Fitness consequences of sex-ratio meiotic drive and female multiple mating in a stalk-eyed fly, *Teleopsis dalmanni*

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I, Lara Meade, 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 work.

Abstract

Meiotic drive genes are a class of segregation distorter that gain a transmission advantage in heterozygous males by causing degeneration of non-carrier sperm. This advantage must be balanced by fertility or viability costs if drive is to remain at stable frequencies in a population. A reduction in male fertility due to sperm destruction reduces the fitness of the rest of the genome, accordingly mechanisms to circumvent the effects of drive may evolve. Such adaptations will have implications for how likely it is that drive will persist. The primary theme of this thesis has been examining fertility consequences of meiotic drive in a Malaysian stalk-eyed fly, *Teleopsis dalmanni*. I demonstrate that drive carrier males are not sperm limited, despite the destruction of half their sperm. They produce ejaculates with sperm numbers equivalent to wildtype male ejaculates. Furthermore, drive males achieve this with greatly enlarged testes. However, resources are not unlimited; drive males also have reduced body size, and reduced accessory glands and eyespan for their body size. Accessory gland size limits male mating frequency, and male eyespan is a sexually selected trait used in female choice and male-male competition. I discuss how these patters fit with theoretical models that predict males should invest in producing an optimal ejaculate according to levels of expected sperm competition, even if they are low-fertility males.

A second interrelated theme of this thesis has been to examine the benefits of polyandry, female mating with multiple males, using wild-caught individuals. Polyandry is widespread across many taxa and almost ubiquitous in insects. However, there is much debate around its proximate and ultimate causes. There

are many costs associated with mating and so polyandry requires an adaptive explanation. I utilise data on wild-caught *T. dalmanni* to explore how natural variation amongst females and males influences fertility gains for females.

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Chapter 1

Introductory Material

1.1 Overview

Selfish genetic elements are found across a large array of taxa. Here I introduce some of the many different forms of selfish genetic element that have been discovered. I focus primarily on segregation distorters that manipulate spermatogenesis to cause meiotic drive. These meiotic drivers generally act through an "actor-responder" system, causing the destruction of sperm that carry the sensitive responder locus, thus the driver, with an insensitive responder, gains a transmission advantage. Much theoretical and empirical work seeks to describe the selective forces that allow these drivers to persist in natural populations without reaching fixation or, in the case of sex linked drivers, causing population extinction. I introduce potential direct fertility and viability costs to being a carrier of drive in both males and females, and also discuss indirect consequences at both the individual and population level. To illustrate this, I highlight one particular species, *Drosophila simulans*, which has been found to carry a number of cryptic drivers on the X chromosome. I discuss the importance of understanding the costs and benefits of polyandry—female multiple mating with multiple males. While the costs that females incur from remating may be intuitive, such as time and energy costs, the benefits are less well understood, especially where females are limited by their investment in gametes. Many potential benefits to polyandry have been proposed, including the ability for females to avoid intrage-

nomic conflict such as arises from meiotic drive. I introduce the study organism that my research has utilised, a species of Malaysian stalk-eyed fly. Two sister species of stalk-eyed fly have so far been found to carry X-linked meiotic drive, and I utilise one of them, *Teleopsis dalmanni*, to address questions about the evolution of meiotic drive and female multiple mating. This species is pertinent for addressing these research questions, as *T. dalmanni* populations harbour an X-linked driver at stable frequencies of around 20%, and furthermore females are highly promiscuous. This introduction concludes by outlining the content and aims of the further chapters.

1.2 Selfish Genetic Elements

Selfish genetic elements subvert normal patterns of replication to increase their representation in the next generation (Burt and Trivers, 2006). They are ubiquitous in eukaryotic genomes (Hurst and Werren, 2001; Burt and Trivers, 2006; Werren, 2011) and examples include transposable elements, supernumerary B chromosomes, meiotic drivers and post-segregation killers, including cytoplasmically transmitted organisms such as *Wolbachia* (Hurst and Werren, 2001). They are expected to evolve wherever there is the potential to exploit transmission between the generations, even at the expense of fitness of the rest of the genome. Segregation distorters, otherwise called meiotic drivers, are a particular type of selfish genetic element that manipulate gametogenesis (Lyttle, 1991; Hurst and Werren, 2001; Burt and Trivers, 2006). They exhibit what is referred to as meiotic drive, where during meiosis one of a pair of heterozygous alleles (or heteromorphic chromosomes) is represented in the gametes in a greater proportion than is expected from Mendelian segregation, and at the expense of the non-distorting partner. This may be through "true" meiotic drive, where there is preferential segregation towards the functional pole as in female oogenesis; alternatively, the drive element may cause the destruction of non-carrier gametes, or even the destruction of the whole genome, as in the parasitoid wasp *Nasonia vitripennis* where diploid (female) eggs are converted to haploid (male) eggs

through the destruction of the paternal genome (Beukeboom, 1994).

1.2.1 Meiotic drive

There are many examples of segregation distorters existing as polymorphisms in a wide range of taxa. Predominantly they generate a transmission advantage in heterozygous male carriers, for example “Sperm killers” such as autosomal distorters *SD* in *Drosophila melanogaster* (Tokuyasu et al., 1977) and the *t*-complex in house mice (Ardlie, 1998), Y-linked drivers in *Aedes* and *Culex* mosquitoes (Wood and Newton, 1991) and X-linked drivers in a multitude of *Drosophila* species and other dipterans (Novitski et al., 1965; James and Jaenike, 1990; Cobbs et al., 1991; Presgraves et al., 1997; Cazemajor et al., 2000; Wilkinson and Sanchez, 2001; Keais et al., 2017). Furthermore there are “Spore killers” such as the autosomal *Sk* in *Neurospora* fungi (Harvey et al., 2014), and “Pollen killers” found in a number of plant species (Cameron and Moav, 1956; Loegering and Sears, 1963; Sano, 1990; Taylor and Ingvarsson, 2003). It is these gamete killer drive systems that I discuss in further detail. Female meiotic drive occurs when homologous chromosomes are differentially transmitted to the egg rather than polar bodies during meiosis (Lindholm et al., 2016). Examples of female meiotic drive are rare as research has focused predominantly on male drivers that have observable phenotypic effects. Female meiotic drive has been uncovered through hybridisation in the Monkey-flower (Fishman and Saunders, 2008), and through close examination of segregation patterns in the chicken (Axelsson et al., 2010). The fact that most discoveries of meiotic drive have been made in well studied species such as *Drosophila*, as well as relatively recent discoveries of novel segregation distorters in a range of other well studied organisms (Axelsson et al., 2010; Corbett-Detig and Hartl, 2012), suggest that many more drivers remain to be found.

Sex-ratio meiotic drive has the potential to be a more influential evolutionary force than autosomal drive as it may be more likely to invade (Frank, 1991; Hurst and Pomiankowski, 1991). “Killer” meiotic drive requires the action of two loci, a drive locus and a sensitivity locus. Reduced recombination between

the sex chromosomes facilitates linkage between the drive and sensitivity locus whereas an autosomal driver is only likely to invade if it arises in close proximity to a resistant sensitivity locus. Meiotic drive elements are often found within inversions or regions of low recombination (James and Jaenike, 1990; Johns et al., 2005; Dyer et al., 2007, 2013; Reinhardt et al., 2014); however, inversions are not an essential prerequisite for sex-chromosome drive and there are some observed exceptions (James and Jaenike, 1990; Capillon and Atlan, 1999). Sexual antagonism may also favour reduced recombination on the sex chromosomes and lead to masculinisation of the driving sex chromosome. For a drive polymorphism to be maintained in equilibrium, the transmission advantage must be counterbalanced by fertility or viability costs. If these costs are shared by the sexes, rather than being borne solely by males (the sex in which drive occurs), the driving allele will be more fit in males, and less fit in females, than the non-driving allele. Consequently, alleles that fare better in males will tend to become associated with the drive haplotype so that natural selection favours reduced recombination here also (Rydzewski et al., 2016). Observation bias can also lead to a greater discovery rate of sex-ratio meiotic drive systems compared to autosomal drive. Sex-ratio meiotic drive produces an easily observable phenotype, especially when drive transmission is strong. Autosomal drive, on the other hand, is often discovered through observing lower than expected frequency of recessive lethal mutations segregating an a population, as well as their effect on offspring numbers (Lyon, 2003; Larracuente and Presgraves, 2012). In *Drosophila*, sex-chromosome meiotic drive has been recorded in at least 15 species, but autosomal meiotic drive has been described only in *D. melanogaster* (Lyttle, 1991; Jaenike, 2001). Furthermore, autosomal meiotic drivers that have been identified are associated with inversions (*t*-complex in *Mus*: Lyon et al., 1988; *Sk* in *Neurospora*: Harvey et al., 2014) and/or located close to the centromere where recombination is suppressed, as in the case of the *SD* complex in *D. melanogaster* (Temin et al., 1991). Reduced recombination will facilitate the accumulation of linked alleles which help maintain the drive

polymorphism, but will also allow the accumulation of deleterious mutations.

1.2.2 Costs to fertility and viability of drive

A major focus of discussion and research has been the investigation of selection forces that maintain stable frequencies of drive polymorphisms in natural populations (Wu, 1983*a*; Haig and Bergstrom, 1995; Jaenike, 1996, 1999, 2001; Zeh and Zeh, 1996; Taylor and Jaenike, 2002; Price and Wedell, 2008; Holman et al., 2015; Lindholm et al., 2016). A stable polymorphism will result from the balance between the transmission advantage gained by drive in males, countered by the deleterious effects of drive on the fitness of carriers. There are a multitude of deleterious effects caused by drive, so we need to understand these in order to explain the basis of how a stable polymorphism might arise. For example, drive has deleterious consequences in males (the sex in which drive occurs) but also in females, so both direct and indirect effects of drive need consideration in both sexes. This may lead to evolutionary change to ameliorate the negative effects of drive, and such adaptation may feed-back in making it more or less likely that drive persists in the long term. Drive also has fitness consequences at the level of the population, which may further affect persistence of the driver.

The most obvious cost of drive occurs through the reduction of drive carrier male fertility due to the production of non-functioning sperm. Plentiful evidence exists for the production of inviable sperm during or after spermatogenesis (Policansky and Ellison, 1970; Hauschteck-Jungen and Maurer, 1976; Brown et al., 1989; Wood and Newton, 1991; Taylor, 1996; Presgraves et al., 1997; Cazemajor et al., 2000; Wilkinson and Sanchez, 2001; Tao et al., 2007*b*). This can be detrimental to male fertility, and drive carrier males in *D. recens*, *D. simulans* and *D. neotestacea* have all been shown to exhibit reduced fertility compared to wildtype males after a single mating, and even more so when mating rate is high (Jaenike, 1996; Atlan et al., 2004; Pinzone and Dyer, 2013). In many other species, including *D. melanogaster*, *D. pseudoobscura*, *D. quinaria*, and the stalk-eyed fly *T. dalmanni*, drive males exhibit reduced fertility at high mating rates (Peacock and Erickson, 1965; Wu, 1983*b*; Jaenike, 1996; Wilkinson et al.,

2006). Impaired fertility is often particularly severe when drive male sperm are in direct competition with wildtype sperm (Taylor et al., 1999; Wilkinson and Fry, 2001; Atlan et al., 2004; Wilkinson et al., 2006; Angelard et al., 2008; Price et al., 2008*a*). A reduction in drive male fertility by one half exactly balances the twofold advantage of drive alleles, and prevents their spread. While there is much evidence that drive reduces sperm production, the reduction in fertility typically does not reach 50% (Price and Wedell, 2008). For example, there is often no difference between drive and wildtype male fertility after a single mating (Peacock and Erickson, 1965; Wu, 1983*c*; Jaenike, 1996; Taylor et al., 1999; Fry and Wilkinson, 2004; Wilkinson et al., 2006; Verspoor et al., 2016). Nonetheless, impaired fertility can have an indirect negative impact on females when they mate with drive males, as they may receive too few sperm to maximise their fertility (Haig and Bergstrom, 1995; Zeh and Zeh, 1996). This is likely to be particularly important in species where females mate only once, or infrequently. Likewise, if populations are female biased—possibly as a result of X-linked drive—male mating rate will be high, causing males to suffer from ejaculate limitation with potential knock-on negative effects on female fertility (Jaenike, 1996). Despite these predicted deleterious effects on females, investigations either experimental or under natural conditions have been lacking, so it is not clear how important such deleterious effects are.

As well as a direct impact on male fertility from non-carrier sperm destruction, there may be pleiotropic effects of drive expression. Since drive involves the disruption of the normal process of gametogenesis, an additional reduction in the quality of surviving carrier sperm may occur. For example in wheat (*Triticum aestivum*), pollen which do not carry the segregation distorter are inviable, while carrier pollen also have reduced fertilisation capacity due to major increases in meiotic abnormalities (Nasuda et al., 1998). Most evidence for damage to carrier gametes comes from fertility observations under conditions of sperm competition, where drive males are found to be worse sperm competitors than expected, even after taking into account reduced sperm numbers (Wilkin-

son and Fry, 2001; Fry and Wilkinson, 2004; Wilkinson et al., 2006; Angelard et al., 2008; Price et al., 2008*a*). Drive might also have negative effects in tissues other than the gonads if the genes involved are pleiotropically expressed during somatic development. For example, the *SD* drive locus in *D. melanogaster* is a partial duplication of *RanGAP* (*Ran GTPase activating protein*) which is also a general cellular nuclear transport protein, involved in spindle assembly, nuclear envelope assembly and chromosome segregation in mitosis, and a range of other cellular functions (Larracuente and Presgraves, 2012), so plausibly it could affect a number of other somatic functions. However, this possibility has not been investigated, as the literature on meiotic drive has been centred on characterising the molecular mechanisms causing the drive phenotype rather than any side-effects of the genes involved.

Drivers are often located within low frequency inversions (Lyon et al., 1988; James and Jaenike, 1990; Johns et al., 2005; Dyer et al., 2007, 2013; Reinhardt et al., 2014; Harvey et al., 2014). Inversions dramatically suppress recombination because pairing is partially inhibited during synapsis or because crossovers give rise to unbalanced gametes (Kirkpatrick, 2010). Any segregating beneficial mutations that arise in different individuals can only be brought together through recombination (Fisher, 1930; Muller, 1932). Furthermore, without recombination, negative interactions between different beneficial mutations can reduce the probability of their fixation (Hill-Robertson interference, Hill and Robertson, 1966). These effects are likely to be manifest in both males and females as in the case of autosomal drive in house mice, where multiple inversions in the *t*-complex have allowed the accumulation of recessive mutations that result in either homozygote male sterility or prenatal death (Lyon, 1986, 2003; Sutter and Lindholm, 2015).

In the case of X chromosome drive, deleterious recessives on the X are effectively dominant in males due to the hemizygosity of the X chromosome. Deleterious recessive mutations are thus selected against, and will accumulate at lower rates than on the autosomes. However, for a low frequency X-linked

driver (James and Jaenike, 1990; Jaenike, 1996; Wilkinson et al., 2003; Price et al., 2014), selection is weaker as its effective population size is low, reducing the rate of removal of deleterious X-linked recessive mutations. Furthermore, when associated with inversions, a driving X will have reduced rates of recombination in females, similar to the Y chromosome or an asexual organism (Barton and Charlesworth, 1998), leading to stronger Muller's ratchet effects (Muller, 1964; Felsenstein, 1974) and allowing selective sweeps to increase the frequency of deleterious alleles. Selective sweeps of drive will be difficult to detect as an increase in frequency due to selfish drive can leave a molecular signature similar to that left by an increase in frequency due to positive selection. A selective sweep of a sex-linked distorter is also likely to cause population extinction, so the sweep cannot be detected. In *D. pseudoobscura*, the drive chromosome causes drive males to have lower egg to adult viability relative to wildtype males (Curtsinger and Feldman, 1980; Beckenbach, 1996). There does not appear to be any viability loss in *T. dalmanni* (Johns et al., 2005; Wilkinson et al., 2006), despite evidence that the drive locus is associated with inversions (Paczolt et al., 2017). However, viability differences between genotypes were not directly tested for, and inferred *post hoc*.

While male-specific deleterious mutations are selected against, female-specific deleterious mutations are likely to accumulate. Given the typically low frequency of drive, homozygotes rarely arise and so there is little selection against recessive deleterious mutations that negatively affect female fitness. For example, in *D. recens* the driving X is associated with a complex set of inversions that completely suppress recombination between the drive and wildtype chromosome, and the drive chromosome is fixed with recessive mutations that cause female sterility (Jaenike, 1996; Dyer et al., 2007). Conversely, in *D. neotestacea*, there is no evidence for reduced homozygote female fitness, but there is evidence of recombination between the drive and wildtype X chromosomes (Dyer et al., 2013). Establishing whether the accumulation of female deleterious effects has had a direct impact on the low frequency of drive, or if

low drive frequency has enabled the accumulation of these mutations, will be difficult to disentangle.

1.2.3 Sperm competition

Poor sperm (or pollen) competitive ability is a recurring feature of drive males in many systems (Taylor et al., 1999; Wilkinson and Fry, 2001; Wilkinson et al., 2006; Angelard et al., 2008; Price et al., 2008*a*). Taylor and Jaenike (2002) suggest that sperm competition plays a prominent role in determining drive frequency if drive male success in sperm competition declines with male mating rate. The spread of X-linked drive will cause increasing bias in the population sex ratio towards females. Under these conditions, males can be expected to increase their mating rate, which may exacerbate the disadvantage of drive males during sperm competition. However, because of sex ratio distortion, females will decrease their average mating rate which will in turn decrease the incidence of sperm competition, to the benefit of drive males. Taylor and Jaenike (2002) modelled this and found that the combination of forces can lead to a stable equilibrium of drive and wildtype chromosomes if the success of drive males in sperm competition declines disproportionately with male mating rate. However, if female mating rate dominates the dynamics, drive will spread to fixation causing extinction of the population. Extinction is more likely in small populations and where environmental conditions cause fluctuations in population density. It should also be noted that the model makes a number of simplifying assumption that may invalidate its conclusions. For example, the model only considers the probability of a single or double mating, and does not allow males to evolve allocation strategies based on ejaculate size versus the number of matings.

1.2.4 Meta-population dynamics

Stable frequencies of X-linked drive could be maintained though meta-population dynamics—a meta-population structure with a global equilibrium frequency of a driving X chromosome maintained by a balance between extinction and founding of local populations. However, this hypothesis has not been

formally modelled and is not well supported empirically. In *Drosophila* species, the observed variation in drive is generally uniform or clinal, rather than the expected checker-board pattern, and long-term studies reveal no directional changes in drive frequency (Jaenike, 2001). For example in *D. pseudoobscura*, the latitudinal cline in X-linked drive frequency has remained broadly stable for at least 70 years (Price et al., 2014). Furthermore, theoretical models suggest that it is also not biologically plausible for autosomal *t*-haplotypes in *Mus musculus* (Durand et al., 1997).

1.2.5 Evolution of suppressors

Because drive occurs at a cost to the rest of the genome, suppressors of drive are predicted to evolve (Stalker, 1961; Wu, 1983*a*). For autosomal drive, suppression could arise though recombination, creating chromosomes with the wildtype drive locus but the insensitive responder locus. Alternatively, suppressor chromosomes could arise through mutational loss of the drive function while retaining the insensitive locus. In the autosomal *SD* system in *D. melanogaster*, "immune" chromosomes which lack the drive allele but are insensitive to drive have been studied in laboratory populations, and are thought to have arisen both as rare recombinants and from deletion events (Larracuente and Presgraves, 2012).

According to Fisher's (1930) sex ratio theory, autosomal or Y-linked suppressors of X-linked drive should evolve and thereby achieve stable drive polymorphism by increasing production of high fitness male offspring (Wu, 1983*a*). There is evidence for autosomal and Y- (or X-) linked suppressors in many systems (Stalker, 1961; Wood and Newton, 1991; Carvalho and Klaczko, 1993; Presgraves et al., 1997; Carvalho et al., 1997; Cazemajor et al., 1997; Tao et al., 2007*b*). However, there are also systems where no evidence of suppression has been found, such as *D. pseudoobscura* and *D. neotestacea* (Dyer, 2012). Furthermore, drive may spread rapidly and not give time for suppressors to evolve without other mechanisms keeping the spread in check.

1.2.6 *Drosophila simulans*

To illustrate the complex dynamics of drive, it is worth considering the variety of X-linked drive systems uncovered in *D. simulans* (Faulhaber, 1967; De Magalhães et al., 1985; Merçot et al., 1995; Dermitzakis et al., 2000; Tao et al., 2001). In this species, three independent systems have been confirmed through hybridisation ("Winters", Dermitzakis et al., 2000), introgression ("Durham", Tao et al., 2001) or population crosses ("Paris", Merçot et al., 1995). The best studied of all drive systems is the Paris sex-ratio system. It was uncovered accidentally from laboratory crosses of individuals from different geographic regions of Seychelles archipelago and New Caledonia (Merçot et al., 1995). The Paris X appears to have a relatively recent origin (Derome et al., 2004), and it is not associated with any complex chromosomal inversions (Montchamp-Moreau et al., 2006). Drive in the Paris system involves two loci, Dp^{SR} and *Wlasta*, and the latter has been identified as heterochromatin protein 1 D2 (*HP1D2*) (Helleu et al., 2016). Distortion is a consequence of dysfunctional *HP1D2* alleles failing to prepare the Y chromosome for meiosis.

In *D. simulans* the Paris X has a global distribution, with frequencies as high as 60% in some geographically isolated populations (Atlan et al., 1997) and there is evidence for a selective sweep (Derome et al., 2004). However, most populations are found at a $\sim$1:1 sex ratio, and this is due to the existence of multiple suppressors on both the autosomes and the Y chromosome (Merçot et al., 1995; Cazemajor et al., 1997; Atlan et al., 2003). The Paris X is not invasive in experimental populations where suppressors have been removed, and it was eliminated in less than 17 generations from three replicate populations (Capillon and Atlan, 1999). This seems to be due to the negative impact on male fertility caused by the driver, which must exceed the transmission advantage under certain circumstances. The action of unsuppressed drive causes most Y chromosomes to fail to undergo disjunction during the second meiotic division (Cazemajor et al., 2000), and the loss of Y-bearing sperm is not compensated by the over-production of X-bearing sperm (Montchamp-Moreau and

Joly, 1997). At low mating frequencies (access to 2 females over 1 week), drive males carrying an unsuppressed driving X chromosome have similar fertility to wildtype males (Merçot et al., 1995; Capillon and Atlan, 1999; Atlan et al., 2004). However in both single mating conditions and when left with 10 females over 24 hours, wildtype males have significantly higher fertility than drive males (Atlan et al., 2004; Angelard et al., 2008). Furthermore, drive males are unable to displace wildtype male sperm in sperm competition, while wildtype males can displace drive sperm (Atlan et al., 2004; Angelard et al., 2008). Overall, fertility reductions during sperm competition suggest that rapid spread of the driving X is only possible when population density is low and individuals are unlikely to mate frequently.

The Winters system was uncovered through interspecific hybridisation of *D. sechellia* and *D. simulans* (Dermitzakis et al., 2000). Distortion requires two genes, the X-linked genes *Dox* (*Distorter on the X*), and *MDox* (*Mother of Dox*), from which *Dox* was derived through duplication (Tao et al., 2007*a*). They are not located within an inversion, and so are unlikely to be associated with deleterious mutations (Kingan et al., 2010). A dominant autosomal suppressor *Nmy* (*Not much yang*) is a retrotransposon copy of *Dox* on chromosome 3R, and suppresses *Dox* through an RNA interference mechanism (Tao et al., 2007*b*). When the autosomal suppressor *Nmy* has been removed, males produce strongly female biased offspring (Tao et al., 2007*a*). During meiosis, the Y chromosome undergoes normal disjunction; however, post-meiotic spermatogenesis of the Y-bearing gametes is disrupted (Tao et al., 2007*b*).

A worldwide screen of 78 Y chromosomes has additionally revealed the existence of resistant Y chromosomes (Branco et al., 2013). A diversity of Y chromosomes produced a range of offspring sex-ratios, between 63% – 98% female progeny, and expression levels of over 200 testes-specific genes in males with resistant Y chromosomes were found to be upregulated. Resistant Y chromosomes may act through epigenetic mechanisms not requiring the expression of protein-coding genes, as it was found that the Y chromosome also modulated

expression in XXY females, in which the Y-linked protein-coding genes are not transcribed.

The Durham system was uncovered by introgressing regions of the third chromosome from *D. mauritiana* into *D. simulans* (Tao et al., 2001). Males homozygous for the introgression produced strongly female biased progeny, and had low fertility. There was no effect on embryo viability, and all introgression lines had the same *D. simulans* cytoplasm. Consequently, the *D. simulans* allele *Tmy* (*Too much yin*), appears to be a dominant autosomal suppressor of an X-linked distorter.

1.3 Polyandry

Multiple mating (with multiple males, polyandry) is costly for females. Costs include exposure to disease, predation, male harm, as well as energy and time costs (Rowe, 1994; Arnqvist and Nilsson, 2000; Crudgington and Siva-Jothy, 2000; Blanckenhorn, 2002; Rönn et al., 2006; Ashby and Gupta, 2013). Female reproductive potential is thought to be achieved after one or a few matings (Bateman, 1948) due to constraints in their greater investment in offspring. In some insect mating systems females are able to obtain sufficient sperm to ensure fertility throughout their reproductive life by mating only once (Arnqvist and Nilsson, 2000; Arnqvist and Andrés, 2006; South and Arnqvist, 2008), or multiple times but over a single short period (Boomsma et al., 2005). However, in many mating systems females must remate throughout their adult life in order to maintain fertility (Ridley, 1988; Fox, 1993; Fjerdingstad and Boomsma, 1998; Drnevich et al., 2001; Chevrier and Bressac, 2002; Wedell et al., 2002; Wang and Davis, 2006). Much empirical work has been aimed at uncovering the benefits to polyandry that must outweigh the costs. Potential benefits to females include direct benefits to female survival, fecundity and fertility (Yasui, 1998; Arnqvist and Nilsson, 2000; Hosken and Stockley, 2003), as well as indirect genetic benefits through increasing the genetic diversity or quality of offspring (Yasui, 1998; Slatyer et al., 2012). Furthermore, polyandry is likely to enable females to avoid intragenomic

conflict such as that imposed by meiotic drive elements (Haig and Bergstrom, 1995; Zeh and Zeh, 1996).

1.3.1 The evolution of polyandry as a female response to drive

As described above, drive males tend to perform poorly in sperm competition. There is evidence in support of polyandry as a mechanism that inhibits the spread of drive. In laboratory populations of *D. pseudoobscura* that contained drive, female remating caused the frequency of the driver to fall rapidly (Price et al., 2010). Furthermore, limiting females to only a single mating was associated with increased risk of population extinction due to extreme sex-ratio bias, while only a single additional mating was sufficient to create a significant reduction in drive frequency. Just as female multiple mating may protect a population against the spread of drive, so the presence of drive may promote the evolution of polyandry as a female response to the negative fitness consequences of mating with a drive male (Haig and Bergstrom, 1995; Zeh and Zeh, 1997; Wedell, 2013; Holman et al., 2015).

For females, mating with a drive male could be costly for multiple reasons. Drive males may be sperm depleted and so fail to maximise female fertility. Drive males may also be of lower fitness as their sperm performs relatively poorly in competition, and furthermore cause females to produce female offspring in a female-biased population. In particular, mating with a drive male is detrimental for females heterozygous for drive when homozygotes are inviable or sterile. In laboratory lines of *D. pseudoobscura*, females evolved higher frequencies of multiple mating when a meiotic driver (that negatively affects sperm competitive ability) was present in the population (Price et al., 2008*b*). Rates of polyandry also correlate with drive frequencies across natural populations of *D. pseudoobscura* (Price et al., 2014) and *D. neotestacea* (Pinzone and Dyer, 2013), and polyandry correlates with drive frequency between closely related species of stalk-eyed fly (Wilkinson et al., 2003). It is very difficult to disentangle whether the frequency of drive observed in a population is determined by the level of

polyandry, or if the level of polyandry is determined by drive. Furthermore, variation in female mating frequency will be caused by various aspects of the life history of the species under study. In order to understand the causal relationship between drive and female mating frequency, it is also imperative to understand the range of factors that influence the evolution of polyandry.

1.4 Stalk-eyed flies

Hypercephaly is the defining character of diopsids (order: Diptera). The head capsule is elongated into eyestalks, causing the lateral displacement of the eyes and antennae to the end of the stalks. Elongated heads, as well as antlers, have evolved in several Dipteran families, however diopsids are unique in that both sexes of all known species in this family (of which there are currently over 150 characterised, and nearer 300 estimated) exhibit hypercephaly (Wilkinson and Dodson, 1997). In some species, the sexes are monomorphic for eyespan (the distance between the outermost tips of the eyebulbs), and this is believed to be the ancestral state (Baker and Wilkinson, 2001). The number of optical components in each compound eye (ommatidia) increases with eyespan, providing a naturally selected advantage of increased binocular field of vision, at the expense of a loss of spatial resolution (de la Motte and Burkhardt, 1983; Buschbeck and Hoy, 1998). In many diopsid species, eyespan is sexually dimorphic, and this dimorphism has evolved independently on multiple branches of the phylogeny (Baker and Wilkinson, 2001). In all cases, male eyespan is larger than female eyespan on average, and males have a larger eyespan to body ratio. It has been shown that this dimorphism is influenced by both male-male competition (Panhuis and Wilkinson, 1999; Small et al., 2009) and female choice (Burkhardt and de la Motte, 1988; Wilkinson and Reillo, 1994; Cotton et al., 2010). Furthermore, within the genus *Teleopsis* (synonymous with *Cyrtodiopsis*, Meier and Baker, 2002), two sister species *T. dalmanni* and *T. whitei*, harbour meiotic drive systems (Presgraves et al., 1997). It is unknown whether drivers are present in other clades. Male eyespan has been suggested to pro-

vide females with a signal of the presence of the meiotic driver in *T. dalmanni* (Presgraves et al., 1997; Wilkinson et al., 1998*b*; Cotton et al., 2014).

T. dalmanni and *T. whitei* have very similar life histories and ecology, and they are sympatric across some of their southeast Asian distribution range (Wilkinson et al., 2003). Both species live in rainforest habitat, feeding on fungus, mould and yeast from decaying leaf vegetation, where eggs are also laid (Wilkinson and Dodson, 1997). As with other dimorphic (but not monomorphic) species, adults aggregate in nocturnal clusters resulting in a lek style mating system (Wilkinson and Dodson, 1997; Cotton et al., 2010). This likely reduces the chances that females remain unmated due to low encounter rates—in comparison to *T. quinqueguttata* that do not carry drive and do not aggregate nocturnally (Burkhardt and de la Motte, 1985; Wilkinson et al., 2003). Adults congregate on root hairs overhanging eroded banks of rainforest streams. Males arrive at these sites before dusk and guard them aggressively, competing with each other, and typically the male with the largest eyespan wins the encounter (de la Motte and Burkhardt, 1983; Lorch et al., 1993; Panhuis and Wilkinson, 1999; Small et al., 2009). At dusk, females assess the sites and tend to roost and mate with males with larger eyespan (Wilkinson and Reillo, 1994; Cotton et al., 2010). In the wild, mating predominantly occurs at dawn, where males mate with females who have settled on their root hair. Males mate multiply, and a large *T. whitei* male may mate up to 20 times (Burkhardt et al., 1994), with *T. dalmanni* males mating fewer times (Cotton et al., 2015), and females in both species have been observed to mate multiply each morning in a laboratory setting (Wilkinson et al., 1998*a*; Baker et al., 2001*a*). However, wild observations, or even molecular evidence is lacking. Microsatellite evidence would be beneficial for estimating wild polyandry rates where observations are difficult to take, however they may underestimate rates if males have the same genotype or if some males contribute small amounts of sperm. In dimorphic lekking species, males produce small spermatophores in comparison to males of monomorphic species (Kotrba, 1996), and females are sperm limited and fail to fertilise many

of their eggs (Baker et al., 2001*b*; Cotton et al., 2010).

1.4.1 *Teleopsis dalmanni*

My research uses *T. dalmanni* as a model organism to understand how meiotic drive can be maintained in natural populations, as well as to specifically examine the benefits of female multiple mating. *T. dalmanni* populations exhibit X chromosome meiotic drive (Presgraves et al., 1997; Wilkinson et al., 2003; Cotton et al., 2014; Paczolt et al., 2017). The drive element, located on the X chromosome (X^{SR}), prevents the proper formation and function of Y-bearing sperm during spermatogenesis, and carrier males predominantly produce > 90% female biased broods (Presgraves et al., 1997; Wilkinson and Sanchez, 2001). Spermatogenesis is generally similar to that of *Drosophila* (Presgraves et al., 1997). Maturation of spermatids occurs in bundles where, for X^{SR} carriers, the heads of Y-bearing spermatids fail to elongate. Drive males may produce a few male offspring and these males are fully fertile (Presgraves et al., 1997). The frequency of drive in a wild population of *T. dalmanni* has been observed at stable frequencies of $\sim$20% over many generations (Presgraves et al., 1997; Wilkinson et al., 2003; Cotton et al., 2014; Paczolt et al., 2017).

The meiotic drive element is located within at least one inversion, and there is support for a single origin of the X^{SR} chromosome (Paczolt et al., 2017). The X^{SR} chromosome is over 500,000 years old and appears to be evolving independently of the standard X chromosome (X^{ST}) in an otherwise shared genetic background. X^{ST} and X^{SR} show widespread divergence (Christianson et al., 2011; Cotton et al., 2014; Reinhardt et al., 2014; Paczolt et al., 2017), with little or no recombination (Wilkinson et al., 2005; Paczolt et al., 2017), showing both fixed differences and differential expression (Reinhardt et al., 2014). Despite its age and impact on spermatogenesis and brood sex-ratio, there is minimal evidence for suppression of drive. Previous work has indicated the presence of Y-linked modifiers, and that Y suppression is genetically linked to eyespan, so female preference for drive resistance could evolve (Wilkinson et al., 1998*a*,*b*). However, further work addressing this found no effect of the Y chromosome on

relative eyespan (eyespan after variation in thorax size is taken into account) (Wolfenbarger and Wilkinson, 2001), and this hypothesis is not supported by theory, which suggests that when resistance is Y-linked, female choice for drive-resistant males is disadvantageous (Reinhold et al., 1999). Further work has failed to find clear evidence for drive suppression in *T. dalmanni* (Johns et al., 2005; Paczolt et al., 2017).

Furthermore, despite the estimated low recombination rates between X^{SR} and X^{ST}, homozygous drive females do not exhibit effects of deleterious recessive mutations on fecundity, fertility or survival (Wilkinson et al., 2006). Drive males, however, may have reduced eyespan. Wilkinson et al. (1998*b*) found that selecting lines (carrying drive) for increased male eyespan to body size ratio produced fewer males with female biased broods after selection. Subsequently, allele size of a microsatellite marker, *ms395*, was found to associate with both brood sex-ratio and male relative eyespan in wild files; furthermore, male relative eyespan decreased with increasingly female biased brood sex-ratio in the wild (Cotton et al., 2014). These findings indicate that in *T. dalmanni* female choice for male eyespan could be maintained partly by the presence of meiotic drive, and as a corollary the spread of drive hindered, where loci for drive and loci for male eyespan both reside within the same inversion (Lande and Wilkinson, 1999).

The number of normal sperm bundles in the testis is reduced in drive males as a result of the failed elongation of Y-bearing sperm (Presgraves et al., 1997). This does not seem to have any negative impact on drive male fertility at low mating frequencies (Wilkinson et al., 2006). Jaenike (1996) suggests that this is expected, and that drive male fertility should suffer at high mating frequencies, where sperm limitation exacerbates the difference in drive and wildtype male fertility. Indeed, there does appear to be some evidence to support this: when males are allowed to mate with 4 or 8 females over a 3 week or 1 week period, drive males exhibit a reduction in fertility compared to wildtype males of $\sim$0.7 (Wilkinson and Sanchez, 2001; Wilkinson et al., 2006). However this reduction

has only been demonstrated under high mating frequencies that are beyond what a male would expect to encounter in nature (Lorch et al., 1993; Wilkinson and Reillo, 1994; Cotton et al., 2015). Furthermore, this reduction in fertility may be ascribed to the reduced mating rate of drive males (Wilkinson et al., 2003, S. Finnegan, *unpublished data*), rather than sperm limitation, and mating rate is determined by the availability of male accessory gland products in *T. dalmanni* (Baker et al., 2003; Rogers et al., 2005*a*,*b*). Sperm are produced over long periods of time (Baker et al., 2003) and mature sperm are probably stored in the vas deferens (Wolfner, 1997), while accessory gland products are used up quickly and replenished over much shorter time scales (Rogers et al., 2005*b*).

Drive *T. dalmanni* males appear to be poorer sperm competitors than wildtype males, and may be even worse than expected through sperm death alone. Sperm competition between wildtype males seems to be determined by random sperm mixing (Corley et al., 2006), while drive males mating second in competition with a wildtype male achieve only $\sim$0.25 paternity share (Wilkinson et al., 2006), which is the same as the overall paternity share estimated for *D. pseudoobscura* (Price et al., 2008*a*). This is well below the critical theoretical threshold allowing stable polymorphisms to persist (Taylor and Jaenike, 2002), and suggests that there must be a balance between sperm competition levels and female remating rate, for example, females may frequently spend time laying eggs before remating with a different male. Assuming sperm mixing and that drive males have half as many sperm as wildtype males in their ejaculate, one would expect a driving paternity share of 0.33 for *T. dalmanni* drive males. This would suggest that X^{SR} sperm have reduced fertilisation capacity, perhaps due to imperfect targeting of damage to Y sperm during spermatogenesis (Price and Wedell, 2008), and furthermore in *T. whitei*, a single mating followed by the seminal fluid only of a wildtype male elicits a reduction in fertility (Fry and Wilkinson, 2004). Taylor and Jaenike (2002) suggest that if drive male success in sperm competition declines disproportionately with male mating rate, then sperm competition can maintain a stable drive polymorphism. How drive male performance

in sperm competition changes with mating rate is currently unknown.

In the wild and the laboratory, female *T. dalmanni* are highly promiscuous, and the cost of mating appears to be low (Wilkinson et al., 1998*a*; Reguera et al., 2004). Females are sperm limited and fail to fertilise a large portion of their eggs—males transfer few sperm per copulation (Wilkinson et al., 2005; Rogers et al., 2006) encased in a small spermatophore that is unlikely to confer any non-sperm benefits (Kotrba, 1996). In the laboratory, females remate to gain direct fertility benefits (Baker et al., 2001*a*) and this is influenced by female quality (Rogers et al., 2006). It is unknown how female mating rate varies in the wild, if it is influenced by drive frequency or other ecological factors, and how it impacts sperm competition.

1.5 Structure of the thesis

The thesis is comprised of this introduction (Chapter 1), three "results" chapters presenting novel empirical findings, a "methods" chapter and a discussion (Chapter 6) which recapitulates the main findings and highlights priorities for future research to build upon my findings. Chapter 2 and 3 examine the effects of meiotic drive in *T. dalmanni* male sperm production capacity and fertility. Chapter 4 examines the utility of microsatellite and INDEL markers for identifying X^{SR}. Chapter 5 investigates the fertility benefits of female multiple mating in wild *T. dalmanni* females.

1.5.1 Chapter 2

A little explored possibility is the likelihood that sexual selection on male investment in sperm per ejaculate will lead to an adaptive response in drive males, and as a consequence drive male fertility will not suffer as predicted. Models examining the evolution of male ejaculate allocation suggest that males with a fertility disadvantage will compensate by producing competitive ejaculates in response to the expected levels of sperm competition. I test this possibility in *T. dalmanni* by analysing sperm storage within two female sperm storage organs, after females have mated with either a drive or wildtype male. Furthermore, I look at

sperm numbers in storage on a male's third mating. I discover that drive males are not sperm limited even after mating multiple times, despite the destruction of sperm during spermatogenesis. This study suggests that conventional assumptions about the effects of drive on male fertility need to be examined more closely. The laboratory work included in this chapter was carried out with the aid of two MSci students (Deidre Dinneen and Ridhima Kad) and a summer student (Dominic Lynch), funded by an award from the Association for the study of Animal Behaviour (UK).

1.5.2 Chapter 3

The effect of meiotic drive on non-carrier sperm during spermatogenesis has severe implications for drive male fertility, and consequently on the fitness of the rest of the genome. However, the previous chapter demonstrates that *T. dalmanni* drive males are not sperm limited. In this chapter, I suggest a mechanism by which drive males are able to maintain high numbers of sperm, such that males are capable of producing an adaptive response when they are carriers of drive, and reduce the impact of drive on their fertility. I discuss possible patterns of investment by *T. dalmanni* in the context of theoretical models that predict that males should invest in the production of an optimal ejaculate according to levels of expected sperm competition, even if they are low-resource or low-fertility males, but at the expense of the number of matings that they can achieve. The laboratory work included in this chapter was carried out with the aid of an MSci student (Ridhima Kad).

1.5.3 Chapter 4

There is an abundance of empirical work seeking to understand the drive system in *T. dalmanni*. However it remains the case that genetic markers that can reliably distinguish between drive and wildtype males are sorely needed. In this chapter, I report on four X-linked markers (one microsatellite and three INDEL markers) and describe the extent to which they covary with brood sex-ratio and male morphology, and their reliability for use in identifying drive and wildtype

males from both wild and laboratory samples. I also discuss patterns that point to the possibility of recombination or gene conversion between drive and wild-type X chromosomes. The wild samples utilised in this chapter were collected by Alison Cotton.

1.5.4 Chapter 5

Polyandry is widespread across many taxa, and almost ubiquitous in insects, but this conflicts with the traditional idea that females are constrained by their comparatively large investment in gametes and offspring. In this chapter I take a novel approach, utilising wild-caught individuals, to explore how natural variation among females and males influences fertility gains for females. Wild females were captured and their fertility assessed across time to elucidate any negative effects to female fertility that arise from not remating. Further, females were allowed a single additional mating with a wild-caught male. In a follow-up experiment, the males were manipulated to either be fully fertile, or to have a history of recent mating, in order to examine the impact of variation in male mating history on female fertility. This chapter includes my integrated analysis of data collected from a pair of experiments, the first collected by Elizabeth Harley, and the second by Alison Cotton and James Howie. It has been published in *Ecology & Evolution*: Meade, L., Harley, E., Cotton, A., Howie, J. M., Pomiankowski, A. && Fowler, K. (2017). Variation in the benefits of multiple mating on female fertility in wild stalk-eyed flies. *Ecology & Evolution*. 7:10103-10115. doi: 10.1002/ece3.3486 (Appendix F).

1.5.5 Chapter 6

In this chapter, I summarise the main findings and discussion of the prior chapters. Further, I highlight directions that future research could take to advance our understanding of the evolution of meiotic drive and female multiple mating.

1.5.6 Chapter 7

Here I present additional information pertaining to the previous chapters, as well as a copy of the published version of Chapter 5. Appendix A is the supplemen-

tary information for Chapter 2, presenting model tables and effect size estimates for models reported in this chapter. Similarly, Appendix B is the supplementary information for Chapter 3. Appendix C is the extended methods for Chapter 3, describing the addition of a diet treatment. Appendix **??** provides the supplementary information for the analyses including this additional variable. Appendix D provides the supplementary information for Chapter 4. Appendix E provides the supplementary information for Chapter 5, and lastly, Appendix F is a copy of the published version of this chapter, published in 2017 in *Ecology & Evolution*.

Chapter 2

Adaptive maintenance of ejaculate size in the face of sperm destruction by meiotic drive

Abstract

Meiotic drive genes are a class of segregation distorter that cause the degeneration of non-carrier sperm in heterozygous drive males. It is predicted that the spread of drive alleles through populations could be slowed as a result of sperm limitation of drive males. However, it is likely that sexual selection on male investment in sperm per ejaculate will lead to an adaptive response in drive males. Males that have a fertility disadvantage are expected to compensate by producing competitive ejaculates in response to the expected levels of sperm competition. I test whether males have strategically altered their ejaculate to adapt to the presence of meiotic drive in a species that carries X chromosome meiotic drive, the stalk-eyed fly *Teleopsis dalmanni*. I find that drive males are able to compensate for their loss of sperm. Females mated to drive or standard males store equal numbers of sperm in their long-term storage organs, even after a male's third mating. Additionally, I find evidence to confirm that these sperm are viable, as numbers of sperm that migrated to the short-term storage organ (and site of fertilisation) did not differ between drive and standard males, over two days after mating. This study suggests that conventional assumptions about the effects of drive on male fertility need to be examined more closely. These results have important implications for hypotheses about how stable and/or low frequencies of meiotic drive are maintained in wild populations. Future research needs to determine exactly how drive males compensate for failed spermatogenesis, and how such compensation may trade-off with investment in other fitness traits.

2.1 Introduction

Meiotic drive genes are a class of segregation distorter which manipulate the products of gametogenesis so as to bias transmission in their favour (Burt and Trivers, 2006; Lindholm et al., 2016). Both autosomal and sex chromosome meiotic drive predominantly take place in males that are heterozygous for the distorter (Taylor and Ingvarsson, 2003) and negatively affect the formation of non-carrier sperm during spermatogenesis (Price and Wedell, 2008). The driver enjoys a transmission advantage while the individual suffers reduced fertility and other fitness deficits (Jaenike, 2001; Price and Wedell, 2008; Lindholm et al., 2016). Sex-ratio (SR) meiotic drive occurs when the driver is located on the X-chromosome and acts against Y-bearing gametes (or vice versa), producing a distorted brood sex ratio. Meiotic drive has been observed in a diverse range of taxa, including plants, fungi, mammals and insects, being particularly common in a range of intensively studied mosquito and *Drosophila* species (Taylor, 1999; Jaenike, 2001; Burt and Trivers, 2006).

Males heterozygous for a driving element (drive males) are expected to be sperm limited compared to wildtype males, as half of their sperm is dysfunctional. Abnormal sperm development in drive males has been reported for *Aedes* and *Culex* mosquitoes carrying Y-drive (Wood and Newton, 1991), *D. melanogaster* and house mice carrying autosomal drive (Lyttle, 1991), as well as multiple species of *Drosophila* and other dipterans carrying X-chromosome drive (Novitski et al., 1965; Tokuyasu et al., 1977; Cobbs et al., 1991; Presgraves et al., 1997; Cazemajor et al., 2000; Wilkinson and Sanchez, 2001; Keais et al., 2017). It has been predicted that this deficit slows the spread of drive through populations, as drive males are unable to deliver as many sperm per copulation as wildtype males, and suffer disproportionately when there is sperm competition (Taylor and Jaenike, 2002; Holman et al., 2015).

However, this view fails to consider the likelihood that selection on females and males leads to adaptive responses to ameliorate the negative effects of drive. Both theoretical and empirical work suggests that meiotic drive has

favoured higher levels of female multiple mating (Haig and Bergstrom, 1995; Price et al., 2010, 2014; Holman et al., 2015). Polyandry will hinder the fertilisation success of drive males if the surviving sperm of drive males are poor post-copulatory competitors. Here I investigate an evolutionary response in males, where alterations to the ejaculate allocation might allow drive males to cope better with the detrimental effect of reduced sperm numbers. Male ejaculate allocation strategies are predicted to evolve in response to the average degree of sperm competition faced by an ejaculate (Parker, 1990, 1998; Wedell et al., 2002). Models that examine the co-evolution of male optimal ejaculate expenditure with the degree of sperm competition in a population, predict that males with varying resource levels are generally not expected to vary in their levels of investment per ejaculate (Tazzyman et al., 2009). This is because in any particular mating all males expect to encounter the same levels of sperm competition, and it is this that determines how to optimally allocate their ejaculate (i.e. on the assumption that males do not know how often individual females have mated previously or will mate in the future). Males with greater resources simply invest in a larger number of copulations. From this perspective, drive males could be viewed as males with fewer resources to allocate to reproduction because they waste a proportion of their resources on non-functional sperm, with the prediction that they will make the same allocation per ejaculate as wildtype males, but simply reduce their number of matings. A similar conclusion was reached by Engqvist (2012) in a model that considered the allocation strategies of fertile and sub-fertile males. This predicted that under sperm competition, sub-fertile males should make good their deficit by investing more in an ejaculate than males with standard fertility. Drive males are sub-fertile males, in the sense that a proportion of the sperm that they produce are non-functional (Engqvist, 2012). Consequently, selection for optimal male ejaculate strategies results in drive males increasing their investment per ejaculate and thus the number of viable sperm in their ejaculate. If this is the case, there is no longer a straightforward expectation that drive males should be worse sperm competitors, or that

drive males should have lower fertility per copulation.

I test these ideas using the Malaysian stalk-eyed fly species *Teleopsis dalmanni*. Male carriers of X^{SR} (SR males) produce strongly female-biased broods due to the failed maturation of Y-bearing sperm during spermatogenesis (Presgraves et al., 1997; Wilkinson and Sanchez, 2001). One-half of all sperm produced are expected to be non-functional. Populations of *T. dalmanni* have stable frequencies of X^{SR} ($\sim$10 – 30%), that have persisted across many generations (Wilkinson et al., 2003; Cotton et al., 2014), and drive is also found in its sister species *T. whitei* (Presgraves et al., 1997; Baker et al., 2001*b*). This suggests that there has been ample time for selection to have driven adaptation of ejaculate allocation in drive males. Male *T. dalmanni* transfer few sperm per copulation (Wilkinson et al., 2005; Rogers et al., 2006) as they partition their ejaculate across many matings, a strategy to cope with females that mate repeatedly with multiple males (Wilkinson et al., 1998*a*). Consequently, male ejaculate investment is likely to have been selected in response to the expected level of multiple mating and resulting sperm competition. Furthermore, there is no clear advantage to males in mating first or second in this species (Corley et al., 2006) and so total numbers of sperm are likely to be indicative of a male's sperm competitive ability, as is assumed in sperm competition models based on a fair raffle (Parker, 1998; Wedell et al., 2002; Tazzyman et al., 2009; Engqvist, 2012). There is also no reason to believe that males expect differing levels of sperm competition; whilst some males may mate with more attractive females that gain more matings, these females will also be more fecund (Rogers et al., 2006). Additionally, work on *Diasemopsis meigenii*, an African species of stalk-eyed fly, has demonstrated that males alter sperm number in response to female quality, but do not adjust their ejaculate allocation with variation in male condition (Harley et al., 2013). This provides empirical support for the prediction that males with greater resources do not invest in more sperm per ejaculate (Tazzyman et al., 2009). However, *D. meigenii* is only distantly related to *T. dalmanni* (Baker et al., 2001*a*; Meier and Baker, 2002; Kotrba and Balke, 2006), has a

considerably different life history and reproductive morphology (Kotrba, 1995), and lacks any evidence of meiotic drive—hence it is not a useful model for the questions I want to address here.

