractive males.

Chapter 4

Multiple mating by female insects is widespread, and numerous explanations for the phenomenon have been proposed. One benefit that has attracted significant attention is that of fertility assurance: females have been observed to need to mate multiply in order to attain high fertility. Here I conduct the first test of the fertility

assurance hypothesis in a wild insect population, using *T. dalmanni*. I asked whether receiving an additional mating alleviates sperm limitation in wild females. In our experiment one group of females received a single additional mating, while a control group received an interrupted, and therefore unsuccessful, mating. Females that received an additional mating did not lay more fertilised eggs in total, nor did they lay proportionately more fertilised eggs. Female fertility declined significantly through time, demonstrating that females were sperm limited. However, receipt of an additional mating did not significantly alter the rate of this decline. My data suggest that the fertility consequences of a single additional mating were small. I discuss this effect (or lack thereof), and suggest that it is likely to be attributed to small ejaculate size, a high proportion of failed copulations, and the presence of X-linked meiotic drive in this species.

The material in this chapter has been published: Harley, E., Fowler, K. & Cotton, S. 2010. No detectable fertility benefit from a single additional mating in wild stalk-eyed flies. *PLoS* ONE, 5, 12.

Chapter 5

Highly fertile females whose sperm storage organs are relatively full may well experience smaller mating-related fertility benefits than sperm-limited females whose storage organs are close to empty. I extend the design from chapter 4 to examine this hypothesis, by isolating wild *T. dalmanni* females from males to cause sperm depletion before allowing a single additional mating. I also examine a corollary of this hypothesis: namely, that female sexual receptivity will be negatively correlated with the number of sperm stored. My investigation used a laboratory population of *T.*

dalmanni because assays of wild flies proved unsuccessful. The magnitude of fertility increase after a single additional mating was negatively correlated with the degree of fertility before mating indicating that increased sperm storage does reduce the direct benefit of an additional mating. However, I found mixed evidence for a change in sexual receptivity with sperm-limitation. Sperm-limited females mated sooner than recently mated females, but there was no difference in the duration or number of matings during a one hour period. Female re-mating rate may be determined by factors other than quantity of sperm stored. I consider these findings in the context of the possible functions of the different elements of the sperm storage system in *T. dalmanni*. I also found evidence that male invest more sperm in matings with large eyespan females. Following the single additional mating, female fertility was significantly correlated with eyespan, a possible phenotypic indicator of fecundity. Direct assessment of the number of sperm stored in the ventral receptacle of wild female *T. dalmanni* showed a positive association between female fecundity and number of sperm stored.

Chapter 6

My results from chapters 2 and 3, combined with recent mathematical models (Tazzyman et al., 2009, Appendix 3) show evidence of variation in male ejaculate allocation strategy relative to attractiveness. In this chapter I place this variation in mating strategy within the context of a trade-off between reproduction and soma, by asking whether variation in male reproductive strategy is reflected in male investment of adult-derived nutrients (dietary lipids). Male eyespan, as the determinant of sexual attractiveness, may mediate the trade-off between

reproduction and soma. Large eyespan males constrain their somatic investment in favour of increased current reproduction, while small eyespan males favour investment in soma. As males also show evidence of tailoring their ejaculate investment relative to female size in *T. dalmanni*, I also examine how female size determines the outcome of reproductive trade-offs. As with the males, large females may invest more in current reproduction, while small females invest in the future. While there was evidence to suggest that large eyespan males retain fewer lipids within their soma than small eyespan males, this relationship was not affected by diet treatment, and I found no evidence of increased reproductive investment in the form of accessory gland and testes lengths. This suggests that investment patterns in these reproductive traits are fixed during the larval stage. Investment in sperm number, quality and ejaculate protein production may be better indicators of the cost of reproduction than organ length in this species. Starved females produced zero eggs, while well-fed females were highly fecund. I found no evidence of a female size-mediated trade-off between reproduction and soma: under starvation conditions large females show no higher fecundity than small females. Female reproductive investment appears to be determined by energy thresholds, below which it is either impossible or unviable to invest resources into egg production. Environmental manipulation studies like these can provide initial insight into the nature of reproductive trade-offs. I discuss various possibilities for more sophisticated examinations of both the consequences and the underlying physiology of reproductive trade-offs in *T. dalmanni*.

Chapter 7

This chapter includes a brief summary of the findings of my thesis. It places the key outcomes in their general context and outlines a variety of ways for future research to build upon my findings.

Chapter 8

There are five appendices. Appendix 1 provides details of the software programme that I use to calculate sperm content using pixel counting and is exploited in the experiments described in chapters 2 and 3. The model of optimal sperm allocation during single matings, relevant to chapter 2, is summarized in Appendix 2. Details of Samuel Tazzyman's model of the strategy of sperm depletion are provided in Appendix 3 and are relevant to chapter 3's investigation of the consequences of multiple mating for ejaculate allocation. Appendix 4 is a reprint of a published paper, based on the material described in chapter 4, about the apparent lack of a fertility benefit from a single additional mating in wild stalk-eyed flies. Appendix 5 provides a summary of the effect sizes associated with analyses described in chapter 5.

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2

Ejaculate allocation strategies in the stalk-eyed fly *Diasemopsis meigenii*

Abstract

The costs associated with producing ejaculates place intense selection upon males to allocate their limited resources strategically during single matings. As a result males may bias their ejaculate allocation to the most fecund females, potentially using a secondary sexual characteristic to infer the reproductive quality of their partner. The phenotype-linked fertility hypothesis proposes that male functional fertility may be advertised via a phenotypic signal, explaining female preference for highly sexually ornamented males. Conversely, highly attractive males may actually constrain their ejaculate allocation per-mating so as to participate in a greater number of mating events. I examine these ideas in the African stalk-eyed fly, *Diasemopsis meigenii.* I asked whether male ejaculate allocation strategy during single matings was influenced by either male eyespan or female size. Female size was an accurate predictor of fecundity, and males mated for longer with and transferred more sperm to large females. This suggests that males gain a selective advantage by over-investing in these females. However, despite large eyespan males having larger internal reproductive organs, I found no correlation between male eyespan and the quantity of ejaculate transferred. I consider how female mate preference for large male eyespan can continue to be adaptive despite the lack of obvious direct benefits during a single mating.

2.1 Introduction

Traditional sperm competition theory predicts that male fertilisation success following a mating is determined by the number of sperm transferred to the female (Parker, 1970, Wedell et al., 2002, Pizzari and Parker, 2009). Male ejaculate is likely to be limited by the costs of producing energetically expensive sperm and accessory fluids (Dewsbury, 1982, Moore et al., 2004), and by the depletion of their reserves in prior matings (Nakatsuru and Kramer, 1982, Preston et al., 2001). So it is expected that males will strategically adjust their ejaculates to maximize the number of matings and fertilisation success they can achieve given the limited resources they have to expend on reproduction. Males are also expected to evolve to be sensitive to a range of female characters that reflect female reproductive value, for example: age, size or mating history (Parker et al., 1999, Martin and Hosken, 2002, Lupold et al., 2011). Males may also respond to demographic features that reflect the likely intensity of sperm competition, for example: phase of mating season, male dominance and the sex ratio (Wedell and Cook, 1999, Bretman et al., 2010, Ingleby et al., 2010).

The strategic allocation of ejaculate by males could result in impaired fertility amongst females if males restrict the amount of sperm contained in a single ejaculate (Royer and McNeil, 1993, Svensson et al., 1998). It is a well-established phenomenon that a single mating is rarely sufficient to fertilise all of a female's eggs (Ridley, 1988, Arnqvist and Nilsson, 2000). Reduced female fertility is also possible if females are selected to restrict the number of matings due to fitness disadvantages associated with multiple mating (Chapman et al., 1995, Stutt and Siva-Jothy, 2001,

Crudgington and Siva-Jothy, 2000). Under these circumstances female preference for mating with the most fertile males will be selectively advantageous (Rogers et al., 2006). Direct assessment of male fertility is unlikely. But it is possible that males advertise their reproductive quality. This idea has come to be known as the 'phenotype-linked fertility' (PLF) hypothesis, and proposes that exaggerated male sexual ornaments act as indicators of male reproductive quality (Sheldon, 1994, Iwasa and Pomiankowski, 1999). The PLF hypothesis can be framed within the handicap principle, with the association between male ornament size and fertility assumed to arise because both traits are strongly condition-dependent (Pizzari et al., 2004, Rogers et al., 2008).

The PLF hypothesis predicts that attractive males transfer larger ejaculates during mating and females gain fertility benefits through their choice of mate (Pizzari et al., 2004). However, a recent model of sperm competition, in which males vary both in the quantity of resources they can allocate to reproduction (R) and also in the cost of obtaining a mate (c), comes to a different conclusion (Tazzyman et al., 2009). The analysis models sperm competition between males as a fair raffle proportional to the amount of sperm per ejaculate. It calculates the ESS resources allocated to a mating (s) given the expected number of matings as $R / (c + s)$, the resources allocated to reproduction divided by the total cost per mating (i.e. the cost of obtaining a mating added to the resources allocated to a mating). Under these assumptions, males with a lower cost of obtaining a mating will value their matings less. These males are expected to mate more often and to constrain their ejaculate investment per mating, resulting in smaller ejaculates relative to those of competitors who experience a higher cost of obtaining a partner. In contrast, a male's optimal ejaculate

expenditure does not vary with respect to the amount of resources allocated to reproduction (assuming cost per mating is fixed). In species where females exert mate choice using male sexual ornaments, attractive males (i.e. those with low costs of obtaining a mating) are predicted to invest less per mating. Unattractive males experiencing a high cost are expected to produce larger ejaculates (Tazzyman et al., 2009). Where males also vary in their resources allocated to reproduction, I expect no effect on ejaculate size except when these resources covary with the costs of obtaining a mate.

The PLF hypothesis predicts that ejaculate investment will be positively associated with male attractiveness, while the model of strategic allocation (Tazzyman et al. 2009) predicts a negative association. In order to test these mutually exclusive predictions, I investigated male ejaculate allocation in a model insect species, the stalk-eyed fly *Diasemopsis meigenii.* These flies are characterised by the lateral displacement of their eyebulbs on long stalks. Eyespan is sexually dimorphic, with males having more widely displaced eyes than females (Baker and Wilkinson, 2001) and is subject to sexual selection through female choice for large male ornamentation (Burkhardt and Delamotte, 1988, Wilkinson et al., 1998, Cotton et al., 2006). Male eyespan is a highly condition dependent trait in *D. meigenii (Bellamy et al., In review)* and other stalk-eyed fly species (David et al., 1998, Bjorksten et al., 2001, Cotton et al., 2004). In the related stalk-eyed fly species *Teleopsis dalmanni*, eyespan is a reliable indicator of the size of male internal reproductive organs (Rogers et al., 2008 and Cotton field data 2010). A number of studies have shown that male stalk-eyed flies are sperm limited (Fry and Wilkinson, 2004, Rogers et al., 2005, Rogers et al., 2006), resulting in reduced female fertility and long term sperm

depletion (Baker et al., 2001, Rogers et al., 2006, Cotton et al., 2010, Harley et al., 2010).

I began by asking whether male eyespan acted as a signal of male reproductive investment by measuring testes and accessory gland length. I also examined the effect of male eyespan upon sperm length. Male strategic allocation of ejaculate was measured in recently mated females by calculating the size and sperm content of the spermatophore transferred. I asked how males allocate their ejaculates to larger, more fecund females by comparing the quantities of ejaculate transferred to large and small size females during a single mating. The PLF and strategic allocation hypotheses (Tazzyman et al., 2009) make contrasting predictions about how male ejaculate investment varies with male attraction, so I compared ejaculate allocation in a single mating was compared amongst large and small eyespan males. Finally I briefly extend the modelling results published previously (Tazzyman et al., 2009) to include potential variation in male ejaculate investment relative to female fecundity.

2.2 Materials and Methods

2.2.1 Experimental flies

Eggs were collected from a laboratory population of *D. meigenii* and placed in groups of 13-20 into Petri dishes that contained a moist cotton pad and approximately 0.4g of ground sweetcorn food medium. These conditions created a high stress larval environment, resulting in large eyespan variation between emerging flies (Rogers et al., 2006). Adult flies were sorted into single sex groups

and housed in 11 litre Perspex containers containing a moist cotton wool lining and *ad libitum* ground sweetcorn food. Only sexually mature adult flies, aged 8-10 weeks post eclosion, were used in the experiments.

Eyespan (defined as the distance between the outer tips of the eyestalks) was measured to a tolerance of 0.01mm (ImageJ v.5, NIH, USA) in adult flies (Cotton et al., 2004). As female *D.meigenii* select males based upon the length of their eyespans (Cotton et al., 2006), I divided males into large eyespan (≥7.40 mm) and small eyespan (≤7.20 mm) classes. In females the eyespan trait is not subject to sexual selection but is strongly correlated with body size, so females were also divided into large (≥5.40 mm) and small (≤5.20 mm) classes based upon their eyespan measurements. Other flies were discarded. The experimental flies were transferred to individual 500ml containers lined with a moist cotton pad, and given fresh ground sweetcorn food every 2-3 days.

2.2.2 Reproductive investment

Female reproductive investment was measured by monitoring reproductive output. Female containers were lined with a sheet of blue paper so that the eggs were visible. The number of eggs laid was counted every 2-3 days for a period of 10 days. Data was collected from large (n = 43) and small (n = 30) females.

Male reproductive investment was measured by accessory gland and testis size. Sexually mature males were anaesthetised on ice. The accessory glands and testes were dissected out in phosphate-buffered saline (PBS) solution and transferred to a

glass slide. Images of each organ were captured using a digital camera attached to a dissecting microscope at 10x magnification. The length of each pair of organs was measured by tracing a midline bisecting the length of the organ (Rogers et al., 2005). Both accessory glands and testes were measured and the mean of each pair was used in subsequent analyses (Baker et al., 2003). Data was collected from large ($n = 23$) and small ($n = 22$) eyespan males.

I measured the length of sperm stored in the testes of large ($n = 15$) and small ($n = 13$) eyespan males. Male testes were dissected out as described above, and gently ruptured to release the sperm bundles. I measured the lengths of four mature sperm bundles and used the mean of each quartet in the analyses.

2.2.3 Ejaculate investment per mating

Two separate experiments were carried out to investigate strategic allocation of ejaculate. In the first experiment, I tested whether variation in female size results in different size or quality of ejaculate transferred. Males (all large eyespan) were mated once to either a large ($n = 119$) or a small ($n = 91$) female. In the second experiment, I tested whether variation in male eyespan results in different size or quality of ejaculate. Large ($n = 110$) or small ($n = 104$) eyespan males were mated singly to large virgin females.

In both experiments, matings were conducted by transferring a male into a female's container at dawn (approx. 0900 hours). The time to copulation and the duration of the copulation were recorded to the nearest second. A mating was defined as genital

engagement for longer than 150 seconds, the length of time known to be needed for sperm transfer to take place (Harley, unpublished data). Females sometimes rejected mating attempts by males (Cotton et al., 2006) or the male disengaged after less than 150 seconds (typically within 20 seconds). In either case, this was recorded as a rejection. The male was allowed to make further mating attempts for up to half an hour. If a mating had still not happened, the female (male) was replaced in the male (female) eyespan (size) variation experiment, and the procedure repeated. The individual replaced was drawn from the same size class. If there was still no successful mating after a further half hour, that individual was removed from the study. All males and females used were sexually mature virgins and were only used once.

Immediately following the mating, the female was anaesthetised on ice and her reproductive tract dissected out into 25% glycerol/PBS (pH 7.2). A coverslip was placed gently over the reproductive tract and the spermatophore was viewed using a DIC-equipped binocular microscope. Photographs were taken at 400x magnification using a Nikon CoolPix digital camera. Male *D. meigenii* transfer sperm to females in a spermatophore envelope of accessory proteins (Kotrba, 1996). Spermatophore area was measured to the nearest $0.0001mm^2$ (ImageJ v5, NIH, USA). The number of sperm contained in a spermatophore is impossible to quantify as the sperm are tightly coiled into a dense mass. Using digital images I calculated the area of the spermatophore occupied by sperm pixels (Appendix 1).

2.2.4 Statistical analyses

I used F-tests to evaluate the effect of female size (large and small) upon: fecundity, the area of the spermatophore transferred, the area of sperm in the spermatophore, and copulation duration. I also evaluated the effect of male eyespan (large and small) on the same variables (excluding fecundity), in addition to accessory gland, testes, and sperm bundle length. General Linear Models (GLMs) were used to examine the association between copulation duration and area or sperm content of the spermatophore transferred. I tested whether sperm content and spermatophore area were positively correlated and when appropriate, included spermatophore area as a covariate in GLMs testing for variation in sperm content. I used an exponential distribution to transform the time to copulation data, to control for the non-normal distribution of this variable. I tested for an association between time to copulation and spermatophore characteristics, and also for an association between occurrence of rejection (measured as occurring or not) and time to copulation, copulation duration and spermatophore characteristics. A χ^2 contingency analysis was used to determine the effect of male eyespan and female size upon the occurrence of rejection. All analyses were performed using JMP statistical software (SAS, USA).

2.2.5 Model of optimal sperm allocation

A prior mode of sperm allocation (Tazzyman et al., 2009) was adapted to consider variation in male ejaculate investment relative to female fecundity. Briefly, males have a quantity of resources R to allocate to mating. They are subject to a cost c which describes the quantity of resources they expend in order to obtain each

mating. Their strategy then consists of the quantity s of resources that they allocate to each mating. Since the number of matings they can afford will be $n(s \mid R,c) = R / (c + s)$, the smaller the value of s the more matings a male can afford. However, the success per mating is a function $v(s)$ which increases with s. For details of the function see Tazzyman et al (2009).

In the original model (Tazzyman et al., 2009) all females were assumed to be identical. Here I adapt this framework by assuming there are two types of female which differ in fecundity. Normal females, which make up a proportion q of the population of females, have fecundity 1. Fecund females, which make up a proportion $1 - q$ of the population of females, have fecundity $1 + h$. The new model assumes that the two types of female are identical in mating preference. Males are assumed to be able to detect the difference in female fecundity, and to adopt independent ejaculate allocation strategies for each type of female (s_1 for normal females and s_2 for fecund females). Using the techniques set out in (Tazzyman et al., 2009), I derive the ESS strategies s_1 and s_2.

2.3 Results

2.3.1 Reproductive investment

To assess whether large females have higher reproductive value to males, I measured female fecundity. Large females laid significantly more eggs during a 10 day period than small females ($F_{1,71} = 12.7725$, $p = 0.0006$; fig. 1). To assess whether large eyespan males have higher reproductive capacity, I measured their

testes and accessory glands. Large eyespan males had significantly larger testes ($F_{1,43}$ = 6.5223, p = 0.0143; fig. 2A) and larger accessory glands ($F_{1,37}$ = 9.2252, p = 0.0041; fig. 2B) than small eyespan males. Sperm bundle length was consistent across the two groups of males, with no effect of male eyespan size class observed (L males: 1.8922 ± 0.0217mm, S males: 1.8715±0.0233mm, $F_{1,26}$ = 0.4219, p = 0.5217).

2.3.2 Effect of female size on male ejaculate investment

Males did not vary the size of spermatophore transferred during mating in relation to female size ($F_{1,158}$ = 0.0422, p = 0.8375; fig. 3A). However, males transferred spermatophores with greater absolute sperm content when mating with large females ($F_{1,158}$ = 5.4511, p = 0.0208, fig. 3A). Spermatophore area and sperm content were highly positively correlated (r = 0.489, n = 160, p <0.0001), so I repeated this test with spermatophore area as a covariate, and still found that sperm content differed between the female size classes, with large females receiving relatively more sperm (L females: 0.0342±0.0024 mm^2, S females: 0.0247±0.0024 mm^2; $F_{1,157}$ = 7.8705, p = 0.0057).

Males took less time to start copulating with large females ($\chi^2_{1,208}$ = 10.4090, p = 0.0013; fig. 4A) and copulated for longer with large females ($F_{1,208}$ = 5.4625, p = 0.0204; fig. 4A). Neither time to copulation nor copulation duration had a significant effect upon spermatophore area (time to copulation $\chi^2_{1,158}$ = 0.0001, p = 0.9963; copulation duration: $F_{1,158}$ = 0.2277, p = 0.6339), absolute sperm content (time to copulation: $F_{1,158}$ = 0.0058, p = 0.9394; copulation duration: $F_{1,158}$ = 0.0165, p =

0.8981) or relative sperm content (time to copulation: $F_{1,157} = 0.0054$, $p = 0.9412$; copulation duration: $F_{1,157} = 0.0142$, $p = 0.9049$).

2.3.3 Effect of male eyespan on male ejaculate investment

Male eyespan had no significant effect upon spermatophore area ($F_{1,158} = 3.7101$, $p = 0.0559$; fig. 3B). As this was border-line significant, I examined the distributions for outliers, but found that their exclusion reduced the difference ($F_{1,1556} = 1.6715$, $p = 0.1980$) as they all belonged to small eyespan males ($n = 3$). Neither did male eyespan influence absolute sperm content ($F_{1,158} = 0.7355$, $p = 0.3924$; fig. 3B). As in the female size variation experiment, spermatophore area and sperm content were highly positively correlated ($r = 0.461$, $n = 160$, $p < 0.0001$). After taking account of spermatophore area, there was still no difference in relative sperm content associated with male eyespan ($F_{1,157} = 0.0006$, $p = 0.9799$).

Male eyespan had no significant effect upon time to copulation ($\chi^2_{1,212} = 0.0798$, $p = 0.7775$, fig. 4B), but it did affect copulation duration as small eyespan males copulated for longer than large eyespan males ($F_{1,212} = 4.0814$, $p = 0.0446$, fig. 4B). Neither time to copulation or copulation duration had a significant effect upon spermatophore area (time to copulation: $F_{1,158} = 0.0231$, $p = 0.8794$; copulation duration: $F_{1,158} = 0.0026$, $p = 0.9590$), absolute sperm content (time to copulation: $F_{1,158} = 0.4344$, $p = 0.5108$; copulation duration: $F_{1,158} = 0.0060$, $p = 0.9382$) or relative sperm content (time to copulation: $F_{1,157} = 0.9205$, $p = 0.3388$; copulation duration: $F_{1,157} = 0.1800$, $p = 0.6719$).
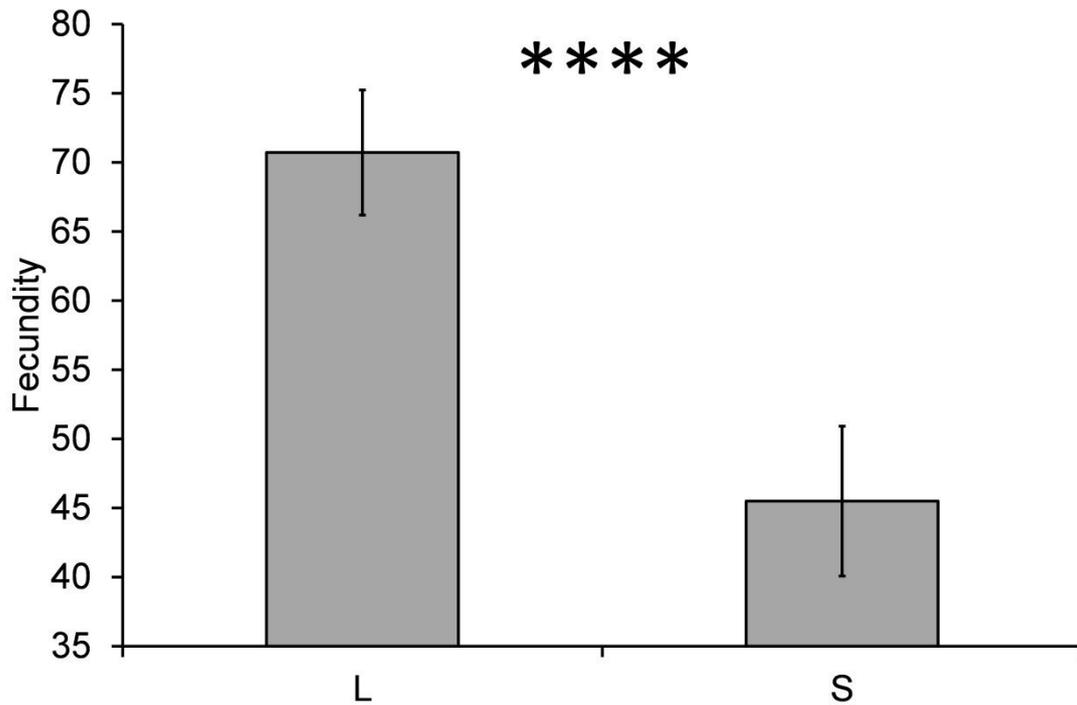
Fig. 1. Effects of female eyespan class (L and S) upon mean female fecundity over a 10 day period. Error bars show ± SEM. Degree of significance is shown using asterisks (****: $p<0.0001$).
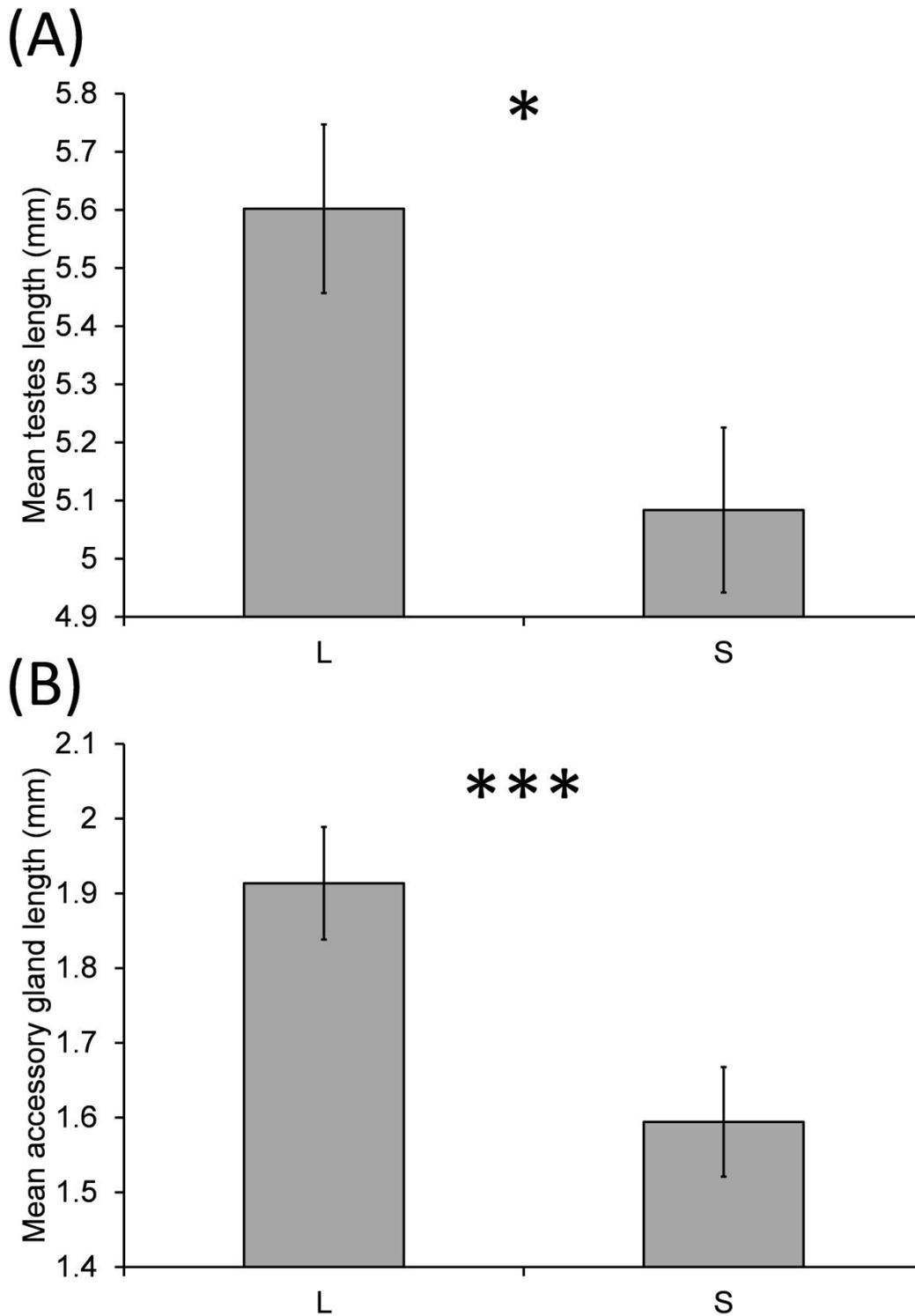
Fig. 2. The relationship between (A) male eyespan class (L and S) and mean testis length (mm), and (B) male eyespan class (L and S) and mean accessory gland length (mm). Error bars show ± SEM. Degree of significance is shown using asterisks (*: p<0.05; ***: p<0.001).
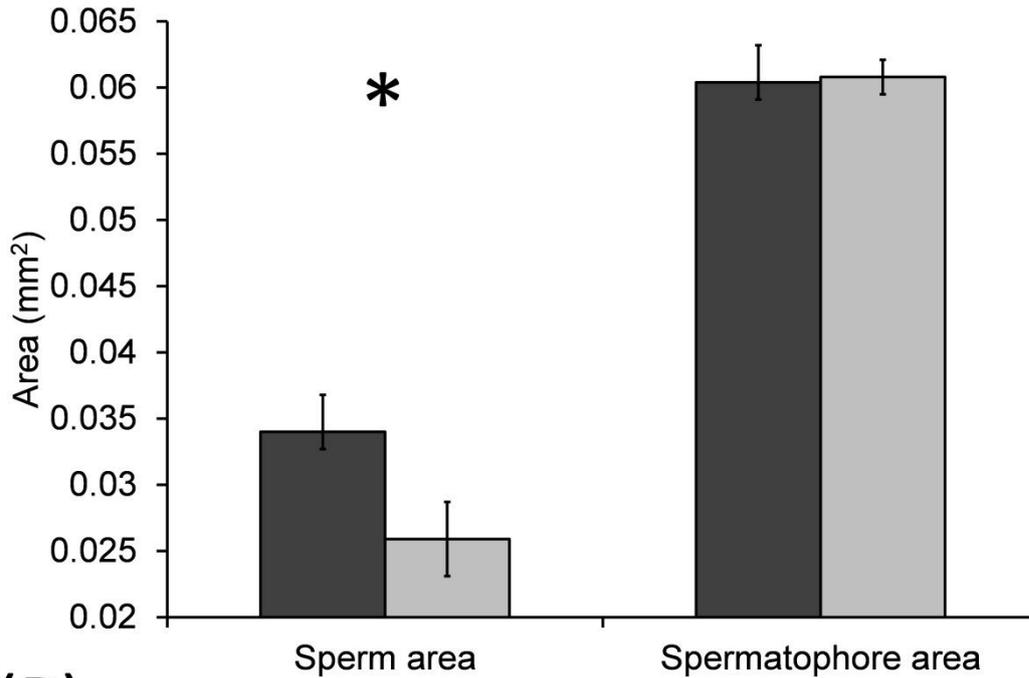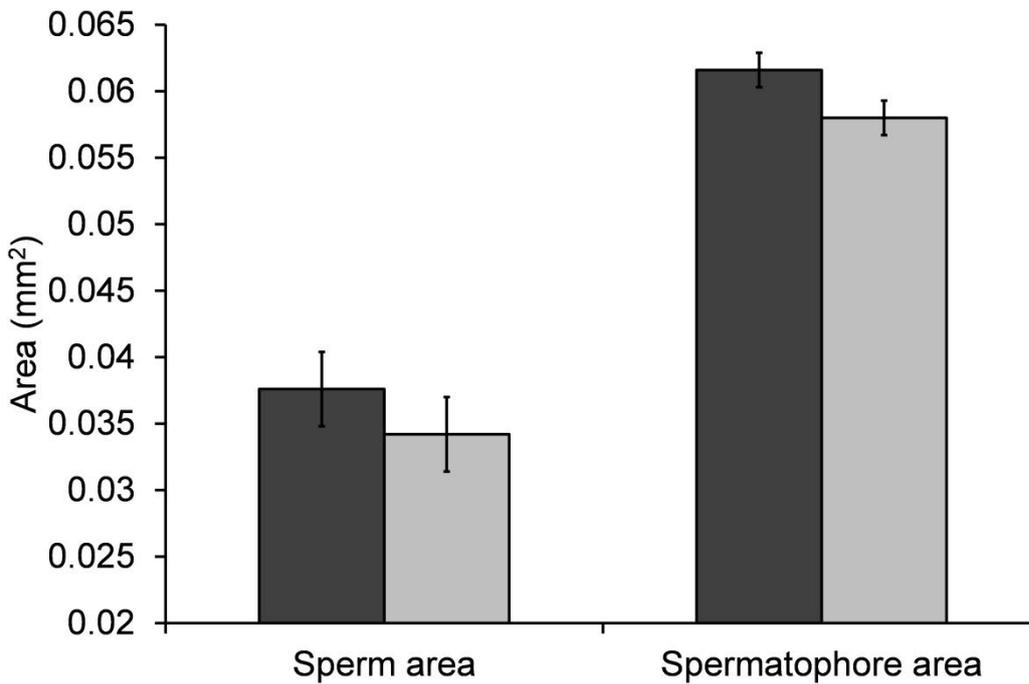
Fig. 3. Effects of female size **(A)** and male eyespan **(B)** variation (large eyespan: dark bars; small eyespan: light bars) upon spermatophore area (mm^2) and absolute sperm content (mm^2). Error bars show ±SEM. Significant differences between eyespan classes are shown with an asterisk (*: p <0.05).
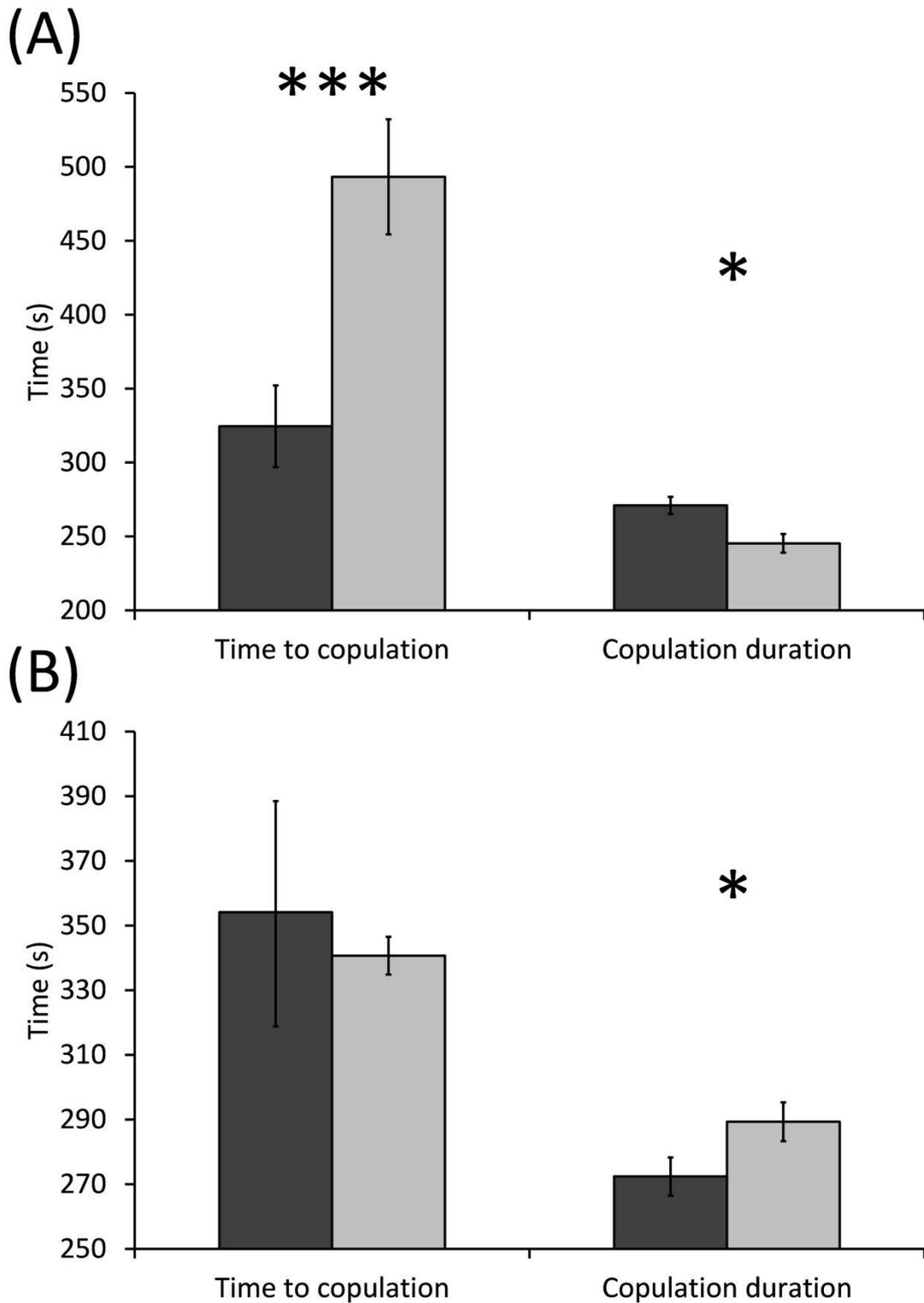
Fig. 3. The effect of female size **(A)** and male eyespan **(B)** variation (large eyespan class: dark bars; small eyespan class: light bars) upon time to copulation and copulation duration. Error bars show ±SEM. Significant differences between eyespan classes are shown with asterisks (*: $p<0.05$, ***: $p<0.001$).

