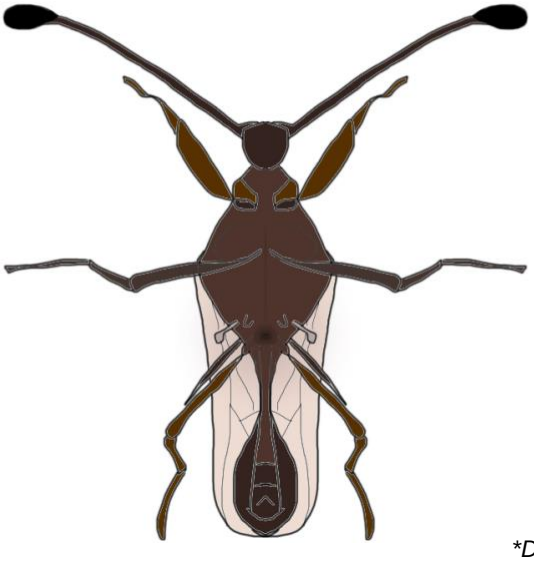
Fertility and genomic consequences of meiotic drive in *Teleopsis dalmanni*



**Drawing credit: Sasha Bradshaw*

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

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“HAVE YOU PRESSED THE BUTTON, IS IT SUBMITTED?!” — POM, 16:59 March 2023—1 minute to the thesis submission deadline.
“JUST DO IT. NOW.” — the POM motto.

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Ernest Bates
Grandad
September 1935 – February 2012

Abstract

Meiotic drivers distort the segregation of alleles during meiosis to bias transmission in their favour. In males, this occurs through the degradation of non-carrier sperm. Meiotic drive is associated with negative fitness effects both in male and female carriers, and changes in the host genome as drive variants are found in chromosomal inversions. In this thesis, I explore the themes of fertility costs incurred by carrying a driving X chromosome and its maintenance in the population, using *Teleopsis dalmanni* as a model system.

I first examine the effects of meiotic drive on sperm competition; an important component of post-copulatory selection in this species, where sperm from different males are stored in the female reproductive tract and compete to fertilise eggs. I use double-mating trials – in which a female is mated with one drive and one non-drive male – to determine the competitive ability of drive males under low competition. Next, I mate females multiply to a competitor male, either drive or non-drive, and assess the fertility success of a male of the alternative genotype. This measures drive male offensive and defensive capabilities under stronger competition, i.e., when the female's storage organs contain more rival sperm.

Next, I turn to investigate the effects of drive on components of female fertility. I determine the age at sexual maturity and fecundity for non-carrier, heterozygous (one driving X chromosome) and homozygous (two driving X chromosomes) females. I examine how these effects might constrain the population frequency of drive.

Finally, I develop a method to improve the annotation of the *T. dalmanni* genome. Following an improved genome assembly for this species, annotation is needed to link genome sequences to the biology of drive. The resulting annotation set will aid in future studies of the genomic differences between carrier and non-carrier flies.

Impact statement

Meiotic drive genes are selfish genetic elements that distort Mendelian patterns of inheritance to bias transmission in their favour. X-linked drive genes disable gametes bearing the alternate sex chromosome in the heterogametic sex. I investigate the fitness effects associated with an X-linked drive system using the stalk-eyed fly, *Teleopsis dalmanni*. In males, *Sex Ratio*, (SR) destroys Y-bearing sperm, so females who mate with SR-carrying males sire all-female broods. Yet, in wild populations of *T. dalmanni*, the frequency of SR is stabilised at around 20%. In this thesis, I examine the factors that lead to this equilibrium, focusing on the impact of SR on fertility in both sexes.

In Chapter 2, I show that drive males sire the same number of offspring as their non-drive competitors. This work is in preparation for submission to *Evolution* and has been presented at several conferences: Evolution 2022 in Cleveland, ESEB 2022 in Prague and PopGen 2023 in London. In Chapter 3, I build on this finding by increasing the level of sperm competition such that it more closely reflects wild populations of *T. dalmanni*. I demonstrate that drive sperm perform as well as non-drive sperm, even under intense competition. This agrees with recent evidence from our group that drive males have adapted to mitigate the fitness cost of drive, and my results in chapter 2. In combination, these lines of evidence point to the success of drive male sperm; likely a major factor in contributing to the high frequency of drive in natural populations. In Chapter 4, I examine the effects of SR on female fertility. I demonstrate for the first time that fecundity is negatively affected by SR, with some evidence of recessivity. Negative effects of drive that are heightened in homozygotes lead to negative frequency-dependent selection, limiting its spread. The importance of this is magnified in *T. dalmanni* due to the success of drive males under sperm competition. Finally, I present a novel approach to genome annotation that I developed in collaboration with the Dessimoz group at the University of the Lausanne. This work is in preparation for submission to *Bioinformatics* and was presented at the 2022 SIB conference in Biel. I demonstrate that this approach performs well compared to state-of-the-art methods and will employ it to annotate new *T. dalmanni* genome assemblies. Having drive and non-drive genome annotations will improve our understanding of the genomic effects of meiotic drive in this species.

This work has broader relevance beyond improving our understanding of the *T. dalmanni* meiotic drive system. Recently, there has been a surge in interest in utilising meiotic drive systems for population control of invasive or disease vector species. Drive dynamics in *T. dalmanni* can inform the development of artificial gene drive systems by highlighting the contribution of different factors to stabilising drive gene frequencies in natural populations. Finally, the genome annotation tool I present in Chapter 5 addresses a challenging aspect of genome annotation and will be publicly available for use in annotating the new genome assemblies of various species.

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Chapter 1. General Introduction

Background: Meiotic drive and sperm competition

In sexually reproducing organisms, most alleles from either parent are transmitted to offspring at a rate of 50%. Adaptive alleles can spread under the forces of natural and sexual selection. However, some selfish 'drive' genes act to subvert this Mendelian pattern of inheritance: they bias transmission in their favour to spread in a population without conferring a fitness benefit to their carrier, often even at a cost to the rest of the genome. One prominent class of these drivers is the meiotic drive genes, which act during gametogenesis to suppress the development of gametes carrying the alternative allele (Sandler and Novitski 1957). With such a huge transmission advantage, one might expect these genes to always rapidly spread to fixation; a state which, once reached, precludes easy detection. However, if a drive gene reduces carrier fitness, negative selection limits its transmission to offspring. In particular, if fitness costs increase with copy number – i.e., if higher fitness costs are incurred by homozygotes over heterozygotes – frequency-dependent negative selection can lead to a stable polymorphism. This is because as the drive gene spreads within a population, the number of homozygous individuals increases and thus the fitness of drive-carrying individuals declines, increasing the cost associated with carrying the drive gene (Larner et al. 2019). When drive genes are linked to a sex chromosome (usually the homogametic sex chromosome), it is more important that an equilibrium is reached, in order to prevent population extinction that would result from fixation (Gershenson 1928; Hamilton 1967).