During copulation, male *T. dalmanni* pass a spermatophore containing sperm and seminal fluid into the female reproductive tract, where it is attached to the base of the spermathecal ducts (Kotrba, 1996). Sperm from the spermatophore move up the ducts and into the spermathecae. The spermathecae are sclerotised sacs of which there is a singlet (one sac) and a doublet (two sacs) (Kotrba, 1995; Presgraves et al., 1999). These are used for long-term sperm storage, and female *T. dalmanni* can continue to lay fertilised eggs for around three weeks after a single mating (Rogers et al., 2006). To be used in the fertilisation of an egg, sperm must move from the spermathecae to the ventral receptacle (VR) (Kotrba, 1993). The VR has multiple pouches, each of which can store a single coiled sperm, and the proportion of sperm stored in the VR is predictive of male fertilisation success (Rose et al., 2014).

To calibrate SR male investment per ejaculate, I considered measuring spermatophore size and content. The spermatophore size in *T. dalmanni* can be measured (Rogers et al., 2006), but the structure is very small and compact (Kotrba, 1996) and individual sperm numbers are impossible to quantify, even in the much larger spermatophores of *D. meigenii* (Harley et al., 2013). Instead I examined the number of sperm stored in the spermathecae. Spermathecae can be dissected from the female reproductive tract and gently crushed to release stored sperm, that can then be accurately counted. This should reflect the number of sperm transferred, even in SR males. Non-viable Y-bearing sperm are not passed over to the female in the male spermatophore in the related *T. whitei* (Fry and Wilkinson, 2004), and have not been seen in *T. dalmanni* (*personal observation*). I also looked at sperm in the VR, to examine sperm viability. For a male to successfully achieve fertilisation, sperm must be able to survive in the spermathecae (i.e. long-term storage) and then migrate to the VR. To gauge this, I examined the proportion of the VR pouches that were filled at $\sim$6 and at

$\sim$54 hours after a single mating with an SR or an ST male.

In addition, males may adopt ejaculate allocation strategies depending on female quality (Wedell et al., 2002; Reinhold et al., 2002; Harley et al., 2013). The female quality that most obviously affects male fitness is fecundity. In *T. dalmanni* fecundity is positively correlated with female eyespan (Rogers et al., 2006), and males prefer to mate with large eyespan females (Cotton et al., 2015) and give them larger spermatophores (Rogers et al., 2006). So, I used variation in female eyespan as an indicator of fecundity, and counted the sperm in the spermathecae as a measure of SR and ST sperm allocation. I expected males to allocate a greater quantity of sperm per copulation to large females compared to small females, as has been found in the related stalk-eyed fly *D. meigenii* (Harley et al., 2013). I tested the proposition that SR males have adapted to the loss of sperm and also allocate higher number of sperm to high quality females, in accordance with allocation strategies predicted to counteract their fertility disadvantage (Tazzyman et al., 2009; Engqvist, 2012). In a final experiment, males were mated sequentially to three virgin females and I examined sperm numbers in the spermathecae of the females of each of the first, second and third mating, to test whether SR males become sperm depleted quicker than ST males, or if their allocation strategies diverge after multiple matings.

If sperm numbers within females mated to SR males are half those within females mated to ST males, this would support the traditional view that drive males are sub-fertile and matings with them will lead to low female fertility. However, if counts of sperm in females mated to SR males approach those of ST males this would indicate compensatory increase in investment per ejaculate. It would support the hypothesis that SR males have adapted to the loss of sperm caused by drive and maintain their fertility and post-copulatory competitive ability by increasing investment per ejaculate (Tazzyman et al., 2009; Engqvist, 2012).

2.2 Methods

2.2.1 Stock source and maintenance

Flies for the standard stock (ST-stock) population were collected (by S. Cotton and A. Pomiankowski) in 2005 from the Ulu Gombak valley, Peninsular Malaysia (3°19'N 101°45'E). Subsequently, flies were maintained in high density cages (> 200 individuals) to minimise inbreeding. This population has been regularly monitored and does not contain meiotic drive.

Flies for the meiotic drive stock (SR-stock) population were collected from the same location as the ST-stock, in Malaysia in 2012 (by A. Cotton and S. Cotton). To establish and maintain a stock with meiotic drive, a standard protocol was followed (Presgraves et al., 1997). Briefly: wild males (of unknown genotype) were mated to ST-stock females and their offspring (F1) were collected. When an F1 brood was female biased (80% female, > 10 offspring), it was assumed that the father was a carrier of the sex-ratio distorting X^{SR} chromosome, so that the F1 female offspring had genotype X^{SR}/X^{ST}. When sexually mature (> 4 weeks, Baker et al., 2003), F1 X^{SR}/X^{ST} females were mated with ST-stock males and their offspring (F2) were collected. X^{SR}/X^{ST} females and ST-stock males were housed in cage populations of $\sim$100 individuals at 1:1 sex-ratio. Male F2 offspring are expected to be 50:50 X^{SR}/Y:X^{ST}/Y as they inherit either an X^{SR} or X^{ST} chromosome from their mother. F2 males were subsequently mated to ST-stock females to identify those males carrying X^{SR}, and the process repeated.

Even though there was error in the assignment of individuals as carriers of X^{SR}, the process maintains the X^{SR} chromosome in this stock. Over generations the SR phenotype has become more distinct as the stock maintenance procedure selected for female biased broods, so most SR-stock males now produce only female offspring, or at least > 95% female biased broods. Note that because the SR-stock maintenance involves back-crossing to ST-stock males and females, the autosomes, Y-chromosome and mitochondrial backgrounds are homogenised across the two stocks. For brevity, we hereafter refer to X^{SR}/Y

and X^{ST}/Y males as SR and ST males respectively.

The stock populations were kept at 25°C, with a 12:12 h dark: light cycle and fed puréed sweetcorn twice weekly. Fifteen-minute artificial dawn and dusk periods were created by illumination from a single 60-W bulb at the start and end of the light phase. Experimental flies were collected from egg-lays placed in the stock population cages. Egg-lays consist of damp cotton-wool and excess puréed sweetcorn contained in a Petri dish. After eclosion, adult flies were measured for eyespan and thorax length using ImageJ (v1.46) and separated by sex prior to sexual maturity (< 3 weeks after eclosion). Eyespan was defined as the distance between the outer tips of the eyes (Hingle et al., 2001*a*). Thorax length was measured ventrally from the anterior tip of the prothorax along the midline to the joint between the metathoracic legs and the thorax (Rogers et al., 2008).

2.2.2 Sperm in the spermathecae

At the time of testing, all flies were > 6 weeks old and so had reached sexual maturity (Baker et al., 2003). Experimental virgin females were isolated from the ST-stock population prior to sexual maturity and sorted into two groups based on size—large (eyespan $\geq$ 6mm) or small (eyespan 4.1 – 5.2mm). Intermediate eyespan females were discarded. Experimental, non-virgin males were taken from the SR-stock population and are the resulting offspring of X^{SR}/X^{ST} females and ST-stock males from crosses outlined above. They were held in 500ml pots without access to females for at least 48 hours prior to testing, so were not sperm or accessory gland product depleted, as testes and accessory glands are known to recover to full size within 24 hours of mating (Rogers et al., 2005*b*). To test for differences in sperm number, and to examine male sperm allocation dependent on female quality, at artificial dawn experimental males were placed with either a large or small female and allowed to mate. Matings were observed and recorded to the nearest second. They were then kept in isolation for at least 48 hours, and subsequently mated to a female from the opposite size-class.

Mated females were anaesthetised on ice and their reproductive tract were

removed $\sim$4 – 6 hours after mating. The spermathecae were isolated and placed on a glass microscope slide with 15μl 4% formaldehyde and incubated on ice for 20 min. The spermathecae were then washed in a drop of phosphate buffered saline (PBS) and placed into 10 μl of dead stain (5% 2mM propidium iodide, Sperm Viability kit, L-7011; Molecular Probes, Eugene, OR, diluted 1:20 in PBS). A cover slip was placed over the sample and the spermathecae were gently crushed. Fluorescing dead sperm were counted at 1000x magnification under an oil immersion lens using a fluorescence filter. The total number of sperm were counted three times and an average taken. Most males (N = 62) were phenotyped through offspring sex-ratio counts, with a few genotyped using INDEL markers (N = 5), see below.

2.2.3 Sperm in the VR

To examine the sperm that migrated to the ventral receptacle (VR), a similar protocol was followed as above for assays of spermathecae. At artificial dawn males were mated once to either a large or small female. Dissections were performed $\sim$4 – 9 hours after mating, however, the whole female internal reproductive tract was simply dissected into PBS and a cover slip placed over the sample. The total number of empty pouches in the VR, as well as the number of pouches that contained sperm, were counted at 1000x magnification using an oil immersion lens. The overall presence or absence of sperm was also recorded by checking the rest of the female reproductive tract (spermathecae, spermathecal ducts, base of ducts). In a further experiment, females were mated as described and dissected two days after mating ($\sim$51 – 58 hours) and the VR examined as before. From the early period, 118 males were phenotyped through offspring sex-ratio counts, and 121 were genotyped using INDEL markers. From the later period, 195 males were phenotyped, and 54 were genotyped.

2.2.4 Sperm depletion

To examine male sperm depletion over successive matings, a similar protocol was followed to that used to look at sperm in the spermathecae. Females were

all mid-sized with eyespan between 5.25mm and 5.95mm (small and large eyespan females were excluded). At artificial dawn males were introduced successively to three virgin females and allowed to mate. After a male had mated to the first female, he was removed and placed with the second female, and subsequently with a third female. Females were dissected and the sperm stored in the spermathecae were counted, as with the previous assay of spermathecae.

2.2.5 Phenotyping and genotyping

Experimental males were either classified as SR or ST using phenotypic offspring counts, or genotypic INDEL markers. Phenotyped males were kept with three non-focal females for up to 4 weeks and egg-lays, consisting of damp cotton-wool and excess puréed sweetcorn contained in a Petri dish, were collected twice weekly. Males were subsequently stored in ethanol at -20°C. Males were classified as SR or ST through offspring counts by testing for deviations from a 1:1 sex ratio using 2 tests on offspring counts greater than 10. Males with a brood sex-ratio diverging significantly ($P < 0.05$) from 1:1 and with $\geq$ 65% female bias were classified as SR. Males that did not deviate from a 1:1 brood sex-ratio were classified as ST.

Alternatively, males were classified as SR and ST males using two INDEL markers, *comp162710* and *cnv395*. These markers are X-linked and were developed from sequenced drive and non-drive populations from Kanching (3°18'N 101°37'E) and Ulu Gombak (J. Reinhardt and G.S. Wilkinson, *personal communication*). Their allele sizes segregate into two categories—large and small. SR males have a *comp162710* allele length of 201 bp, while ST males have a longer allele of 286 bp. Similarly, SR males have a *cnv395* allele length of $\sim$330 bp and ST males of $\sim$362 bp. A genotype can be assigned, based on the size category of the allele. In phenotyped laboratory samples from the SR-stock population (see Chapter 4), the probability that a male with an SR allele from either one of these markers has at least a strong SR phenotype (brood sex-ratio deviating significantly from 1:1 and female-bias > 90%) is 0.83, and the probability that they have at least a weak SR phenotype (brood sex-ratio deviating significantly

from 1:1 and female-bias > 65%) is 0.91.

For each sample, half a thorax was crushed and digested in 250μl digestion solution (20mM EDTA, 120mM NaCl, 50mM Tris-HCL, 1% SDS, pH 8.0) and 10μl proteinase K (10mg ml^{-1}), and the samples incubated for $\sim$12hrs at 55°C. Proteins were precipitated out with 300μl of 4M ammonium acetate and spun at 13000rpm for 10min. The supernatant was aspirated into 1ml absolute ethanol to precipitate out the DNA, which was pelleted by spinning at 13000rpm for 10min. The DNA pellet was washed in 500ml of 70% ethanol and allowed to dry before being stored in 50μl T10 E0.1 buffer at -20°C. PCR reactions were performed on a 2720 Thermal Cycler (Applied Biosystems, Woolston, UK) in 2μl samples, containing 1μl QIAGEN Mastermix (QIAGEN, Manchester, UK), 1μl Primer mix and 1μl DNA (dried). All primers were at a 0.2μM concentration. PCR reactions had an initial denaturing stage of 95°C for 15min followed by 45 cycles of 94°C for 30sec, 60°C for 1min 30sec and 72°C for 1min 30sec. This was completed by an elongation step of 60°C for 30min. The Applied Biosystems ABI3730 Genetic Analyzer was used to visualise the microsatellites, with a ROX500 size standard. GENEMAPPER 4.0 was used to assign microsatellite allele sizes. Genotyping was carried out at the NERC Biomolecular Analysis Facility at the University of Sheffield.

2.2.6 Statistical analysis

All tests were carried out in R version 3.31 (R Core Team, 2016). First, to test if SR and ST males differed in their ability to mate successfully and achieve sperm storage, analyses on the presence or absence of sperm within the spermathecae (coded as 1 or 0) were fitted using generalised linear mixed effects models (GLMMs) with a binomial error distribution and a logit link function using the *glmer* function from the package *lme4* (Bates et al., 2015). GLMMs with a Poisson error distribution and a log link function were used to look at total sperm counts in successfully mated females (those with sperm present in the spermathecae). These models included male thorax length, eyespan, female size (small or large), male type (SR or ST) and their interactions as fixed effects,

and male ID as a random effect to account for repeated measures. Spermathecal sperm count data were over-dispersed and so an additional observational level random effect (OLRE) was added to account for this (Harrison, 2014).

Analyses of the presence or absence of sperm in the VR, or anywhere in the female reproductive tract, were fitted as a function of male thorax length, eyespan, male type, female size and their interactions, using generalised linear models (GLMs) with a binomial error distribution and a logit link function using the *glm* function. All models looking at sperm in the VR did not include male ID as a random effect, as males did not have repeated measures. The number of pouches in the VR was analysed as a function of female size. Pouch counts were normally distributed (Shapiro-Wilk test $P > 0.2$) and analysed using a linear model using the *lm* function. As VR pouch count depended on female size (see Results), sperm counts in the VR were analysed as proportion data (filled pouches, empty pouches) as a function of male thorax length, eyespan, female size, male type and their interactions in a GLM. These data were over-dispersed and so a quasi-binomial error distribution was used.

Lastly, I report on male latency to mate and copulation duration depending on male genotype and female size. Latency to mate was modelled in a Poisson GLM or a Poisson GLMER if male ID was included as a random effect. To account for over-dispersion a quasi- distribution was used, or an OLRE included. Copulation duration was modelled in LM, or an LMER if male ID was included as a random effect, and excluding extreme values (> 150 seconds, N = 16 matings across all experiments).

Model simplification was performed by stepwise removal of nonsignificant factors by comparing models of decreasing complexity based on Akaike information criterion. All models, except for LMs analysing VR pouch counts, included male thorax to control for male body size, as well as residual male eyespan. Residual eyespan are the residuals from an LM after the variation in eyespan explained by thorax size has been removed (Dormann et al., 2013). All reports of eyespan use residuals. Across all the analyses, the addition of female size

as a covariate made no difference to any other relationships and so this is not further commented on. P values were calculated using the *Anova* function from the car package (Fox and Weisberg, 2011). Model tables and effect sizes can be found in Appendix A.

2.3 Results

2.3.1 Sperm in the spermathecae

Females were dissected in an early period of 5.402 ± 0.527 (mean ± SD, N = 109) hours after mating. Sperm was present in the spermathecae of most mated females (N = 97). There was no effect on sperm presence of male thorax length (χ^2_1 = 0.522, P = 0.47), male eyespan (χ^2_1 = 0.265, P = 0.606), or male drive type (χ^2_1 = 0.733, P = 0.392). On average, females that mated successfully had 89.62 ± 69.166 (mean ± SD) sperm stored in their spermathecae. There was no effect on the number of stored sperm of male thorax length (χ^2_1 = 1.075, P = 0.3), male eyespan (χ^2_1 = 1.783, P = 0.182), or male drive type (χ^2_1 = 0.222, P = 0.638, Fig. 2.1). Female size did not influence the chance that a female had sperm present (χ^2_1 = 1.31, P = 0.253), or the total number of sperm (χ^2_1 = 0.47, P = 0.493), in the spermathecae.

2.3.2 Sperm in the VR

In a second set of experiments, I measured sperm presence in the ventral receptacle (VR). Experimental females were dissected at two time points, either an early period shortly after mating as above (mean ± SD 5.672 ± 1.403 hours, N = 239), or a late period two days after mating (mean ± SD 54.032 ± 1.57 hours, N = 246). I looked at sperm presence in the VR of females that had a successful mating (sperm observed in the spermathecae and/or VR; early N = 170, late N = 214). Sperm presence in the VR did not vary with male thorax size in either the early (χ^2_1 = 0.539, P = 0.463) or late period (χ^2_1 = 0.005, P = 0.944), male eyespan in the early period (χ^2_1 = 0.23, P = 0.632), nor male drive type in the early (χ^2_1 = 1.392, P = 0.238). However, there was an interaction between male eyespan and drive type in the late period (eyespan x male drive

type χ^2_1 = 9.528, P = 0.002). Females were less likely to have sperm in their VR when mated to SR males with small eyespan. This interaction was in the same direction but not significant in the early period (χ^2_1 = 1.303, P = 0.254). There was no effect of female size on sperm presence in the VR in the early period (χ^2_1 = 1.19, P = 0.096), but in the late period, small females were more likely to have sperm present in the VR than large females (χ^2_1 = 6.404, P = 0.011 small: 83%, large: 67%).

In addition to recording sperm presence in the VR, the number of pouches filled and empty were recorded. Pouch number increased with female size ($F_{1,451}$ = 94.224, P < 0.001; mean ± s.e. small: 31.913 ± 0.374, N = 188, large: 36.776 ± 0.328, N = 265), so the proportion of filled pouches was used in further analyses (including only females with sperm in the VR, early: N = 74, late: N = 156). The proportion of pouches filled did not depend on male thorax length in the early ($F_{1,70}$ = 2.206, P = 0.142) or late period ($F_{1,152}$ = 0.015, P = 0.902), male eyespan in the early ($F_{1,70}$ = 0.935, P = 0.337) or late period ($F_{1,152}$ = 0.667, P = 0.415), nor male drive type in the early ($F_{1,70}$ = 0.486, P = 0.488, 15.148%) or late period ($F_{1,152}$ = 0.366, P = 0.546, 15.214%, Fig. 2.2). Again, there was no effect of female size in the early ($F_{1,69}$ = 0.015, P = 0.904) or late period ($F_{1,151}$ = 0.161, P = 0.689).

Sperm presence was recorded in the spermathecae as well as the VR. The distribution of sperm between these two sperm storage organs was not random in either the early (Pearson's Chi-squared test: χ^2 = 38.465, P < 0.001, N = 239) or late period (χ^2 = 60.657, P < 0.001, N = 246). When sperm were present in the VR, it was also always present in the spermathecae (with a single exception in the early period where four sperm were observed in the VR of a female and none in the spermathecae). However, the reverse was not always true when sperm was present in the spermathecae in either the early (sperm presence in the spermathecae: 71%, VR: 31%, both: 31%) or late (spermathecae: 87%, VR: 63%, both: 63%) time periods. Of the females in which sperm was present in the spermathecae (early N = 170, late N = 214), the proportion of females

with sperm present in the VR increased from the early (43.5%) to the late period (72.9%, χ^2_1 = 35.469, P < 0.001).

2.3.3 Sperm depletion

In the final experiment, males (N = 46) were mated sequentially to three different females (N = 135 matings). On average, females that mated successfully had 147.1 ± 81.507 (mean ± SD) sperm stored in their spermathecae. As in the previous experiments, there was no difference in the number of females where sperm was present due to either male thorax length (χ^2_1 = 0.354, P = 0.552) or male eyespan (χ^2_1 = 0.644, P = 0.422). Similarly, there was no difference due to mating order (χ^2_1 = 1.739, P = 0.187) or between male drive types (χ^2_1 = 0.087, P = 0.767).

Across all successful matings (i.e. from males that had at least one mating where sperm were present, N = 118), sperm number did not differ across mating order (χ^2_1 = 0.303, P = 0.582). However, there were interactions with male thorax length. There was an interaction between male thorax length and drive type (thorax x male drive type χ^2_1 = 5.57, P = 0.018). When females were mated to ST males, sperm counts were lower for those mated to small thorax males, however, sperm counts in females mated to SR males remained high even for those mated to small thorax SR males. There was an additional interaction between male thorax length and eyespan (thorax x eyespan χ^2_1 = 5.851, P = 0.016). For both male types, sperm counts decreased with male eyespan when male thorax was small, but sperm counts increased with male eyespan when male thorax was large. There was no interaction between male drive type and mating order (χ^2_1 = 3.047, P = 0.081).

Looking across those males that had up to three contiguous matings resulting in sperm stored in the spermathecae (N = 103), sperm numbers did not vary with male thorax length (χ^2_1 = 2.712, P = 0.100), male eyespan (χ^2_1 = 0.003, P = 0.96), mating order (χ^2_1 = 0.158, P = 0.691) or between male drive types (χ^2_1 = 1.163, P = 0.281). There was no interaction between male drive type and mating order (χ^2_1 = 3.200, P = 0.074).

2.3.4 Mating latency and mating duration

In two experiments, SR males took longer to start mating than ST males ("sperm in the spermathecae": χ^2_1 = 7.669, P = 0.006, N = 97; "sperm in the VR (early)": $F_{1,164}$ = 5.314, P = 0.022). In one experiment, all males took longer to mate when presented with a small female ("sperm in the spermathecae": χ^2_1 = 5.963, P = 0.015, N = 97). Generally, copulation duration did not differ between SR and ST males, or between small and large females, however, in one instance, ST males copulated for longer than SR males when females were small ("sperm in the VR (early)": $F_{1,159}$ = 5.266, P = 0.023). Including latency duration and copulation time in models did not alter any of the results above.

2.4 Discussion

Male meiotic drive typically involves the dysfunction and loss of half of the gametes produced (Burt and Trivers, 2006; Price and Wedell, 2008). Whilst this is beneficial to the drive element itself because it specifically excludes non-carrier sperm, in many systems it leads to a reduction in fertility of drive males (Peacock and Erickson, 1965; Jaenike, 1996; Atlan et al., 2004; Wilkinson et al., 2006; Angelard et al., 2008; Price et al., 2012; Pinzone and Dyer, 2013), particularly under conditions of sperm competition (Wilkinson and Fry, 2001; Atlan et al., 2004; Angelard et al., 2008; Price et al., 2008*a*). In some cases, drive has been demonstrated to result in a reduction in the amount of sperm transferred to females at mating (*D. melanogaster*: Peacock and Erickson, 1965; *D. simulans*: Angelard et al., 2008; *D. pseudoobscura*: Price et al., 2008*a*). However, contrary to these observations, I find no evidence that SR male ejaculates have reduced sperm numbers in *T. dalmanni*. In female spermathecae, the long-term primary storage organs, sperm numbers were not different between those of females mated to SR and ST males. Likewise, transfer of sperm to the VR, a small organ that sperm migrate to prior to subsequent use in fertilisation, was also similar for SR and ST male sperm, over both short and longer time frames (after two days). Furthermore, sperm numbers in storage were similar for fe-

males mated to SR and ST males even after the males had mated multiple times. Across all sampled organs and time points, SR males had comparable amounts of sperm to ST males per ejaculate.

These findings in *T. dalmanni* challenge the conventional assumption that drive acts like a genetic disease that causes disruption of normal male reproductive activity. This static view ignores the possibility of adaptive responses to drive that ameliorate its negative effects and restore organismal fitness. Such evolutionary change may be unlikely if drive has arisen recently (e.g. SR in *D. simulans,* Derome et al., 2004) or persists at such a low frequency that it is likely to exert little selective effect (e.g. SR in *D. recens* and *D. quinaria*, Jaenike, 1996). But this is not the case for X^{SR} in *T. dalmanni* where the current form of drive is estimated to be over half a million years old (Paczolt et al., 2017). Meiotic drive has a longer history in this group as it is present in the sister species *T. whitei* that diverged around 3.5 million years ago (Christianson et al., 2005; Swallow et al., 2005). In addition, X^{SR} occurs at a significant frequency of $\sim$20% in many populations (Wilkinson et al., 2003; Cotton et al., 2014). The long-term persistence of drive at a significant frequency in this lineage seems likely to have created a selective environment favouring adaptive changes in reproductive behaviour in order to tolerate the presence of drive.

There is considerable theoretical work on optimal ejaculate expenditure relevant to understanding investment strategies in drive males (Parker, 1998; Tazzyman et al., 2009; Engqvist, 2012). Given that sperm competition follows a fair raffle, and that males lack information about previous or future female mating patterns, then all males face the same risk and intensity of sperm competition (Parker, 1998). It follows that a male's optimal ejaculate size per mating is independent of the resources that he has available to allocate to reproduction (Tazzyman et al., 2009). Males with more resources deliver the same ejaculate size per mating as poorly resourced males, but simply mate more often (Tazzyman et al., 2009). If we consider drive males to have half the resources for reproduction compared to wildtype males, then they are expected to pro-

duce ejaculates of similar size (in order to be able to optimise their success in sperm competition) but simply mate less often. A subsequent more explicit version of this modelling argument, considers two types of males (sub-fertile vs. fertile) (Engqvist, 2012). Given that drive males produce half as many sperm as wildtype, that sperm competition is common and that drive is at a relatively high frequency, then the ejaculate size of the two male types is predicted to be similar. Drive males may even be expected to invest more per ejaculate, given stochasticity in the expected number of additional mates, because drive males invest in fewer matings and so gain less from dividing their reproductive resource when females have mated less than expected (Engqvist, 2012). These findings, which are the first to explicitly measure the effect of meiotic drive on ejaculate investment, mirror these predictions. I found no difference between drive and wildtype male in either ejaculate size or storage (Fig. 2.1 – 2.3). I did not find higher input from drive males, though this is likely to reflect the limits of sample size and high variability observed.

Males in multiple species can distinguish between virgin and mated females (Thomas, 2011). However, in a mating system like in *T. dalmanni*, where females mate throughout their lifetime, virgins are likely to be rare. There is no evidence that males can distinguish recently mated females from sperm depleted females, although this needs to be further investigated. Alternatively, females may differ predictably in their likelihood to remate. For example, large fecund females may mate at a high rate. However in reality this may be difficult to predict as these females will also have more eggs and so may not represent an arena of high sperm competition compared to small females with fewer eggs that mate at a lower rate.

The capacity of drive males to compensate for failed spermatogenesis in order to match wildtype male investment must come at the cost of other potential resource expenditures. The most obvious cost is the number of matings that a male can achieve, which is the trade-off modelled in recent theoretical work (Tazzyman et al., 2009; Engqvist, 2012). This is evident in *T. dalmanni* as

X^{SR} males have been shown to mate at lower rates than wildtype males (Wilkinson et al., 2003; S. Finnegan, *unpublished data*), also documented in other meiotic drive systems (Price et al., 2012; Verspoor et al., 2016). Lower mating rates rather than fewer sperm per mating may explain why females mated to drive males suffer reduced fertility. This effect is only seen when males are housed with many females, and must achieve high mating frequencies, as has been reported in *T. dalmanni* (Wilkinson and Sanchez, 2001; Wilkinson et al., 2006), as well as a range of other *Drosophila* systems (*D. melanogaster*: Peacock and Erickson, 1965; *D. pseudoobscura*: Wu, 1983*a*; *D. quinaria* and *D. recens*: Jaenike, 1996; *D. simulans*: Capillon and Atlan, 1999; Atlan et al., 2004; *D. neotestacea*: Pinzone and Dyer, 2013). There may be additional trade-offs associated with meiotic drive. In *T. dalmanni*, X^{SR} is linked to smaller relative eyespan (Wilkinson et al., 1998*b*; Cotton et al., 2014), meaning that X^{SR} males tend to be less attractive to females than X^{ST} males. This may mean that the life history of X^{SR} males has evolved to expect fewer mating opportunities, so that maintaining their ejaculate size per copulation leads to less cost incurred from being denied matings. These ideas need both further theoretical and empirical investigation.

These results suggest that the assumption that drive males are weak post-copulatory competitors due to producing fewer viable sperm, should not be a standard assumption. However, drive male sperm may generally be of poorer quality than wildtype male sperm, as the action of the drive element during spermatogenesis may also damage carrier sperm (Newton et al., 1976; Nasuda et al., 1998; Price and Wedell, 2008). This has implications for hypotheses that propose polyandry to be a mechanism that prevents the spread of drive (Haig and Bergstrom, 1995; Zeh and Zeh, 1996; Taylor and Jaenike, 2002; Holman et al., 2015), and empirical studies, mostly in *Drosophila* species, that link polyandry with the prevention of the spread of drive (Beckenbach, 1978; Wilkinson et al., 2003; Price et al., 2010, 2014). Drive males have been found to be worse sperm competitors than expected, even after taking into account their

reduced numbers of sperm, in *Drosophila* (Angelard et al., 2008; Price et al., 2008*a*) and also in *T. whitei* (Fry and Wilkinson, 2004) and *T. dalmanni* (Wilkinson et al., 2006). However, I find no evidence that SR sperm is less viable in *T. dalmanni*, as SR and ST male sperm were equally capable of migrating to the VR, even after two days (Fig. 2.2). This points to the importance of other non-sperm components as being fundamental for the differences in SR and ST male fertility. In particular, accessory gland products have been shown to play major roles in various aspects of sperm storage and fertilisation in insects, including sperm competition (Avila et al., 2011, 2015; Perry et al., 2013). In *T. whitei*, SR male sperm has been claimed to have reduced capacity to fertilise eggs in the presence of ST male seminal fluid (Fry and Wilkinson, 2004), but such an effect has not been confirmed in other systems. Furthermore, mating rates in *T. dalmanni* are significantly associated with accessory gland size (Baker et al., 2003; Rogers et al., 2005*a*,*b*), with similar patterns found in *D. melanogaster* (Markow et al., 1978; Herndon et al., 1997) and small residual eyespan reliably predicts smaller accessory gland size (Rogers et al., 2008).

Further work is needed to establish how SR males can provide females with equivalent amounts of sperm as ST males, despite the failure of Y-bearing sperm to mature during spermatogenesis. The amount of sperm stored in female spermathecae correlates with male testis size in *T. dalmanni* (Fry, 2006), and data from other species demonstrates that selection on increased sperm production capacities promotes larger testis size (Price and Wedell, 2008). Accordingly an increase in testis size may be a mechanism by which SR males could produce large numbers of viable sperm, and may lead to trade-offs with other traits such as accessory gland size and secondary sexual signal traits such as exaggerated eyestalks (Emlen and Nijhout, 2000). Little is known about the function of accessory gland products in *T. dalmanni*. It seems unlikely that they delay female remating (Grant et al., 2002), but in *T. whitei* SR male sperm are incapacitated by the seminal fluid of ST males (Fry and Wilkinson, 2004) and may play a role in sperm viability. Further work should aim to verify whether the

performance of SR males is limited by their ability to produce accessory gland products of sufficient quantity or quality, as is implied by their lower mating frequency relative to that of ST males (Wilkinson et al., 2003).

Hypotheses that seek to explain the maintenance of drive polymorphisms generally assume that drive males produce fewer viable sperm than wildtype males, and have reduced fertility and are poorer sperm competitors. However, this neglects the possibility that drive males have adapted to their sub-fertile condition and are able to compensate. Theoretical models that look at the evolution of male ejaculate allocation do not predict sub-fertile males to invest any less per ejaculate than do standard males. I show here that drive males can indeed compensate, because their ejaculates are able to match those of standard males in sperm number, even after a third mating, and their sperm are capable of migrating from storage to the site of fertilisation. Further work is needed to ascertain exactly how these drive males are able to compensate for failed spermatogenesis, and how this influences investment in other important aspects of male fertility such as the costly non-sperm components of male ejaculate and the production of primary and secondary sexual traits.

2.5 Figures

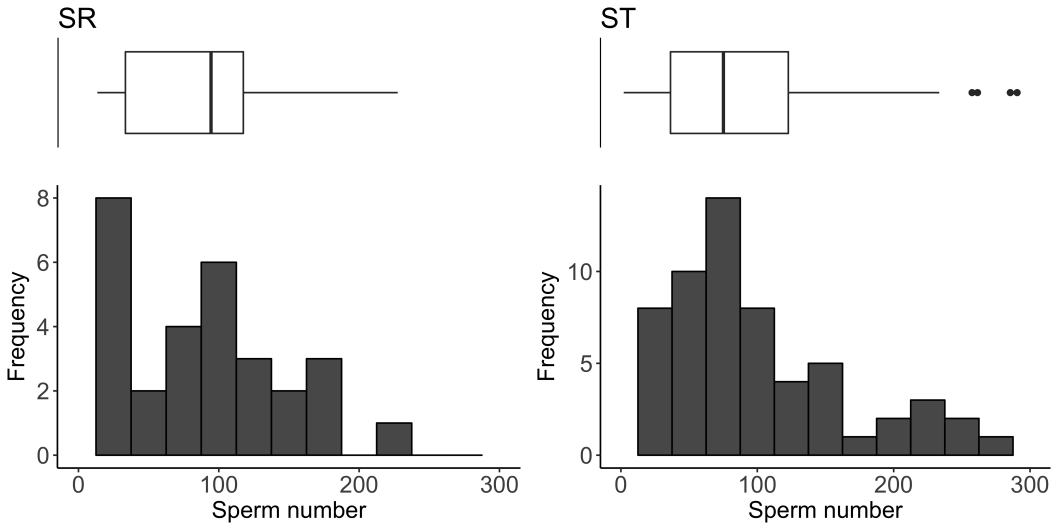

Figure 2.1: Number of sperm in female spermathecae after a single mating with either an SR (left) or ST (right) male. Upper: boxplots (first to third quartile) with median line and whiskers (1.5 IQR), and lower: frequency distribution of sperm counts.

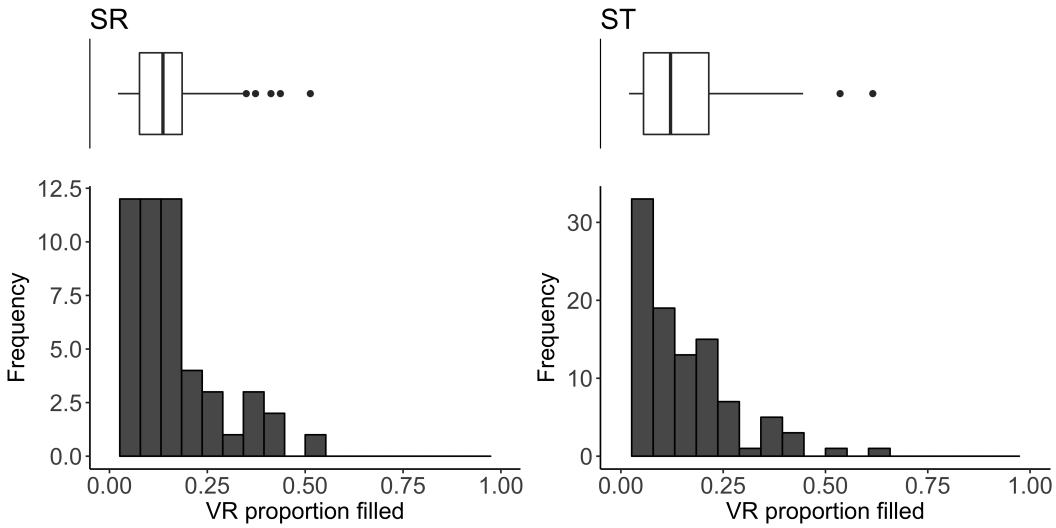

Figure 2.2: Proportion of pouches in the ventral receptacle (VR) filled with sperm after a single mating with an SR (left) or ST (right) male, two days after mating (late period). Upper: boxplots (first to third quartile) with median line and whiskers (1.5 IQR), and lower: frequency distribution of proportions.

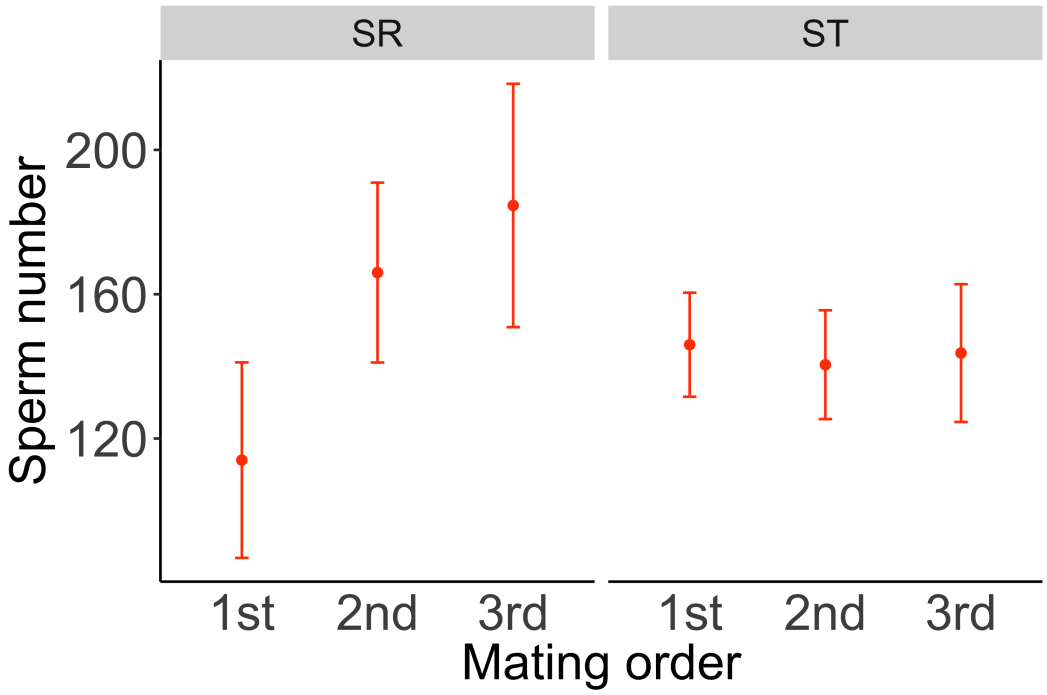

Figure 2.3: Number of sperm stored in the female spermathecae after a SR (left) or ST (right) male's first, second or third mating. Points show mean ± s.e. Data presented includes only those males that have up to three contiguous matings that resulted in sperm storage in the spermathecae.

Chapter 3

Adaptive compensation in fertility to meiotic drive in a stalk-eyed fly

Abstract

Meiotic drivers are selfish genetic elements that subvert normal Mendelian segregation, biasing transmission in their favour, and have been observed in a wide range of taxa. Meiotic drive negatively impacts the formation of non-carrier sperm during spermatogenesis, with severe implications for drive male fertility. However, in many species fertility does not suffer as expected, and drive males produce ejaculates that contain similar numbers of sperm to wildtype male ejaculates. Whilst the drive element enjoys a transmission advantage, the consequential reduction in male fertility reduces the fitness of the rest of the genome, and so mechanisms to circumvent drive are expected to evolve. In this chapter, I suggest a mechanism by which drive males are able to maintain high numbers of sperm and propose that males can produce an adaptive response when they are carriers of drive, and reduce the impact of drive on their fertility. I examine this possibility in the Malaysian stalk-eyed fly, *Teleopsis dalmanni*. Male carriers of X-linked meiotic drive produce predominantly female offspring due to the destruction of Y-bearing sperm. However, drive carrying *T. dalmanni* males are not sperm limited. I demonstrate that drive males have larger testes than wildtype males and additionally are able to achieve high fertility. Furthermore, there appears to be a trade-off in investment in testis size against investment in body size, and in primary and secondary sexual traits. Drive males are smaller than wildtype males on average, and have smaller accessory glands and smaller eyespan than than expected for their body size. Both accessory gland size and eyespan are traits linked to male mating frequency. These patterns in *T. dalmanni* fit with theoretical models that predict males should invest in producing an optimal ejaculate according to levels of expected sperm competition, even if they are low-resource or low-fertility males, but at the expense of the number of matings that they can achieve.

3.1 Introduction

Meiotic drivers are a well-known category of selfish genetic elements that subvert normal Mendelian segregation during gametogenesis in diploid organisms, and cause the biased transmission of a gene or chromosome over its homologous partner (Burt and Trivers, 2006; Lindholm et al., 2016). They have been observed in a range of taxa including plants, fungi, mammals, Hymenoptera and particularly in Dipteran species including mosquitoes, stalk-eyed flies and *Drosophila* (Hurst and Pomiankowski, 1991; Presgraves et al., 1997; Taylor, 1999; Jaenike, 2001). Drivers are passed to more than half and sometimes to all of the offspring of heterozygous carriers, and consequently can rapidly spread through populations even if they lower the fitness of the individual in other respects (Lindholm et al., 2016). Populations are often observed to be polymorphic for meiotic drive, with frequencies remaining stable over long periods of time (Jaenike, 2001; Lindholm et al., 2016), indicating that there must be sufficient counter selection to oppose the spread of drive.

Much theoretical and empirical work has attempted to understand how stable frequencies of drive are maintained in natural populations (Wu, 1983*a*; Haig and Bergstrom, 1995; Jaenike, 1996; Zeh and Zeh, 1996; Jaenike, 1999, 2001; Taylor and Jaenike, 2002; Price and Wedell, 2008; Holman et al., 2015; Lindholm et al., 2016). Autosomal and sex chromosome meiotic drive predominantly occur in heterozygous males (Taylor and Ingvarsson, 2003) and so negatively affect the formation of non-carrier sperm during spermatogenesis, and indeed, degenerate sperm have been observed from the testes of drive males in multiple systems (Tokuyasu et al., 1977; Brown et al., 1989; Presgraves et al., 1997; Cazemajor et al., 2000; Wilkinson and Sanchez, 2001; Shahjahan et al., 2006; Owusu-Daaku et al., 2007; Tao et al., 2007*b*; Keais et al., 2017). This loss of sperm is often associated with a fertility disadvantage for carrier males (Price and Wedell, 2008), for example in *D. pseudoobscura* drive males have a fertility reduction relative to wildtype of $\sim$0.5 (Price et al., 2012) and similarly, in the "Paris" system in *D. simulans* the reduction is $\sim$0.41 (Angelard et al., 2008).

However, in many cases, despite this loss of sperm, there is at most only a minor reduction in drive male fertility (Peacock and Erickson, 1965; Wood and Newton, 1991; Beckenbach, 1996; Jaenike, 1996; Capillon and Atlan, 1999; Wilkinson and Sanchez, 2001; Wilkinson et al., 2006; Verspoor et al., 2016). In *Teleopsis whitei* and *D. melanogaster*, this has specifically been demonstrated to be the result of drive males producing ejaculates that contain similar numbers of sperm to wildtype male ejaculates (Peacock and Erickson, 1965; Fry and Wilkinson, 2004). In addition, in Chapter 2, I demonstrate that this is also the case for *T. dalmanni*. In multiple systems, it appears that drive males are able to produce similar numbers of viable sperm as wildtype males, translating into high fertility, despite the loss of sperm during spermatogenesis.

These patterns of drive male fertility in fact demonstrate similarities with predictions of sperm competition theory, which suggest that males with fewer resources or low intrinsic fertility, such as drive males, should invest similarly to a highly resourced or highly fertile male in numbers of sperm per ejaculate, in response to expected levels of post-copulatory competition (Parker, 1998; Tazzyman et al., 2009; Engqvist, 2012). There are some reports of these patterns in species without drive (Lewis et al., 2011; Harley et al., 2013), and it may be likely that drive males are able to adaptively compensate for failed spermatogenesis and so are not constrained by loss of sperm. Males that have half the resources to allocate to reproduction (as we could consider drive males to be) are expected nonetheless to optimise their success in sperm competition and produce ejaculates of similar size to males with greater resources, but to mate less often (Tazzyman et al., 2009). Furthermore, drive males may alternatively be viewed as intrinsically less fertile, where for the same level of investment in an ejaculate, drive male fertility is lower as a proportion of their sperm are non-functional. Here sub-fertile drive males must invest more sperm than wildtype males per ejaculate to compete (Engqvist, 2012). Reproduction is costly for males (Wedell et al., 2002; Lewis et al., 2008) and so we can expect that males will have to trade-off between ejaculate size and the mating rate (Parker, 1982).

If this is the case we may expect males that carry drive to invest heavily in functions that increase sperm production, such as testis, and reduce investment in those that promote high mating frequencies.

Sperm numbers are positively correlated with male testis size in multiple species (Møller, 1989; Pitnick and Markow, 1994; Gage, 1994; Pitnick, 1996; Stockley et al., 1997; Fry, 2006), and increased testis size is a correlated response to selection on increased sperm production capacity (Price and Wedell, 2008). Investing in increased testis size could be a potential mechanism by which drive males are able to increase sperm production and produce competitive ejaculate despite the loss of sperm during spermatogenesis.

To produce competitive ejaculates, drive males must produce equal numbers of viable sperm as wildtype males. However, as resources are inevitably finite an additional investment in increased production must lead to reduced investment elsewhere. Differences in drive and wildtype fertility are often only found under circumstances involving multiple mating (Peacock and Erickson, 1965; Wu, 1983*b*; Jaenike, 1996; Atlan et al., 2004; Wilkinson et al., 2006; Price et al., 2012; Pinzone and Dyer, 2013). This suggests that, in these cases, drive males invest in few but good quality matings and consequently invest less than wildtype males in traits that enable males to mate more frequently, such as primary and secondary sexual ornaments, and courtship behaviour. There is some evidence that this is the case in several drive systems. In the stalk-eyed fly, *T. dalmanni*, reduced eyespan, a sexual ornament, is linked to X drive (Wilkinson et al., 1998*b*; Cotton et al., 2014). Furthermore, in house mice, male carriers of the *t*-complex have smaller seminal vesicles (Sutter and Lindholm, 2016), which are know to become reduced in size with repeated matings (Sutter et al., 2016), and have a reduced ability to be able to maintain territories (Carroll et al., 2004). Lastly, in *D. pseudoobscura*, males carrying X drive appear to be slower to achieve matings (Verspoor et al., 2016).

In *T. dalmanni*, a drive element is located on the X chromosome (X^{SR}) and causes sex-ratio drive males (SR males) to produce predominantly female-

biased broods (Presgraves et al., 1997; Wilkinson and Sanchez, 2001). Despite this transmission advantage, the frequency of drive has remained broadly stable ($\sim$10 – 30%) in *T. dalmanni* populations over many generations (Wilkinson et al., 2003; Cotton et al., 2014). Biased brood sex-ratios produced by SR males are due to failed production of Y-bearing sperm during spermatogenesis (Presgraves et al., 1997; Wilkinson and Sanchez, 2001), but previous work has demonstrated that SR males are not sperm limited (Chapter 2) and provide females with equal numbers of sperm as ST males, even after multiple matings. I use this species of stalk-eyed fly to make the first investigation of how drive males compensate for the loss of half their sperm and examine other fitness traits relating to mating frequency that may be negatively affected by any increased investment in sperm production.

We now know that SR males are capable of producing ejaculates with large numbers of sperm (Chapter 2), and large testis size may be an indication of how they are able to provide an adaptive response to their drive genotype. I measured the size of male testes, as in this species the amount of sperm stored by a female is correlated with the testis size of her male mate (Fry, 2006), and sperm numbers are frequently reported to correlate with testis size in other species (Møller, 1989; Pitnick and Markow, 1994; Gage, 1994; Pitnick, 1996; Stockley et al., 1997). Large testis size would indicate that SR males can increase their production of sperm to match ST males in viable sperm numbers per ejaculate, in response to their drive status and the expected levels of sperm competition (Tazzyman et al., 2009; Engqvist, 2012).

However, increased investment in sperm production likely leads to a trade-off with the number of matings that an SR male can achieve (Parker, 1982). I tested this by additionally measuring the size of another primary sexual organ, the male accessory glands. Mating rates in *T. dalmanni* are not limited by testis size but instead are positively correlated with accessory gland size (Baker et al., 2003; Rogers et al., 2005*a*,*b*), and accessory glands, but not testes, become depleted after repeated matings (Rogers et al., 2005*b*). Furthermore, accessory

gland proteins are likely to be costly components of the ejaculate in *T. dalmanni*, as they are in other insect species (Linklater et al., 2007; Perry et al., 2013; Crean et al., 2016).

Additionally, I test whether this response to drive has an impact on male fertility. I expect SR males to exhibit large testes in response to drive (Tazzyman et al., 2009), leading to the production of smaller accessory glands as they must trade off investment in sperm production against mating frequency. As a result SR males should generally maintain high fertility like ST males, but when mating frequency is not limited by females, SR male fertility will be reduced if they are unable to mate as frequently as ST males.

Alternatively, large numbers of sperm in SR male ejaculate may simply be explained by the presence of sperm that does not have the same fertilisation capacity as an ST male's sperm. There are various reports where drive males have lower fertility than expected from sperm numbers alone when in competition with a wildtype ejaculate (Taylor, 1999; Wilkinson and Fry, 2001; Atlan et al., 2004; Angelard et al., 2008; Price et al., 2012), and, in *T. whitei*, even when in competition with only the seminal fluid of the rival male (Fry and Wilkinson, 2004). Conversely, previous work does not support the hypothesis of reduced fertilisation capacity in *T. dalmanni*, as equal numbers of sperm reach the female long-term storage organ, the spermathecae, and are equally capable of migrating from the spermathecae to the site of fertilisation (Chapter 2). However, if large numbers of inviable sperm, rather than increased investment in sperm production, do explain sperm numbers, then I would not expect increased testis size in SR males, and would expect generally low SR male fertility.

3.2 Methods

3.2.1 Stock source and maintenance

Flies for the standard stock (ST-stock) population were collected (by S. Cotton and A. Pomiankowski) in 2005 from the Ulu Gombak valley, Peninsular Malaysia (3°19'N 101°45'E). Subsequently, flies were maintained in high density cages

(> 200 individuals) to minimize inbreeding. This population has been regularly monitored and does not contain meiotic drive. Flies for the sex ratio meiotic drive stock population were collected from the same location in Malaysia in 2012 (by A. Cotton and S. Cotton). To establish and maintain a stock with meiotic drive, a standard protocol has been followed (Presgraves et al., 1997). SR-stock males consist of 50:50 SR:ST, where most SR males now produce only female offspring, or at least > 95% female biased broods (see Chapter 2 for details).

The stock populations are kept at 25°C, with a 12:12 h dark: light cycle and fed puréed sweetcorn twice weekly. Fifteen-minute artificial dawn and dusk periods are created by illumination from a single 60-W bulb at the start and end of the light phase. Experimental flies were collected from egg-lays placed in the stock population cages. Egg-lays consist of damp cotton-wool and excess puréed sweetcorn contained in a Petri dish. After eclosion, adult flies were measured for eyespan and thorax length using ImageJ (v1.46) and separated by sex prior to sexual maturity (> 3 weeks after eclosion). Eyespan was defined as the distance between the outer tips of the eyes (Hingle et al., 2001*b*). Thorax length was measured ventrally from the anterior tip of the prothorax along the midline to the joint between the metathoracic legs and the thorax (Rogers et al., 2008).