2.3.4 Female rejection and copulation failure

Females rejected males in 16% of pairings, before eventually accepting them ($n = 68$ out of 424). Rejection significantly increased the time to copulation in both the male eyespan (no rejection: 287.54±25.90s; rejection: 557.50±59.34s; $F_{1,198} = 17.3856$, p <0.0001) and female size (no rejection: 384.52±28.51s; rejection: 555.51±59.54s; $F_{1,191} = 6.6931$, $p = 0.0104$) variation experiments. There was no effect of male eyespan (L males: 18 rejections, S males: 14 rejections; $\chi^2_{1,200} = 0.3440$, p = 0.5575) or female size (L females: 17 rejections, S females: 19 rejections; $\chi^2_{1,193} = 0.2240$, $p = 0.6362$) upon the occurrence of rejection. The occurrence of rejection was found to have no effect upon copulation duration (male eyespan variation, no rejection: 279.43s, rejection: 272.56s, $F_{1,198} = 0.3540$, $p = 0.5526$; female size variation, no rejection: 258.57±4.95s, rejection: 262±11s, $F_{1,191} = 0.0952$, $p = 0.7580$), spermatophore area (male eyespan variation, no rejection: 0.059±0.001mm^2, rejection: 0.059±0.003mm^2, $F_{1,147} = 0.0312$, $p = 0.8600$; female size variation, no rejection: 0.061±0.001mm^2, rejection: 0.057±0.002mm^2, $F_{1,147} = 2.9188$, $p = 0.0897$), absolute sperm content (male eyespan variation, no rejection: 0.036±0.002mm^2, rejection: 0.032±0.006mm^2, $F_{1,147} = 0.3980$, $p = 0.5291$; female size variation, no rejection: 0.029±0.002mm^2, rejection: 0.027±0.0048mm^2, $F_{1,147} = 0.2038$, $p = 0.6524$) or relative sperm content (male eyespan variation, no rejection: 0.036±0.002mm^2, rejection: 0.032±0.005mm^2, $F_{1,146} = 0.2215$, $p = 0.6386$; female size variation, no rejection: 0.029±0.002mm^2, rejection: 0.031±0.004mm^2, $F_{1,146} = 0.4550$, $p = 0.5010$).

Almost 25% of matings (n = 104 out of 424) did not result in successful spermatophore transfer. In these cases the spermatophore was either misshapen and empty, or completely absent. I found no effect of male eyespan (L males: 30 failures, S males: 24 failures; $\chi^2_{1,214}$ = 0.4990, p = 0.4800) or female size (L females: 27 failures, S females: 23 failures; $\chi^2_{1,210}$ = 0.2240, p = 0.6214) upon the occurrence of copulation failure.

2.3.5 Model of optimal sperm allocation

Using an evolutionary game theory approach it can be shown (see Appendix 2) that for all males, the ESS strategy (s_1^*, s_2^*) has the feature that $s_2^* = (1 + h)s_1^*$. Since fecund females are $(1 + h)$ times more fecund than normal females, males value matings with them as being $(1 + h)$ times more valuable. Thus at the ESS, males invest $(1 + h)$ times ejaculate per mating. As shown by the original model (Tazzyman et al., 2009), at the ESS, ejaculate investment increases with the cost of obtaining a mating (c) (i.e. decreases with male attraction). This investment is independent of the quantity of resources R that a male has to allocate to reproduction.

2.4 Discussion

Ejaculate limitation places a significant selective pressure on males to invest their reproductive resources strategically, typically directing more ejaculate to females with higher reproductive value (reviewed in Wedell et al., 2002). In *Diasemopsis meigenii*, I found that female size was strongly positively correlated with female fecundity. So my expectation was that males should direct more sperm to larger

females. To formalize this hypothesis, I added variation in female fecundity to a model of sperm allocation that already incorporates sperm competition (Tazzyman et al., 2009), and showed that males should allocate higher quantities of sperm to more fecund females. My experiments largely confirm this prediction. I found that males allocated more sperm to large females. I found that sperm content was correlated with spermatophore size, so I estimated the relative sperm content transferred and found that this too was positively associated with female size. This means that for a given spermatophore size, more sperm were transferred to large females. However, there was no difference in spermatophore size transferred to large and small females.

I also investigated whether variation in male sexual attractiveness, as determined by male eyespan, altered male ejaculate allocation. There was no difference in spermatophore size, the amount of sperm transferred, or in the relative amount of sperm transferred, between large and small eyespan males. The phenotype-linked fertility (PLF) hypothesis (Trivers, 1972, Sheldon, 1994) suggests that females prefer to mate with males bearing larger sexual ornaments as these males are capable of investing more resources into each mating, resulting in increased fertility benefits for females. My results do not support this prediction of the PLF hypothesis.

The PLF hypothesis has been formally investigated in a sperm competition model in which males varied in attraction (the costs of gaining a mating) and in the resources they have to allocate to reproduction (Tazzyman et al., 2009). This theoretical analysis also failed to support the PLF hypothesis. The model found that attractive males constrain their investment per mating as they have more mating opportunities,

predicting that attractive males produce smaller ejaculates or fewer numbers of sperm per mating (Tazzyman et al., 2009). When males differed in the resources committed to reproduction, they were not found to alter ejaculate allocation per mating. Instead, males with greater resources were predicted to mate more often (Tazzyman et al., 2009). These observations are relevant here as I found that male eyespan was a predictor of both of testis and accessory gland size in *D. meigenii.*. Consequently, in *D. meigenii,* male attraction and resources allocated to reproduction are positively correlated. A similar finding was reported in *Teleopsis dalmanni,* another stalk-eyed species (Rogers et al., 2008). Though findings like these have been interpreted as supporting the PLF hypothesis (e.g. Pizzari et al., 2004, Rogers et al., 2008, Small, 2009) this is not a reasonable deduction as reproductive organ size may scale with attraction in order to allow more attractive males to successfully mate more often rather than to increase their ejaculate size per mating. As a result, the number of matings and ejaculate size are likely to be coupled to condition, as has been demonstrated in *T. dalmanni* (Rogers et al., 2008).

Male eyespan did significantly influence copulation duration: small eyespan males mated for longer than large eyespan males. This could be indicative of increased investment per mating by unattractive males, as predicted by Tazzyman *et al* (2009). However, I found that copulation duration was not significantly associated with either spermatophore size or sperm content. This suggests that variation in male copulation duration is associated with factors beyond simple ejaculate transfer. One possibility is that large eyespan males are subject to selection to reduce the amount of time they spend per mating, so as to exploit other mating opportunities. My observations suggest that unattractive small males benefit in some way from longer

copulations. Perhaps longer copulation duration ensures that a greater proportion of sperm are transferred to storage. Males may engage in longer copulations with large females, as I observed, for similar reasons. This possibility will be worth further investigation.

I measured quantity of sperm as the proportional area within the spermatophore that contained sperm pixels, rather than the more commonly reported value of sperm number. Consequently I cannot be certain that the observed differences in male ejaculate allocation strategy are due to differences in sperm number. The mass of sperm in the spermatophore was typically highly tangled and overlaid (see fig. 5), so I could only estimate the area of the spermatophore in which sperm were present. It is possible that sperm length varied across female size classes, and this contributed to the differences observed. However, my examination of sperm bundle length does not suggest that there is much variation in the length of sperm between large and small eyespan males. It seems far more plausible that the differences observed are due to sperm number. As well as sperm, males transferred accessory gland proteins in the spermatophore. The role of these ejaculate proteins (aside from spermatophore formation) remains poorly understood in stalk-eyed flies (Kotrba, 1996). Accessory gland proteins are likely to have important post-copulatory roles in stalk-eyed flies, as has been observed in *D. melanogaster* (Chapman, 2001). Quantification of accessory gland proteins would be useful in order to determine whether spermatophore protein content covaries positively with sperm number. The proteins contained in an ejaculate are also likely to be an important component of male ejaculate allocation strategy.
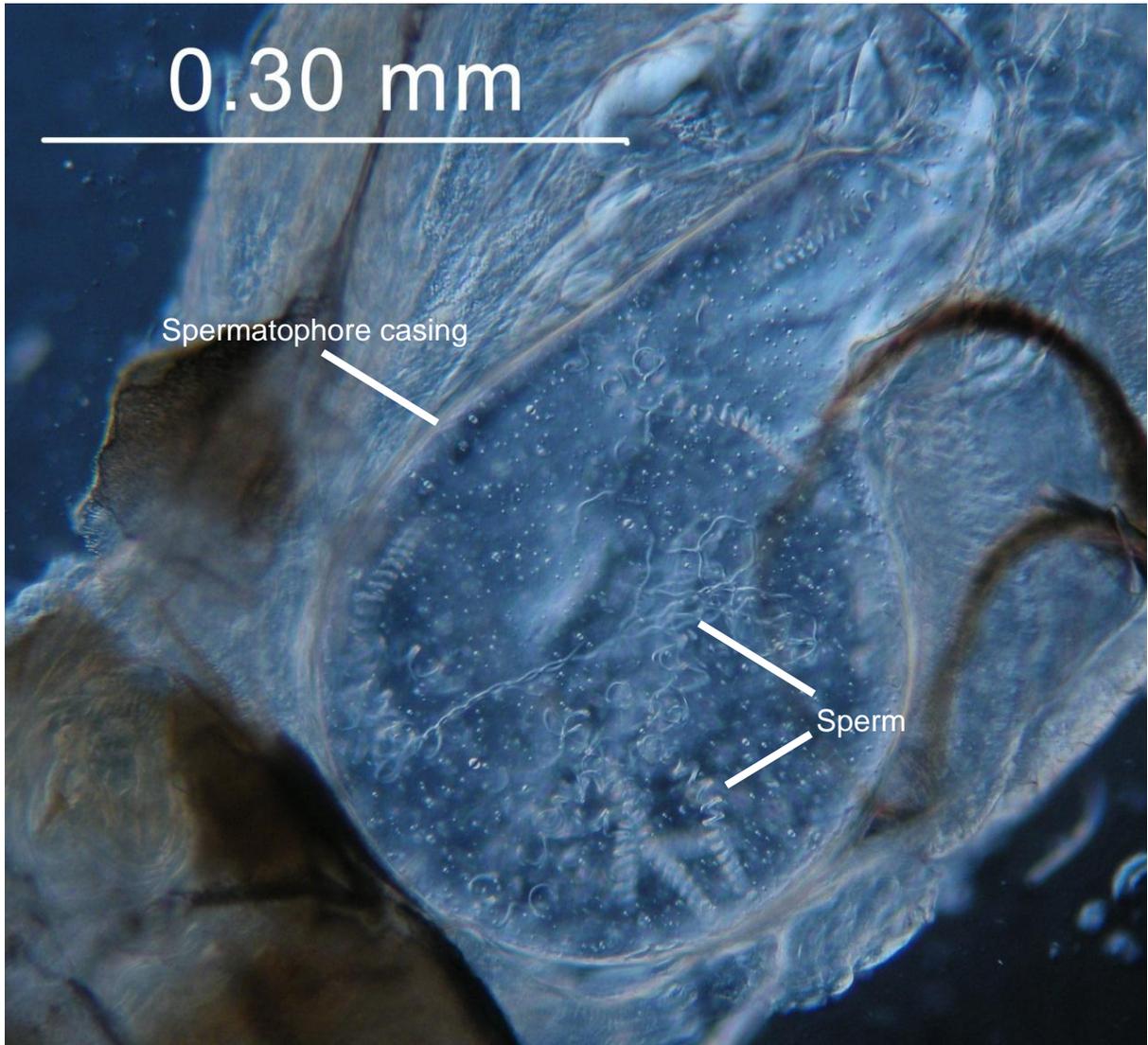
Fig. 4. The spermatophore of *D. meigenii*, photographed in the female reproductive tract at 200x magnification. Boundary of the spermatophore, and individual spermatids are labelled.

My results do not support the PLF hypothesis, but neither do they completely support the alternative hypothesis proposed by Tazzyman *et al.* (2009). This model predicts that attractive males should invest less per mating, whereas there should be no effect of variation in male resources on sperm allocation (even if sperm allocation co-varies with male attraction). Eyespan is known to increase male attractiveness in *D. meigenii*, but I found no evidence that large eyespan attractive males reduced their ejaculate investment as predicted (Tazzyman et al., 2009). This finding suggests that the model (Tazzyman *et al.* 2009) does not fully capture the selective pressures operating on sperm allocation strategy. One important possibility to consider is multiple mating. Both male and female stalk-eyed flies, including *D. meigenii,* are highly promiscuous. Multiple mating is likely to influence male ejaculate allocation, as small eyespan males will not get many opportunities to mate multiply compared with large eyespan males. Therefore ejaculate investment patterns are expected to reflect how quickly individuals can replenish depletion of their ejaculate reserves (Wedell et al., 2002). This needs to be investigated both theoretically and empirically.

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3

Multiple mating and ejaculate depletion in *Diasemopsis meigenii*

Abstract

Males are not capable of producing unlimited ejaculates, and as a result may transfer smaller ejaculates later in the breeding season than in earlier matings. Previous studies of ejaculate limitation and depletion, including my work in chapter 2, have focussed upon mean investment per mating of a male. The potential for sperm depletion to be an adaptive strategic choice by males across multiple mating events has thus far been overlooked. A new model, in which males have limited resources for sperm and are unsure of the number of matings they will receive in a breeding season, proposes that the optimal strategy is to invest fewer resources in each successive mating. The pattern of this adaptive depletion is sensitive to both the quantity of resources a male has, and the likelihood of him obtaining each successive mating. This likelihood is strongly associated with male sexual attractiveness, as more attractive males are more likely to successfully attract partners. Here I examine these theoretical predictions with an empirical assessment of ejaculate depletion in the African stalk-eyed fly, *Diasemopsis meigenii*. Over three sequential matings both large (attractive) and small (unattractive) eyespan males showed evidence of depletion through a decrease in the size and sperm content of the spermatophore transferred. Male attractiveness had a significant effect upon the rate of depletion, with unattractive, small eyespan showing more extreme depletion than attractive males. I consider the implications of this finding for our understanding of female preference for attractive male phenotypes, and the potential impact of sperm depletion upon the outcome of post copulatory sperm competition.

3.1 Introduction

Ejaculate depletion, defined as a reduction in reproductive investment per mating by a male during successive matings, is an important and not well-understood area of post-copulatory sexual selection. Investing in the production of large, competitive ejaculates comes with significant energetic costs (Dewsbury, 1982, Wedell et al., 2002), and as a result males are predicted to strategically allocate their resources to maximise the number of fertilisations they achieve. By strategically allocating their ejaculates to maximise their fertilisation opportunities, males could bias fertilisation success away from males who are more successful in pre-copulatory sexual selection. For example, Soay rams that are successful in overt contests and mate more frequently as a result are significantly sperm depleted by the end of the breeding season (Preston et al., 2001). This results in significantly decreased siring success for highly attractive males later in the breeding season (Preston et al., 2001).

Depletion might also affect the nature of female choice, particularly in cases where females are believed to select mates based on fertilisation ability (Tazzyman et al., 2012). The phenotype-linked fertility hypothesis (Sheldon, 1994) predicts that females select males upon the basis of a sexual signal from which they can indirectly infer male fertility. Variation in male condition, which may be detectable by females, can also influence male ejaculate production. Perry and Rowe (Perry and Rowe, 2010) provide some of the first evidence of condition dependent male ejaculate allocation: high condition male ladybird beetles *Adalia bipuncta* transferred larger ejaculates than low condition males. While females of this species do not appear to

select males upon the basis of an attractive phenotype, male condition may still be detectable through olfactory cues. Typically male strategy has been characterised by a single value: the expected quantity of resources that the male will invest per mating (Parker, 1970, Parker, 1990a, Parker, 1990b, Mesterton-Gibbons, 1999, Wedell et al., 2002, Williams et al., 2005, Tazzyman et al., 2009, Parker and Pizzari, 2010). However, investigations of per mating ejaculate investment do not provide an adequate framework for examining ejaculate depletion as they do not directly consider the possibility of ejaculate depletion.

Ejaculate depletion has generally been seen as maladaptive, as males are not always able to invest ejaculates according to their strategic optimum. Surprisingly little thought has been directed at the possibility that depletion may be an adaptive response given the limitation of a finite reservoir of resources available to invest in mating. The optimal level of investment in a mating may differ depending on the quantity of resources remaining, and how many more matings a male is likely to obtain. In a new model (see Appendix 3), Tazzyman investigates this possibility, and makes predictions about the patterns of depletion expected from males of differing levels of attractiveness and quantities of resources available for investment in mating. The model assumes that males have a limited pool of resources to be used in ejaculate production in a given mating season, and must assign these resources to maximise their reproductive success without knowing the exact number of matings they are likely to obtain. Optimal allocation of resources will always result in decreasing quantities of resources being allocated to sequential matings until all resources are used, at which point the male invests nothing. The more likely a future

mating is the more resources a male should allocate to it, so that more attractive males are expected to deplete at a slower rate than less attractive males.

In natural populations, the quantity of resources and male attractiveness are likely to covary, possibly through condition-dependent association. If, for example, females are able to ascertain the size of a male's ejaculate production potential at the start of a mating season, then higher quantities of resources would lead to increased attractiveness. Alternately, both the production of ejaculate and the search for partners require significant energetic investment, potentially resulting in a trade-off between resources allocated to these traits. Where males have spent more effort in obtaining matings, and are more attractive, then lower quantities of resources for ejaculate production may be observed. The model's predictions are relatively simple with regard to increasing resources – increased resources means higher allocation of ejaculate to each mating – and thus the prediction concerning the rate of depletion of attractive and unattractive males should still hold in either case.

Stalk-eyed flies can be used to examine the influence of male phenotypic attractiveness upon ejaculate investment, as males are highly sexually ornamented and produce easily quantifiable ejaculates. The eyes of both males and females are located on long stalks projecting laterally from the head capsule, and the length of the "eyespan" (distance between the eyebulbs) is subject to intense sexual selection via female choice, with females preferring to roost and mate with large eyespan males (Burkhardt and Delamotte, 1988, Cotton et al., 2006). Males show evidence of both sperm (Fry, 2006, Rogers et al., 2006) and ejaculate limitation (Rogers et al., 2005), a phenomenon that has been shown to result in reduced female fertility

(Rogers et al., 2006, Baker et al., 2001, Cotton et al., 2010, Harley et al., 2010). I tested the predictions of Tazzyman et al.'s model using the African stalk-eyed fly *Diasemopsis meigenii.* All Diptera transfer sperm via a spermatophore (Kotrba, 1996), a sperm-containing envelope of accessory proteins secreted by glands in the male reproductive tract. Two distinct ejaculate components can be measured from the spermatophore: the overall size of the ejaculate (spermatophore area) and the quantity of sperm transferred (sperm area). While the model primarily considers sperm depletion (see Appendix 3), it requires only that fewer resources for fertilising females are invested in each mating: this could apply to both spermatophore size and quantity of sperm.

To empirically examine how male attractiveness influences ejaculate limitation I subjected male *D. meigenii* to a fixed number of sequential matings and measured the quantity of ejaculate transferred at each mating. By only allowing males a short recovery time between matings I attempted to experimentally induce ejaculate limitation by placing significant mating-induced demands upon the reproductive resources available to males. In this study I examined male investment in two ejaculate traits: overall ejaculate size (spermatophore area) and sperm content (sperm area). I varied male attractiveness using the sexually dimorphic eyespan trait, assigning males to either large (attractive) or small (less attractive) eyespan classes. As *D. meigenii* males are known to invest sperm relative to female size (chapter 2) I standardised female size using the homologous eyespan trait. I compared the ejaculates transferred by large and small eyespan males to determine whether males altered their investment strategy relative to the attractive eyespan trait, and provide an empirical test for the new theoretical predictions.

3.2 Materials and methods

3.2.1 Experimental flies

Eggs were collected from a laboratory-reared and maintained population of outbreeding *D. meigenii*, and placed into Petri dishes containing a moist cotton pad and approximately 0.4g of ground sweetcorn food medium (containing nipagin mould inhibitor). These conditions created a high stress larval environment, resulting in wide variation in both male and female eyespans. At 8-9 weeks post eclosion, eyespan (the distance between the outer tips of the eyestalks) and thorax length (distance between the top of the head and the joint between the metathoracic legs) were measured prior to the experiment to a tolerance of 0.01mm (ImageJ v.5, NIH, USA). Sexually mature males were divided into large eyespan (≥7.40 mm) and small eyespan (≤7.20 mm) classes as female *D. meigenii* select males based upon the length of their eyespans (Cotton et al., 2006). In females the eyespan trait is strongly correlated with body size (chapter 2), so I used it as a proxy for size, and divided females into large (≥5.40 mm) and small (≤5.20 mm) classes. Experimental flies were placed in individual 500ml containers. Non-virgin experimental males were generated by exposing males to two stock females for 48 hours. The stock females were then removed and the male was left in isolation for a further 48 hours before being used in the study. Experimental females were kept singly and so were virgins at the start of the experiment.

3.2.2 Mating observations

At dawn large and small eyespan males were mated sequentially to a minimum of one and a maximum of three females. I attempted to mate all males to three females, but some were unable to mate more than once or twice (see Results for details). The final sample size of males that had successfully mated more than once was $n = 42$ large eyespan males and $n = 37$ small eyespan males. For the bulk of the analyses I only included those males that had successfully mated three times (L: $n = 30$; S: $n = 15$). However, I also asked whether those males that did not mate three times showed any particular differences in spermatophore area or sperm content characteristics that might explain their failure to mate multiply (see Results section).

Matings were conducted by introducing the male into the female's container. I recorded time until copulation, copulation duration and the number of rejections made by the female. If a male failed to mate within the half hour period, he was removed and placed in the container of a new female for a further half hour. If a male failed to mate with two females in a row (i.e. no matings for one hour) he was either considered to have finished (if he had successfully mated previously) or he was removed from the study (if he had no prior successful matings).

3.2.3 Spermatophore dissections

Following the completion of a mating, the female was removed and anaesthetised on ice. The female reproductive tract was dissected out at 10x magnification into a 25% glycerol/PBS solution on a clean glass slide, and covered with a coverslip. The

spermatophore was viewed and photographed at x200 magnification using a Nikon CoolPix camera attached to a binocular DIC microscope. Spermatophore area was measured from the images to the nearest $0.0001mm^2$ using ImageJ (v5, NIH, USA). I measured sperm content of the spermatophores using the same pixel counting method as in chapter two (see Appendix 1).

In total I photographed $n = 167$ spermatophores. Of these $n = 68$ spermatophores were recorded at mating one (large males: $n = 35$; small males: $n = 33$), $n = 57$ recorded at mating two (large males: $n = 33$; small males: $n = 24$) and $n = 42$ recorded at mating three (large males: $n = 28$; small males: $n = 14$).

3.2.4 Statistical analysis

I began by examining those males who mated successfully three times. I used GLMs to examine the effects of male eyespan class (large or small) and mating order upon the area and contents (measured as sperm area) of the spermatophores transferred during multiple matings. I also measured the relative area of the spermatophore that was filled with sperm, by including spermatophore area as a covariate in models examining variation in sperm content. All males contributed three spermatophores to the study, so to control for this pseudo-replication I incorporated male identity as a random factor (shrunk by REML estimation) into models explaining mating order effects to control for non-independence of within male measures. I tested for an effect of the interaction between male eyespan and mating number to determine whether male eyespan influenced the rate of spermatophore area and sperm content change with multiple mating. I also compared the morphologies of spermatophores

transferred by large and small eyespan males at each of the matings in the sequence. Finally, I looked at the data collected from all males, including those who mated less than three times. I asked whether male eyespan influenced the mean number of matings achieved, mean copulation duration and the occurrence of failed matings (empty or misshapen spermatophores). I also asked whether those males that only mated once showed significant ejaculate differences when compared with those males that mated more than once.

3.3 Results

3.3.1 Spermatophore area and content

Comparing the data from large and small eyespan males that had mated three times, I observed that across the three matings large eyespan males transferred larger spermatophores than small eyespan males (L: 0.0707 ± 0.0026mm^2, S: 0.06062 ± 0.0037mm^2; $F_{1.39.09} = 4.8273$, $p = 0.0340$). This relationship persisted when I accounted for the effect of mating order ($F_{1,40.15} = 5.2907$, $p = 0.0267$). There was a significant 21% decrease in spermatophore area from mating 1 to mating 3 (parameter estimate = - 0.0080, intercept = 0.0834, $F_{1,72.4} = 41.4016$, $p < 0.0001$). Large male spermatophore area declined by 17% (parameter estimate = - 0.0064, intercept = 0.0836, $F_{1,48.15} = 30.6414$, $p <0.0001$), whereas small eyespan spermatophore area declined by 32% (parameter estimate = - 0.0112, intercept = 0.0836, $F_{1,24.36} = 15.5347$, $p = 0.0006$).

Over the three consecutive matings, there was not a greater decline in the spermatophore size in small eyespan males than large (male eyespan x mating number interaction, $F_{1,73.14} = 3.2436$, $p = 0.0758$). This was due to small non-significant differences spermatophore area transferred at mating one (L: $0.0761\pm0.0031mm^2$, S: $0.0708\pm0.0044mm^2$; $F_{1,36} = 0.9934$, $p = 0.3256$) and mating two (L: $0.0741\pm0.0033mm^2$, S: $0.0685\pm0.0050mm^2$; $F_{1,37} = 0.8766$, $p = 0.3552$). However, by the third mating, small eyespan males were transferring considerably smaller spermatophores than large eyespan males (L: $0.0635\pm0.0029mm^2$, S: $0.0482\pm0.0041mm^2$; $F_{1,40} = 9.2958$, $p = 0.0041$; fig.1A).

The difference between large and small eyespan males in absolute sperm content were in the same direction as those for spermatophore size, but was weaker and not significantly greater in large eyespan males (L: $0.0426\pm0.0041mm^2$; S: $0.0338\pm0.0058mm^2$, $F_{1,39.36} = 1.5319$, $p = 0.2232$), and this lack of difference remained even after the addition of mating order ($F_{1,49.81} = 1.7152$, $p = 0.1978$). Absolute sperm content decreased by 31% across the three consecutive matings (parameter estimate = - 0.0076, intercept = 0.0551; $F_{1,71.61} = 9.7445$, $p = 0.0026$). The decline in absolute sperm content was 28% for large eyespan males (parameter estimate = - 0.0073, intercept = 0.0570, $F_{1,44.99} = 7.3536$, $p = 0.0094$) and 35% for small eyespan males (parameter estimate = - 0.0080, intercept = 0.0504, $F_{1,26.48} = 2.6762$, $p = 0.1137$), though the latter reduction was not significant. There was no difference between the reduction seen in large and small eyespan males when taken as a whole ($F_{1,71.3} = 0.0247$, $p = 0.8755$; fig. 1B) or for each mating separately: mating one ($F_{1,35} = 0.5041$, $p = 0.4824$), mating two ($F_{1,36} = 2.2975$, $p = 0.1383$) or mating three ($F_{1,38} = 0.6947$, $p = 0.4098$).
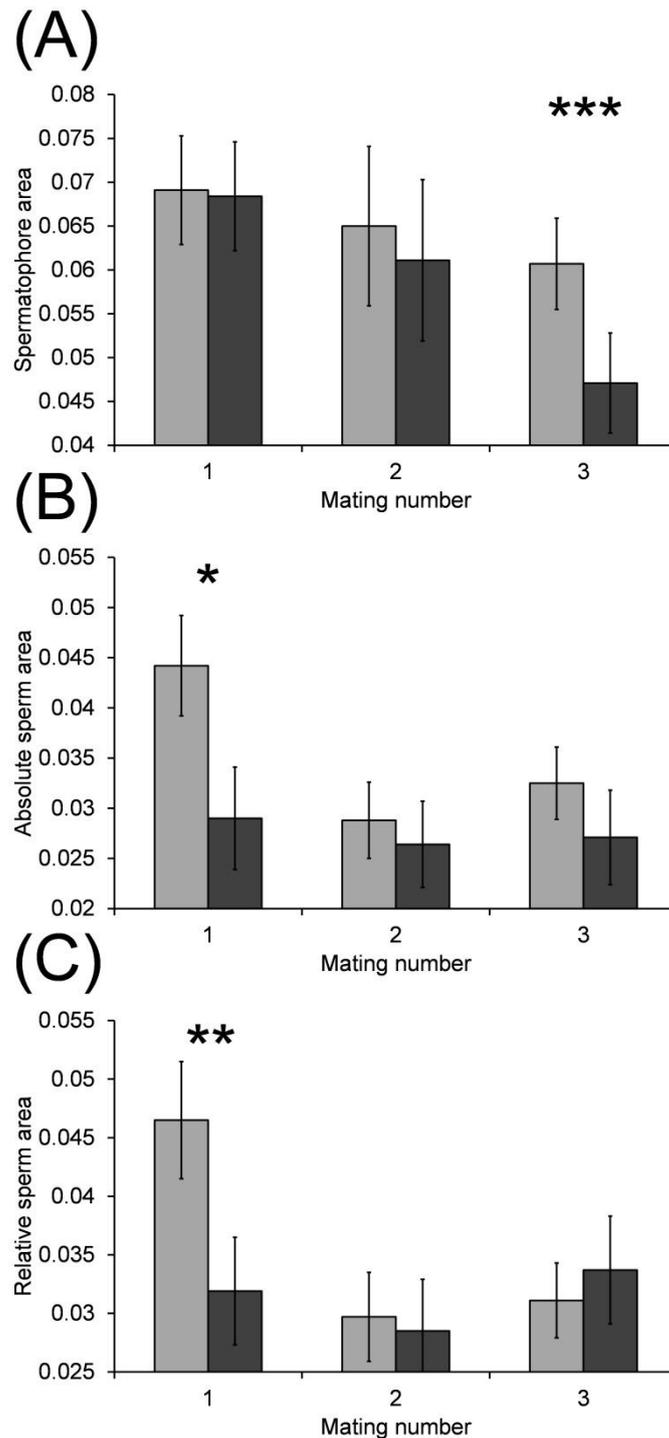
**Fig. 1.** The effect of mating number upon spermatophore area **(A)**, absolute sperm content **(B)** and relative sperm content **(C)** (all in mm^2). Pale bars represent large eyespan males; dark bars represent small eyespan males. Error bars show LSM ± SEM. Significant differences between male eyespan classes at the different matings are shown by asterisks (*: $p = 0.05$, **: $p = 0.01$, ***: $p = 0.001$).