Sex-linked drive typically reduces the production of heterogametic sex (usually male) through preferential killing of gametes that do not carry the driving chromosome. Perhaps counterintuitively, populations are able to maintain sex-linked drive at a stable frequency without failing. Moreover, if the drive gene causes a moderately female biased sex ratio of 65-70%, the skew is not sufficiently high to pose an extinction risk and may even be beneficial, increasing population productivity (Mackintosh et al. 2021). Equilibrium can be achieved even in the absence of suppressors; genes whose products disable the mechanisms of the driving gene. The most well-documented cases of this are among the *Diptera* taxa, for example, *Drosophila pseudoobscura* and *Drosophila neotestacea*, which both maintain an X-linked drive polymorphism (X^D) at a stable frequency. In *D. pseudoobscura*, females homozygous for X^D suffer the greatest reproductive fitness costs, resulting in negative frequency dependent selection that acts to prevent fixation (Larner et al. 2019). In *D. neotestacea*, polyandry has been suggested to promote stabilisation of X^D frequencies: females mate multiply when X^D is rare, and when males mate multiply the fitness of X^D males is reduced compared to non-carrying ('standard') males (Pinzone and Dyer 2013). Whilst Pinzone and Dyer (2013) found no evidence that polyandry evolved in response to drive, or that it was sufficient to prevent fixation, they were able to demonstrate that higher polyandry was associated with lower levels of X^D in natural populations. A theoretical study by Holman *et al.* (2015) supports this theorised role for polyandry, demonstrating that it can contribute to preventing fixation of X^D in combination with other factors, such as increased fitness costs for homozygotes (Holman et al. 2015). In short, these studies exemplify two major factors contributing to stabilisation of drive in *Diptera* populations: 1) deleterious homozygous effects in females, where the homozygous female suffers reduced fitness and 2) incomplete compensation for reduced male

fitness, where drive-carrying males suffer reduced fertility (Jaenike 2001; Burt and Trivers 2006). The latter is the focus of this work.

Male drive-carriers ('drive' males) have long been hypothesised to be less fit than their non-carrying ('standard') male counterparts due to sperm loss; the half of their sperm that inherit a Y chromosome are destroyed by the drive gene (Policansky 1974; Price and Wedell 2008). The effect of this reduced fertility manifests during sperm competition; a phenomenon that occurs when sperm from multiple males compete to fertilise an ovum. It is often high among insect species, where sperm from multiple males are likely to overlap due to pre-adaptations to polyandrous mating systems. Namely, females have specialised sperm storage organs in which sperm from different matings are preserved for several days post-copulation (Parker 1970). Sperm competition can be envisaged as a 'raffle', in which sperm are tickets to a fertilisation lottery. A raffle is either 'loaded' – where a male's mating order contributes to his paternity chances (male precedence) – or 'fair' – where each male's sperm mix randomly and have equal chances of success (sperm mixing). Investigations into sperm competition involve mating virgin females with two males (known as double mating trials) and measuring the resulting paternity share for each male. Typically, the outcome of each trial is expressed as the proportion of offspring sired by the second male (termed the P_2 value), which is indicative of the mode of sperm competition: male precedence or sperm mixing (Parker 1970). These general double mating trials are adapted to test the effect of drive on sperm competitive ability: a drive and standard male are each mated with a standard female, and P_2 is calculated alongside the proportion of offspring sired by the drive male.

Double mating trial studies in several *Diptera* – the majority of which have been performed in *Drosophila* and *Teleopsis* species – provide empirical evidence that drive males are outperformed by standard males under sperm competition, as drive males sire fewer offspring than standard males per double mating (reviewed in: (Verspoor et al. 2020). In *Drosophila,* sperm competition occurs through male precedence; the second male to mate gains the largest paternity share (Price 1997; Price et al. 1999; Simmons 2002). Studies of *Drosophila pseudoobscura* and *Drosophila simulans* demonstrate that drive males have a disproportionally lower P_2 value than standard males, implying the drive element has deleterious effects on the offensive capabilities of drive bearing sperm (Atlan et al. 2004; Angelard et al. 2008; Price et al. 2008). These authors postulate that this is due to the sperm loss caused by the drive gene-mediated killing of Y-bearing sperm, meaning they transfer fewer sperm to females per ejaculate. Additionally, Angelard *et al*. (2008) found evidence that drive male sperm were released from the female reproductive tract in the absence of second mating, suggesting that drive-bearing sperm can be identified by females. Perhaps surviving drive-bearing sperm incur damage from the sperm killing mechanism. For these reasons, it has long been the consensus in the literature that sperm from drive males ought to be outcompeted by the sperm from standard males. As such, the level of polyandry in a population is predicted to be one of the factors mediating post copulatory selection against drive (Price and Wedell 2008; Verspoor et al. 2020).

However, new evidence has begun to emerge that suggests drive males are not as disadvantaged during sperm competition as previously assumed. Firstly, the sperm loss associated with sperm killing by the drive element is unlikely to have a marked negative impact on anisogamous species as gamete production is not limited in males,

and studies have demonstrated that drive-carrying species of the *Diptera* can compensate for sperm loss by developing larger sperm-producing organs (Beckenbach 1996; Meade et al. 2020). Though it should be noted that *Teleopsis* males have adapted to a high degree of polyandry by partitioning their ejaculate into smaller packages with fewer sperm, drive males compensate for sperm loss and produce the equivalent low number per ejaculate as standard males (Baker et al. 2001; Wilkinson et al. 2005; Rogers et al. 2006; Meade et al. 2019, 2020).

This interplay between drive and sperm competition is explored here using the stalk-eyed fly species, *Teleopsis dalmanni*, as a model system (taxonomy note: *Teleopsis whitei and Teleopsis dalmanni* were formerly considered members of the *Cyrtodiopsis* group. *Cyrtodiopsis* was synonymised with *Teleopsis* in 2002; (Baker and Meier 2002)). *T. dalmanni* carries an X-linked '*Sex Ratio'* drive element (SR), which is also been documented in the closely related stalk-eyed fly species, *Teleopsis whitei* (Presgraves et al. 1997). Its distortion effect manifests through the degeneration of Y-bearing (i.e., non-carrier) sperm, producing 90-100% female offspring broods (Presgraves et al. 1997). The frequency of SR within *T. dalmanni* and *T. whitei* populations appears to be stable at approximately 8-20% and 34-36%, respectively (Presgraves et al. 1997; Wilkinson et al. 1998, 2003; Paczolt et al. 2017). As sperm mixing is the major mechanism of competition in the two stalk-eyed fly species known to carry SR, drive male (SR male) sperm are assumed to be outperformed by standard male (ST male) sperm regardless of mating order (Lorch et al. 1993; Wilkinson and Fry 2001; Corley et al. 2006). Though studies by Wilkinson *et al.* (2001, 2006) support this view, more recent evidence suggests that SR males can compete with standard males during sperm competition as they transfer similar numbers of sperm per ejaculate to a female's sperm storage organs (see chapter 2 for review) (Meade et al. 2019, 2020). Therefore, the long-held hypotheses regarding lower fertility in SR males might not be true in *T. dalmanni*.