3.2.2 Mating treatment

On day 1, prior to the mating treatment, experimental males were housed in mixed-sex cages. Males were all > 6 weeks old and had reached sexual maturity (Baker et al., 2003). Cages were set up in 16 batches (two 12 L cages per batch) over a period of 46 days, and each cage contained 15 – 20 males, with the same number of females. On day 6 the females were switched between cages, and on day 12 the females were removed and discarded. On the evening of day 14, males were divided into three treatment groups. The first two groups of males were housed individually in the upper chamber of mating pots (Cotton et al., 2015). In the lower chamber, separated from the male by a card partition, either

one (*x1*) or five (*x5*) experimental females were added. These females were virgin, > 6 weeks old, and had medium – large eyespan ($\geq$ 5.7mm). On day 15, just before dawn, the card partitions were removed and males and females could mix and mate. After 10 hours, males and females were separated. Males were then kept overnight in individual 500ml pots. The third group of males (unmated) were not exposed to experimental females and were simply moved individually to 500ml pots on day 14. From day 1 to day 14 males were fed one of two diets, either puréed sweet corn or a 25% sugar solution (see Appendix C; Rogers et al., 2008). These diets did reduce accessory gland size but had no impact on male fertility (see Appendix B) and are not further reported on.

3.2.3 Male reproductive organ size and fertility

At dawn on day 16, males of all three groups were anaesthetised on ice for dissection. Male testes and accessory glands were dissected into a small amount of PBS on a glass microscope slide and a cover slip added (Baker et al., 2003; Rogers et al., 2005*b*, 2008, Fig. 3.1). Uncoiled organs were visualised using differential interference contrast microscopy and images were photographed at 50x magnification using a monochrome microscope camera and QCapture Pro imaging software (v7.0). Organ area was measured using ImageJ (v1.50i) by tracing the outline of the organ to give a longitudinal surface area. The area of a single randomly chosen testis was measured and the area of both accessory glands were measured (Baker et al., 2003; Rogers et al., 2005*b*). Males were stored in ethanol at -20°C prior to genotyping.

After mating, experimental females were transferred to 500ml pots and allowed to lay eggs. The bases of pots consisted of damp cotton-wool covered with blue tissue paper and females were fed with puréed sweetcorn in a quartered plastic weigh-boat. Eggs were collected every 2 – 3 days for 14 days, and were allowed to develop in a Petri dish with moist cotton-wool for at least a further 3 days. Fecundity was recorded through egg counts of eggs laid on the tissue paper. Hatching success was used as an estimate for fertility. Eggs were observed under a light microscope at 10x magnification; fertilised eggs that

have hatched appear as empty chorion cases. Additionally, eggs were counted as fertilised if they failed to hatch but showed clear signs of development (brown horizontal striations in the chorion and early mouthpart formation, Baker et al., 2001*a*). Unfertilised eggs show no signs of development.

3.2.4 Genotyping

Males were genotyped at the NERC Biomolecular Analysis Facility, University of Sheffield. Two INDEL markers, *comp162710* and *cnv395*, were used to identify SR and ST males. These markers are X-linked and were developed from sequenced drive and non-drive populations from Kanching (3°18'N 101°37'E) and Ulu Gombak (J. Reinhardt and G.S. Wilkinson, *personal communication*). SR males have a *comp162710* allele length of 201 bp, while ST males have a longer allele of 286 bp. Similarly, SR males have a *cnv395* allele length of $\sim$330 bp and ST males of $\sim$362 bp (see Chapter 4).

To extract DNA, a protocol was followed *sensu* Bruford et al. (1998). For each sample, half a thorax was crushed and digested in 250μl digestion solution (20mM EDTA, 120mM NaCl, 50mM Tris-HCL, 1% SDS, pH 8.0) and 10μl proteinase K (10mg ml^{-1}), and the samples incubated for $\sim$12hrs at 55°C. Proteins were precipitated out with 300μl of 4M ammonium acetate and spun at 13000rpm for 10min. The supernatant was aspirated into 1ml absolute ethanol to precipitate out the DNA, which was pelleted by spinning at 13000rpm for 10min. The DNA pellet was washed in 500ml of 70% ethanol and allowed to dry before being stored in 50μl T10 E0.1 buffer at -20°C. PCR reactions were performed on a 2720 Thermal Cycler (Applied Biosystems, Woolston, UK) in 2μl samples, containing 1μl QIAGEN Mastermix (QIAGEN, Manchester, UK), 1μl Primer mix and 1μl DNA (dried). All primers were at a 0.2μM concentration. PCR reactions had an initial denaturing stage of 95°C for 15min followed by 45 cycles of 94°C for 30sec, 60°C for 1min 30sec and 72°C for 1min 30sec. This was completed by an elongation step of 60°C for 30min. The Applied Biosystems ABI3730 Genetic Analyzer was used to visualise the microsatellites, with a ROX500 size standard. GENEMAPPER 4.0 was used to assign microsatellite

allele sizes.

3.2.5 Statistical analysis

All tests were carried out in R version 3.32 (R Core Team, 2016). I first tested if male genotypes differed in their morphological and reproductive traits. To test if male genotypes differed in thorax length I analysed thorax length as a function of genotype (ST, SR) in a linear model (LM). Thorax length was transformed (squared) to normalise the distribution of the errors. To test if genotypes differed in eyespan, eyespan was modelled in a LM as a function of thorax length, genotype and their interaction. To test if male testis and accessory gland size differed between genotypes I analysed both testis size and accessory gland size as functions of mating group (*unmated*, *x1*, *x5*), thorax length, eyespan and genotype, and up to their three-way interactions in linear mixed effect models (LMMs), including batch as a random effect. Model selection was performed by stepwise removal of nonsignificant factors by comparing models of decreasing complexity based on Akaike information criterion. As well as differences in relative trait sizes between genotypes, differences in absolute trait sizes are also reported by inspecting models with body size excluded.

Next I tested if females mated to SR or ST males differed in total and proportion fertility. I analysed total fertility (total number of fertile eggs) and proportion fertility (fertile eggs, non-fertile eggs) as functions of mating group (*x1*, *x5*), thorax length, eyespan, genotype and the interaction between mating group and genotype, in generalised linear mixed effects models (GLMMs) and including batch as a random effect. Total fertility was modelled in a GLMM using a Poisson error distribution and a log link function, while proportion fertility was modelled using a binomial error distribution and logit link function. These data were over dispersed, and in all GLMMs an observation-level random effect was added to account for this (Harrison, 2014). Across all GLMMs, egg count data were only used if males had a minimum of 11 days of egg collections. Fecundity and fertility data were also excluded where females laid no eggs or fertility was $< 2\%$.

I next determined which reproductive traits were important predictors of total fertility, proportion fertility and fecundity (total number of eggs). Total and proportion fertility were modelled as before, and fecundity was modelled in a GLMM with Poisson error distribution and with batch and observation level as random effects. Models included mating group, thorax length, eyespan, testis size, accessory gland size and genotype, and up to all three-way interactions. Model selection was performed as before by stepwise removal of nonsignificant factors, with the stipulation that thorax length was always included when eyespan, testis size or accessory gland size remained in the model.

To avoid collinearity of male morphological and reproductive traits with body size, models used residual values for eyespan, testis size and accessory gland size. For example, residual eyespan are the residuals from an LM after the variation in eyespan explained by body size has been removed (Dormann et al., 2013). Continuous variables in models predicting fecundity and fertility were centered and rescaled using the *scale* function in R, as egg counts and male traits are on disparate scales. P values were calculated with type II tests or type III tests where significant interactions were present, using the *Anova* function from the *car* package (Fox and Weisberg, 2011). Model tables and effect sizes of all analyses are reported in Appendix B.

3.3 Results

3.3.1 SR morphological and reproductive trait size

SR males had smaller thorax length compared to ST males ($F_{1,357}$ = 8.745, P = 0.003; mean ± s.e. SR: 2.29 ± 0.013mm; ST: 2.336 ± 0.009mm), smaller eyespan on average ($F_{1,357}$ = 45.631, P < 0.001; mean ± s.e. SR: 8.048 ± 0.046mm; ST: 8.402 ± 0.031mm) and smaller eyespan after controlling for thorax length ($F_{1,355}$ = 5.868, P = 0.016; Fig. 3.2).

Despite their small size, SR males had larger testes than ST males on average ($F_{1,280.16}$ = 73.796, P < 0.001; mean ± s.e. SR: 1.873 ± 0.04mm^2; ST: 1.486 ± 0.022mm^2, Fig. 3.3a). In contrast, SR males had smaller accessory

glands ($F_{1,335.36}$ = 16.353, P < 0.001; mean ± s.e. SR: 0.296 ± 0.008mm^2; ST: 0.35 ± 0.008mm^2, Fig. 3.4a). When accounting for body size, SR male testis size remained larger than ST male testis ($F_{1,282.78}$ = 99.982, P < 0.001, Fig. 3.3b), and SR male accessory gland size remained smaller ($F_{1,334.03}$ = 7.801, P = 0.006, Fig. 3.4b).

Testis size increased with thorax length ($F_{1,283.71}$ = 6.697, P = 0.010, Fig. 3.3a) and additionally with male relative eyespan ($F_{1,283.99}$ = 15.354, P < 0.001, Fig. 3.3b). Accessory gland size did not change with male thorax length ($F_{1,335.63}$ = 0.639, P = 0.406, Fig. 3.4a), but increased with male relative eyespan ($F_{1,335.69}$ = 8.971, P = 0.003, Fig. 3.4b).

3.3.2 SR fertility

SR and ST males did not differ in total (χ^2_1 = 2.146, P = 0.120, N = 215, Fig. 3.5) or proportion fertility (χ^2_1 = 2.469, P = 0.116, N = 215). Males mating with five females achieved higher total fertility than those mating with a single female (χ^2_1 = 43.698, P < 0.001, N = 215), but were unable to fertilise as high a proportion of eggs (χ^2_1 = 6.021, P = 0.014, N = 215). The interaction between the number of females (one or five) and genotype did not influence total (χ^2_1 = 0.591, P = 0.442, N = 215) or proportion fertility (χ^2_1 = 1.377, P = 0.241, N = 215). There was also no effect of male thorax length on total (χ^2_1 = 0.688, P = 0.407, N = 215), or proportion fertility (χ^2_1 = 1.268, P = 0.260, N = 215). Similarly, there was no effect of male relative eyespan on total (χ^2_1 = 1.439, P = 0.230, N = 215) or proportion fertility (χ^2_1 = 0.017, P = 0.895, N = 215).

3.3.3 Reproductive trait size and fertility

Male testis size was an important predictor of fertility. Testis size predicted total (χ^2_1 = 6.216, P = 0.013, N = 165, Fig. 3.6) and proportion fertility (χ^2_1 = 6.216, P = 0.013, N = 165), where males with larger relative testis size had higher fertility. The addition of testis size did not alter the relationship between genotype and total (χ^2_1 = 0.018, P = 0.895, N = 173) or proportion fertility (χ^2_1 = 0.260, P = 0.610, N = 173). There was no interaction between testis size and genotype

predicting total (χ^2_1 = 0.164, P = 0.686, N = 173) or proportion fertility (χ^2_1 = 0.617, P = 0.432, N = 173). Total fertility increased with accessory gland size only for large eyespan males (interaction relative eyespan x accessory glands χ^2_1 = 7.133, P = 0.008, N = 173, Fig. 3.7). Accessory gland size did not predict proportion fertility (χ^2_1 = 0.160, P = 0.689, N = 165).

3.3.4 Fecundity

The *x5* group laid more eggs than the *x1* group (χ^2_1 = 70.210, P < 0.001, N = 199; mean ± s.e. *x1*: 95 ± 4.92, *x5*: 179 ± 6.94). Females mated to males with both large eyespans and large accessory glands for their body size laid the most eggs, while females mated to males with only large eyespan or large accessory glands did not produce more eggs than males with smaller traits (interaction relative eyespan x accessory gland χ^2_1 = 3.995, P = 0.046, N = 199). No other male traits influenced fecundity (χ^2_1 < 0.130, P > 0.7, N = 199).

3.4 Discussion

Drive males are expected to be sperm limited, as non-carrier sperm are rendered inviable (Price and Wedell, 2008). However, a common pattern between drive systems is that there is no, or only equivocal, direct evidence for drive male sperm limitation and there are many examples where drive males exhibit high fertility, and produce ejaculates with large numbers of sperm (Peacock and Erickson, 1965; Wood and Newton, 1991; Capillon and Atlan, 1999; Taylor, 1999; Jaenike, 1996; Verspoor et al., 2016, Chapter 2). In this chapter, I provide an explanation as to how drive males achieve this, despite the fact that drive causes the destruction of sperm. I present the first evidence that males adapt to drive by increasing their investment in primary sexual organs in order to increase sperm production. I show that this results in a negative trade-off, reducing investment in traits that promote high mating frequencies. Accessory glands, eyespan and body size are reduced in drive males, and both accessory gland size and eyespan are traits linked to male mating frequency.

Reduced fertility due to drive reduces the fitness of the rest of the genome

and so mechanisms to circumvent drive are expected to evolve, for example through the evolution of suppressors of drive. There is an extensive literature on the scope for genetic elements that interfere and suppress the action of drive in males (Atlan et al., 1997; Presgraves et al., 1997; Dyer, 2012; Larracuente and Presgraves, 2012; Branco et al., 2013). Suppressors have been found on the Y chromosome when drive is linked to the X chromosome (Carvalho et al., 1997; Cazemajor et al., 1997; Branco et al., 2013), as well as suppressors throughout the rest of the genome (Carvalho and Klaczko, 1993; Atlan et al., 2003; Tao et al., 2007*b*). The drive system in *T. dalmanni* is relatively ancient at around 500,000 years since the divergence of X^{ST} and X^{SR} (Paczolt et al., 2017), so there has been ample time for an adaptive response to evolve, but no clear evidence of autosomal suppression. Y-linked suppressors have been suggested (Presgraves et al., 1997; Wilkinson et al., 1998*b*), however later work has not found any evidence for suppression in this species (Reinhold et al., 1999; Wolfenbarger and Wilkinson, 2001; Paczolt et al., 2017). Evidence for suppression has been found in many systems (Stalker, 1961; Tokuyasu et al., 1977; Gummere et al., 1986; Hauschteck-Jungen, 1990; Wood and Newton, 1991; Carvalho and Klaczko, 1993; Carvalho et al., 1997; Cazemajor et al., 1997; Atlan et al., 2003; Tao et al., 2007*b*), but it is not the rule as no evidence of suppressors has been uncovered in *D. pseudoobscura* or *D. neotestacea* (Dyer, 2012). A mechanism that has yet to be evaluated is the possibility that males can produce an adaptive response to being a carrier of drive, and subvert the impact of drive on male fertility. In *T. dalmanni*, populations harbour X-linked drive (Presgraves et al., 1997; Wilkinson et al., 2003; Cotton et al., 2014; Paczolt et al., 2017) and SR males produce ejaculates with equivalent numbers of sperm as ST male ejaculates, even after multiple matings (Chapter 2). Here I clearly demonstrate that drive males are able to achieve these high sperm numbers through greatly enlarged testes (Fig. 3.3). Testis size in this (Fry, 2006), and many other insect species (Møller, 1989; Gage, 1994; Pitnick and Markow, 1994; Pitnick, 1996; Stockley et al., 1997), correlates with sperm number, and

so this is a likely mechanism by which males can increase sperm production.

Enlarged testis size in SR males appears to be an effective mechanism for ensuring high fertility, as testis size was a good predictor of male fertility. Males with large testes had high total fertility (Fig. 3.6) and high proportion fertility. Testis and accessory gland size are usually highly correlated in size (Baker et al., 2003, this study). However here I found that this was not the case for SR males—as SR male testis size increased, accessory gland size remained small, and SR males had small accessory glands overall (Fig. 3.4). This mirrors the findings that SR males were on average smaller than ST males, with smaller thorax and eyespan (Fig. 3.2), and additionally had smaller eyespan and accessory glands than expected for their body size. Other examples in non-drive systems demonstrate trade-offs between male testis size and investment in traits that impact male pre-copulatory mating success such as courtship behaviour and sexual ornaments (Droney, 1998; Simmons and Emlen, 2006; Yamane et al., 2010; Somjee et al., 2015). In *T. dalmanni*, male courtship behaviour is minimal (de la Motte and Burkhardt, 1983; Wilkinson and Dodson, 1997). However, accessory glands limit male mating frequency (Baker et al., 2003; Rogers et al., 2005*a*,*b*) and accordingly SR males mate less frequently than ST males (Wilkinson et al., 2003; S. Finnegan, *unpublished data*). Furthermore, the sexual ornament male eyespan is important in male mating success as both in the laboratory and in the wild, females prefer to roost and mate with large eyespan males (Wilkinson and Reillo, 1994; Hingle et al., 2001*a*; Cotton et al., 2010) and large eyespan males tend to win in male-male competition (Panhuis and Wilkinson, 1999). How the disparities between drive male testis size, accessory gland size and eyespan influence courtship behaviour is currently unknown and requires further investigation. Here I show for the first time that SR males have reduced body size, and show that SR males have smaller eyespan and relative eyespan, corroborating previous links between male eyespan and drive (Presgraves et al., 1997; Wilkinson et al., 1998*b*; Cotton et al., 2014).

Predictions from theoretical analyses suggest that males should invest in

their ejaculate in response to the expected levels of sperm competition (Parker, 1998). This remains the expectation even when males are low-resource or low-fertility males (Tazzyman et al., 2009; Engqvist, 2012). When males have fewer resources to invest they must still produce ejaculates that are competitive, but sacrifice the number of ejaculates they can make and hence reduce their mating frequency (Tazzyman et al., 2009). Similarly, males that are intrinsically of low fertility (for example a portion of their sperm are non-viable), must increase their investment to produce adequate ejaculates at a cost to mating frequency (Engqvist, 2012). Consequently, males that have adapted by investing more to the production of sperm, from their finite resources committed to reproduction, will have fewer resources for other reproductive functions that remain necessary for high mating frequencies. We see exactly this pattern in drive carrier *T. dalmanni* males—males have reduced body size, as well as reduced eyespan and accessory glands, even when body size is accounted for. Conversely, drive males have large testes. Consequently, two traits that are linked to male mating frequency—accessory gland size and eyespan—are reduced in drive males, suggesting that drive males have a life history that involves fewer mating opportunities.

Currently it is uncertain what causes this pattern of increased testes size, but reduction in traits related to mating frequency. It appears that SR males adopt a different investment strategy in their reproductive and somatic traits compared to ST males. A simple model describes a finite amount of resource for reproduction, distributed between the production of sperm and non-sperm components of ejaculate. Increased investment in sperm inevitably leads to decreased investment in accessory gland products. Reduced body size and eyespan may stem from the accumulation of deleterious mutations within the inversion on X^{SR}. Deleterious mutations can be purged through recombination between X^{SR} and X^{ST}. This requires double crossover events to produce viable mitotic products (Navarro et al., 1997), and gene flow between the X^{SR} and X^{ST} is estimated to be very low (Johns et al., 2005; Paczolt et al., 2017). This will have

a negative effect on these traits that are costly and highly condition-dependent (David et al., 2000; Cotton et al., 2004*a*; Bellamy et al., 2013). Mutations producing condition-dependent reduction in male size will result in SR males being relatively unattractive and able to gain fewer mating opportunities. Consequently, investment in accessory glands which serve to enable higher mating rates will give lower returns than diversion of resources into larger testes, allowing SR males to compete under conditions of high sperm competition. These ideas, and how these trade-offs impact drive equilibrium frequency, will need further investigation, in particular under the mating conditions that occur in nature.

How might drive males mechanistically adjust investment in testes when they carry meiotic drive? Transcriptomic analyses comparing sexually mature males have identified a large number of genes with differential expression in SR male testes (approximately equal numbers showing up and down regulation), and these genes are disproportionately X-linked (Reinhardt et al., 2014). This indicates that genetic linkage between the drive element and alleles beneficial to its transmission constitutes a plausible mechanism by which SR males increase investment in testis production. The structure of the driving X chromosome is not known in *T. dalmanni*, except that one or more inversions cover a large fraction of the chromosome, resulting in low recombination and reduced gene flow between X^{SR} and X^{ST} (Johns et al., 2005; Paczolt et al., 2017). Trapping of alleles within the X^{SR} inversion(s) avoids sexual conflict that would arise if these alleles were able to move freely between the different X haplotypes.

In the case of *T. dalmanni*, I show that it is no longer a surprise that drive males can achieve high fertility. Indeed, in this study drive males are able to fertilise just as many eggs as standard males, not only when given access to a single female, but also when allowed to mate with five females (Fig. 3.5). This is in contrast to some previous findings demonstrating that drive *T. dalmanni* males have reduced fertility at high mating rates (Wilkinson and Sanchez, 2001; Wilkinson et al., 2006). However, the experimental design of these prior studies differed from the current experiment in vital ways. Primarily, the mating condi-

tions imposed are far away from the natural range of experiences that a male would expect to encounter (Cotton et al., 2010, 2015). Typically, males copulate with the same set of females on his lek at dusk and at dawn, before dispersal (Lorch et al., 1993; Wilkinson and Reillo, 1994). However one study presented males with 8 females continuously over a period of one week, and reported a reduction in fertility compared to standard males of $\sim$0.68 (Wilkinson et al., 2006), while another presented males with 4 females over 3 weeks and reported a fertility reduction of $\sim$0.74 (Wilkinson and Sanchez, 2001). This indicates that fertility differences in *T. dalmanni* can be found between drive and standard males, but only under extreme conditions of very high mating frequencies, much higher than is typical of natural populations.

On the other hand, certain extreme conditions may be closer to those experienced in the wild, and their impact needs to be examined. For example, larval and adult diet is likely to be highly variable (Wilkinson and Reillo, 1994; Felton, 1996), as we can infer from the high variability in relative eyespan and female fecundity in wild-caught compared to laboratory flies (Cotton et al., 2014; Meade et al., 2017). A trade-off between ejaculate size and number will most likely be more extreme under dietary stress where resources become limiting, and males are under greater pressure to allocate resources optimally. In a number of species, diet quality and quantity has a significant impact on the the production of secondary sexual traits such as male ornaments (Hooper et al., 1999; Hill, 2000; Cotton et al., 2004*a*; Devigili et al., 2013; Johns et al., 2014), as well as primary sexual traits such as testis and accessory gland size (Ward and Simmons, 1991; Droney, 1998; Baker et al., 2003; Rogers et al., 2008; Joseph et al., 2016). In *T. dalmanni*, both larval and pre-maturity diet manipulations are known to have a large impact on traits such as male eyespan, accessory gland and testis size (Baker et al., 2003; Cotton et al., 2004*a*; Rogers et al., 2008). In this study, I manipulated post-maturity adult diet. This had the desired effect of reducing accessory gland size (see Appendix B), whilst not altering male thorax length or eyespan as these traits are determined during larval growth and

reflect resources available during this developmental period. However the manipulation had no impact or either ST or SR male fertility. This suggests two things, firstly that accessory gland size—and male mating frequency—was not vital over this 10 hour mating period, supported by the finding that accessory gland size was not a main predictor of fertility. Secondly, that the diet manipulation was not extreme enough to illicit a response in even SR male fertility, despite already have reduced accessory glands. Future work would aim at determining SR male sensitivity to manipulations of larval, pre- and post- maturity diet quality.

Accessory gland products appear to be energetically limiting and their quality is highly variable with factors including male age, mating history and environment, independent of sperm quality (Perry et al., 2013; Crean et al., 2016). Accessory gland size is the limiting factor in male mating frequency in insect species such as *D. melanogaster* (Bangham et al., 2002; Linklater et al., 2007), *D. pseudoobscura* (Crudgington et al., 2009) and in *T. dalmanni* (Baker et al., 2003; Rogers et al., 2005*a*,*b*), and accessory gland products have been shown to play vital roles in various aspects of insect fertilisation (Perry et al., 2013; Avila et al., 2011, 2015). Importantly, the role of accessory gland products in the fertility of drive males has not yet been established. Where resources are limiting, differences may be uncovered between drive and wildtype male fertility that was not revealed here. Diet is likely to be an important ecological factor for male fitness, but its impact on the fertility of drive and wildtype males and primary sexual trait size has not been examined.

This study found an interaction that hints at differences between SR and ST male fertility. Male fertility increased with accessory gland size, only for males with large eyespan for their body size (Fig. 3.7). There was no specific effect of genotype here, however, while SR males have small accessory glands and small eyespan, indicating that they may be less able to achieve as high fertility as ST males under certain conditions, particularly in situations that are time-constrained (requiring high mating frequency) or allowing female choice. Further

work is needed to explicitly focus on how variation in male eyespan and organ size relates to fertility between SR and ST males, for example by standardising eyespan and body size across all focal males and examining their reproductive organ size and fertility.

Drive causes the destruction of non-carrier sperm during spermatogenesis, and this can leave clear evidence in the testes of drive males (Policansky and Ellison, 1970; Tokuyasu et al., 1977; Brown et al., 1989; Presgraves et al., 1997; Cazemajor et al., 2000; Wilkinson and Sanchez, 2001; Shahjahan et al., 2006; Owusu-Daaku et al., 2007; Tao et al., 2007*b*; Keais et al., 2017). However, despite this, the fertility differences between drive and wildtype males are not clear cut. How then can this be explained? Here I demonstrate that drive males can increase the production of viable sperm through investment in testes. Drive males have larger testes than wildtype males in *T. dalmanni*, and indeed drive males can achieve high fertility. These patterns fit with theoretical models that examine the evolution of male ejaculate allocation, and predict that males should invest optimally per ejaculate, but that those males with fewer resources will forgo high mating frequency as a consequence. I additionally show that male traits that are associated with mating frequency—accessory gland size and eyespan—are both reduced in male drive carriers. Further work is needed to determine the precise ecologically relevant conditions under which drive male fertility is pushed to its limits. It will be important to assess the impact of variation in time scales, mating frequency and diet quality.

3.5 Figures

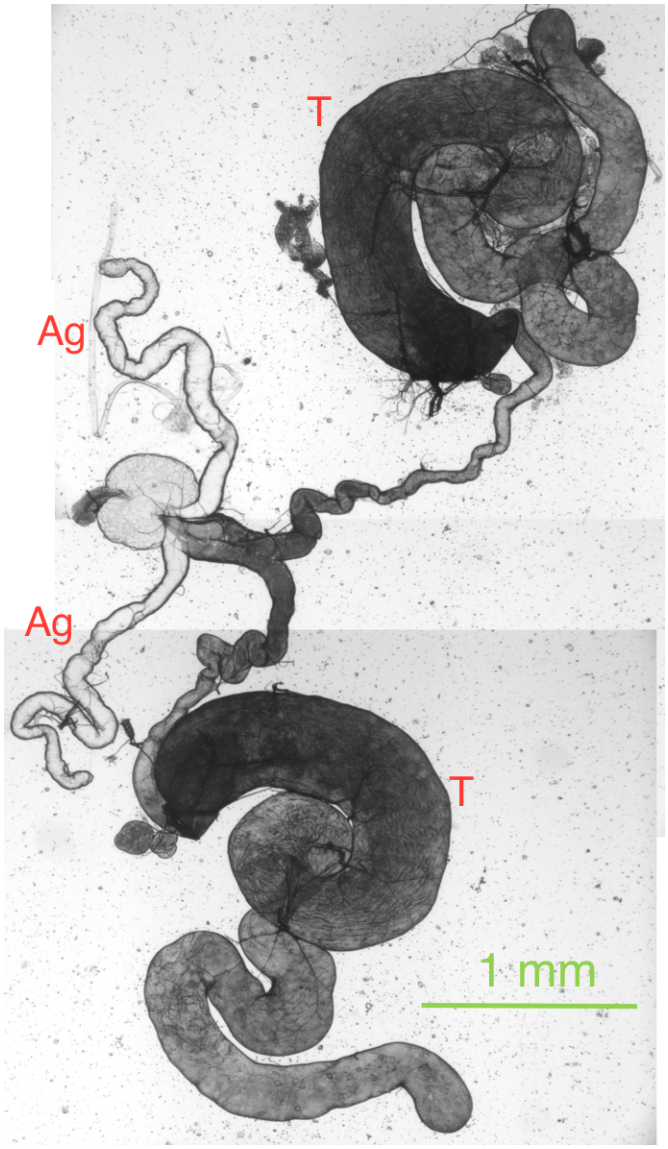

Figure 3.1: *T. dalmanni* testes (T) and accessory glands (Ag). Dissected and uncoiled organs were visualised using differential interference contrast microscopy at 50x magnification, and images were photographed using a monochrome microscope camera.

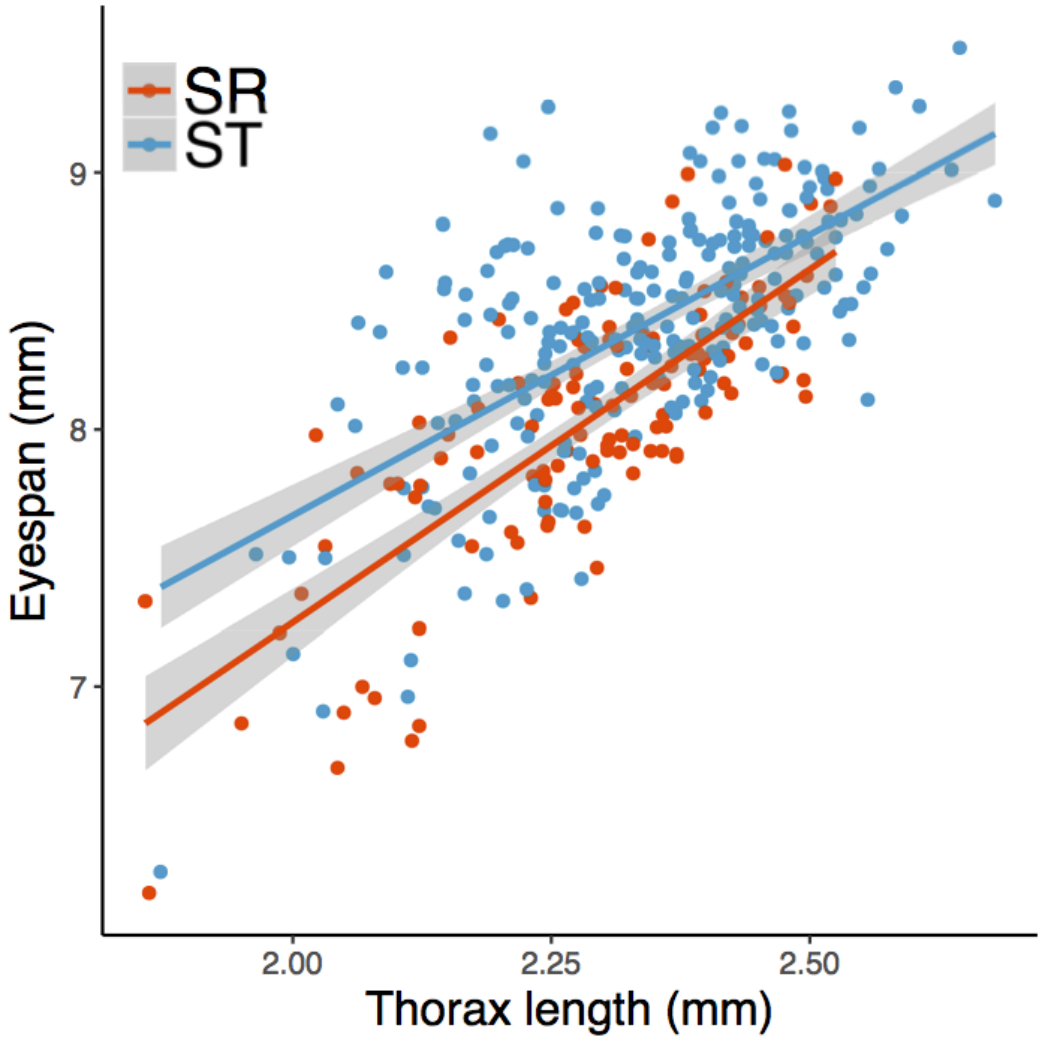

Figure 3.2: Male eyespan (mm) against male thorax length (mm) for SR (red) and ST (blue) males. Eyespan increases with thorax length and SR males have small eyespans for their body size compared to ST males across all thorax lengths. Grey shading shows ± 1 s.e.

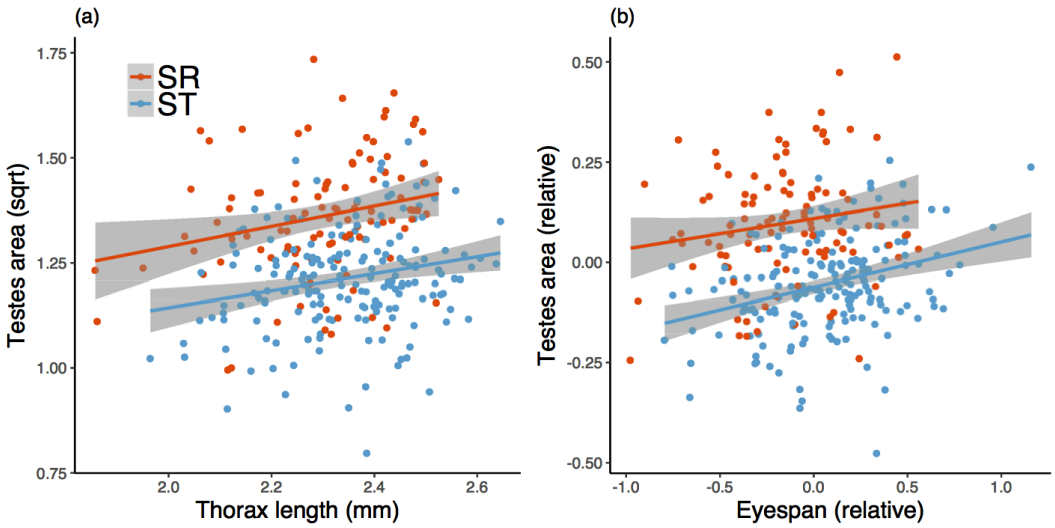

Figure 3.3: Male testis area against (a) thorax length and (b) relative eyespan, for SR and ST males. Testis area increased with male thorax length. When variation due to male thorax length is accounted for (relative testis area and relative eyespan), eyespan provides additional information on male testis area. SR males had larger testis size for their thorax length and eyespan than ST males. Grey shading shows ± 1 s.e.

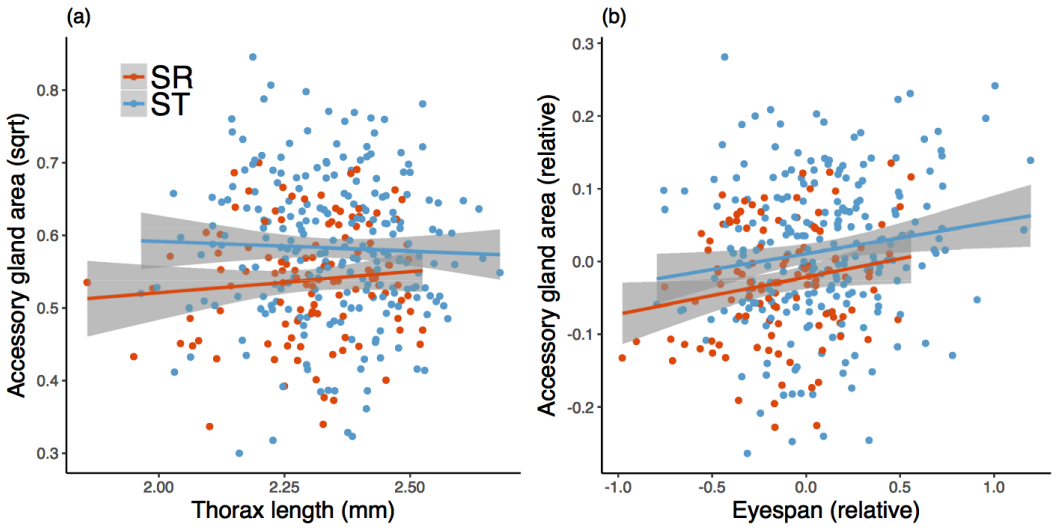

Figure 3.4: Male accessory gland area against (a) thorax length and (b) relative eyespan, for SR and ST males. Accessory gland area did not change with male thorax length. When variation due to male thorax length is accounted for (relative accessory gland area and relative eyespan), eyespan provides information on male accessory gland area. SR males had smaller accessory glands for their thorax length and for their eyespan than ST males. Grey shading shows ± 1 s.e.

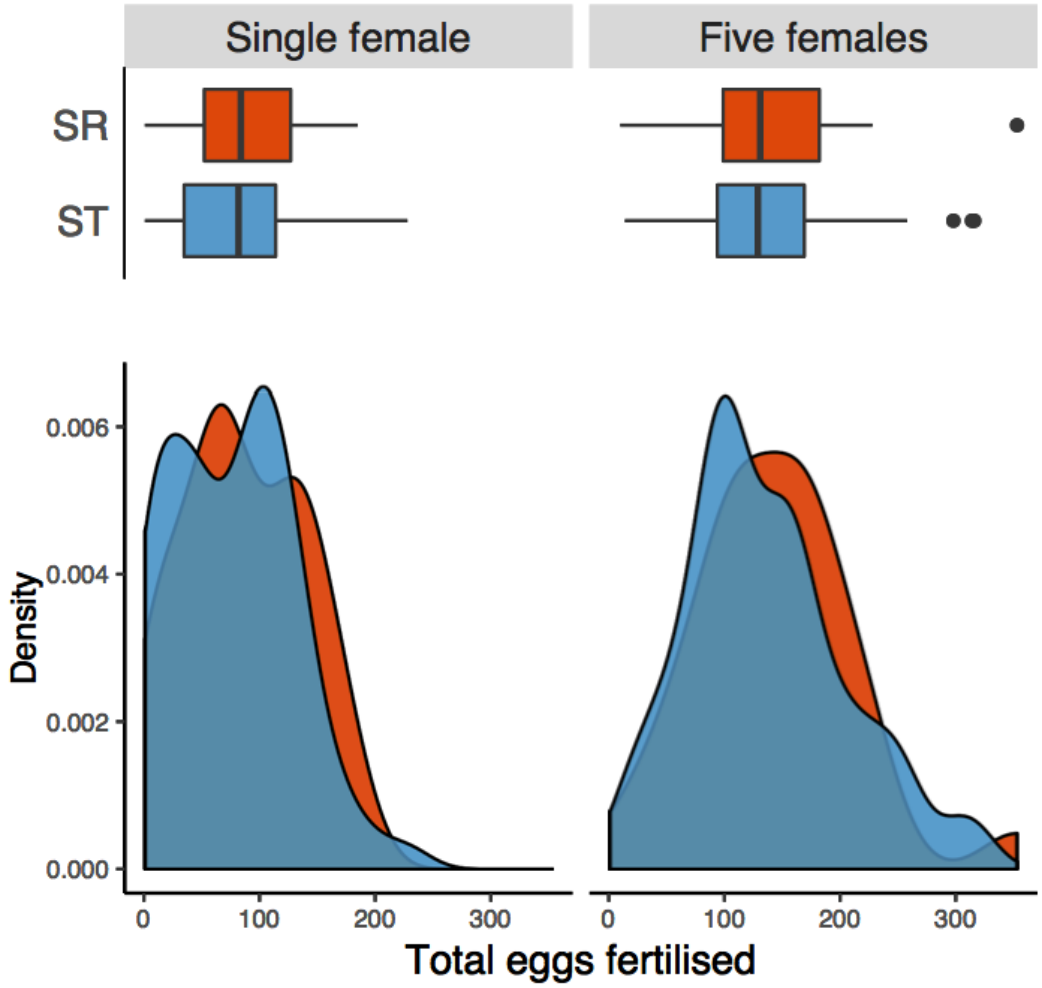

Figure 3.5: Upper panel: Box plots (median and interquartile range) of the total number of eggs that were fertilised by SR (red) and ST (blue) males when allowed to mate with one or five females. Lower panel: Kernel probability density of data for SR (red) and ST (blue) males. SR and ST males did not differ in the number of eggs they fertilised when mating to a single female or five females.

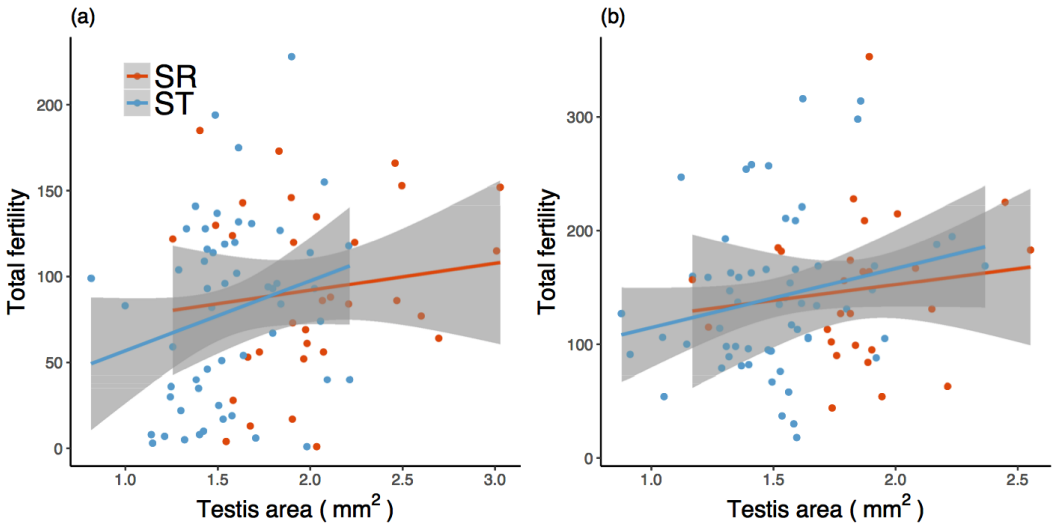

Figure 3.6: Male testis area against total fertility when males were mated to (a) a single female or (b) five females. Total fertility increased with testis size, and this was the case for both SR (red) and ST (blue) males. Grey shading shows ± 1 s.e.

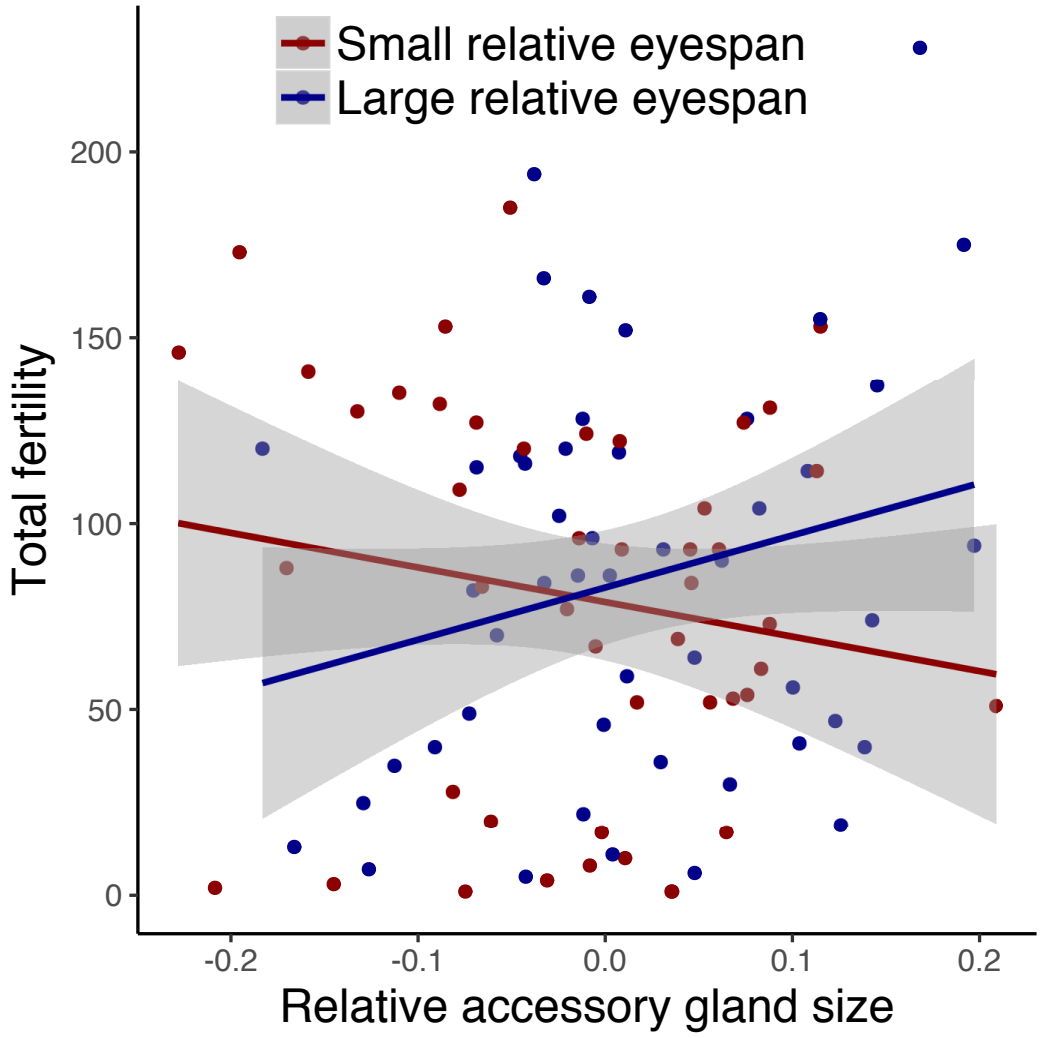

Figure 3.7: Male relative accessory gland size against total fertility when males have wither small relative eyespan (< mean relative eyespan, dark red) or large relative eyespan (dark blue). Total fertility increased with accessory gland size only for males with large eyespan for their body size. Grey shading shows ± 1 s.e.

Chapter 4

The use of microsatellite and INDEL markers to detect sex ratio meiotic drive in *Teleopsis dalmanni*

Abstract

Wild populations of the stalk-eyed fly *Teleopsis dalmanni* exhibit X chromosome meiotic drive, which causes female biased brood sex-ratios in male carriers. There is an abundance of empirical work seeking to understand this drive system, however it remains the case that genetic markers that can reliably distinguish between drive and wildtype males are needed. Here I report on the attributes of four X-linked markers (one microsatellite and three INDEL markers). I describe their relationship with brood sex-ratio and male morphology, and their reliability for use in identifying drive and wildtype males from both wild and laboratory samples. Allele size of all four markers exhibit a directional relationship with brood sex-ratio, and INDEL *comp162710* allele size is especially reliable at accurately distinguishing drive and wildtype males. Furthermore, the frequency distribution of allele size may be informative about the evolutionary history of the driving X chromosome. I report on these patterns, which indicate almost complete segregation of drive and wildtype alleles, as well as on exceptions that point to the possibility of recombination or gene conversion between drive and wildtype X chromosomes.

4.1 Introduction

Wild populations of the stalk-eyed fly *Teleopsis dalmanni* exhibit X chromosome meiotic drive and up to 30% of wild males exhibit a significantly female biased brood sex-ratio (Wilkinson et al., 2003; Presgraves et al., 1997; Cotton et al., 2014; Paczolt et al., 2017). The drive element, located on the X chromosome, prevents the proper formation and function of Y-bearing sperm during spermatogenesis (Presgraves et al., 1997; Wilkinson and Sanchez, 2001). With this transmission advantage, the driving X can rapidly increase in frequency even if it lowers the fitness of the individual, and furthermore can potentially cause population extinction due to the rarity of males (Hamilton, 1967). However, the frequency of drive in wild populations of *T. dalmanni* has in fact been observed at stable frequencies of $\sim$20% over many generations (Presgraves et al., 1997; Wilkinson et al., 2003; Cotton et al., 2014; Paczolt et al., 2017). Accordingly, there has been many studies examining potential mechanisms that may explain the prevalence of drive in wild populations (Wilkinson et al., 1998*b*; Wilkinson and Sanchez, 2001; Wilkinson et al., 2003; Johns et al., 2005; Wilkinson et al., 2006; Cotton et al., 2014).

To conduct efficient experiments on drive, it is vital to have a convenient and accurate method of distinguishing drive from wildtype males. A predominant conventional method is through offspring counts and performing χ^2 tests of significance on deviations from a 1:1 sex ratio. However, this labour-intensive and time consuming method is limited in multiple ways. Firstly, a sample of at least 10 offspring is needed perform a χ^2 test (Cochran, 1952), so it is not possible to assign a phenotype to less prolific males. Furthermore, various factors may influence brood sex-ratio independent of drive, causing false identification of drive and wildtype males. For example, selection may operate on larval survival and thus alter sex ratios. This may lead to variation in wildtype male brood sex-ratio, sufficient to emulate drive. There may also be suppressors of drive that prevent distortion of the brood sex-ratio, thereby leading a drive male to be identified as wildtype (Presgraves et al., 1997). Brood sex-ratios are also of lim-

ited use in identifying females that are heterozygous or homozygous for meiotic drive. Finally, and importantly, there are many situations in which there is no opportunity for males to sire offspring, for example, when collecting flies in the field, or when there is a need to use laboratory males in experiments as soon as they are sexually mature or as virgins. These factors severely limit the utility of offspring counts as a means of identifying individuals that carry meiotic drive.

An alternative approach is to use genetic markers that can reliably predict the phenotype of individuals. The task of finding useful markers is feasible because the sex ratio distortion X chromosome (X^{SR}) shows widespread divergence from the standard X chromosome (X^{ST}) (Christianson et al., 2011; Cotton et al., 2014; Reinhardt et al., 2014; Paczolt et al., 2017). Furthermore, recombination between X^{SR} and X^{ST} is rare or absent (Johns et al., 2005; Paczolt et al., 2017). A number of X-linked microsatellite markers were identified for *T. dalmanni* which showed association with meiotic drive (Johns et al., 2005). A further investigation using wild collected flies found that only one of the four microsatellites, *ms395*, was predictive of the drive phenotype in *T. dalmanni*, albeit with rather high error (Cotton et al., 2014). Here I further investigate locus *ms395*, along with three additional INDEL markers, *comp162710*, *cnv395* and *cnv125*. Using data from wild-caught and laboratory reared flies, I assess these markers for their predictive power of male phenotype.

The consistency of each marker can be informative about the evolutionary history of X^{SR}. A marker is consistent when phenotypes are represented by particular alleles. Males exhibit four prominent brood sex-ratio phenotypes. Firstly, males can exhibit a standard phenotype, ST, and produce unbiased brood sex-ratios. Alternatively, males can produce a female-biased brood sex-ratio and these are described as SR males. SR males can be subdivided into SR-strong and SR-weak indicating the extremity of the bias. Lastly, males can produce male-biased brood sex-ratios, described here as MB males. I expect that X^{ST} carrier males will exhibit the ST phenotype, while X^{SR} carrier males will exhibit the SR-strong phenotype. Offspring sex ratios have a large variance, how-

ever it is possible that modifiers of X^{SR} produce SR-weak or MB phenotypes. Male-biased brood sex-ratios have been described previously (Presgraves et al., 1997; Wilkinson et al., 1998*b*), and have been suggested to be the result of a Y-linked modifier of X^{SR}. It is highly unlikely that modifiers still exist in laboratory samples as stock maintenance has selected for medium-strong drive, however they may be found in wild samples. Patterns of allele distribution may provide evidence of drive modification.