The analyses were repeated with spermatophore area as a covariate, in order to look for difference in the relative sperm content of spermatophores. Relative sperm area showed no change with mating number ($F_{1,86.93} = 0.3990$, $p = 0.5293$), and no difference between large and small eyespan males ($F_{1,41.34} = 0.0175$, $p = 0.8953$). All other comparisons were also not significant ($p > 0.05$).

17% of spermatophores transferred during this study were empty, and had no evidence of sperm being present ($n = 27$, of 163 spermatophores). The occurrence of empty spermatophores was not associated with the large and small eyespan classes ($\chi^2_1 = 0.2770$, $n = 163$, $p = 0.5985$), or mating order ($\chi^2_2 = 1.2510$, $n = 163$, $p = 0.5351$).

In order to check whether the spermatophores without sperm influenced the statistics above, individual males who transferred empty spermatophores were removed and the analyses repeated. There was no change to the relationships reported above for spermatophore area. However, many of the absolute sperm content outcomes were different. As before, large eyespan males transferred more sperm in their spermatophores than small males, but this difference was now significant (L: $0.0546 \pm 0.0032 mm^2$, S: $0.0409 \pm 0.0046 mm^2$; $F_{1,22.78} = 6.0480$, $p = 0.0220$). The effect persisted when the mating order effect was accounted for ($F_{1,21.92} = 5.6501$, $p = 0.0266$). Multiple mating resulted in a 30% decrease in sperm content (parameter estimate = -0.0094, intercept = 0.0686, $F_{1,45.45} = 10.9950$, $p = 0.0018$). Both large and small eyespan males experienced significant decreases in sperm content with mating order. Large male sperm content decreased by 22% (parameter estimate: -0.0069, intercept: 0.0682, $F_{1,26} = 4.3825$, $p = 0.0462$) during the three matings, while

small males experienced a 44% decrease (parameter estimate: -0.0139, intercept: 0.0691, $F_{1,17.52}$ = 7.1027, p = 0.0160). There was no eyespan x mating order interaction upon change in sperm content ($F_{1,46.13}$ = 1.4170, p = 0.2400). But as with the spermatophore area results, there were only small non-significant differences between males at matings one (L: 0.0639±0.0049mm^2, S: 0.0581±0.0049mm^2; $F_{1,25}$ = 0.4606, p = 0.5036) and two (L: 0.0505±0.0053mm^2, S: 0.0350±0.0082mm^2; $F_{1,25}$ = 2.5259, p = 0.1246), whereas at mating three large eyespan males transferred spermatophore containing more sperm than small eyespan males (L: 0.0501±0.0052mm^2, S: 0.0302±0.0072mm^2; $F_{1,27}$ = 4.9905, p = 0.0340; fig.2). All contrasts using spermatophore size as a covariate remained non-significant ($p >$ 0.05).

3.3.2 Mating frequency, copulation duration and mating failure

I compared males that mated three times with those that could not. Males that only managed to mate once (n = 14) during the experiment transferred smaller spermatophores during the first mating than males who mated two (n = 20) or three times (one mating: 0.0610±0.0043mm^2, two matings: 0.0740±0.0040mm^2, three matings: 0.0743±0.0026mm^2; $F_{2,65}$ = 3.7078, p = 0.0299). In addition, males that could only mate once transferred spermatophores with lower absolute sperm content at mating one than males who mated two or three times (one mating: 0.0189±0.0072mm^2, two matings: 0.0382±0.0070mm^2, three matings: 0.0509±0.0045mm^2; $F_{2,63}$ = 7.1908, p = 0.0015). This effect remained after controlling for spermatophore area ($F_{2,62}$ = 4.2587, p = 0.0185).
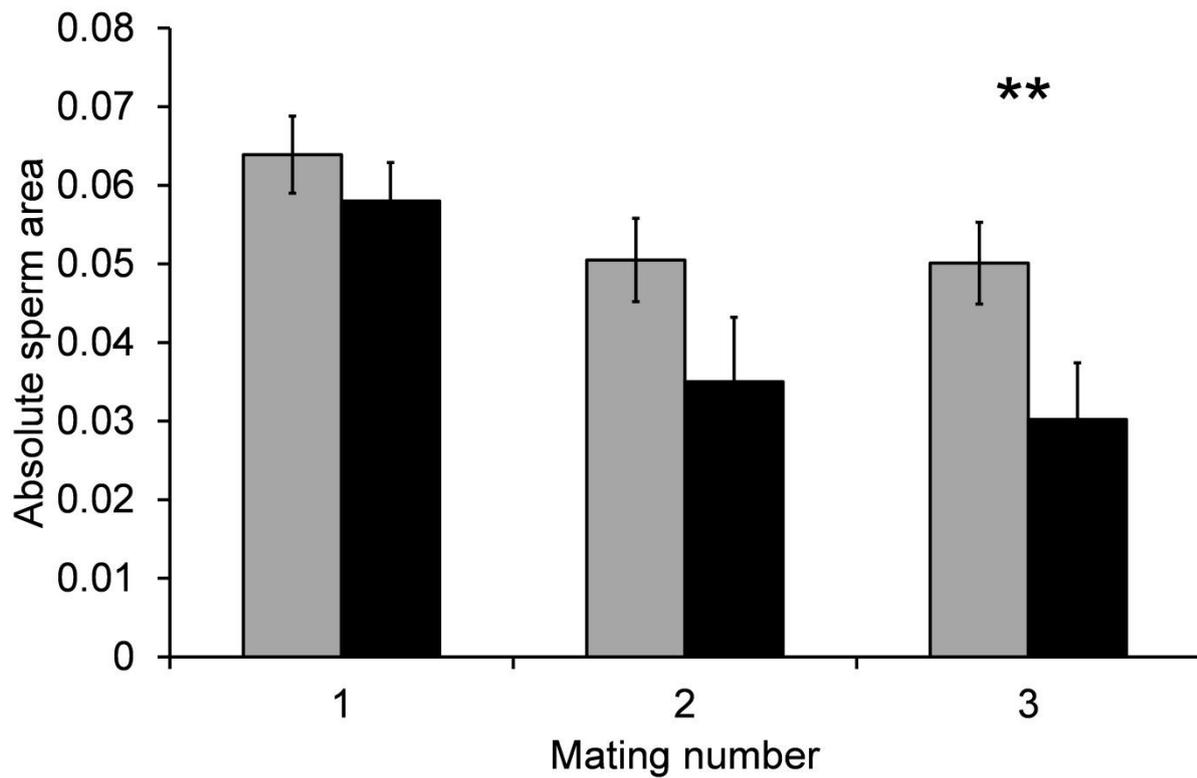
Fig. 2. The effect of mating number upon absolute sperm area (mm^2), with individuals who transferred one or more empty spermatophores removed. Pale bars represent large eyespan males; dark bars represent small eyespan males. Error bars show LSM ± SEM. Significant differences between male eyespan classes at the different matings are shown by asterisks (**: $p = 0.01$).

Using the data collected from all males (those who mated one or more times), I asked whether males that were unable to mate three times differed from other males. Male mating frequency was related to eyespan. Males with large eyespan on average mated with 2.61±0.12 females, while small eyespan males mated with 2.14±0.12 females ($\chi^2_2 = 8.087$, $n = 79$, $p = 0.0175$). Another way of measuring this effect is to count the number of males that successfully mated three times, which was 71% (30/42) for large eyespan males compared to only 41% (15/37) for small eyespan males.

The average copulation duration (mean ± s.d.) in this study was 305.85±88.61 seconds. Copulation duration did not differ significantly between the two eyespan classes ($F_{1,74.62} = 1.7808$, $p = 0.1861$), or with the order of mating ($F_{1,129.6} = 0.5010$, $p = 0.4803$). The duration of the copulation did not have a significant effect upon spermatophore area ($F_{1,161} = 0.4110$, $p = 0.5224$), absolute ($F_{1,135.6} = 0.6581$, $p = 0.4187$) or relative sperm content ($F_{1,139.8} = 1.5122$, $p = 0.2209$).

During the experiment I observed that 11% ($n = 20/189$ matings) of matings resulted in misshapen spermatophores. There was no significant difference between the number of misshapen ($\chi^2_1 = 0.425$, $n = 189$, $p = 0.5145$) spermatophores transferred by large eyespan males and small eyespan males. There was also no significant effect of mating order upon the number of misshapen spermatophores transferred ($\chi^2_1 = 1.066$, $n = 189$, $p = 0.3017$).

3.4 Discussion

Ejaculate depletion across multiple matings may be an adaptive strategy in males to conserve limited resources. If males allocate more resources to future matings based upon an increased likelihood of engaging in those matings, then attractive males are predicted to deplete their resources at a slower rate than do unattractive males. I provide empirical support for this hypothesis in the stalk-eyed fly *D. meigenii.*

In this study, the sizes and contents of spermatophores transferred by *D. meigenii* males show evidence for eyespan-dependent strategic depletion strategies. Large and small eyespan males showed evidence of depletion, but the unattractive, small eyespan males showed greater depletion of spermatophore area by their third mating. Initially we did not find this relationship for sperm content. However when males that produced empty spermatophores were excluded from the analysis, sperm content followed a similar pattern to that seen for spermatophore area; by their third mating, depletion of sperm transfer was greater in small eyespan males compared to large eyespan males. These results suggest that unattractive males, with small eyespans, invest more heavily in early mating attempts and are more strikingly depleted by a third mating. In contrast, attractive large eyespan males appear to maintain their investment level per ejaculates across three matings, resulting in a much weaker reduction in spermatophore area and sperm size. These observations are in line with the predictions made by Tazzyman's model (Appendix 3) of adaptive depletion.

The effects of sperm depletion and male adaptive strategy on the outcome of sperm competition are not well understood, as competition between ejaculates is typically examined on a per-mating basis and not in the context of multiple matings. The predictions of a new model of adaptive ejaculate depletion by males (Appendix 3) and the results of my experiment have significant consequences for our understanding of how the outcome of sperm competition is determined. A previous investigation of sperm competition in *D. meigenii* (Bellamy, 2012) has shown no evidence of sperm precedence, but with multiple matings male precedence may well be affected by mating history. For example, an attractive but depleted male may find himself at a serious fertilising disadvantage when in competition with an unattractive male who has yet to mate. Under these circumstances, unattractive males may be able to bias fertilisation success away from attractive males by investing a larger ejaculate. An experimental design in which a focal male engages in sperm competition with either a less depleted or a more depleted rival could be employed to address this question. The paternity of offspring, determined through the use of microsatellite markers (Bellamy, 2012) would show whether there are significant fitness consequences associated with sperm depletion. For example, transferring increasingly smaller spermatophores containing fewer sperm may significantly impair male reproductive success through lower female fertility, or by reduced levels of sperm competition. Future investigations could examine how depletion impacts upon female reproductive output by measuring the fertility of females mated to depleted and non-depleted males.

I only found evidence for adaptive depletion of sperm once those males that produced one or more empty spermatophores (i.e. containing no sperm) were

removed from the dataset. The change in pattern of results after exclusion of empty spermatophores needs to be evaluated. Empty spermatophores could be an experimental artefact, possibly resulting from a change in female receptivity during mating. Females might be able to terminate the copulation before sperm transfer takes place, resulting in an empty spermatophore. However, this is unlikely because the majority of female assessment appears to take place prior to and during the early stages of copulation. Females will actively reject matings from males that they find unattractive (Cotton et al., 2006), and once a male and female are in copula, I have observed that females may then reject the male after around 15 to 20 seconds of mating have elapsed. No visible ejaculate is transferred during this time. Females may be subjecting males to a short 'probationary' period at the start of copulation, and will terminate the copulation if an otherwise attractive male is found to be lacking in some way. This rejection could be based on a lack of physical stimulation in the reproductive tract, or on chemical cues transmitted by the male that are only detectable through direct contact. In a species with such a long copulation time as *D. meigenii* (>150s is required for successful sperm transfer, Harley, unpublished data), successful early mate assessment is vital in order to avoid the cost of a lengthy mating with an undesirable partner. Terminating the mating after a number of minutes have elapsed would be an unnecessarily costly behaviour.

Failure to transfer sperm may also be a natural feature of *D. meigenii* populations. In chapter 2 I note that around 25% of matings failed due to sperm transfer failure. These observations are mirrored in *T. dalmanni*, in which one third of single copulations resulted in zero fertility (Baker et al., 2001). Furthermore, similar failure rates have been observed in other insect species that transfer sperm via an internal

spermatophore (Lamunyon, 2000). However, it should be noted that all of these examples are laboratory studies. Determining whether empty spermatophores are a natural feature of *D. meigenii* mating is impossible without an assessment of natural infertility and copulation failure among males. Given the ambiguity surrounding the nature of empty spermatophore transfer, the dual analytical approach employed in this chapter is entirely suitable. In the future, further studies should aim to clarify the precise nature and effect of empty spermatophores.

As well as evidence for adaptive depletion strategies associated with male attractiveness, my results also suggest that male mating rate is inhibited by a shortage of ejaculate components. When I examined the first spermatophores transferred, I observed that males who only mated once during the experiment transferred a smaller spermatophore containing fewer sperm than those males that mated two or three times. Males may refrain from engaging in additional matings if they do not have sufficient resources available to construct spermatophores that will provide a competitive advantage in post-copulatory competition. The cost of avoiding mating may be balanced by the increased likelihood of paternity that can be gained through waiting and replenishing their available ejaculate components. However, the males used in this study were highly unlikely to suffer from depleted ejaculate components. Although they were previously mated, they had been allowed sufficient time to recover their ability to construct spermatophores. Some males may naturally produce smaller spermatophores than others. Thus far, this chapter has only considered the environmental components of ejaculate depletion (e.g. energetic constraints in accessory peptide and sperm production). However, the development of inbred lines of *D. meigenii* provides the opportunity to determine whether there are

genetic components to spermatophore production, which might result in variation between males that is independent of attractiveness. The experimental design reported in this chapter could be repeated using males from different inbred lines paired with outbred females from a stock population. Larger variation in spermatophore construction and depletion between the inbred lines than within them would indicate that ejaculate production has a genetic component. This type of experimental design would permit the estimation of the genetic covariances of spermatophore traits with other male traits such as attractiveness (male eyespan) and with female traits such as mate preference which determine the long term response to selection.

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4

No detectable fertility benefit from a single additional mating in wild stalk-eyed flies

Abstract

Multiple mating by female insects is widespread, and the explanation(s) for repeated mating by females has been the subject of much discussion. Females may profit from mating multiply through direct material benefits that increase their own reproductive output, or indirect genetic benefits that increase offspring fitness. One particular direct benefit that has attracted significant attention is that of fertility assurance, as females often need to mate multiply to achieve high fertility. This hypothesis has never been tested in a wild insect population. Female Malaysian stalk-eyed flies (*Teleopsis dalmanni*) mate repeatedly during their lifetime, and have been shown to be sperm limited under both laboratory and field conditions. Here I ask whether receiving an additional mating alleviates sperm limitation in wild females. In our experiment one group of females received a single additional mating, while a control group received an interrupted, and therefore unsuccessful, mating. Females that received an additional mating did not lay more fertilised eggs in total, nor did they lay proportionately more fertilised eggs. Female fertility declined significantly through time, demonstrating that females were sperm limited. However, receipt of an additional mating did not significantly alter the rate of this decline. My data suggest that the fertility consequences of a single additional mating were small. I discuss this effect (or lack thereof), and suggest that it is likely to be attributed to small ejaculate size, a high proportion of failed copulations, and the presence of X-linked meiotic drive in this species.

4.1 Introduction

Multiple mating in insects is a widespread phenomenon that has attracted much attention and for which many explanations have been proposed (Thornhill and Alcock, 1983, Ridley, 1988, Arnqvist and Nilsson, 2000, Jennions and Petrie, 2000, Tregenza and Wedell, 2000, 2002, Zeh and Zeh, 2006). From a classical perspective, males are expected to increase their fitness by mating multiply with many females, while females are assumed to require only one or a few matings to maximise their fertility (Bateman, 1948, Arnqvist and Nilsson, 2000). However, multiple mating by females is characteristic in many species, despite the potentially large costs that females incur from doing so (Thornhill and Alcock, 1983). As a result much work has focussed on female re-mating to seek explanations for this apparently paradoxical behaviour (Ridley, 1988, Arnqvist and Nilsson, 2000).

Multiple mating by females is likely to be costly as a result of ecological risks (Thornhill and Alcock, 1983, Rowe, 1994, Webberley and Hurst, 2002), costs derived from the act of mating itself (Chapman et al., 1995, Crudgington and Siva-Jothy, 2000, Stutt and Siva-Jothy, 2001), and even increased rates of polyspermy or the expression of adaptations for sperm competition that reduce fertility (Eberhard, 1996). Advantages of mating multiply are usually classed as either direct or indirect (genetic) benefits (reviews in Arnqvist and Nilsson, 2000, Jennions and Petrie, 2000). Females may derive direct benefits from multiple copulations when males provide nuptial gifts that enhance female fitness directly (Thornhill and Alcock, 1983). Alternatively, if males transfer insufficient sperm in a single ejaculate to fertilize all of a female's eggs (Thornhill and Alcock, 1983, Ridley, 1988), or if fertility is limited by

substances other than sperm that are also transferred during mating (Boggs and Gilbert, 1979, Butlin et al., 1987), then multiple mating increases female fitness directly by assuring long-term fertility (Ridley, 1988, Arnqvist and Nilsson, 2000). Indirect benefits to females may also ensue if multiple mating results in the production of genetically superior offspring, for example if sperm competition engenders fertilisation of eggs by genetically superior or more compatible males (Olsson et al., 1996, Tregenza and Wedell, 1998).

There is good evidence from many insect species that multiply-mated females have higher fertility than those that have only mated once (e.g. stalk-eyed fly, Baker et al., 2001, seed bug, Wang and Davis, 2006). Such comparisons clearly demonstrate that multiple mating confers fertility advantages. However, why do females who have already mated multiply continue to do so? It is currently unclear whether additional matings by females that have already mated multiply also increase fertility; for example, re-mating frequency had inconsistent effects on fertility in dung flies (Hosken et al., 2003), leaf beetles (Orsetti and Rutowski, 2003) and field crickets (Gershman, 2007). One might expect any immediate fertility benefit of an additional mating to decline with the frequency of female re-mating (e.g. Gershman, 2007), if fertility approaches a maximum or if a female's sperm storage reaches capacity. Nonetheless, re-mating may still be important in the longer term if it maintains high fertility by replenishing used, lost, or dead sperm. Under such circumstances, correlations between mating frequency and fertility will be relatively uninformative about the adaptive value of female re-mating, as they reveal little about sperm depletion over time in females. To detect this 'hidden' fertility advantage of multiple

mating requires experimental intervention that allows detection of temporal declines in fertility when females are denied additional matings (e.g. Cotton et al., 2010).

The majority of investigations into multiple mating and its associated benefits in insects have been conducted under laboratory conditions (e.g. Arnqvist and Nilsson, 2000). Laboratory studies allow powerful, systematic and controlled investigations of mating behaviour and its consequences. However, laboratory conditions are also largely uniform and potentially unrepresentative of the natural environment under which mating traits originally evolved. In order to fully understand the forces that shape female mating behaviour, I need to also address questions concerning the benefits of female remating under natural conditions. This study is the first to examine fertility benefits associated with female multiple mating in a wild insect population.

Here, I investigate whether females gain fertility benefits from additional mating in a wild population of the polygamous Malaysian stalk-eyed fly, *Teleopsis dalmanni* (Diptera, Diopsidae). Stalk-eyed flies are characterised by lateral extensions of the head capsule, on which their eyes are located. In *T. dalmanni* the distance between the eyes (eyespan) is a sexually dimorphic trait, with males having greatly exaggerated eyespans compared to females (Baker and Wilkinson, 2001). They form nocturnal lekking aggregations at dawn and dusk, during which copulations take place (Burkhardt and de la motte, 1985, Small et al., 2009). Fights between males for control of these aggregations are typically won by individuals with greater eyespan (Small et al., 2009), and females prefer to roost and mate with large

eyespan males (Wilkinson and Reillo, 1994, Hingle et al., 2001a, Hingle et al., 2001b, Cotton et al., 2010).

Both sexes of *T. dalmanni* are highly promiscuous and mate at high frequencies (Wilkinson et al., 1998, Reguera et al., 2004, Rogers et al., 2005a). Male mating rate is heritable (Rogers et al., 2005a), but there is no evidence that female mating rate is genetically correlated with that of males (Grant et al., 2005). Females usually have low fertility, and continually mated females lay a higher percentage of fertile eggs than females mated three times or those mated only once (81%, 62% and 40% respectively, Baker et al., 2001). This suggests that females re-mate to obtain direct fertility benefits, at least in laboratory populations. There is no evidence for fertility advantages arising from polyandry, as distinct from multiple mating, in this species (Baker et al., 2001). The act of mating *per se* does not appear to be particularly costly, in terms of lifespan and lifetime fecundity, in *T. dalmanni* (Reguera et al., 2004). However, multiple mating may incur other, ecological costs (e.g. Thornhill and Alcock, 1983, Rowe, 1994, Webberley and Hurst, 2002). Low fertility in female *T. dalmanni* is likely the result of sperm-limitation (Baker et al., 2001). Males transfer few sperm during a single copulation (~ 65, ref Wilkinson et al., 2005, ~142, ref Rogers et al., 2006), and spermatophores are small (Kotrba, 1996) and unlikely to provide females with non-sperm resource benefits. Low fertility and chronic sperm limitation has also been documented in a wild *T. dalmanni* population. In a recent field study, Cotton et al. [24] found that only around 55% of eggs laid by wild females were fertilised, and that fertility declined with time when females were denied access to males. This implies that females face sperm-limitation over both the short and long-term.

I used a wild *T. dalmanni* population to test whether non-virgin wild females that received a single mating had higher fertility than a control group of wild females that received an interrupted and incomplete mating. While manipulating the control group in this way may seem counter-intuitive, the intention of this design was to focus upon the effect of a natural mating upon female reproductive output. I therefore decided to compare females that were permitted to mate naturally with females that were known to be sexually mature and receptive, but were prevented from mating. This approach allowed me to examine the fertility benefits of performing an additional mating, in an *n*+1 versus *n* mating design, where *n* is the mating frequency of experimental females prior to the start of the experiment. Given the low levels of female fertility and severe sperm limitation observed previously in this wild population (Cotton et al., 2010), I asked whether a single copulation confers a significant reproductive advantage to a female by a) increasing fertility, and b) slowing the rate at which fertility declines when a female is housed in isolation.

4.2 Materials and Methods

Fieldwork was carried out in Ulu Gombak, Peninsular Malaysia (3°19' N, 101°45' E) during March and September 2009. Observations of females, conducted by E.H. and S.C., took place during dusk (1800 to 1930 hours) at three distinct lekking areas (LD, BW and UBW). These sites were located along two adjacent tributaries of the Gombak River that were within 100 metres of each other.

To estimate the effect of a single mating on female reproductive output, I experimentally manipulated matings between wild flies. To ensure that they were sexually mature and receptive, all focal females were chosen once they had begun copulation, defined as engagement of male and female genitalia. At this point they were randomly assigned to one of two groups. Mated (M) females ($n = 43$) were allowed to continue mating before being captured. Matings were classified as successful when copulation lasted >30s; this ensured that complete spermatophore transfer had occurred (Lorch et al., 1993, Corley et al., 2006). Interrupted mating (IM) ($n = 44$) females were separated from their mate and captured before 30s of copulation had elapsed. Matings were interrupted by using a pencil or paintbrush to gently separate the male and female. Un-manipulated females from the mated group, which copulated for <30s were reclassified into the interrupted female group. Interrupted copulations do not result in sperm transfer, although they may lead to the transfer of seminal fluid (Fry and Wilkinson, 2004). Females were captured from the leks by aspiration into a plastic bag, and transferred into individual 500ml containers within one hour of capture. These containers were lined with a moist cotton pad and a tissue paper base, and females were fed every two days with pureed banana.

Eggs laid on the tissue paper bases were collected from the containers every two days for 10 days following capture, and allowed to develop for a further five days in a Petri dish containing a moist cotton pad and pureed banana. Egg fertility was estimated by scoring hatching success under a light microscope at 10x magnification. Fertilised eggs that have hatched appear as empty chorion cases, while unfertilised eggs are full and show no signs of development. Sometimes fertilised eggs failed to hatch, but still showed signs of development (e.g. horizontal

striations in the chorion and early mouthpart formation) (Baker et al., 2001). These eggs were recorded as fertile.

Once egg collection was completed, females were killed and stored in ethanol. On return to the UK, females were measured for eyespan and thorax length using a monocular microscope and the image analysis programme ImageJ (Version 1.43e; National Institutes of Health, USA). Eyespan was defined as the distance between the outer tips of the eyes, and thorax length was measured along a midline from the base of the head to the joint between the metathoracic legs and the thorax (Cotton et al., 2010). Both measurements were made to an accuracy of 0.01mm.

4.2.1 Statistical Analyses

I evaluated the factors that affected female reproductive output using general linear models (GLMs). Female reproductive output was measured as number of eggs laid (fecundity) and number of eggs fertilised (absolute fertility). Since absolute fertility and fecundity were highly correlated ($F_{1,85} = 1522.773$, $p < 0.0001$), I also estimated the relative number of eggs fertilised (relative fertility) by including fecundity as a covariate in GLMs explaining variation in absolute fertility. Females that failed to lay any eggs during the observation period ($n = 8$) were not included in the final models.

I found significant geographic variation in all aspects of female reproductive output (three sites: BW, UBW and LD, $n = 7$, 34 and 46 females respectively; all $F_{2,67} \geq$ 4.3526, all $p \leq 0.0167$), and as a result sample site was included in all models as a main effect. All site interactions were found to be non-significant so were not

included in the models. I also investigated whether female morphology (eyespan and thorax length) had significant effects upon reproductive output.

I asked whether a single additional mating had any significant effects on female reproductive output by looking at treatment differences (i.e. n+1 matings in the M group versus n matings in the IM group) in fecundity, and absolute and relative fertility. I used data summed over the 10-day collection period to estimate overall reproductive output during the experiment. I also report the number of fertilised eggs as a percentage of total fecundity during the experiment for each group. Note that the sample site effect was not significant ($F_{2,64}$ = 2.5372, p = 0.0870), and hence not included in the test of percentage fertility. Changes in the reproductive output of wild females during enforced time in captivity have previously been reported (Cotton et al., 2010). To determine whether fecundity and (relative) fertility changed over the captivity period (5 x 2-day egg collections), I included assay period as an ordinal factor (time in captivity) in the GLMs. Since eggs were collected from each female five times during the sample period, I included female identity as a random factor (shrunk by REML estimation) to account for non-independence of within-female measures.

All statistical analyses were performed using JMP software (version 5.0.1a, SAS Institute Inc.).

4.3 Results

I found no significant difference between mated and interrupted females in terms of their total fecundity ($F_{1,63} = 0.0466$, $p = 0.8298$), absolute fertility ($F_{1,63} = 0.1759$, $p = 0.6763$) or relative fertility ($F_{1,62} = 0.5762$, $p = 0.4507$) over the 10 day sample period (Fig. 1). The percentage of fertile eggs in the mated group did not differ significantly from that in the interrupted group (mean%±SE: mated = 83.02±2.55, unmated = 78.09±2.75, $F_{1,65} = 1.7297$, $p = 0.1931$).

All aspects of female reproductive output varied significantly with time in captivity, but in different ways (Fig. 2). Both fecundity and absolute fertility showed significant peaks on day four of observation (Fig. 2A and 2B; fecundity: $F_{4,282} = 7.5978$, p <0.0001; absolute fertility: $F_{4,194} = 5.9830$, $p = 0.0001$). This effect is probably a short-term response to capture, and subsequent acclimatisation to captivity. In agreement with a previous study (Cotton et al., 2010) I observed that relative fertility declined significantly with time (Fig. 2C; $F_{4,193} = 6.9897$, p <0.0001; Fig. 2D shows percentage fertility changes through time). This is most likely the result of females being sperm limited after isolation from males (Baker et al., 2001, Rogers et al., 2008, Cotton et al., 2010).

If a single mating were able to alleviate sperm-limitation, I would expect differences between treatment groups; fertility in recently mated females should show less of a decline over time compared to that of interrupted females. However I did not observe this, as there was no significant interaction between treatment and time for relative fertility ($F_{4,188} = 0.5580$, $p = 0.6934$). Similarly, the treatment × time interactions were
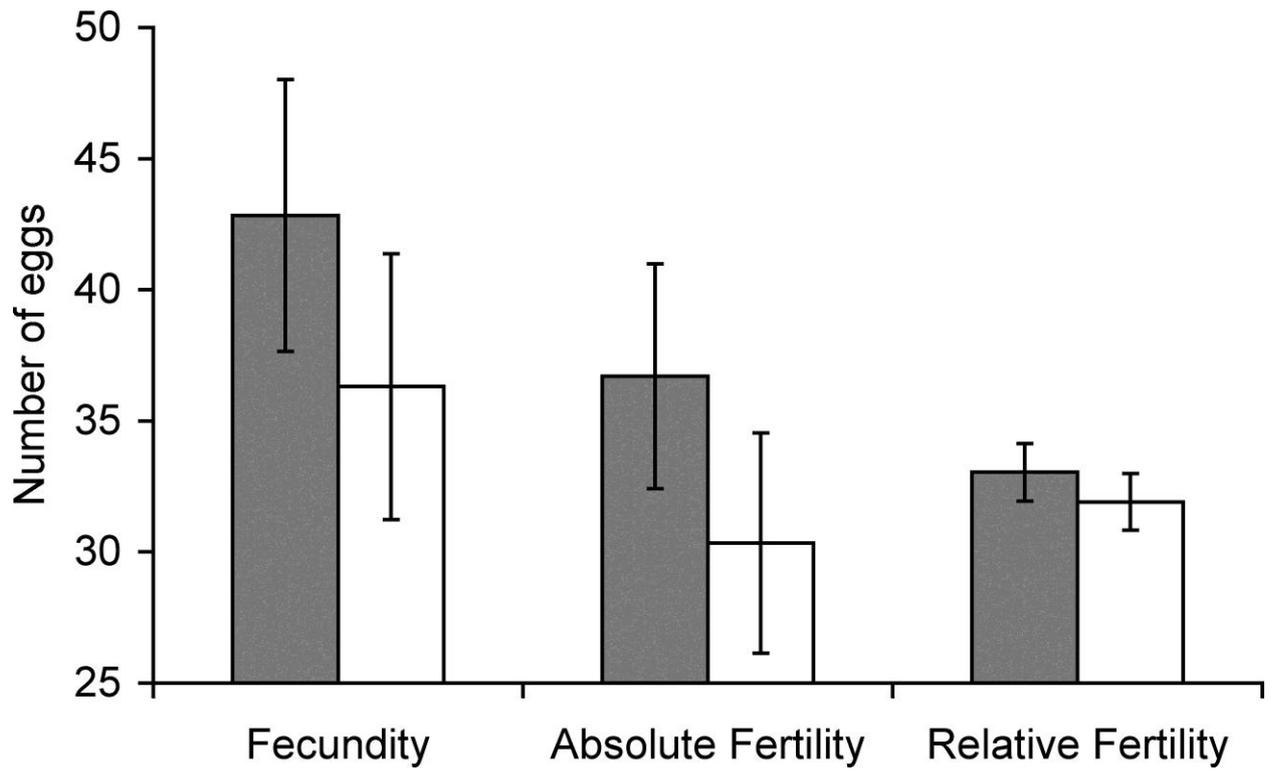
Fig. 1. Fecundity, absolute fertility and relative fertility, summed over the 10-day observation period, for females that received either a single additional mating (shaded bars) or an interrupted mating (open bars). Data displayed as least squares means ± SE.

Fig. 2. Changes in fecundity **(A)**, absolute fertility **(B)** relative fertility **(C)** and percentage fertility **(D)** over time in captivity for females that received a single additional mating (shaded bars) or an interrupted mating (open bars). Time periods that are not connected by the same letter are significantly different (Tukey HSD comparison of pooled (mated plus interrupted) means). Data displayed as least squares means ± SE.

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also non-significant for fecundity ($F_{4, 277}$ = 0.3317, p = 0.8565) and absolute fertility ($F_{4,189}$ = 0.2761, p = 0.8932), supporting the view that a single mating does not have an appreciable effect on female reproductive output (Fig. 2).

Neither female eyespan (fecundity: $F_{1,58}$ = 1.2314, p = 0.2717; absolute fertility: $F_{1,58}$ = 1.4236, p = 0.2377; relative fertility: $F_{1,57}$ = 0.2065, p = 0.6513) nor thorax length (fecundity: $F_{1,58}$ = 0.8337, p = 0.3650; absolute fertility: $F_{1,58}$ = 0.2266, p = 0.6359; relative fertility: $F_{1,57}$ = 2.3604, p = 0.1300) had significant effects upon female reproductive output.