1.1 Thesis outline

This work aims to contribute to the body of research in this area by testing the offensive and defensive capabilities of SR male sperm in *T. dalmanni* to elucidate if sperm competition is a mechanism contributing to the stabilisation of SR at the frequencies of ~20% observed in wild populations (Presgraves et al. 1997; Wilkinson et al. 1998, 2003; Paczolt et al. 2017). In Chapter 2, sperm competition is first considered in a simple system of reciprocal matings, where a male of each genotype, ST/SR, is mated once with the same standard female on subsequent days. The mode of competition – mixing or precedence – is discussed along with the success of each genotype in each position. In Chapter 3, the system of sperm competition increases in complexity to more closely replicate conditions found in the wild. An ST female is maximally mated with a single male of either genotype over a period of 7 days, before she is mated singularly with an SR or ST male, in order to assess ST/SR sperm performance when a female's storage organs are occupied by sperm from another male. In Chapter 4, the focus shifts away from sperm competition to other sexual traits that may be negatively impacted by meiotic drive. Chapter 4 discusses a future work planned to test the effect of SR on time to sexual maturity – an important measure of reproductive fitness – in females. Chapter 5 is a departure from empirical studies and instead is bioinformatics-focused. This work was conducted in collaboration with Prof Christophe

Dessimoz at the Swiss Institute of Bioinformatics and involved developing a new approach to genome annotation. This approach has been successful in our proof of principle testing and will be used to annotate the new *T. dalmanni* genome assembly completed in December 2022. A more accurate annotation of the *T. dalmanni* genome might help us elucidate the genomic effects of SR.

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Chapter 2. Meiotic drive does not impede success in sperm competition in the stalk-eyed fly, *Teleopsis dalmanni*

Title page

BRIEF COMMUNICATION
Meiotic drive does not impede success in sperm competition in the stalk-eyed fly, *Teleopsis dalmanni*
Running title: meiotic drive does not impede sperm success

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Conflict of Interest
The authors declare no conflicts of interest.

Author Contributions
SB, LM and AP conceived the study. SB and LM carried out experiments and SB collected the data. SB, LM and AP analysed the data. SB and AP wrote the paper. All authors read, reviewed and agreed on the submitted version.

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Data Archiving
The data that support the findings of this study are openly available in the Dryad at https://doi.org/10.5061/dryad.mkkwh713g.

2.1 Abstract

Male X-linked meiotic drive systems, which cause the degeneration of Y-bearing sperm, are common in the Diptera. Sperm killing is typically associated with fitness costs that arise from the destruction of wildtype sperm and collateral damage to maturing drive sperm, resulting in poor success under sperm competition. We investigate X-linked meiotic drive fertility in the stalk-eyed fly, *Teleopsis dalmanni*. Drive male paternity was measured in double mating trials under sperm competition against a wildtype male. Drive males sired the same number of offspring as wildtype males, both when mated first or second. This is the first evidence that drive males can compete equally with non-drive males in double matings, challenging the assumption that drive males inevitably suffer reduced fertility. The finding is in accord with previous work showing that the number of sperm per ejaculate transferred to females during non-competitive single matings does not differ between drive and wildtype males, which is likely due to the adaptive evolution of enlarged testes in drive males. Future experiments will determine whether the competitive ability of drive males is maintained under higher rates of female remating likely to be experienced in nature.

Key words
meiotic drive, stalk-eyed fly, sperm competition, multiple mating

2.2 Introduction

Meiotic drive causes the unequal transmission of genes to the next generation, violating Mendelian laws of segregation (Gershenson 1928; Sandler and Novitski 1957). In the extreme, the driver entirely excludes wildtype alleles and is transmitted to all offspring (Searle and de Villena 2022; Wolf et al. 2022). X-linked drivers are common among Diptera species and lead to the dysfunction of Y-bearing sperm and the production of female-only broods (Policansky 1974; Newton et al. 1976; James and Jaenike 1990; Hurst and Pomiankowski 1991; Presgraves et al. 1997; Jiggins et al. 1999). Such a significant transmission advantage could potentially lead to population extinction due to the lack of males (Hamilton 1967; Hatcher et al. 1999; Mackintosh et al. 2021). However, the fitness costs associated with carrying drive genes often result in negative frequency-dependent selection, which limits their spread (Lindholm et al. 2016; Finnegan et al. 2019).

One factor that strongly impacts the spread of meiotic drive genes is reduced fertility (Zanders and Unckless 2019). Males with drive not only lose wildtype gametes but typically suffer pleiotropic “collateral damage” that reduces the activity or number of mature drive sperm, leading to poor outcomes, especially under sperm competition (Price and Wedell 2008). This deficit is likely to be prominent in insects that possess reproductive organs specialised for long-term storage of viable sperm, increasing interactions between ejaculates (Parker 1970). Evidence from sperm competition studies of X-linked meiotic drive systems in *Drosophila* species supports this prediction. In *D. pseudoobscura,* SR drive males sire fewer offspring than standard males in double mating trials (Price et al. 2008a). Drive males have a disproportionally lower success both in their ability to defend against other sperm as the first (P1) male or to displace sperm already in storage as the second (P2) male (Price et al. 2008a). A similar pattern occurs in *D. simulans* with reduced success in P1 and P2 positions for drive males, and preferential drive male sperm ejection from the female reproductive tract even without competition from the second male’s sperm (Atlan et al. 2004; Angelard et al. 2008). It has been suggested that increased female polyandry evolves to undermine the success of drive sperm and an experimental evolution study in *D. pseudoobscura* and a double mating experiment in *D. recens* support this possibility, linking the frequency of drive with the rate of multiple mating (Haig and Bergstrom 1995; Zeh and Zeh 1997; Price et al. 2008b; Courret et al. 2019; Dyer and Hall 2019).

In this paper, we investigate this association between X-linked meiotic drive and reduced male fertility using the X-linked SR meiotic drive system in the stalk-eyed fly, *Teleopsis dalmanni.* Stalk-eyed fly females store sperm in the spermathecae (long-term storage organs) after mating, before sperm migrate to the ventral receptacle, where they are individually packaged into pouches prior to release into the oviduct for fertilisation of mature eggs (Kotrba 1995; Presgraves et al. 1999). In several stalk-eyed fly species, the main mode of sperm competition is sperm mixing, rather than male precedence (Lorch et al. 1993; Wilkinson et al. 1998a; Corley et al. 2006; Bellamy 2012). Double mating trials appear to confirm that drive males should be poor competitors as drive (SR) males sired fewer offspring than wildtype (ST) males (Wilkinson and Fry 2001; Wilkinson et al. 2006). However, several factors raise concerns about a simplistic interpretation of these findings. The first study was an inter-population cross of Malaysian and Thai flies. It was carried out before genetic

markers had been developed, and used variation in leg colour to assign parentage, which has an unknown error-rate (Wilkinson and Fry 2001). In addition, this study was in the conger *T. whitei* which may well have a different pattern of sperm competition than in *T. dalmanni.* The second study is in *T. dalmanni* and reported a lower SR male paternity using double mating trials (Wilkinson et al. 2006). However, this effect was limited to broods in which all offspring were sired by a single parent, that were less frequently fathered by SR males. There was no difference in SR and ST paternity in mixed paternity broods. In addition, this experiment only considered the competitive ability of SR males when mating second. This means that defensive traits of SR sperm and ejaculate were not assessed, so it is unclear whether the lack of success of SR males is general or limited to lower sperm precedence when mating second.