X^{SR} arose from X^{ST} through one or several inversions in the X chromosome (Paczolt et al., 2017), potentially trapping particular alleles in X^{SR}. Alternatively, mutants may have arisen and swept to fixation on either chromosome. As X^{SR} has a lower effective population size, it will be susceptible to higher levels of fixation due to random genetic drift as there will be weaker selective constraint and lower recombination rates compared to X^{ST} (Kirkpatrick, 2010). In these situations, it can be expected that X^{ST} and X^{SR} alleles will have diverged, with X^{ST} males potentially carrying alleles distinct from those on X^{SR} and *vice versa*. However, this pattern may not emerge for several reasons. Allelic diversity may have existed prior to the inversion and may be retained, especially in the more numerous population of X^{ST} chromosomes. Mutations could also regenerate allelic diversity, particularly in microsatellites. Lastly, gene conversion and recombination could occur between X^{ST} and X^{SR}. For viable recombinant products, double crossovers within the inverted region must occur, and both gene conversion and double crossover events are known to occur in other inversion systems (Navarro et al., 1997; Nóbrega et al., 2008; Stevison et al., 2011; Pieper and Dyer, 2016; Knief et al., 2016). So even though recombination rates are estimated to be low between X^{SR} and X^{ST} chromosomes (Johns et al., 2005; Paczolt et al., 2017), it is possible that “unique” alleles are exchanged between the two chromosomes.

In *T. dalmanni*, like many stalk-eyed flies, both males and females exhibit hypercephaly where the head capsule is elongated to form eyestalks (Wilkinson and Dodson, 1997). Males have greatly exaggerated eyespan, the distance be-

tween the outermost edge of the eye bulbs (Burkhardt and de la Motte, 1985). Eyespan acts as a sexual ornament that has evolved through sexual selection, both as a trait used in female mate choice (Wilkinson and Reillo, 1994; Cotton et al., 2010) and male antagonistic interactions (Small et al., 2009). The X-linked microsatellite *ms395* has been associated with male eyespan (Cotton et al., 2014), and an X-linked quantitative trait locus has been shown to be physically associated with a major locus for eyespan (Johns et al., 2005). Consequently, it has been suggested that sexual signalling may be impaired by drive but that female preference for larger eyespan could limit the spread of drive in wild populations. This hypothesis would gain support if genetic markers for drive follow this pattern of association with both male eyespan and brood sex-ratio.

4.2 Methods

4.2.1 Wild males

A total of 31 wild males were collected in September 2009 from 5 sites in the Ulu Gombak valley, Peninsular Malaysia (3°19'N 101° 45'E), described in Cotton et al. (2014), 3°19'13.83"N, 101°45'52.85"E), Upper Blair Witch (UBW, 3°19'10.93"N, 101°45'53.10"E), Upper Lazy Dog (ULD, 3°19'16.37"N, 101°45'58.96"E), Cascade (C, 3°19'20.86"N, 101°45'46.68"E) and Quarry (Q, 3°18'23.15"N, 101°44'36.29"E). A further 91 males from 8 sites were collected in August (N = 31) and March (N = 60) 2012: Lazy Dog (LD, 3° 19'16.69"N, 01° 45'56.28"E), BW, UBW, Mihaly (M, 3°19'22.64"N, 101°45'41.88"E), Rubbish (R, 3°18'36.63"N, 101°44'43.15"E), Q, Upper Quarry (UQ, 3° 18'21.67"N, 101° 44'42.05"E), Wasp (W). Drive phenotype information was produced by allowing males to mate freely with laboratory wildtype females and offspring collected and sexed, as described in Cotton et al. (2014). Males were subsequently stored in 100% ethanol at -20°C.

4.2.2 Laboratory males

To create a standard wildtype stock (ST-stock) population, individuals were originally collected (by S. Cotton and A. Pomiankowski) in 2005 from the Ulu Gom-

bak valley and maintained in high density cages (> 200 individuals) to minimise inbreeding. This population has been regularly monitored and does not contain meiotic drive.

Flies were collected in 2012 (by A. Cotton and S. Cotton) from the Ulu Gombak valley to create a sex ratio meiotic drive stock (SR-stock) population. To establish and maintain a stock with meiotic drive, a standard protocol was followed (Presgraves et al., 1997). Briefly: wild males (of unknown genotype) were mated to ST-stock females and their offspring collected. When an F1 brood was female biased (80% female, > 10 offspring) it was assumed that the father was a carrier of the X^{SR} chromosome, so that the F1 female offspring had genotype X^{SR}/X^{ST}. When sexually mature (> 4 weeks, Baker et al., 2003), F1 females were mated with ST-stock males and their F2 offspring collected. F2 male offspring are expected to be 50:50 X^{SR}/Y:X^{ST}/Y as they inherit either an X^{SR} or X^{ST} chromosome from their mothers. F2 males were mated to ST-stock females and the process repeated. Even though there was error in the assignment of individuals as carriers of X^{SR}, the process maintains the X^{SR} chromosome in this stock. Over generations the SR phenotype has become more distinct as the stock maintenance procedure selected for female biased broods, so most SR-stock males now produce only female offspring, or at least > 95% female biased broods. Note that because the SR-stock maintenance involves back-crossing to ST-stock males and females, the autosomes, Y-chromosome and mitochondrial backgrounds are homogenised across the two stocks. For brevity, I hereafter refer to X^{SR}/Y and X^{ST}/Y males as X^{SR} and X^{ST} males respectively.

The stock populations were kept at 25°C, with a 12:12 h dark:light cycle and fed puréed sweetcorn twice weekly. Fifteen-minute artificial dawn and dusk periods were created by illumination from a single 60-W bulb at the start and end of the light phase.

Males were measured for eyespan and thorax length using ImageJ (v1.46) and separated by sex prior to sexual maturity (< 3 weeks after eclosion). Eyespan was defined as the distance between the outer tips of the eyes (Hingle

et al., 2001*a*). Thorax length was measured ventrally from the anterior tip of the prothorax along the midline to the joint between the metathoracic legs and the thorax (Rogers et al., 2008).

To produce offspring to determine male brood sex-ratio, males were kept with three non-focal females for up to 4 weeks and egg-lays, consisting of damp cotton-wool and excess puréed sweetcorn contained in a Petri dish, were collected twice weekly. Eggs were allowed to develop into pupae and offspring were collected and sexed until no more offspring emerged from the egglay. Males were subsequently stored in 100% ethanol at -20°C.

4.2.3 Phenotype assignment

Significant ($P < 0.05$) deviation from a 1:1 sex ratio was tested for using χ^2 tests on offspring counts with a minimum of 10. Males were subsequently assigned one of four phenotype categories (MB, SR-weak, SR-strong, ST) with additional specific requirements of their brood sex-ratios: (1) MB, males with brood sex-ratios deviating significantly from 1:1 and with male-biased brood sex-ratios of 35% female or less; (2) SR-weak, males with brood sex-ratios deviating significantly from 1:1 and with female-biased brood sex-ratios of between 65% – 90% female; (3) SR-strong, males with brood sex-ratios deviating significantly from 1:1 and with female-biased brood sex-ratios of 90% female or greater; (4) ST, males with a brood sex-ratios that does not deviate from 1:1, or males that do deviate significantly, but do not fulfil any of the criteria above. A sample size of 10 detects SR-strong with high power of 0.8 or greater (low type II error). There is low power to detect SR-weak and MB with this sample size, and so males that produce broods that are weakly different from a 1:1 ratio will be classed as ST. If SR-weak and MB phenotypes are produced through modification of X^{SR} then ST males will be more likely to segregate with SR-strong due to misclassification of SR-weak and MB males, making this a conservative test of the utility of these markers in distinguishing SR-strong.

4.2.4 Genotyping

Three X-linked INDEL markers (*comp162710*, *cnv395* and *cnv125*) were developed from sequenced drive and non-drive populations from Kanching (3° 18'N 101° 37'E) and Ulu Gombak valley, Peninsular Malaysia (J. Reinhardt and G.S. Wilkinson, *personal communication*). The product sizes of these markers clearly segregate into two categories—small and large. These alleles were reported to co-segregate with ST and SR phenotype males: for *comp162710* ST segregates with the large allele (286 bp) and SR with the small allele (201 bp), for *cnv395* ST segregates with the large allele (362 bp) and SR with the small allele (330 bp), and for *cnv125* ST segregates with the large allele (358 bp) and SR with the small allele (129 bp). The *ms395* locus has previously been shown to have an association with the drive phenotype in wild males (Cotton et al., 2014), where large *ms395* alleles (>218 bp) are associated with female-biased broods.

Wild males from September 2009 had their DNA extracted and were genotyped for *ms395* by Cotton et al. (2014). For all other samples a standard protocol was followed to extract DNA (Bruford et al., 1998). For each sample, half a thorax was crushed and digested in 250μl digestion solution (20mM EDTA, 120mM NaCl, 50mM Tris-HCL, 1% SDS, pH 8.0) and 10μl proteinase K (10mg ml^{-1}), and the samples incubated for $\sim$12hrs at 55°C. Proteins were precipitated out with 300μl of 4M ammonium acetate and spun at 13000rpm for 10min. The supernatant was aspirated into 1ml absolute ethanol to precipitate out the DNA, which was pelleted by spinning at 13000rpm for 10min. The DNA pellet was washed in 500ml of 70% ethanol and allowed to dry before being stored in 50μl T10 E0.1 buffer at -20°C. PCR reactions were performed on a 2720 Thermal Cycler (Applied Biosystems, Woolston, UK) in 2μl samples, containing 1μl QIAGEN Mastermix (QIAGEN, Manchester, UK), 1μl Primer mix and 1μl DNA (dried). All primers were at a 0.2μM concentration. PCR reactions had an initial denaturing stage of 95°C for 15min followed by 45 cycles of 94°C for 30sec, 60°C for 1min 30sec and 72°C for 1min 30sec. This was completed by an elongation step of 60°C for 30min. The Applied Biosystems ABI3730 Ge-

netic Analyzer was used to visualise the microsatellites, with a ROX500 size standard. GENEMAPPER 4.0 was used to assign microsatellite allele sizes.

4.2.5 Statistical analysis

Analyses were carried out in R version 3.31 (R Core Team, 2016). Only males that produced at least 10 offspring were included in the analysis. The relationship between allele size and brood sex-ratio for each X-linked locus was examined using generalised linear models (GLMs). Offspring counts were analysed as proportion data (total female off-spring, total male offspring) in binomial GLMs. These models assess sex ratio bias, while accounting for brood size. The data was over-dispersed, so models were fitted with a quasi-binomial error distribution and a logit link function. *ms395* allele size was included as a nominal variable, split into groups of 10 base pairs, as in Cotton et al. (2014). The allele sizes of the three INDEL markers segregate into two distinct size groups (Fig. 4.2), and so allele size for *comp162710*, *cnv395* and *cnv125* were split into two groups of small and large alleles. I subsequently split *ms395* alleles into large and small depending in whether they were > 218 or not (Cotton et al., 2014), and for each locus I examined the frequency distribution of allele size groups between brood sex ratio phenotype categories using Fisher's exact test.

I also examined the relationship between brood sex-ratio and male body size measures—thorax length (as a measure of body size), absolute eyespan and relative eyespan (after accounting for body size variation), as above using a quasi-binomial GLM and offspring counts as proportion data. Furthermore, I examined the relationship between allele size and male thorax length, absolute eyespan and relative eyespan. Loci were split as before into large and small alleles, with the exception of *ms395* which was split depending on whether alleles were > mean allele size or not. In wild samples, stream site was included as a random effect because sites may differ in their frequencies of allele size. The association between allele size and body size measures were tested using generalised linear mixed effects models (GLMMs) in wild samples and GLMs in laboratory samples, using a binomial error distribution and logit link function. All

model tables and effect sizes can be found in Appendix D.

Allele size groups were evaluated for their consistency at predicting phenotype categories. Loci are consistent within a category when most individuals of that category carry the same size allele. Consistency was calculated as the absolute value of the frequency of small alleles (p) minus the frequency of large alleles (q), $|p-q|$, per phenotype category. A value of 0 indicates complete inconsistency, where a phenotype is equally likely to carry a small or large allele. A value of 1 indicates complete consistency, where all members of a phenotype category carry the same size allele.

Linear discriminant analysis (LDA) was additionally used to examine how separation is achieved between the phenotype categories with loci size information (large or small), using the *lda* function from the *MASS* package.

4.3 Results

4.3.1 Wild males

As reported previously by Cotton et al. (2014) for the samples taken in September 2009, 22.6% of males produced significantly sex ratio biased broods (7/31). This was similar to the collections in March 2012 (24.2%, 15/62) and August 2012 (26.8%, 11/41). Families with significant sex-ratio distortion were mostly female biased (25/134), but a few (8/134) were male biased. After applying the criteria for defining male phenotype category, 7 males were defined as MB, 6 as SR-weak, 16 as SR-strong and 105 as ST (Fig. 4.1a).

4.3.1.1 Brood sex-ratio and allele size in wild samples

Using information on locus *ms395* for the 29 males genotyped by Cotton et al. (2014) as well as that for an additional 87 wild males, the previously reported relationship with the *ms395* locus remained ($F_{5,115}$ = 10.238, P < 0.001, Fig. 4.2a), where males with larger *ms395* alleles produced more female biased broods. These alleles segregate into two groups, small: 191 – 218 bp and large: 224 – 250 bp, and small and large alleles were not distributed randomly between phenotypes (Fisher's exact test P < 0.001, Table 4.1). The allele distribution for

ST did not differ from that of MB (P = 1), but did differ from that of SR-weak (P = 0.029) and SR-strong (P < 0.001). The allele distribution of SR-strong differed from that of MB (P = 0.038) but not that of SR-weak (P = 0.62). Small *ms395* alleles were highly predictive of an ST phenotype in wild samples (85%, Table 4.1). Large *ms395* alleles were less strongly predictive of SR-strong, but appear in general to be associated with female sex ratio bias (73% SR-strong or SR-weak), and not with male sex ratio bias (0% MB), though the sample of the latter group was small (N = 7).

Allele size for locus *comp162710* had a relationship with offspring sex ratio ($F_{1,85}$ = 61.523, P < 0.001, Fig. 4.2b), where males with small alleles tended to produce more female biased broods. The allele sizes of this locus segregate into two product lengths, small: 201 bp or large: 287 bp (and 290 bp, a single male), and again small and large alleles were not distributed randomly between phenotypes (Fisher's exact test P < 0.001, Table 4.1). The allele distribution of ST did not differ from that of MB (P = 1) or of SR-weak (P = 0.082), but did differ from that of SR-strong (P < 0.001). The allele distribution of SR-strong differed from that of MB (P = 0.031) but not of SR-weak (P = 0.523). Large *comp162710* alleles were highly predictive of an ST phenotype (87%, Table 4.1), while small alleles were predictive of an SR-strong phenotype (75%).

The *cnv395* locus had a relationship with offspring sex ratio ($F_{1,87}$ = 31.274, P < 0.001, Fig. 4.2). The allele sizes of this locus segregate into two groups, small: 331 – 335 bp and large: 358 – 370 bp, and small and large alleles were not distributed randomly between phenotypes (Fisher's exact test P < 0.001, Table 4.1). Again, the allele distribution of ST did not differ from that of MB (P = 0.447), or of SR-weak (P = 0.261), but did differ from that of SR-strong (P < 0.001). The allele distribution of SR-strong did not differ from that of MB (P = 0.103) or of SR-weak (P = 0.491). Large *cnv395* alleles were predictive of an ST phenotype (87%, Table 4.1).

Lastly, locus *cnv125* also had a relationship with brood sex-ratio ($F_{1,71}$ = 8.98, P = 0.004, Fig. 4.2c). Allele sizes at this locus also segregate into two

groups, small: 128 bp and large: 350 – 363 bp, and small and large alleles were not distributed randomly between phenotypes (Fisher's exact test P = 0.011, Table 4.1). The allele distribution of ST did not differ from that of MB (P = 0.059) or of SR-weak (P = 1), but did differ from that of SR-strong (P = 0.019). The allele distribution of SR-strong did not differ from that of MB (P = 1) or of SR-weak (P = 0.5). Large *cnv125* alleles were highly predictive of an ST phenotype (90%, Table 4.1).

4.3.1.2 Male morphology, allele size and brood sex-ratio in wild samples

Brood sex-ratio was not predicted by male thorax length ($F_{1,129}$ = 0.225, P = 0.636), absolute eyespan ($F_{1,129}$ = 0.21, P = 0.648) or residual eyespan ($F_{1,129}$ = 1.141, P = 0.288). Furthermore, for all markers, allele size did not predict male thorax length (*ms395*: χ^2_1 = 0.003, P = 0.958, N = 116; *comp162710*: χ^2_1 = 0.834, P = 0.361, N = 86, Fig. 4.3a; *cnv395*: χ^2_1 = 0.409, P = 0.522, N = 88; *cnv125*: χ^2_1 = 0.154, P 0.694, N = 72), absolute eyespan (*ms395*: χ^2_1 = 0.335, P = 0.563, N = 116; *comp162710*: χ^2_1 = 2.086, P = 0.149, N = 86; *cnv395*: χ^2_1 = 0.232, P = 0.63, N = 88; *cnv125*: χ^2_1 = 0.251, P = 0.617, N = 72) or relative eyespan (*ms395*: χ^2_1 = 0.769, P = 0.38, N = 116; *comp162710*: χ^2_1 = 1.128, Fig. 4.3b, P = 0.288, N = 86; *cnv395*: χ^2_1 = 1.467, P = 0.226, N = 88; *cnv125*: χ^2_1 = 0.111, P = 0.739, N = 72).

4.3.1.3 Allele size consistency in wild samples

Allele sizes were generally consistent for ST samples for all markers, but less so for locus *cnv395* and locus *cnv125* (Table 4.1, Fig. 4.2a – d). Almost all ST males carried a single allele size for *ms395* (consistency = 0.93, N = 92), *comp162710* (consistency = 0.97, N = 69), *cnv395* (consistency = 0.63, N = 71) and *cnv125* (consistency = 0.52, N = 58).

Allele sizes within SR-strong samples were much less consistent (Table 4.1, Fig. 4.2a – d). However, apart from *ms395* (consistency = 0.09, N = 11), the consistency of the SR-strong allele size was in favour of the alternative allele

size group to that for ST samples, for *comp162710* (consistency = 0.33, N = 9), *cnv395* (consistency = 0.5, N = 8) and *cnv125* (consistency = 0.43, N = 7). It is possible that these consistency values would improve with larger samples. SR-weak and MB males also had small sample sizes, but in both cases their allele size tended to co-segregated with the patterns for ST (Table 4.1, Fig. 4.2a – d).

4.3.1.4 Linear discriminant analysis

The first discriminant axis of three accounted for 90% of the separation between the phenotype categories in wild samples. *comp162710* contributed most to the discriminant function: -1.38 * *ms395* + 2.99 * *comp162710* + 0.70 * *cnv395* - 0.15 * *cnv125*. Leave-one-out cross-validation produced poor predictions of SR-strong (3/6), but good predictions of ST (53/56). The best predictions were produced by LDA including *comp162710* (SR-strong: 6/9, ST: 68/69) or *cnv395* (SR-strong: 6/8, ST: 68/69) only. Predictions were not improved through the addition of any other loci to the LDA. Both SR-male and SR-weak males were almost always predicted to be ST.

4.3.1.5 Amplification

Amplification success varied across the four loci. Out of the 91 males examined for all four loci, 96% amplified for *ms395*, 95% for *comp162710*, 97% for *cnv395* and 79% for *cnv125*. Samples that failed to amplify for one locus tended to amplify for the other loci, apart from *cnv125*.

4.3.2 Laboratory males

From the laboratory stocks, 35.8% of males produced significantly sex ratio biased broods (229/639). As with wild samples, families with significant sex-ratio distortion were mostly female biased (222/639), but a smaller number were significantly male biased (7/639). After applying the criteria for defining male phenotype category, 4 males were defined as MB, 28 as SR-weak and 174 as SR-strong and 433 as ST (Fig. 4.1b).

4.3.2.1 Brood sex-ratio and allele size in laboratory samples

The relationship reported above for the *ms395* locus in wild males is also seen in laboratory samples ($F_{3,178}$ = 48.076, P < 0.001, Fig. 4.2e), where males with larger alleles produced more female biased broods. As in the wild samples, these alleles segregate into two groups, small: 197 – 206 bp and large: 227 – 247 bp. 22 alleles that were seen in the wild samples are no longer present, as well as the appearance of two alleles that were not seen in wild samples (227 bp and 246 bp). Small and large alleles were not distributed randomly between phenotypes (Fisher's exact test P < 0.001, Table 4.2). The allele distribution of ST did not differ from that for MB (P = 1) or SR-weak (P = 0.202), but did differ from that of SR-strong (P = 0.018). The allele distribution of SR-strong differed from that of MB (P = 0.018) and SR-weak (P < 0.001). Just as in wild samples, small *ms395* alleles were highly predictive of an ST phenotype in laboratory samples (86%, Table 4.2, Fig. 4.2e). Unlike wild samples, large *ms395* alleles were highly predictive of the SR-strong phenotype (85%). In addition, there was no association of *ms395* with SR-weak. Once again the large allele was not predictive of male sex ratio bias (0% MB).

The relationship between offspring sex ratio and *comp162710* allele size remained ($F_{1,195}$ = 401.35, P < 0.001, Fig. 4.2f), so that males with small alleles produced more female biased broods (Table 4.2). The allele sizes of laboratory samples segregate into two size categories, small: 201 bp and large: 286 – 287 bp, including an additional allele size that was not seen in wild samples (286 bp) and the 290 bp allele size is absent. Small and large alleles were not distributed randomly between phenotypes (Fisher's exact test P < 0.001, Table 4.2). The allele distribution of ST did not differ from that of MB (P = 1), but did differ from that of SR-weak (P = 0.013) and SR-strong (P < 0.001). The allele distribution of SR-strong differed from that of MB (P < 0.001) and of SR-weak (P < 0.001). Large *comp162710* alleles were highly predictive of an ST phenotype (87%, Table 4.2, Fig. 4.2f), and small alleles of an SR-strong phenotype (83%), as in wild samples.

The *cnv395* locus also had a relationship with offspring sex ratio ($F_{1,186}$ = 150.18, P < 0.001, Fig. 4.2g). As with wild samples, the allele sizes at this locus segregate into two groups, small: 331 bp and large: 360 – 370 bp. But again some alleles are missing (333 bp, 335 bp, 358 bp, 359 bp and 362 bp). Small and large alleles were not distributed randomly between phenotypes (Fisher's exact test P < 0.001, Table 4.2). The allele distribution of ST did not differ from that of MB (P = 1) or SR-weak (P = 0.126), but did differ from SR-strong (P < 0.001). The allele distribution of SR-strong differed from MB (P = 0.001) and SR-weak (P < 0.001). Large *cnv395* alleles were highly predictive of an ST phenotype (85%, Table 4.2, Fig. 4.2g), as were small alleles of an SR-strong phenotype (83%).

cnv125 showed no relationship with brood sex-ratio ($F_{1,114}$ = 1.614, P = 0.207, Fig. 4.2h), however allele sizes at this locus still segregate into two groups in laboratory samples (small: 128 bp and large: 359 – 361 bp), with the loss of some large alleles seen in wild samples. There was some indication that small and large alleles are not distributed randomly between phenotypes (Fisher's exact test P = 0.049, Table 4.2). The allele distribution of ST did not differ from that of MB (P = 0.548) or SR-weak (P = 1), but did differ from SR-strong (P = 0.018). The allele distribution of SR did not differ from that of MB (P = 1) or SR-weak (P = 0.085). Despite no clear statistical association, large *cnv125* alleles remained predictive of an ST phenotype (80%, Table 4.2, Fig. 4.2h), although less so than in wild samples, as only a single SR-strong male carried a large allele (Table 4.2, Fig. 4.2h).

4.3.2.2 Male morphology, allele size and brood sex-ratio in laboratory samples

Unlike in wild samples, brood sex-ratio was predicted by male thorax length ($F_{1,635}$ = 4.25, P = 0.04), with smaller males having more female-biased broods. Absolute eyespan also had a negative relationship with brood sex-ratio ($F_{1,635}$ = 6.157, P = 0.013) but relative eyespan did not do so ($F_{1,635}$ = 1.928, P = 0.166). In laboratory samples, *ms395* allele size did not predict thorax length ($F_{1,177}$

= 2.01, P = 0.158), absolute eyespan ($F_{1,177}$ = 1.592, P = 0.209) or relative eyespan ($F_{1,177}$ = 0.05, P = 0.823). *comp162710* allele size did not predict thorax length ($F_{1,194}$ = 0.811, P = 0.369, Fig. 4.3c). However, males with larger *comp162710* alleles had larger absolute eyespan ($F_{1,194}$ = 5.678, P = 0.018) and larger relative eyespan ($F_{1,194}$ = 7.987, P = 0.005, Fig. 4.3d). *cnv395* allele size did not predict thorax length ($F_{1,185}$ = 0.229, P = 0.633) or absolute eyespan ($F_{1,185}$ = 0.835, P = 0.362), however males with larger *cnv395* alleles had larger relative eyespan ($F_{1,185}$ = 4.24, P = 0.041). Unlike the other markers, *cnv125* allele size did predict thorax length ($F_{1,113}$ = 6.956, P = 0.01), as males with larger alleles had longer thorax length. Allele size similarly predicted absolute eyespan ($F_{1,113}$ = 4.206, P = 0.043), but not relative eyespan ($F_{1,113}$ = 0.005, P = 0.945).

4.3.2.3 Allele size consistency in laboratory samples

In laboratory samples, allele sizes were highly consistent for ST males for all markers except *cnv125* (Table 4.2, Fig. 4.2e – h). As in wild samples, almost all ST males carried a single allele size for *ms395* (consistency = 0.99, N = 143), *comp162710* (consistency = 0.91, N = 147) and *cnv395* (consistency = 0.97, N = 140). However, contrary to wild samples, ST males tended to carry either a small or a large, *cnv125* allele (consistency = 0.28, N = 78). Overall, allele sizes within SR-strong samples were much more consistent in laboratory than in wild samples, and remained biased towards the alternative allele size group to ST samples (Table 4.2, Fig. 4.2e – h). SR-strong samples tended to carry a single allele for *ms395* (consistency = 0.47, N = 15), *comp162710* (consistency = 0.92, N = 25), *cnv395* (consistency = 0.81, N = 21) and *cnv125* (consistency = 0.88, N = 17). As with wild samples, SR-weak and MB male allele size tended to co-segregate with ST (Table 4.2, Fig. 4.2e – h).

4.3.2.4 Linear discriminant analysis

The first discriminant axis of three accounted for 99.8% of the separation between the phenotype categories in laboratory samples. As in wild samples,

comp162710 contributed most to the discriminant function: 1.05 * *ms395* + 11.23 * *comp162710* - 0.09 * *cnv395* - 0.07 * *cnv125*. Leave-one-out cross-validation produced poor predictions of SR-strong (1/10) and of ST (18/72) when *cnv125* was included in the analysis. However, phenotype predictions were good with *cnv125* removed (SR-strong: 18/19, ST: 128/131), as well as when only *comp162710* was included (SR-strong: 24/25, ST: 145/147). Both SR-male and SR-weak males were almost always predicted to be ST.

4.3.2.5 Amplification

Amplification success varied across the four loci. For the 211 males examined, 85% for *ms395*, 93% amplified for *comp162710*, 89% for *cnv395* and 55% for *cnv125*. Where samples failed to amplify for *comp162710* (N = 15), all samples also failed for *ms395*, indicating minor technical issues because these loci were amplified in a multiplex. In contrast, all 15 amplified for *cnv395*.

4.4 Discussion

The main aim of this study was to develop markers that are useful for differentiating drive and wildtype males. To be of value for that purpose, markers must associate with brood sex-ratio and be a reliable predictor of a phenotype category. Here I evaluated four X-linked markers (one microsatellite and three INDEL markers) and found that all four markers associate with brood sex-ratio and have some predictive value (Table 4.1 and 4.2). For wild samples, all four markers, *ms395*, *comp162710*, *cnv395* and *cnv125*, reliably predict ST phenotypes (85 – 90%). However, only *comp162710* reliably predicts an SR-strong phenotype (75%). Large alleles of microsatellite *ms395* to some extent predict SR-strong (55%), but the other two INDELS, *cnv395* and *cnv125*, have low power (22% and 29%). Laboratory samples follow similar patterns, but the predictability is higher, probably due to the SR-stock and ST-stock breeding regimes having removed rare allele/phenotype combinations. In laboratory samples, all loci are good at predicting ST (80 – 87%) while *comp162710* now joins *ms395* and *cnv395* in reliably predicting the SR-strong phenotype (83 – 85%). *cnv125* remains unin-

formative because while almost all SR-strong males have the same sized allele, most ST males also have this allele. We can conclude that *cnv125* has no utility in defining SR-strong and is not a worthwhile marker to be used for either wild or laboratory analyses. Furthermore, markers *ms395*, *comp162710* and *cnv395* amplified well in both wild and laboratory samples, while amplification rates for *cnv125* were poor in comparison.

If ST and SR-strong phenotypes revealed by offspring ratios from wild samples accurately reflect X^{ST} and X^{SR} chromosomal states, then the pattern of allele distribution may be informative about the evolutionary history of X^{SR}. Within ST males, allele size is consistent for *ms395* and *comp162710*; almost all ST males carry an allele of the same size category for these loci (Table 4.1). In contrast, within SR-strong males, none of the 4 loci show high consistency. However, the allele frequency distribution among ST and SR-strong males is different across all loci. Only three ST males carry unexpected alleles for the two otherwise consistent loci, *ms395* and *comp162710*. Complete segregation is less likely in microsatellites, as they have high rates of copy number change and can undergo sharp switches in length (Schlötterer, 2000), and two of these three males differ only for *ms395*. The last male carries all four alleles expected of an SR-strong phenotype. This may be an indication of drive suppression where this sample is a carrier of X^{SR}, hence it has all the associated alleles, but autosomal suppression or a resistant Y prevents distortion of the brood sex-ratio. This sample has a brood sex ratio of 0.40 but a weak power to detect distortion (power = 0.33). Previous studies have reported evidence for suppression of X^{SR} (Presgraves et al., 1997; Wilkinson et al., 1998*b*) and autosomal or Y-linked suppressors of X drive are expected to evolve because they increase the production of high fitness male offspring (Fisher, 1930; Wu, 1983*a*). Strong segregation of alleles can be expected for loci on X^{SR}, given that this chromosome does not recombine with X^{ST} and has been at low frequency so that it rarely undergoes intra-chromosomal recombination. This leads to weaker purifying selection, greater rates of hitchhiking and higher levels of drift (Kirkpatrick,

2010). This explanation holds also for the microsatellite *ms395*, which has differential frequency between phenotypes. However, it is unclear how high numbers of microsatellite sequence repeats are maintained or if there is some selective or other force that favours accumulation on X^{SR}.

Alleles on X^{ST} are polymorphic for INDELs *cnv395* and *cnv125*, with a bias towards large alleles. Conversely, INDEL alleles on X^{SR} tend to be smaller than the alleles on X^{ST}. This is consistent with polymorphism of small and large alleles preceding the origin of the X^{SR} inversion. The inversion that typifies X^{SR} likely originated in a unique event and would have trapped particular alleles on X^{SR}, explaining why their frequency is distinct from those on X^{ST}. Furthermore, this suggests that small INDELs arose on X^{SR} as deletions, after the divergence of these two chromosomes, and then spread by drift close to fixation. However, this does not explain the high frequency of large INDELs on X^{SR} and this could indicate that rare double crossovers or gene conversions between X^{SR} and X^{ST} can occur in females, switching parts of the X chromosome between the two. The estimated recombination rate between X^{ST} and X^{SR} chromosomes is very low, but non-zero (Johns et al., 2005). If such events occur, they will have a particular frequency distribution across the chromosome, reducing gene exchange near the breakpoints while rates of exchange remain high towards the centre (Navarro et al., 1997). To distinguish between homoplasic mutations on the X^{SR} and recombination events with X^{ST}, as well as the extent to which recombination is suppressed, it would be necessary to have genomic data from X^{SR} and X^{ST} chromosomes. Gene flow between X^{ST} and X^{SR} would create the potential for X^{SR} to purge deleterious mutations as well as to accumulate beneficial ones. Evidence of gene flow between the driving and non-driving X chromosome in *D. neotestacea* has been found despite the presence of large chromosomal inversions (Dyer et al., 2013; Pieper and Dyer, 2016), and homozygous females are fully fertile. This is in contrast to other systems, such as in *D. recens* (Dyer et al., 2007) and the *t* complex in house mice (Lyon, 2003) where there is little evidence of gene flow and the driver is associated with deleterious recessive

mutations.

SR-weak and MB male phenotypes generally segregate with allelic states that mirror X^{ST}, with the exception of two SR-weak males that do not carry alleles associated with an ST phenotype. One explanation for this is that these phenotypes are an artefact of the high variability in brood sex-ratios of both X^{ST} and X^{SR} males, and so both these genotypes can produce SR-weak phenotypes. Alternatively, factors other than X^{SR} could contribute to weakly female-biased or male-biased sex ratios, while an SR-weak phenotype in carriers of X^{SR} may be an indication of autosomal suppressors or a resistant Y. Crosses would be valuable in uncovering additional meiotic drive elements or the presence of modifiers of drive. Crosses and reciprocal crosses between families exhibiting an unexplained phenotype and stock cultures could elucidate whether the presence of the X, Y or autosomes are necessary for the phenotype to occur, and also indicate if there is modification of the trait.

Previous studies have suggested that meiotic drive has contributed to the evolution and maintenance of female mate choice (Wilkinson et al., 1998*a*). Females prefer to mate with males with large residual eyespan (Cotton et al., 2015) and links have been made between meiotic drive and reduced residual eyespan in males (Wilkinson et al., 1998*a*; Johns et al., 2005; Cotton et al., 2014). In laboratory samples, *comp162710* allele size predicted male relative eyespan (Fig. 4.3d). This locus is highly predictive of sex-ratio phenotype, and ST and SR-strong males consistently carry alternative *comp162710* alleles (Table 4.2). Males with small alleles had a female-biased brood sex ratio and also small relative eyespan. This relationship between allele size and male relative eyespan was in the same direction in wild samples (Fig. 4.3b), but was not significant. In wild samples there are comparatively few samples carrying small *comp162710* and larger field samples may reveal a stronger relationship. However, contrary to expectation (Wilkinson et al., 1998*a*; Lande and Wilkinson, 1999; Johns et al., 2005; Cotton et al., 2014), brood sex-ratio was not predicted by male relative eyespan in either wild or laboratory samples. This suggests that this is not a

continuous relationship, but rather that X^{SR} males as a group have reduced relative eyespan and also produce an SR-strong phenotype. Additionally, there is high variability in brood sex-ratio, and other factors may also contribute to sex-ratio bias. For example, Y suppression of drive may reduce and even reverse sex ratio bias and also associate with male eyespan (Wilkinson et al., 1998*a*).

This study demonstrates that microsatellite and INDEL markers can be used to reliably predict sex-ratio phenotype. In both wild and laboratory samples INDEL *comp162710* can replace offspring counts as a method of distinguishing between wildtype and drive males, and furthermore will be able to identify females that carry X^{SR}. Two of the markers (*ms395*, *comp162710*) show clear, but not perfect, segregation between ST and SR-strong phenotypes in wild samples, with a third (*cnv395*) in laboratory samples. Samples that do not segregate into expected categories may be examples of gene conversion, recombination or suppression, or may represent ancestral allelic variation that existed prior to the inversion. Some evidence was found to support the hypothesis that meiotic drive has contributed to the evolution of female mate choice—in laboratory samples males with small *comp162710* alleles had more female biased broods, but also had smaller eyespans. This pattern was not recovered in wild samples, likely due to a restricted sample size. However further investigation is required to determine if this effect is large enough to provide females with a consistent indicator of drive status in natural populations.

4.5 Figures

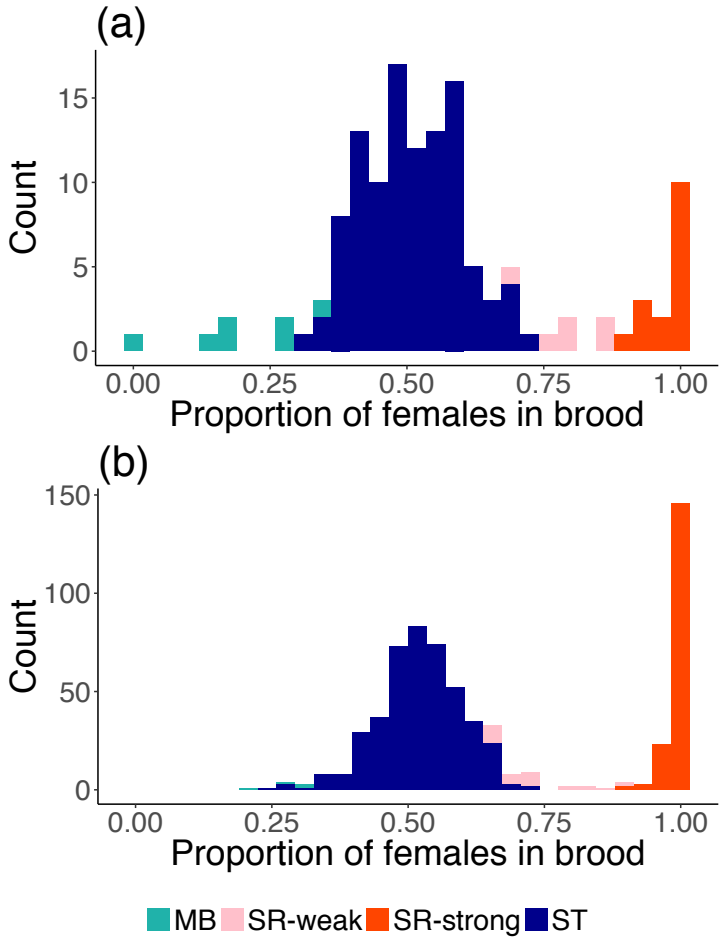

Figure 4.1: Histograms of brood sex-ratios (BSR), given as proportion of females, for (a) wild-caught (N = 130) and (b) laboratory males (N = 636). Males are categorised according to their brood sex ratio by testing for significant ($P < 0.05$) deviations from a 1:1 sex ratio using χ^2 tests on offspring counts greater than 10. Males are categorised as MB (green): $P < 0.05$ and BSR < 0.35; SR-weak (pink): $P < 0.05$ and BSR = 0.65 – 0.9; SR-strong (red): $P < 0.05$ and BSR > 0.9; ST (dark blue): $P > 0.05$ or does not fulfil the criteria for any other category.

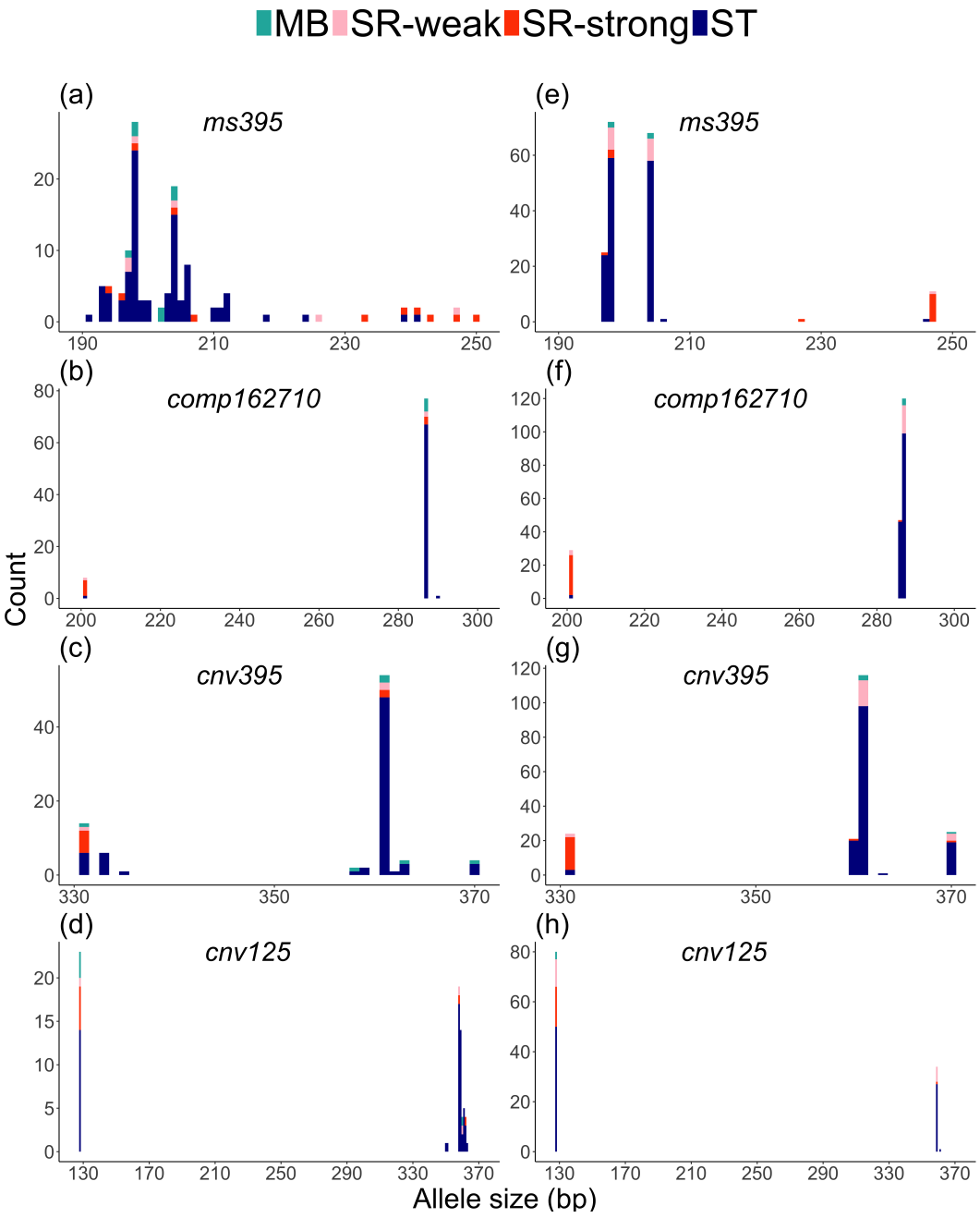

Figure 4.2: Histograms of allele sizes for wild-caught (a – d) and laboratory (e – h) males (*ms395* wild N = 116, laboratory N = 179; *comp162710* wild N = 86, laboratory N = 196; *cnv395* wild N = 88, laboratory N = 186; *cnv125* wild N = 72, laboratory N = 115). Males are categorised according to their brood sex-ratio (BSR), given as proportion of females, by testing for significant ($P < 0.05$) deviations from a 1:1 sex ratio using χ^2 tests on offspring counts greater than 10. Males are categorised as MB (green): $P < 0.05$ and BSR < 0.35; SR-weak (pink): $P < 0.05$ and BSR = 0.65 – 0.9; SR-strong (red): $P < 0.05$ and BSR > 0.9; ST (dark blue): $P > 0.05$ or does not fulfil the criteria for any other category.

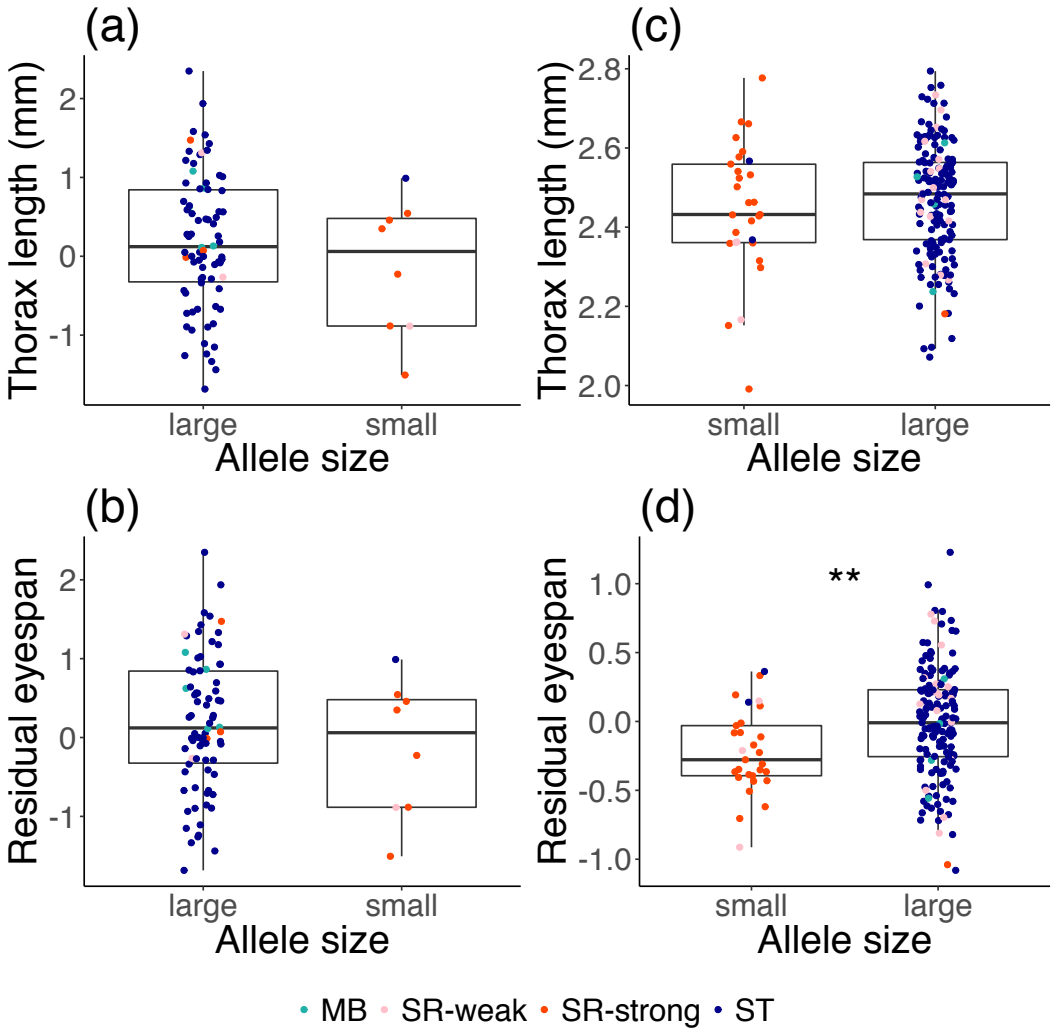

Figure 4.3: Boxplots (median ± interquartile range) showing male thorax length in (a) wild-caught and (c) laboratory samples, and male residual eyespan in (b) wild-caught and (d) laboratory samples, for males carrying large or small *comp162710* alleles. Laboratory males with small *comp162710* alleles had smaller residual eyespan than those with larger alleles (d, P = 0.005). Points are coloured according to male brood sex-ratio phenotype category. Males are categorised according to their brood sex ratio by testing for significant (P < 0.05) deviations from a 1:1 sex ratio using χ^2 tests on offspring counts greater than 10. Males are categorised as MB (green): P < 0.05 and BSR < 0.35; SR-weak (pink): P < 0.05 and BSR = 0.65 – 0.9; SR-strong (red): P < 0.05 and BSR > 0.9; ST (dark blue): P > 0.05 or does not fulfil the criteria for any other category.

4.6 Tables

Table 4.1: Allele distribution between phenotypes in wild samples. Allele sizes for each locus (microsattelite *ms395* and INDELs *comp162710*, *cnv395* and *cnv125*) segregate into two size groups—small and large. Allele size consistency indicates whether each phenotype category (ST, SR-strong, SR-weak and MB) tends to be represented by one or both allele size groups. A consistency value of 0 (not consistent) means samples may carry an allele of either size, while a value of 1 (consistent) indicates that samples are represented by a single allele size group.

locus (wild)	allele	ST	SR-strong	SR-weak	MB	*%ST*	*%SR-strong*
	small	89	5	4	7	*85*	*4.8*
ms395	large	3	6	2	0	*27*	*55*
	allele size consistency	*0.93*	*0.09*	*0.33*	*1*		
	small	1	6	1	0	*13*	*75*
comp162710	large	68	3	2	5	*87*	*3.8*
	allele size consistency	*0.97*	*0.33*	*0.33*	*1*		
	small	13	6	1	1	*62*	*29*
cnv395	large	58	2	2	5	*87*	*3*
	allele size consistency	*0.63*	*0.5*	*0.33*	*0.67*		
	small	14	5	1	3	*61*	*22*
cnv125	large	44	2	2	1	*90*	*4.1*
	allele size consistency	*0.52*	*0.43*	*0.33*	*0.5*		

Table 4.2: Allele distribution between phenotypes in laboratory samples. Allele sizes for each locus (microsattelite *ms395* and INDELs *comp162710*, *cnv395* and *cnv125*) segregate into two size groups—small and large. Allele size consistency indicates whether each phenotype category (ST, SR-strong, SR-weak and MB) tends to be represented by one or both allele size groups. A consistency value of 0 (not consistent) indicates that samples may carry an allele of either size, while a value of 1 (consistent) means samples are represented by a single allele size group.

locus (lab)	allele	ST	SR-strong	SR-weak	MB	*%ST*	*%SR-strong*
	small	142	4	16	4	*86*	*2.4*
ms395	large	1	11	1	0	*7.7*	*85*
	allele size consistency	*0.99*	*0.47*	*0.88*	*1*		
	small	2	24	3	0	*6.9*	*83*
comp162710	large	145	1	17	4	*87*	*0.6*
	allele size consistency	*0.91*	*0.92*	*0.7*	*1*		
	small	2	19	2	0	*8.7*	*83*
cnv395	large	138	2	19	4	*85*	*1.2*
	allele size consistency	*0.97*	*0.81*	*0.81*	*1*		
	small	50	16	11	3	*63*	*20*
cnv125	large	28	1	6	0	*80*	*2.9*
	allele size consistency	*0.28*	*0.88*	*0.29*	*1*		

Chapter 5

Variation in the benefits of multiple mating on female fertility in wild stalk-eyed flies

Abstract

Polyandry, female mating with multiple males, is widespread across many taxa and almost ubiquitous in insects. This conflicts with the traditional idea that females are constrained by their comparatively large investment in each offspring, and so should only need to mate once or a few times. Females may need to mate multiply to gain sufficient sperm supplies to maintain their fertility, especially in species in which male promiscuity results in division of their ejaculate amongst many females. Here I take a novel approach, utilising wild-caught individuals to explore how natural variation among females and males influences fertility gains for females. I studied this in the Malaysian stalk-eyed fly species *Teleopsis dalmanni*. After an additional mating, females benefit from greatly increased fertility (proportion fertile eggs). Gains from multiple mating are not uniform across females; they are greatest when females have high fecundity or low fertility. Fertility gains also vary spatially, as I find an additional strong effect of the stream from which females were collected. Responses were unaffected by male mating history (males kept with females or in male-only groups). Recent male mating may be of lesser importance because males in many species, including *T. dalmanni*, partition their ejaculate to maintain their fertility over many matings. This study highlights the importance of complementing laboratory studies with data on wild-caught populations, where there is considerable heterogeneity between individuals. Future research should focus on environmental, demographic and genetic factors that are likely to significantly influence variation in individual female fecundity and fertility.