4.4 Discussion

One of the most compelling explanations for the occurrence of multiple mating in female insects is that they acquire direct fertility benefits from such behaviour (Ridley, 1988, Arnqvist and Nilsson, 2000). Females often suffer from sperm-limitation, and there are numerous examples in which multiply mated females have higher fertility than once mated females (e.g. Baker et al., 2001, Wang and Davis, 2006). However, evidence for continued fertility benefits from additional matings by females that have already re-mated is equivocal (e.g. Hosken et al., 2003, Orsetti and Rutowski, 2003, Gershman, 2007). A previous field study on the same Malaysian population of *T. dalmanni* assayed here showed that wild females had low fertility (~55%) and were highly sperm limited (Cotton et al., 2010). Males transfer few sperm during copulation (Rogers et al., 2006, Wilkinson et al., 2005), and additional non-sperm direct benefits are unlikely as spermatophores are small (Kotrba, 1996). Given these extreme attributes, I asked whether receiving an

additional single mating could alleviate sperm-limitation and confer significant reproductive advantages upon wild *T. dalmanni* females.

I used an *n*+1 *versus n* mating design to evaluate the fertility benefits that arise from an additional mating, relative to the background level of mating in the population. All of the females analysed were fecund and observed to begin copulating under natural field conditions with their mate of choice, showing that they were both sexually mature and receptive to mating. Contrary to a previous, laboratory-based, study on the fertility benefits of multiple mating in *T. dalmanni* (Baker et al., 2001), I found no evidence for continued fertility benefits from additional mating in wild females, measured using absolute or relative values, or when expressed as a percentage of the total number of eggs laid. This was surprising, since around one fifth of eggs laid were infertile. I also observed a significant decrease in fertility during the time in captivity when females were unable to re-mate. This indicates that they suffered from sperm limitation (see also Cotton et al., 2010). However, the rate of this decline in fertility was unaffected by an additional mating, which implies that a single mating was unable to mitigate sperm limitation. Therefore, against a background of natural mating behaviour, I found no detectable fertility benefits associated with a single mating.

Variability in the background level of mating (*n*) in our experimental design could potentially have obscured any fertility effects caused by the additional experimental mating. However, females in each group were chosen at random from the population, so there is no reason to believe that *n* differed significantly between the mated (*n*+1) and interrupted mating (*n*) treatments. However, this assumption is hard

to confirm. Nonetheless, this apparent shortcoming of our design has the advantage that it frames the effect of a single additional mating relative to the natural mating background. Thus the fertility benefit (or lack thereof) that I observed is a realistic estimate of that gained by the average female in the population.

Why were fertility benefits from a single additional mating not observed, despite the presence of unfertilised eggs and high sperm-limitation? One possibility comes from the observation that male *T. dalmanni* transfer few sperm during a single mating (Rogers et al., 2006, Wilkinson et al., 2005), and around a third of matings do not result in sperm transfer at all, despite lasting for longer than 30 seconds (Baker et al., 2001, see also Fry and Wilkinson, 2004). A similarly high proportion of failed copulations has been reported in other insects that transfer sperm via spermatophores; for example, in a noctuid moth species 20% of copulations between virgins failed to transfer any sperm to the female's storage organs (Lamunyon, 2000). In stalk-eyed flies the proportion of copulations that fail to transfer sperm is currently unknown under field conditions. However, if the failure rate is similar to that observed in the laboratory (Baker et al., 2001), then it is perhaps unsurprising that no difference was detected in the fertility of females mated n+1 times relative to those that mated n times. Under such circumstances, females may mate at high frequencies (Reguera et al., 2004, Rogers et al., 2005b, Wilkinson et al., 1998) in order to accumulate fertility over many matings. Future work should explore the number of additional matings required to significantly elevate female fertility relative to the background level, for example in an *n+i versus n* mating design, where $i > 1$.

Any effect on fertility of low sperm number and a high proportion of failed copulations may be further exacerbated by the presence of an X-linked meiotic drive element (Presgraves et al., 1997). The element is reported to occur in around 13-17% of males derived from wild populations of *T. dalmanni*, and is also found in its sister species, *T. whitei* (Presgraves et al., 1997). Meiotic drive disrupts spermatogenesis, impairing the elongation of Y-carrying sperm and thus reducing their ability to fertilise (Wilkinson and Sanchez, 2001, Wilkinson et al., 2006, Johns and Wilkinson, 2007). As a result, females mated to drive-carrying males suffer from impaired fertility (Wilkinson and Sanchez, 2001, Wilkinson et al., 2006). In *T. whitei* the ejaculate of non-drive males can reduce the competitive ability of sperm from drive males (Fry and Wilkinson, 2004, Wilkinson and Fry, 2001). If this is also the case in *T. dalmanni,* then females may mate multiply in order to counteract the detrimental effects associated with mating with drive-bearing males. Indeed, higher frequencies of female multiple mating have been observed in laboratory populations of both *T. dalmanni* and *T. whitei* where meiotic drive is also present (Wilkinson et al., 2003).

In spite of the factors that may constrain fertility, females in our study nonetheless exhibited higher fertility (~80%) than those in a previous study on the same population (~55%, Cotton et al., 2010). This suggests that there is temporal variation in either mating rate, male fertility, or both. Since the fertility derived from a single additional mating might be expected to decrease as existing fertility approaches a maximum, or if a female's sperm storage reaches capacity, our lack of discovery of fertility benefits may reflect the (relatively) high overall fertility in the population. In addition, since fertility is correlated with female mating history and sperm-limitation (Baker et al., 2001), females with relatively empty sperm storage organs would be

expected to gain greater fertility benefits from an additional mating than females whose storage organs are full. It would therefore be informative to quantify the fertility added by a single mating in other populations or at different times, in which the average background level of fertility is lower than that of the sample studied here.

Why do females continue to mate if they do not benefit from increased fertility? There are two, non-mutually exclusive, solutions. First, females may remate in order to accumulate fertility over many matings (see above). Second, there may be indirect benefits associated with multiple mating if females are polyandrous (Jennions and Petrie, 2000, Ivy and Sakaluk, 2005). While polyandry is weakly associated with increased fertility (Simmons, 2005) (although there is no evidence for this in stalk-eyed flies, Baker et al., 2001), it also allows sperm competition, which can promote fertilisation by genetically superior or more compatible males (Madsen et al., 1992, Olsson et al., 1996, Simmons et al., 2006, Tregenza and Wedell, 1998, Tregenza and Wedell, 2002). A suite of microsatellite markers is available for *T. dalmanni* (Wright et al., 2004) that allows paternity to be assigned to offspring (Corley et al., 2006). Therefore future studies should determine whether wild *T. dalmanni* females are indeed polyandrous and to what degree, or whether they simply mate repeatedly with the male who has control of their chosen lekking site. Controlled mating investigations under laboratory conditions should also explore whether the offspring of polyandrous females have greater (post-hatch) viability than those of monandrous females.

In conclusion, I was unable to detect any fertility benefits from a single additional mating in a wild population of promiscuous stalk-eyed flies. This effect is most likely

attributable to small ejaculate size, the high proportion of failed copulations, and the

presence of X-linked meiotic drive (Baker et al., 2001, Wilkinson and Sanchez, 2001,

Wilkinson et al., 2006). Other, indirect, benefits may also result from polyandry

(Jennions and Petrie, 2000, Simmons et al., 2006, Tregenza and Wedell, 1998), but

these hypotheses have yet to be tested in wild populations. Males appear to derive

few fertility benefits from a single mating, as mating once with a female does not

significantly increase reproductive output, although assignment of paternity to

offspring is required to test this hypothesis sufficiently (Bretman et al., 2004,

Simmons et al., 2006). Nonetheless, our data suggest that the high mating rate

observed in both sexes of this species may be an adaptation to accrue fertility over

many matings.

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5

Sperm storage in wild *Teleopsis dalmanni*: effects on reproductive output and female receptivity

Abstract

My results from chapter 4 (Harley et al., 2010) raised a number of questions for further study. This work showed that the sperm transferred during a single additional mating was not sufficient to alleviate sperm depletion. Females in the study had relatively high fertility (~80%), suggesting that these females were storing relatively large numbers of sperm. In this chapter I examine the prediction that females with relatively empty sperm storage organs gain greater fertility benefits upon receipt of additional sperm. I also examine a by-product of this prediction: that sperm depleted females may be more receptive to additional matings than recently mated females, as they need additional matings to assure their fertility. Finally I directly examine sperm storage in wild females, examining associations with female fecundity and morphology. My results show that sperm-limitation is a phenomenon in wild *T. dalmanni* populations, which can be significantly alleviated by additional mating. However, in a laboratory investigation I observed no change in sexual receptivity associated with sperm depletion, suggesting that there are benefits to mating beyond the simple receipt of sperm that drive the evolution of female remating rate in this species. Males appeared to allocate more sperm to highly fecund females, inferred through female fertility changes and a significant association between sperm storage and female fecundity in wild flies. I discuss my findings and the possibility of alternative benefits of multiple mating in the context of the *T. dalmanni* sperm storage system.

5.1 Introduction

It has been widely found in insects that sperm from a single mating is insufficient to fertilise all of a female's available eggs (Ridley, 1988, Arnqvist and Nilsson, 2000). In order to prolong the availability of sperm when copulation opportunities are rare or sperm availability is limited the majority of insect females store sperm within specialised regions of the reproductive tract, commonly referred to as spermathecae (Pitnick et al., 1999). Storage extends the survival of sperm within the female reproductive tract long after a mating has taken place (e.g. *Drosophila melanogaster*, 2 weeks, Neubaum and Wolfner, 1999a; stalk-eyed fly *Diasemopsis meigenii,* 3-11 days, Small, 2009), and prevents gametes from being wasted through lack of fertilisation opportunities (Qazi et al., 2003). Storing sperm enables females to increase their fertility by coordinating ovulation with the release of sperm from spermathecae.

Female control over sperm release has been observed directly in a few cases. In female locusts the presence of an egg in the genital tract stimulates a neural loop that controls spermathecal contraction (Clark and Lange, 2001), and in *Drosophila melanogaster* females regulate the release of sperm from their spermathecae relative to the availability of suitable oviposition sites (Qazi and Hogdal, 2010). It has been suggested that coordination of egg and sperm interaction may also reduce the occurrence of non-viable fertilisations resulting from polyspermy (Qazi et al., 2003).

There is a wealth of empirical evidence demonstrating that the physical presence of sperm in the female reproductive tract, whether in storage or not, reduces sexual

receptivity. For example, in the cockroach *Supella longipalpa* (Smith and Schal, 1990) and *Blatella germanica* (Liang and Schal, 1994) the release of sexual pheromones signal female willingness to mate. Pheromone release is reduced by the presence of a spermatophore in the reproductive tract, and completely inhibited when sperm are stored. The response appears to vary with the quantity of sperm store. Female medflies that store few sperm during their first mating are likely to have higher short term receptivity when compared to females that stored greater numbers of sperm (Mossinson and Yuval, 2003).

Two potential mechanisms for a sperm-induced reduction in sexual receptivity are pressure within the sperm storage organs, exerted by the presence or movement of sperm, and distension of the walls of the reproductive tract and/or storage organs (reviewed in Qazi et al., 2003). Introducing saline to the reproductive tract of female cabbage butterflies (*Pieris rapae*) causes a decrease in sexual receptivity similar to that caused by mating (Obara et al., 1975), and tsetse fly sexual receptivity can be reduced by inserting glass beads into the uteri of virgin females to cause distension (Gillott and Langley, 1981). As a result, females whose sperm storage organs are relatively full should be less receptive to further mating than sperm limited females.

Compared to wild studies, laboratory conditions allow for rigorously controlled studies of female sperm storage, fertility and behaviour on a large, systematic scale. However, the uniform nature of laboratory investigations is generally unrepresentative of the natural environment in which the traits in question evolved. Extrapolating the results of laboratory investigations to natural populations remains difficult (Demont et al., 2011), as often it is unclear whether the laboratory conditions

reflect those in nature (Bretman and Tregenza, 2005). In chapter 4, I used a wild population of the stalk-eyed fly *Teleopsis dalmanni* to study multiple mating fertility benefits under natural conditions. For these investigations I return to the same wild populations to determine how receiving additional sperm affects the fertility of sperm depleted females. I attempted to study how sperm depletion influenced female sexual receptivity in the same populations, but my methods were not successful. As a result I used a captive laboratory population to test the latter prediction.

T. dalmanni females are highly sperm limited and must mate multiply to maintain their fertility (Baker et al., 2001). Males transfer small ejaculates that do not appear to provide any non-sperm benefits (Kotrba, 1996), and the number of sperm stored in the spermathecae after a single mating are very small (~65, Wilkinson et al., 2005, ~142, Rogers et al., 2006). As discussed in chapter 4, males and females form nocturnal aggregations at dawn and dusk during which matings take place (Burkhardt and Delamotte, 1985, Small et al., 2009). Females prefer to mate and roost with the most attractive, large eyespan males (Wilkinson and Reillo, 1994, Hingle et al., 2001a, Hingle et al., 2001b).

Female *T. dalmanni* have highly complex reproductive tracts (fig.1). During mating males transfer their sperm directly into the female bursa copulatrix in a spermatophore: a protein envelope secreted by the male accessory glands into the female reproductive tract (Kotrba, 1996). Female Diptera, including *T. dalmanni*, typically have two types of sperm storage organs: spermathecae and a seminal/ventral receptacle (Pitnick et al., 1999). Rates of sperm loss from the spermathecae are typically slower than those from the ventral receptacle, indicating
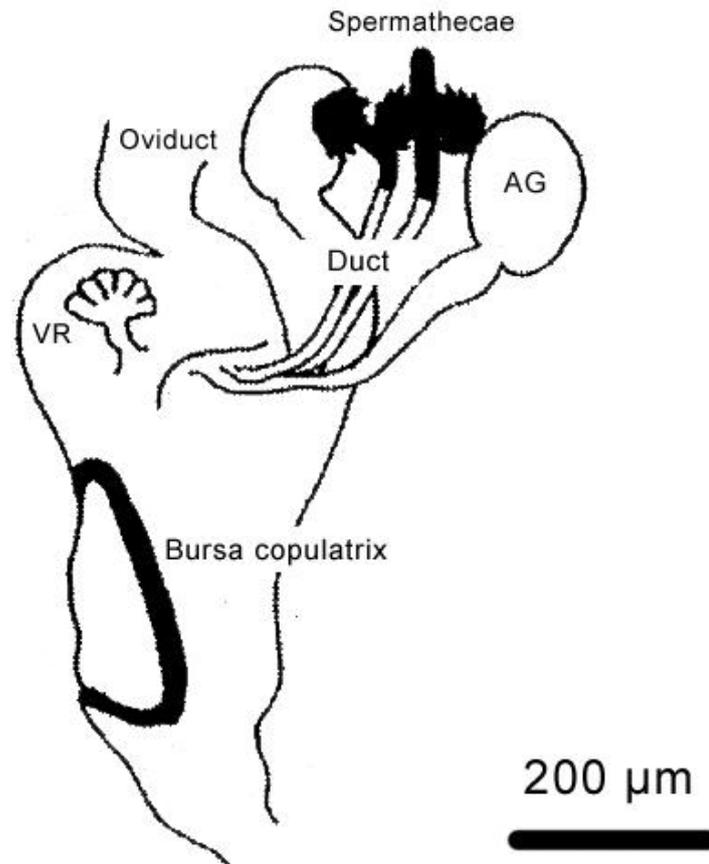
Fig. 1. The reproductive tract of female *T. dalmanni*. Spermatophores are transferred into the bursa copulatorix, and sperm move into storage in the spermathecae. Sperm move into the ventral receptacle (VR) and spermathecae for storage, and the ventral receptacle is believed to be the site of egg fertilisation. The ducts from the spermathecae and female accessory glands (AG) open into the bursa copulatorix. Adapted from Presgraves et al. 1999.

that the spermathecae are long term storage organs (Pitnick et al., 1999). *T. dalmanni* females have one paired spermathecal organ and one singlet, which are connected to the bursa copulatrix via two spermathecal ducts. While the exact functions of the individual organs are not known in *T. dalmanni*, it is possible to infer how sperm move through the female reproductive tract from extensive investigations conducted in *D. melanogaster*. The ventral receptacle retains the vast majority of sperm transferred during mating, and sperm are released from the receptacle more or less exclusively during the first few days after mating (reviewed in Qazi et al. 2003). Once the receptacle becomes depleted sperm are released from the spermathecae for fertilisation.

In chapter 4 I showed that highly fertile female *T. dalmanni*, whose sperm storage organs may be either full or close to full, do not gain additional fertility from a single mating. Fertility of these females was observed to decline with time in captivity, in isolation from males (Harley et al., 2010). Since fertility is correlated with mating history and degree of sperm limitation (Baker et al., 2001), I predicted that females with relatively empty sperm storage organs will gain greater direct fertility benefits from an additional mating. I aim to quantify the fertility added by a single mating in sperm limited females, whose fertility has been reduced through isolation from mating opportunities.

Given the strong association between fertility and sperm limitation in this species (Baker et al., 2001), I use female fertility as an indirect estimate of the quantity of sperm present in storage. In this chapter I also examine sperm storage directly in the same wild populations of *T. dalmanni*. In a pilot investigation I attempted to quantify

the number of sperm stored in spermathecae using fluorescent staining. This approach did not prove successful under field conditions, so instead I examined sperm number in the ventral receptacle. I asked whether the contents of the ventral receptacle associated with the number of mature oocytes contained in the ovaries, as there is evidence from both laboratory (Rogers et al., 2006) and field (Jameson-Cotton et al., unpublished data) studies demonstrating that males prefer to mate with large, potentially more fecund females. In addition I examine other potential correlates of sperm storage, including female morphological traits, namely eyespan and body size.

5.2 Materials and Methods

5.2.1 Female sperm depletion and direct benefits

Fieldwork was carried out in Ulu Gombak, Peninsular Malaysia (3°19' N, 101°45' E) during August 2010. Females and males were collected at dusk from leks found at 13 different stream sites. Individuals were aspirated into plastic bags, and transferred into individual 500ml containers within one hour of capture. These containers were lined with a moist cotton pad and a tissue paper base, and individuals were fed every two days with pureed banana. All females/males were housed in isolation.

I determined that sperm depletion was taking place by recording female reproductive output, measured as fecundity and fertility, over a 12 day period. Eggs laid on the tissue paper bases were collected from the containers every two days and allowed to develop for a further five days in Petri dishes containing a moist cotton pad and

pureed banana. Egg fertility was estimated by scoring hatching success under a light microscope at 10x magnification. Fertilised eggs that have hatched appear as empty chorion cases, while unfertilised eggs are full and show no signs of development. Sometimes fertilised eggs failed to hatch, but still showed signs of development (e.g. horizontal striations in the chorion and early mouthpart formation) (Baker et al., 2001). These eggs were recorded as fertile.

Once females had undergone 12 days of isolation and sperm depletion, I assessed whether a single additional mating could alleviate female sperm limitation. Mating observations were conducted at dawn (0730 – 0800 hours) and dusk (1800 – 1930 hours) using specialised mating containers comprised of two 500ml chambers separated by a removable card partition (fig. 2). 12 hours prior to the mating observations, a male was placed in the top chamber, and a female in the bottom, with the partition in place to prevent them from interacting. At the start of an observation period the card partition was removed and the pair were observed until a copulation longer than 30s took place, in order to ensure that sperm transfer had occurred (Lorch et al., 1993, Corley et al., 2006). Females were then returned to their original 500ml containers and their reproductive output was monitored for a further eight days, using the same method as described above.

Once egg collections were complete, the females were killed and stored in ethanol for transport back to the UK. Female eyespan (distance between the outer tips of the eyes) and thorax length (distance from base of the head to the joint between the meta-thoracic legs and the thorax) were measured upon return to the UK. Both measurements were made to an accuracy of 0.01mm, using a monocular

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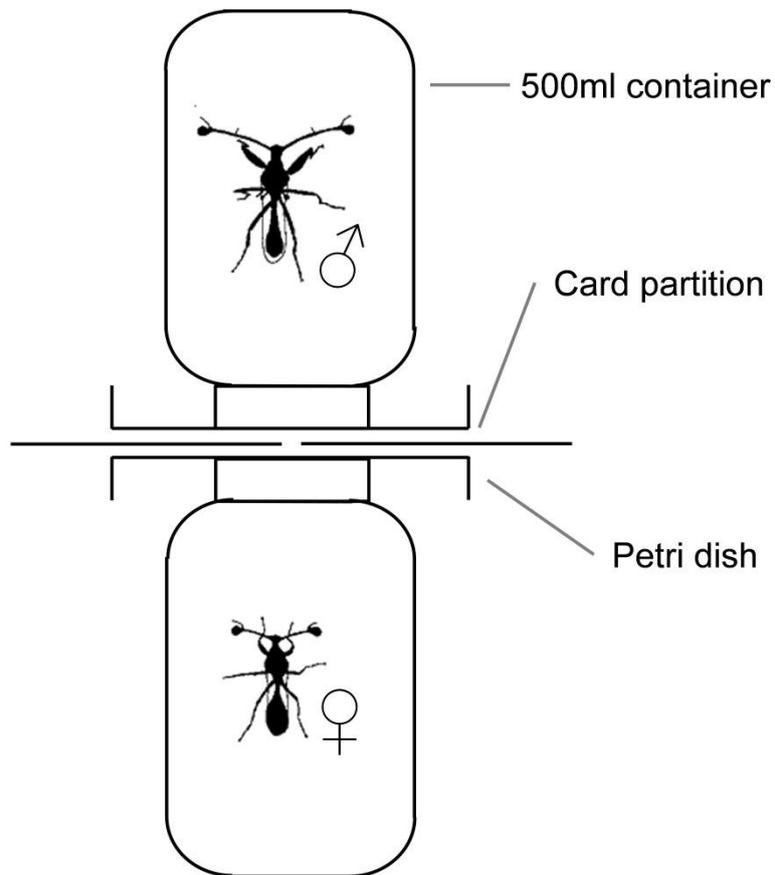
Fig. 2. The design of the mating chambers used during experiments 5.2.1 and 5.2.2. A male was placed in the top container and a female in the bottom container, separated by a removable card partition.

microscope and the image analysis software Image J (Version 1.43e, National Institutes of Health, USA).

5.2.2 Sperm depletion and female sexual receptivity

Field investigations

I conducted two field-based pilot investigations of sperm depletion effects upon female sexual receptivity during February 2011 at the Ulu Gombak field site (as described above). All females were captured as described in section 5.2.1 and subjected to 12 days of sperm depletion in the field laboratory. After 12 days, half of the females were housed with a single male for 24 hours to allow for one or more matings to take place (RM), while the other half continued to be isolated from males. I gave females the opportunity to mate multiply, rather than allowing for one controlled mating, to ensure the greatest difference between the depleted (D) and recently mated (RM) groups.

These females were then treated in two different ways in order to contrast D and RM sexual receptivity. In the first study I returned females to their original stream sites at dawn (0600 hours), and attempted to place them at a lek site containing a single male and no other females. On the evening prior to the dawn observations I selected these lek sites and cleared all females and excess males from them. I was unable to record many interactions between the focal female and the lek-owning male, because in the vast majority of cases, the female left the lek immediately. In the second study, I used the mating chamber technique described in section 5.2.1, with

observations once again conducted at dawn. Thus method proved unsuccessful because the majority of the pairs did not mate/interact at all, resulting in a sample size that was too small to analyse.

Laboratory investigation

Following these pilot studies, I decided to use a laboratory population of *T. dalmanni* to determine whether sperm depleted females were more receptive to further matings than recently mated females. The flies were collected from the Gombak sites in 2005 (by S. Cotton and A. Pomiankowski), and have been maintained in cage culture (20x20x30cm, 11L Perspex containers) with an approximate 1:1 sex ratio in groups of 100-200 to minimise inbreeding. The population is housed at 25°C with a 12:12 hour dark:light cycle. Artificial dawn and dusk are simulated by the automatic slow raising and dimming of the lights at the beginning and end of the cycle. In using these flies my intention was to allow females to mate at a "natural" rate, and thus provide a realistic simulation of natural sperm storage.

Sexually mature, non-virgin, large eyespan (>5.00mm) females ($n = 94$) were taken from the outbreeding stock population and isolated from males for 12 days. This treatment ensured that the females were sperm limited. On the thirteenth day the selected females were randomly assigned to one of two groups. Depleted (D) females continued in isolation from males for a further 24 hours; recently mated (RM) females were housed with an age-matched sexually mature non-virgin male for a further 24 hours and allowed to mate.

Receptivity assays were conducted using the mating chamber technique described above. An age-matched, sexually mature, large eyespan (>7.50mm) male was placed in the top chamber, and the focal female in the bottom 12 hours prior to the start of the experiment. The cardboard partition was removed at dawn (approx. 09:00) and each pair was observed for one hour. The number of copulations and rejections were recorded. The duration of copulations was recorded to the nearest whole second. Double blind observations were conducted by assigning each pair a random numerical identifier.

5.2.3 Sperm storage and female fecundity

Finally, I directly assessed potential associations between sperm storage and female fecundity and morphology. Fieldwork was again carried out at the Ulu Gombak field site (as described above) during September 2010 and February 2011.

To quantify ventral receptacle contents, females ($n = 268$) were collected at night (20:00 onwards) from 12 different stream sites. Entire leks were captured by enclosing them in a plastic bag. The number of males and females present at each lek was recorded. In the field laboratory, the females were anaesthetised on ice. The paired ovaries were dissected out into PBS and the number of mature eggs counted. The female reproductive tract was also dissected out into PBS, and transferred to a clean glass slide with a small drop of 25% glycerol/75% PBS solution. The tract was covered with a coverslip and the edges of the coverslip were painted down with clear nail polish to prevent it from being dislodged during transit. The remains of the females were stored in ethanol and brought back to London.

The ventral receptacle samples were viewed at 630x magnification using a DIC-equipped binocular microscope to determine the presence/absence of sperm. I counted the total number of pouches present in each ventral receptacle, and the total number of pouches that contained sperm. Female eyespan (distance between the outer tips of the eyes) and thorax length (distance from base of the head to the joint between the meta-thoracic legs and the thorax) were measured. Both measurements were made to an accuracy of 0.01mm, using a monocular microscope and the image analysis software Image J (Version 1.43e, National Institutes of Health, USA).

5.2.4 Statistical analysis

I asked whether an additional mating resulted in increased direct benefits for sperm limited females, using General Linear Models (GLMs). Female reproductive output was measured as fecundity (number of eggs laid) and absolute fertility (number of eggs fertilised). Measurements of fertility are highly susceptible to fluctuations in fecundity as the two were significantly correlated ($r^2 = 0.1203$; $F_{1,42} = 5.7438$, $p = 0.0211$), so I also estimated the relative number of eggs fertilised (relative fertility) by including fecundity as a covariate in GLMs explaining variation in absolute fertility.

Previous field experiments have shown that the reproductive output of wild females varies significantly with time in captivity (Cotton et al., 2010, Harley et al., 2010). There is typically a short-term response and subsequent acclimatisation to captivity, with low fecundity on day 2 and a higher than normal peak in fecundity on day 4 (Cotton et al., 2010, Harley et al., 2010). I observed the same pattern in this

investigation ($F_{9, 353}$ = 10.8532, $p<0.0001$), so I excluded days 2 and 4 from the subsequent analyses. I asked whether fecundity and fertility changed significantly with time during the experiment by including assay period as a continuous factor in the GLMs. My previous work has shown a strong effect of stream site upon reproductive output (Harley et al., 2010), so stream site was included as a covariate in reproductive output models.

I examined reproductive output during the 8 days before and after the single additional mating (4 x 2-day egg collections) to determine whether females showed signs of sperm limitation and increase in response to mating. Since eggs were collected 8 times in total from each female during the course of the experiment, I included female identity as a random factor (shrunk by REML estimation) to account for non-independence of within female measures. I compared per diem reproductive output before and after the mating, and reproductive output on days 12 and 15 (either side of the additional mating on day 13) to determine whether there were fertility differences.

I asked whether less fertile females experience a larger fertility increase after a mating. I calculated the difference between daily reproductive output before and after the single additional mating for each female. As some difference values were negative, distributions were transformed by adding a constant value (*a*) such that the minimum value plus (*a*) equalled unity. Differences were calculated for the fecundity and fertility of each female. The difference values were transformed using natural logarithms to normalise their distribution. We asked whether total fecundity and absolute fertility before the single mating were correlated with their corresponding

transformed differences. Pre-mating daily values of fecundity and fertility were also transformed by the addition of (*a*). GLMs were used to test the association between reproductive output prior to the single mating and the reproductive output differences. This made it possible to account for the effects of stream site and female morphology upon reproductive output, by including them as covariates in the models.

I evaluated the effect of female morphology (eyespan and thorax length) and stream location upon per diem female reproductive output during the 12 days preceding the single additional mating, and the 8 days following the single mating.

A χ^2 contingency test was used to discover whether sperm depleted (D) females were more receptive to additional matings than recently mated (RM) females. *F*-tests were used to assess whether female mating status (D or RM) altered the number and mean duration of matings that occurred. I also examined mating status differences in mean copulation duration, time between each copulation and time to the first copulation.

Further GLMs were used to evaluate the factors affecting the size and contents of the ventral receptacle, and the number of eggs in ovaries (fecundity). I excluded from the analyses any females that were found to be sexually immature ($n = 92$). Ventral receptacle size was measured as total number of pouches. Number of sperm stored in the receptacle was estimated by counting the number of pouches that were filled. I estimated the relative number of filled pouches by including total number of pouches in models explaining variation in number of filled pouches. I asked whether ventral receptacle size, contents and female fecundity varied significantly with female

eyespan, thorax length and stream of origin. A stepwise elimination was used to test for additional effects of female eyespan over thorax length, and vice versa. I also used GLMs to determine whether the number of eggs in the ovaries predicted either the size of the ventral receptacle or the contents of the ventral receptacle.

Parameter estimates and intercepts for all tests are given in Appendix 5. All statistical analyses were performed using JMP software (version 9.0, SAS Institute Inc.).

5.3 Results

5.3.1 Female sperm depletion and direct benefits

The fecundity of females in captivity did not show a significant decline across the first 8 day period of the experiment ($F_{1,134} = 0.0463$, $p = 0.8300$; fig. 3A). After the single additional mating on day 13, fecundity declined ($F_{1,110} = 4.8781$, $p = 0.0293$), but this was largely accounted for by a peak output on day 15. The additional mating was not associated with a difference in average fecundity per day when the pre-mating period (first 8 days) was compared to the post-mating period (second 8 days) (per diem fecundity before: 2.0349±0.4159 eggs and after: 2.1142±0.4184 eggs, $F_{1,37.52} = 0.0545$, $p = 0.8167$). Nor was there any difference when comparing day 12 and day 15, the time points adjacent to the additional mating (day 12: 4.5972±1.4041 eggs; day 15: 6.2980±1.4727 eggs, $F_{1,39.93} = 1.7323$, $p = 0.1956$).

Female fertility (measured as the number of eggs laid that hatched) was low and highly variable. Over the first 8 days of the study, the mean ± s.d. fertility was 31.6911±33.3252% (95% CI 21.5593 – 41.8229%, range 0-100%). This remained the case even when females with 10 eggs or fewer were excluded from the analysis (35.0783±34.4731%, 95% CI 23.2364 – 46.9202%, range 0-100%). In contrast to fecundity, both absolute fertility ($F_{1,77.91}$ = 8.1639, p = 0.0055; fig. 3B) and relative fertility ($F_{1,75.11}$ = 14.1332, p = 0.0003; fig. 3C) declined over the first 8 day period. But after the additional mating, neither absolute fertility ($F_{1,57.66}$ = 1.4920, p = 0.2269; fig. 3B) nor relative fertility ($F_{1,56.93}$ = 0.0010, p = 0.9750; fig. 3C) declined.

The additional mating was associated with an increase in absolute fertility ($F_{1,38.14}$ = 4.1775, p = 0.0479) and relative fertility per day ($F_{1,39.42}$ = 11.8014, p = 0.0014) when the pre-mating period (first 8 days) was compared to the post-mating period (second 8 days). Comparing day 12 and day 15, the time points adjacent to the additional mating, there was a large increase in absolute fertility ($F_{1,24.09}$ = 7.9538, p = 0.0095) and relative fertility per day ($F_{1,25.79}$ = 4.9168, p = 0.0356).