In addition, a profound challenge arises from recent findings that SR males transfer similar numbers of sperm per ejaculate (Meade et al. 2019, 2020). This was measured in females both in the spermathecae and the ventral receptacle after matings with SR or ST males, as well as after up to three sequential matings by a single male (Meade et al. 2019). Furthermore, when egg counts were used to measure fertility after single matings, it did not differ for females mated to SR or ST males (Meade et al. 2020). Dissection of adult SR males reveals that they have greatly enlarged testes which allow sperm delivery and fertility to be maintained despite the destruction of sperm caused by meiotic drive (Meade et al. 2019, 2020). This challenges the conventional view that drive males are weak competitors, and specifically, the finding of a competitive deficit of drive males in double mating trials.

Here, the competitive success of SR males was measured in a standard sperm competition assay using reciprocal double mating trials in which the SR male mated first followed by the ST male, or vice versa. This allowed an assessment of the SR male's success in both the offensive and defensive role and revealed whether there is first or last male sperm precedence. Even though multiple mating well above two is the norm in *T. dalmanni* stalk-eyed flies (Baker et al., 2001, 2003; Chapman et al. 2005), the simplicity of the double mating trial allows clear assessment of whether SR sperm suffer a disadvantage when in competition with ST sperm when the two males mate equally. The offspring arising from these trials were collected and genotyped at the larval stage to determine the proportion of offspring sired by SR males. This enabled the study to avoid confounds in paternity share relating to egg to adult viability differences, which have recently been shown to disadvantage SR-carrying larvae (Finnegan et al. 2019).

2.3 Methods

2.3.1 Stock populations

Flies for the standard stock (ST-stock) population carry only the wildtype X chromosome (X^{ST}). They were collected (by S. Cotton and A. Pomiankowski) in 2005 from the Ulu Gombak valley, Peninsular Malaysia (3°19′N 101°45′E). They have since been maintained in high-density cages (> 200 individuals) to minimise inbreeding and are regularly monitored to ensure they do not contain meiotic drive.

The meiotic drive stock (SR-stock) population is composed of females that are homozygous for a sex-ratio distorting X chromosome (X^{SR}). They were derived from flies collected in 2012 (by A. Cotton and S. Cotton) from the same location as the ST-

stock. X^{SR}/Y males produce 100% female offspring due to transmission distortion. The X^{SR} female stock is maintained by crossing X^{SR}/X^{SR} females with X^{ST}/Y males to produce X^{SR}/Y drive males, who are then mated to the X^{SR}/X^{SR} females to generate the next generation of the SR-stock females. The outcrossing to ST males from the ST-stock ensures that the two stocks only differ in their X chromosomes and are homogenised for autosomal content.

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

2.3.2 Experimental populations

Experimental ST (X^{ST}/Y) and SR males (X^{SR}/Y) were drawn from the ST-stock and SR-stock, respectively. They were housed separately in cages of ~50 individuals until sexually mature, in groups of similar age (6-8 weeks). ST-stock females were added to these cages at an equal sex ratio for > 3 days to allow males to mate. The females were then removed and discarded. Experimental males were then kept in single-sex groups for a further 3-6 days to allow their accessory glands to return to full size (Rogers et al. 2005).

Experimental ST females (X^{ST}/X^{ST}) were drawn from the ST-stock. All experimental females were virgins, 6-8 weeks old, and had reached sexual maturity (Baker et al. 2003). ST females were anaesthetised on ice and their eyespans were measured (see below method) to exclude small flies and limit variation in size and fecundity that could influence sperm allocation strategies in males (Cotton et al. 2015). Only large females with an eyespan >5.4mm were used in mating trials (range 5.4 – 5.8 mm).

2.3.3 Sperm competitiveness of SR and ST males

Mating trials were conducted to measure the competitiveness of SR and ST males. On the day preceding each assay, experimental females were housed singly in 500ml clear plastic containers with a moist cotton wool base. On the trial day, a single male was added to each container approximately 15mins after dawn, as this is the period during which mating is most likely (Chapman et al. 2005). Males were allowed to mate, defined as a copulation lasting ≥ 30s, as durations shorter than this are usually insufficient for sperm transfer (Rogers et al. 2006; Cotton et al. 2015). The mating duration was recorded. If no mating was observed after 15min, the male was moved to a new container with a new female. If mating still did not occur after a further 15min, the male was discarded. The original unmated female was used again and placed with another male. If this did not result in a copulation after 15min, the female was discarded.

A second mating was performed 24h later, following the same protocol. Again, if the male failed to copulate with the female after 30mins, he was replaced, and if mating still did not occur, the female was discarded. The mating failure rate was extremely low: one ST male failed to mate on day 1 (P1), one SR male failed to mate on day 2 (P2) and one female was discarded as she failed to mate with any male. Females were mated either to an SR male followed by an ST male, or an ST male followed by an SR male. Once females had been double mated, the containers were lined with a

fresh, moist cotton wool base and 1tsp puréed sweetcorn, which was collected and renewed every 2-3 days for 2 weeks. This kept larval density low, maximising survival. Bases were stored in Petri dishes at 25ºC. In total, 62 females were successfully mated twice: 30 to an SR male first and 32 to an ST male first. For ease, these matings were carried out in two batches, one week apart.

After mating, experimental males were removed and frozen, and their eyespan and thorax length were measured under a Leica microscope using ImageJ (v1.46; Schneider et al. 2012). Eyespan was defined as the distance between the outer tips of the eyes (Hingle et al. 2001). Thorax length was defined as the distance 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).

2.3.4 Progeny genotyping

Petri dishes were examined for larvae one week after collection. Larvae that had developed to be large enough to be seen by eye were transferred to a 96-well plate. Each Petri dish was then examined daily to collect the remaining growing larvae until there was no further evidence of their presence. Each well of the plate contained 100µL digestion solution (20mM EDTA, 120mM NaCl, 50mM Tris-HCL, 1% SDS, pH 8.0) and 4µL proteinase K (10mg ml−1). A standard protocol was adapted to extract and purify DNA from larvae (see **SI1: supplementary methods** for details; protocol from Burke et al., 1998). The X-linked INDEL marker *comp162710* was used to identify offspring of ST and SR fathers, due to its reported accuracy in determining phenotype (>90%; Meade *et al.* 2019). X^{ST} carries a large allele (286 bp), whereas X^{SR} carries a small allele (201 bp).

Nine females produced no offspring. A further two females produced low numbers of offspring (2, 6), of which none and one were successfully genotyped, respectively. In 7/31 cases, the mating order was P1 ST—P2 SR, and in 4/31 cases the mating order was P1 SR—P2 ST. There was no mating order effect on failure to produce genotyped offspring (Fisher exact test $P = 0.508$). These 11 females were removed from further analysis.