5.1 Introduction

Female mating with multiple males (polyandry) is found widely across many taxa (mammals: Ginsberg and Huck, 1989; Clutton-Brock, 1989; birds: Griffith et al., 2002; fishes: Avise et al., 2002; general: Jennions and Petrie, 2000; Zeh and Zeh, 2001) and is almost ubiquitous in insects (Arnqvist and Nilsson, 2000). Whilst multiple mating is expected in males, as their reproductive success typically increases with the number of matings, it is less clearly beneficial for females. Female reproductive potential is thought to be realised after one or a few matings (Bateman, 1948), as females are assumed to be constrained by the greater investment they make in each of their offspring. This has led to an extensive literature considering potential benefits to females from multiple mating, in terms of increases to female survival, fecundity and fertility (Arnqvist and Nilsson, 2000; Hosken and Stockley, 2003; Yasui, 1998), and whether polyandry may be a mechanism to quell or mitigate intragenomic conflicts (Haig and Bergstrom, 1995; Zeh and Zeh, 1996). Additionally females may gain indirect genetic benefits through increasing the genetic diversity or quality of offspring, but these are likely to be of secondary importance when females gain direct benefits from multiple mating (Slatyer et al., 2012; Yasui, 1998).

However, rather less attention has been given to considering how variation amongst females impacts on the benefits of multiple mating. For instance, how do female condition, fecundity or prior mating history alter the fitness consequences of further matings or polyandrous matings? Greater study in this area is needed in order to uncover the contexts in which multiple mating benefits, harms or has no effect on females (House et al., 2009; Toft and Albo, 2015; Wright et al., 2013). In addition, there has been an over-reliance on laboratory matings to investigate the consequence of multiple mating. While laboratory studies allow control and standardisation (e.g. using virgins), assays may not fully reflect the natural history of mating experienced by females and males. Laboratory studies need to be complemented by experiments conducted on wild-caught individuals, in situations that more closely replicate the natural range of conditions

of female and male encounters.

Here, I apply these principles to consider the consequences of multiple mating on female fertility in the Malaysian stalk-eyed fly *Teleopsis dalmanni*, when females vary in the degree of sperm limitation. In insects, it is widely found that sperm acquired in a single mating is insufficient to fertilise all of a female's eggs (Ridley, 1988; Wedell et al., 2002). To maintain fertility, females may need to mate multiply to gain sufficient sperm supplies for egg laying throughout their adult life (Fjerdingstad and Boomsma, 1998; Chevrier and Bressac, 2002) or remate at regular intervals as sperm supplies dwindle (Drnevich et al., 2001; Fox, 1993; Wang and Davis, 2006). This implies that the fertility benefits of female remating will change with fluctuating environmental factors, such as the operational sex ratio, food availability and the fertility of previous mates (Fox, 1993; Arnqvist and Nilsson, 2000; Cordero and Eberhard, 2003; Pitcher et al., 2003; Crean and Marshall, 2009; Rogers et al., 2008; Navara et al., 2012; Tuni et al., 2013). In line with this view, females may be able to modify their mating rates in response to changing circumstances that affect the relative costs and benefits of mating (Wilgers and Hebets, 2012; Boulton and Shuker, 2016).

There are two important fluctuating factors that are likely to regulate the direct benefits to female fertility of an additional mating. First is current female sperm limitation. Female insects have internal sperm storage organs where sperm are kept and used to fertilise eggs long after mating (Kotrba, 1995; Eberhard, 1996; Pitnick et al., 1999; Orr and Brennan, 2015). The current fertility status of a female will change over time; as females use up their sperm reserves or as sperm die, female fertility will probably decrease. Consequently, females that have mated recently or have full sperm storage organs will likely gain less benefit from an additional mating than sperm depleted females.

Second, the increase in female fertility from an additional mating may be influenced by the male's investment. Individual males have finite resources and their investment in ejaculates is predicted to be shaped by the trade-off with the number of matings (Parker, 1982). There is good evidence that males increase

their allocation to females that have higher reproductive value (Engqvist and Sauer, 2001; Wedell et al., 2002; Rogers et al., 2006; Kelly and Jennions, 2011; Perry et al., 2013). Likewise, in many situations, males increase their ejaculate size when females are subject to greater sperm competition (Wedell et al., 2002; Kelly and Jennions, 2011). It has been suggested that the quality of an ejaculate that a female receives may positively correlate with male condition (Sheldon, 1994; Iwasa and Pomiankowski, 1999), though firm evidence for this is lacking (Pizzari et al., 2004; Fitzsimmons and Bertram, 2013; Harley et al., 2013; Mautz et al., 2013). Conversely dominant or attractive males may invest fewer sperm per mating as they have more opportunities to mate and so need to divide their ejaculate into smaller packages per female (Warner et al., 1995; Jones, 2001; Tazzyman et al., 2009). In many cases, female fertility suffers when the male has recently mated (Wedell and Ritchie, 2004; Torres-Vila and Jennions, 2005; Perez-Staples et al., 2008; Levin et al., 2016). The net effect is that female sperm limitation will vary with male mating strategy depending on the female's value to the male, the condition or attractiveness of the male and his recent mating history. As a result, the direct fertility benefit that a female gains from an extra mating will not be a static quantity but will depend on the context in which mating takes place.

I examined how these two factors alter the benefits of female remating by means of experimentation in the wild using the Malaysian stalk-eyed fly *Teleopsis dalmanni* (Diptera, Diopsidae). Both sexes in this species are highly promiscuous (Wilkinson et al., 1998*a*). Females typically have low fertility measured by egg hatch, both in the laboratory and in the wild (Baker et al., 2001*a*; Cotton et al., 2010). One of the main factors contributing to this infertility is that males have evolved to partition their ejaculates between many females. As a consequence, males transfer few sperm in a single copulation ($\sim$65: Wilkinson et al., 2005; $\sim$142: Rogers et al., 2008) leading to females being sperm limited (Baker et al., 2001*a*). Thus females must remate in order to raise their fertility (Baker et al., 2001*a*). As well as few sperm, the small size of male ejaculates is

unlikely to provide any non-sperm benefits (Kotrba, 1996).

Given these patterns in stalk-eyed flies, I expect to find that female *T. dalmanni* remate to gain direct fertility benefits from an additional mating. To distinguish between male and female effects as sources of variation in changes to female fertility, I report two experiments using wild caught *T. dalmanni* females. Prior mating histories of females and males cannot be controlled in field experiments. However, females in these two experiments were kept isolated from males in order that females became sperm depleted, to some extent. I then evaluated the effect of an additional mating on female fertility and expected that sperm depleted females should receive direct fertility benefits from an additional mating. To explore the impact of past male mating experience on the ability of males to confer fertility on females, in a second experiment, prior mating rate and state of sperm depletion of wild caught males was varied by keeping them for several days either with females or in male-only groups. I then evaluated the fertility gain of females mated to these two types of male. These experiments allow the examination, using wild-caught individuals with backgrounds of natural variation, of the extent of female and male effects on fertility.

5.2 Materials and Methods

5.2.1 Experiment 1: Gains from an additional mating

Fly collections took place in February 2011 from eleven stream sites in the Ulu Gombak valley, Peninsular Malaysia (3°19'N 101°45'E). Females and males were collected on day zero at dusk from lek sites on the edge of forest streams at several stream sites adjacent to tributaries of the Gombak River. Individuals were aspirated into plastic bags and within one hour of capture, males and females were transferred to individual 500 ml containers lined with a moist cotton-wool and tissue paper base. Flies were fed every two days with puréed banana.

Female fecundity was recorded from counts of eggs deposited on the tissue paper base, which were collected and renewed every two days. Eggs were

allowed to develop for a further five days in Petri dishes containing a moist cotton pad. 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. If fertilised eggs failed to hatch, but showed signs of development (horizontal striations in the chorion and early mouthpart formation), they were recorded as fertile (Baker et al., 2001*a*).

On day 13 after capture, each female was given a single additional mating with a male collected at the same time as the female. This time period was chosen to allow females to become sperm depleted prior to mating. Matings were carried out in mating chambers, each made up of two 500 ml cells, separated by a removable card partition, and a single string running the length of the chamber provided a suitable roosting site (Cotton et al., 2015, Fig. 5.1). In the evening, a male was placed in the upper cell and the focal female in the lower cell. The following morning (after $\sim$12 hours), the card partition was removed and the pair observed until a successful copulation took place, classed as lasting 30 s or more, to ensure that sperm transfer had occurred (Lorch et al., 1993; Corley et al., 2006). Males were only used once. The remated females were then re-housed as before and their reproductive output was monitored from day 15 every two days for a further eight days. The females were then killed and stored in ethanol. Female eyespan (distance between the outer tips of the eyes, Hingle et al., 2001*a*) and thorax length (distance from base of the head to the joint between the meta-thoracic legs and the thorax, Rogers et al., 2008) were measured to an accuracy of 0.01 mm, using a monocular microscope and the image analysis software ImageJ, version 1.43e (Schneider et al., 2012). In total, fertility was recorded for N = 45 females across the full sampling periods before and after the extra mating.

5.2.2 Experiment 2: Investigation of female and male effects

A second experiment was carried out using flies collected from five stream sites in the Ulu Gombak valley in July/August 2012. Individuals were collected as

above. Females were housed individually in 500 ml containers and their reproductive output was recorded as in the first experiment. Males were placed in large 1500 ml containers either with a mix of males and non-focal females allowing them to mate freely (sperm depleted), or only with other males (non-sperm depleted). Isolation from females allows males to replenish their sperm stores (Rogers et al., 2005*b*). Fly density was standardised across these two treatments, each pot containing a total of 10 flies, either a 1:1 ratio of males to females (sperm depleted) or 10 males (non-sperm depleted). On the evening of day 12, a focal female and male were placed in a mating container (Fig. 5.1) and allowed to have an additional mating following the protocol above, except that males did not have an isolated over-night period. Females were placed either with a sperm depleted male (N = 19) or a non-sperm depleted male (N = 17). After the additional mating, females were re-housed and their subsequent reproductive output was recorded every two days from day 14 over the following eight days, and morphometric measures taken as before.

5.2.3 Statistical analysis

Female sperm depletion was determined by the decline in female fertility over the eight days before the single additional mating (comprising four egg counts) as well as over the eight days after the additional mating (again, four egg counts). To test whether an additional mating resulted in increased fecundity or fertility, I compared the total individual reproductive output over the 8 days before (days 5 – 12) and after (days 14 – 21 in the first experiment; days 13 – 20 in the second experiment) mating, as well as total individual reproductive output on the days immediately before (days 11 – 12) and after the additional mating (day 14 – 15 in the first experiment; day 13 – 14 in the second experiment). Lastly, I examined whether the direction of change in individual fertility was positive, or negative/unchanged, and tested the degree to which individual proportion fertility changed depended on female pre-mating fecundity or fertility.

All tests were carried out in R, version 3.31 (R Core Team, 2016) and are reported (including effect sizes) in Appendix E. I analysed female reproductive

output (fecundity and fertility) using generalised linear mixed effects models (GLMMs) using the *lme4* package (Bates et al., 2015). Fecundity (number of eggs laid) and fertility (number of fertile eggs laid) were modelled in a GLMM with a Poisson distribution and log link function. In addition, egg counts were modelled as proportion data with a binomial distribution (fertile eggs, non-fertile eggs) and logit link function. I modelled the direction of change in individual fertility using a GLMM with a binomial distribution, where change was coded as 1s and 0s (increase, decrease/unchanged). I tested the change in proportion fertility (proportion after mating minus proportion before mating) using a linear mixed effects model (LMM). Reported P-values were computed by model comparison using ANOVA. Percentage fertility is described with the exclusion of females that laid fewer than 10 eggs.

Previous work showed a strong effect of stream site upon reproductive output (Harley et al., 2010), so I included stream site was as a random factor in reproductive output models—both in the first and second experiment. Variation between stream sites is reported for fecundity, fertility and proportion fertility for the first experiment, where females were collected across 11 stream sites. They are not reported for the second experiment, as there was a more limited sample of only 5 stream sites, so any conclusions based on such a small sample would not be trustworthy. Where appropriate, female identity was included as a random factor to account for the non-independence of multiple female measures. Variation between females is reported as a factor similar to stream sites.

The data was found to be over-dispersed and to account for this, I included an observation-level random effect (OLRE) in all models (except for those modelling change), rather than a quasi- distribution, as results can be unreliable when using both random effects and a quasi-distribution (Harrison, 2014, 2015). The improvement in model fit from the addition of OLRE was checked through model comparison. OLRE may perform poorly in binomial models, so the parameter estimates of these models were checked against those from the comparable beta-binomial model using the *glmmADMB* package (Fournier et al., 2012;

Skaug et al., 2016) to confirm robustness (Harrison, 2015).

Female eyespan and thorax length are known to be strong proxies for fecundity (Cotton et al., 2004*a*; Rogers et al., 2006) and were highly correlated with female fecundity and fertility (Spearman's rank $\rho > 0.3$, $P < 0.01$). For both experiments, I repeated all analyses with female eyespan and thorax as covariates. This did not alter any of the results (see Appendix E). For simplicity, the final models reported in the results did not include these covariates.

I examined reproductive output over the 8 days before and 8 days after mating, excluding days 2 and 4 from all analyses. Previous studies have reported that reproductive output of recently caught *T. dalmanni* females typically falls in the short term (day 2) after mating, followed by a peak (day 4) before settling to a more steady level (Cotton et al., 2010; Harley et al., 2010). The same pattern was observed in this investigation (data not shown). Females that died or escaped during the observation period were excluded from the analyses (8 of 45 females in the first experiment; 2 of 36 females in the second experiment), as was a single female that failed to lay any eggs during the observation period in the first experiment.

5.3 Results

5.3.1 Experiment 1: Gains from an additional mating

5.3.1.1 Variation in fecundity

Fecundity was highly variable between females both in the pre-mating (days 5 – 12, mean ± s.d. per day = 2.17 ± 2.48; range = 0.13 – 11.13, N = 36; χ^2_1 = 5.3291, N = 144, P = 0.0210) and post-mating periods (days 14 – 21, mean ± s.d. per day = 2.42 ± 2.93, range = 0 – 11.88, N = 36; χ^2_1 = 24.5018, N = 144, P < 0.0001). Female fecundity did not change over the pre-mating period (χ^2_1 = 1.1815, N = 144, P = 0.2770, Fig. 5.2a), and there was no consistent directional change in fecundity over the whole 17-day period of the experiment (χ^2_1 = 1.2586, N = 288, P = 0.2619).

Female fecundity did not differ when individual reproductive output was

compared across the pre-mating and post-mating periods (χ^2_1 = 0.1001, N = 72, P = 0.7517), and was not different between the days immediately before (days 11 – 12) and immediately after (days 14 – 15) the extra mating (χ^2_1 = 2.4907, N = 72, P = 0.1145, Fig. 5.3a). Lastly, I examined differences in fecundity across streams. There was also no effect of stream site on fecundity in the pre-mating (χ^2_1 = 2.8652, N = 144, P = 0.0905) or post-mating periods (χ^2_1 = 0.0676, N = 144, P = 0.7948).

5.3.1.2 Variation in fertility

The pattern for individual female fertility in the pre-mating period (days 5 – 12), showed considerable variation among females, both in the absolute number of fertile eggs laid (mean ± s.d. per day = 0.66 ± 1.02; range = 0 – 4.75, N = 36; χ^2_1 = 5.7493, N = 84, P = 0.0165) and proportion fertility (mean ± s.d. per day = 35.7057 ± 32.5241%, range = 0 – 86.3636%, N = 17; χ^2_1 = 20.5766, N = 84, P < 0.0001), and this extended into the post-mating period (days 14 – 21) for female absolute fertility (mean ± s.d. per day = 1.53 ± 2.59, range = 0 – 10.5, N = 36; χ^2_1 = 9.7932, N = 85, P = 0.0018) but not proportion fertility (mean ± s.d. = 58.5037% ± 33.0920%, range = 0 – 100%, N = 21; χ^2_1 = 3.4542, N = 85, P = 0.0631). In contrast to fecundity, across the pre-mating period there was a decline in absolute (χ^2_1 = 8.4502, N =84, P = 0.0037, Fig. 5.2b) and proportion fertility (χ^2_1 = 17.5402, N = 84, P < 0.0001, Fig. 5.2c). Note that it was important to examine proportion fertility as there was a positive relationship between total female fertility and fecundity both in the pre-mating (χ^2_1 = 5.9894, N = 36, P = 0.0144) and post-mating periods (χ^2_1 = 22.6367, N = 32, P < 0.0001).

Comparing total fertility over the whole pre-mating and post-mating periods, absolute fertility did not change after the additional mating (χ^2_1 = 3.5892, N = 68, P = 0.0582), however proportion fertility increased (χ^2_1 = 5.1530, N = 68, P = 0.0232). The percentage of females with low fertility (< 20% total egg hatch) dropped from 38% to 19%, whereas the proportion with high fertility (> 70% total egg hatch) rose from 24% to 48% (Fig. 5.4a). Comparing across a closer period of time, there was a distinct increase in the days around the extra mating (days

11 – 12 to days 14 – 15), both absolute (χ^2_1 = 10.0766, N = 41, P = 0.0015, Fig. 5.3b) and proportion fertility increased (χ^2_1 = 15.5344, N = 41, P < 0.0001, Fig. 5.3c).

The direction of change in total individual fertility after the additional mating (increase or decrease/unchanged) did not depend on female fecundity (χ^2_1 = 2.2001, N = 32, P = 0.1380). However, when female fertility was accounted for, females with higher fecundity were more likely to have a positive change in fertility after the additional mating (χ^2_1 = 18.3375, N = 32, P < 0.0001). In addition, females with low fertility were more likely to benefit from the additional mating (χ^2_1 = 5.8261, N = 32, P = 0.01579). This greater effect of pre-mating fertility persisted after accounting for differences in individual female fecundity (χ^2_1 = 21.9635, N = 32, P < 0.001).

A similar examination was made using the change in proportion fertility between the pre- and post-mating periods (Fig. 5.5). Females with high pre-mating fecundity had a larger positive change in their proportion fertility post mating (χ^2_1 = 7.5575, N = 32, P = 0.0060), and this result remained when female fertility was accounted for (χ^2_1 = 12.842, N = 32, P < 0.0001). Female pre-mating fertility had no effect on the change in proportion fertility (χ^2_1 = 2.0648, N = 32, P = 0.1507). However, once fecundity was accounted for, female pre-mating fertility did have an effect (χ^2_1 = 7.349, N = 32, P = 0.0067), as females that fertilised few of their eggs had a larger positive change in proportion fertility then females that were already fertilising relatively more.

Finally, I examined differences in fertility across streams. In the pre-mating period, there was variation between stream sites in absolute (χ^2_1 = 5.8958, N = 84, P = 0.0152) and proportion fertility (χ^2_1 = 4.3233, N = 84, P = 0.0376). After the additional mating, absolute fertility no longer differed between stream sites (χ^2_1 = 1.4439, N = 85, P = 0.2295), but variation in proportion fertility persisted despite the extra mating (χ^2_1 = 5.5951, N = 85, P = 0.0180).

5.3.2 Experiment 2: Investigation of female and male effects

To investigate potential male effects on fertility gain amongst females, a second experiment was carried out. Females were mated once either with a sperm depleted male that had been held for the previous two weeks with multiple females or with a non-sperm depleted male that had been held in a male-only container.

5.3.2.1 Variation in fecundity

The pattern for female fecundity was broadly similar to that of the previous experiment (Fig. 5.6a and 5.7a, see Appendix E). There was no effect of male type on total fecundity before versus after the additional mating (male type x before/after interaction, χ^2_1 = 0.4838, N = 68, P = 0.4867), or for the contrast of the days immediately before and after the additional mating, days 11 – 12 and 13 – 14 (χ^2_1 = 0.5267, N = 68, P = 0.4680).

5.3.2.2 Variation in fertility

Fertility also showed a broadly similar pattern to the previous experiment (Fig. 5.4b, 5.6b and 5.7b, see Appendix E). At the end of the pre-mating period individual absolute fertility was comparable to that of the low absolute fertility in the previous experiment (1.7368 ± 2.6634 and 1.9355 ± 2.4074, experiment 1 and experiment 2, mean ± s.d., days 11 – 12). Proportion fertility was also similar to the previous experiment prior to mating (19% and 21%, experiment 1 and experiment 2, days 11 – 12). Comparing total fertility in the pre-mating and post-mating periods, absolute (χ^2_1 = 12.5805, N = 66, P < 0.0001) and proportion fertility (χ^2_1 = 12.4228, N = 66, P < 0.0001) increased after the additional mating. Likewise, between the days immediately prior (days 11 – 12) and immediately after (days 13 – 14) the additional mating there was an increase in absolute (χ^2_1 = 23.8148, N = 62, P < 0.0001, Fig. 5.7b) and proportion fertility (χ^2_1 = 27.0669, N = 62, P < 0.0001, Fig. 5.7c).

The direction of change in individual fertility was more likely to be positive for more fecund females (χ^2_1 = 4.7193, N = 32, P = 0.0298), but not after female fertility was accounted for (χ^2_1 = 0.1939, N = 32, P = 0.6597). Females with low

pre-mating fertility were more likely to have a positive change (χ^2_1 = 8.2079, N = 32, P = 0.0042). However again, after accounting for fecundity, pre-mating fertility did not predict the direction of change (χ^2_1 = 3.6824, N = 32, P = 0.0550).

Change in proportion fertility between the pre-mating and post mating periods did not depend on pre-mating fecundity (χ^2_1 = 0.0476, N = 32, P = 0.8274), but when female fertility was controlled for, more fecund females had a more positive change in proportion fertility (χ^2_1 = 4.4386, N = 32, P = 0.0351). Change in proportion fertility likewise did not depend on pre-mating fertility (χ^2_1 = 3.1064, N = 32, P = 0.0780). In addition, when the analysis was repeated and fecundity was accounted for, females with low fertility prior to mating also had a more positive change in proportion fertility (χ^2_1 = 7.4975, N = 32, P = 0.0062).

Comparing the 8 days before and after the additional mating, male type was unrelated to the increase in absolute (male type x before/after interaction, χ^2_1 = 0.6327, N = 66, P = 0.4264) and proportion fertility (χ^2_1 = 2.6744, N = 66, P = 0.1020). Likewise comparing the days immediately before (day 12) and after the additional mating (day 14), male type had no effect on the increase in absolute (χ^2_1 = 0.0027, N = 62, P = 0.9589) or proportion fertility (χ^2_1 = 0.2317, N = 62, P = 0.6303). There was no effect of male type on either the direction of change in fertility (χ^2_1 = 0.2076, N = 32, P = 0.6487) or the degree of change in proportion fertility (χ^2_1 = 0.4654, N = 32, P = 0.4951).

5.4 Discussion

There are abundant studies investigating the direct fertility benefits from multiple mating (Haig and Bergstrom, 1995; Zeh and Zeh, 1996; Yasui, 1998; Arnqvist and Nilsson, 2000; Hosken and Stockley, 2003; Slatyer et al., 2012). However there is currently minimal focus on how these benefits vary between individuals and across time, or in particular contexts like associations with the degree of polyandry and female age or experience (House et al., 2009; Wright et al., 2013; Toft and Albo, 2015). In addition, experiments evaluating direct benefits of multiple mating have rarely been carried out amongst individuals sampled from

wild populations, in ways that examine the encounters likely to occur between females and males in nature.

In this study, I aimed to redress these deficits by assessing fecundity and fertility in wild-caught stalk-eyed flies, and how these benefits vary with the time since the last mating (and, as a corollary, whether there is a cost of a failure to remate that increases with time). Females from laboratory populations of *T. dalmanni* have been shown to benefit from multiple mating (Baker et al., 2001*a*). But the experience of flies under laboratory conditions are inevitably very different from those in wild populations, for example in terms of population density, food availability and exposure to parasites/predators. Moreover, laboratory studies of stalk-eyed flies and other species have utilised virgin males and females in remating assays, in order to standardise prior mating experience (Baker et al., 2001*a*; Tregenza and Wedell, 2002; Bayoumy et al., 2015; Burdfield-Steel et al., 2015; Chelini and Hebets, 2016; Droge-Young et al., 2016). However, virgins are rare in nature in species in which males and females readily remate, and this is particularly true of stalk-eyed flies in which adult fertility persists for many weeks (Rogers et al., 2006). All of these factors point to the necessity for controlled experiments using wild-caught individuals with backgrounds of natural variation.

Female sperm limitation is likely to be an important fluctuating factor that regulates the direct fertility benefits to females from multiple mating. In some insect mating systems females only mate once (Arnqvist and Nilsson, 2000; Arnqvist and Andrés, 2006; South and Arnqvist, 2008) or mate multiple times but over a single short period (Boomsma et al., 2005). These restricted mating patterns provide sufficient sperm to ensure female fertility throughout her reproductive life. However, in many other insect species, sperm acquired in a single mating or mating period is insufficient to fertilise all of a female's eggs (Ridley, 1988; Wedell et al., 2002). Consequently females necessarily need to remate throughout their adult life, as sperm supplies diminish through use and with time (Fjerdingstad and Boomsma, 1998; Fox, 1993; Drnevich et al., 2001; Chevrier and Bressac, 2002; Wang and Davis, 2006). I demonstrate that this form of re-

productive life history typifies *T. dalmanni* stalk-eyed fly females collected from the wild. Females from the two collections, in 2011 and 2012, had mean female fertility of 46% or 32% respectively shortly after they were initially captured (days 5 – 6), and this declined to $\sim$20% in both cases over the following week (days 11 – 12; Fig. 5.2 and Fig. 5.6). An additional mating after 12 days markedly changed fertility, causing a substantially larger proportion of their eggs to be fertilised, 61% and 48%, immediately after the additional mating (Fig. 5.3 and Fig. 5.7). In contrast, female fecundity was unchanged by an additional mating (Fig. 5.3 and Fig. 5.7) and remained consistent across the whole of the study period, although with a fair degree of stochastic variation (Figs 5.2 and 5.6). Accordingly, negative and positive changes in fertility can be ascribed to females being able to fertilise a smaller or larger proportion of their eggs, rather than due to fluctuations in the number of eggs laid.

I show an overall increase in fertility, however I additionally make the novel finding that the increase in fertility was not uniform between individual females. Females with low pre-mating fertility were more likely to benefit from an additional mating, as were females with high fecundity. After taking account of variation in pre-mating fecundity, it is apparent that females were able to fertilise a larger proportion of their eggs if they initially had low fertility. Similarly, after taking account of variation in pre-mating fertility, females gained more in fertility from an additional mating if they were highly fecund. These outcomes reveal a strong context-dependence in the benefit of additional matings. Low prior fertility is indicative that females were subject to sperm depletion, and high fecundity is indicative of the need for greater numbers of stored sperm, both seemingly addressed by the additional mating. To test these predictions, direct measurements of sperm numbers within females will be necessary. This is possible in female stalk-eyed flies which retain sperm in spermathecae that act as long-term storage organs, and the ventral receptacle, a small structure to which sperm move and are stored individually within pouches (capacity $\sim$16 – 40 sperm) prior to release for fertilisation of an egg (Kotrba, 1993; Rose et al.,

2014).

The results here contrast with those of a previous study carried out on the same population (Harley et al., 2010). In that study, females were collected from the wild at lek mating sites and half were immediately allowed a single additional mating. Both groups showed a decline in fertility through time, as in the current study. However, there was no difference in fertility between females that received an extra mating on capture and those that did not. What explains the divergence from the current study? The striking difference is that females were unusually fertile, $\sim$80% over the first 10 days in captivity, both among females with and females without the extra mating (Harley et al., 2010). This degree of fertility is comparable to the levels achieved in laboratory populations when females are given the opportunity to mate repeatedly (Baker et al., 2001*a*). This failure of an additional mating to enhance female fertility echoes the finding that fertility gains from an extra mating are weaker when females already have high fertility. In the current study, average fertility was much lower, around $\sim$30% fertility in both years of this study. Hence there was plenty of opportunity for an extra mating to benefit female fertility. I suspect this low level is the norm as an earlier census also from the same area in Malaysia reported 36% fertility (Cotton et al., 2010).

Several inferences can be made from these studies of wild-caught females. First, they confirm there is a cost of a failure to remate as the proportion of fertile eggs laid declines with time when females are unable to remate. Second, an additional mating has a greater beneficial effect when females already have low fertility. The most obvious proximate reason for this is that many wild females are sperm limited, either because they had not mated recently, not mated at a sufficiently high rate or because sperm allocation by males was considerably limited. These explanations could be directly assessed in the future by counting sperm in female sperm storage organs in wild caught females and after matings with wild caught males. This could be complemented by observing mating rates in the wild, and relating these measures to natural fertility levels. A third infer-

ence from the current experiments is that the fertility benefits to females vary between individuals, stream sites, across matings and fluctuate through time. In some contexts, individual females may be limited by the availability of mating opportunities, whereas in others, they may become increasingly limited by their own fecundity.

The source of variation in fertility between individuals in the wild is currently undefined. It is likely that variable factors such as population density and sex ratio are important, particularly as they will affect female and male mating rates. Similarly, environmental conditions such as food availability can influence mating rates (Kotiaho et al., 2001; Rogers et al., 2005*b*, 2008), male fertility (Perry and Rowe, 2010; Perry et al., 2013; O'Dea et al., 2014; Bunning et al., 2015) and female fecundity (Awmack and Leather, 2002; Stewart et al., 2005; Cotton et al., 2015; Levin et al., 2016). While in certain contexts an additional mating may be clearly beneficial for female fertility, I show that this is not always the case and there is a need to test females under a range of contexts that reflect those experienced under natural conditions. Only then can the full force of remating on female fertility be understood.

Other significant factors to consider are variation in male mating strategy and male quality as they may have a significant influence on the benefit that females obtain from remating. Males can adjust their ejaculate investment in response to female reproductive value (Engqvist and Sauer, 2001; Wedell et al., 2002; Rogers et al., 2006; Kelly and Jennions, 2011; Perry et al., 2013), and investment may positively correlate with male condition (Sheldon, 1994; Iwasa and Pomiankowski, 1999; but see: Pizzari et al., 2004; Harley et al., 2013; Fitzsimmons and Bertram, 2013; Mautz et al., 2013) or negatively with male dominance or attractiveness (Warner et al., 1995; Jones, 2001; Tazzyman et al., 2009). I explicitly evaluated the importance of variation in recent male mating experience, contrasting males that had multiple opportunities to mate, with those that had been deprived of females. Rather surprisingly, there was no difference in fertility gains from extra matings with either type of male (Fig. 5.7b,c). This

reveals that male allocation of ejaculate is tailored to repeated mating, and the replenishment of resources occurs on a short time scale. Males partition their ejaculate in order to copulate with many females each day (Small et al., 2009); spermatophore size is very small in *T. dalmanni* (Kotrba, 1996) and males transfer few sperm in a single ejaculate ($\sim$100, Wilkinson et al., 2005; Rogers et al., 2006). Partitioning of ejaculate is presumably a mechanism for males to maintain fertility over successive matings (Wedell et al., 2002; Linklater et al., 2007). In addition, male reproductive activity is scheduled in a highly concentrated burst each day, as lek holding males mate with females that have settled with them overnight before they disperse at dawn (Chapman et al., 2005; Cotton et al., 2010). To cope with this pattern of sexual activity, males replenish their accessory glands and hence their ability to produce ejaculate within 24 hours (Rogers et al., 2005*b*). In this system, prior mating activity has no or a minimal effect on a male's ability to mate effectively. However, I only assessed female fertility gains after the first mating by a male. It might still be the case that prior mating experience could affect the ability of males to deliver ejaculate in subsequent matings or even to be able to mate repeatedly. In the wild, it is notable that females often leave lek sites before mating if the male is preoccupied in matings with other females (A. Pomiankowski, *personal observation*). This suggests that fertility gains may fall with subsequent matings, but this remains to be investigated. Again, this points to the complexity of context underpinning the benefits associated with remating.

Another cause of variation in male fertility and ejaculate allocation, other than recent mating history, is meiotic drive (Wilkinson et al., 2006). An X-linked meiotic drive system is present in these populations of *T. dalmanni* (Cotton et al., 2014) and causes the degeneration of Y-bearing sperm and the production of female-biased broods (Presgraves et al., 1997). Drive male fertility may be impaired due to this dysfunction, resulting in the transfer of fewer sperm. Consequently, mating with a drive male may not provide a female with the same fertility benefit as mating with a standard male. There is evidence that females mated

to drive males have lower fertility, particularly when males are mating at high frequencies (Wilkinson et al., 2003, 2006) and that drive males are poor sperm competitors (Wilkinson et al., 2006). In this study, I found that several females failed to raise their fertility after mating (Fig. 5.5), and in fact had lower fertility than prior to mating. Mating with a drive male could potentially produce this pattern. Future research should evaluate explicitly how an extra mating with a drive male impacts on female fertility amongst wild-caught flies, when males and females are in their natural condition. It would also be of interest to investigate the hypothesis that multiple mating is an evolved mechanism by which females dilute the negative effects of mating with a drive male (Haig and Bergstrom, 1995; Zeh and Zeh, 1996), both to ensure fertility and because any male progeny produced in a female-biased population will have increased fitness (Fisher, 1930; Holman et al., 2015).

I used wild-caught flies to capture the natural variation between individuals, an approach that has been much neglected. It is important to dig deeper into the life history of *T. dalmanni* to further understand the environmental and population level variables that affect the benefits to additional matings. For example, we know that there is much variation in female fecundity and fertility between stream sites. What we have yet to elucidate is how streams differ—do they vary in food availability and quality, rainfall, humidity, temperature, population density or sex ratio? Are these factors stable or fluctuating? Which have the most influence on female fecundity and fertility? I show that females with low fertility and high fecundity benefit the most from mating; improved knowledge of the conditions experienced by individuals throughout their lifetime will further our understanding on when and why it is beneficial for females to remate.

In conclusion, this study has demonstrated that female sperm storage and depletion since the previous mating are key selection forces driving the benefits and evolution of mating rates in the wild. Females are generally sperm-limited due to the minimal male sperm investment in individual copulations (Wilkinson et al., 2005; Rogers et al., 2006), so females gain direct fertility benefits from

multiple mating both in the laboratory (Baker et al., 2001*a*) and in wild populations. However, these gains are not uniform between females and are contingent on female fecundity and fertility. In a broader context, stalk-eyed fly reproductive activity is governed by a co-evolutionary spiral of exaggerated mating rates. Females have evolved high levels of multiple mating because their fertility is subject to sperm-limitation. The resulting higher levels of multiple mating by males, especially those that are attractive to females, has led to the evolutionary corollary of finer partitioning of ejaculate, that has only exacerbated sperm-limitation and the benefits of multiple mating. The various studies of stalk-eyed fly fertility in the wild (Cotton et al., 2010; Harley et al., 2010, this study) demonstrate both high variation (across space and time, and between individuals) and now also context dependence in benefits to remating. They highlight the importance of complementing laboratory studies with those using wild populations, where natural mating rates may be very different. Further studies will disentangle whether other factors such as variation in age, condition, attractiveness, a range of environmental variables and the presence of meiotic drive are important as well, and allow a better understanding of the range of forces that influence female and male mating behaviour.

5.5 Figures

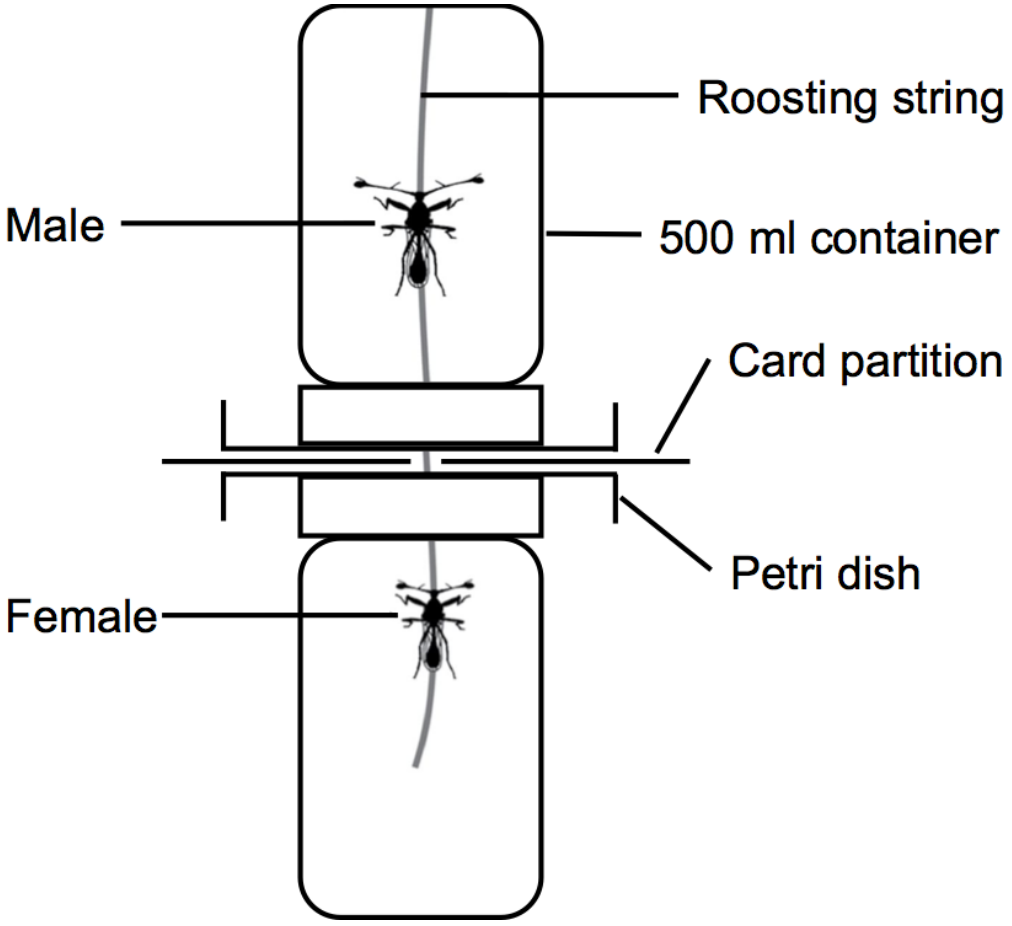

Figure 5.1: Mating chambers composed of two 500 ml cells, separated by a removable card partition. A single string runs the whole length of the chamber, providing a suitable roosting site. A male was placed in the upper cell and a female in the lower cell. The card partition was removed and the pair was allowed to mate once, before being separated.

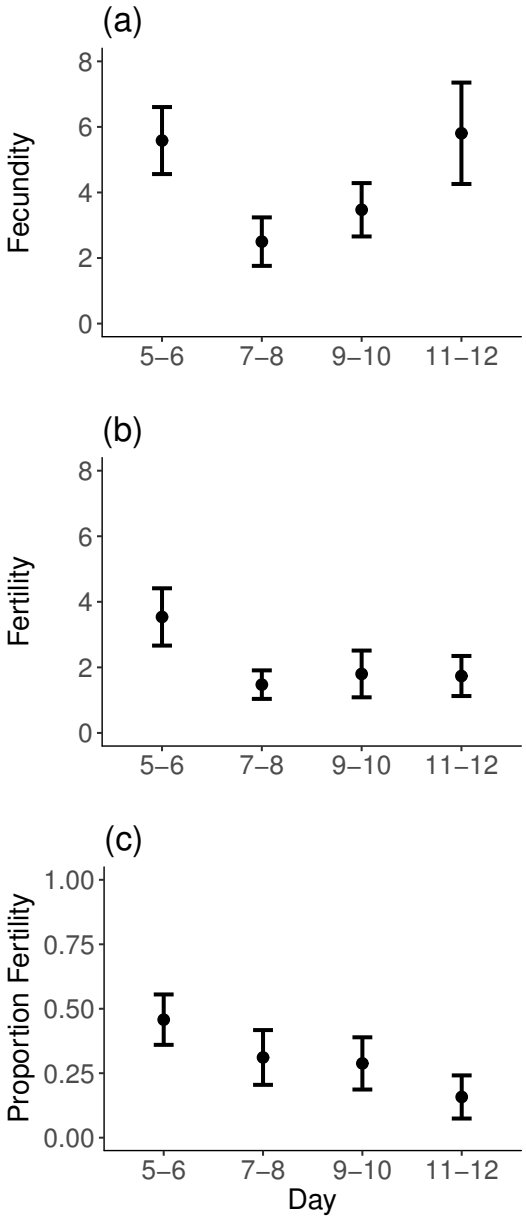

Figure 5.2: Pre-mating female reproductive output through time (mean ± standard error). Mean (a) fecundity, (b) fertility and (c) proportion fertility per two-days, over an eight-day period. Flies were captured at dusk on day zero.

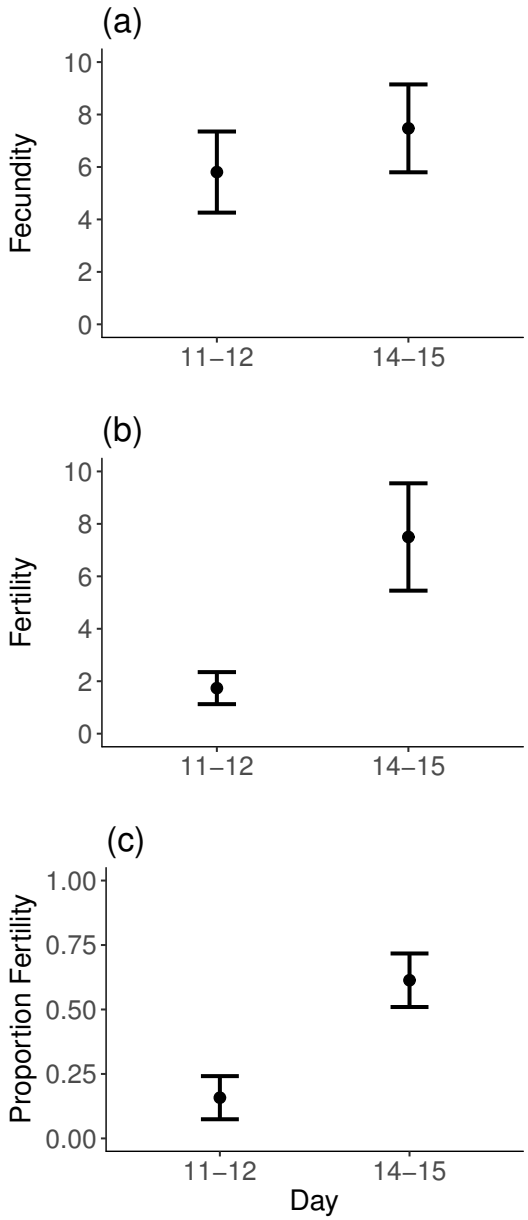

Figure 5.3: Reproductive output immediately before and immediately after mating (mean ± standard error). Mean (a) fecundity, (b) fertility and (c) proportion fertility on days 11 – 12 and days 14 – 15. Females were captured at dusk on day zero and mating occurred at dawn on day 13.

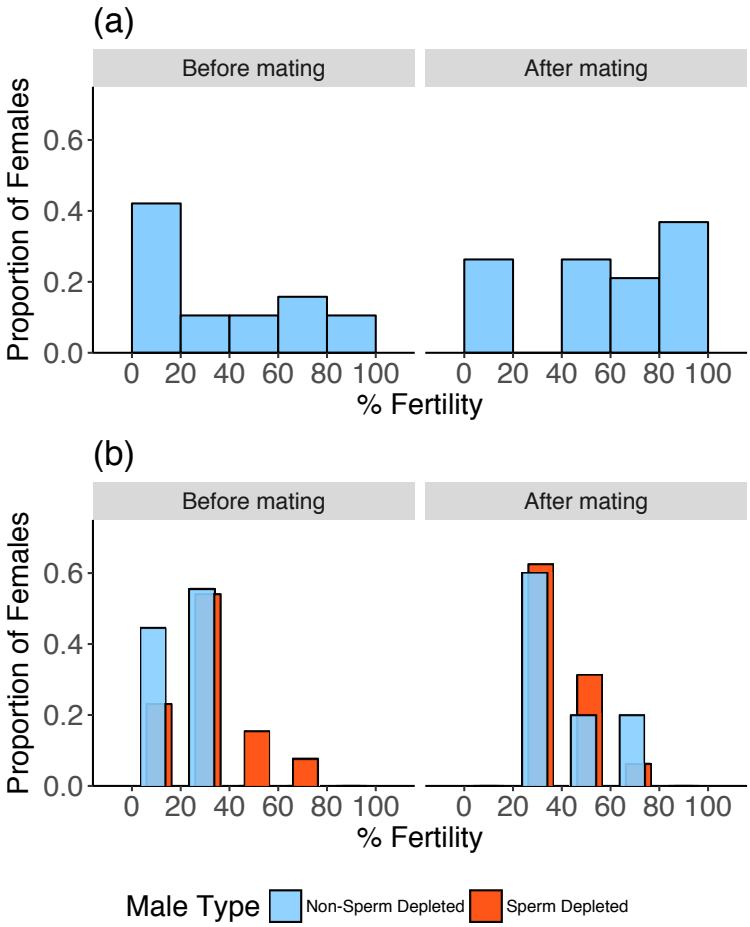

Figure 5.4: The distribution of percentage fertility (total eggs hatched / total eggs laid) for females in the 8 days before, and 8 days after the extra mating in (a) experiment 1 and (b) experiment 2. Females used in experiment 2 were either mated to a sperm depleted (orange) or a non-sperm depleted male (light blue). Plots exclude females who laid fewer than 10 eggs over each period.

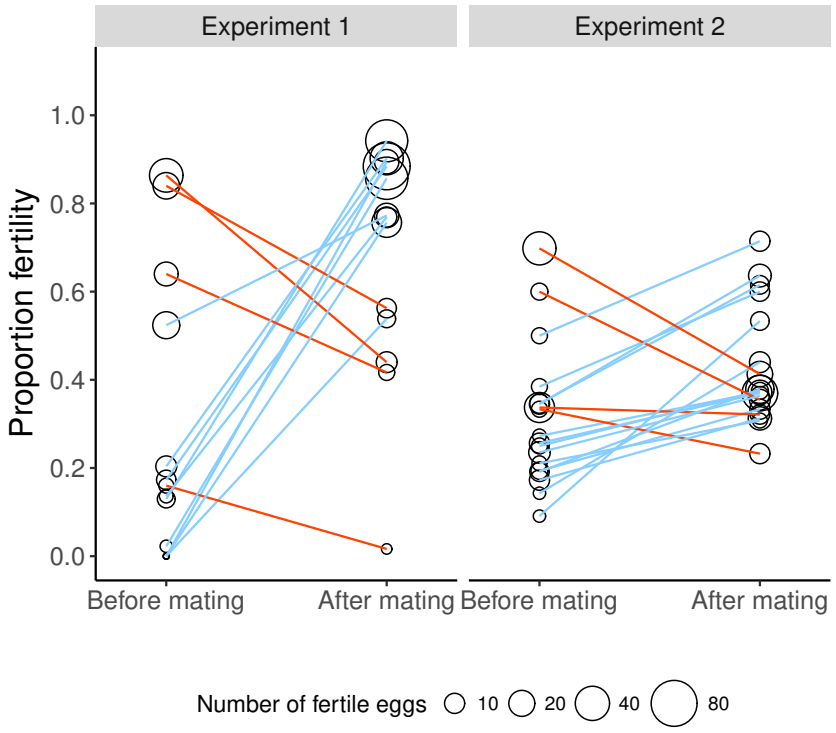

Figure 5.5: Total proportion fertility in the 8 days before, and 8 days after the extra mating in experiment 1 and experiment 2. Lines are individual females, coloured by slope: increased fertility (light blue), decreased fertility (orange). Circle size indicates the total absolute number of fertile eggs laid by each female. Plots exclude females that laid fewer than 10 eggs either before or after the extra mating.

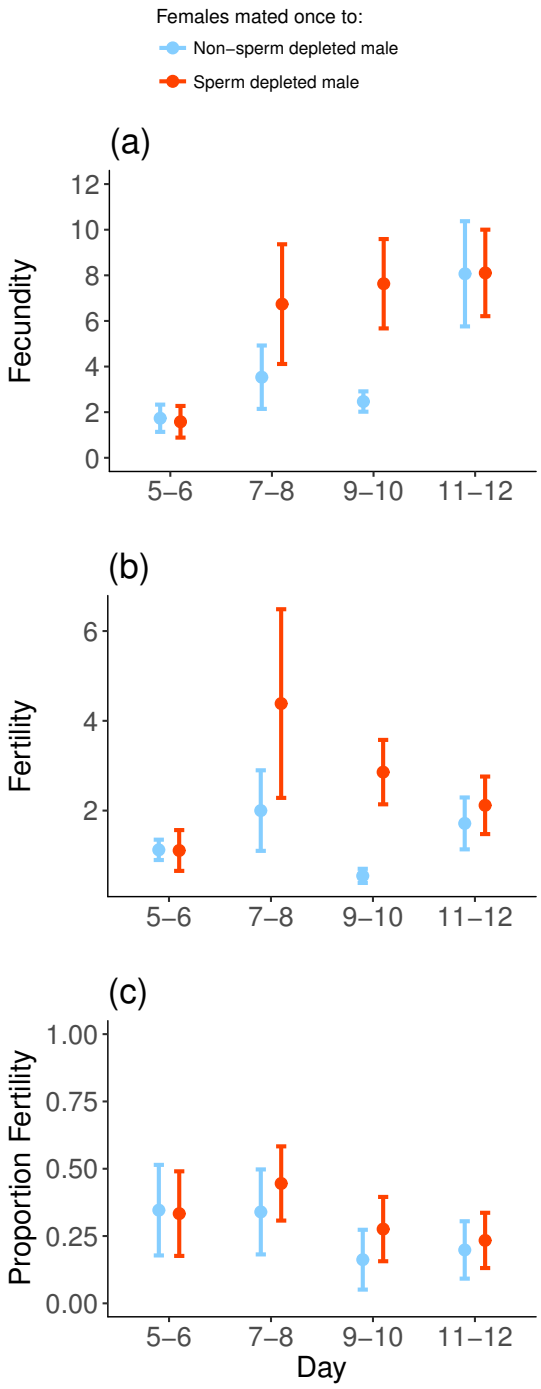

Figure 5.6: Pre-mating female reproductive output of mean (a) fecundity, (b) fertility and (c) proportion fertility per two-days through time (mean ± standard error). Females from the sperm-depleted (orange) or non-sperm depleted male (light blue) treatment are shown separately. Flies were captured at dusk on day zero.