I also asked whether absolute reproductive output per day before the single mating influenced the degree of change experienced by females as a result of the single mating. Female fecundity before the single mating showed no association with the difference in fecundity (fig. 4A: $F_{1,36}$ = 0.1046, p = 0.7483). Absolute fertility before the single mating was significantly negatively correlated with the difference in fertility (fig. 4B: $F_{1,36}$ = 5.2937, p = 0.0273), showing that less fertile females gained greater fertility benefits as a result of the single mating. The same negative association was present for relative female fertility before the single mating ($F_{1,29}$ = 3.6109, p =
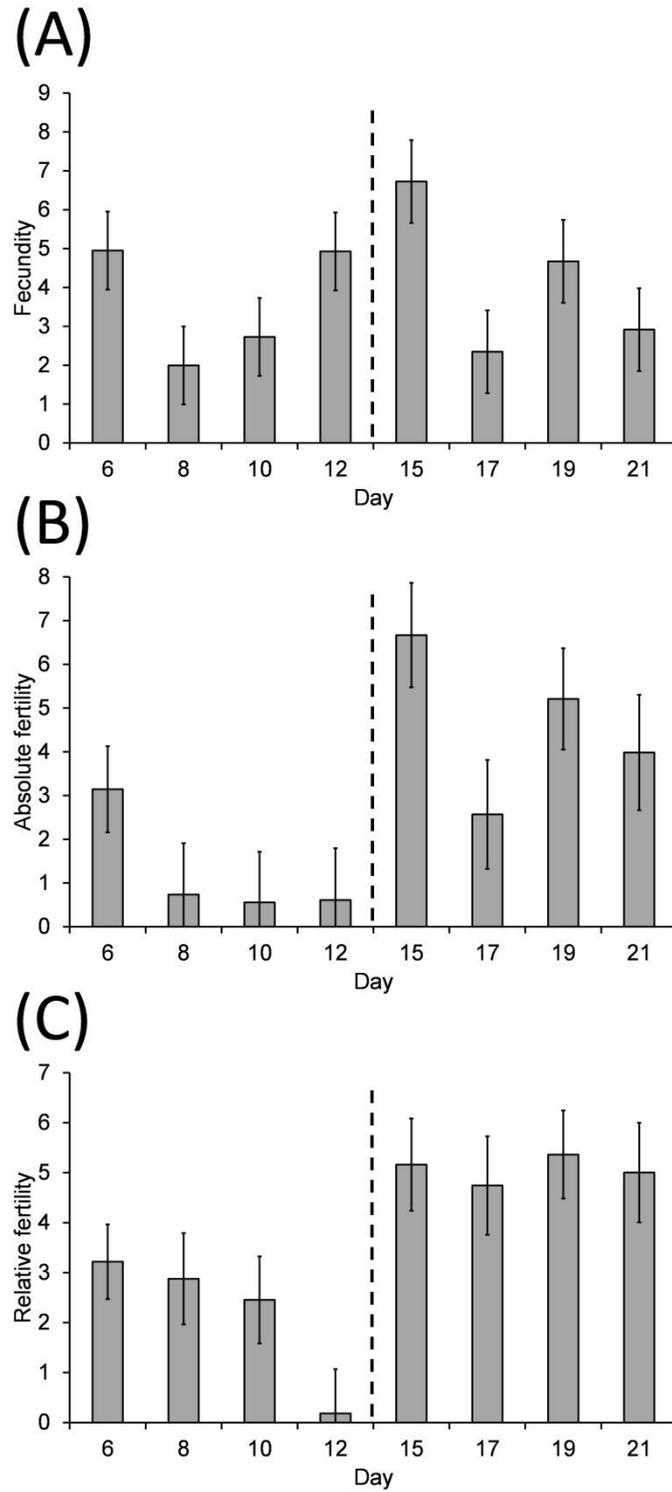
Fig. 3. Variation in LSM female fecundity (A), absolute fertility (B) and relative fertility (C) during the experiment. Black dashed line indicates timing of single additional mating. Error bars show ±SEM.
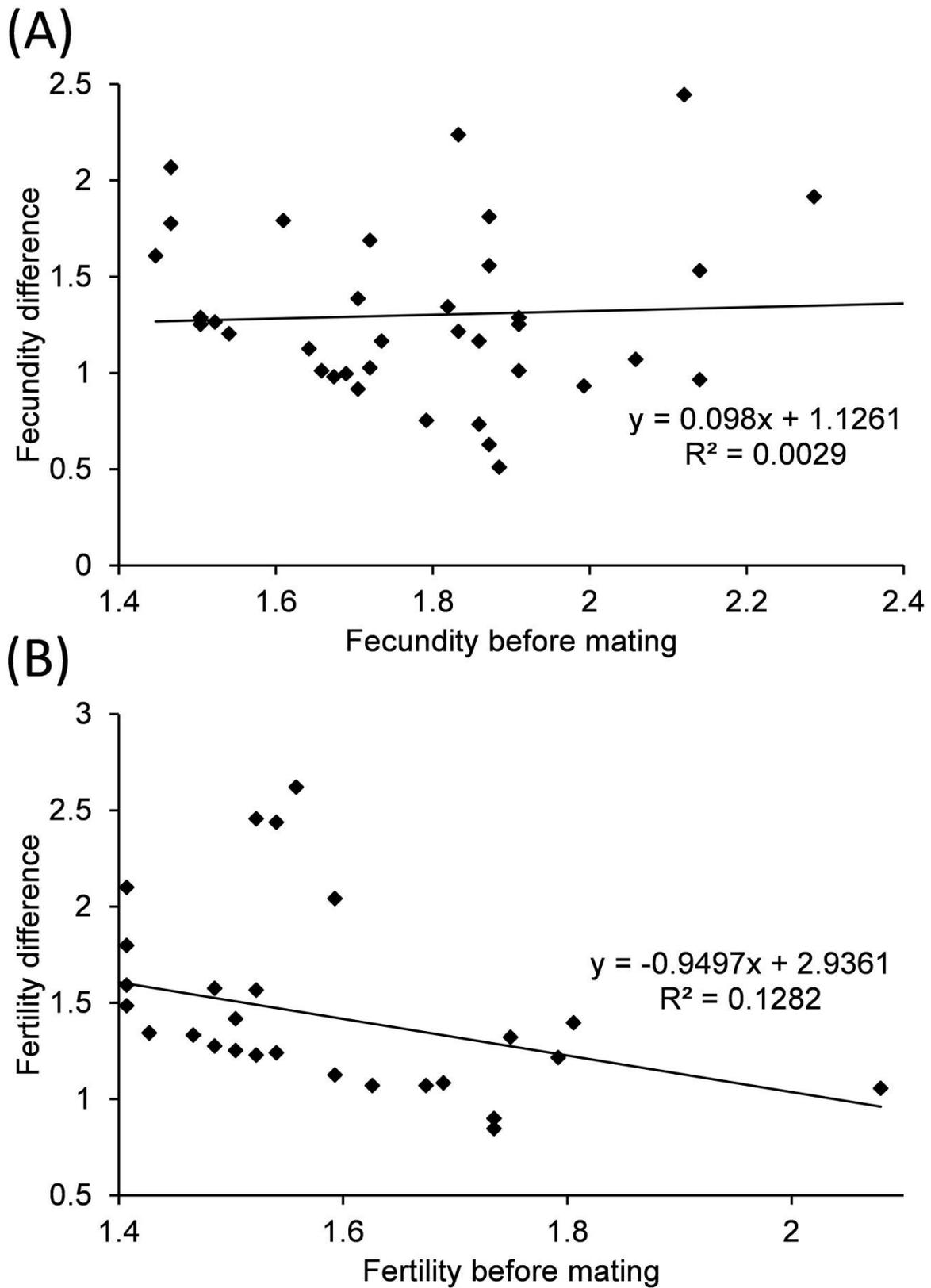
Fig. 4. Relationship between per diem reproductive output before mating, and the reproductive output differences for (A) fecundity and (B) and fertility.

0.0674), albeit non-significant. However, the lack of statistical association was caused by a single outlying female. Removing this female resulted in a strong negative association between relative fertility and the degree of fertility change experienced ($F_{1,28} = 26.1866$, $p <0.0001$), confirming that females with lower relative fertility gained greater fertility benefits as a result of the single mating.

I observed no geographic variation in female fecundity across the 13 stream sites either before ($F_{10,33} = 1.5905$, $p = 0.1530$) or after the additional mating ($F_{10,26} = 1.1230$, $p = 0.3827$). In contrast, before the additional mating, geographic variation explained a substantial amount of the overall variation absolute fertility ($r^2 = 0.4753$, $F_{10,33} = 2.9892$, $p = 0.0086$) and relative fertility ($r^2 = 0.5056$, $F_{10,32} = 2.4942$, $p = 0.0243$). However, the additional mating abolished this geographic pattern (absolute fertility: r^2, $F_{10,26} = 0.9990$, $p = 0.4695$; relative fertility: r^2, $F_{10,26} = 1.0100$, $p = 0.4622$).

Female eyespan was positively correlated with female fecundity both before ($F_{1,40} = 5.2119$, $p = 0.0278$) and after ($F_{1,35} = 10.594$, $p = 0.0025$) the single additional mating. Before the additional mating neither aspect of fertility was associated with female eyespan (absolute fertility: $F_{1,39} = 0.0212$, $p = 0.5005$; relative fertility: $F_{1,39} = 0.0454$, $p = 0.8325$). Following the additional mating, female eyespan was positively correlated with absolute fertility ($F_{1,34} = 6.1689$, $p = 0.0181$), but not with relative fertility ($F_{1,33} = 0.0031$, $p = 0.9562$).

5.3.2 Sperm depletion and female sexual receptivity

Sperm depleted females took less time to engage in their first copulation than recently mated females (fig. 5; D: 1082.1935±131.2053s; RM: 1463.6667±127.1673; $F_{1,62}$ = 4.3587, p = 0.0409). Copulation duration did not differ significantly between sperm depleted and recently mated females (D: 57.5485±2.1317s; RM: 56.8197±2.0325s; $F_{1,61}$ = 0.0612, p = 0.8054), nor did the time between copulations (D: 387.8425±30.6825; R: 418.0952±33.0280; $F_{1,22}$ = 0.0200, p = 0.8890). In addition, the mean number of matings that occurred also did not differ between the two groups (D: 3.6875±0.4935 matings; RM: 3.4130±0.5041 matings; $F_{1,92}$ = 0.1514, p = 0.6981). I found that the time to first mating had a strong negative effect on the number of matings in the recently mated group ($F_{1,31}$ = 19.7307, $p<0.0001$), but not in the depleted group ($F_{1,28}$ = 2.3603, p = 0.1357). After taking time to mating into account as a GLM covariate, the mean number of matings still did not differ between the depleted and recently mated females (D: 5.5054±0.4623 matings; RM: 5.1162±0.4399 matings; $F_{1,60}$ = 0.3575, p = 0.5522).

5.3.3 Sperm storage and female fecundity

Despite the geographic fertility variation observed in my earlier study (chapter 4, Harley et al., 2010), I found no effect of stream site upon the number of pouches in the ventral receptacle ($F_{11,162}$ = 1.0479, p = 0.4071), the number of filled pouches (absolute: $F_{11,162}$ = 1.7023, p = 0.0770, relative: $F_{11,161}$ = 1.7593, p = 0.0652) or female fecundity ($F_{11,163}$ = 0.6920, p = 0.7449). Nonetheless, stream was included as a covariate in further analyses due to the previous observations.
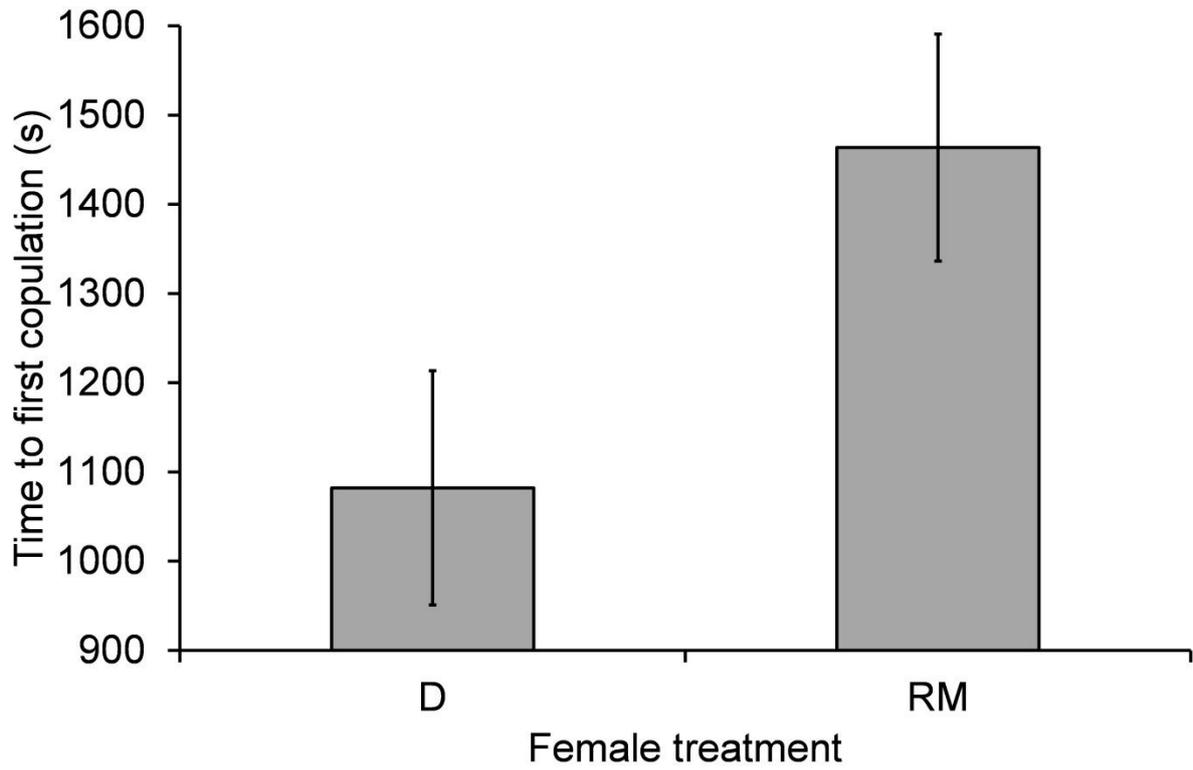
Fig. 5. Time taken until first copulation for sperm depleted (D) and recently mated (RM) females. Error bars show ±SEM.

Amongst wild caught flies the ventral receptacle contained on average 27.3086±5.7141 pouches (mean±s.d, $n = 175$), with a range of 11- 47 pouches. The total number of pouches increased with female eyespan ($r^2 = 0.1142$, $F_{1,171} = 23.2951$, $p <0.0001$). The mean (±s.d.) number of ventral receptacle pouches that were filled with sperm was 8.7886±7.3848, with a range 0 - 100% relative to the total number of pouches. The absolute number of filled pouches was strongly related to the total number of pouches ($F_{1,173} = 23.6738$, $p<0.0001$). However, there was no association of the number of sperm present in the ventral receptacle in either absolute or relative terms (taking account of total pouches) with female eyespan (absolute: $F_{1,171} = 0.3493$, $p = 0.5553$; relative: $F_{1,170} = 0.0517$, $p = 0.8205$).

Female fecundity, measured on dissection as the number of mature eggs in ovaries, was found to increase with female eyespan ($F_{1,172} = 30.7522$, $p < 0.0001$) and total number of pouches in the ventral receptacle ($F_{1,173} = 10.3164$, $p = 0.0016$). As these variables covary (see above) I repeated the analysis with a GLM that included eyespan as a covariate. I found no independent relationship between fecundity the number of pouches in the ventral receptacle ($F_{1,171} = 2.8256$, $p = 0.0946$). However, I did find a positive association between female fecundity and both the absolute ($F_{1,172} = 7.8440$, $p = 0.0057$) and relative ($F_{1,171} = 4.2549$, $p = 0.0406$) number of filled ventral receptacle pouches. These relationships held when eyespan was included as a covariate (absolute: $F_{1,171} = 8.0018$, $p = 0.0052$; relative: $F_{1,170} = 5.6214$, $p - 0.0178$).

5.4 Discussion

I extended my investigation of female fertility in wild stalk-eyed flies (Harley et al., 2010) by examining the prediction that females with relatively empty sperm storage organs gain greater fertility benefits upon receipt of additional sperm. In line with previous studies, wild female *T. dalmanni* fecundity did not vary with time in captivity (Harley et al., 2010) and was not affected by the additional mating. Also in line with previous work, wild females were shown to have low (~35%) and highly variable (22 – 47%) fertility, which declined with time in captivity in terms of both the absolute number of fertilised eggs and the relative number of fertile eggs laid (Cotton et al., 2010, Harley et al., 2010). However, unlikely Harley et al. 2010, the sperm received from single additional mating caused a significant increase in female fertility (absolute and relative). I also found that the increase in fertility post-mating was positively correlated with existing female fertility as less fertile females experienced a significantly higher elevation in their fertility as a result of mating than did highly fertile females (both for absolute and relative fertility).

These results suggest that females in the wild are generally sperm-limited, giving rise to low levels of fertility. This view is supported by the finding that infertility increased through time, presumably because the lack of mating exacerbated the level of sperm depletion. Female sperm-limitation is a natural feature of wild populations, shown by the large geographic range in fertility before mating (3% - 88%) as well as the variation in the number of sperm stored in the ventral receptacle (2 – 13 sperm). Why this variation between different stream sites exists is currently unknown. Mating rate may directly covary with population density. Alternately, some

streams have a greater number of available lek sites (Harley, personal observation), which could provide more mating opportunities for females. Further studies should examine the effect of lek site number and population density upon female mating rate and subsequent fertility.

Laboratory populations of *T. dalmanni* typically mate at high rates (4 - 8 times per hour, Wilkinson and Reillo, 1994), which is presumed to occur in order for females to replenish their sperm levels. My findings suggest that sperm depletion from the storage organs acts as a stimulus for re-mating in wild *T. dalmanni* also. However, if re-mating rate is solely determined by number of sperm stored then female re-mating would occur with a periodicity of 13 days, when the sperm benefits of receiving an additional mating are highest. The mating rate of wild females could be quantified by marking individual females so that their visits and behaviour at different lek sites can be recorded on a daily basis. Given the laboratory evidence a low re-mating rate is highly unlikely in wild females, suggesting that there are additional non-sperm benefits associated with multiple mating. This hypothesis is further supported by the lack of reduced sexual receptivity observed in recently mated females. While I observed reduced time to first copulation in sperm-limited females compared to recently mated females, I could find no other indicators of increased receptivity in sperm-limited females: number of copulations and mean copulation duration were the same for recently mated and sperm-limited females. This agrees with a similar study in *T. dalmanni* (Grant et al., 2002), which found no effect of mating upon female receptivity.

The effect of mating upon receptivity is by no means consistent across insect taxa. While there is extensive evidence from *D. melanogaster* that mating reduces sexual receptivity in females (reviewed in Qazi et al., 2003), the story is much more variable in other species. Female Mediterranean fruit flies are more likely to re-mate if they store few sperm from their first partner (Mossinson and Yuval, 2003), but the receptivity of Queensland fruit fly females did not associate with the number of sperm stored (Harmer et al., 2006). In these examples, female receptivity may be inhibited by the physical presence of accessory fluids in the reproductive tract rather than sperm. However, this effect does not account for the lack of mating-induced female receptivity decline in *T. dalmanni*. Females storing large quantities of sperm may be equally receptive to further matings as sperm-limited females if they can gain genetic benefits as a result of mating and/or avoid the detrimental effects of X-linked meiotic drive (both factors discussed in chapter 4).

The complexity of the *T. dalmanni* female sperm storage system may enable females to select genetically superior sperm by spatially isolating the ejaculates of different males and selecting preferred sperm for fertilisation (Hellriegel and Ward, 1998, Pitnick et al., 1999, Qazi et al., 2003). Females may well mate beyond the point of diminishing fertility returns (Gershman, 2007) in order to counteract the reduced-fertility effects of meiotic drive-carrying sperm (Wilkinson and Sanchez, 2001) or to find a more genetically compatible partner (Zeh and Zeh, 2006). While the exact mechanism of sperm storage in *T. dalmanni* is not known, it is possible to infer potential functions from studies in related species or those with similar reproductive architecture. The *T. dalmanni* female reproductive tract is highly similar to that of the yellow dung fly *Scatophaga stercoraria*, with two distinct spermathecal

organs (a doublet and a singlet). *S. stercoraria* females store different proportions of sperm from a second male in the different spermathecae, which appears to be regulated by muscular activity in the female reproductive tract (Hellriegel and Bernasconi, 2000).

Further evidence of female regulation of sperm storage comes from *D. melanogaster*. Genetically manipulated *D. melanogaster*, with female bodies and 'masculinised' nervous systems, stored fewer sperm than control females, and the majority of sperm stored were in the ventral receptacle (Arthur et al., 1998). The *T. dalmanni* female reproductive tract is likely to have a similarly active role in manipulating sperm competition. A suite of microsatellite markers exist for *T. dalmanni* (Wright et al., 2004), which may make identifying the sperm of rival males in the reproductive tract a possibility for the future. However, isolating a sperm sample from the reproductive tract is very difficult. Alternately, paternity could be used as an indirect measure of how "preferred" a male's sperm is.

If females are subjected to unwanted copulations through male coercion, multiple mating and increased reproductive tract complexity may be adaptations to counteract the receipt of unwanted sperm (Hellriegel and Bernasconi, 2000). The occurrence of forced copulations may also explain why there was no effect of recent mating upon female sexual receptivity. In the South African stalk-eyed fly, *Diasemopsis meigenii*, females exhibit an unambiguous rejection response in the presence of unwanted males (Cotton et al., 2006), such that copulations cannot be forced. The reproductive tract of *D. meigenii* is greatly simplified compared to that of *T. dalmanni*; spermathecal function has been lost and the ventral receptacle is

enlarged and appears to function as the primary organ of sperm storage. Atrophied spermathecae and enlarged ventral receptacles have also been observed in other *Diasemopsis* species (Presgraves et al., 1999).

An alternate explanation for the fertility changes observed during this investigation could be due to a male sperm effect. The males for this investigation were collected at the same time as the females, and housed in the field laboratory for 12 days before being mated to the experimental females. Males in the wild could be close to sperm exhaustion, caused by sustained multiple mating with minimal recovery time. Isolation from females during the study, and the beneficial conditions provided in the laboratory could have resulted in increased male fitness. This is supported by the observation that female fertility did not decline over the 8 days following the additional mating. In contrast, fertility declines in the pre-mating phase of the experiment, suggesting that wild males transfer smaller numbers of sperm per ejaculate than laboratory males. To distinguish between male and female effects, further field investigations should examine how male health associates with the fertility of their partners. One way to test this would be to compare the fertility of females mated to either recently captured males, or males that have been housed in the laboratory for a length of time.

I found a positive correlation between the number of sperm stored in the ventral receptacles of wild *T. dalmanni* and the number of mature eggs in their ovaries. This suggests that males invest more sperm into matings with more fecund females; an effect that I have already reported in the South African stalk-eyed fly *D. meigenii* (chapter 2). This hypothesis is supported by the finding that following a single

additional mating, the fertility of wild female *T. dalmanni* was positively correlated with female eyespan, a potential phenotypic advertisement of fecundity. I found no association between the number of sperm stored and female eyespan. It is possible that the design failed to fully quantify the number of sperm in the reproductive tract. The importance of the ventral receptacle as a sperm storage site is assumed based upon studies using *D. melanogaster* (reviewed in Qazi et al. 2003). However, there is evidence in *T. dalmanni* that males may attempt to force their sperm into the spermathecae. During mating a process on the top of the male intromittent organ is forced into the opening of the spermathecal ducts (Kotrba and Huber, 2011), suggesting that sperm may be preferentially deposited close to or even within the duct openings. The length of the ducts appears to covary with intromittent process length across multiple species, including *T. dalmanni* (Kotrba and Huber, 2011). This has been interpreted as a female evolutionary response to possible male manipulation of sperm storage. As a result the spermathecae may be a more important sperm storage region for males. The number of chambers in the ventral receptacle is far smaller than current estimates of ejaculate size (average of 27 pouches vs. ~100 sperm transferred). Males may risk wasting their sperm if they try to force them into the ventral receptacle, particularly if the sperm from another male are already present. Further studies could determine whether males preferentially deposit sperm in the spermathecae or the ventral receptacle. This could be done by examining the relative numbers of sperm stored in both sets of organs following the first mating of a virgin female.

My results suggest that sperm storage and sperm depletion potentially have an important role in the evolution of mating rate in wild female *T. dalmanni*. Fertility and

sperm storage are naturally variable, and as a result there are obvious direct benefits of multiple mating. However, there may also be indirect benefits of multiple mating, as females may be able to use the complexity of their reproductive tracts to spatially isolate the sperm of competing males. Such a system may allow females to select genetically superior sperm, promote sperm competition or protect females from unwanted sperm obtained in forced copulations. While the techniques for detailed sperm storage examination in stalk-eyed flies are still in their infancy, there are a number of possibilities available for indirectly inferring how females use multiple ejaculates. *T. dalmanni*, both as a wild and a laboratory study system, are an ideal species in which to continue these investigations.

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6

Dietary stress and trade-offs between lipid provisioning and reproductive strategy

Abstract

Theoretical and empirical studies show evidence of variation in male ejaculate allocation strategy relative to attractiveness. In this chapter I place this variation in mating strategy within the context of a trade-off between reproduction and soma using the South East Asian stalk-eyed fly *Teleopsis dalmanni*. I ask whether variation in male reproductive strategy is reflected in male investment of adult-derived nutrients (dietary lipids). Male eyespan, as the determinant of sexual attractiveness, may mediate the trade-off between reproduction and soma. Large eyespan males constrain their somatic investment in favour of increased current reproduction, while small eyespan males favour investment in soma. As males also show evidence of tailoring their ejaculate investment relative to female size in *T. dalmanni*, I also examine how female size determines the outcome of reproductive trade-offs. As with the males, large females may invest more in current reproduction, while small females invest in the future. I found very little evidence, in both males and females, to support the possibility of an eyespan-mediated trade-off between reproduction and soma. Environmental manipulation studies like these can provide initial insight into the nature of reproductive trade-offs, but do not ultimately provide insight into the underlying physiology. I discuss various possibilities for more sophisticated examinations of both the consequences and the underlying physiology of reproductive trade-offs in *T. dalmanni*.

6.1 Introduction

Differential allocation of limited resources has traditionally been viewed as one of the major constraints determining the evolution of life-history traits (Harshman and Zera, 2007). Where two or more traits share a common resource pool, trade-offs between allocation and acquisition arise, such that investing in one trait requires neglecting others. (Stearns, 1989). Classic trade-off examples have considered the association between longevity and other aspects of life history such as fertility: investing in increased longevity causes reduced fertility and vice versa. *Drosophila melanogaster* females selected for increased longevity suffer reduced early fecundity (Rose, 1984), and female red deer *Cervus elaphus* experienced survival costs associated with lactation (Clutton Brock et al., 1989). In their simplest form trade-offs can be illustrated using the "Y" allocation model (fig. 1), where incoming resources are distributed between two competing traits. How these resources are allocated depends upon such external factors as fluctuating environmental conditions that require plastic resource investment strategies. For example, Colorado potato beetles histolyse their flight muscles prior to overwintering, suggesting that maintaining flight muscles under low-nutrient conditions may prove a significant energy drain (Zera and Denno, 1997).

The cost of reproduction, defined as a negative association between increased reproduction and increased survival, is one of the most prominent life history trade-offs (Stearns, 1989). Within the framework of the Y allocation model, organisms allocate a finite resource pool to reproduction and somatic growth, distributing resources evenly between the two or favouring one over the other. Which trait is

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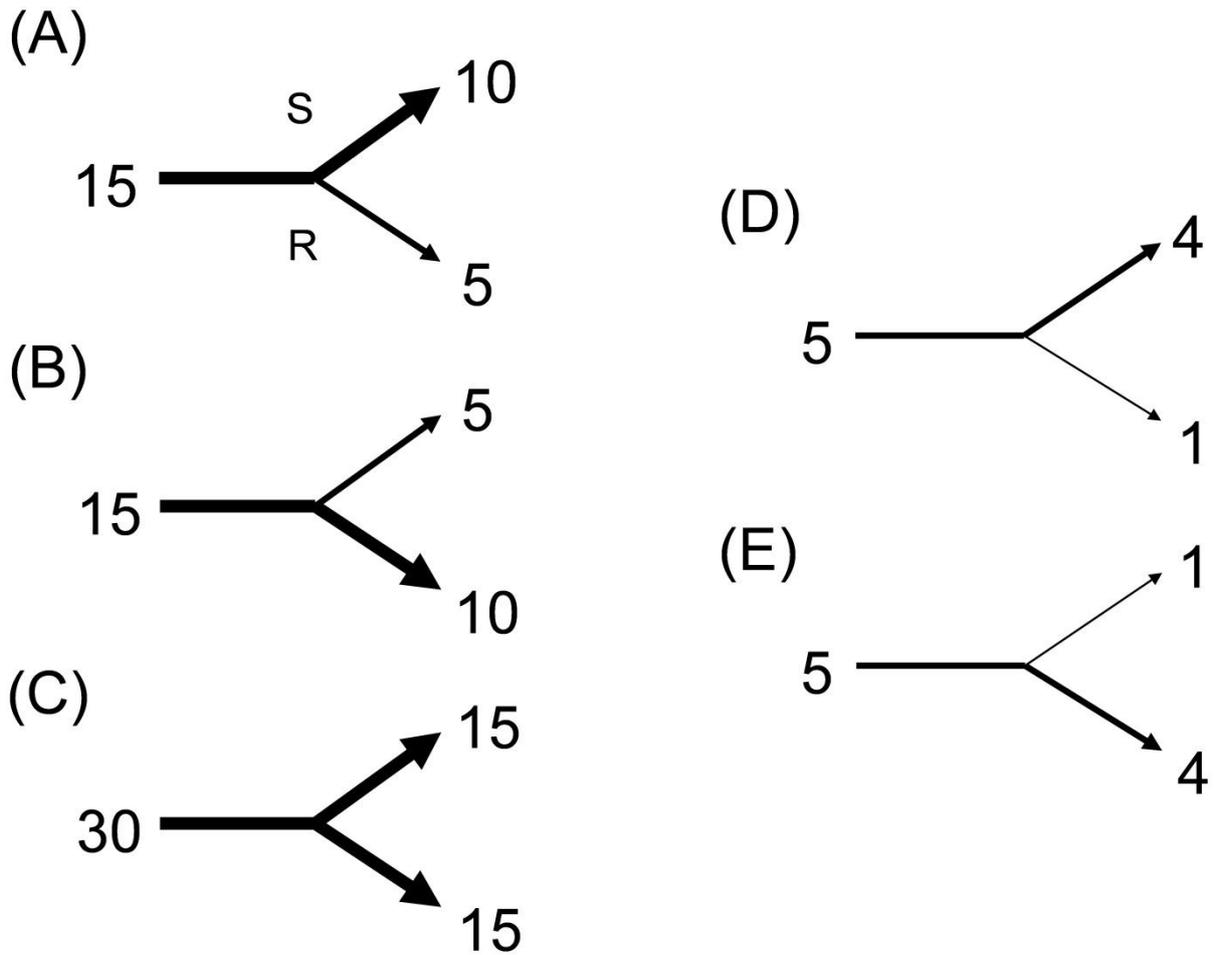
Fig. 1. Diagrammatic representation of limited resource allocation: the "Y allocation" model. Each diagram illustrates resource input (value on the left of the tree) and the pattern of resource allocation reproduction (R) and soma (S) (the values on the right of each tree). In trees (A) to (C) the maximum physiological cost of R or S equal 15. Trees (A) and (B) represent differential allocation of a limited resource. (C) illustrates how increased resource input can remove the need for a trade-off by meeting the physiological requirements of both R and S, and trees (D) and (E) illustrate how reduced nutrient input exacerbates the trade-off.

Adapted from Zera and Harshman (2001).

favoured can depend upon a range of factors. Organisms that have a defined breeding season will increase reproductive investment during this period, and favour soma during the remainder of the year. Alternately, under highly heterogeneous environmental conditions, organisms will favour somatic investment in harsh conditions in order to survive long enough for conditions to improve. Insulin signalling has been proposed as a key factor regulating this trade-off (reviewed in Harshman and Zera, 2007) as it is known to have a role in mediating the relationship between reproduction and survival. Harshman and Zera (2007) propose that insulin signalling may act to integrate the signals from the endpoints of the Y allocation model, such as food input and physiological signals from the reproductive organs and the rest of the body (summarised in fig. 1).

The discrepancy in gamete size between the sexes has traditionally been viewed as a major determinant of the cost of reproduction; males produce small, cheap sperm while females produce few, costly eggs (Bateman, 1948). In females, egg production typically makes high energetic demands upon available resources. A negative association between female survival and egg production has frequently been observed in female insects (e.g. *Drosophila melanogaster,* Partridge et al. 1987, Chapman et al. 1994; *Ceratitis capitata*, Chapman et al. 1998). In *Bicyclus anynana* butterflies, the eggs of well-fed females have higher hatching success than those fed on plain sucrose solution (Geister et al., 2008). The same energetic costs of reproduction have been largely overlooked in males, but there is evidence of a trade-off between reproduction and lifespan in males (Partridge and Farquhar, 1981). Synthesising large numbers of accessory proteins can generate non-trivial costs for males (Dewsbury, 1982). In addition there is also evidence to suggest that

spermatogenesis itself may carry energetic costs. For example, male *Plodia*

interpunctella moths reared on a low-protein diet showed reduced sperm number

compared with well-fed males (Gage and Cook, 1994), and significant trade-offs

between sperm number and sperm length have been identified in eleven *Drosophila*

species (Pitnick, 1996). Findings like these are in contrast to the traditional, but now

largely discredited, view that sperm production is energetically inexpensive. The

costs associated with ejaculate production place significant selection upon males to

be strategic with their allocation of accessory fluids and sperm to each mating.

Thus far, little consideration has been given to the possibility that variation in

reproductive strategy may result in intra-male variation in the trade-off between

reproduction and soma. In chapters 2 and 3 I discussed the evidence that males

may alter their reproductive strategy relative to their own sexual phenotype

(modelled in Tazzyman et al., 2009). Such intra-male variation in mating strategy

could also result in varying resource allocation within the framework of the

reproduction versus soma trade-off model (fig. 1). In a high nutrient environment,

both attractive and unattractive males will be able to invest equally in soma and

reproduction (fig. 1C). However, under low resource input, an attractive male might

be expected to favour investment in reproduction over soma (fig. 1B) in order to

maximise current mating opportunities. In contrast, unattractive males may constrain

their investment in current reproduction in order to increase survival (fig. 1A). These

contrasting investment strategies are likely to be exacerbated by further reduction in

nutrient input (fig. 1D and 1E).