Not all offspring collected over the two-week period were genotyped for logistical reasons. On average, 39.8 (range 0-116) offspring were collected and 21.9 (range 0-59) offspring were genotyped per female — a total of 1161 successful PCRs. The 96-well plates were genotyped without regard to the offspring of particular females as they were collected on particular days. This approach led to a high correlation between offspring production and genotyping ($\rho = 0.872$, $n = 51$, $P < 0.001$).

2.3.5 Statistical methods

All tests were carried out in R version 4.1.2 (R core team 2021). To test if mating order or genotype affected the number of offspring sired by each male, P1:P2 offspring (the number of offspring sired by P1 relative to the number of offspring sired by the P2 male) or ST:SR offspring (the number of offspring sired by the ST relative to the number of offspring sired by the SR male) were fitted as the response variable in Generalised Linear Models (GLMs) with a binomial error distribution. The response variables were coded using the R *cbind* function. Count data of offspring sired by each

male was used in the binomial analysis rather than one male's paternity proportion to account for the variable sample size of offspring assigned to each male (larger sample sizes provide better estimates), as used by others (Dobler et al. 2022). It is not possible to treat mating order and genotype in a single “global” model combining genotype and mating order as each female’s offspring are derived from only two males who have both a genotype and mating order. Hence the binomial analysis (y_1, y_2) enters offspring either according to mating order (y_1=P1, y_2=P2) or genotype (y_1=ST, y_2=SR) in two separate analyses. As the GLMs were over-dispersed, a quasi-binomial error distribution was used.

Models of the following form were fitted to investigate the impact of mating order:
(y_1=ST offspring, y_2=SR offspring) ~ mating position of the SR male (whether the order was SR — ST or ST— SR) + fixed effects + quasi-binomial error term

And models of the following form were fitted to investigate the impact of male genotype:
(y_1=P1 offspring, y_2=P2 offspring) ~ male genotype of the P2 male (whether the second male was ST or SR) + fixed effects + quasi-binomial error term

Tests were repeated without females that had ≤ 10 offspring genotyped. The number of larvae collected and the batch in which the matings were performed were assessed as potential confounding variables. In addition, the data was split in two, considering offspring number of SR/ST or in the P1/P2 role, with linear models on genotype. In order to assess the power of the experiment to detect differences in mating order or genotype, the same GLM statistic was calculated with up to a 10-fold increase in sample size on re-sampled data (with replacement). 1,000 repeats were performed at each sample size, and resulting GLM statistics examined for evidence of difference in paternity due to mating order or genotype (see **SI4** for detailed method description and code).

The effect of male thorax length (a proxy for body size) and relative eyespan (the variation in eyespan after controlling for thorax length) were also considered in the analysis. Both traits are strongly condition-dependent and indicators of male genetic and phenotypic quality (David et al. 2000; Cotton et al. 2015; Howie et al. 2019). Whether these male trait sizes differed between genotypes was tested by fitting thorax length and relative eyespan as the response variable in linear models. In addition, whether mating duration differed by mating order and genotype was tested by fitting mating duration as a response variable in linear models, and by its inclusion as a fixed effect in GLMs with the number of offspring sired by each male. Full statistical analyses are reported in the **Supplementary Material** (**SI2** and **SI3**).

2.4 Results

2.4.1 Male fertility

In total, 62 females were reciprocally mated to males of each genotype. 51 females had offspring that were successfully genotyped (between 4-59; 27 P1 SR—P2 ST and 24 P1 ST—P2 SR) and of these, 47 females had ≥10 genotyped offspring (23 P1 SR—P2 ST and 24 P1 ST—P2 SR). For two of the reciprocal matings, one mating was 29 secs in duration; these matings were included in the subsequent analysis as in both cases the male in question produced offspring.

The distribution of proportions sired by the two males was flat, including offspring broods that were exclusively sired by either the P1 or P2 male (Figure 2.1A) or by either the ST or SR male (Figure 2.1B), with means around equality (mean ± sd P2 male = 0.522 ± 0.327, SR male = 0.575 ± 0.316). Using offspring numbers (rather than proportions), there was no effect of mating order ($F_{1,49}$ = 1.307, P = 0.259; Figure 2.2A) or genotype ($F_{1,49}$ = 0.196, P = 0.660; Figure 2.2B) on the number of offspring sired by each male. Nor was there an effect of genotype when the data was split in halves, either with the SR male in the P1 role ($F_{1,49}$ = 0.002, P = 0.963), or in the P2 role ($F_{1,49}$ = 0.434, P = 0.513). An additional test added total number of offspring collected as a covariate as it varied between females (mean ± sd; 48.196 ± 22.735 offspring; range 6 – 116 offspring), but its inclusion didn't alter the main effects of mating order or genotype (P > 0.05; see **SI2**). Likewise, the main effects were unchanged when batch number was included as a covariate (P > 0.05; see **SI2**). The results of these tests were also unchanged after the exclusion of the 4 females that had less than 10 offspring genotyped (see **SI3**).

In 11 of the 47 cases with ≥ 10 offspring genotyped, one male sired more than 0.95 of the offspring, with no difference between male mating position (4 sired by the P1 male, and 7 sired by the P2 male, $F_{1,9}$ = 0.986, P = 0.351) or male genotype (8 sired by the SR male and 3 were sired by the ST male, $F_{1,9}$ = 0.841, P = 0.383). When these extreme cases were excluded, there was still no effect of mating order ($F_{1,32}$ = 0.094, P = 0.761) or male genotype ($F_{1,32}$ = 0.589, P = 0.448) on the number of offspring sired.

To assess the power of the data to detect differences, the data was resampled (with replacement) using a 1 to 10-fold increase in sample size compared to the original data (1,000 repeats for each fold increase, **SI4**). As expected, the fraction of runs with significant differences (at P < 0.05) increased with sample size. The increase was marked for mating order with a P2 advantage evident at a 4-fold increase in sample size (95% confidence interval 0.207 - 4.567 in favour of P2). However, the increase was minor for genotype and there was no advantage to either genotype even with a 10-fold increase in sample size (95% confidence interval -0.789 - 3.667 in favour of SR).

2.4.2 Male trait size and mating duration

Thorax length was smaller in SR than ST males (mean ± se, SR = 2.190 ± 0.023mm, N = 49, ST = 2.297 ± 0.025mm, N = 50; $F_{1,97}$ = 9.783, P = 0.002). Eyespan is strongly colinear with thorax ($F_{1,97}$ = 167.242, P < 0.001) and was likewise smaller in SR males (SR = 7.304 ± 0.111mm, ST = 7.897 ± 0.115mm; $F_{1,97}$ =13.766, P < 0.001; Figure 2.S1). However, relative eyespan did not differ between genotypes ($F_{1,96}$ = 3.734, P = 0.056; Figure 2.S2). As thorax length differed between genotypes, it was added as a covariate, but there was still no effect of mating order ($F_{1,44}$ = 1.161, P = 0.287) or male genotype ($F_{1,44}$ = 0.369, P = 0.547) on the number of offspring sired by each male.