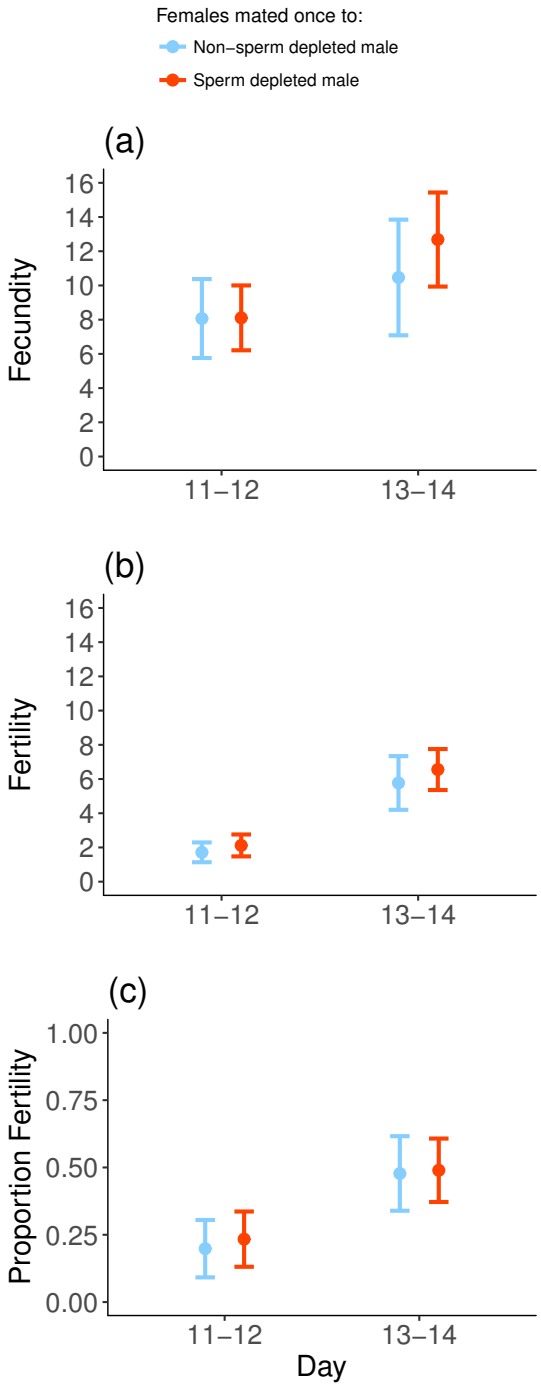

Figure 5.7: Reproductive output immediately before and immediately after mating (mean ± standard error) where females received an extra mating from either a sperm-depleted (orange) or non-sperm depleted (light blue) male. Mean (a) fecundity, (b) fertility and (c) proportion fertility on days 11 – 12 and days 13 – 14. Females were captured at dusk on day zero and mating occurred on the evening of day 12.

Chapter 6

General Discussion

6.1 Overview

Meiotic drive genes are a class of segregation distorter that cause the degeneration of non-carrier sperm in heterozygous drive males (Taylor and Ingvarsson, 2003; Burt and Trivers, 2006; Lindholm et al., 2016). This gives the driver a transmission advantage that must be balanced by fertility or viability costs if it is to remain at stable frequencies in a population. One such cost could be sperm limitation due to sperm destruction of non-carrier sperm, and possibly also reduced fertilisation capacity of carrier sperm (Jaenike, 1996; Taylor and Jaenike, 2002; Price and Wedell, 2008). However, whilst the drive element enjoys a transmission advantage, the consequential reduction in male fertility reduces the fitness of the rest of the genome, and so mechanisms to circumvent the effects of drive may evolve. Males that have a fertility disadvantage are expected to compensate by producing competitive ejaculates in response to the expected levels of sperm competition (Tazzyman et al., 2009; Engqvist, 2012). In which case, the fertility costs that could inhibit drive transmission become less apparent. This may be particularly pertinent in a species that only produce small ejaculates, such as the Malaysian stalk-eyed fly *Teleopsis dalmanni* (Kotrba, 1996; Wilkinson et al., 2005; Rogers et al., 2008). The primary theme of this thesis has been the examination of the fertility consequences of being a carrier of meiotic drive in *T. dalmanni*, and testing if and how males can respond adap-

tively. I have examined the effects of drive on male ejaculate allocation, fertility and investment in primary and secondary sexual organs.

Polyandry, female mating with multiple males, is widespread across many taxa and almost ubiquitous in insects (Ginsberg and Huck, 1989; Clutton-Brock, 1989; Jennions and Petrie, 2000; Arnqvist and Nilsson, 2000; Zeh and Zeh, 2001; Griffith et al., 2002; Avise et al., 2002). However, there are many costs associated with mating (Rowe, 1994; Arnqvist and Nilsson, 2000; Crudgington and Siva-Jothy, 2000; Blanckenhorn, 2002; Rönn et al., 2006; Ashby and Gupta, 2013), and so polyandry needs an adaptive explanation. Females may need to mate multiply to gain sufficient sperm supplies to maintain their fertility, especially in species in which male promiscuity results in division of their ejaculate amongst many females. I utilised data on wild-caught *T. dalmanni* to explore how natural variation among females and males influences fertility gains for females.

6.2 Summary of findings

6.2.1 Chapter 2: Adaptive maintenance of ejaculate size in the face of sperm destruction by meiotic drive

Meiotic drive typically involves sperm destruction (Burt and Trivers, 2006; Price and Wedell, 2008). This is beneficial to the drive element because it prevents non-carrier sperm from taking part in fertilisation. However, it can also lead to a reduction in overall fertility for drive males (Novitski et al., 1965; Jaenike, 1996; Atlan et al., 2004; Wilkinson et al., 2006; Angelard et al., 2008; Price et al., 2012; Pinzone and Dyer, 2013), as well as in sperm competition (Wilkinson and Fry, 2001; Atlan et al., 2004; Angelard et al., 2008; Price et al., 2008*a*). In a minority of cases, direct examination of the numbers of sperm transferred to females at mating have been made, and confirmed that numbers are reduced in the ejaculate of drive males (Novitski et al., 1965; Angelard et al., 2008; Price et al., 2008*a*). Consequently, drive males are viewed as unavoidably low-fertility, sperm-limited males. However, this static view ignores the possibility of adaptive responses to drive that ameliorate its negative effects and restore organismal

fitness. In fact, theoretical models that look at the evolution of male ejaculate allocation do not predict sub-fertile males, such as drive males, to invest any less per ejaculate than standard males (Tazzyman et al., 2009; Engqvist, 2012). Using the stalk-eyed fly *T. dalmanni*, I have found evidence contrary to traditional views of drive, and in support of theoretical predictions. I examined sperm numbers in the long-term primary sperm storage organs (spermathecae) of female *T. dalmanni*, and found that the numbers of sperm in storage ($\sim$147) were not different between females mated singly to SR and ST males. Furthermore, sperm numbers in storage were similar for females mated to SR and ST males even after the males had mated multiple times. Mating in *T. dalmanni* involves the transfer of sperm and other non-sperm components parcelled into a spermatophore, which is attached to the base of the spermathecal ducts (Kotrba, 1996). Sperm then proceed to migrate along the ducts and into the spermathecae. Sperm must then move from the primary storage organs to the site of fertilisation, the ventral receptacle (VR, Kotrba, 1993). This is a much smaller organ containing $\sim$35 pouches, each of which can hold a single sperm. The entrance to this organ lines up with the gonopore of an egg as the egg passes out of the oviduct, thus it is vital for sperm to attain storage here if they are to be used in fertilisation. I found that the transfer of sperm to the VR was also similar for SR and ST male sperm, over both short and longer time frames (after two days), suggesting that SR males not only attain similar sperm storage but that the sperm have normal fertilisation capacity. These findings challenge the conventional assumption that drive acts like a genetic disease that causes disruption of normal male reproductive activity.

6.2.2 Chapter 3: Adaptive compensation in fertility to meiotic drive in a stalk-eyed fly

As outlined in the previous section, drive males are conventionally expected to be sperm limited, as non-carrier sperm are rendered inviable. However, there is nonetheless a recurring pattern in the literature that direct evidence for drive male sperm limitation is equivocal. In fact, there are plenty of examples where

drive males have comparable fertility to wildtype males, and produce ejaculates with large numbers of sperm (Peacock and Erickson, 1965; Wood and Newton, 1991; Jaenike, 1996; Taylor, 1999; Capillon and Atlan, 1999; Verspoor et al., 2016), including in *T. dalmanni*, as demonstrated in Chapter 2.

These frequent instances of compensation require an explanation as to how drive males can achieve this, despite the destruction of sperm during spermatogenesis. At a general evolutionary level mechanisms to circumvent drive—or the effects of drive—are expected to evolve, as impaired fertility due to drive reduces the fitness of the rest of the genome. In this chapter, I propose such a mechanism, and present the first evidence that males adapt to drive by increasing their investment in primary sexual organs to increase sperm production. This chapter demonstrates that drive males can achieve the high sperm numbers described in Chapter 2 through increased production in greatly enlarged testes. Testis size in this (Fry, 2006), and many other insect species (Møller, 1989; Pitnick and Markow, 1994; Gage, 1994; Pitnick, 1996; Stockley et al., 1997) correlates with sperm number, and so this is a credible mechanism by which males can increase sperm production and circumvent the negative consequences of drive for fertility. Furthermore, I found that testis size was a good predictor of fertility, and drive male total and proportion fertility was not reduced compared to standard male fertility, even at high mating frequencies. However, resources are not unlimited, and other examples from non-drive systems demonstrate trade-offs between testis size and investment in traits that impact on male pre-copulatory mating success (Droney, 1998; Simmons and Emlen, 2006; Yamane et al., 2010; Somjee et al., 2015). In this chapter I show for the first time that drive *T. dalmanni* males are generally smaller than standard males, but they also have smaller accessory glands and eyespan than expected for their body size. Male accessory gland size limits male mating frequency in *T. dalmanni* (Baker et al., 2003; Rogers et al., 2005*a*,*b*), and drive males mate less frequently than standard males (Wilkinson et al., 2003; S. Finnegan, *unpublished data*). Furthermore, the sexual ornament, male eyespan, is important in

male mating success, female preference and male-male competition (Wilkinson && Reillo 1994; Panhuis && Wilkinson 1999; Hingle, Fowler && Pomiankowski 2001a; Cotton et al. 2010). These patterns in *T. dalmanni* fit with theoretical models which predict that males should invest in producing optimal ejaculate according to levels of expected sperm competition, even if they are low-resource or low-fertility males, but at the expense of the number of matings that they can achieve (Tazzyman et al., 2009; Engqvist, 2012).

6.2.3 Chapter 4: The use of microsatellite and INDEL markers to detect sex ratio meiotic drive in *Teleopsis dalmanni*

The primary goal of this chapter was to describe markers that are useful for identifying the presence of X^{SR} in both wild and laboratory samples. I evaluated the relationship between male brood sex-ratio and allele sizes of four X-linked markers—one microsatellite marker, *ms395*, and three INDEL markers, *comp162710*, *cnv395* and *cnv125*. I found all four markers to have an association with brood sex-ratio in both wild and laboratory samples, and furthermore, allele sizes segregated non-randomly between sex-ratio phenotype categories. Males were assigned to phenotype categories according to the direction and strength of their brood sex-ratio bias, ranging from male biased (MB), through unbiased (ST), to female biased (SR-weak and SR-strong). Consequently, these markers are suitable candidates to asses for predictability of drive phenotype. In wild samples, all four markers reliably predicted male phenotype category (85 – 90%), while only *comp162710* reliably predicted an SR-strong phenotype, where 75% of males with a small *comp162710* allele were SR-strong. The microsatellite *ms395* has previously been reported to be associated with brood sex-ratio (Cotton et al., 2014), and I found that large alleles for this marker predicted SR-strong but only to some extent (55%). Laboratory samples followed similar patterns, but their predictability was higher, reflecting the likelihood that SR-stock and ST-stock breeding regimes remove

rare allele/phenotype combinations. In laboratory samples, all loci were good at predicting ST (80 – 87%), and *ms395*, *comp162710* and *cnv395* all reliably predicted SR-strong (83 – 85%). Combined with amplification rates for each marker, *comp162710* is the most useful and informative marker for identifying X^{SR} in both wild and laboratory samples.

6.2.4 Chapter 5: Variation in the benefits of multiple mating on female fertility in wild stalk-eyed flies

To date, there has been minimal focus on how the benefits of polyandry vary between individuals or across time, or in particular contexts like associations with the degree of polyandry and female age or experience (House et al., 2009; Wright et al., 2013; Toft and Albo, 2015). These factors will be particularly pertinent in wild populations, which will certainly be very different from laboratory populations, for example in terms of population density, food availability and exposure to parasites and predators. Furthermore, experiments evaluating direct benefits of female multiple mating have rarely been carried out amongst individuals sampled from wild populations. In this chapter I aimed to redress these deficits by assessing fecundity and fertility in wild-caught *T. dalmanni*. In this chapter, I demonstrated that female fertility suffers if females are unable to remate, and after an additional mating was permitted, females were able to fertilise a substantially larger proportion of their eggs. I additionally made the novel finding that the increase in fertility was not uniform between individual females. Females were particularly likely to benefit from an additional mating if they had low pre-mating fertility or if they were highly fecund. This reveals a strong context-dependence in the benefit of additional matings, and there may be many factors in the wild that influence female fertility and fecundity. It is likely that variable factors such as population density and sex ratio are important, particularly as they will affect female and male mating rates. Similarly, environmental conditions such as food availability can influence mating rates (Kotiaho et al., 2001; Rogers et al., 2005*b*, 2008), male fertility (Perry and Rowe, 2010; Perry et al., 2013; O'Dea et al., 2014; Bunning et al., 2015) and female fecun-

dity (Awmack and Leather, 2002; Stewart et al., 2005; Cotton et al., 2015; Levin et al., 2016). I did explicitly evaluate the importance of variation in recent male mating history, contrasting males that had multiple opportunities to mate, with those that had been deprived of females, and found that this variation in males had no influence on female fertility. Prior mating activity appears to have no or minimal effect on a male's ability to mate effectively, and this is likely due to males tailoring their ejaculate allocation to a pattern of repeated mating (Kotrba, 1996; Small et al., 2009).

6.3 Discussion

Chapters 2 and 3 demonstrate, for the first time, evidence of a host adaptation to meiotic drive where evolutionary change improves the fitness of carrier males through a compensatory increase in the production of viable sperm. A large body of work over many decades has revealed the ubiquity of meiotic drivers across many species, and researchers have endeavoured to understand the forces that determine their abundance in natural populations (Sturtevant and Dobzhansky, 1936; Wu, 1983*a*; Lyttle, 1991; Zeh and Zeh, 1996; Jaenike, 2001; Taylor and Jaenike, 2002; Price and Wedell, 2008; Holman et al., 2015; Lindholm and Price, 2016). The diversity of adaptive responses has been so far under-explored, yet may have significant relevance for predictions of drive frequency in natural populations. Genetic suppressors of drive in males, female multiple mating and female mate choice, are all mechanisms that are likely to reduce the transmission advantage gained by drive. In contrast, I present the first evidence for an adaptive response to drive that will intensify, rather than prevent, the transmission of the driver.

One of the main features of drive in males is reduced fertility due to the dysfunction of non-carrier sperm. This predicts that mechanisms to suppress drive are likely to be strongly favoured. Genetic suppressors of drive have been widely documented in a range of species (Tokuyasu et al., 1977; Gummere et al., 1986; Hauschteck-Jungen, 1990; Wood and Newton, 1991; Tao et al., 2007*b*). In some

cases, suppressors appear to spread to fixation and can entirely mask the underlying activity of drive elements, that are revealed through crosses with naïve populations (Dermitzakis et al., 2000; Tao et al., 2001). However, segregating suppressors are not an inevitable feature of drive systems, as they appear to be lacking in a range of species, including *D. pseudoobscura*, *D. neotestacea*, *D. recens* (Dyer et al., 2007; Dyer, 2012), as well as stalk-eyed flies (Reinhold et al., 1999; Wolfenbarger and Wilkinson, 2001; Paczolt et al., 2017).

Another adaptive response is the evolution of increased levels of female multiple mating to reduce the probability that drive sperm fertilise her eggs (Haig and Bergstrom, 1995; Zeh and Zeh, 1997; Wedell, 2013). An experimental evolution investigation in *D. pseudoobscura* found that females evolved higher frequencies of multiple mating when a meiotic driver (that negatively affects sperm competitive ability) was present in the population (Price et al., 2008*b*), and female remating has also been shown to cause driver frequency to fall rapidly in *D. pseudoobscura* (Price et al., 2010) and *Mus musculus* (Manser et al., 2017). There is suggestive data correlating the coincidence of the rate of polyandry and meiotic drive in clines of two *Drosophila* species (Pinzone and Dyer, 2013; Price et al., 2014), however the full extent to which drive influences the rate of multiple mating in natural systems remains to be demonstrated. Chapter 5 shows that female *T. dalmanni* mate to gain direct fertility benefits, but we do not know how SR influences the incidence of polyandry. It appears unlikely that SR promotes polyandry because *T. dalmanni* females can avoid the detrimental fitness cost of mating with a low-fertility male, as Chapters 2 and 3 show that SR males are capable of producing high numbers of sperm. Females may remate to reduce the chances for fertilising her eggs with SR sperm. This would only be effective if SR males are comparatively poor sperm competitors due to factors other than sperm number. This is quite possible, as drive-carrying sperm may be damaged as a by-product of the action of drive (Newton et al., 1976; Nasuda et al., 1998; Price and Wedell, 2008), and furthermore, accessory gland products may have important function in sperm competition (Avila et al., 2015; Perry et al., 2013;

Avila et al., 2011; Fry and Wilkinson, 2004) and SR *T. dalmanni* males have reduced accessory glands (Chapter 3). Females may also evolve mechanisms to avoid mating with a drive male in the first place. In house mice, it is possible that females could avoid mating with males that carry an autosomal drive element *t*, through detecting unique major histocompatibility (MHC) alleles which are physically linked to the *t* haplotype (Silver, 1985; Lindholm et al., 2013). However, evidence for this remains unclear, with contradictory findings (Lindholm and Price, 2016). In stalk-eyed flies, meiotic drive has been linked to small eyespan, which may allow females to avoid mating with carrier males through assessing this trait (Wilkinson et al., 1998*b*; Cotton et al., 2014), and I found further evidence that drive is linked to small eyespan and small body size in Chapters 3 and 4. Perhaps an intriguing comparison that should made is between the two species of *T. dalmanni*. *T. dalmanni* has been split into two reproductively isolated species which are indistinguishable by eye. They are unofficially called "*T. dalmanni*-1", the species used in this thesis, and "*T. dalmanni*-2", a species which does not exhibit strong brood sex ratio distortion (Christianson et al., 2005; Paczolt et al., 2017). Currently differences between these species in the strength of sexual selection and the preponderance of female multiple mating is unknown.

In contrast to other host adaptations studied, increased investment in sperm production will intensify, rather than prevent, the transmission of the driver. Male *T. dalmanni* carrying the X^{SR} drive chromosome produce strongly female-biased broods (Cotton et al., 2014; Paczolt et al., 2017; Chapter 4). This demonstrates the extent of sperm destruction, and is of a strength that is comparable to that observed in other systems (James and Jaenike, 1990; Ardlie and Silver, 1996; Cazemajor et al., 2000; Jaenike, 2001; Tao et al., 2007*a*; Larracuente and Presgraves, 2012; Keais et al., 2017). Despite this, in Chapter 3 I find that SR males are able to achieve high fertility. When mating to a single female over an extended period of 10 hours, both SR and ST males are able to fertilise a high proportion of eggs laid. From Chapter 2, I know that on a single mating SR and ST males deliver equivalent numbers of sperm to females, and

SR males also have equivalent sperm numbers by a third mating. Clearly SR males are able to deliver the numbers of viable sperm required in 10 hours to fertilise most eggs a single female can lay over 14 days. Allowing males access to more females starts to remove female fecundity as a limitation to male fertility, and when mating with five females, males fertilise a smaller proportion of eggs laid. However, I found that SR males perform just as well as ST males in this task. In these chapters I have demonstrated that SR males are able to produce sufficient viable sperm, and succeed at the more challenging task of fertilising most of five females' eggs, despite the destruction of half their sperm.

However, to maximise fertility over a limited time, males must produce sufficient numbers of sperm and mate at a maximal frequency to deliver the sperm. I found a dramatic trade-off between SR male testis and accessory gland size, as SR male accessory glands were small for their already small body size. Previous work shows male mating rate is phenotypically correlated (Baker et al., 2003) and genetically linked (Rogers et al., 2005*a*) with accessory gland size. Accordingly, SR males have been observed to mate at lower frequency than ST males (Wilkinson et al., 2003; S. Finnegan, *unpublished data*). Furthermore, SR males are generally small and have small eyespan for their body size, demonstrated in Chapters 3 and 4. Reduction in secondary sexual ornament size in *T. dalmanni* will additionally impact upon male mating frequency as male eyespan is important in determining success in male-male antagonistic interactions (Panhuis and Wilkinson, 1999; Small et al., 2009), and is a sexual ornament used by females in their mate choice (Panhuis and Wilkinson, 1999; Hingle et al., 2001*a*; Cotton et al., 2010).

These reduced traits are likely to be highly influential in situations where males are under time constraints to mate, and where females are able to choose between males. This is exactly how males are likely to compete in the wild. Males and females gather at lek sites, where males compete for roosting sites. Females assess males and roost with their chosen male overnight, with large eyespan males attracting more females (Wilkinson and Reillo, 1994; Cotton

et al., 2010). Males mate with the females on their root hair over a brief half hour period, where females are also likely to depart if they fail to gain a mating (A. Pomiankowski, *personal observation*). Large attractive males that can mate at high frequency will mate with more females, and these factors will bias mating success toward ST males. Consequently, predictions of drive equilibrium frequency where dive carriers can compensate for their disadvantage though adaptations such as increased investment in sperm production will not be intuitive and must be formally modelled.

X^{SR} in *T. dalmanni* is estimated to be over half a million years old (Paczolt et al., 2017). In addition, X^{SR} is not rare, with a frequency of $\sim$20% across many generations (Wilkinson et al., 2003; Cotton et al., 2014; Paczolt et al., 2017). The long-term persistence of drive at a significant frequency in this lineage seems likely to have created a selective environment favouring adaptive changes in reproductive behaviour in order to tolerate its presence. Other older systems, such as SR in *D. pseudoobscura* which is estimated to be 1 million years old (Kovacevic and Schaeffer, 2000), and the *t* haplotype in house mice which has persisted for over 1.5 million years (Hammer and Silver, 1993), do not present evidence for a similar adaptive response in sperm allocation. In house mice, drive males are poor at sperm competition, as measured by number of offspring sired (Manser et al., 2011; Sutter and Lindholm, 2015), but surprisingly there is no evidence for any alternative reproductive tactics in behaviour or morphology (Sutter and Lindholm, 2016). In *D. pseudoobscura* the sperm of drive carrier males are also poorer in competition relative to the sperm of wildtype males (Price et al., 2008*a*, 2014), and drive males transfer fewer than half the number of sperm in a single mating compared to wildtype males (Price et al., 2008*a*). Why these deleterious effects of drive have not led to an adaptive response is unclear. Both of these haplotypes are associated with inversions (Sturtevant and Dobzhansky, 1936; Hammer et al., 1989). Inversions could attract linked genes through rare recombination events, however genetic exchange may be limited in *D. pseudoobscura* as at least one of three inversions is small (Kovacevic and Schaeffer,

2000). Conversely, the *t* complex comprises of four major, non-overlapping inversions that completely suppresses recombination across the entire length of chromosome 17 (Artzt et al., 1982; Hammer et al., 1989) where many recessive lethal mutations have accumulated, forming distinct haplotypes (Silver, 1985). It is possible that the low frequency and patchy distribution of the *t* complex in wild populations limits selection on linked genes that enable changes to sperm allocation (Ardlie and Silver, 1998). However, in *D. pseudoobscura*, SR exists along a latitudinal cline, reaching high and stable frequencies of up to 30% in its southern reach (Price et al., 2014). Neither of these drive systems is associated with suppressors of drive, as there is no genetic resistance in *D. pseudoobscura* (Policansky and Dempsey, 1978), and suppressors of the *t* complex do not appear to be widespread (Ardlie and Silver, 1996). These patterns suggest that there may only be weak selection for genes controlling compensatory mechanism, either in sperm allocation or suppressors of drive. This further highlights the difficulty in assessing the impact of adaptive responses to drive, which may may depend on a plethora of factors, including population density, population spacial structure and sperm competition intensity.

In this thesis have demonstrated for the first time that drive males can increase the production of viable sperm and maintain fertility despite sperm destruction through investment in testes. This investment in sperm production leads to a trade-off with mating frequency, where drive males suffer due to reduced accessory glands. I additionally show that male drive carriers exhibit reduced eyespan, a trait that plays a vital role in pre-copulatory competition and mating frequency. These patterns fit with theoretical models examining the evolution of male ejaculate allocation that predict that males will invest optimally per ejaculate irrespective of their resources or intrinsic fertility. Furthermore they add to the literature that reports change in testis size as an evolutionary response to sperm competition. Male adaptations to the presence of meiotic drive must be considered in theoretical analyses of the spread and equilibrium frequency of drive. Specifically, how female multiple mating and meiotic drive

interact when drive carrier males compensate for loss of sperm needs both empirical and theoretical treatment. Further work is needed to determine more precisely the ecologically relevant conditions under which drive male fertility is pushed to its limits.

6.4 Future directions

This thesis has explored key questions relating to the evolution of meiotic drive and polyandry. In this section, I identify some important areas that have been highlighted by this research as in need of further study. Future work utilising *T. dalmanni* will now have the advantage of being able to use reliable markers for drive, in particular INDEL marker *comp162710*, which have been described in Chapter 4.

6.4.1 Sperm competition and polyandry in *Teleopsis dalmanni*

In Chapters 2 and 3 I have shown that *T. dalmanni* males that carry drive (SR males) are fully capable of producing large numbers of sperm as they have large testes, and are able to achieve high fertility. However, previous work has demonstrated that SR males are poor sperm competitors compared to standard males (ST), where SR males achieve $\sim$0.25 paternity share (Wilkinson et al., 2006). In the closely related sister species, *T. whitei*, SR males achieve only $\sim$0.1 paternity share (Wilkinson and Fry, 2001), and poor sperm competitive ability of drive males is a recurring theme (*Silene latifornia* (*alba*): Taylor et al., 1999; *D. simulans*: Atlan et al., 2004; Angelard et al., 2008; *D. pseudoobscura*: Price et al., 2008*a*; *Mus musculus domesticus*: Manser et al., 2017). Poor sperm competitive ability may help to explain how drive is maintained at stable frequencies in *T. dalmanni* populations. What is currently unknown is how important this deficit actually is for SR *T. dalmanni* males—what is the risk of sperm competition in the wild? While estimates of male mating frequency in the wild have been reported (Cotton et al., 2015), we only have detailed reports of female mating frequency from laboratory populations. Female *T. dalmanni* in the laboratory

are highly promiscuous, for example Reguera et al. (2004) showed that virgin females housed with three males mated $\sim$5 times per hour (over 1.5 hours), and continued to mate throughout the day. However, mating rates in laboratory populations may vary drastically from the rates of natural ones (Burton-Chellew et al., 2007). In wild *T. dalmanni*, individuals gather in nocturnal lekking aggregations, generally with only one male and multiple females per lek (Cotton et al., 2010, 2015). This suggests then that over this timescale, males may not expect to be in competition with other males' sperm. Indeed, *T. dalmanni* males do not exhibit the classic traits expected for high levels of sperm competition. They produce small spermatophores (Kotrba, 1996) and mating appears to have no influence on female propensity to remate (Harley, 2013) or egg-laying (Reguera et al., 2004), and copulation duration is short and with no advantage to males mating first or second (Corley et al., 2006). However, as Chapter 5 demonstrates, wild females do need to remate over time, and *across* mating sessions females will mate with different males. Females return nightly to mate, but move on average $\sim$1m between aggregation sites, while males tend to return to the same site each night (Wilkinson et al., 1998*b*). Firstly, accurate reports on female mating rates with multiple males in the wild are needed, with particular emphasis on how this affects the risk and intensity of sperm competition. Further, there is currently no data demonstrating how SR male fertility is affected in competition over these more ecologically relevant timescales, and examining this is a necessary next step in order to understand how drive polymorphisms can be maintained in natural populations.

If SR males are worse sperm competitors than ST males, as seems intuitive, then polyandry could be an effective mechanism by which females can avoid fertilising their eggs with X^{SR} sperm (Zeh and Zeh, 1996). Furthermore, polyandry may prevent the invasion of drive through a population (Haig and Bergstrom, 1995; Taylor and Jaenike, 2002; Holman et al., 2015). Across geographical clines of *D. pseudoobscura*, high rates of polyandry are associated with lower frequencies of SR in the population (Price et al., 2014), and in labo-

ratory populations, as little as one single additional mating prevents the spread of SR, while monogamous lines become extinct due to extremely biased sex ratio (Price et al., 2010). How does the frequency of drive across populations vary with female mating rate in *T. dalmanni*? Wilkinson et al. (2003) compared female mating rates in *T. dalmanni* originating from four different populations, one near the Ulu Gombak valley on peninsular Malaysia and three in Indonesia. They did not find any difference in female mating rates between populations, but the populations also showed little variation in drive frequency. Conversely, along the Ulu Gombak valley itself, populations at various stream sites along a distance of $\sim$3.5km are known to differ widely in drive frequency (Cotton et al., 2014). A more precise examination of SR frequency, using an INDEL marker (*comp162710*) described in Chapter 4, and female mating rates along the valley, as well as more distant populations, may elucidate as yet undescribed trends. This should involve both direct observations of leks in the wild, and controlled experiments in the laboratory where aspects of female quality can also be accounted for.

6.4.2 The effects of diet quality and variability on drive

In *T. dalmanni*, both larval and adult diet can have far reaching effects in both males and females. Poor quality larval diet decreases mean and increases the variance of body size and relative eyespan (David et al., 2000; Cotton et al., 2004*b*). Pre-maturity adult diets effect the growth of testis and accessory gland size, and limiting the growth of accessory glands increases the time it takes for males to reach sexual maturity (Baker et al., 2003; Rogers et al., 2008). Furthermore, poor quality diet reduces female fecundity (Hingle et al., 2001*b*). In the wild, diet is likely to be highly variable (Wilkinson and Reillo, 1994; Felton, 1996), as we can infer from the high variability in relative eyespan and female fecundity in wild-caught compared to laboratory flies (Cotton et al., 2014; Meade et al., 2017). Furthermore, *T. whitei* larvae produce an aggressive response towards competitors when larval density is high (de la Motte and Burkhardt, 1983) and this might extend to *T. dalmanni*, though no evidence for this currently exists.

Costs to fertility and viability of drive in *T. dalmanni* have only been tested in laboratory flies experiencing high quality food. Perhaps consequently, little evidence for fertility and viability costs of drive have been uncovered. Viability differences have not been directly examined in this species, but indirect evidence does not suggest that large differences will be found under standard laboratory conditions (Johns et al., 2005; Wilkinson et al., 2006). However, differences are predicted to exist. The drive locus is located within an inversion, which suppresses recombination and will allow the acquisition of deleterious recessive mutations (Wilkinson et al., 2005; Kirkpatrick, 2010; Paczolt et al., 2017). Homozygote viability especially will likely be reduced, as there is infrequent opportunity for selection to act when drive is at low frequency and homozygotes are rare. Furthermore, in Chapter 3 I show that SR males suffer from reduced body size, relative eyespan and accessory gland size. Accessory gland size places a limit on male mating frequency (Baker et al., 2003; Rogers et al., 2005*a*,*b*), and indeed, SR males do seem to mate at lower rates than ST males (Wilkinson et al., 2003; S. Finnegan, *unpublished data*). However, I found no difference between SR and ST male fertility when they had access to five females for over 10 hours, a situation which should allow a high mating rate. Future work should aim to readdress the deficit of testing fertility and viability in ecologically relevant environments, which could include variation in food quality, population density and temperature.

6.5 Conclusion

In this thesis, I have highlighted the need to test for adaptive responses to the negative implications of being a drive carrier. This will change how we understand the evolutionary consequences of meiotic drive systems. Furthermore, the use of testing in more ecologically relevant environments will be vital for future research on both meiotic drive and polyandry. Only then will we be able to fully understand the ecological dynamics of meiotic drive systems, the proximate and ultimate causes of polyandry, and how they interact.

Chapter 7

Appendices

Appendix A

Chapter 2: Supplementary Information

I present model tables and effect size estimates for models reported in Chapter 2.

A.1 Sperm in the spermathecae

A.1.1 Sperm presence in the spermathcea

```
glmer(sperm presence ~ thorax + residual eyespan +
    drive type + (1|id), family = binomial)
```

```
Analysis of Deviance Table (Type II Wald chisquare tests)

Response: sperm.presence
                  Chisq Df Pr(>Chisq)
thorax           0.5220  1     0.4700
residual_eyespan 0.2653  1     0.6065
drive_type       0.7328  1     0.3920

                 Estimate Std. Error
(Intercept)        -0.394      5.425
thorax              1.691      2.341
residual_eyespan   -0.467      0.906
drive_typeST       -0.880      1.028
```

N = 109

A.1.1.1 Sperm presence in the spermathecae, including female size

```
glmer(sperm presence ~ thorax + residual eyespan +
    drive type + female size + (drive type + females size)^2
    + (1|id), family = binomial)
```

```
Analysis of Deviance Table (Type II Wald chisquare tests)

Response: sperm.presence
                Chisq Df Pr(>Chisq)
thorax          0.522  1      0.470
residual_eyespan 0.265  1      0.606
drive_type      0.733  1      0.392

                          Estimate Std. Error
(Intercept)                  9.788     14.431
thorax                       1.415      5.467
residual_eyespan            -0.647      2.136
drive_typeST                -5.788      4.801
female_sizeS                -5.323      4.511
drive_typeST:female_sizeS    8.202      5.211
```

N = 109

A.1.2 Sperm number in the spermathecae

```
glmer(sperm number ~ thorax + residual eyespan +
    drive type + (1|id) + (1 | OLRE), family = poisson)
```

```
Analysis of Deviance Table (Type II Wald chisquare tests)

Response: sperm.number
                Chisq Df Pr(>Chisq)
thorax          1.075  1      0.300
residual_eyespan 1.783  1      0.182
drive_type      0.222  1      0.638

                Estimate Std. Error
(Intercept)        2.624      1.490
thorax             0.641      0.618
residual_eyespan   0.312      0.233
drive_typeST      -0.108      0.229
```

N = 97

A.1.2.1 Sperm number in the spermathecae, including female size

```
glmer(sperm number ~ thorax + residual eyespan + drive type +
    female size + (female size + drive type)^2 + (1|id) +
    (1 | OLRE), family = poisson)
```

```
Analysis of Deviance Table (Type II Wald chisquare tests)

Response: sperm.number
                       Chisq Df Pr(>Chisq)
thorax                 1.184  1      0.277
residual_eyespan       1.615  1      0.204
drive_type             0.267  1      0.605
female_size            0.470  1      0.493
drive_type:female_size 0.071  1      0.789

                          Estimate Std. Error
(Intercept)                  2.448      1.511
thorax                       0.672      0.618
residual_eyespan             0.298      0.234
drive_typeST                -0.062      0.311
female_sizeS                 0.227      0.379
drive_typeST:female_sizeS   -0.121      0.452
```

N = 97

A.2 Sperm in the VR in the early period

A.2.1 Sperm presence in the VR

```
glm(VR sperm presence ~ thorax + residual eyespan +
    drive type + (residual eyespan + drive type)^2,
    family = binomial)
```

```
Analysis of Deviance Table (Type II tests)

Response: VR_sperm_presence
                           LR Chisq Df Pr(>Chisq)
male_thorax                   0.539  1      0.463
residual_eyespan              0.230  1      0.632
drive_type                    1.392  1      0.238
residual_eyespan:drive_type   1.303  1      0.254
```

```
                              Estimate Std. Error
(Intercept)                     -2.359      2.687
male_thorax                      0.783      1.070
residual_eyespan                 0.997      1.150
drive_typeST                     0.280      0.405
residual_eyespan:drive_typeST   -1.390      1.237
```

N = 170

A.2.1.1 Sperm presence in the VR, including female size

```
glm(VR sperm presence ~ thorax + residual eyespan +
    drive type + female size +
    (residual eyespan + drive type)^2, family = binomial)
```

```
Analysis of Deviance Table (Type II tests)

Response: VR_sperm_presence
                           LR Chisq Df Pr(>Chisq)
male_thorax                   0.660  1      0.417
residual_eyespan              0.337  1      0.562
drive_type                    1.633  1      0.201
female_size                   2.776  1      0.096 .
residual_eyespan:drive_type   1.189  1      0.276
---
```

```
                              Estimate Std. Error
(Intercept)                     -2.387      2.702
male_thorax                      0.872      1.078
residual_eyespan                 0.899      1.146
drive_typeST                     0.329      0.408
female_sizeS                    -0.529      0.320
residual_eyespan:drive_typeST   -1.330      1.233
```

N = 170

A.2.2 Proportion of pouches filled in the VR

```
glm(cbind(filled pouch count, empty pouch count) ~ thorax +
    residual eyespan + drive type, family = quasibinomial)
```

```
Analysis of Deviance Table (Type II tests)

Response: cbind(filled_pouch, empty_pouch)
Error estimate based on Pearson residuals

                    SS Df     F Pr(>F)
male_thorax      15.01  1 2.206  0.142
residual_eyespan  6.36  1 0.935  0.337
drive_type        3.31  1 0.486  0.488
Residuals       476.08 70

                Estimate Std. Error
(Intercept)       -5.555      2.528
male_thorax        1.444      0.986
residual_eyespan   0.413      0.427
drive_typeST       0.275      0.403
```

N = 74

A.2.2.1 Proportion of pouches filled in the VR, including female size

```
glm(cbind(filled pouch count, empty pouch count) ~ thorax +
    residual eyespan + drive type + female size,
    family = quasibinomial)
```

```
Analysis of Deviance Table (Type II tests)

Response: cbind(filled_pouch, empty_pouch)
Error estimate based on Pearson residuals

                    SS Df     F Pr(>F)
male_thorax      15.10  1 2.190  0.143
residual_eyespan  6.21  1 0.900  0.346
drive_type        3.40  1 0.494  0.485
female_size       0.10  1 0.015  0.904
Residuals       475.66 69

                Estimate Std. Error
(Intercept)       -5.562      2.545
male_thorax        1.450      0.994
residual_eyespan   0.410      0.431
drive_typeST       0.281      0.408
female_sizeS      -0.038      0.315
```

N = 74

A.3 Sperm in the VR in the late period

A.3.1 Sperm presence in the VR

```
glm(VR sperm presence ~ thorax + residual eyespan +
    drive type + (residual eyespan + drive type)^2,
    family = binomial)
```

```
Analysis of Deviance Table (Type II tests)

Response: VR_sperm_presence
                            LR Chisq Df Pr(>Chisq)
male_thorax                    0.005  1      0.944
residual_eyespan               0.872  1      0.351
drive_type                     0.425  1      0.514
residual_eyespan:drive_type    9.528  1      0.002 **
---
```

```
                             Estimate Std. Error
(Intercept)                     1.277      3.227
male_thorax                    -0.093      1.328
residual_eyespan                1.936      0.993
drive_typeST                    0.076      0.367
residual_eyespan:drive_typeST  -3.412      1.168
```

N = 214

A.3.1.1 Sperm presence in the VR, including female size

```
glm(VR sperm presence ~ thorax + residual eyespan +
    drive type + female size +
    (residual eyespan + drive type)^2, family = binomial)
```

```
Analysis of Deviance Table (Type II tests)

Response: VR_sperm_presence
                            LR Chisq Df Pr(>Chisq)
male_thorax                    0.110  1      0.740
residual_eyespan               0.860  1      0.354
drive_type                     0.450  1      0.502
female_size                    6.404  1      0.011 *
residual_eyespan:drive_type    9.275  1      0.002 **
---
```

```
                              Estimate Std. Error
(Intercept)                     -0.350      3.322
male_thorax                      0.453      1.362
residual_eyespan                 1.968      1.014
drive_typeST                     0.076      0.374
female_sizeS                     0.893      0.367
residual_eyespan:drive_typeST   -3.422      1.187
```

N = 214

A.3.2 Proportion of pouches filled in the VR

```
glm(cbind(filled pouch count, empty pouch count) ~ thorax +
    residual eyespan + drive type, family = quasibinomial)
```

```
Analysis of Deviance Table (Type II tests)

Response: cbind(filled_pouch, empty_pouch)
Error estimate based on Pearson residuals

                     SS  Df     F Pr(>F)
male_thorax        0.06   1 0.015  0.902
residual_eyespan   2.56   1 0.667  0.415
drive_type         1.41   1 0.366  0.546
Residuals        583.64 152

                 Estimate Std. Error
(Intercept)        -1.851      1.404
male_thorax         0.071      0.577
residual_eyespan    0.193      0.237
drive_typeST       -0.095      0.157
```

N = 156

A.3.2.1 Proportion of pouches filled in the VR, including female size

```
glm(cbind(filled pouch count, empty pouch count) ~ thorax +
    residual eyespan + drive type + female size,
    family = quasibinomial)
```

```
Analysis of Deviance Table (Type II tests)
```

```
Response: cbind(filled_pouch, empty_pouch)
Error estimate based on Pearson residuals

                     SS  Df     F Pr(>F)
male_thorax        0.14   1 0.037  0.848
residual_eyespan   2.45   1 0.635  0.427
drive_type         1.37   1 0.355  0.552
female_size        0.62   1 0.161  0.689
Residuals        582.28 151

                 Estimate Std. Error
(Intercept)        -1.978      1.444
male_thorax         0.113      0.588
residual_eyespan    0.189      0.237
drive_typeST       -0.094      0.157
female_sizeS        0.061      0.152
```

N = 156

A.4 Sperm presence in the VR across early and late periods

```
glm(VR sperm presence ~ thorax + residual eyespan +
    drive type + time period, family = binomial)
```

```
Analysis of Deviance Table (Type II tests)

Response: VR_sperm_presence
                 LR Chisq Df Pr(>Chisq)
male_thorax         0.031  1      0.861
residual_eyespan    0.853  1      0.356
drive_type          1.362  1      0.243
time_period        35.469  1     <2e-16 ***
---


                 Estimate Std. Error
(Intercept)        -0.826      1.975
male_thorax         0.140      0.800
residual_eyespan   -0.288      0.312
drive_typeST        0.286      0.245
time_periodLate     1.284      0.221
```

N = 384

A.5 VR pouch number

```
lm(pouch number ~ female size)
```

```
Analysis of Variance Table

Response: pouch_number
             Df  Sum Sq Mean Sq F value    Pr(>F)
female_size   1  2600.1  2600.2  94.224 < 2.2e-16 ***
Residuals   451 12445.6    27.6
---


             Estimate Std. Error
(Intercept)    36.775      0.323
female_sizeS   -4.862      0.501
```

N = 453

A.6 Sperm depletion

A.6.1 Sperm presence in the spermathcea

```
glmer(sperm presence ~ thorax + residual eyespan +
    mating order + drive type + (1|id),
    family = binomial)
```

```
Analysis of Deviance Table (Type II Wald chisquare tests)

Response: sperm_presence
                 Chisq Df Pr(>Chisq)
thorax           0.3537  1     0.5520
residual_eyespan 0.6445  1     0.4221
mating_order     1.7392  1     0.1872
drive_type       0.0874  1     0.7675

                Estimate Std. Error
(Intercept)        5.581      6.667
thorax            -1.664      2.798
residual_eyespan  -1.073      1.337
mating_order       0.496      0.376
drive_typeST       0.328      1.109
```

N = 135

A.6.2 Sperm number in the spermathecae (across sequential matings)

```
glmer(sperm number ~ male thorax + residual eyespan +
    mating order * male type + (1|id) + (1 | OLRE),
    family = poisson)
```

```
Analysis of Deviance Table (Type II Wald chisquare tests)

Response: sperm_number
                       Chisq Df Pr(>Chisq)
thorax                 2.712  1      0.100 .
residual_eyespan       0.003  1      0.959
mating_order           0.158  1      0.691
male_type              1.163  1      0.281
mating_order:male_type 3.200  1      0.074 .
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
                         Estimate Std. Error
(Intercept)                 2.546      1.083
thorax                      0.728      0.442
residual_eyespan            0.010      0.196
mating_order                0.332      0.186
male_typeST                 0.501      0.430
mating_order:male_typeST   -0.369      0.206
```

N = 103

A.6.3 Sperm number in the spermathecae (across all matings)

```
glmer(sperm number ~ thorax + residual eyespan +
    mating order + drive type + (1|id) + (1 | OLRE),
    family = poisson)
```

```
Analysis of Deviance Table (Type II Wald chisquare tests)

Response: sperm_number
                       Chisq Df Pr(>Chisq)
thorax                 1.437  1      0.231
```

```
residual_eyespan        0.125  1      0.723
mating_order            0.509  1      0.475
drive_type              2.440  1      0.118
thorax:residual_eyespan 6.357  1      0.012 *
thorax:drive_type       5.829  1      0.016 *
mating_order:drive_type 3.047  1      0.081 .
---
Signif. codes:  0 ’***’ 0.001 ’**’ 0.01 ’*’ 0.05 ’.’ 0.1 ’ ’ 1
```

	Estimate	Std. Error
(Intercept)	5.735	1.590
thorax	−0.609	0.674
residual_eyespan	−6.270	2.468
mating_order	0.309	0.164
drive_typeST	−4.428	2.023
thorax:residual_eyespan	2.617	1.038
thorax:drive_typeST	2.029	0.840
mating_order:drive_typeST	−0.319	0.183

N = 118

Appendix B

Chapter 3: Supplementary Information

I present model tables and effect size estimates for models reported in Chapter 3.