The significant energetic costs associated with egg production (e.g. Chapman et al., 1994, Chapman et al., 1998, Partridge et al., 1987, Geister et al., 2008) could result in intra-female reproductive strategy trade-offs that are similar to those of males. A female can maximise her reproductive success through increased egg investment, particularly if males allocate their ejaculates based on a female phenotypic indicator of fecundity such as size (chapter 2, reviewed in Wedell et al., 2002). When forced into a trade-off between reproduction and soma, more fecund females may invest more dietary resources into egg production because they are more likely to get the sperm to fertilise them with. Under the same conditions less fecund females may forgo the expense of egg production when subject to dietary stress in order to prolong lifespan.

South East Asian stalk-eyed flies *Teleopsis dalmanni* are holometabolous, and are an ideal model species in which to study intra-sex variation in resource allocation. As adults, stalk-eyed flies depend entirely upon exogenous nutrient sources for growth and reproduction. Eyespan and all other external morphological traits are permanently fixed at eclosion (Buschbeck et al., 2001), but the size of male internal reproductive organs and their rate of growth is positively associated with dietary quality (Rogers et al., 2008). Male sexual attractiveness is determined by eyespan: females prefer to roost and mate with large eyespan males (Burkhardt and Delamotte, 1988). There is also evidence that ejaculate production is energetically costly for males. Sperm-limitation is a known feature of this species, with ejaculate sperm numbers estimated to be low (Rogers et al., 2006, Wilkinson et al., 2005) and females suffering declining fertility with time in isolation from males (chapter 4, chapter 5, Cotton et al., 2010, Harley et al., 2010). Males make a significant

investment of accessory proteins into each mating in the form of a spermatophore (Kotrba, 1996), and the rate of accessory fluid replenishment appears to be major factor determining male mating rate (Rogers et al., 2005b). As a result there is likely to be selection for intra-male variation in ejaculate allocation strategy. Less is known about the selective forces that shape female sexual development, but there is evidence that males prefer to mate with larger, more fecund females (Jameson-Cotton et al., in prep) and may even allocate larger ejaculates to preferred females (chapter 5). As female fecundity is positively associated with female eyespan (Rogers et al., 2006), males may well use this signal when determining ejaculate allocation.

In this study I use extractable lipid content as a measure of somatic investment. Lipids are an important energy store in insects, providing energy dense fuel that can be rapidly mobilised (reviewed in Lease and Wolf, 2011). The vast majority of species depend upon exogenous lipid sources for reproduction and development as they are unable to synthesise complex fatty acids *de novo* (Clayton, 1964). Lipids are primarily stored in the fat body, an aggregate of cells forming lobes or sheets of tissue spread throughout the body that is unique to insects (Beenakkers et al., 1985, Law and Wells, 1989). As well as storing lipids, fat body cells also synthesise and secrete large amounts of essential proteins (Arrese and Soulages, 2010). The lipid composition of the whole insect is considered to be an accurate reflection of the fat body content (Beenakkers et al., 1985). The simplest method of measure body lipid content is to apply a solvent-based extraction method (adapted from Ballard et al., 2008). While this approach is an effective way to quantify male investment in soma, solvents are not capable of distinguishing between the lipids sequestered in eggs

and those from somatic tissues. As a result this is not ideal for studying female somatic lipid investment, as any lipids present in oocytes would also be extracted. Currently there are no solvent-based extraction methods, like that of diethyl ether, which can distinguish between somatic and reproductive lipid investment in female *T. dalmanni*. I considered bypassing this shortcoming by dissecting out the female ovaries before placing females into the lipid extraction procedure. However, this is a highly invasive procedure that results in significant loss of haemolymph and as a result the majority of the circulating lipids. In the absence of a suitable alternative, I use a solvent extraction method to measure the body lipid content of female *T. dalmanni* as an approximation of investment in soma. In the Discussion I consider the flaws in this measure, and propose future protocol developments that could considerably improve our ability to quantify female somatic investment.

I used dietary manipulation to generate an adult trade-off between investment in soma (estimated as insect body lipid content) and in reproductive function (estimated as reproductive morphology and life-history trait values), which can then be examined through phenotypic observations. Laboratory-reared stalk-eyed flies are fed on pureed sweetcorn, so the availability of exogenous lipids can be manipulated by varying the percentage of corn contained in the food media. I measured reproductive investment in males as their achieved accessory gland and testis length, and in females as the number of mature eggs present in the ovaries. Larger, potentially more attractive flies may invest more in reproduction when subject to dietary stress. I tested whether eyespan and diet treatment had any effect upon my measures of reproductive investment. I measured somatic investment in adults as

their extractable lipid dry weight, and determined whether diet treatment affected how different size of flies allocated their internal resources.

6.2 Materials and methods

6.2.1 Experimental flies

In order to generate a large range of body sizes and eyespans in the sample of adult flies, I reared larvae under high nutritional stress (Cotton et al., 2004). Groups of between 13-30 eggs were placed on filter paper overlaying moist cotton wool with 0.39g of pureed sweetcorn in a Petri dish (Rogers et al., 2008).

At eclosion adult males and female were assigned to one of three diet treatments, consisting of a homogenous mixture of sucrose (25% sucrose solution and 3% carbomethylcellulose) and either 0%, 25% or 100% sweetcorn (% corn by mass). I chose these percentages in order to generate a wide range in reproductive organ lengths (Rogers et al., 2008). Reproductive organs are small at eclosion, but continue to mature and grow up to and beyond the point of sexual maturity. Adult diet during maturation is known to affect final trait values (Rogers et al., 2008). Flies were housed in mixed-sex groups to sexual maturity (defined as 28 days) in 11L clear Perspex containers lined with moist cotton wool.

At 28 days post-eclosion, flies were anesthetised on ice sexed into separate male and female groups by checking for presence or absence of an ovipositor. Morphological measurements were made of eyespan (the distance between the

outer tips of the eyestalks) and thorax length (the distance between the base of the head and a joint between the meta-thoracic legs and the thorax) (Rogers et al., 2006, Cotton et al., 2010), to a tolerance of 0.01mm using a video microscope and Image J (v. 1.45s, NIH). Flies were then randomly assigned to one of two groups to assess either their reproductive or somatic investment.

During the 28 days between eclosion and inclusion in the experiment, the mortality of both males and females was much higher in the 25% and 0% diet treatments than in the 100% treatment and this resulted in extremely uneven sample sizes among the treatments. Mean eyespan was also highly variable between the treatments, as mortality occurred more frequently in smaller flies. To correct for these issues I first reduced the variation in eyespan between the diet treatments, by excluding individuals from the 25% and 100% diet treatments that fell outside the upper and lower limits for eyespan in the 0% treatment. This approach reduced the range of eyespan measures, but did not eliminate variation in eyespan from the treatments. Secondly, I adjusted for sample size by randomly selecting individuals from within the corrected 25% and 100% diet treatment datasets to match the number of individuals in the 0% treatment. Details of the resampling for individual male and female experiments are described in the relevant sections below (6.2.3 and 6.2.4).

6.2.2 Reproductive investment

Anaesthetised males (0% $n = 26$; 25% $n = 68$; 100% $n = 99$) and females (0% n = 33; 25% n = 114; 100% n = 116) had their reproductive tracts dissected out into phosphate buffered saline (pH 7.5). I placed the paired testes and uncoiled

accessory glands of each male onto a glass slide in PBS and photographed them at 10x magnification using a Nikon CoolPix digital camera. A midline bisecting the middle of each organ was measured to a tolerance of 0.0001mm using ImageJ (v1.43e; NIH, USA), and a mean value for testis length and accessory gland length was calculated for use in the analyses.

For females, I placed the paired ovaries onto a glass slide in PBS and counted the number of mature eggs present at 10x magnification. I selected four eggs at random and photographed them using a Nikon CoolPix digital camera at 10x magnification. The length and width of each egg was measured to a tolerance of 0.0001mm using ImageJ (v1.43e; NIH, USA), and mean values of egg length and width were calculated.

I corrected for the large differences in mean male eyespan between diet treatments by calculating the upper and lower limits of the 0% diet treatment (the group with the smallest sample size), and then excluding those individuals from the 25% and 100% treatments that fell outside of those limits. Then, from within the male group, where the sample size of 0% males was $n = 26$ (mean eyespan: 8.2381±0.0976mm), I randomly selected $n = 30$ males from each of the 25% (final mean eyespan: 8.1927±0.0759mm) and 100% (final mean eyespan: 8.0167±0.0614mm) diet treatments. In the females the 0% sample size was $n = 33$ (mean eyespan: 5.5248±0.0571mm), so I randomly selected $n = 35$ females from each of the 25% (final mean eyespan: 5.5751±0.0387mm) and 0% diet (final mean eyespan: 5.4651±0.0494mm) treatments.

6.2.3 Somatic investment

The body lipid content of males (n = 810) and females (n = 960) was measured by washing their bodies in diethyl ether to dissolve any lipids present and calculating the difference in weight before and after treatment. In order to generate a detectable change in dry weight after the ether treatment I grouped flies from each diet treatment into single-sex replicates each containing five size-matched flies (males: 0% n = 15, 25% n = 42, 100% n = 85; females: 0% n = 19, 25% n = 61, 100% = 112). Flies were approximately size-matched using their relative eyespan, defined as the ratio eyespan and thorax length (Wilkinson, 1993), by selecting groups of adjacent individuals from the full range of the distribution. This gave a reasonably wide range in mean eyespan across groups.

To extract lipids from the replicates I used a solvent-based extraction method (described in Ballard et al., 2008). All replicates were flash frozen and stored at -80°C. After 20 minutes thawing each replicate was placed in an individual 10ml glass tube and oven-dried at 100°C for 48 hours. The dry weight of the replicate (minus tube) was recorded to the nearest 0.0001g. The replicate was returned to the glass tube, covered in diethyl ether and gently rocked for 24 hours in order to dissolve the lipids contained in the bodies. The diethyl ether was removed, and the replicates were dried again at 100°C for a further 40 hours to remove any residual chemical. The final weight of the replicate was recorded to the nearest 0.0001g. The difference between weight before diethyl ether treatment and weight after was calculated to determine the weight of lipids contained in each replicate.

I adjusted the data for mean eyespan differences between diet treatments in the same way as described in section 6.2.2. I matched the sample sizes of the 25% and 100% diet treatments with that of the 0% treatment (where the number of replicates was the lowest). For the males I selected $n = 20$ replicates from each of the 25% (final mean eyespan: 8.1180±0.0525mm) and 100% (final mean eyespan: 8.1081±0.0479mm). The 0% sample size was $n = 15$ replicates (mean eyespan: 8.1132±0.0593mm). For females, the 0% diet treatment (mean eyespan: 5.4226±0.0373mm) contained $n = 18$ replicates, so I selected $n = 20$ replicates each from the 25% (final mean eyespan: 5.4296±0.0211mm) and 100% (final mean eyespan: 5.3665±0.0368mm) diet treatments.

6.2.4 Statistical analysis

I began by examining the 'natural' relationship between eyespan and reproductive and somatic investment in both males and females in the absence of dietary stress, using those 100% diet flies that were not selected in sections 6.2.2 and 6.2.3. Using general linear models (GLMs). I examined the effect of male eyespan upon accessory gland and testis length. Male eyespan is strongly correlated with body size, so I calculated the effect of relative male eyespan by including male body size (thorax length) as a covariate. For females I examined the effect of eyespan upon fecundity. In females the eyespan trait is also strongly associated with body size, but is not subject to sexual selection. As a result I did not include body size as a covariate in the female models. I assessed the effect of male and female eyespan upon somatic investment by using the mean eyespan of male and female replicates,

and the total lipid content of a five-fly replicate. I also estimated relative lipid content

by including the total dry weight replicates before ether treatment as a covariate in

total lipid content models. As in the reproductive data, I examined the effect of

relative male eyespan (but not female) by including mean replicate thorax length as

a covariate.

I also used GLMs to test for diet treatment (0%, 25% and 100%) effects upon

reproductive investment in both males and females. I asked whether diet treatment

influenced the total lipid content of male and female replicates. As before I included

eyespan (or mean replicate eyespan) as a covariate in both male and female

models, and in the male models I tested for an effect of relative eyespan by including

thorax length (or mean thorax length) as a covariate. Finally I tested for an effect of

the interaction between diet treatment and eyespan upon reproductive and somatic

traits.

6.3 Results

6.3.1 Background reproductive and somatic investment information

Males

Both male accessory gland and testis length were positively associated with male

eyespan (accessory glands: parameter estimate: 0.1144, intercept: 0.7866; $F_{1,67}$ =

6.1455, p = 0.0157; testes: parameter estimate: 0.2429, intercept: 1.8598; $F_{1,67}$ =

15.6689, p = 0.0002). When body size is included as a covariate in these models,

the association with accessory gland length disappeared ($F_{1,66}$ = 2.1120, p = 0.1509) but testis length remained positively associated with male eyespan ($F_{1,66}$ = 13.6117, p = 0.0005).

Absolute measures of male somatic investment (total dry weight and absolute lipid content of the male replicates) increased with male eyespan ($F_{1,63}$ = 198.7748, p <0.0001; $F_{1,63}$ = 17.0637, p = 0.0001, respectively). Including body size did not alter the association between eyespan and total replicate weight ($F_{1,62}$ = 86.1372, p <0.0001), but removed that with absolute lipid content ($F_{1,62}$ = 2.5026, p = 0.1187). The eyespan of male replicates was negatively associated with relative lipid weight (parameter estimate: -0.0002±0.0001g, intercept: 0.0005±0.0003; $F_{1,62}$ = 11.8833, p = 0.0010). This effect remained when body size was included as a covariate ($F_{1,61}$ = 15.2548, p = 0.0002).

Females

Female reproductive investment (measured as fecundity) increased with female eyespan (parameter estimate: 3.3818±1.45272, intercept: -9.5376±7.5885; $F_{1,74}$ = 5.4193, p = 0.0227). Absolute measures of female somatic investment (total dry weight and absolute lipid content of the female replicates) increased with female eyespan. The total dry weight of female replicates was positively associated with mean replicate eyespan (parameter estimate: 0.0046±0.0003, intercept: -0.0147±0.0015; $F_{1,90}$ = 271.8073, p <0.0001), as was absolute lipid content (parameter estimate: 0.0006±0.0001, intercept: -0.0023±0.0007; $F_{1,90}$ = 20.4202, p <0.0001). Relative lipid weight was negatively associated with female eyespan

(parameter estimate: -0.0010±0.0002, intercept: 0.0029±0.0007; $F_{1,89}$ = 27.5846, p <0.0001).

6.3.2 Male reproductive investment

Male reproductive investment, measured as accessory gland and testis length, was significantly associated with diet treatment. Well-fed, 100% corn diet males had longer accessory glands (100%: 1.7636±0.0645mm, 25%: 1.4281±0.0645mm, 0%: 1.1780±0.0693mm; $F_{2,83}$ = 19.4484, p <0.0001) and testes (100%: 3.8619±0.1015mm, 25%: 3.5692±0.1015mm, 0%: 3.5045±0.1091mm, $F_{2,83}$ = 3.3843, p = 0.0386) than males fed on 25% or 0% corn diets. This association remained significant when absolute eyespan was included as a model covariate (accessory glands: $F_{2,82}$ = 21.5687, p <0.0001; testes: $F_{2,82}$ = 5.5705, p = 0.0054), and when relative eyespan was included as a covariate (accessory glands: $F_{2,81}$ = 19.9434, p <0.0001; testes: $F_{2,81}$ = 4.9611, p = 0.0093).

Male reproductive investment was also associated with male eyespan. There was a positive association of absolute eyespan with testis length (parameter estimate: 0.3145, intercept: 1.0904; $F_{1,84}$ = 4.8299, p = 0.0307) but not with accessory gland length (parameter estimate: 0.0366, intercept: 1.1956; $F_{1,84}$ = 0.0955, p = 0.7581). However relative eyespan was positively associated with both accessory gland length ($F_{1,81}$ = 5.3267, p = 0.0236) and testis length ($F_{1,81}$ = 10.8768, p = 0.0014). Taking into account the strong effect of diet treatment did not change these associations, either for absolute eyespan (accessory glands: $F_{1,82}$ = 3.3034, p =

0.0728; testes: $F_{1,82}$ = 9.0699, p = 0.0035) or relative eyespan (accessory glands: $F_{1,81}$ = 5.3267, p = 0.0236; testes: $F_{1,81}$ = 10.8768, p = 0.0014).

There was no absolute eyespan x diet treatment effect upon either accessory gland length ($F_{2,80}$ = 1.4450, p = 0.2418) or testis length ($F_{2,80}$ = 1.7619, p = 0.1783). Nor was there a relative eyespan x diet treatment effect on accessory gland length ($F_{2,79}$ = 1.1048, p = 0.3363) or testis length ($F_{2,79}$ = 1.3509, p = 0.2649).

6.3.3 Male somatic investment

The total dry weight of male replicates increased significantly with diet treatment (100%: 0.0107±0.0003g, 25%: 0.0071±0.0003g, 0%: 0.0059±0.0003g; $F_{2,52}$ = 77.5941, p <0.0001). This effect remained when mean replicate absolute eyespan ($F_{1,51}$ = 87.0663, p <0.0001) and relative eyespan ($F_{2,50}$ = 81.6540, p <0.0001) were included as covariates. The absolute lipid content of replicates also increased with diet treatment (100%: 0.0005±0.0001g, 25%: 0.0003±0.0001g, 0%: 0.0003±0.0001g; $F_{2,52}$ = 5.0861, p = 0.0096). Again, this effect remained significant when absolute eyespan ($F_{2,51}$ = 4.9887, p = 0.0105) and relative eyespan ($F_{2,50}$ = 5.0754, p = 0.0098) were included as covariates. Relative lipid content (lipids as a proportion of dry weight) showed no variation with diet treatment ($F_{2,51}$ = 1.2946, p = 0.2829), and this remained the case after controlling for the effects of both absolute eyespan ($F_{2,50}$ = 1.6713, p = 0.1983) and relative eyespan ($F_{2,49}$ = 1.5065, p = 0.2318).

Absolute eyespan was not significantly associated with total dry weight ($F_{1,53}$ = 1.4169, p = 0.2392), absolute lipid weight ($F_{1,53}$ = 0.0005, p= 0.9825) or relative lipid

weight ($F_{1,52}$ = 0.5460, p = 0.4633). When I accounted for the effect of diet treatment however, total dry weight increased with absolute eyespan ($F_{1,51}$ = 7.0142, p = 0.0107), but absolute lipid weight ($F_{1,51}$ = 0.0011, p = 0.9735) and relative lipid weight ($F_{1,50}$ = 1.2982, p = 0.2600) did not. Including relative eyespan as a covariate did not result in an association for either absolute ($F_{1,50}$ = 0.0051, p = 0.9435) or relative ($F_{1,49}$ = 1.1898, p = 0.2807) lipid content.

There was no effect of either the absolute or relative eyespan x diet treatment interaction on absolute lipid content (absolute: $F_{2,49}$ = 1.0211, p = 0.3677; relative: $F_{2,48}$ = 1.0487, p = 0.3583). Relative lipid content on the other hand was significantly affected by the absolute eyespan x diet treatment interaction ($F_{2,48}$ = 3.3109, p = 0.0450), and the relative eyespan x diet treatment interaction ($F_{2,47}$ = 3.3106, p = 0.0452). These values are only borderline significant. When the different diet treatments were examined individually there was no association between eyespan and relative lipid content in any of the treatment groups (100%: $F_{2,17}$ = 2.8568, p = 0.1092; 25%: $F_{1,17}$ = 2.3901, p = 0.1405; 0%: $F_{1,12}$ = 1.4186, p = 0.2567). There was no obvious positive or negative trend of relative lipid content with relative male eyespan either (100%: $F_{1,16}$ = 3.1893, p = 0.0931; 25%: $F_{1,16}$ = 2.4539, p = 0.1368; 0%: $F_{1,11}$ = 1.0991, p = 0.3169).

6.3.4 Female reproductive investment

Female fecundity, measured as number of mature eggs contained in the ovaries, was significantly associated with diet treatment. 100% diet females had more mature eggs in their ovaries than females fed on either 25% or 0% diets (100%:

11.2857±0.9888 eggs, 25%: 1.0286±0.9888 eggs, 0%: 0.3636±1.0182 eggs; $F_{2,100}$ = 37.9034, p <0.0001). This effect remained significant when I included absolute eyespan ($F_{2,99}$ = 41.6687, p <0.0001) as a covariate.

Fecundity was not associated with female eyespan (parameter estimate: 2.0834, intercept: -7.2028; $F_{1,101}$ = 0.6109, p = 0.4363). However, after accounting for the diet treatment as a covariate, there was a positive relationship between female fecundity and eyespan (parameter estimate: 4.6449, intercept: -25.2986; $F_{1,99}$ = 5.3420, p = 0.0229).

There was a significant eyespan x diet treatment effect upon female fecundity ($F_{2,97}$ = 5.3248, p = 0.0064). When the diet treatments were examined individually, I found no association between female eyespan and fecundity in either the 0% (parameter estimate: -0.1361, intercept: 1.1155; $F_{1,31}$ = 0.0526, p = 0.8202) or 25% diet treatments (parameter estimate: 0.1321, intercept: 0.2920; $F_{1,33}$ = 0.0041, p = 0.9490). Only well-fed, 100% diet females showed a significant positive association between eyespan and fecundity (parameter estimate: 13.0773, intercept: -60.1834; $F_{1,33}$ = 6.2012, p = 0.0180).

6.3.5 Female somatic investment

Well-fed 100% diet female replicates had greater dry weight than female replicates from the 25% or 0% diet treatments (100%: 0.0101±0.0003g, 25%: 0.0081±0.0003, 0%: 0.0077±0.0003g; $F_{2,55}$ = 19.1338, p <0.0001). This effect remained when mean replicate absolute eyespan ($F_{2,54}$ = 29.1916, p <0.001) was included as a covariate.

Absolute lipid content in female replicates also increased with diet treatment (100%: 0.0010±0.0001g, 25%: 0.0006±0.0001g, 0%: 0.0008±0.0001g; $F_{2,55} = 4.9433$, $p = 0.0106$). This relationship persisted when absolute eyespan ($F_{2,54} = 5.8640$, $p = 0.0050$) was taken into account. Relative lipid content decreased with increasing diet treatment. 0% diet female replicates had a higher relative lipid content than either the 25% or 100% diet treatments (100%: 0.0007±0.0001g, 25%: 0.0007±0.0001, 0%: 0.0010±0.0001g; $F_{2,54} = 4.1937$, $p = 0.0203$). Again, this effect was not altered by the inclusion of absolute eyespan ($F_{2,53} = 4.4159$, $p = 0.0168$) as a covariate.

The total dry weight of female replicates showed no association with absolute eyespan ($F_{1,56} = 3.3343$, $p = 0.0732$). But after the inclusion of diet treatment as a covariate, female dry weight increased with eyespan (parameter estimate: 0.0043, intercept: -0.0158; $F_{1,54} = 16.2184$, $p = 0.0002$). The absolute lipid content showed no association with absolute eyespan ($F_{1,56} = 0.8170$, $p = 0.3699$), and this effect remained non-significant even after the inclusion of diet treatment ($F_{1,54} = 2.5260$, $p = 0.1178$). Relative lipid content also showed no association with mean eyespan ($F_{1,55} = 0.0480$, $p = 0.8274$), and this effect was not changed by the inclusion of diet treatment as a covariate ($F_{1,53} = 0.5659$, $p = 0.4552$).

Finally, the interaction between female absolute eyespan x diet treatment had no significant effect upon either absolute ($F_{2,52} = 1.2041$, $p = 0.3082$) or relative lipid content ($F_{2,51} = 0.1048$, $p = 0.9007$).

6.4 Discussion

I examined how the trade-off between reproductive and somatic investment varied between males and females in the stalk-eyed fly *T. dalmanni*. I investigated the trade-off by manipulating diet quality, rearing adult males and females on high, medium and low sweetcorn content diets. Reproductive investment was recorded as reproductive organ length in males, and egg number. I used extractable body lipid content as a measure of somatic investment in males, and as an approximate measure in females (as solvent-based lipid extraction methods cannot distinguish between lipids sequestered in soma and those present in eggs).

6.4.1 Males

I predicted that large eyespan males would disproportionately invest in reproductive traits when subject to dietary stress. Larger eyespan males are more attractive and so are likely to benefit more from investment in current reproduction rather than future reproduction. In contrast, small eyespan males attract fewer females, and so probably gain more by a relatively greater investment in soma. Under benign adult conditions (100% diet) I observed that reproductive organ length increased with male eyespan. I predicted that this difference in organ length would be exacerbated by dietary stress (0% and 25% diets), with large eyespan males producing larger accessory glands and testes, representing increased investment in reproduction compared to small males.

I found that male eyespan associated positively with reproductive organ length in all diet treatments, with no change in this relationship when males were subjected to dietary stress. This result was also observed by Rogers et al. (2008), who proposed the explanation that the essential relationship between male eyespan and reproductive organ length is established during the larval stage. As a result adult diet manipulation may change the size of the organs (as observed) but not the nature of the association between eyespan and organ length. Stress during the larval environment may induce long-lasting physiological changes affecting the allocation of resources in the adult. For example, in *D. melanogaster* high larval density induces the expression of a heat-shock protein that ultimately leads to increased adult longevity (Sorensen and Loeschcke, 2001). In *T. dalmanni*, males that invest in the production of a large eyespan at the larval stage may as a result have adopted a life-history strategy that requires the production of large reproductive organs.

Male reproductive organ length may not be an adequate measure of male reproductive investment. While the size of male testes may be an indicator of the number of sperm present, this measure gives no indication of whether spermatogenesis is successful, or whether any sperm are in fact viable (Dowling and Simmons, 2012, Simmons, 2012). The same could be true for accessory glands. The length of accessory glands is strongly associated with male mating frequency in *T. dalmanni* (Baker et al., 2003, Rogers et al., 2005a, Rogers et al., 2005b), but again length is no indicator of the quality of the contents. Successful sperm transfer is dependent upon successful spermatophore construction using proteins secreted by the accessory glands (Kotrba, 1996). The rate of copulation failure has been recorded at 30% in *T. dalmanni* in males fed on a normal diet (Baker et al., 2001).

While starved males will have a reduced mating rate regardless of eyespan, due to their impaired accessory gland length, the percentage of failed matings may well increase with decreasing diet quality. If sperm quality is similarly tied to diet quality, then female infertility may also rise when females are paired with starved males. Future investigations should examine how decreasing resource availability affects male ability to produce functional ejaculates.

Male reproductive organs continue to develop after eclosion. Males become sexually mature at approximately 28 days post eclosion (Baker et al., 2003), which coincides with a plateau in accessory gland growth. However, testis length continues to increase beyond this period, finally reaching a plateau at approximately 43 days (Rogers et al., 2008). As a result the trade-off between reproduction and soma may be exacerbated in older males by the increase in sperm production, assuming a positive association between the size of the testes and the quantity of sperm that they contain. Future investigations should examine whether the relationship between eyespan and reproductive organs (and by extension the relationship between eyespan and body lipid content) change with increasing male age, under different dietary regimes.

As predicted, 100% diet males were in better condition than those that had been fed on 25% and 0% diets. I found that well-fed males were heavier, both in terms of dry weight and weight of lipids, than males from the starvation treatments. Male eyespan was also an important predictor of overall weight. As would be expected, large eyespan males were heavier than small. However, large eyespan males did not have higher quantities of body lipids than small eyespan males. This could be indicative of

variation in lipid investment relative to male eyespan: large males expend lipids in short-term reproductive investment, while small males constrain their utilisation of lipids in order to increase lifespan. Eyespan dependent variation in expenditure strategies could account for the lack of an observable effect. However, I found no clear effect of diet treatment upon the relationship between male eyespan and body lipid content, making this hypothesis difficult to confirm. A more sophisticated examination of the underlying physiology of lipid storage and utilisation should be applied in future investigations to address this hypothesis. I discuss potential methods for this later in this section.

6.4.2. Females

I predicted that highly fecund, large eyespan females would always invest more resources into egg production when compared with small females. As for the males, when access to dietary lipids is unrestricted the differences between large and small eyespan females will be smaller than under harsh diet conditions. However, females experienced a significant energetic cost of reproduction, as under harsh dietary conditions they were unable to produce large numbers of eggs. The majority of 0% females failed to produce any eggs at all, suggesting that the quantity of lipids present is not sufficient to allow for egg production. This hypothesis is supported by the fact that well-fed females had higher absolute body lipid content (including lipids sequestered in eggs) than starved females. My results also show that 0% females have the highest quantity of body lipids relative to their existing dry weight, compared to better fed females. Starved females are sequestering lipids rather than using them. However, this investigation gives no evidence that different sized females vary

their egg production strategy relative to their diet. Starvation simply eliminates a female's ability to produce eggs at all, suggesting that there is a minimum resource threshold for egg production. Eggs that are too small may fail to hatch, or may be too poorly provisioned to produce viable offspring. Future studies could compare the hatching success of eggs from females on the 25% and 100% diet treatments, and examine the relative viability of any resulting offspring.

One caveat of the analyses of trait patterns in females is that the lipid extraction method fails to fully distinguish between lipids sequestered in soma and those present in eggs. This is likely to inflate the estimates of somatic lipids in the 25% and 100% diet treatments (as these two treatments actually produced eggs). As 25% females produce 1-2 eggs on average, the contribution of egg-sequestered lipids to the overall lipid content is likely to be small. The opposite is likely to be true in the 100% treatment, where fecundity and egg size are both high. As a result, there are likely to be far fewer somatic lipids present in 100% females than this investigation has reported. As there are no solvent-based lipid extraction methods that can distinguish between somatic lipids and oocyte lipids, future studies need to consider alternate methods of estimating female somatic investment.

In *D. melanogaster* it is possible to use genetic mutants to remove elements of wild type reproductive function. For example, 'ovaryless' females can be generated via a genetic mutant that lays eggs lacking in germ cells (Bownes and Reid, 1990). These eggs ultimate develop into agametic adults; females with no ovaries. In the context of reproductive trade-offs, being able to isolate or remove reproductive effects in this non-invasive way is extremely valuable. Sadly such elegant genetic solutions are

currently unavailable for *T. dalmanni*, but there are other possible techniques that could be applied in future studies. Other insect studies have examined the quantities of circulating lipids and lipoproteins (lipid transport molecules) in haemolymph samples (e.g. Woodring et al., 1994, Lorenz et al., 1999). Samples can be extracted from the insect body without (in theory) affecting other lipid carrying organs, and as a result could effectively isolate somatic lipid investment from egg lipids.

6.4.3 General Discussion

Studies that examine trade-offs by manipulating environmental conditions and measuring phenotypic responses are limited by their inability to determine the underlying physiology of life-history trade-offs. Thus far these effects have rarely been studied in stalk-eyed flies. One study, in which a juvenile hormone (JH) analogue was topically applied to final instar larvae, suggested that JH induces a trade-off between eyespan and gonad development in male larvae (Fry, 2006). Investing in eyespan development during the final larval instar compromises the development of the reproductive organs. However, this study has a number of flaws. Our understanding of how hormones influence the expression of complex phenotypic traits in insects is currently limited (Zera, 2007). As a result, topical application of JH is unlikely to implicitly demonstrate that JH alone is mediating the trade-off. Without a full characterisation of the *T. dalmanni* JH receptors (Towlson, 2009) and a molecular measurement of the direct effects of JH (Zera, 2007) it is erroneous to assume a central role for JH in mediating a trade-off between eyespan and reproductive organ size.

As mentioned earlier, techniques exist for assessing the lipid content of insect haemolymph, and similar protocols now exist that can directly measure the haemolymph concentrations of circulating hormones (Zera, 2007). The insulin concentration of haemolymph in particular is believed to be a key mediator of the trade-off between reproduction and soma (Zera and Harshman, 2001, Harshman and Zera, 2007). Studies could begin by examining whether there are differences in insulin concentration between different male eyespans. This would begin to determine whether variation in male lipid utilisation strategies is responsible for the lack of association between male eyespan and body lipid content. Extending this experimental design to include dietary manipulation could provide valuable insight into the underlying physiology of reproductive trade-offs in stalk-eyed flies.

Ultimately, while environmental and phenotypic studies like this are a reasonable starting point for examining life history trade-offs, they do not provide all of the answers. Phenotypic approaches do not supply information about the underlying physiology of the trade-off, and these cannot be elucidated purely through phenotypic analysis. In the absence of suitable genetic models, future studies of trade-offs in *T. dalmanni* could begin to examine the detailed effects of hormones as mediators of reproductive trade-offs.

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7

General discussion

7.1 Overview

In this thesis I have used two species of stalk-eyed fly to test hypotheses concerning

male ejaculate limitation, and the resulting impact upon male mating strategy, female

fertility and reproductive trade-offs. Alongside traditional laboratory approaches, I

have also incorporated field experiments in order to place my findings within the

context of natural populations. Here, I first provide a summary of my principal

findings in a manner that describes how the results from each chapter relate to each

other. Secondly I propose possible avenues for further research arising from the

topics presented.

7.2 Summary of principal findings

7.2.1 Male ejaculate expenditure strategy

When offspring production is partly limited by the ability of males to produce

ejaculates, males are expected to distribute their reproductive effort in a manner that

optimises their opportunities to gain fertilisations. I have used two approaches to test

this hypothesis using both *Diasemopsis meigenii* (chapters 2 and 3) and *Teleopsis*

dalmanni (chapter 5). In order to determine the reproductive value of a partner,

males require an external phenotypic indicator of female quality. I showed that in

both *D. meigenii* (chapter 2) and *T. dalmanni* (chapter 5) female egg production is

positively associated with size, and provide evidence that males of both species

preferentially allocate more sperm to large females during single matings. In *D.*

meigenii this effect was determined through direct assessment of the sperm content

of spermatophores transferred during mating (chapter 2). Males transferred similar sized spermatophores to large and small females, but spermatophores transferred to large females contained more sperm. In *T. dalmanni* I used female fertility as an indirect indicator of male sperm investment in a wild population (chapter 5), and demonstrated that large females were more fertile following a single mating. I also showed that the number of sperm stored in the ventral receptacles of female *T. dalmanni* increased with female fecundity, implying that males invest more sperm in matings with highly fecund females.