Mating duration did not differ with mating order (mean ± se, P1 = 63.94 ± 3.43 sec, P2 = 73.45± 3.43 sec; $F_{1,100}$ = 0.943, P = 0.334) or genotype (ST = 60.82± 2.36 sec, SR = 76.57sec ± 9.42sec, $F_{1,100}$ = 2.627, P = 0.108). Mating duration did not affect the number of offspring sired by the P2 male ($F_{1,48}$ = 0.022, P = 0.882), but P1 males with

a shorter mating duration sired a greater number of offspring ($F_{1,48}$ = 4.082, P = 0.049). Mating duration did not affect the number of offspring sired by the SR male ($F_{1,48}$ = 0.246, *P* = 0.622) or the ST male $F_{1,48}$ = 3.366, *P* = 0.073). Given its inconsistent effect on the number of offspring sired, the mating durations of the two males were added as covariates, but there was still no effect of mating order ($F_{1,47}$ = 1.208, P = 0.277) or genotype ($F_{1,47}$ = 0.071, P = 0.791) on the number of offspring sired.

2.5 Discussion

Our study provides little support for the idea that males carrying X-linked meiotic drive are at a disadvantage under sperm competition due to sperm loss and other deleterious effects of meiotic drive on sperm function (Courret et al. 2019; Verspoor et al. 2020). Here, the paternity of SR males did not differ from ST males overall, nor in the P1 or P2 positions considered separately. This challenges the general pattern which has been reported across the Diptera (Policansky 1974; Newton et al. 1976; James and Jaenike 1990; Hurst and Pomiankowski 1991; Presgraves et al. 1997; Jiggins et al. 1999; Price et al. 2008a; Dyer and Hall 2019). It is also in opposition to previous evidence of lower drive male paternity in stalk-eyed fly double-mating experiments, which were discussed in the Introduction (Wilkinson and Fry, 2001; Wilkinson *et al.* 2006). Our results are robust to a number of potential confounding factors: matings were performed between flies from the same population, offspring paternity was assessed using highly accurate genetic markers, larvae were used to assess paternity – which reduces the impact of lower egg-adult viability in SR females – and double matings were carried out with SR males in the first and second mating position to reliably assess sperm precedence. Furthermore, the findings here align with those of Meade *et al.* (2019, 2020), who showed that sperm numbers transferred to females and the resulting fertility do not differ in single matings by SR and ST males.

Our results do not invalidate previous findings, which likely reflect genuine experimental differences. The study of Wilkinson and Fry (2001) was carried out on the closely related species *T. whitei*, which also carries X-linked SR meiotic drive that is thought to have evolved prior to the divergence of these two species (Presgraves et al. 1997; Meier and Baker 2002). Genetic markers for drive have not been identified in *T. whitei* (G. S. Wilkinson, personal communication), implying a small inversion is associated with drive in this species, unlike the multiple inversions that cover most of the *T. dalmanni* SR X chromosome (Wilkinson et al. 2006; Christianson et al. 2011; Reinhardt et al. 2014, 2023; Paczolt et al. 2017). This means that few X-linked genes are in linkage disequilibrium with those that control drive, potentially limiting the possibility of compensatory testes enlargement and explaining why *T. whitei* drive males have reduced fertility under sperm competition. The second study of Wilkinson *et al.* (2006) used a similar double mating design in *T. dalmanni* (although only with SR males in the P2 role). As in this study, it reported no difference between SR and ST success in mixed paternity broods. However, in single-parent broods (where only one male fathered offspring), there were 11 from the ST male and only 3 from the SR male (rate 14/40 = 35%). In this study we found the pattern was reversed with 3 from the ST male and 8 from the SR male (rate 11/51 = 22%). There were experimental design differences that might be important. In particular, Wilkinson *et al.* (2006) took experimental males from mixed sex cages with no control over prior mating, whereas we kept males without females for several days to allow their accessory glands to return to full size (Rogers et al. 2005). This could explain the higher rate of single

parent broods in Wilkinson *et al.* (2006). However, combining across these two studies, we conclude that there can be little confidence that there is a large deficit in SR male single-parent broods. This is consistent with previous work which showed no difference in the failure rate of sperm transfer to the spermatheca of females mated once either to ST or SR males (Meade *et al.* 2019).

In line with earlier work on sperm competition in stalk-eyed flies, there was no effect of mating order on paternity, suggesting that the sperm of the first and second male simply mix and there is no sperm precedence in *T. dalmanni* (Wilkinson and Fry 2001; Corley et al. 2006; Bellamy 2012). Corley *et al.* (2006) found evidence of a trimodal P2 distribution, centred around equal paternity as well as a strong bias to either the first or second male (double matings with ST males). This contrasts with the flat distribution shown here (Figure 2.1). The difference could be due to the multiple mating design used by Corley *et al.* (2006), in which each female was mated three times with the first and second males. A trimodal pattern was also reported in a double mating design in the distantly related South African stalk-eyed fly species *Diasemopsis meigenii*, where extreme paternity bias was explained by the failure of sperm transfer after a single copulation (Bellamy 2012). Whatever the explanation, none of these studies support the idea of a competitive advantage associated with mating position in stalk-eyed flies.

The lack of difference found in this study may be limited by sample size (n = 51), like all statistical comparisons. We addressed this by re-sampling the data with up to a 10-fold inflation in sample size. This increased the likelihood of finding a mating order difference (favouring P2 at a 4-fold increase in sample size) to a much greater extent than a genotypic difference (no difference even at a 10-fold increase in sample size). Given that these comparisons rely on the same distribution of the data, they allow us to conclude that if there is a difference in the paternity gain due to genotype, it is of a lower order than that relating to mating order, and there is no evidence to support the hypothesis of a competitive disadvantage associated with drive (if anything, the data favours a SR advantage). Our approach is not wholly satisfactory as re-sampling maintains the distribution of offspring genotyped per female which was variable (95% confidence range 19-26), although to some extent this is accounted for by the binomial tests. A re-sampling of this distribution would inevitably require further assumptions and end-up being contrived. We adopted this approach to frame our conclusions within the limitations of the data collected.