B.1 SR morphological and reproductive trait size

B.1.1 Body size (thorax)

```
lm(body size^2 ~ genotype)
```

```
Anova Table (Type II tests)

Response: thorax^2
           Sum Sq  Df F value Pr(>F)
genotype    3.686   1   8.745  0.003 **
Residuals 150.490 357
---
```

```
            Estimate Std. Error t value
(Intercept)    5.479      0.042 130.179
genotypeSR    -0.214      0.072  -2.957
```

N = 359

B.1.2 Eyespan

```
lm(eyespan ~ thorax + genotype)
```

```
Anova Table (Type III tests)

Response: eyespan
               Sum Sq  Df F value Pr(>F)
(Intercept)     9.396   1  77.309 <2e-16 ***
thorax         22.642   1 186.296 <2e-16 ***
genotype        0.713   1   5.868  0.016 *
thorax:genotype  0.507   1   4.175  0.042 *
Residuals      43.145 355
---
```

```
                  Estimate Std. Error t value
(Intercept)          3.295      0.375   8.793
thorax               2.186      0.160  13.649
genotypeSR          -1.541      0.636  -2.422
thorax:genotypeSR    0.563      0.275   2.043
```

N = 359

B.1.2.1 Eyespan (absolute)

```
lm(eyespan ~ genotype)
```

```
Anova Table (Type II tests)

Response: eyespan
          Sum Sq  Df F value    Pr(>F)
genotype  10.042   1  42.631 < 2.2e-16 ***
Residuals 84.090 357
---
```

```
            Estimate Std. Error t value
(Intercept)    8.402      0.031 267.072
genotypeSR    -0.354      0.054  -6.529
```

N = 359

B.1.3 Testes

```
lmer(testis ~ females + thorax + resid.eyespan +
     genotype + (1 | batch))
```

```
Analysis of Deviance Table (Type II Wald F tests with Kenward-Roger df)

Response: testis
                   F Df Df.res Pr(>F)
females        4.179  2 274.12  0.016 *
thorax         6.697  1 283.71  0.010 **
resid.eyespan 15.354  1 283.99 <2e-16 ***
genotype      99.982  1 282.78 <2e-16 ***
---


              Estimate Std. Error t value
(Intercept)      0.568      0.327   1.737
females1         0.131      0.047   2.802
females5         0.056      0.047   1.177
thorax           0.358      0.137   2.608
resid.eyespan    0.224      0.057   3.947
genotypeSR       0.409      0.041  10.052
```

N = 290

B.1.3.1 Testes (absolute)

```
lmer(testis ~ females + genotype +  (1 | batch))
```

```
Analysis of Deviance Table (Type II Wald F tests with Kenward-Roger df)

Response: testis
              F Df Df.res Pr(>F)
females   3.937  2 274.53  0.021 *
genotype 73.796  1 280.16 <2e-16 ***
---


            Estimate Std. Error t value
(Intercept)    1.426      0.053  26.792
females1       0.130      0.048   2.703
females5       0.053      0.048   1.093
genotypeSR     0.337      0.039   8.615
```

N = 290

B.1.4 Accessory glands

```
lmer(accessory.gland ~ thorax + resid.eyespan +
    genotype  + (1 | batch))
```

```
Analysis of Deviance Table (Type II Wald F tests with Kenward-Roger df)

Response: accessory.gland
                 F Df Df.res Pr(>F)
thorax       0.693  1 335.63  0.406
resid.eyespan 8.971  1 335.69  0.003 **
genotype      7.801  1 334.03  0.006 **
---

             Estimate Std. Error t value
(Intercept)     0.261      0.101   2.590
thorax          0.036      0.043   0.836
resid.eyespan   0.052      0.017   3.016
genotypeSR     -0.036      0.013  -2.804
```

N = 340

B.1.5 Accessory glands (including diet)

```
lmer(accessory.gland ~ diet + thorax + resid.eyespan +
    genotype  + (1 | batch))
```

```
Analysis of Deviance Table (Type II Wald F tests with Kenward-Roger df)

Response: accessory.gland
                 F Df Df.res Pr(>F)
diet         4.592  1 321.50  0.033 *
thorax       1.098  1 334.48  0.296
resid.eyespan 8.555  1 334.87  0.004 **
genotype     7.349  1 332.69  0.007 **
---

             Estimate Std. Error t value
(Intercept)     0.251      0.100   2.504
dietSucrose    -0.024      0.011  -2.144
thorax          0.045      0.043   1.053
resid.eyespan   0.051      0.017   2.944
genotypeSR     -0.035      0.013  -2.721
```

N = 340

B.1.5.1 Accessory glands (absolute)

```
lmer(accessory.gland ~ genotype  + (1 | batch))
```

```
Analysis of Deviance Table (Type II Wald F tests with Kenward-Roger df)

Response: accessory.gland
              F Df Df.res    Pr(>F)
genotype 16.353  1 335.36 < 2.2e-16 ***
---


            Estimate Std. Error t value
(Intercept)    0.349      0.011  31.371
genotypeSR    -0.049      0.012  -4.059
```

N = 340

B.1.5.2 Accessory glands (absolute, including diet)

```
lmer(accessory.gland ~ diet + genotype  + (1 | batch))
```

```
Analysis of Deviance Table (Type II Wald F tests with Kenward-Roger df)

Response: accessory.gland
              F Df Df.res Pr(>F)
diet      4.727  1 323.24   0.03 *
genotype 15.789  1 334.08 <2e-16 ***
---


            Estimate Std. Error t value
(Intercept)    0.361      0.012  28.894
dietSucrose   -0.024      0.011  -2.175
genotypeSR    -0.048      0.012  -3.988
```

N = 340

B.1.6 Fecundity

```
glmer(total.eggs ~ females + thorax + resid.eyespan +
    resid.accessory.gland + genotype +
    resid.eyespan:resid.accessory.gland + (1 |OLRE) +
    (1 | batch))
```

```
Analysis of Deviance Table (Type III Wald chisquare tests)

Response: fecundity
                                     Chisq Df Pr(>Chisq)
(Intercept)                       3926.294  1     <2e-16 ***
females                             70.210  1     <2e-16 ***
thorax                               0.130  1      0.719
resid.eyespan                        0.007  1      0.934
resid.accessory.gland                0.048  1      0.826
resid.eyespan:resid.accessory.gland  3.995  1      0.046 *
---

                                   Estimate Std. Error z value
(Intercept)                           4.369      0.070  62.660
females5                              0.717      0.086   8.379
thorax                               -0.018      0.049  -0.360
resid.eyespan                        -0.004      0.048  -0.082
resid.accessory.gland                 0.010      0.045   0.219
resid.eyespan:resid.accessory.gland   0.081      0.041   1.999
```

N = 199

B.2 SR fertility

B.2.1 Total fertility

```
glmer(fertility ~ females + thorax + resid.eyespan +
    genotype + females * genotype + (1 | batch) +
    (1 | OLRE))
```

```
Analysis of Deviance Table (Type II Wald chisquare tests)

Response: fertility
                 Chisq Df Pr(>Chisq)
females         43.698  1     <2e-16 ***
thorax           0.688  1      0.407
```

```
resid.eyespan     1.439  1      0.230
genotype          2.416  1      0.120
females:genotype  0.591  1      0.442
---
```

```
                    Estimate Std. Error z value
(Intercept)            3.893      0.106  36.880
females5               0.853      0.144   5.928
thorax                 0.051      0.061   0.830
resid.eyespan          0.076      0.063   1.199
genotypeSR             0.316      0.190   1.666
females5:genotypeSR   -0.201      0.262  -0.769
```

N = 215

B.2.2 Proportion fertility

```
glmer(cbind(fert, unfert) ~ females + thorax +
    resid.eyespan + genotype + females * genotype +
    (1|batch) + (1|OLRE))
```

```
Analysis of Deviance Table (Type II Wald chisquare tests)

Response: cbind(fertility, fecundity - fertility)
                Chisq Df Pr(>Chisq)
females         6.021  1      0.014 *
thorax          1.268  1      0.260
resid.eyespan   0.017  1      0.895
genotype        2.469  1      0.116
females:genotype 1.377  1      0.241
---
```

```
                    Estimate Std. Error z value
(Intercept)            1.759      0.195   9.006
females5              -0.345      0.249  -1.388
thorax                 0.130      0.115   1.126
resid.eyespan          0.015      0.112   0.132
genotypeSR             0.673      0.345   1.950
females5:genotypeSR   -0.532      0.453  -1.173
```

N = 215

B.2.3 Fecundity

```
glmer(total.eggs ~ females + thorax + resid.eyespan +
    genotype + females * genotype + (1 |OLRE) +
    (1 | batch))
```

```
Analysis of Deviance Table (Type II Wald chisquare tests)

Response: fecundity
                  Chisq Df Pr(>Chisq)
females          78.719  1     <2e-16 ***
thorax            0.297  1      0.586
resid.eyespan     1.562  1      0.211
genotype          1.079  1      0.299
females:genotype  0.096  1      0.757
---
```

```
                     Estimate Std. Error z value
(Intercept)             4.303      0.075  57.269
females5                0.776      0.103   7.562
thorax                  0.024      0.044   0.545
resid.eyespan           0.057      0.045   1.250
genotypeSR              0.132      0.136   0.975
females5:genotypeSR    -0.058      0.187  -0.310
```

N = 215

B.3 Reproductive trait size and fertility

B.3.1 Total fertility

```
glmer(fertility ~ females + thorax + resid.eyespan +
    resid.testis + resid.accessory.gland +
    resid.eyespan:resid.accessory.gland + (1 | batch) +
    (1 | OLRE))
```

```
Analysis of Deviance Table (Type III Wald chisquare tests)

Response: fertility
                                        Chisq Df Pr(>Chisq)
```

```
(Intercept)                          2188.421  1     <2e-16 ***
females                                43.547  1     <2e-16 ***
thorax                                  0.509  1      0.475
resid.eyespan                           0.208  1      0.648
resid.testis                            6.216  1      0.013 *
resid.accessory.gland                   0.032  1      0.858
resid.eyespan:resid.accessory.gland     7.133  1      0.008 **
---
```

```
                                   Estimate Std. Error z value
(Intercept)                           4.096      0.088  46.781
females5                              0.762      0.115   6.599
thorax                                0.046      0.064   0.714
resid.eyespan                         0.029      0.063   0.456
resid.testis                          0.148      0.059   2.493
resid.accessory.gland                 0.011      0.060   0.179
resid.eyespan:resid.accessory.gland   0.165      0.062   2.671
```

N = 165

B.3.2 Total fertility: testis and genotype

```
glmer(fertility ~ females + thorax + resid.eyespan +
    resid.testis * genotype + (1 | batch) + (1 | OLRE))
```

```
Analysis of Deviance Table (Type III Wald chisquare tests)

Response: fertility
                        Chisq Df Pr(>Chisq)
(Intercept)          1483.845  1     <2e-16 ***
females                43.563  1     <2e-16 ***
thorax                  0.068  1      0.794
resid.eyespan           0.008  1      0.927
resid.testis            4.159  1      0.041 *
genotype                0.018  1      0.895
resid.testis:genotype   0.164  1      0.686
---
```

```
                      Estimate Std. Error z value
(Intercept)              4.119      0.107  38.521
females5                 0.778      0.118   6.600
thorax                   0.017      0.065   0.261
resid.eyespan           -0.007      0.075  -0.091
```

```
resid.testis                0.203      0.099   2.039
genotypeSR                 -0.023      0.175  -0.132
resid.testis:genotypeSR    -0.056      0.138  -0.404
```

N = 173

B.3.3 Proportion fertility

```
glmer(cbind(fert, unfert) ~ females + thorax +
    resid.eyespan + resid.testis +
    resid.eyespan:resid.testis +  (1|batch) + (1|OLRE))
```

```
Analysis of Deviance Table (Type III Wald chisquare tests)

Response: cbind(fertility, fecundity - fertility)
                            Chisq Df Pr(>Chisq)
(Intercept)                205.835  1     <2e-16 ***
females                      9.620  1      0.002 **
thorax                       1.816  1      0.178
resid.eyespan                0.724  1      0.395
resid.testis                16.646  1     <2e-16 ***
resid.accessory.gland        0.160  1      0.689
resid.eyespan:resid.testis   3.867  1      0.049 *
---
```

```
                           Estimate Std. Error z value
(Intercept)                   2.156      0.150  14.347
females5                     -0.601      0.194  -3.102
thorax                        0.138      0.102   1.348
resid.eyespan                -0.093      0.109  -0.851
resid.testis                  0.421      0.103   4.080
resid.accessory.gland         0.041      0.103   0.400
resid.eyespan:resid.testis    0.222      0.113   1.966
```

N = 165

B.3.4 Proportion fertility: testis and genotype

```
glmer(cbind(fert, unfert) ~ females + thorax +
    resid.eyespan + resid.testis * genotype +  (1|batch) +
    (1|OLRE))
```

```
Analysis of Deviance Table (Type III Wald chisquare tests)

Response: cbind(fertility, fecundity - fertility)
                            Chisq Df Pr(>Chisq)
(Intercept)                133.484  1     <2e-16 ***
females                      6.268  1      0.012 *
thorax                       2.421  1      0.120
resid.eyespan                2.674  1      0.102
resid.testis                12.198  1     <2e-16 ***
genotype                     0.260  1      0.610
resid.eyespan:resid.testis   4.889  1      0.027 *
resid.testis:genotype        0.617  1      0.432
---


                           Estimate Std. Error z value
(Intercept)                   2.141      0.185  11.554
females5                     -0.497      0.199  -2.504
thorax                        0.162      0.104   1.556
resid.eyespan                -0.200      0.122  -1.635
resid.testis                  0.612      0.175   3.493
genotypeSR                   -0.148      0.290  -0.510
resid.eyespan:resid.testis    0.257      0.116   2.211
resid.testis:genotypeSR      -0.187      0.238  -0.786
```

N = 173

Appendix C

Chapter 3: Extended Methods

Experimental males described in Chapter 3 were additionally subject to a post-maturity dietary stress treatment, using an established food manipulation (Rogers et al., 2008; Cotton et al., 2015) with either low or high protein content. Previous work has established that SR males are not limited in sperm number across multiple matings (Chapter 2) suggesting SR males are able to compensate for failed spermatogenesis of half their sperm. Furthermore, low quality diet may increase the extremity of any trade-off as resources become more limiting. It is already known in this study species that a low protein diet reduces accessory gland size (Baker et al., 2003; Rogers et al., 2008) and increases the time taken for males to reach sexual maturity because its impact is to stunt the growth rate of accessory glands (Baker et al., 2003). Accordingly, males subjected to dietary (protein reduction) stress may have reduced accessory gland size.

C.1 Diet and mating treatments

From day 1 to day 14 males were additionally fed one of two diets, *high* or *low*. These diets had no impact on male fertility and are not reported on the main text. After stepwise removal of variables bassed in AIC diet only remains in models predicting accessory gland size (see Appendix B). The *high* diet was made from puréed sweetcorn. The *low* diet was a sucrose food deficient in protein, consisting of 25% sugar solution with 3% carboxymethylcellulose (Rogers et al., 2008).

Carboxymethylcellulose is an indigestible starch used to give the sucrose solution the viscosity of puréed sweetcorn. On starting the treatment males were all > 6 weeks old and so had reached sexual maturity (Baker et al., 2003). Consequently the diet treatments could not alter male ornaments—eyespan—or thorax length which are dependant on resources gained during the larval stage and fixed soon after eclosion (David et al., 1998, 2000), and could not affect the maturation of primary sexual organs (testes and accessory glands) which gain maximum size by $\sim$5 weeks on an unrestricted diet (Baker et al., 2003; Rogers et al., 2008).

As described in the main text, cages were set up in 16 batches (Two 12 L cages per batch, one *high* and one *low*) over a period of 46 days, and each cage contained 15 – 20 males with females $\sim$1:1 ratio. Cages were provided ad libitum amounts of one of the two diet treatments. Females were switched between the *high* and *low* treatment cage pairs on day 6. This minimised any effects of diet on female mating behaviour and ensured that within each pair of cages, males had prior exposure to the same set of females. Females were removed and discarded on day 12.

On the evening of day 14, experimental males were divided into three groups (unmated, *x1* and *x5*), as described in the main text, and were allowed to mate and/or were dissected.

Appendix D

Chapter 4: Supplementary Information

I present model tables and effect size estimates for models reported in Chapter 4.

D.1 Wild males

D.1.1 Brood sex-ratio and allele size in wild samples

D.1.1.1 *ms395*

```
glm(cbind(female offspring, male offspring) ~
    ms395 allele size, family = quasibinomial)
```

```
                   Df Deviance Resid. Df Resid. Dev      F    Pr(>F)
NULL                                  115     836.92
ms395_allele_size_n  5   248.52       110     588.40 10.238 < 2.2e-16 ***
---
```

```
                              Estimate Std. Error
(Intercept)                      0.175      0.088
ms395_allele_size_n(200,210]    -0.093      0.135
ms395_allele_size_n(210,220]    -0.107      0.301
ms395_allele_size_n(220,230]     0.178      0.571
ms395_allele_size_n(230,240]     2.446      1.144
ms395_allele_size_n(240,250]     1.741      0.333
```

D.1.1.2 *comp162710*

```
glm(cbind(female offspring, male offspring) ~
    comp162710 allele size, family = quasibinomial)
```

```
                      Df Deviance Resid. Df Resid. Dev      F    Pr(>F)
NULL                                     85     815.16
comp162710_allele_size  1   345.78        84     469.38 61.523 < 2.2e-16

NULL
comp162710_allele_size ***
---

                            Estimate Std. Error
(Intercept)                    0.095      0.074
comp162710_allele_sizeSmall    2.167      0.350
```

D.1.1.3 *cnv395*

```
glm(cbind(female offspring, male offspring) ~
    cnv395 allele size, family = quasibinomial)
```

```
                  Df Deviance Resid. Df Resid. Dev      F    Pr(>F)
NULL                                 87     814.99
cnv395_allele_size  1    197.4        86     617.59 31.274 < 2.2e-16 ***
---

                        Estimate Std. Error
(Intercept)                0.073      0.083
cnv395_allele_sizeSmall    1.100      0.208
```

D.1.1.4 *cnv125*

```
glm(cbind(female offspring, male offspring) ~
    cnv125 allele size, family = quasibinomial)
```

```
                  Df Deviance Resid. Df Resid. Dev    F Pr(>F)
NULL                                 71     706.73
cnv125_allele_size  1   67.122        70     639.61 8.98  0.004 **
---

                        Estimate Std. Error
(Intercept)                0.129      0.110
cnv125_allele_sizeSmall    0.567      0.191
```

D.1.2 Male morphology, allele size and brood sex-ratio in wild samples

D.1.2.1 Brood sex-ratio and thorax and eyespan

```
glm(cbind(female offspring, male offspring) ~ thorax +
    residual eyespan, family = quasibinomial)
```

```
                 Df Deviance Resid. Df Resid. Dev     F Pr(>F)
NULL                                129     989.24
thorax            1    1.369       128     987.87 0.225  0.636
residual_eyespan  1    6.952       127     980.92 1.141  0.288
```

```
                 Estimate Std. Error
(Intercept)         0.146      0.276
thorax              0.074      0.152
residual_eyespan   -0.074      0.070
```

D.1.2.2 *ms395* and body size

Thorax

```
lmer(thorax ~ ms395 allele size + (1|stream))
```

```
Analysis of Deviance Table (Type II Wald chisquare tests)

Response: thorax
                  Chisq Df Pr(>Chisq)
ms395_allele_size 0.003  1      0.958
```

```
                       Estimate Std. Error
(Intercept)               1.973      0.129
ms395_allele_sizeSmall   -0.005      0.087
```

N = 116

Absolute eyespan

```
lmer(eyespan ~ ms395 allele size + (1|stream))
```

```
Analysis of Deviance Table (Type II Wald chisquare tests)

Response: eyespan
                  Chisq Df Pr(>Chisq)
ms395_allele_size 0.335  1      0.563
```

```
                       Estimate Std. Error
(Intercept)               7.180      0.309
ms395_allele_sizeSmall    0.157      0.272
```

N = 116

Relative eyespan

```
lmer(residual eyespan ~ ms395 allele size + (1|stream))
```

```
Analysis of Deviance Table (Type II Wald chisquare tests)

Response: residual_eyespan
                  Chisq Df Pr(>Chisq)
ms395_allele_size 0.769  1       0.38

                       Estimate Std. Error
(Intercept)              -0.173      0.264
ms395_allele_sizeSmall    0.172      0.196
```

N = 116

D.1.2.3 *comp162710* and body size

Thorax

```
lmer(thorax ~ cnv395 allele size + (1|stream))
```

```
Analysis of Deviance Table (Type II Wald chisquare tests)

Response: thorax
                       Chisq Df Pr(>Chisq)
comp162710_allele_size 0.834  1      0.361

                            Estimate Std. Error
(Intercept)                    1.758      0.047
comp162710_allele_sizeSmall   -0.140      0.154
```

N = 86

Absolute eyespan

```
lmer(eyespan ~ comp162710 allele size + (1|stream))
```

```
Analysis of Deviance Table (Type II Wald chisquare tests)

Response: eyespan
                       Chisq Df Pr(>Chisq)
comp162710_allele_size 2.086  1      0.149

                            Estimate Std. Error
(Intercept)                    7.184      0.131
comp162710_allele_sizeSmall   -0.620      0.429
```

N = 86

Relative eyespan

```
lmer(residual eyespan ~ comp162710 allele size + (1|stream))
```

```
Analysis of Deviance Table (Type II Wald chisquare tests)

Response: residual_eyespan
                       Chisq Df Pr(>Chisq)
comp162710_allele_size 1.128  1      0.288

                            Estimate Std. Error
(Intercept)                    0.192      0.097
comp162710_allele_sizeSmall   -0.337      0.317
```

N = 86

D.1.2.4 *cnv395* and body size

Thorax

```
lmer(thorax ~ cnv395 allele size + (1|stream))
```

```
Analysis of Deviance Table (Type II Wald chisquare tests)

Response: thorax
                   Chisq Df Pr(>Chisq)
cnv395_allele_size 0.246  1       0.62

                        Estimate Std. Error
(Intercept)                1.629      0.094
cnv395_allele_sizeSmall    0.061      0.123
```

N = 88

Absolute eyespan

```
lmer(eyespan ~ cnv395 allele size + (1|stream))
```

```
Analysis of Deviance Table (Type II Wald chisquare tests)

Response: eyespan
                   Chisq Df Pr(>Chisq)
cnv395_allele_size 0.301  1      0.583

                        Estimate Std. Error
(Intercept)                6.971      0.269
cnv395_allele_sizeSmall   -0.197      0.359
```

N = 88

Relative eyespan

```
lmer(residual eyespan ~ cnv395 allele size + (1|stream))
```

```
Analysis of Deviance Table (Type II Wald chisquare tests)

Response: residual_eyespan
                   Chisq Df Pr(>Chisq)
cnv395_allele_size 1.309  1      0.253

                        Estimate Std. Error
(Intercept)                0.254      0.288
cnv395_allele_sizeSmall   -0.293      0.256
```

N = 88

D.1.2.5 *cnv125* and body size

Thorax

```
lmer(thorax ~ cnv125 allele size + (1|stream))
```

```
Analysis of Deviance Table (Type II Wald chisquare tests)

Response: thorax
                   Chisq Df Pr(>Chisq)
cnv125_allele_size 0.154  1      0.694
```

```
                        Estimate Std. Error
(Intercept)                1.729      0.082
cnv125_allele_sizeSmall    0.042      0.108
```

N = 72

Absolute eyespan

```
lmer(eyespan ~ cnv125 allele size + (1|stream)
```

```
Analysis of Deviance Table (Type II Wald chisquare tests)

Response: eyespan
                   Chisq Df Pr(>Chisq)
cnv125_allele_size 0.251  1      0.617
```

```
                        Estimate Std. Error
(Intercept)                6.749      0.391
cnv125_allele_sizeSmall    0.146      0.292
```

N = 72

Relative eyespan

```
lmer(residual eyespan ~ cnv125 allele size + (1|stream))
```

```
Analysis of Deviance Table (Type II Wald chisquare tests)

Response: residual_eyespan
                   Chisq Df Pr(>Chisq)
cnv125_allele_size 0.111  1      0.739
```

```
                        Estimate Std. Error
(Intercept)               -0.096      0.368
cnv125_allele_sizeSmall    0.067      0.203
```

N = 72

D.2 Laboratory males

D.2.1 Brood sex-ratio and allele size in laboratory samples

D.2.1.1 *ms395*

```
glm(cbind(female offspring, male offspring) ~
    ms395 allele size, family = quasibinomial)
```

```
                  Df Deviance Resid. Df Resid. Dev      F    Pr(>F)
NULL                                 178    1429.64
ms395_allele_size_n  3   624.45       175     805.19 48.076 < 2.2e-16 ***
---
```

```
                             Estimate Std. Error
(Intercept)                     0.279      0.051
ms395_allele_size_n(200,210]   -0.080      0.084
ms395_allele_size_n(220,230]    2.665      1.234
ms395_allele_size_n(240,250]    2.849      0.385
```

D.2.1.2 *comp162710*

```
glm(cbind(female offspring, male offspring) ~ ]
    comp162710 allele size, family = quasibinomial)
```

```
                     Df Deviance Resid. Df Resid. Dev      F    Pr(>F)
NULL                                   195    2288.24
comp162710_allele_size  1   1619.4       194     668.82 401.35 < 2.2e-16

NULL
comp162710_allele_size ***
---
```

```
                            Estimate Std. Error
(Intercept)                    0.203      0.039
comp162710_allele_sizeSmall    2.909      0.216
```

D.2.1.3 *cnv395*

```
glm(cbind(female offspring, male offspring) ~
    cnv395 allele size, family = quasibinomial)
```

```
                  Df Deviance Resid. Df Resid. Dev      F    Pr(>F)
NULL                                 186    1652.34
cnv395_allele_size  1    771.5       185     880.85 150.18 < 2.2e-16 ***
---
```

```
                        Estimate Std. Error
(Intercept)                0.285      0.046
cnv395_allele_sizeSmall    2.245      0.241
```

D.2.1.4 *cnv125*

```
glm(cbind(female offspring, male offspring) ~
    cnv125 allele size, family = quasibinomial)
```

```
                  Df Deviance Resid. Df Resid. Dev     F Pr(>F)
NULL                                114     1218.0
cnv125_allele_size  1   13.151       113     1204.9 1.614  0.207
```

```
                        Estimate Std. Error
(Intercept)                0.389      0.150
cnv125_allele_sizeSmall    0.219      0.172
```

D.2.2 Male morphology, allele size and brood sex-ratio in laboratory samples

D.2.2.1 Brood sex-ratio and thorax and eyespan

```
glm(cbind(female offspring, male offspring) ~
    thorax +
    residual eyespan, family = quasibinomial)
```

```
                Df Deviance Resid. Df Resid. Dev     F Pr(>F)
NULL                              635      12157
thorax           1   60.233       634      12096 4.250  0.040 *
residual_eyespan 1   27.317       633      12069 1.928  0.166
---
```

```
                Estimate Std. Error
(Intercept)        1.957      0.607
thorax            -0.527      0.247
residual_eyespan  -0.133      0.096
```

D.2.2.2 *ms395* and body size

Thorax

```
lm(thorax ~ ms395 allele size)
```

```
Analysis of Variance Table

Response: thorax
                   Df Sum Sq Mean Sq F value Pr(>F)
ms395_allele_size   1  0.044   0.044    2.01  0.158
Residuals         177  3.850   0.022

                       Estimate Std. Error
(Intercept)               2.455      0.016
ms395_allele_sizeSmall    0.031      0.022
```

Absolute eyespan

```
lm(eyespan ~ ms395 allele size)
```

```
Analysis of Variance Table

Response: eyespan
                   Df Sum Sq Mean Sq F value Pr(>F)
ms395_allele_size   1  0.654   0.654   1.592  0.209
Residuals         177 72.774   0.411

                       Estimate Std. Error
(Intercept)               8.215      0.071
ms395_allele_sizeSmall    0.121      0.096
```

Relative eyespan

```
lm(residual eyespan ~ ms395 allele size)
```

```
Analysis of Variance Table

Response: residual_eyespan
                   Df Sum Sq Mean Sq F value Pr(>F)
ms395_allele_size   1  0.008   0.008    0.05  0.823
Residuals         177 26.804   0.151

                       Estimate Std. Error
(Intercept)              -0.024      0.043
ms395_allele_sizeSmall    0.013      0.058
```

D.2.2.3 *comp162710* and body size

Thorax

```
lm(thorax ~ cnv395 allele size)
```

```
Analysis of Variance Table

Response: thorax
                        Df Sum Sq Mean Sq F value Pr(>F)
comp162710_allele_size   1  0.017   0.017   0.811  0.369
Residuals              194  4.181   0.022

                            Estimate Std. Error
(Intercept)                    2.471      0.011
comp162710_allele_sizeSmall   -0.027      0.030
```

Absolute eyespan

```
lm(eyespan ~ comp162710 allele size)
```

```
Analysis of Variance Table

Response: eyespan
                        Df Sum Sq Mean Sq F value Pr(>F)
comp162710_allele_size   1  2.366   2.366   5.678  0.018 *
Residuals              194 80.828   0.417
---

                            Estimate Std. Error
(Intercept)                    8.287       0.05
comp162710_allele_sizeSmall   -0.309       0.13
```

Relative eyespan

```
lm(residual eyespan ~ comp162710 allele size)
```

```
Analysis of Variance Table

Response: residual_eyespan
                        Df Sum Sq Mean Sq F value Pr(>F)
comp162710_allele_size   1  1.171   1.171   7.987  0.005 **
Residuals              194 28.432   0.147
---

                            Estimate Std. Error
(Intercept)                   -0.005      0.030
comp162710_allele_sizeSmall   -0.218      0.077
```

D.2.2.4 *cnv395* and body size

Thorax

```
lm(thorax ~ cnv395 allele size)
```

```
Analysis of Variance Table

Response: thorax
                    Df Sum Sq Mean Sq F value Pr(>F)
cnv395_allele_size   1  0.005   0.005   0.229  0.633
Residuals          185  3.668   0.020

                         Estimate Std. Error
(Intercept)                 2.468      0.011
cnv395_allele_sizeSmall     0.015      0.031
```

Absolute eyespan

```
lm(eyespan ~ cnv395 allele size)
```

```
Analysis of Variance Table

Response: eyespan
                    Df Sum Sq Mean Sq F value Pr(>F)
cnv395_allele_size   1  0.323   0.323   0.835  0.362
Residuals          185 71.471   0.386

                         Estimate Std. Error
(Intercept)                 8.292      0.049
cnv395_allele_sizeSmall    -0.124      0.136
```

Relative eyespan

```
lm(residual eyespan ~ cnv395 allele size)
```

```
Analysis of Variance Table

Response: residual_eyespan
                    Df Sum Sq Mean Sq F value Pr(>F)
cnv395_allele_size   1  0.641   0.641    4.24  0.041 *
Residuals          185 27.965   0.151
---

                         Estimate Std. Error
(Intercept)                 0.010      0.030
cnv395_allele_sizeSmall    -0.175      0.085
```

D.2.2.5 *cnv125* and body size

Thorax

```
lm(thorax ~ cnv125 allele size)
```

```
Analysis of Variance Table

Response: thorax
                    Df Sum Sq Mean Sq F value Pr(>F)
cnv125_allele_size   1  0.125   0.125   6.956   0.01 **
Residuals          113  2.038   0.018
---


                         Estimate Std. Error
(Intercept)                 2.552      0.023
cnv125_allele_sizeSmall    -0.072      0.027
```

Absolute eyespan

```
lm(eyespan ~ cnv125 allele size)
```

```
Analysis of Variance Table

Response: eyespan
                    Df Sum Sq Mean Sq F value Pr(>F)
cnv125_allele_size   1  1.433   1.433   4.206  0.043 *
Residuals          113 38.496   0.341
---


                         Estimate Std. Error
(Intercept)                 8.535      0.099
cnv125_allele_sizeSmall    -0.243      0.118
```

Relative eyespan

```
lm(residual eyespan ~ cnv125 allele size)
```

```
Analysis of Variance Table

Response: residual_eyespan
                    Df Sum Sq Mean Sq F value Pr(>F)
cnv125_allele_size   1  0.001   0.001   0.005  0.945
Residuals          113 15.165   0.134
```

	Estimate	Std. Error
(Intercept)	-0.037	0.062
cnv125_allele_sizeSmall	0.005	0.074

Appendix E

Chapter 5: Supplementary Information

I present model tables and effect size estimates for models reported in Chapter 5.

E.1 Experiment 1: Gains from an additional mating

E.1.1 Variation in fecundity

E.1.1.1 Variation in fecundity over time prior to an additional mating

```
model1 = glmer(Fecundity ~ Day +(1|Stream) +
    (1|Female.ID) + (1|OLRE), data = Before, family = poisson)
```

Model comparison:

	χ^2	P
Stream	2.8652	0.09051
Female ID	5.3291	0.02097
Day	1.1815	0.277

Fixed effects:

```
             Estimate Std.Error z.value      P
(Intercept)  0.84950   0.61180   1.389 0.1650
Day         -0.06627   0.06132  -1.081 0.2798
```

Random effects:

```
           Variance
Female.ID   0.6073
Stream      0.4624
```

Sample size:

```
Observations    Female.ID       Stream
         144           36           11
```

E.1.1.2 Variation in fecundity over time after an additional mating

```
model1 = glmer(Fecundity ~ Day + (1|Stream) +
    (1|Female.ID) + (1|OLRE), data = After, family = poisson)
```

Model comparison:

	χ^2	P
Stream	0.0676	0.7948
Female ID	24.5018	7.424×10^{-7}
Day	4.9927	0.02545

Fixed effects:

```
             Estimate Std.Error z.value      P
(Intercept)   2.5340   1.06000   2.392 0.01678
Day          -0.1291   0.05722  -2.257 0.02402
```

Random effects:

```
          Variance
Female.ID   1.9174
Stream      0.1141
```

Sample size:

```
Observations    Female.ID       Stream
         144           36           11
```

E.1.1.3 Variation in fecundity across all days

```
model1 = glmer(Fecundity ~ Day + (1|Stream) +
    (1|Female.ID) + (1|OLRE), data = All, family = poisson)
```

Model comparison:

	χ^2	P
Day	1.2586	0.2619

Fixed effects:

```
            Estimate Std.Error z.value          P
(Intercept)   0.5232 0.0007437  703.50  0.000e+00
Day          -0.0218 0.0007472  -29.18 4.002e-187
```

Random effects:

```
          Variance
Female.ID   1.2165
Stream      0.2680
```

Sample size:

```
Observations    Female.ID       Stream
         288           36           11
```

E.1.1.4 Total individual fecundity before and after an additional mating

```
model1 = glmer(TotFecundity ~ Group + (1|Stream) +
    (1|Female.ID) + (1|OLRE), data = Total, family = poisson)
```

Model comparison:

	χ^2	P
Group (B/A)	0.1001	0.7517

Fixed effects:

```
             Estimate Std.Error z.value        P
(Intercept)   2.21300    0.2705  8.1810 2.809e-16
Group2.After -0.05855    0.1845 -0.3173 7.510e-01
```

Random effects:

```
          Variance
Female.ID   0.8604
Stream      0.3275
```

Sample size:

```
Observations    Female.ID       Stream
          72           36           11
```

E.1.1.5 Total individual fecundity between day 12 and day 15

```
model1 = glmer(Fecundity ~ Group + (1|Stream) +
    (1|Female.ID) + (1|OLRE), data = Day12v15, family = poisson)
```

Model comparison:

	χ^2	P
Group (12/15)	2.4907	0.1145

Fixed effects:

```
            Estimate Std.Error z.value      P
(Intercept)   0.1171    0.4740   0.247 0.8049
GroupDay15    0.3696    0.2298   1.608 0.1078
```

Random effects:

```
          Variance
Female.ID   4.2621
Stream      0.2425
```

Sample size:

```
Observations    Female.ID       Stream
          72           36           11
```

E.1.2 Variation in fertility

E.1.2.1 Variation in total fertility with total fecundity prior to an additional mating

```
model1 = glmer(TotFertility ~ TotFecundity + (1|Stream) +
    (1|OLRE), data =family = poisson)
```

Model comparison:

	χ^2	P
TotFecundity	5.9894	0.01439

Fixed effects:

```
            Estimate Std.Error z.value      P
```

```
(Intercept)  -0.10130    0.50230 -0.2016 0.84020
TotFecundity  0.03429    0.01489  2.3030 0.02126
```

Random effects:

```
       Variance
Stream   0.7826
```

Sample size:

```
Observations       Stream
          36           11
```

E.1.2.2 Variation in total fertility with total fecundity after an additional mating

```
model1 = glmer(TotFertility ~ TotFecundity + (1|Stream) +
    (1|OLRE), TotalAfter, family = poisson)
```

Model comparison:

	χ^2	P
TotFecundity	22.6367	1.957×10^{-6}

Fixed effects:

```
             Estimate Std.Error z.value        P
(Intercept)   0.31830  0.390100   0.816 4.145e-01
TotFecundity  0.05308  0.009147   5.803 6.497e-09
```

Random effects:

```
       Variance
Stream    0.453
```

Sample size:

```
Observations       Stream
          32           11
```

E.1.2.3 Variation in fertility over time prior to an additional mating

```
model1 = glmer(Fertility ~ Day + (1|Stream) +
    (1|Female.ID) + (1|OLRE), data = Before, family = poisson)
```

Model comparison:

	Chi Sq	P
Stream	5.8958	0.01518
Female ID	5.7493	0.0165
Day	8.4502	0.00365

Fixed effects:

```
            Estimate Std.Error z.value        P
(Intercept)    1.164   0.57970   2.008 0.044670
Day           -0.168   0.05626  -2.986 0.002828
```

Random effects:

```
          Variance
Female.ID   0.5756
Stream      0.7800
```

Sample size:

```
Observations    Female.ID       Stream
          84           36           11
```

E.1.2.4 Variation in proportion fertility over time prior to an additional mating

```
model1 = glmer(Prop.Fertility ~ Day  + (1|Stream) +
    (1|Female.ID) + (1|OLRE), data = Before, family = binomial)
```

Model comparison:

	χ^2	P
Stream	4.3233	0.03759
Female ID	20.5766	5.729×10^{-6}
Day	17.5402	2.813×10^{-5}

Fixed effects:

```
            Estimate Std.Error z.value        P
(Intercept)   0.9179   0.78510   1.169 2.424e-01
Day          -0.2789   0.06416  -4.347 1.378e-05
```

Random effects:

```
          Variance
Female.ID   2.2974
Stream      2.2500
```

Sample size:

```
Observations    Female.ID       Stream
          84           36           11
```

E.1.2.5 Variation in fertility over time after an additional mating

```
model1 = glmer(Fertility ~ Day + (1|Stream) +
    (1|Female.ID) + (1|OLRE), data = After, family = poisson)
```

Model comparison:

	Chi Sq	P
Stream	1.4439	0.2295
Female ID	9.7932	0.001752
Day	2.1722	0.1405

Fixed effects:

```
            Estimate Std.Error z.value      P
(Intercept)   2.0720   1.30100   1.593 0.1112
Day          -0.1043   0.07077  -1.474 0.1405
```

Random effects:

```
          Variance
Female.ID   1.3584
Stream      0.6006
```

Sample size:

```
Observations    Female.ID       Stream
          85           32           11
```

E.1.2.6 Variation in proportion fertility over time after an additional mating

```
model1 = glmer(Prop.Fertility ~ Day + (1|Stream) +
    (1|Female.ID) + (1|OLRE), data = After, family = binomial)
```

Model comparison:

	χ^2	P
Stream	5.5951	0.01801
Female ID	3.4542	0.06309
Day	0.5063	0.4767

Fixed effects:

```
            Estimate Std.Error z.value      P
(Intercept)    1.742    2.9330  0.5941 0.5525
Day           -0.114    0.1613 -0.7068 0.4797
```

Random effects:

```
          Variance
Female.ID   2.5136
Stream      4.0503
```

Sample size:

```
Observations    Female.ID       Stream
          85           32           11
```

E.1.2.7 Total individual fertility before and after an additional mating

```
model1 = glmer(TotFertility ~ Group + (1|Stream) +
    (1|Female.ID) + (1|OLRE), data = Total, family = poisson)
```

Model comparison:

	χ^2	P
Group (B/A)	3.5892	0.05816

Fixed effects:

```
             Estimate Std.Error z.value       P
(Intercept)    0.3536    0.4327  0.8172 0.41380
Group2.After   0.8616    0.4509  1.9110 0.05605
```

Random effects:

```
          Variance
```

```
Female.ID    0.000
Stream       0.751
```

Sample size:

```
Observations    Female.ID       Stream
          68           36           11
```

E.1.2.8 Total proportion individual fertility before and after an additional mating

```
model1 = glmer(Prop.Fertility ~ Group + (1|Stream) +
    (1|Female.ID) + (1|OLRE),data = Total,
    family = binomial)
```

Model comparison:

	χ^2	P
Group	5.153	0.02321

Fixed effects:

```
             Estimate Std.Error z.value       P
(Intercept)    -1.469    0.4929  -2.979 0.00289
Group2.After    1.307    0.5595   2.336 0.01948
```

Random effects:

```
          Variance
Female.ID   0.0000
Stream      0.8011
```

Sample size:

```
Observations    Female.ID       Stream
          68           36           11
```

E.1.2.9 Total individual fertility between day 12 and day 15

```
model1 = glmer(Fertility ~ Group + (1|Stream) +
    (1|Female.ID) + (1|OLRE), data = Day12v15,
    family = poisson)
```

Model comparison:

	χ^2	P
Group (12/15)	10.0766	0.001502

Fixed effects:

```
            Estimate Std.Error z.value         P
(Intercept)  -0.6121    0.5317  -1.151 0.2496000
GroupDay15    1.6650    0.4513   3.689 0.0002251
```

Random effects:

```
          Variance
Female.ID   0.8276
Stream      0.5030
```

Sample size:

```
Observations    Female.ID       Stream
          41           24           10
```

E.1.2.10 Total individual proportion fertility between day 12 and day 15

```
model1 = glmer(Prop.Fertility ~ Group + (1|Stream) +
    (1|Female.ID) + (1|OLRE),Day12v15, family = binomial)
```

Model comparison:

	χ^2	P
Group (12/15)	15.5344	8.102×10^{-5}

Fixed effects:

```
            Estimate Std.Error z.value        P
(Intercept)   -3.207    0.9602  -3.340 8.378e-04
GroupDay15     3.431    0.8366   4.101 4.118e-05
```

Random effects:

```
          Variance
Female.ID   0.0000
Stream      3.6911
```

Sample size:

```
Observations    Female.ID       Stream
          41           24           10
```

E.1.2.11 Direction of change in fertility before and after an additional mating

```
model1a = glmer(FertilityChange ~ FecundityBefore +
    (1|Stream), data = dataBA, family = binomial)
```

```
model1b = glmer(FertilityChange ~ FertilityBefore +
    (1|Stream), data = dataBA, family = binomial)
```

```
model1c = glmer(FertilityChange ~ FecundityBefore +
    FertilityBefore + (1|Stream), data = dataBA,
    family = binomial)
```

Model comparison:

	χ^2	P
Fecundity	2.2001	0.138
Fertility	5.8261	0.01579
Relative Fecundity	18.3375	1.85×10^{-5}
Relative Fertility	21.9635	2.779×10^{-6}

Fixed effects:

	Estimate	Std.Error	z.value	P
(Intercept 1a)	−0.7495	0.653	−1.148	0.2511
1a FecundityBefore	0.03305	0.02603	1.27	0.2042
(Intercept 1b)	0.5516	0.4736	1.165	0.2442
1b FertilityBefore	−0.1559	0.08398	−1.857	0.06335
(Intercept 1c)	−1.156	0.8439	−1.37	0.1708
1c FecundityBefore	0.3001	0.1328	2.26	0.02379
1c FertilityBefore	−1.258	0.6316	−1.992	0.04642

Random effects:

	Variance
1a Stream	0.5952
1b Stream	0.0000
1c Stream	0.0000

Sample size:

Observations	Stream
32	11

E.1.2.12 Degree of change in proportion fertility before and after an additional mating

```
model1a = lmer(PropChange ~  FecundityBefore + (1|Stream),
    data = dataBA)
```

```
model1b = lmer(PropChange ~  FertilityBefore + (1|Stream),
    data = dataBA)
```

```
model1c = lmer(PropChange ~  FecundityBefore +
    FertilityBefore + (1|Stream), data = dataBA)
```

Model comparison:

	χ^2	P
Fecundity	7.5575	0.005976
Fertility	2.0648	0.1507
Relative Fecundity	12.842	3.389×10^{-4}
Relative Fertility	7.3493	0.006709

Fixed effects:

```
                  Estimate Std.Error t.value
(Intercept 1a)     -0.07952    0.1423   13.88
1a FecundityBefore   0.0123  0.003987   26.81

(Intercept 1b)       0.2384    0.1156   8.521
1b FertilityBefore -0.01568   0.01122   14.15

(Intercept 1c)      0.02965     0.113   11.37
1c FecundityBefore  0.01567  0.004105   28.77
1c FertilityBefore -0.03133   0.01049   19.21
```

Random effects:

```
          Variance
1a Stream   0.1157
1b Stream   0.0271
1c Stream   0.0284
```

Sample size:

```
Observations       Stream
          32           11
```

E.2 Experiment 2: Investigation of female and male effects

E.2.1 Variation in fecundity

E.2.1.1 Variation in fecundity over time prior to an additional mating

```
model1 = glmer(Fecundity ~ Day * MaleType + (1|Stream) +
    (1|Female.ID) + (1|OLRE),data = Before, family = poisson)
```

Model comparison:

	χ^2	P
Female ID	23.4754	1.265×10^{-6}
MaleType (SD/NSD) x Day	0.0336	0.8546
MaleType (SD/NSD)	1.0766	0.2994
Day	33.7777	6.178×10^{-9}

Fixed effects:

```
                Estimate Std.Error z.value        P
(Intercept)     -1.89600   0.73010 -2.5970 0.009412
Day              0.26640   0.06956  3.8300 0.000128
MaleTypeSD       0.24610   0.95140  0.2587 0.795800
Day:MaleTypeSD   0.01678   0.09155  0.1833 0.854600
```

Random effects:

```
          Variance
Female.ID   0.9057
```

Sample size:

```
Observations    Female.ID       Stream
         136           34            5
```

E.2.1.2 Variation in fecundity over time and between male types (sperm depleted / non-sperm depleted) after an additional mating

```
model1 = glmer(Fecundity ~ Day * MaleType + (1|Stream) +
    (1|Female.ID) + (1|OLRE),data = After, family = poisson)
```

Model comparison:

	χ^2	P
Female ID	12.3373	4.44×10^{-4}
MaleType (SD/NSD) x Day	0.85	0.3566
MaleType (SD/NSD)	0.3047	0.581
Day	37.8321	7.71×10^{-10}

Fixed effects:

```
                Estimate Std.Error z.value         P
(Intercept)      5.39300   1.29300  4.1690 0.0000306
Day             -0.27120   0.07633 -3.5530 0.0003804
MaleTypeSD       1.75200   1.72600  1.0150 0.3100000
Day:MaleTypeSD -0.09379   0.10180 -0.9214 0.3568000
```

Random effects:

```
           Variance
Female.ID   0.6181
```

Sample size:

```
Observations    Female.ID       Stream
         136           34            5
```

E.2.1.3 Variation in fecundity across all days

```
model1 = glmer(Fecundity ~ Day + (1|Stream) +
    (1|Female.ID) + (1|OLRE), data = All, family = poisson)
```

Model comparison:

	χ^2	P
Day	0.2665	0.6057

Fixed effects:

```
            Estimate Std.Error z.value         P
(Intercept)  0.67970 0.0007596  894.90 0.000e+00
Day          0.01072 0.0007613   14.09 4.572e-45
```

Random effects:

```
           Variance
Female.ID   0.4259
```

Sample size:

```
Observations    Female.ID       Stream
         272           34            5
```

E.2.1.4 Total individual fecundity before and after an additional mating and between male types

```
model1 = glmer(TotFecundity ~ Group * MaleType  +
    (1|Stream) + (1|Female.ID) + (1|OLRE),data = Total,
    family = poisson)
```

Model comparison:

	χ^2	P
MaleType (SD/NSD) x Group (B/A)	0.4838	0.4867
MaleType (SD/NSD)	0.96	0.3272
Group (B/A)	2.0814	0.1491

Fixed effects:

```
                         Estimate Std.Error z.value        P
(Intercept)                2.3240    0.2643   8.790 1.497e-18
Group2.After               0.4160    0.2797   1.487 1.370e-01
MaleTypeSD                 0.4210    0.3489   1.207 2.275e-01
Group2.After:MaleTypeSD   -0.2582    0.3704  -0.697 4.858e-01
```

Random effects:

```
          Variance
Female.ID   0.4262
```

Sample size:

```
Observations    Female.ID       Stream
          68           34            5
```

E.2.1.5 Total individual fecundity between day 12 and day 14 and between male types

```
model1 = glmer(Fecundity ~ Group * MaleType + (1|Stream) +
    (1|Female.ID) + (1|OLRE),data = Day12v14, family = poisson)
```

Model comparison:

	χ^2	P
MaleType (SD/NSD) x Group (12/14)	0.5267	0.468
MaleType (SD/NSD)	0.3591	0.549
Group (12/14)	2.7386	0.09795

Fixed effects:

```
                     Estimate Std.Error z.value        P
(Intercept)          1.606000    0.2904 5.53100 3.189e-08
GroupDay14           0.201300    0.3497 0.57560 5.649e-01
MaleTypeSD           0.005779    0.3861 0.01497 9.881e-01
GroupDay14:MaleTypeSD 0.340500    0.4646 0.73290 4.636e-01
```

Random effects:

```
          Variance
Female.ID   0.3151
```

Sample size:

```
Observations    Female.ID       Stream
          68           34            5
```

E.2.2 Variation in fertility

E.2.2.1 Variation in total fertility with total fecundity prior to an additional mating

```
model1 = glmer(TotFertility ~ TotFecundity + (1|Stream) +
    (1|OLRE), data = TotalBefore, family = poisson)
```

Model comparison:

	χ^2	P
TotFecundity	36.4477	1.568×10^{-9}

Fixed effects:

```
             Estimate Std.Error z.value        P
(Intercept)   0.60720  0.180500   3.364 7.692e-04
TotFecundity  0.03741  0.004727   7.915 2.467e-15
```

Sample size:

```
Observations       Stream
          33            5
```

E.2.2.2 Variation in total fertility with total fecundity after an additional mating

```
model1 = glmer(TotFertility ~ TotFecundity + (1|Stream) +
    (1|OLRE) , TotalAfter, family = poisson)
```

Model comparison:

	χ^2	P
TotFecundity	40.8785	1.62×10^{-10}

Fixed effects:

```
             Estimate Std.Error z.value        P
(Intercept)   1.64400  0.105900  15.510 2.758e-54
TotFecundity  0.01987  0.002248   8.838 9.779e-19
```

Sample size:

```
Observations       Stream
          33            5
```

E.2.2.3 Variation in fertility over time prior to an additional mating

```
model1 = glmer(Fertility ~ Day + (1|Stream) + (1|Female.ID) +
    (1|OLRE), data = Before, family = poisson)
```

Model comparison:

	χ^2	P
Female ID	4.7501	0.0293
Day	0.2539	0.6144

Fixed effects:

```
            Estimate Std.Error  z.value      P
(Intercept) -0.04793   0.56520 -0.08481 0.9324
Day          0.02807   0.05572  0.50380 0.6144
```

Random effects:

```
          Variance
Female.ID    0.378
```

Sample size:

```
Observations    Female.ID       Stream
          95           33            5
```

E.2.2.4 Variation in proportion fertility over time prior to an additional mating

```
model1 = glmer(Prop.Fertility ~ Day + (1|Stream) +
    (1|Female.ID) + (1|OLRE), data = Before, family = binomial)
```

Model comparison:

	χ^2	P
Female ID	4.0869	0.04322
Day	7.9025	0.004937

Fixed effects:

```
            Estimate Std.Error z.value        P
(Intercept)   0.5122   0.51120   1.002 0.316300
Day          -0.1502   0.04971  -3.022 0.002515
```

Random effects:

```
          Variance
Female.ID   0.2882
```

Sample size:

```
Observations    Female.ID       Stream
          95           33            5
```

E.2.2.5 Variation in fertility over time and between male types after an additional mating

```
model1 = glmer(Fertility ~ Day * MaleType + (1|Stream) +
    (1|Female.ID) + (1|OLRE), data = After,
    family = poisson)
```

Model comparison:

	χ^2	P
Female ID	0.327	0.5674
MaleType (SD/NSD) x Day	0.0201	0.8872
MaleType (SD/NSD)	0.1299	0.7186
Day	33.2647	8.043×10^{-9}

Fixed effects:

```
              Estimate Std.Error  z.value         P
(Intercept)     5.7590   1.15400  4.99200 5.982e-07
Day            -0.3067   0.07170 -4.27800 1.886e-05
MaleTypeSD     -0.1442   1.56500 -0.09211 9.266e-01
Day:MaleTypeSD  0.0139   0.09809  0.14170 8.873e-01
```

Random effects:

```
          Variance
Female.ID   0.0424
```

Sample size:

```
Observations    Female.ID       Stream
          97           33            5
```

E.2.2.6 Variation in proportion fertility over time and between male types after an additional mating

```
model1 = glmer(Prop.Fertility ~ Day * MaleType + (1|Stream) +
    (1|Female.ID) + (1|OLRE),data = After, family = binomial)
```

Model comparison:

	χ^2	P
Female ID	0.327	0.5674
MaleType (SD/NSD) x Day	0.1995	0.6552
MaleType (SD/NSD)	0.9201	0.3374
Day	31.2344	2.287×10^{-8}

Fixed effects:

```
                Estimate Std.Error z.value         P
(Intercept)      3.62100   1.16000  3.1220 0.0017980
Day             -0.24750   0.07211 -3.4320 0.0005982
MaleTypeSD       0.47910   1.50100  0.3191 0.7496000
Day:MaleTypeSD -0.04202   0.09388 -0.4476 0.6544000
```

Random effects:

```
          Variance
Female.ID        0
```

Sample size:

```
Observations    Female.ID       Stream
          97           33            5
```

E.2.2.7 Total individual fertility before and after an additional mating and between male types

```
model1 = glmer(TotFertility ~ Group * MaleType + (1|Stream) +
    (1|Female.ID) + (1|OLRE),data = Total, family = poisson)
```

Model comparison:

	χ^2	P
MaleType (SD/NSD) x Group (B/A)	0.6327	0.4264
MaleType (SD/NSD)	1.0901	0.2964
Group (B/A)	12.5805	3.898×10^{-4}

Fixed effects:

```
                        Estimate Std.Error z.value          P
(Intercept)               1.2030    0.2565  4.6890 2.744e-06
Group2.After              0.7751    0.2534  3.0590 2.223e-03
MaleTypeSD                0.4059    0.3045  1.3330 1.826e-01
Group2.After:MaleTypeSD  -0.2669    0.3293 -0.8106 4.176e-01
```

Random effects:

```
          Variance
Female.ID    0.222
```

Sample size:

```
Observations    Female.ID       Stream
          66           34            5
```

E.2.2.8 Total proportion individual fertility before and after an additional mating and between male types

```
model1 = glmer(Prop.Fertility ~ Group * MaleType +
    (1|Female.ID) + (1|OLRE) + (1|Stream), data = Total,
    family = binomial)
```

Model comparison:

	χ^2	P
MaleType (SD/NSD) x Group (B/A)	2.6744	0.102
MaleType (SD/NSD)	0.006	0.9384
Group (B/A)	12.4228	4.241×10^{-4}

Fixed effects:

```
                        Estimate Std.Error z.value        P
(Intercept)              -1.1520    0.2051  -5.616 1.957e-08
Group2.After              0.9001    0.2453   3.670 2.430e-04
MaleTypeSD                0.3224    0.2600   1.240 2.151e-01
Group2.After:MaleTypeSD  -0.5289    0.3125  -1.693 9.054e-02
```

Random effects:

```
          Variance
Female.ID   0.0817
```

Sample size:

```
Observations    Female.ID       Stream
          66           34            5
```

E.2.2.9 Total individual fertility between day 12 and day 14 and between male types

```
model1 = glmer(Fertility ~ Group * MaleType + (1|Stream) +
    (1|Female.ID) + (1|OLRE),data = Day12v14, family = poisson)
```

Model comparison:

	χ^2	P
MaleType (SD/NSD) x Group (12/14)	0.0027	0.9589
MaleType (SD/NSD)	0.3949	0.5297
Group (12/14)	23.8148	1.061×10^{-6}

Fixed effects:

```
                     Estimate Std.Error  z.value         P
(Intercept)            0.2518    0.3090  0.81500 0.4150000
GroupDay14             1.2330    0.3423  3.60300 0.0003143
MaleTypeSD             0.1831    0.3901  0.46940 0.6388000
GroupDay14:MaleTypeSD -0.0227    0.4409 -0.05148 0.9589000
```

Random effects:

```
          Variance
Female.ID   0.2145
```

Sample size:

```
Observations    Female.ID       Stream
          62           34            5
```

E.2.2.10 Total individual proprtion fertility between day 12 and day 14 and between male types

```
model1 = glmer(Prop.Fertility ~ Group * MaleType +
    (1|Stream) + (1|Female.ID) + (1|OLRE), data = Day12v14,
    family = binomial)
```

Model comparison:

	χ^2	P
MaleType (SD/NSD) x Group (12/14)	0.2317	0.6303
MaleType (SD/NSD)	0.1717	0.6786
Group (12/14)	27.0669	1.965×10^{-7}

Fixed effects:

```
                     Estimate Std.Error z.value        P
(Intercept)           -1.4090    0.2925 -4.8190 1.446e-06
GroupDay14             1.4260    0.3541  4.0280 5.633e-05
MaleTypeSD             0.2421    0.3805  0.6363 5.246e-01
GroupDay14:MaleTypeSD -0.2180    0.4513 -0.4831 6.290e-01
```

Random effects:

```
          Variance
Female.ID   0.1289
```

Sample size:

```
Observations    Female.ID       Stream
          62           34            5
```

E.2.2.11 Direction of change in fertility before and after an additional mating

```
model1a = glmer(FertilityChange ~ FecundityBefore +
    (1|Stream), data = dataBA, family = binomial)
```

```
model1b = glmer(FertilityChange ~ FertilityBefore +
    (1|Stream), data = dataBA, family = binomial)
```

```
model1c = glmer(FertilityChange ~ FecundityBefore +
    FertilityBefore + MaleType + (1|Stream), data = dataBA,
    family = binomial)
```

Model comparison:

	χ^2	P
Fecundity	4.7193	0.02983
Fertility	8.2079	0.004171

	χ^2	P
Relative Fecundity	0.1939	0.6597
Relative Fertility	3.6824	0.05499
MaleType (SD/NSD)	0.2076	0.6487

Fixed effects:

```
                  Estimate Std.Error z.value       P
(Intercept 1a)       2.316     1.031   2.246 0.02471
1a FecundityBefore -0.04942   0.02754  -1.794 0.07278

(Intercept 1b)       3.175     1.681   1.889 0.05888
1b FertilityBefore  -0.2983    0.2087   -1.43  0.1528

(Intercept 1c)       3.609     2.021   1.786 0.07418
1c FecundityBefore  0.0316   0.06389  0.4945  0.6209
1c FertilityBefore  -0.444    0.3681  -1.206  0.2278
1c MaleTypeSD       -0.4878      1.09 -0.4475  0.6545
```

Random effects:

```
          Variance
1a Stream   0.3917
1b Stream   1.2593
1c Stream   1.5571
```

Sample size:

```
Observations       Stream
          32            5
```

E.2.2.12 Degree of change in proportion fertility before and after an additional mating and between male types

```
model1a = lmer(PropChange ~  FertilityBefore + (1|Stream),
    data = dataBA)
```

```
model1b = lmer(PropChange ~  FertilityBefore + (1|Stream),
    data = dataBA)
```

```
model1c = lmer(PropChange ~  FecundityBefore +
    FertilityBefore + MaleType + (1|Stream), data = dataBA)
```

Model comparison:

	χ^2	P
Fecundity	0.0476	0.8274
Fertility	3.1064	0.07798
Relative Fecundity	4.4386	0.03514
Relative Fertility	7.4975	0.006179
MaleType (SD/NSD)	0.4654	0.4951

Fixed effects:

```
                     Estimate Std.Error t.value
(Intercept 1a)         0.1297   0.06412   11.95
1a FecundityBefore -0.0005093  0.002123   29.92

(Intercept 1b)         0.1733   0.05553   7.095
1b FertilityBefore  -0.009276  0.005172   29.99

(Intercept 1c)         0.1423   0.06768    15.1
1c FecundityBefore    0.00667  0.003239   27.57
1c FertilityBefore   -0.02193  0.008353    27.2
1c MaleTypeSD         -0.0507   0.07915      28
```

Random effects:

```
          Variance
1a Stream   0.0013
```

```
1b Stream   0.0021
1c Stream   0.0003
```

Sample size:

```
Observations       Stream
          32            5
```

Appendix F

Variation in the benefits of multiple mating on female fertility in wild stalk-eyed flies

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ORIGINAL RESEARCH

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Variation in the benefits of multiple mating on female fertility in wild stalk-eyed flies

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Abstract

Polyandry, female mating with multiple males, is widespread across many taxa and almost ubiquitous in insects. This conflicts with the traditional idea that females are constrained by their comparatively large investment in each offspring, and so should only need to mate once or a few times. Females may need to mate multiply to gain sufficient sperm supplies to maintain their fertility, especially in species in which male promiscuity results in division of their ejaculate among many females. Here, we take a novel approach, utilizing wild-caught individuals to explore how natural variation among females and males influences fertility gains for females. We studied this in the Malaysian stalk-eyed fly species *Teleopsis dalmanni*. After an additional mating, females benefit from greatly increased fertility (proportion fertile eggs). Gains from multiple mating are not uniform across females; they are greatest when females have high fecundity or low fertility. Fertility gains also vary spatially, as we find an additional strong effect of the stream from which females were collected. Responses were unaffected by male mating history (males kept with females or in male-only groups). Recent male mating may be of lesser importance because males in many species, including *T. dalmanni*, partition their ejaculate to maintain their fertility over many matings. This study highlights the importance of complementing laboratory studies with data on wild-caught populations, where there is considerable heterogeneity between individuals. Future research should focus on environmental, demographic and genetic factors that are likely to significantly influence variation in individual female fecundity and fertility.