In addition, new modelling frameworks (Tazzyman et al., 2009, chapter 3) have predicted that males will invest their resources relative to the cost of obtaining additional matings. Highly attractive males, whose mating cost is low, will constrain their investment of ejaculate per mating in order to successful obtain as many matings as possible. The situation is reversed in unattractive males, where the cost of obtaining additional matings is high, and thus males invest more into the current mating. This prediction is in direct contrast to that made by the phenotype-linked fertility (PLF) hypothesis, in which male attractiveness correlates with fertility and thus explains female preference for high value male traits. Using *D. meigenii* I demonstrate that male attractiveness (eyespan) does not influence ejaculate investment, measured as size of spermatophore and area of sperm within, during a single mating (chapter 2). This finding does not support the PLF hypothesis, but neither does it provide complete support for the new model (Tazzyman et al., 2009), suggesting that the model does not fully capture the selective pressures operating on sperm allocation strategy. In section 7.3.1 I discuss factors beyond the simple quantity of ejaculate that may govern the evolution of ejaculate expenditure strategy.

When the model framework is extended to cover multiple sequential matings, attractive males are expected to suffer reduced rates of ejaculate depletion relative to their less attractive counterparts. Using *D. meigenii* again I found that, over the course of multiple matings, the spermatophore size and quantity of sperm transferred by unattractive small eyespan males decreased in size at a much faster rate than those of large eyespan males (chapter 3). These findings have significant consequences for our understanding of sperm competitive outcomes in this species. Studies of sperm competition in *D. meigenii* (Bellamy, 2012) have shown no clear pattern of male sperm precedence. However, sperm depletion offers unattractive males a chance to bias paternity away from attractive males. By over-investing in a few matings, a small eyespan male may have greater precedence in sperm competition than a large eyespan male who has already exhausted his available sperm reserves.

Finally, my results from chapter 5 using wild *T. dalmanni* raise the possibility that male sperm production is associated with male condition. Wild males housed under benign laboratory conditions appeared to transfer enough sperm to prevent female sperm depletion over the course of 8 days. Previously this length of time had proved sufficient to cause significant female sperm depletion, suggesting that under natural conditions wild males may produce fewer sperm or ejaculates that are less able to fertilise.

7.2.2 Female multiple mating and sperm storage

Male sperm limitation can translate into female fertility impairment, as the sperm received during a single mating are rarely sufficient to fertilise all of a female's available eggs. Highly sperm limited females are predicted to mate at a high rate in order to replenish their stores of sperm. Wild *T. dalmanni* are known to mate at extremely high rates (Burkhardt et al., 1994) and females are severely sperm limited (Cotton et al., 2010, Harley et al., 2010). In chapters 4 and 5, I examined the direct fertility benefits obtained by females per mating, using a novel design that induced sperm-limitation through isolation from males. In chapter 4, sexually receptive females either received a mating at the point of capture ($n + 1$, where n is the natural background mating rate), or were transferred directly to captivity with no mating (n). Female fertility declined during the period in captivity, suggesting that sperm were being used and/or lost from the sperm storage organs, but an additional mating had no ameliorating effect upon the rate of fertility decline. These females were observed to be highly fertile, suggesting that females with relatively full sperm storage organs would gain smaller direct benefits per mating than females whose organs were empty. I tested this hypothesis in a second study in chapter 5, and showed that the direct fertility benefit associated with mating was negatively associated with the contents of sperm storage organs (inferred through measuring fertility). Sperm-limited females gained greater fertility benefit than those females whose organs were full (chapter 5). This result shows that a loss of sperm from the storage organs is highly likely to be a trigger for female remating. A corollary of this hypothesis is that highly depleted females will show higher sexual receptivity than those females whose organs are full of sperm. However, in an investigation using a laboratory

population of *T. dalmanni* (chapter 5) I found limited evidence of variation in female

sexual receptivity in relation to level of depletion. This suggests that while multiple

mating does furnish females with direct benefits, there may be additional indirect

benefits that motivate non-depleted females to re-mate.

7.2.3 Mating strategy and reproductive trade-offs

Males experience significant costs associated with ejaculate production, and adopt

differential allocation strategies in order to maximise their fertilisation returns. This

strategy is based upon a trade-off between increased reproduction and increased

survival, or alternately, current reproduction against future reproduction. As male

allocation strategy varies with attractiveness (chapters 2 and 3), I predicted that the

trade-off between reproduction and survival (measured here as somatic lipid

investment) would do the same. In chapter 6 compared the reproductive (accessory

gland and testis lengths) and somatic (extractable body lipid content) of a range of

different size males fed on three different diet treatments ranging from high to low

stress. I used laboratory populations of *T. dalmanni* for this investigation, as previous

studies have extensively documented the growth and development of male

reproductive organs under different dietary stress conditions (Rogers et al., 2008).

I found very little evidence that the trade-off in male investment between

reproduction and soma is mediated by eyespan. Both attractive and unattractive

males retained the same quantity of lipids within their soma, measured as

extractable lipid content, under benign diet conditions. This suggests that

unattractive, small eyespan males may be constraining their utilisation of body lipids,

implying a bias towards somatic investment over reproduction. This strategy may prolong survival long enough to enable an unattractive male to find a willing partner. Attractive males have a higher probability of encountering a willing mate, and so will gain greater fitness advantages by investing in reproduction. However, I found no evidence that dietary stress affected male utilisation of somatic lipids, or any evidence of condition-dependent reproductive investment. High stress diet conditions reduce the size of the reproductive organs, suggesting that investing in large organs represents a significant cost in terms of available resources. Male eyespan associated positively with the size of the reproductive organs, but the nature of this relationship was not altered by dietary stress. There are two possible, non-mutually exclusive possibilities to explain this phenomenon. First, the relationship between eyespan and organ length may well be established during the larval stage, when males 'commit' to producing large or small organs depending upon how much they invest in producing a large eyespan. Second, while reproductive organ length may indicate the quantity of resources available to a male, it is not necessarily an indication of the quality of the organ contents.

In chapter 6 I also applied this reproductive trade-off framework to female *T. dalmanni*. In the same way that large eyespan males are 'programmed' to produce large reproductive organs, so large females may consistently be highly fecund. Accordingly, large females may invest more into short term reproductive gains (i.e. high fecundity, low somatic investment) than small females. However, reduced resource input caused females to stop making eggs entirely, irrespective of size. If egg viability positively associated with female condition (Geister et al., 2008), then

female reproductive investment may well be determined by energy thresholds, below which it is either impossible or unviable to invest resources into egg production.

7.3 Future directions

7.3.1 Ejaculate components

Seminal fluid is known to contain biologically active components that affect male fertility through a wide variety of different mechanisms (Poiani, 2006). Male *D. meigenii* show evidence of strategic accessory protein allocation during mating designed to maximise their reproductive success (chapter 3); suggesting that ejaculate production is metabolically or energetically expensive. In *D. meigenii* (chapter 3) and *T. dalmanni* (Rogers et al., 2005a, Rogers et al., 2005b) the ability of male stalk-eyed flies to produce spermatophores is likely to limit the reproductive success of both males and females. The roles of the various ejaculate components in reproduction are poorly understood in stalk-eyed flies. In other insect species accessory proteins typically function to modify female traits (e.g. sexual receptivity, oogeneis and oviposition, reviewed in Gillott, 2003), but there is no evidence of these effects in stalk-eyed flies (e.g. Reguera et al., 2004). Accessory proteins are a key component of the spermatophore casing (Kotrba, 1996), and as such are essential for successful sperm transfer. Species of stalk-eyed fly from the genus *Diopsis* transfer either a partially formed spermatophore or no spermatophore at all (Kotrba, 1996). Simple anatomical comparisons between these species, and Diopsids that do produce spermatophores such as *D. meigenii* and *T. dalmanni* could help to establish whether spermatophore production is the sole function of the accessory glands. Members of the *Diopsis* genus would be expected to exhibit greatly reduced

accessory glands compared to *Diasemopsis* and *Teleopsis* species if the sole function of the accessory glands is to produce the spermatophore.

Organ length can be a valuable indicator of investment, but this measure gives no indication as to the nature of the contents. Empirical studies have primarily focussed upon the effect that sperm competition has upon the number of sperm in an ejaculate, with little attention paid to variation in ejaculate quality (Snook, 2005). Recent experimental evidence from the field cricket, *Teleogryllus oceanicus,* shows that males are capable of making adjustments to the viability of their sperm relative to the risk of sperm competition (Simmons et al., 2007). In the presence of a rival, males produce ejaculates that contain a greater proportion of viable sperm then when they are isolated from other males. Thus far the effect of rival male presence has not been considered when examining the mating strategies of stalk-eyed flies. Determining whether males regulate their ejaculate expenditure relative to rival male presence could be achieved using a series of very simple experiments. First, rival males could be separated from the focal male using a transparent, permeable (e.g. gauze) partition that allows for the transmission of visual and olfactory cues. Males with rivals may be expected to allocate more sperm to their ejaculates than males without. Alternately, recent results from *T. oceanicus* using *in vitro* mixtures of sperm and seminal fluid have shown the importance of seminal fluid in modulating sperm viability (Simmons and Beveridge, 2011). If increased seminal fluid increases sperm viability, male stalk-eyed flies may allocate larger spermatophores in the presence of rivals. These questions could be initially addressed by mating a male to a female in the presence of a rival, whom the focal male can detect visual and olfactory cues from, but who cannot interfere with the focal male's courtship and mating. This

simple framework could be expanded into a graded series with increasing numbers of rivals present.

Our ability to conduct assessments of male sperm viability in stalk-eyed flies is limited by access to the ejaculates. In crickets, males transfer their spermatophores externally, enabling researchers to readily collect them and isolate the contents (e.g. Simmons and Beveridge, 2011). Stalk-eyed flies transfer their smaller spermatophores internally, and once the spermatophore is present in the reproductive tract removing and examining the contents is extremely difficult. Sperm can be quantified once they are stored by the female, but this approach could easily confuse male allocation strategy with cryptic female choice. One solution could be to isolate samples of sperm from the male seminal vesicle, where mature sperm are stored prior to ejaculation. The effects of a male's seminal fluid upon his own sperm viability could be assessed by combining sperm *in vitro* with accessory gland proteins. These would be obtained by extracting and macerating the accessory glands in order to release the contents. This design could be further extended to examine how male accessory proteins affect the sperm viability of rival males.

Ultimately, a full understanding of stalk-eyed fly accessory gland proteins is not entirely possible without a full characterisation of the genes that are expressed in the accessory glands. Function can be inferred from sequence homology with known accessory gland genes from other species. For example, it may be possible to identify proteins similar to those in *Drosophila melanogaster* that manipulate female sexual receptivity, sperm storage and ovulation (e.g. Wolfner, 2002). Once these sequences are identified, and their chromosomal locations mapped, there exists an

extensive library of microsatellites for *T. dalmanni* (Wright et al., 2004), which could be used to examine the effects of allelic variation in accessory gland genes upon components of male reproductive success. The male eyespan trait has already been mapped in *T. dalmanni* using microsatellite loci (Johns et al., 2005). When genetic tools such as knock-out and knock-down become available in *T. dalmanni*, it will be possible to characterise the precise function of accessory gland genes. A set of microsatellites now also exists for *D. meigenii* (Bellamy L, Fowler K, Burke T, Dawson D and Pomiankowski A, unpublished data, see Bellamy, 2012 for details), and the stalk-eyed fly research group at UCL is currently developing a single-nucleotide polymorphism (SNP) genomic map that can be used to map the genetic locations of key traits.

7.3.2 Sperm storage

Thus far, studies examining sperm competition in stalk-eyed flies have largely considered the female as a simple vessel for sperm interaction. However, the complexity of the reproductive tract, in both *T. dalmanni* (chapter 5) and *D. meigenii* (Harley, personal observation), suggests that the female is anything but a passive player in post-copulatory ejaculate competition. Studies of sperm competition in both *T. dalmanni* and *D. meigenii* (Corley et al., 2006, Bellamy, 2012) have shown male sperm precedence to be highly variable, suggesting that either males do not invest heavily in post-copulatory competitive adaptations, or that there are significant female effects upon the competition outcome.

There is currently no concrete evidence to suggest where the primary location of sperm storage is in either species of stalk-eyed fly. Both Kotrba (1996) and Presgraves et al. (1999) assert that, in *T. dalmanni*, sperm are initially transferred to the spermathecae, and are then 'shunted' (Presgraves et al., 1999) to the ventral receptacle for fertilisation. This hypothesis is supported by recent observations made by Kotrba and Huber (2011), showing that the male intromittent organ of several *Teleopsis* species carries a process that enters the spermathecal duct openings during mating. As female common spermathecal duct length appears to co-evolve with process length, they conclude that females may evolve longer ducts in order to minimise male manipulation during mating. However, in *D. melanogaster*, males appear to preferentially deposit their sperm into the ventral receptacle (Arthur et al., 1998). The simplest way to ascertain how and where sperm move through the reproductive tract in stalk-eyed flies under natural conditions is through direct observation. Mated females (who were previously virgin) could be dissected at regular time points immediately following mating, and the locations of sperm in the reproductive tract (e.g. spermathecal ducts, spermathecae, ventral receptacle etc.) could then be recorded. This approach would be straightforward to adopt given our current methodological abilities.

The evolution of complex sperm storage organs in *T. dalmanni* may well be an adaptation by females to reduce male manipulation of fertilisation (Pitnick et al., 1999). Investigations of insect female control over sperm storage have adopted many different approaches, including nervous system manipulation (Arthur et al., 1998) and muscle inhibition using carbon dioxide (Hellriegel and Bernasconi, 2000). The latter could prove effective for studying male and female sperm storage effects

in stalk-eyed flies. By anaesthetising females using carbon dioxide immediately after mating has concluded, sperm should still move into storage but the female-regulated muscle control of the storage organs will be removed. Comparing sperm movement in anaesthetised females with control females retaining control would show whether females influence the eventual storage location of sperm.

Pitnick et al. (1999) proposed three, non-mutually exclusive hypotheses for the evolution of multiple kinds of sperm storage organs, which could be applied to a comparative study of reproductive tract morphology and function in multiple stalk-eyed fly species. The first two hypotheses explain the evolution of multiple organs types (e.g. spermathecae, ventral receptacle) as resulting from selection to specialise in more than one function. First, one type of sperm storage organ may act as a 'quarantine' chamber where sperm are treated to eliminate possible pathogens (Birkhead et al., 1993). Second, different organ types may be better specialised for either long or short term storage. The first hypothesis predicts that sperm will be consistently moved into one organ type first, a possibility that would be easy to confirm using the direct observation method described above. For the second hypothesis to be true, sperm must be preferentially used from one organ over another to demonstrate differential storage time. This could be achieved by examining sperm depletion from the different sperm storage organs through time.

The third hypothesis proposes that the evolution of multiple types of sperm storage organs is the result of the addition of 'improved' organ types, and potentially is particularly relevant to the study of *D. meigenii*. The hypothesis generates two predictions. First, where the ancestral organs are replaced, rather than continuing to

coexist with the new organ, they will be lost as they are no longer required. Second, where both organ types remain functional, the site of primary sperm storage will shift to the new organ. There are significant physical differences between the reproductive tracts of *T. dalmanni* and *D. meigenii*, suggesting that these two species are subject to different selective pressures over sperm storage. As discussed in chapter 5, *T. dalmanni* females have two spermathecae (a double organ and a singlet) and a ventral receptacle. *D. meigenii* have a ventral receptacle that is significantly enlarged, and two single spermathecae that appear to be atrophied. This observation suggests that *D. meigenii* have adopted an 'improved' organ in the form of the enlarged ventral receptacle. The spermathecae have not been lost, suggesting that they still have a role to play in sperm storage, albeit minor.

Why the ventral receptacle might have displaced the spermathecae in *D. meigenii* is unknown. There is potentially a disadvantage to having just one sperm storage organ (instead of three), as this is predicted to dramatically reduce female control over offspring paternity (Hellriegel and Ward, 1998). However, the efficiency of sperm usage may be increased (Pitnick et al., 1999), resulting in decreased sperm wastage and the occurrence of polyspermy. Sperm in the spermathecae are often tangled in a disorganised mass (Pitnick et al., 1999); by contrast, in *D. meigenii* (and *T. dalmanni*) the ventral receptacle is highly organised with each spermatid being allocated to an individual chamber. Far from reducing female control over paternity, this type of structure could actually increase female control, by potentially allowing females to expel a desired spermatid from its individual pouch at the passing egg. Determining fertilisation efficiency, by examining the numbers of sperm present in the ventral receptacle against the number of fertilised eggs laid, would be one way of

determining whether *D. meigenii* are more efficient in their sperm usage than *T. dalmanni*.

The female rejection response in *D. meigenii* (Cotton et al., 2006) may also act to increase female control over sperm storage, by preventing forced copulations. As a result rejection could serve as a pre-copulatory selection mechanism. This could be determined by a comparative behavioural and physiological examination of the *Diasemopsis* genus. Enlarged ventral receptacles and reduced spermathecae appear to be a feature of the genus (e.g. *Diasemopsis aethiopica,* Presgraves et al., 1999). If the rejection response is also inherent to the genus, and not restricted only to *D. meigenii*, then this would support the pre-copulatory selection hypothesis.

7.3.3 Genetic benefits of female polyandry

My work, and the majority of stalk-eyed fly studies that have preceded it, have focussed upon the direct benefits that females obtain from mating, specifically female fertility. However, as I discuss in chapter 5, fertility assurance does not appear to form the entire basis of female multiple mating. Future investigations should begin to examine the indirect or genetic benefits of polyandry. The hypothesis of good genes – that all females in a population will benefit from mating with a certain male genotype – is almost certainly unrepresentative of the whole story (Jennions and Petrie, 2000). Females may in fact gain greater benefits by mating with a range of male genotypes. Genetic antagonism and intra-genomic conflict are taxonomically widespread, and can be a significant source of genetic incompatibility between mates (Zeh and Zeh, 1996). As a result, polyandry may help females to

bypass incompatibility and increase their fertility. In *Gryllus bimaculatus* crickets, number of partners is a stronger predictor of female fertility than number of matings (Tregenza and Wedell, 1998), showing that certain males may be more genetically compatible with certain females. However, a similar experiment using *T. dalmanni* failed to show that number of partners can increase fertility (Baker et al., 2001).

Alternately, female polyandry may increase offspring viability, ultimately resulting in increased female reproductive success. An extension of the previous experiment could examine whether polyandrous females produce more viable offspring. Allowing the eggs to develop and recording the number of progeny that successfully eclose as adults could show how female polyandry affects offspring survival. Females may also use postcopulatory mechanisms, such as preferential sperm storage, to bias fertilisation in favour of those males that will confer greater offspring viability (Yasui, 1997). As a result, polyandrous females should gain more attractive sons than monandrous females. *D. meigenii* would be an ideal species in which to test this particular hypothesis, as offspring attractiveness could be measured not only through the eyespan of male offspring, but also via female preference for male eyespan leading to elevated rejection rates of unattractive sons relative to those of attractive sons.

7.3.4 Field observations of Diasemopsis meigenii

D. meigenii are rapidly becoming an important model organism for studying sexual selection, from the work on ejaculate allocation and mating strategy presented in this thesis, to detailed examinations of female preference. However, our knowledge of

the fundamental natural history of *D. meigenii* is sorely lacking. Many assumptions about the behaviour of *D. meigenii* have been made based upon existing knowledge about *T. dalmanni*. For example, *T. dalmanni* are known to require a mating longer than 30s for successful sperm transfer to occur (Lorch et al., 1993), and this same logic has been applied to subsequent studies of *D. meigenii* (e.g. Small, 2009). However, during recent studies it has become apparent that *D. meigenii* not only mate for a lot longer than *T. dalmanni* (*T. dalmanni* <1 minute; *D. meigenii* 3 – 9 minutes), but that successful sperm transfer only occurs after at least 150s of mating (Harley et al, unpublished data). This example demonstrates that without a better understanding of the natural behaviour of *D. meigenii*, our ability to interpret experimental results fully is limited. Furthermore we are at risk of making incorrect assumptions based upon the biology of a behaviourally and physically distinct species, and we cannot be entirely sure that our studies and findings are representative of the evolutionary processes at work.

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8

Appendices

Appendix 1: Calculation of sperm content using pixel counting

Sperm overlap with one another within the spermatophore, making sperm number very difficult to quantify. Therefore a programme was designed and written by Brian K. Birge (NASA) for estimating the percentage area of a spermatophore occupied by sperm using the numerical computing environment Matlab.

The programme analyses digital images of spermatophores and counts pixels based upon brightness. Sperm are distinguishable as they are typically paler than the surrounding images. When making measurements I equalised the image contrast and converted the colours to grey scale. The boundary of the spermatophore excluding the tubular neck was manually marked. An arbitrary threshold was then chosen which on visual inspection classified sperm and non-sperm accurately. If the sperm were dispersed through a spermatophore then I were able to define a sub-region of the spermatophore within which to count sperm pixels. This improved accuracy by avoiding the mis-counting of paler pixels as sperm in regions of the spermatophore where visual inspection revealed that there were none.

The programme returned the percentage of the total spermatophore area that was occupied by sperm pixels. Using my independent measurements of sperm content (see Materials and Methods) I converted these percentages into absolute measurements, using the following equation:

$$sperm\ content = \frac{programme\ output\ (\%\ area) \times spermatophore\ area}{100}$$

Appendix 2: Model of optimal sperm allocation during single matings

Model by Samuel J. Tazzyman

Background

Suppose there are two types of females in the population: a proportion q of normal females, and a remaining proportion $1 - q$ of super-fecund females. The normal females have fecundity 1, while the super-fecund females have fecundity $1 + h$.

Strategy

A male's strategy is a vector $\mathbf{s} = (s_1, s_s)$ describing the quantity of resources he will assign to a mating with female types 1 and 2. Since both s_1 and s_2 must be positive real numbers, we can also characterise a male's strategy as being $\mathbf{t} = (s_1, x)$, where x is the (positive real) coefficient such that $s_2 = x \, s_1$.

Males have two key parameters, their quantity of resources R, and the resource cost that they must pay to obtain a mating, c. A male's strategy will be conditional upon these parameters, so that it will be a function $\mathbf{s}[R,c]$. The optimal strategy for a male will also depend upon what the rest of the population is doing. We denote the mean population strategy as $\overline{\mathbf{s}} = (\overline{s}_1, y\overline{s}_1)$, where y is the (positive real) coefficient such that $y\overline{s}_1 = \overline{s}_2$.

Fitness function

The fitness function for a male with resources R, and cost c (hereafter referred to as an (R,c)-male) playing strategy $\mathbf{s}[R,c]$ is defined as

$$W[\mathbf{s},R,c|\overline{\mathbf{s}}] = n[\mathbf{s},R,c] \cdot v[\mathbf{s},R,c|\overline{\mathbf{s}}]$$

where $n[\mathbf{s},R,c]$ is the expected number of matings, and $v[\mathbf{s},R,c|\overline{\mathbf{s}}]$ is the expected success per mating. If we denote the partial derivatives of W with respect to s_1 and to s_2 by W_1 and W_2 respectively, then a strategy $\mathbf{s}$ can only be a best reply to a population mean strategy $\overline{\mathbf{s}}$ if

$$W_1[\mathbf{s},R,c|\overline{\mathbf{s}}] = W_2[\mathbf{s},R,c|\overline{\mathbf{s}}] = 0 ,$$

and we also have that for $i = 1,2$,

$$W_i[\mathbf{s},R,c|\overline{\mathbf{s}}] = n_i[\mathbf{s},R,c] \cdot v[\mathbf{s},R,c|\overline{\mathbf{s}}] + n[\mathbf{s},R,c] \cdot v_i[\mathbf{s},R,c|\overline{\mathbf{s}}]$$

where n_i and v_i refer to partial derivatives with respect to i.

Number of matings

We define the expected number of matings by

$$n[\mathbf{s}, R, c] = \frac{R}{c + qs_1 + (1-q)s_2}.$$

This is the ratio of the quantity of resources that the male possesses to the average quantity of resources he uses per mating. This will be only an approximation to the expected number of matings given $\mathbf{s}$, R, and c, but simulations have shown it to be a reasonable approximation under a range of parameter values (results not shown).

We then have

$$n_1[\mathbf{s}, R, c] = \frac{-qR}{(c + qs_1 + (1-q)s_2)^2},$$

$$n_2[\mathbf{s}, R, c] = \frac{-(1-q)R}{(c + qs_1 + (1-q)s_2)^2},$$

(1)

so that $n_2/n_1 = (1-q)/q$.

Success per mating

The expected success per mating is then defined as

$$v[\mathbf{s}, R, c | \overline{\mathbf{s}}] = \sum_{k=0}^{\infty} \frac{\overline{n}^k e^{-\overline{n}}}{k!} \left(\frac{qs_1}{s_1 + k\overline{s}_1} + \frac{(1-q)(1+h)s_2}{s_2 + k\overline{s}_2} \right).$$

The term in brackets in the summand represents the expected success from mating with a randomly chosen female who also mates with k other males. With a probability q this female is a normal female, and the focal male invests s_1 resources and receives expected success $s_1/(s_1 + k\overline{s}_1)$, which is the proportion of total sperm the female receives that belongs to him. With a probability $(1 - q)$, the female is super-fecund, and the focal male invests s_2 resources and receives expected success $((1+h)s_2)/(s_2 + k\overline{s}_2)$, which is the proportion of total sperm the female receives that belongs to him multiplied by her fecundity coefficient $1 + h$.

We then have that

$$v_1[s_1, R, c|\overline{s}_1] = \sum_{k=0}^{\infty} \frac{\overline{n}^k e^{-\overline{n}}}{k!} \left(\frac{qk\overline{s}_1}{(s_1 + k\overline{s}_1)^2} \right),$$

$$v_2[s_2, R, c|\overline{s}_2] = \sum_{k=0}^{\infty} \frac{\overline{n}^k e^{-\overline{n}}}{k!} \left(\frac{(1-q)(1+h)k\overline{s}_2}{(s_2 + k\overline{s}_2)^2} \right)$$

$$= \sum_{k=0}^{\infty} \frac{\overline{n}^k e^{-\overline{n}}}{k!} \left(\frac{(1-q)(1+h)ky\overline{s}_1}{(xs_1 + ky\overline{s}_1)^2} \right)$$

$$= \frac{1-q}{q} \frac{1+h}{y} v_1\left[(x/y)s_1, R, c|\overline{s}_1 \right]$$

(2)

Analysis

Suppose there exists an evolutionary stable strategy (ESS) $\mathbf{s}^* = (s_1^*, s_2^*)$ for each combination (R,c), and suppose the population is playing this strategy. Then the

mean population strategy will be $\bar{\mathbf{s}}^* = (\bar{s}_1^*, \bar{s}_2^*) = (\bar{s}_1^*, y^*\bar{s}_1^*)$. Since it is an ESS, this strategy must be a best reply to itself, i.e. for all (R,c) combinations,

$$W_1\left[\mathbf{s}^*[R,c], R, c | \bar{\mathbf{s}}^*\right] = W_2\left[\mathbf{s}^*[R,c], R, c | \bar{\mathbf{s}}^*\right] = 0.$$

Since $W_1 = 0$,

$$n_1[\mathbf{s}^*, R, c] \cdot v[\mathbf{s}^*, R, c | \bar{\mathbf{s}}^*] + n[\mathbf{s}^*, R, c] \cdot v_1[s_1^*, R, c | \bar{s}_1^*] = 0,$$

which gives

$$v[\mathbf{s}^*, R, c | \bar{\mathbf{s}}^*] = \frac{-n[\mathbf{s}^*, R, c] \cdot v_1[s_1^*, R, c | \bar{s}_1^*]}{n_1[\mathbf{s}^*, R, c]}.$$

(3)

Also

$$n_2[\mathbf{s}^*, R, c] \cdot v[\mathbf{s}^*, R, c | \bar{\mathbf{s}}^*] + n[\mathbf{s}^*, R, c] \cdot v_2[s_2^*, R, c | \bar{s}_2^*] = 0,$$

which means, from (2) and (3), and then (1),

$$\frac{1+h}{y^*} = \frac{v_1[s_1^*, R, c | \bar{s}_1^*]}{v_1\left[(x^*/y^*)s_1^*, R, c | \bar{s}_1^*\right]}.$$

(4)

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Since v_1 is a sum of positive decreasing functions of s_1, it is itself positive and decreasing in s_1. Equation (4) shows that if $y^* > (1+h)$, then $x^* < y^*$ for all (R,c). But this is a contradiction because then the mean population strategy for the population is not equal to $y^* \bar{s}_2^*$. A similar contradiction occurs if $y^* < (1+h)$. Therefore if an ESS exists it must have $x^* = y^* = 1+h$ for all (R,c): all males must invest (1 + h) times as much sperm in super-fecund females as they do in normal females.

It can be proven that the ESS strategy exists and is always of this form, and also that it will always increase in c and be independent of R, but these details are not necessary here.

Appendix 3: The Strategy of Sperm Depletion

Model by Samuel J. Tazzyman.

We assume each male begins a mating season with a fixed quantity of resources R to invest in sperm. A male's "attractiveness" to females determines the number of matings be can expect to obtain in the season, with more attractive males obtaining more matings on average. We therefore measure attractiveness in terms of the probabilities q_i that a male obtains an ith mating for various i. Since an ith mating is only possible following an $(i-1)$th mating, a male's attractiveness can be described by the infinite sequence $q_1 > q_2 > ... > q_n > ...$ (fig. 1). Given his attractiveness, a male's strategy is a partitioning of his resources into quantities assigned to each successive mating, should it occur. Thus for the ith mating, a male's strategy assigns a quantity s_i of sperm to be invested should he obtain such a mating (which will occur with probability q_i). Therefore a male's strategy can be described by the infinite sequence $s_1, s_2, ..., s_n, ...$, and since there are R (sperm equivalent) resources to be so assigned, we have

$$\sum_{i=1}^{\infty} s_i = R .\qquad(1)$$

The expected fertilization success a male obtains from a mating in which he invests s units of sperm is determined by a function $v[s]$. We assume v is continuous, twice-differentiable, increasing in s, and concave, so that $v'[s]$ exists and is positive, and $v''[s]$ exists and is negative, for all s. This captures features used in most sperm competition models (e.g. the "fair raffle"[22,23]) without having to consider the dynamics

of the competition between sperm explicitly. It allows for our model to be both simple and general.

The fitness of a male having resources R, attractiveness $\{q_i\}$ and using strategy $\{s_i\}$ is given by the expected success $v[s_i]$ over all possible matings:

$$W[\{s_i\}|R,\{q_i\}] = \sum_{i=1}^{\infty} q_i v[s_i]. \qquad (2)$$

We find the unique strategy $\{s_i^*\}$ that maximises fitness (2), given mating probabilities $\{q_i\}$ and resources R, and subject to the constraint (1). This unique solution satisfies: a) after some number of matings J, no sperm will be invested (so that for all $k > J$, $s_k^* = 0$), and b) for each $i \le J$, $s_i^* > 0$, and there is a constant $\lambda > 0$ (depending upon R and $\{q_j\}$) such that

$$q_i v'[s_i^*] = \lambda. \qquad (3)$$

This means no sperm is invested for any mating obtained after the Jth mating. Prior to this cut-off point, the quantity of sperm assigned to each mating will be dependent upon the probability of obtaining that mating, and the marginal benefit of investment in sperm. Since λ is constant for each male, and v is positive and decreasing, the higher the probability of obtaining an ith mating, the more sperm should be invested (equation 3). Because $q_1 > q_2 > ... > q_J > ...$, fewer sperm should be invested in each successive mating prior to the cut-off point J (i.e. $s_1^* > s_2^* > ... > s_J^*$), with no sperm

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assigned to matings after this point. Thus depletion is an adaptive response to a finite sperm reservoir, together with imperfect information concerning the number of matings a male will achieve.

In addition to this basic result we are in a position to make further predictions. The first concerns the quantity of resources R a male has available. Given a specific set of mating probabilities $\{q_i\}$, the constant λ in equation (3) will be a decreasing function of R. This means that as the quantity of resources R increases, the optimal amount of sperm assigned to each mating also increases, except for matings for which no sperm was assigned. The cut-off point J may also increase, so that the optimal strategy may assign non-zero values of sperm to more matings. This means that males with more resources will tend to invest more sperm in each mating, and may also manage more matings before becoming completely depleted (fig. 2).

The second prediction we are able to make concerns male attractiveness, as described by the set of probabilities $\{q_i\}$. A male expecting only a small number of matings is likely to have a sequence of probabilities $\{q_i\}$ that tails off very rapidly. For example, if a male is unlikely to get even a single mating, and is extremely unlikely to get any subsequent matings, we may have $q_1 >> q_2 > q_3 \approx 0$. Under these circumstances, although the exact form of the optimal strategy $\{s_j^*\}$ will depend on the function v, we can say from equation (3) that it will be such that $s_1^* >> s_2^* \geq s_3^* \geq ...$, with $s_2^* = s_3^*$ only in the case where both are zero. On the other hand, for a male expecting a large number of matings, we may have that although $q_1 > q_2 > ... > q_n > ...$, $q_i \approx 1$ for the first few i. In these circumstances, again from (3), the optimal strategy

will be to invest a similar number of sperm in each of the first few matings, so that for example $s_1^* \approx s_2^*$. This logic leads us to predict that (assuming equal quantities of resources are available for sperm production) more attractive males are likely to deplete at a slower rate than less attractive males (fig. 3).