In this study of *T. dalmanni,* sperm competition was assessed under low-stress conditions. Virgin females were mated to two males separated by a 24-hour period. Experimental males were not virgins but had been kept for several days in single-sex groups. The objective was to assess SR and ST males under standardised conditions as a first step to understanding how SR males perform under sperm competition. This is a highly specific experiment, designed to test whether a male gains an advantage after a single competitive mating, either because there is first/last male precedence or variation due to genotype. In the wild, competitive conditions are more complex. Males form leks with multiple females at dusk and then mate in a short period at dawn before dispersal, with occasional matings interspersed during daylight hours (Wilkinson et al. 1998b; Chapman et al. 2005; Cotton et al. 2010, 2015). Females mate repeatedly in a life span that can extend over several months (Wilkinson et al. 1998b; Reguera et al. 2004). Multiple matings are required as males transfer low numbers of sperm per

ejaculate (Wilkinson et al. 2005; Rogers et al. 2006; Meade et al. 2019), several matings are needed to reach maximum fertility (Baker 2001) and sperm usage leads to a quick drop in female fertility over time (Wilkinson et al. 1998a; Meade et al. 2017). Future experiments need to assess the success of single SR and ST male matings in females with a background of multiple mating, closer to the conditions found in nature. There may be differences when female sperm storage organs are saturated compared to the situation with double mating when females are below maximal fertility (Baker 2001). In addition, it will be important to assess the effect of the mating rate which is lower in SR males (Wilkinson et al. 2003; Rogers et al. 2008; Meade et al. 2020). SR males may be less able to compete in populations at high density where there are multiple opportunities to mate, even though sperm transfer does not differ between genotypes in sequential matings over a short period of time (Meade et al. 2019). These further studies will provide a more comprehensive assessment of sperm competition as a factor contributing to the fertility of drive males and its consequences for the frequency of SR in wild populations.

In summary, we demonstrate that meiotic drive is not always associated with male fertility reduction under conditions of sperm competition, even though drive destroys half of carrier-male sperm. The lack of a fertility cost potentially contributes to the relatively high frequency of meiotic drive, which is around 20% in wild populations in *T. dalmanni* (Wilkinson et al. 2003; Cotton et al. 2014; Paczolt et al. 2017). This pattern is unlike other species where drive males do poorly under sperm competition and the spread of drive is reliant on a high frequency of monandrous matings (Price et al. 2008b; Courret et al. 2019; Dyer and Hall 2019). The absence of a fertility cost is likely an evolved response to the loss of sperm caused by meiotic drive, which is supported by the finding in *T. dalmanni* that drive male testes are larger at eclosion, have higher growth rates and are considerably enlarged at maturity (Meade et al. 2020; Bradshaw et al. 2022). We provide strong evidence against the consensus that drive males are outperformed by non-drive males under sperm competition – which suggests that other species should be investigated for evidence of mitigation of drive fertility costs.

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2.7 Figures

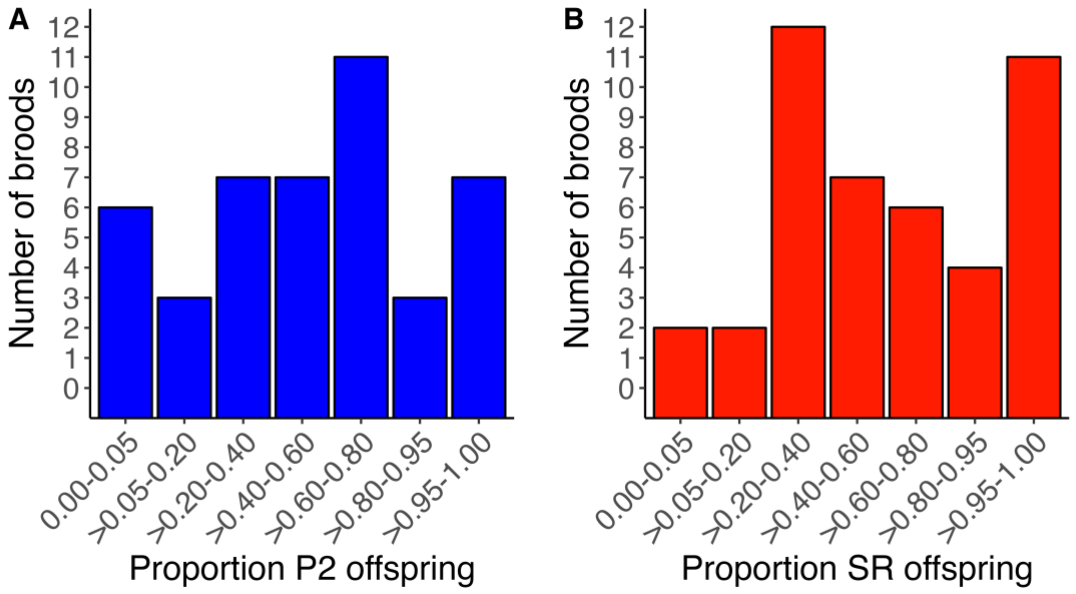

Figure 2.1: A) *The distribution of P2, the proportion of offspring sired by the second male, is shown per brood (blue).* ***B)*** *The distribution of the proportion of offspring sired by the SR male is shown per brood (red).*

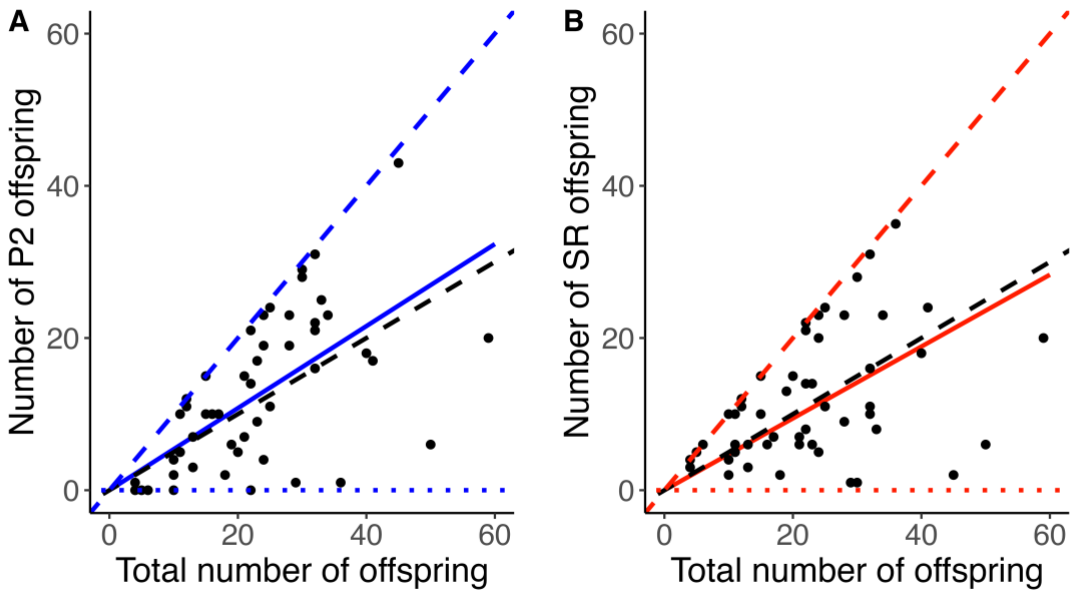

Figure 2.2: *In **A)** Points correspond to the number of P2 offspring against the total number of offspring per brood. The solid blue line represents the regression of the number of P2 offspring against the total number of offspring (ß = 0.539; intercept constrained to zero). The blue dashed line represents P2 = 1.000 (all P2 offspring), the black dashed line represents P2 = 0.500 (equal P1 and P2 offspring), and the blue dotted line represents P2 = 0.000 (all P1 offspring). In **B)** points correspond to the number of SR offspring against the total number of offspring per brood. The solid red line represents the regression of the number of SR offspring against the total number of offspring (ß = 0.472; intercept constrained to zero). The red dashed line represents SR = 1.000 (all SR offspring), the black dashed line represents SR = 0.500 (equal SR and ST offspring), and the red dotted line represents SR = 0.000 (all ST offspring).*