KEYWORDS
Diopsidae, ejaculate partitioning, mating systems, sperm depletion, wild-caught flies

1 | INTRODUCTION

Female mating with multiple males (polyandry) is found widely across many taxa (mammals: Clutton-Brock, 1989; Ginsberg & Huck, 1989; birds: Griffith, Owens, & Thuman, 2002; fishes: Avise, Jones, Walker, & DeWoody, 2002; general: Jennions & Petrie, 2000; Zeh & Zeh, 2001) and is almost ubiquitous in insects (Arnqvist & Nilsson, 2000). While multiple mating is expected in males, as their reproductive success

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typically increases with the number of matings, it is less clearly beneficial for females. Female reproductive potential is thought to be realized after one or a few matings (Bateman, 1948), as females are assumed to be constrained by the greater investment they make in each of their offspring. This has led to an extensive literature considering potential benefits to females from multiple mating, in terms of increases to female survival, fecundity and fertility (Arnqvist & Nilsson, 2000; Hosken & Stockley, 2003; Yasui, 1998), and whether polyandry may be a mechanism to quell or mitigate intragenomic conflicts (Haig & Bergstrom, 1995; Zeh & Zeh, 1996). Additionally, females may gain indirect genetic benefits through increasing the genetic diversity or quality of offspring, but these are likely to be of secondary importance when females gain direct benefits from multiple mating (Slatyer, Mautz, Backwell, & Jennions, 2012; Yasui, 1998).

However rather less attention has been given to considering how variation among females impacts on the benefits of multiple mating. For instance, how does female condition, fecundity, or prior mating history alter the fitness consequences of further matings or polyandrous matings? Greater study in this area is needed in order to uncover the contexts in which multiple mating benefits, harms, or has no effect on females (House, Walling, Stamper, & Moore, 2009; Toft & Albo, 2015; Wright et al., 2013). In addition, there has been an over-reliance on laboratory matings to investigate the consequence of multiple mating. While laboratory studies allow control and standardization (e.g., using virgins), assays may not fully reflect the natural history of mating experienced by females and males. Laboratory studies need to be complemented by experiments conducted on wild-caught individuals, in situations that more closely replicate the natural range of conditions of female and male encounters.

Here, we apply these principles to consider the consequences of multiple mating on female fertility in the Malaysian stalk-eyed fly *Teleopsis dalmanni*, when females vary in the degree of sperm limitation. In insects, it is widely found that sperm acquired in a single mating is insufficient to fertilize all of a female's eggs (Ridley, 1988; Wedell, Gage, & Parker, 2002). To maintain fertility, females may need to mate multiply to gain sufficient sperm supplies for egg laying throughout their adult life (Chevrier & Bressac, 2002; Fjerdingstad & Boomsma, 1998) or remate at regular intervals as sperm supplies dwindle (Drnevich, Papke, Rauser, & Rutowski, 2001; Fox, 1993; Wang & Davis, 2006). This implies that the fertility benefits of female remating will change with fluctuating environmental factors, such as the operational sex ratio, food availability, and the fertility of previous mates (Arnqvist & Nilsson, 2000; Cordero & Eberhard, 2003; Crean & Marshall, 2009; Fox, 1993; Navara, Anderson, & Edwards, 2012; Pitcher, Neff, Rodd, & Rowe, 2003; Rogers, Denniff, Chapman, Fowler, & Pomiankowski, 2008; Tuni, Albo, & Bilde, 2013). In line with this view, females may be able to modify their mating rates in response to changing circumstances that affect the relative costs and benefits of mating (Boulton & Shuker, 2016; Wilgers & Hebets, 2012).

There are two important fluctuating factors that are likely to regulate the direct benefits to female fertility of an additional mating. First is current female sperm limitation. Female insects have internal sperm storage organs where sperm are kept and used to fertilize eggs long after mating (Eberhard, 1996; Kotrba, 1995; Orr & Brennan, 2015; Pitnick, Markow, & Spicer, 1999). The current fertility status of a female will change over time; as females use up their sperm reserves or as sperm die, female fertility will probably decrease. Consequently, females that have mated recently or have full sperm storage organs will likely gain less benefit from an additional mating than sperm-depleted females.

Second, the increase in female fertility from an additional mating may be influenced by the male's investment. Individual males have finite resources and their investment in ejaculates is predicted to be shaped by the trade-off with the number of matings (Parker, 1982). There is good evidence that males increase their allocation to females that have higher reproductive value (Engqvist & Sauer, 2001; Kelly & Jennions, 2011; Perry, Sirot, & Wigby, 2013; Rogers, Grant, Chapman, Pomiankowski, & Fowler, 2006; Wedell et al., 2002). Likewise, in many situations, males increase their ejaculate size when females are subject to greater sperm competition (Kelly & Jennions, 2011; Wedell et al., 2002). It has been suggested that the quality of an ejaculate that a female receives may positively correlate with male condition (Iwasa & Pomiankowski, 1999; Sheldon, 1994), although firm evidence for this is lacking (Fitzsimmons & Bertram, 2013; Harley et al., 2013; Mautz, Møller, & Jennions, 2013; Pizzari, Jensen, & Cornwallis, 2004). Conversely, dominant or attractive males may invest fewer sperm per mating as they have more opportunities to mate and so need to divide their ejaculate into smaller packages per female (Jones, 2001; Tazzyman, Pizzari, Seymour, & Pomiankowski, 2009; Warner, Shapiro, Marcanato, & Petersen, 1995). In many cases, female fertility suffers when the male has recently mated (Levin, Mitra, & Davidowitz, 2016; Perez-Staples, Aluja, Macías-Ordóñez, & Sivinski, 2008; Torres-Vila & Jennions, 2005; Wedell & Ritchie, 2004). The net effect is that female sperm limitation will vary with male mating strategy depending on the female's value to the male, the condition or attractiveness of the male, and his recent mating history. As a result, the direct fertility benefit that a female gains from an extra mating will not be a static quantity but will depend on the context in which mating takes place.

We examined how these two factors alter the benefits of female remating by means of experimentation in the wild using the Malaysian stalk-eyed fly *Teleopsis dalmani* (Diptera, Diopsidae). Both sexes in this species are highly promiscuous (Wilkinson, Kahler, & Baker, 1998). Females typically have low fertility measured by egg hatch, both in the laboratory and in the wild (Baker et al., 2001; Cotton, Small, Hashim, & Pomiankowski, 2010). One of the main factors contributing to this infertility is that males have evolved to partition their ejaculates between many females. As a consequence, males transfer few sperm in a single copulation (~65, Wilkinson, Amitin, & Johns, 2005; ~142, Rogers et al., 2006) leading to females being sperm-limited (Baker et al., 2001). Thus, females must remate in order to raise their fertility (Baker et al., 2001). As well as few sperm, the small size of male ejaculates is unlikely to provide any nonsperm benefits (Kotrba, 1996).

Given these patterns in stalk-eyed flies, we expect to find that female *T. dalmanni* remate to gain direct fertility benefits. To distinguish between male and female effects as sources of variation in changes to female fertility, we report two experiments using wild-caught

T. dalmanni females. Prior mating histories of females and males cannot be controlled in field experiments. However, we initially kept females isolated from males in order that females became sperm-depleted, to some extent. We then evaluated the effect of an additional mating on female fertility and expected that sperm-depleted females should receive direct fertility benefits from an additional mating. To explore the impact of past male mating experience on the ability of males to confer fertility on females, in a second experiment we varied the prior mating rate and state of sperm depletion of wild-caught males by keeping them for several days either with females or in male-only groups. We then evaluated the fertility gain of females mated to these two types of male. These experiments allow us to examine, using wild-caught individuals with backgrounds of natural variation, the extent of female and male effects on fertility.

2 | MATERIALS AND METHODS

2.1 | Experiment 1: Gains from an additional mating

Fly collections took place in February 2011 from eleven stream sites in the Ulu Gombak valley, Peninsular Malaysia (3°19′ N, 101°45′ E). Females and males were collected on day zero at dusk from lek sites on the edge of forest streams at several stream sites adjacent to tributaries of the Gombak River. Individuals were aspirated into plastic bags and within 1 hr of capture, males and females were transferred to individual 500-ml containers lined with a moist cotton wool and tissue paper base. Flies were fed every 2 days with puréed banana.

Female fecundity was recorded from counts of eggs deposited on the tissue paper base, which were collected and renewed every 2 days. Eggs were allowed to develop for a further 5 days in petri dishes containing a moist cotton pad. Fertility was estimated by scoring hatching success under a light microscope at 10 × magnification. Fertilized eggs that have hatched appear as empty chorion cases, while unfertilized eggs are full and show no signs of development. If fertilized eggs failed to hatch, but showed signs of development (horizontal striations in the chorion and early mouthpart formation), they were recorded as fertile (Baker et al., 2001).

On day 13 after capture, each female was given a single additional mating with a male collected at the same time as the female. This time period was chosen to allow females to become sperm-depleted prior to mating. Matings were carried out in mating chambers, each made up of two 500-ml cells, separated by a removable card partition, and a single string running the length of the chamber provided a suitable roosting site (Cotton, Cotton, Small, & Pomiankowski, 2015; Figure 1). In the evening, a male was placed in the upper cell and the focal female in the lower cell. The following morning (after ~12 hr), the card partition was removed and the pair observed until a successful copulation took place, classed as lasting 30 s or more, to ensure that sperm transfer had occurred (Corley et al., 2006; Lorch, Wilkinson, & Reillo, 1993). Males were only used once. The remated females were then rehoused as before and their reproductive output was monitored from day 15 every 2 days for a further 8 days. The females were then killed and stored in ethanol. Female eyespan (distance between the outer tips of the eyes; Hingle, Fowler, & Pomiankowski, 2001) and thorax length (distance from base of the head to the joint between the metathoracic legs and the thorax; Rogers et al., 2008) were measured to an accuracy of 0.01 mm, using a monocular microscope and the image analysis software ImageJ, version 1.43e (Schneider, Rasband, & Eliceiri, 2012). In total, we recorded fertility for $N = 45$ females across the full sampling periods before and after the extra mating.

2.2 | Experiment 2: Investigation of female and male effects

A second experiment was carried out using flies collected from five stream sites in the Ulu Gombak valley in July/August 2012. Individuals were collected as above. Females were housed individually in 500-ml containers, and their reproductive output was recorded as in the first experiment. Males were placed in large 1,500-ml containers either with a mix of males and nonfocal females allowing them to mate freely (sperm-depleted), or only with other males (nonsperm-depleted). Isolation from females allows males to replenish their sperm stores (Rogers, Chapman, Fowler, & Pomiankowski, 2005). Fly density was standardized across these two treatments, each pot containing a total of 10 flies, either a 1:1 ratio of males to females (sperm-depleted) or 10 males (nonsperm-depleted). On the evening of day 12, a focal female and male were placed in a mating container (Figure 1) and allowed to have an additional mating following the protocol above, except that males did not have an isolated overnight period. Females were placed either with a sperm-depleted male ($N = 19$) or a nonsperm-depleted male ($N = 17$). After the additional mating, females were rehoused and their subsequent reproductive output was recorded every 2 days from day 14 over the following 8 days, and morphometric measures taken as before.

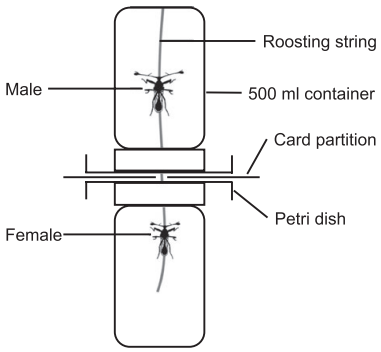

FIGURE 1 Mating chambers composed of two 500 ml cells, separated by a removable card partition. A single string runs the whole length of the chamber, providing a suitable roosting site. A male was placed in the upper cell and a female in the lower cell. The card partition was removed and the pair was allowed to mate once, before being separated

2.3 | Statistical analysis

Female sperm depletion was determined by the decline in female fertility over the 8 days before the single additional mating (comprising four egg counts) as well as over the 8 days after the additional mating (again, four egg counts). To test whether an additional mating resulted in increased fecundity or fertility, the total individual reproductive output over the 8 days before and after mating was compared, as well as total individual reproductive output on the days immediately before (days 11–12) and after the additional mating (days 14–15 in the first experiment; days 13–14 in the second experiment). Lastly, we examined whether the direction of change in individual fertility was positive, or negative/unchanged, and tested the degree to which individual proportion fertility changed depended on female premating fecundity or fertility.

All tests were carried out in R, version 3.31 (R Core Team, 2016), and are reported (including effect sizes) in the Appendix S1. Analyses were carried out of female reproductive output (fecundity and fertility), using generalized linear mixed-effects models (GLMMs) using the *lme4* package (Bates, Mächler, Bolker, & Walker, 2015). Fecundity (number of eggs laid) and fertility (number of fertile eggs laid) were modeled in a GLMM with a Poisson distribution and log link function. In addition, egg counts were modeled as proportion data with a binomial distribution (fertile eggs, nonfertile eggs) and logit link function. We modeled the direction of change in individual fertility using a GLMM with a binomial distribution, where changes were coded as 1 s and 0 s (increase, decrease/unchanged). Change in proportion fertility (proportion after mating minus proportion before mating) was tested using a linear mixed-effects model (LMM). Reported *p*-values were computed by model comparison using ANOVA. Percentage fertility is described with the exclusion of females that laid fewer than 10 eggs.

Previous work showed a strong effect of stream site upon reproductive output (Harley, Fowler, & Cotton, 2010), so stream site was included as a random factor in reproductive output models—both in the first and second experiments. Variation between stream sites is reported for fecundity, fertility, and proportion fertility for the first experiment, where females were collected across 11 stream sites. They are not reported for the second experiment, as there was a more limited sample of only five stream sites, so any conclusions based on such a small sample would not be trustworthy. Where appropriate, female identity was included as a random factor to account for the nonindependence of multiple female measures. Variation between females is reported as a factor similar to stream sites.

The data were found to be overdispersed and to account for this, an observation-level random effect (OLRE) was used in all models (except for those modeling change), as results can be unreliable when using both random effects and a quasi-distribution (Harrison, 2014, 2015). The improvement in model fit from the addition of OLRE was checked through model comparison. OLRE may perform poorly in binomial models, so the parameter estimates of these models were checked against those from the comparable beta-binomial model using the *glmmADMB* package (Fournier et al., 2012; Skaug, Fournier, Bolker, Magnusson, & Nielsen, 2016) to confirm robustness (Harrison, 2015).

Female eyespan and thorax length are known to be strong proxies for fecundity (Cotton, Fowler, & Pomiankowski, 2004; Rogers et al., 2006) and were highly correlated with female fecundity and fertility (Spearman's rank $\rho > 0.3$, $p < .01$). For both experiments, we repeated all analyses with female eyespan and thorax as covariates. This did not alter any of the results (see Appendix S1). For simplicity, the final models reported in the results did not include these covariates.

Reproductive output was examined over the 8 days before and 8 days after mating, excluding days 2 and 4 from all analyses. Previous studies have reported that reproductive output of recently caught *T. dalmanni* females typically falls in the short term (day 2) after mating, followed by a peak (day 4) before settling to a more steady level (Cotton et al., 2010; Harley et al., 2010). The same pattern was observed in this investigation (data not shown). Females that died or escaped during the observation period were excluded from the analyses (eight of 45 females in the first experiment; two of 36 females in the second experiment), as was a single female that failed to lay any eggs during the observation period in the first experiment.

3 | RESULTS

3.1 | Experiment 1: Gains from an additional mating

3.1.1 | Variation in fecundity

Fecundity was highly variable between females both in the premating (days 5–12, mean ± *SD* per day = 2.17 ± 2.48; range = 0.13–11.13, $N = 36$; $\chi^2 = 5.3291$, $N = 144$, $p = .0210$) and postmating periods (days 14–21, mean ± *SD* per day = 2.42 ± 2.93, range = 0–11.88, $N = 36$; $\chi^2 = 24.5018$, $N = 144$, $p < .0001$). Female fecundity did not change over the premating period ($\chi^2 = 1.1815$, $N = 144$, $p = .2770$, Figure 2a), and there was no consistent directional change in fecundity over the whole 17-day period of the experiment ($\chi^2 = 1.2586$, $N = 288$, $p = .2619$).

Female fecundity did not differ when individual reproductive output was compared across the premating and postmating periods ($\chi^2 = 0.1001$, $N = 72$, $p = .7517$), and was not different between the days immediately before (days 11–12) and immediately after (days 14–15) the extra mating ($\chi^2 = 2.4907$, $N = 72$, $p = .1145$, Figure 3a). Lastly, we examined differences in fecundity across streams. There was also no effect of stream site on fecundity in the premating ($\chi^2 = 2.8652$, $N = 144$, $p = .0905$) or postmating periods ($\chi^2 = 0.0676$, $N = 144$, $p = .7948$).

3.1.2 | Variation in fertility

The pattern for individual female fertility in the premating period (days 5–12), showed considerable variation among females, both in the absolute number of fertile eggs laid (mean ± *SD* per day = 0.66 ± 1.02; range = 0–4.75, $N = 36$; $\chi^2 = 5.7493$, $N = 84$, $p = .0165$) and proportion fertility (mean ± *SD* per day = 35.7057 ± 32.5241%,

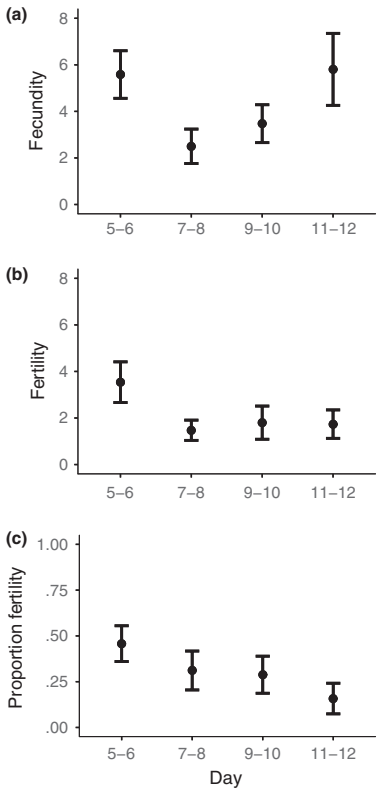

FIGURE 2 Premating female reproductive output through time (mean ± *SE*). Mean (a) fecundity, (b) fertility, and (c) proportion fertility per 2 days, over an 8-day period. Flies were captured at dusk on day zero

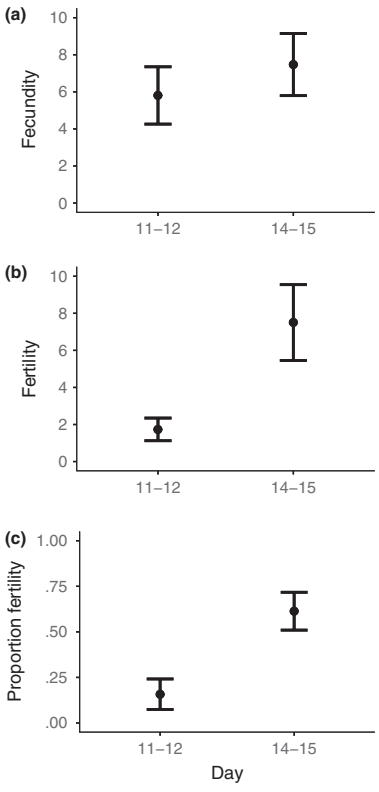

FIGURE 3 Reproductive output immediately before and immediately after mating (mean ± *SE*). Mean (a) fecundity, (b) fertility, and (c) proportion fertility on days 11–12 and days 14–15. Females were captured at dusk on day zero, and mating occurred at dawn on day 13

range = 0–86.3636%, $N = 17$; $\chi^2 = 20.5766$, $N = 84$, $p < .0001$), and this extended into the postmating period (days 14–21) for female absolute fertility (mean ± *SD* per day = 1.53 ± 2.59, range = 0–10.5, $N = 36$; $\chi^2 = 9.7932$, $N = 85$, $p = .0018$) but not proportion fertility (mean ± *SD* = 58.5037% ± 33.0920%, range = 0–100%, $N = 21$; $\chi^2 = 3.4542$, $N = 85$, $p = .0631$). In contrast to fecundity, across the premating period there was a decline in absolute ($\chi^2 = 8.4502$, $N = 84$, $p = .0037$, Figure 2b) and proportion fertility ($\chi^2 = 17.5402$, $N = 84$, $p < .0001$, Figure 2c). Note that it was important to examine proportion fertility as there was a positive relationship between total female fertility and fecundity both in the premating ($\chi^2 = 5.9894$, $N = 36$, $p = .0144$) and postmating periods ($\chi^2 = 22.6367$, $N = 32$, $p < .0001$).

Comparing total fertility over the whole premating and postmating periods, absolute fertility did not change after the additional mating ($\chi^2 = 3.5892$, $N = 68$, $p = .0582$); however, proportion fertility increased ($\chi^2 = 5.1530$, $N = 68$, $p = .0232$). The percentage of females with low fertility (<20% total egg hatch) dropped from 38% to 19%, whereas the proportion with high fertility (>70% total egg hatch) rose from 24% to 48% (Figure 4a). Comparing across a closer period of time, there was a distinct increase in the days around the extra mating (days 11–12 to days 14–15), both absolute ($\chi^2 = 10.0766$, $N = 41$, $p = .0015$, Figure 3b) and proportion fertility increased ($\chi^2 = 15.5344$, $N = 41$, $p < .0001$, Figure 3c).

The direction of change in total individual fertility after the additional mating (increase or decrease/unchanged) did not depend on female fecundity ($\chi^2 = 2.2001$, $N = 32$, $p = .1380$). However, when female fertility was accounted for, females with higher fecundity were more likely to have a positive change in fertility after the additional mating ($\chi^2 = 18.3375$, $N = 32$, $p < .0001$). In addition, females with low fertility were more likely to benefit from the additional mating ($\chi^2 = 5.8261$, $N = 32$, $p = .01579$). This greater effect of premating fertility persisted after accounting for differences in individual female fecundity ($\chi^2 = 21.9635$, $N = 32$, $p < .001$).

A similar examination was made using the change in proportion fertility between the pre- and postmating periods (Figure 5). Females with high premating fecundity had a larger positive change in their proportion fertility postmating ($\chi^2 = 7.5575$, $N = 32$, $p = .0060$),

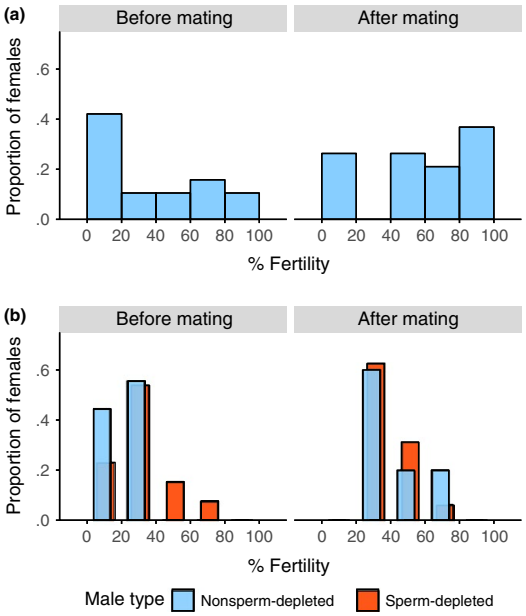

FIGURE 4 The distribution of percentage fertility (total eggs hatched / total eggs laid) for females in the 8 days before, and 8 days after the extra mating in (a) experiment 1 and (b) experiment 2. Females used in experiment 2 were either mated to a sperm-depleted (orange) or a nonsperm-depleted male (light blue). Plots exclude females who laid fewer than 10 eggs over each period

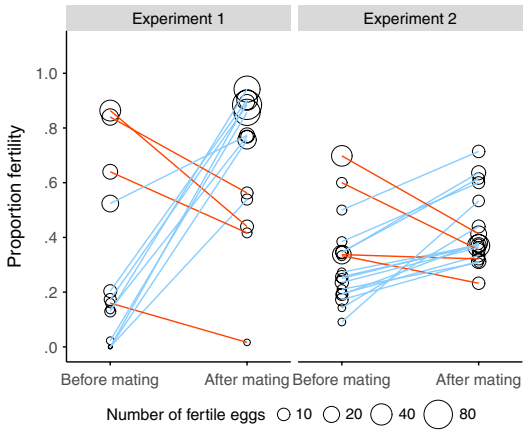

FIGURE 5 Total proportion fertility in the 8 days before, and 8 days after the extra mating in experiment 1 and experiment 2. Lines are individual females, colored by slope: increased fertility (light blue), decreased fertility (orange). Circle size indicates the total absolute number of fertile eggs laid by each female. Plots exclude females that laid fewer than 10 eggs either before or after the extra mating

and this result remained when female fertility was accounted for (χ^2 = 12.842, N = 32, p < .0001). Female premating fertility had no effect on the change in proportion fertility (χ^2 = 2.0648, N = 32, p = .1507). However, once fecundity was accounted for, female premating fertility did have an effect (χ^2 = 7.349, N = 32, p = .0067), as females that fertilized few of their eggs had a larger positive change in proportion fertility than females that were already fertilizing relatively more.

Finally, we examined differences in fertility across streams. In the premating period, there was variation between stream sites in absolute (χ^2 = 5.8958, N = 84, p = .0152) and proportion fertility (χ^2 = 4.3233, N = 84, p = .0376). After the additional mating, absolute fertility no longer differed between stream sites (χ^2 = 1.4439, N = 85, p = .2295), but variation in proportion fertility persisted despite the extra mating (χ^2 = 5.5951, N = 85, p = .0180).

3.2 | Experiment 2: Investigation of female and male effects

To investigate potential male effects on fertility gain among females, a second experiment was carried out. Females were mated once either with a sperm-depleted male that had been held for the previous 2 weeks with multiple females or with a nonsperm-depleted male that had been held in a male-only container.

3.2.1 | Variation in fecundity

The pattern for female fecundity was broadly similar to that of the previous experiment (Figure 6a and 7a, see Appendix S1). There was no effect of male type on total fecundity before versus after the additional mating (male type × before/after interaction, χ^2 = 0.4838, N = 68, p = .4867), or for the contrast of the days immediately before and after the additional mating, days 11–12 and 13–14 (χ^2 = 0.5267, N = 68, p = .4680).

3.2.2 | Variation in fertility

Fertility also showed a broadly similar pattern to the previous experiment (Figure 4b, 6b and 7b, see Appendix S1). At the end of the premating period, individual absolute fertility was comparable to that of the low absolute fertility in the previous experiment (1.7368 ± 2.6634 and 1.9355 ± 2.4074, expt. 1 and expt. 2, mean ± SD, days 11–12). Proportion fertility was also similar to the previous experiment prior to mating (19% and 21%, expt. 1 and expt. 2, days 11–12). Comparing total fertility in the premating and postmating periods, absolute (χ^2 = 12.5805, N = 66, p < .0001) and proportion fertility (χ^2 = 12.4228, N = 66, p < .0001) increased after the additional mating. Likewise, between the days immediately prior (day 11–12) and immediately after (days 13–14) the additional mating, there was an increase in absolute (χ^2 = 23.8148, N = 62, p < .0001, Figure 7b) and proportion fertility (χ^2 = 27.0669, N = 62, p < .0001, Figure 7c).

The direction of change in individual fertility was more likely to be positive for more fecund females (χ^2 = 4.7193, N = 32, p = .0298), but not after female fertility was accounted for (χ^2 = 0.1939, N = 32,

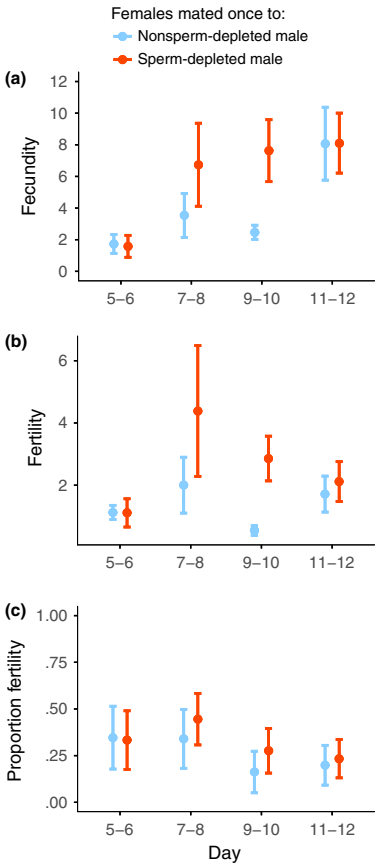

FIGURE 6 Premating female reproductive output of mean (a) fecundity, (b) fertility, and (c) proportion fertility per 2 days through time (mean ± *SE*). Females from the sperm-depleted (orange) or nonsperm-depleted male (light blue) treatment are shown separately. Flies were captured at dusk on day zero

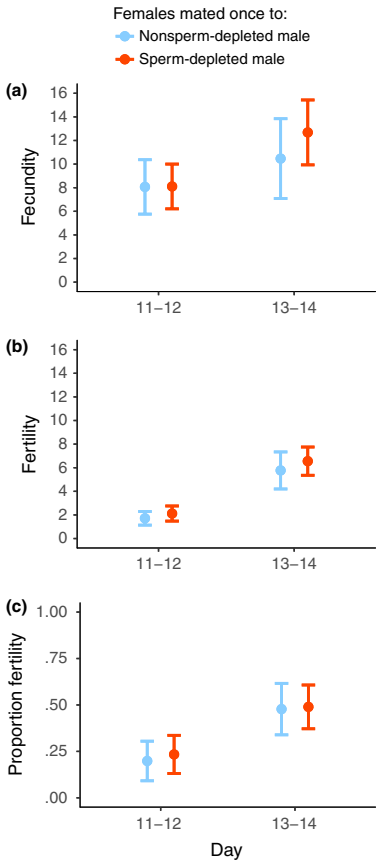

FIGURE 7 Reproductive output immediately before and immediately after mating (mean ± *SE*) where females received an extra mating from either a sperm-depleted (orange) or nonsperm-depleted (light blue) male. Mean (a) fecundity, (b) fertility, and (c) proportion fertility on days 11–12 and days 13–14. Females were captured at dusk on day zero and mating occurred on the evening of day 12

p = .6597). Females with low premating fertility were more likely to have a positive change (χ^2 = 8.2079, N = 32, p = .0042). However again, after accounting for fecundity, premating fertility did not predict the direction of change (χ^2 = 3.6824, N = 32, p = .0550).

Change in proportion fertility between the premating and postmating periods did not depend on premating fecundity (χ^2 = 0.0476, N = 32, p = .8274, Figure 5), but when female fertility was controlled for, more fecund females had a more positive change in proportion fertility (χ^2 = 4.4386, N = 32, p = .0351). Change in proportion fertility likewise did not depend on premating fertility (χ^2 = 3.1064, N = 32, p = .0780). In addition, when the analysis was repeated and fecundity was accounted for, females with low fertility prior to mating also had a more positive change in proportion fertility (χ^2 = 7.4975, N = 32, p = .0062).

Comparing the 8 days before and after the additional mating, male type was unrelated to the increase in absolute (male type × before/after interaction, χ^2 = 0.6327, N = 66, p = .4264) and proportion fertility (χ^2 = 2.6744, N = 66, p = .1020). Likewise comparing the days immediately before (day 12) and after the additional mating (day 14), male type had no effect on the increase in absolute (χ^2 = 0.0027, N = 62, p = .9589) or proportion fertility (χ^2 = 0.2317, N = 62, p = .6303). There was no effect of male type on either the direction of change in fertility (χ^2 = 0.2076, N = 32, p = .6487) or the change in proportion fertility (χ^2 = 0.4654, N = 32, p = .4951).

4 | DISCUSSION

There are abundant studies investigating the direct fertility benefits from multiple mating (Arnqvist & Nilsson, 2000; Haig & Bergstrom,

1995; Hosken & Stockley, 2003; Slatyer et al., 2012; Yasui, 1998; Zeh & Zeh, 1996). However, there is currently minimal focus on how these benefits vary between individuals and across time, or in particular contexts like associations with the degree of polyandry and female age or experience (House et al., 2009; Toft & Albo, 2015; Wright et al., 2013). In addition, experiments evaluating direct benefits of multiple mating have rarely been carried out among individuals sampled from wild populations, in ways that examine the encounters likely to occur between females and males in nature.

In this study, we aimed to redress these deficits by assessing fecundity and fertility in wild-caught stalk-eyed flies, and how these benefits vary with the time since the last mating (and, as a corollary, whether there is a cost of a failure to remate that increases with time). Females from laboratory populations of *T. dalmanni* have been shown to benefit from multiple mating (Baker et al., 2001). But the experience of flies under laboratory conditions is inevitably very different from those in wild populations, for example, in terms of population density, food availability, and exposure to parasites/predators. Moreover, laboratory studies of stalk-eyed flies and other species have utilized virgin males and females in remating assays, in order to standardize prior mating experience (Baker et al., 2001; Bayoumy, Michaud, & Bain, 2015; Burdfield-Steel, Auty, & Shuker, 2015; Chelini & Hebets, 2016; Droge-Young, Belote, Eeswara, & Pitnick, 2016; Tregenza & Wedell, 2002). But virgins are rare in nature in species in which males and females readily remate, and this is particularly true of stalk-eyed flies in which adult fertility persists for many weeks (Rogers et al., 2006). All of these factors point to the necessity for controlled experiments using wild-caught individuals with backgrounds of natural variation.

Female sperm limitation is likely to be an important fluctuating factor that regulates the direct fertility benefits to females from multiple mating. In some insect mating systems females only mate once (Arnqvist & Andrés, 2006; Arnqvist & Nilsson, 2000; South & Arnqvist, 2008) or mate multiple times but over a single short period (Boomsma, Baer, & Heinze, 2005). These restricted mating patterns provide sufficient sperm to ensure female fertility throughout her reproductive life. However, in many other insect species, sperm acquired in a single mating or mating period is insufficient to fertilize all her eggs (Ridley, 1988; Wedell et al., 2002). Consequently, females necessarily need to remate throughout their adult life, as sperm supplies diminish through use and with time (Chevrier & Bressac, 2002; Drnevich et al., 2001; Fjerdingstad & Boomsma, 1998; Fox, 1993; Wang & Davis, 2006). We demonstrate that this form of reproductive life history typifies *T. dalmanni* stalk-eyed fly females collected from the wild. Females from the two collections, in 2011 and 2012, had mean female fertility of 46% or 32%, respectively, shortly after they were initially captured (days 5–6), and this declined to ~20% in both cases over the following week (days 11–12; Figures 2 and 6). An additional mating after 12 days markedly changed fertility, causing a substantially larger proportion of their eggs to be fertilized, 61% and 48%, immediately after the additional mating (Figures 3 and 7). In contrast, female fecundity was unchanged by an additional mating and remained consistent across the whole of the study period, although with a fair degree of stochastic variation (Figures 2 and 6). Accordingly, negative and positive changes in fertility can be ascribed to females being able to fertilize a smaller or larger proportion of their eggs, rather than due to fluctuations in the number of eggs laid.

We show an overall increase in fertility; however, we additionally make the novel finding that the increase in fertility was not uniform between individual females. Females with low premating fertility were more likely to benefit from an additional mating, as were females with high fecundity. After taking account of variation in premating fecundity, it is apparent that females were able to fertilize a larger proportion of their eggs if they initially had low fertility. Similarly, after taking account of variation in premating fertility, females gained more in fertility from an additional mating if they were highly fecund. These outcomes reveal a strong context dependence in the benefit of additional matings. Low prior fertility is indicative that females were subject to sperm depletion, and high fecundity is indicative of the need for greater numbers of stored sperm, both seemingly addressed by the additional mating. To test these predictions, direct measurements of sperm numbers within females will be necessary. This is possible in female stalk-eyed flies, which retain sperm in spermathecae that act as long-term storage organs, and the ventral receptacle, a small structure to which sperm move and are stored individually within pouches (capacity ~16–40 sperm) prior to release for fertilization of an egg (Kotrba, 1993; Rose, Brand, & Wilkinson, 2014).

The results here contrast with those of a previous study carried out on the same population (Harley et al., 2010). In that study, females were collected from the wild at lek mating sites and half were immediately allowed a single additional mating. Both groups showed a decline in fertility through time, as in the current study. However, there was no difference in fertility between females that received an extra mating on capture and those that did not. What explains the divergence from the current study? The striking difference is that females were unusually fertile, ~80% over the first 10 days in captivity, both among females with and females without the extra mating (Harley et al., 2010). This degree of fertility is comparable to the levels achieved in laboratory populations when females are given the opportunity to mate repeatedly (Baker et al., 2001). This failure of an additional mating to enhance female fertility echoes our finding that fertility gains from an extra mating are weaker when females already have high fertility. In the current study, average fertility was much lower, around ~30% fertility in both years of this study. Hence, there was plenty of opportunity for an extra mating to benefit female fertility. We suspect this low level is the norm as an earlier census also from the same area in Malaysia reported 36% fertility (Cotton et al., 2010).

We can make several inferences from these studies of wild-caught females. First, they confirm there is a cost of a failure to remate as the proportion of fertile eggs laid declines with time when females are unable to remate. Second, an additional mating has a greater beneficial effect when females already have low fertility. The most obvious proximate reason for this is that many wild females are sperm-limited, either because they had not mated recently, not mated at a sufficiently high rate or because sperm allocation by males was considerably

limited. These explanations could be directly assessed in the future by counting sperm in female sperm storage organs in wild-caught females and after matings with wild-caught males. This could be complemented by observing mating rates in the wild, and relating these measures to natural fertility levels. A third inference from the current experiments is that the fertility benefits to females vary between individuals, stream sites, across matings and fluctuate through time. In some contexts, individual females may be limited by the availability of mating opportunities, whereas in others, they may become increasingly limited by their own fecundity.

The source of variation in fertility between individuals in the wild is currently undefined. It is likely that variable factors such as population density and sex ratio are important, particularly as they will affect female and male mating rates. Similarly, environmental conditions such as food availability can influence mating rates (Kotiaho, Simmons, & Tomkins, 2001; Rogers et al., 2005, 2008), male fertility (Bunning et al., 2015; O'Dea, Jennions, & Head, 2014; Perry & Rowe, 2010; Perry et al., 2013) and female fecundity (Awmack & Leather, 2002; Cotton et al., 2015; Levin et al., 2016; Stewart, Morrow, & Rice, 2005). While in certain contexts an additional mating may be clearly beneficial for female fertility, we show that this is not always the case and there is a need to test females under a range of contexts that reflect those experienced under natural conditions. Only then can the full force of remating on female fertility be understood.

Other significant factors to consider are variation in male mating strategy and male quality as they may have a significant influence on the benefit that females obtain from remating. Males can adjust their ejaculate investment in response to female reproductive value (Engqvist & Sauer, 2001; Kelly & Jennions, 2011; Perry et al., 2013; Rogers et al., 2006; Wedell et al., 2002) and sperm competition (Kelly & Jennions, 2011; Wedell et al., 2002), and investment may positively correlate with male condition (Iwasa & Pomiankowski, 1999; Sheldon, 1994; but see Fitzsimmons & Bertram, 2013; Harley et al., 2013; Mautz et al., 2013; Pizzari et al., 2004) or negatively with male dominance or attractiveness (Jones, 2001; Tazzyman et al., 2009; Warner et al., 1995). We explicitly evaluated the importance of variation in recent male mating experience, contrasting males that had multiple opportunities to mate, with those that had been deprived of females. Rather surprisingly, there was no difference in fertility gains from extra matings with either type of male (Figure 7b,c). This reveals that male allocation of ejaculate is tailored to repeated mating, and the replenishment of resources occurs on a short time scale. Males partition their ejaculate in order to copulate with many females each day (Small, Cotton, Fowler, & Pomiankowski, 2009); spermatophore size is very small in *T. dalmanni* (Kotrba, 1996), and males transfer few sperm in a single ejaculate (~100, Rogers et al., 2006; Wilkinson et al., 2005). Partitioning of ejaculate is presumably a mechanism for males to maintain fertility over successive matings (Linklater, Wertheim, Wigby, & Chapman, 2007; Wedell et al., 2002). In addition, male reproductive activity is scheduled in a highly concentrated burst each day, as lek-holding males mate with females that have settled with them overnight before they disperse at dawn (Chapman, Pomiankowski, & Fowler, 2005; Cotton et al., 2010). To cope with this pattern of sexual activity, males replenish their accessory glands and hence their ability to produce ejaculate within 24 hr (Rogers et al., 2005). In this system, prior mating activity has no or a minimal effect on a male's ability to mate effectively. However, we only assessed female fertility gains after the first mating by a male. It might still be the case that prior mating experience could affect the ability of males to deliver ejaculate in subsequent matings or even to be able to mate repeatedly. In the wild, it is notable that females often leave lek sites before mating if the male is pre-occupied in matings with other females (A. Pomiankowski, personal observation). This suggests that fertility gains may fall with subsequent matings, but this remains to be investigated. Again, this points to the complexity of context underpinning the benefits associated with remating.

Another cause of variation in male fertility and ejaculate allocation, other than recent mating history, is meiotic drive (Wilkinson, Johns, Kelleher, Muscedere, & Lorsong, 2006). An X-linked meiotic drive system is present in these populations of *T. dalmanni* (Cotton, Földvári, Cotton, & Pomiankowski, 2014) and causes the degeneration of Y-bearing sperm and the production of female-biased broods (Presgraves, Severance, & Wilkinson, 1997). We expect drive male fertility to be reduced due to this dysfunction resulting in the transfer of fewer sperm. Consequently, mating with a drive male may not provide a female with the same fertility benefit as mating with a standard male. There is evidence that females mated to drive males have lower fertility, particularly when males are mating at high frequencies (Wilkinson, Swallow, Christianson, & Madden, 2003; Wilkinson et al., 2006) and that drive males are poor sperm competitors (Wilkinson et al., 2006). In this study, we found that several females failed to raise their fertility after mating (Figure 5), and in fact had lower fertility than prior to mating. Mating with a drive male could potentially produce this pattern. Future research should evaluate explicitly how an extra mating with a drive male impacts on female fertility among wild-caught flies, when males and females are in their natural condition. It would also be of interest to investigate the hypothesis that multiple mating is an evolved mechanism by which females dilute the negative effects of mating with a drive male (Haig & Bergstrom, 1995; Zeh & Zeh, 1996), both to ensure fertility and because any male progeny produced in a female-biased population will have increased fitness (Fisher, 1930; Holman, Price, Wedell, & Kokko, 2015).

We used wild-caught flies to capture the natural variation between individuals, an approach that has been much neglected. It is important to dig deeper into the life history of *T. dalmanni* to further understand the environmental and population-level variables that affect the benefits to additional matings. For example, we know that there is much variation in female fecundity and fertility between stream sites. What we have yet to elucidate is how streams differ—do they vary in food availability and quality, rainfall, humidity, temperature, population density, or sex ratio? Are these factors stable or fluctuating? Which have the most influence on female fecundity and fertility? We show that females with low fertility and high fecundity benefit the most from mating; improved knowledge of the conditions experienced by individuals throughout their lifetime will further our understanding of when and why it is beneficial for females to remate.

In conclusion, this study has demonstrated that female sperm storage and depletion since the previous mating are key selection forces driving the benefits and evolution of mating rates in the wild. Females are generally sperm-limited due to the minimal male sperm investment in individual copulations (Rogers et al., 2006; Wilkinson et al., 2005), so females gain direct fertility benefits from multiple mating both in the laboratory (Baker et al., 2001) and in wild populations. However, these gains are not uniform between females and are contingent on female fecundity and fertility. In a broader context, stalk-eyed fly reproductive activity is governed by a co-evolutionary spiral of exaggerated mating rates. Females have evolved high levels of multiple mating because their fertility is subject to sperm limitation. The resulting higher levels of multiple mating by males, especially those that are attractive to females, have led to the evolutionary corollary of finer partitioning of ejaculate, which has only exacerbated sperm limitation and the benefits of multiple mating. The various studies of stalk-eyed fly fertility in the wild (Cotton et al., 2010; Harley et al., 2010; this study) demonstrate both high variation (across space and time, and between individuals) and now also context dependence in benefits to remating. They highlight the importance of complementing laboratory studies with those using wild populations, where natural mating rates may be very different. Further studies will disentangle whether other factors such as variation in age, condition, attractiveness, a range of environmental variables, and the presence of meiotic drive are important as well, and allow a better understanding of the range of forces that influence female and male mating behavior.

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CONFLICT OF INTEREST

None declared.

AUTHORS' CONTRIBUTIONS

EH, AP, and KF conceived the original project and methodology; EH, AC, and JMH collected the data; LM analyzed the data; LM, AP, and KF led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

DATA ACCESSIBILITY

Raw data have been archived in the Dryad Digital Repository: https://doi.org/10.5061/dryad.pk5vd.

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SUPPORTING INFORMATION

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