Since the predictions concerning the quantity of resources a male possesses are relatively simple (if a male's resource level increases, he should simply invest more sperm in each mating and possibly last a greater number of matings before total depletion), our prediction that more attractive males should deplete at a slower rate should hold even in cases where males have different quantities of resources.
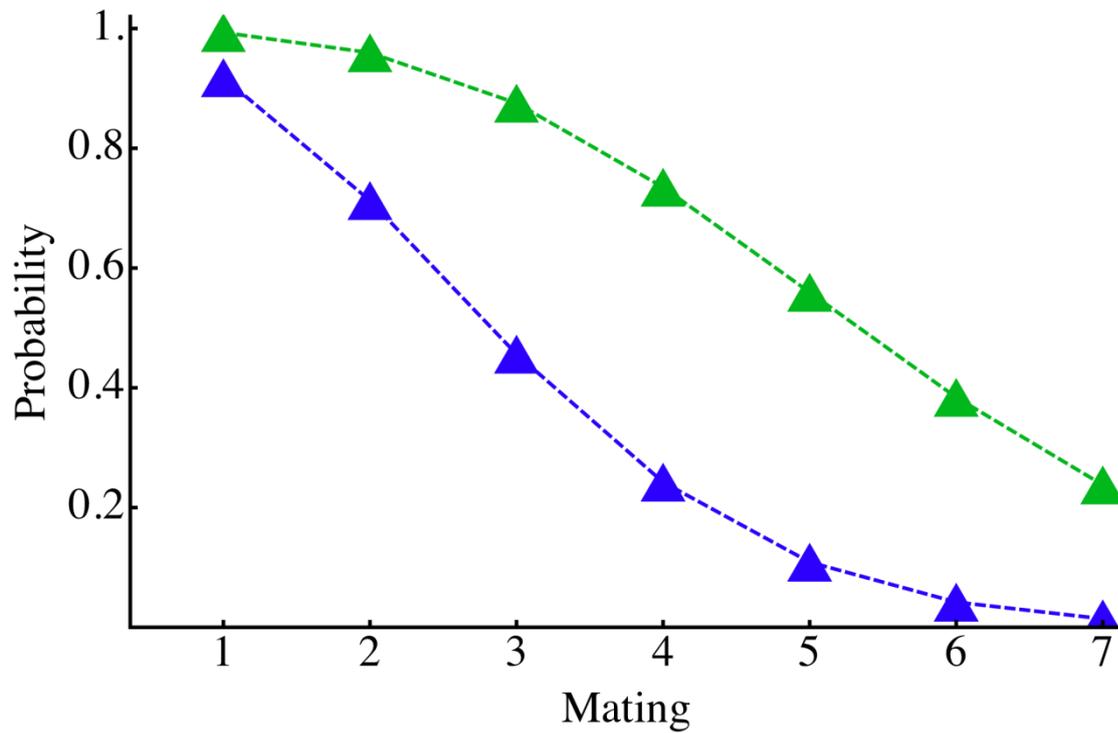
Fig. 1. Two examples of possible sets $\{q_i\}$ describing attractiveness. The sets are infinite, but have only been displayed up to i = 7. The green male is more attractive than the blue male, as he has a higher probability of achieving a first, second, third, fourth, fifth, sixth and seventh mating (and indeed of all other matings too).
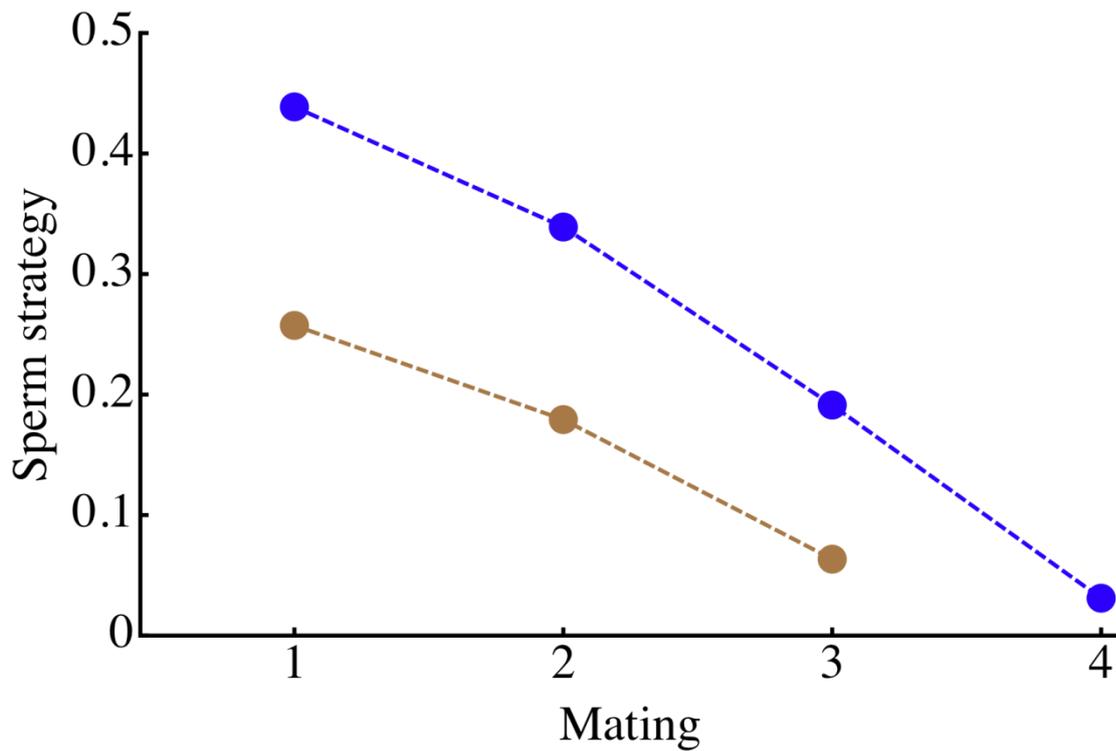
Fig. 2. Optimal strategies of two males with the same attractiveness $\{q_i\}$, but different levels of resources R. The blue male has twice as many resources as the brown male. The strategies have been plotted up to the last mating into which they invest a non-zero quantity of sperm. Thus the brown strategy invests zero sperm in the fourth mating, and both invest nothing after the fourth. The blue strategy invests more in every mating, and also lasts longer before total depletion occurs.
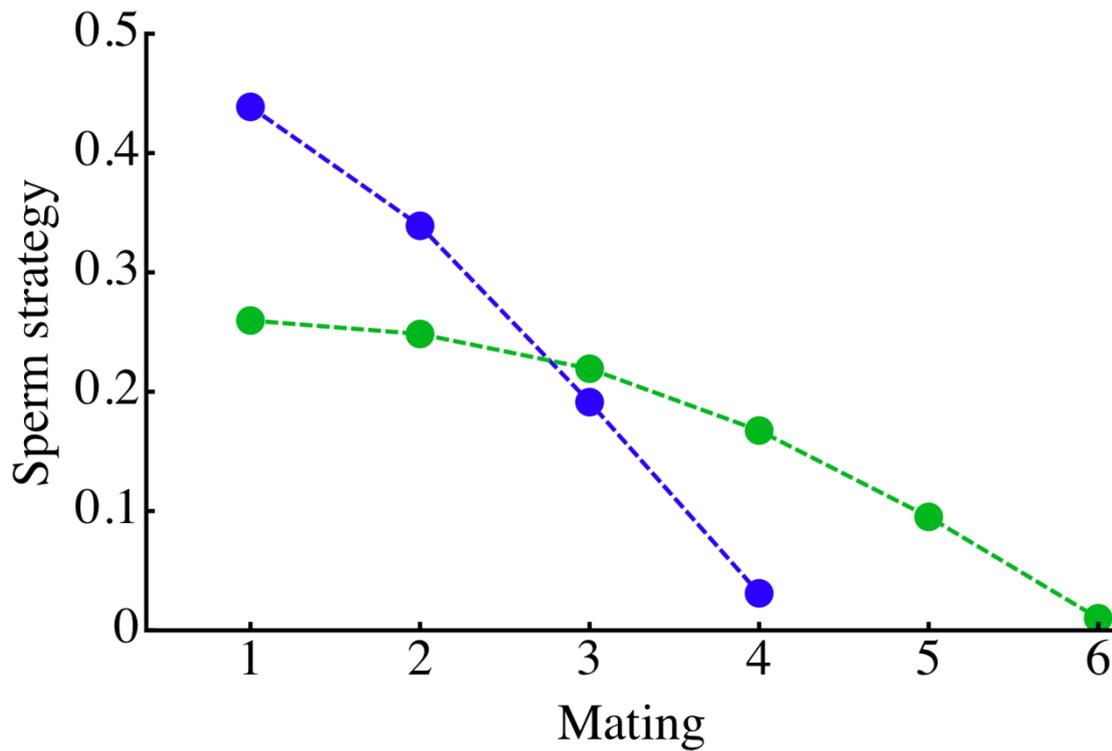
Fig. 3. Optimal strategies of two males with the same level of resources R, but different attractiveness $\{q_i\}$. The colours correspond to the different attractiveness sets seen in Figure 1. The strategies have been plotted up to the last mating into which they invest a non-zero quantity of sperm. The green male is more attractive and depletes at a slower rate than the blue male. The blue male invests more in the first two matings but less after as he depletes more rapidly. The crossover point is due to the fact that both males have R resources, and so the sum of their investments must be equal. Even if resource level differed across males, however, the more attractive green male would deplete at a slower rate.

PLoS one

No Detectable Fertility Benefit from a Single Additional Mating in Wild Stalk-Eyed Flies

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Abstract

Background: Multiple mating by female insects is widespread, and the explanation(s) for repeated mating by females has been the subject of much discussion. Females may profit from mating multiply through direct material benefits that increase their own reproductive output, or indirect genetic benefits that increase offspring fitness. One particular direct benefit that has attracted significant attention is that of fertility assurance, as females often need to mate multiply to achieve high fertility. This hypothesis has never been tested in a wild insect population.

Methodology/Principal Findings: Female Malaysian stalk-eyed flies (*Teleopsis dalmanni*) mate repeatedly during their lifetime, and have been shown to be sperm limited under both laboratory and field conditions. Here we ask whether receiving an additional mating alleviates sperm limitation in wild females. In our experiment one group of females received a single additional mating, while a control group received an interrupted, and therefore unsuccessful, mating. Females that received an additional mating did not lay more fertilised eggs in total, nor did they lay proportionately more fertilised eggs. Female fertility declined significantly through time, demonstrating that females were sperm limited. However, receipt of an additional mating did not significantly alter the rate of this decline.

Conclusions/Significance: Our data suggest that the fertility consequences of a single additional mating were small. We discuss this effect (or lack thereof), and suggest that it is likely to be attributed to small ejaculate size, a high proportion of failed copulations, and the presence of X-linked meiotic drive in this species.

Introduction

Multiple mating in insects is a widespread phenomenon that has attracted much attention and for which many explanations have been proposed [1;2;3;4;5;6;7]. From a classical perspective, males are expected to increase their fitness by mating multiply with many females, while females are assumed to require only one or a few matings to maximise their fertility [3;8]. However, multiple mating by females is characteristic in many species, despite the potentially large costs that females incur from doing so [1]. As a result much work has focussed on female re-mating to seek explanations for this apparently paradoxical behaviour [2;3].

Multiple mating by females is likely to be costly as a result of ecological risks [1;9;10], costs derived from the act of mating itself [11;12;13], and even increased rates of polyspermy or the expression of adaptations for sperm competition that reduce fertility [14]. Advantages of mating multiply are usually classed as either direct or indirect (genetic) benefits [3;4]. Females may derive direct benefits from multiple copulations when males provide nuptial gifts that enhance female fitness directly [1]. Alternatively, if males transfer insufficient sperm in a single ejaculate to fertilize all of a female's eggs [1;2], or if fertility is limited by substances other than sperm that are also transferred

during mating [15;16], then multiple mating increases female fitness directly by assuring long-term fertility [2;3]. Indirect benefits to females may also ensue if multiple mating results in the production of genetically superior offspring, for example if sperm competition engenders fertilisation of eggs by genetically superior or more compatible males [17;18].

There is good evidence from many insect species that multiply-mated females have higher fertility than those that have only mated once [19;20]. Such comparisons clearly demonstrate that multiple mating confers fertility advantages. However, why do females who have already mated multiply continue to do so? It is currently unclear whether additional matings by females that have already mated multiply also increase fertility; for example, re-mating frequency had inconsistent effects on fertility in dung flies [21], leaf beetles [22] and field crickets [23]. One might expect any immediate fertility benefit of an additional mating to decline with the frequency of female re-mating [23], if fertility approaches a maximum or if a female's sperm storage reaches capacity. Nonetheless, re-mating may still be important in the longer term if it maintains high fertility by replenishing used, lost, or dead sperm. Under such circumstances, correlations between mating frequency and fertility will be relatively uninformative about the adaptive value of female re-mating, as they reveal little about

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sperm depletion over time in females. To detect this 'hidden' fertility advantage of multiple mating requires experimental intervention that allows detection of temporal declines in fertility when females are denied additional matings [24].

The majority of investigations into multiple mating and its associated benefits in insects have been conducted under laboratory conditions [3]. Laboratory studies allow powerful, systematic and controlled investigations of mating behaviour and its consequences. However, laboratory conditions are also largely uniform and potentially unrepresentative of the natural environment under which mating traits originally evolved. In order to fully understand the forces that shape female mating behaviour, we need to also address questions concerning the benefits of female remating under natural conditions. This study is the first to examine fertility benefits associated with female multiple mating in a wild insect population.

Here, we investigate whether females gain fertility benefits from additional mating in a wild population of the polygamous Malaysian stalk-eyed fly, *Teleopsis dalmanni* (Diptera, Diopsidae). Stalk-eyed flies are characterised by lateral extensions of the head capsule, on which their eyes are located. In *T. dalmanni* the distance between the eyes (eyespan) is a sexually dimorphic trait, with males having greatly exaggerated eyespans compared to females [25]. They form nocturnal lekking aggregations at dawn and dusk, during which copulations take place [26;27]. Fights between males for control of these aggregations are typically won by individuals with greater eyespan [27], and females prefer to roost and mate with large eyespan males [24;28;29;30].

Both sexes of *T. dalmanni* are highly promiscuous and mate at high frequencies [31;32;33]. Male mating rate is heritable [33], but there is no evidence that female mating rate is genetically correlated with that of males [34]. Females usually have low fertility, and continually mated females lay a higher percentage of fertile eggs than females mated three times or those mated only once (81%, 62% and 40% respectively, [19]). This suggests that females re-mate to obtain direct fertility benefits, at least in laboratory populations. There is no evidence for fertility advantages arising from polyandry, as distinct from multiple mating, in this species [19]. The act of mating *per se* does not appear to be particularly costly, in terms of lifespan and lifetime fecundity, in *T. dalmanni* [32]. However, multiple mating may incur other, ecological costs [1;9;10]. Low fertility in female *T. dalmanni* is likely the result of sperm-limitation [19]. Males transfer few sperm during a single copulation (~ 65 [35]; ~142 [36]), and spermatophores are small [37] and unlikely to provide females with non-sperm resource benefits. Low fertility and chronic sperm limitation has also been documented in a wild *T. dalmanni* population. In a recent field study, Cotton et al. [24] found that only around 55% of eggs laid by wild females were fertilised, and that fertility declined with time when females were denied access to males. This implies that females face sperm-limitation over both the short and long-term.

We used a wild *T. dalmanni* population to test whether non-virgin wild females that received a single mating had higher fertility than a group of wild females that received an interrupted and incomplete mating. This approach allowed us to examine the fertility benefits of performing an additional mating, in an *n*+1 versus *n* mating design, where *n* is the mating frequency of females prior to the start of the experiment. We define females in the interrupted (*n*) mating group as controls and those allowed an additional (*n*+1) mating as experimental females. Given the low levels of female fertility and severe sperm limitation observed previously in this wild population [24], we asked whether a single copulation confers a significant reproductive advantage to a female

by a) increasing fertility, and b) slowing the rate at which fertility declines when a female is housed in isolation.

Materials and Methods

Fieldwork was carried out in Ulu Gombak, Peninsular Malaysia ($3°19'$ N, $101°45'$ E) during March and September 2009. Observations of females, conducted by E.H. and S.C., took place during dusk (1800 to 1930 hours) at three distinct lekking areas (LD, BW and UBW). These sites were located along two adjacent tributaries of the Gombak river that were within 100 metres of each other.

To estimate the effect of a single mating on female reproductive output, we experimentally manipulated matings between wild flies. To ensure that they were sexually mature and receptive, all focal females were chosen once they had begun copulation, defined as engagement of male and female genitalia. At this point they were randomly assigned to one of two groups. Mated (M) females ($n = 43$) were allowed to continue mating before being captured. Matings were classified as successful when copulation lasted >30s; this ensured that complete spermatophore transfer had occurred [38;39]. Interrupted mating (IM) ($n = 44$) females were separated from their mate and captured before 30s of copulation had elapsed. Matings were interrupted by using a pencil or paintbrush to gently separate the male and female. Un-manipulated females from the mated group, which copulated for <30s were reclassified into the interrupted female group. Interrupted copulations do not result in sperm transfer, although they may lead to the transfer of seminal fluid [40]. Females were captured from the leks by aspiration into a plastic bag, and transferred into individual 500 ml containers within one hour of capture. These containers were lined with a moist cotton pad and a tissue paper base, and females were fed every two days with pureed banana.

Eggs laid on the tissue paper bases were collected from the containers every two days for 10 days following capture, and allowed to develop for a further five days in a Petri dish containing a moist cotton pad and pureed banana. Egg fertility was estimated by scoring hatching success under a light microscope at 10x magnification. Fertilised eggs that have hatched appear as empty chorion cases, while unfertilised eggs are full and show no signs of development. Sometimes fertilised eggs failed to hatch, but still showed signs of development (e.g. horizontal striations in the chorion and early mouthpart formation) [19]. These eggs were recorded as fertile.

Once egg collection was completed, females were killed and stored in ethanol. On return to the UK, females were measured for eyespan and thorax length using a monocular microscope and the image analysis programme ImageJ (Version 1.43c; National Institutes of Health, USA). Eyespan was defined as the distance between the outer tips of the eyes, and thorax length was measured along a midline from the base of the head to the joint between the metathoracic legs and the thorax [24]. Both measurements were made to an accuracy of 0.01 mm.

We evaluated the factors that affected female reproductive output using general linear models (GLMs). Female reproductive output was measured as number of eggs laid (fecundity) and number of eggs fertilised (absolute fertility). Since absolute fertility and fecundity were highly correlated ($F_{1,85} = 1522.773$, $p<0.0001$), we also estimated the relative number of eggs fertilised (relative fertility) by including fecundity as a covariate in GLMs explaining variation in absolute fertility. Females that failed to lay any eggs during the observation period ($n = 8$) were not included in the final models.

We found significant geographic variation in all aspects of female reproductive output (three sites: BW, UBW and LD, $n = 7$,

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34 and 46 females respectively; all $F_{2,67} \geq 4.3526$, all $p \leq 0.0167$), and as a result sample site was included in all models as a main effect. All site interactions were found to be non-significant so were not included in the models. We also investigated whether female morphology (eyespan and thorax length) had significant effects upon reproductive output.

We asked whether a single additional mating had any significant effects on female reproductive output by looking at treatment differences (i.e. $n+1$ matings in the M group versus n matings in the IM group) in fecundity, and absolute and relative fertility. We used data summed over the 10-day collection period to estimate overall reproductive output during the experiment. We also report the number of fertilised eggs as a percentage of total fecundity during the experiment for each group. Note that the sample site effect was not significant ($F_{2,64} = 2.5372$, $p = 0.0870$), and hence not included in the test of percentage fertility. Changes in the reproductive output of wild females during enforced time in captivity have previously been reported [24]. To determine whether fecundity and (relative and percentage) fertility changed over the captivity period (5×2-day egg collections), we included assay period as an ordinal factor (time in captivity) in the GLMs. Since eggs were collected from each female five times during the sample period, we included female identity as a random factor (shrunk by REML estimation) to account for non-independence of within-female measures.

All statistical analyses were performed using JMP software (version 5.0.1a, SAS Institute Inc.).

Results

We found no significant difference between mated and interrupted females in terms of their total fecundity ($F_{1,63} = 0.0466$, $p = 0.8298$), absolute fertility ($F_{1,63} = 0.1759$, $p = 0.6763$) or relative fertility ($F_{1,62} = 0.5762$, $p = 0.4507$) over the 10 day sample period (Fig. 1). The percentage of fertile eggs in the mated group did not differ significantly from that in the interrupted group (mean% ± SE: mated = 83.02 ± 2.55, unmated = 78.09 ± 2.75, $F_{1,65} = 1.7297$, $p = 0.1931$).

All aspects of female reproductive output varied significantly with time in captivity, but in different ways (Fig. 2). Both fecundity and absolute fertility showed significant peaks on day four of observation (Fig. 2A and 2B; fecundity: $F_{4,282} = 7.5978$, $p < 0.0001$; absolute fertility: $F_{4,194} = 5.9830$, $p = 0.0001$). This effect is probably a short-


Figure 1. Fecundity, absolute fertility and relative fertility, summed over the 10-day observation period, for females that received either a single additional mating (shaded bars) or an interrupted mating (open bars). Data displayed as least squares means ± SE.
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term response to capture, and subsequent acclimatisation to captivity. In agreement with a previous study [24] we observed that relative fertility declined significantly with time (Fig. 2C; $F_{4,193} = 6.9897$, $p < 0.0001$). We found the same pattern when we examined percentage fertility (Fig. 2D; $F_{4,193} = 12.1636$, $p < 0.0001$). This is most likely the result of females being sperm limited after isolation from males [19;24;41].

If a single mating were able to alleviate sperm-limitation, we would expect differences between treatment groups; fertility in recently mated females should show less of a decline over time compared to that of interrupted females. However we did not observe this, as there was no significant interaction between treatment and time for both relative ($F_{4,188} = 0.5580$, $p = 0.6934$) and percentage fertility ($F_{4,188} = 0.9161$, $p = 0.4557$). Similarly, the treatment × time interactions were also non-significant for fecundity ($F_{4, 277} = 0.3317$, $p = 0.8565$) and absolute fertility ($F_{4,189} = 0.2761$, $p = 0.8932$), supporting the view that a single mating does not have an appreciable effect on female reproductive output (Fig. 2).

Neither female eyespan (fecundity: $F_{1,58} = 1.2314$, $p = 0.2717$; absolute fertility: $F_{1,58} = 1.4236$, $p = 0.2377$; relative fertility: $F_{1,57} = 0.2065$, $p = 0.6513$; percentage fertility: $F_{1,58} = 0.0764$, $p = 0.7832$) nor thorax length (fecundity: $F_{1,58} = 0.8337$, $p = 0.3650$; absolute fertility: $F_{1,58} = 0.2266$, $p = 0.6359$; relative fertility: $F_{1,57} = 2.3604$, $p = 0.1300$; percentage fertility: $F_{1,58} = 0.0108$, $p = 0.9176$) had significant effects upon female reproductive output.

Discussion

One of the most compelling explanations for the occurrence of multiple mating in female insects is that they acquire direct fertility benefits from such behaviour [2;3]. Females often suffer from sperm-limitation, and there are numerous examples in which multiply mated females have higher fertility than once mated females [19;20]. However, evidence for continued fertility benefits from additional matings by females that have already re-mated is equivocal [21;22;23]. A previous field study on the same Malaysian population of *T. dalmanni* assayed here showed that wild females had low fertility (~55%) and were highly sperm limited [24]. Males transfer few sperm during copulation [35;36], and additional non-sperm direct benefits are unlikely as spermatophores are small [37]. Given these extreme attributes, we asked whether receiving an additional single mating could alleviate sperm-limitation and confer significant reproductive advantages upon wild *T. dalmanni* females?

We used an $n+1$ *versus* n mating design to evaluate the fertility benefits that arise from an additional mating, relative to the background level of mating in the population. All of the females analysed were fecund and observed to begin copulating under natural field conditions with their mate of choice, showing that they were both sexually mature and receptive to mating. Contrary to a previous, laboratory-based, study on the fertility benefits of multiple mating in *T. dalmanni* [19], we found no evidence for continued fertility benefits from additional mating in wild females, measured using absolute or relative values, or when expressed as a percentage of the total number of eggs laid. This was surprising, since around one fifth of eggs laid were infertile. We also observed a significant decrease in fertility during the time in captivity when females were unable to re-mate. This indicates that they suffered from sperm limitation [24]. However, the rate of this decline in fertility was unaffected by an additional mating, which implies that a single mating was unable to mitigate sperm limitation. Therefore, against a background of natural mating behaviour,
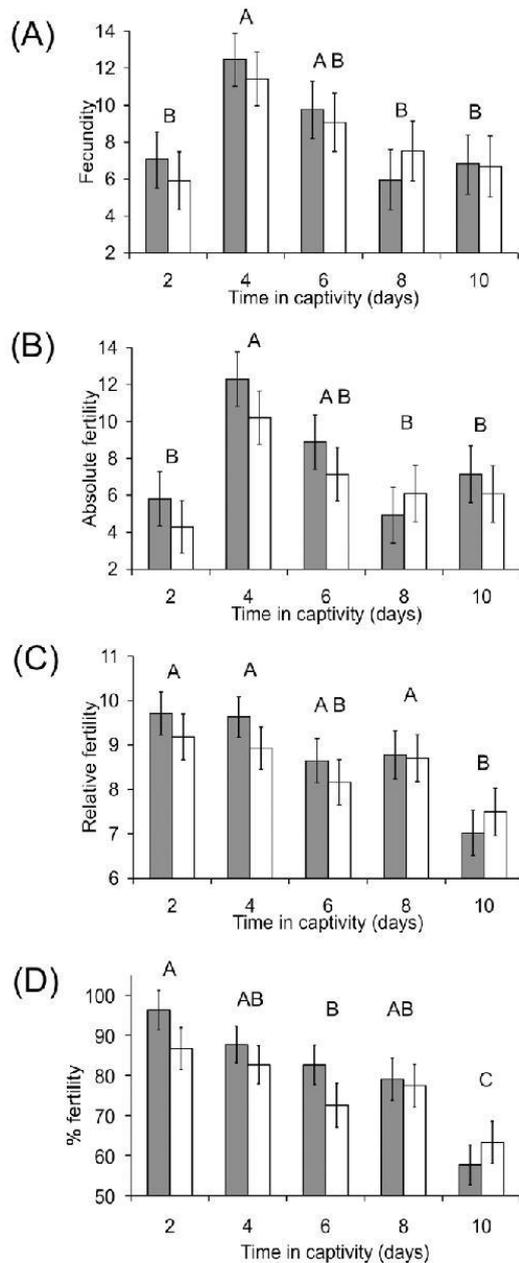
Figure 2. Changes in fecundity (A), absolute fertility (B) relative fertility (C) and percentage fertility (D) over time in captivity for females that received a single additional mating (shaded bars) or an interrupted mating (open bars). Time periods that are not connected by the same letter are significantly different (Tukey HSD comparison of pooled (mated plus interrupted) means). Data displayed as least squares means ± SE.
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we found no detectable fertility benefits associated with a single mating.

Variability in the background level of mating (n) in our experimental design could potentially have obscured any fertility effects caused by the additional, experimental, mating. However, females in each group were chosen at random from the population, so there is no reason to believe that n differed significantly between the mated ($n+1$) and interrupted mating (n) treatments. However, this assumption is hard to confirm. Nonetheless, this apparent shortcoming of our design has the advantage that it frames the effect of a single additional mating relative to the natural mating background. Thus the fertility benefit (or lack thereof) that we observed is a realistic estimate of that gained by the average female in the population.

Why did we not observe fertility benefits from a single additional mating, despite the presence of unfertilised eggs and high sperm-limitation? One possibility comes from the observation that male *T. dalmanni* transfer few sperm during a single mating [35;36], and around a third of matings do not result in sperm transfer at all, despite lasting for longer than 30 seconds [19;40]. A similarly high proportion of failed copulations has been reported in other insects that transfer sperm via spermatophores; for example, in a noctuid moth species 20% of copulations between virgins failed to transfer any sperm to the female's storage organs [42]. In stalk-eyed flies the proportion of copulations that fail to transfer sperm is currently unknown under field conditions. However, if the failure rate is similar to that observed in the laboratory [19], then it is perhaps unsurprising that no difference was detected in the fertility of females mated $n+1$ times relative to those that mated n times. Under such circumstances, females may mate at high frequencies [31;32;43] in order to accumulate fertility over many matings. Future work should explore the number of additional matings required to significantly elevate female fertility relative to the background level, for example in an $n+i$ *versus* n mating design, where $i>1$.

Any effect on fertility of low sperm number and a high proportion of failed copulations may be further exacerbated by the presence of an X-linked meiotic drive element [44]. The element is reported to occur in around 13–17% of males derived from wild populations of *T. dalmanni*, and is also found in its sister species, *T. whitei* [44]. Meiotic drive disrupts spermatogenesis, impairing the elongation of Y-carrying sperm and thus reducing their ability to fertilise [45;46;47]. As a result, females mated to drive-carrying males suffer from impaired fertility [45;46]. In *T. whitei* the ejaculate of non-drive males can reduce the competitive ability of sperm from drive males [40;48]. If this is also the case in *T. dalmanni*, then females may mate multiply in order to counteract the detrimental effects associated with mating with drive-bearing males. Indeed, higher frequencies of female multiple mating have been observed in laboratory populations of both *T. dalmanni* and *T. whitei* where meiotic drive is also present [49].

In spite of the factors that may constrain fertility, females in our study nonetheless exhibited higher fertility (~80%) than those in a previous study on the same population (~55% [24]). This suggests that there is temporal variation in either mating rate, male fertility, or both. Since the fertility derived from a single additional mating might be expected to decrease as existing fertility approaches a maximum, or if a female's sperm storage reaches capacity, our lack of discovery of fertility benefits may reflect the (relatively) high overall fertility in the population. In addition, since fertility is correlated with female mating history and sperm-limitation [19], females with relatively empty sperm storage organs would be expected to gain greater fertility benefits from an additional mating than females whose storage organs are full. It would

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therefore be informative to quantify the fertility added by a single mating in other populations or at different times, in which the average background level of fertility is lower than that of the sample studied here.

Why do females continue to mate if they do not benefit from increased fertility? There are two, non-mutually exclusive, solutions. First, females may remate in order to accumulate fertility over many matings (see above). Second, there may be indirect benefits associated with multiple mating if females are polyandrous [4;50]. While polyandry is weakly associated with increased fertility [51] (although there is no evience for this in stalk-eyed flies [19]), it also allows sperm competition, which can promote fertilisation by genetically superior or more compatible males [6;17;18;52;53]. A suite of microsatellite markers is available for *T. dalmanni* [54] that allows paternity to be assigned to offspring [39]. So future studies should determine whether wild *T. dalmanni* females are indeed polyandrous and to what degree, or whether they simply mate repeatedly with the male who has control of their chosen lekking site. Controlled mating investigations under laboratory conditions should also explore whether the offspring of polyandrous females have greater (post-hatch) viability than those of monandrous females.

In conclusion, we were unable to detect any fertility benefits from a single additional mating in a wild population of promiscuous stalk-eyed flies. This effect is most likely attributable to small ejaculate size, the high proportion of failed copulations, and the presence of X-linked meiotic drive [19;45;46]. Other, indirect, benefits may also result from polyandry [4;18;53], but these hypotheses have yet to be tested in wild populations. Males appear to derive few fertility benefits from a single mating, as mating once with a female does not significantly increase reproductive output, although assignment of paternity to offspring is required to test this hypothesis sufficiently [53;55]. Nonetheless, our data suggest that the high mating rate observed in both sexes of this species may be an adaptation to accrue fertility over many matings.

Acknowledgments

Author Contributions

Conceived and designed the experiments: EH KF SC. Performed the experiments: EH SC. Analyzed the data: EH SC. Contributed reagents/materials/analysis tools: SC. Wrote the paper: EH KF SC.

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Appendix 5: Table of effect sizes for Chapter 5

Model	Effect size	Intercept
Female sperm depletion and direct benefits		
Fecundity, days 6 – 12	0.0333	3.5324
Absolute fertility, days 6 – 12	- 0.3126	4.3953
Relative fertility, days 6 – 12	- 0.3577	3.5211
Fecundity, days 15 – 21	- 0.4554	12.2969
Absolute fertility, days 15 – 21	- 0.3819	10.6396
Relative fertility, days 15 – 21	- 0.0057	- 0.7379
Female eyespan vs. fecundity, days 6 – 12	0.9282	- 2.3576
Female eyespan vs. fecundity, days 15 – 21	2.2507	- 9.0131
Female eyespan vs. absolute fertility, days 15 – 21	1.8157	- 7.6070
Female eyespan vs. relative fertility, days 15 – 21	0.0890	- 0.6919
Female sexual receptivity		
Time to first mating vs. number of matings	- 0.0019	7.6834
Sperm storage and female fecundity		
Total VR pouches vs. filled VR pouches	0.2685	24.9492
Female eyespan vs. total VR pouches	2.5999	13.8315
Female eyespan vs. absolute filled VR pouches	0.9699	3.7749
Female eyespan vs. relative filled VR pouches	-0.2234	- 2.5732

Female fecundity vs. female eyespan	4.1246	- 10.1191
Female fecundity vs. total VR pouches	0.3337	2.2078
Female fecundity vs. absolute filled VR pouches	0.2132	6.3747
Female fecundity vs. relative filled VR pouches	0.1458	-3.7781