Chapter 3. Meiotic drive does not impede success under high sperm competition in *Teleopsis dalmanni*

3.1 Abstract

In *Teleopsis dalmanni*, male carriers of X-linked drive lose half their sperm — those that bear the Y chromosome. Hence, they have been predicted to sire fewer offspring under sperm competition with non-drive males. However, recent work has shown they transfer the same numbers of viable sperm per ejaculate to female sperm storage organs because they compensate for sperm loss by investing in larger testes. As a result, drive males can compete with non-drive males in a singly mated female. However, wild *T. dalmanni* females are sperm limited, and so mate multiply to maximise their reproductive output. Whether drive males can maintain their reproductive success in a multiply mated female has yet to be investigated. Here, we maximally mate females with a drive or non-drive male, then singularly with a male of the opposite genotype, and examine the paternity of the offspring she produces. In doing so, we determine the offensive and defensive capabilities of drive and non-drive male sperm. We find both drive and non-drive males perform best in the first mating position, where they mate multiple times with the female. Importantly, male genotype did not affect reproductive success — drive males are not disadvantaged compared to non-drive males on encountering a female full of rival male sperm. The competitiveness of drive male sperm is doubtless a factor that contributes to the high prevalence of the SR variant in wild populations of *T. dalmanni.*

3.2 Introduction

In the wild, *Teleopsis dalmanni* remate regularly over a period of many weeks. Females are sperm-limited and so must mate multiply to maximise their reproductive output (Baker 2001; Cotton et al. 2010). In the laboratory, females will remate several times a day and have lifespans of months (Reguera et al. 2004). The results presented in Chapter 2 show that in reciprocal double mating trials where each male is given a single mating opportunity with a female, SR males are equally as likely as ST males to sire offspring. In order to elucidate if this pattern carries over into sperm competition in wild populations of *T. dalmanni*, it is essential to determine whether SR males also do as well in maximally mated females. In this chapter, experiments are carried out to ask whether the defensive and offensive qualities of SR sperm differ from those of ST sperm. This is tested under strong competition when a female is maximally mated with a single type of male and then exposed to a single copulation with the other genotype of male. Does SR male sperm succeed when confronted with a female who has previously mated many times? As well as being of interest to the particular situation found in stalk-eyed flies, the experiments address a widely held view that drive males are particularly poor at performing under the increased sperm competition resulting from high rates of female remating, which leads to selection for increased levels of female polyandry (Jaenike 1996; Zeh and Zeh 2001; Price et al. 2008a,b).

In experiment 1, a female was initially housed with a ST male for a long period (one week). An extended mating period was chosen as previous experiments show that high fertility levels are only attained after a female has mated many times (Baker 2001). This can be achieved in the laboratory when females are housed with males under constrained conditions like those implemented in this study because male *T. dalmanni* will mate several times per day, and females show very little resistance to male mating attempts (Chapman et al. 2005). In the wild, mating mainly occurs at dawn on leks sites. Females mate once with the lek holder male and then disperse. However, females will also mate sporadically during the day and sometimes at dusk when females join lek sites (Chapman et al. 2005). This pattern of mating is repeated daily. The upshot is that typical adult females in the wild have mated multiple times, and this was achieved in the experimental design by allowing a female to mate with a ST male over a 7-day period. We then assessed the paternity gain of a SR that was mated once to these females. The performance of the SR male in the P2 position was measured as the number of offspring sired, thereby determining the “offensive” capability of SR sperm was determined. The rationale for starting with this combination is that the SR genotype is less common than ST in the wild (SR is maintained at around 20% in *T. dalmanni* populations), so SR male matings mainly occur with females that have already mated many times, mainly with ST males. We test to see whether SR sperm can displace ST sperm in a female whose sperm storage organs are already charged with ST sperm.

A complementary experiment was also undertaken with the pattern of mating reversed. In this case, a female was initially housed with an SR male for a long period (one week) and then mated once to an ST male. As such, this experiment examines the “defensive” capabilities of SR sperm. That is, how well does SR sperm from prior matings resist displacement by rival sperm from further matings by other males, which are likely to be the more common ST males? In this chapter, I report on my initial analysis of this experiment. Once complete, this work will contribute to our

understanding of whether SR males can sire offspring when in strong competition with ST males and, more widely, if this could be a factor in maintaining the selfish SR variant at a stable frequency in wild *T. dalmanni* populations.

3.3 Methods

3.3.1 Stocks

Flies for the standard stock (ST-stock) population carry only the wildtype X chromosome (X^{ST}). They were collected (by S. Cotton and A. Pomiankowski) in 2005 from the Ulu Gombak valley, Peninsular Malaysia (3°19′N 101°45′E). They have since been maintained in high-density cages (> 200 individuals) to minimise inbreeding and are regularly monitored to ensure they do not contain meiotic drive.

The meiotic drive stock (SR-stock) population is composed of females that are homozygous for a sex ratio distorting X chromosome (X^{SR}). They were derived from flies collected in 2012 (by A. Cotton and S. Cotton) from the same location as the ST-stock. X^{SR}/Y males produce 100% female offspring due to transmission distortion. The X^{SR} female stock is maintained by crossing X^{SR}/X^{SR} females with X^{ST}/Y males to produce X^{SR}/Y drive males, who are then mated to the X^{SR}/X^{SR} females to generate the next generation of the SR-stock females. The outcrossing to ST males from the ST-stock ensures that the two stocks only differ in their X chromosomes and are homogenised for autosomal content.

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

3.3.2 Experimental fly generation

Experimental ST (X^{ST}/Y) and SR males (X^{SR}/Y) were drawn from the ST-stock and SR-stock, respectively. They were housed separately in cages of ~50 individuals until sexually mature, in groups of similar age (6-8 weeks). ST-stock females were added to these cages at an equal sex ratio for > 3 days to allow males to mate and lose their virgin status. The females were then removed and discarded. Experimental males were then kept in single-sex groups for a further 3-6 days to allow their accessory glands to return to full size (Rogers et al. 2005).

Experimental ST females (X^{ST}/X^{ST}) were drawn from the ST-stock. All experimental females were virgins, 6-8 weeks old, and had reached sexual maturity (Baker et al. 2003). ST females were anaesthetised on ice, and their eyespans were measured (see below method). Small flies were excluded to limit variations in size and fecundity that could influence sperm allocation strategies in males (Cotton et al. 2015). Only large females with an eyespan >5.4mm were used in mating trials (range 5.4 – 5.8mm).

3.3.3 SR and ST male sperm competitiveness with a maximally mated female

To allow the experimental female to become maximally mated, she was housed with a single ST or SR male in a 500ml clear plastic container with a moist cotton wool base for 7 days. On the morning of day 7, the male was removed and frozen for measuring.

Three days later, a single male was added to each container approximately 15mins after dawn, as this is the period during which mating is most likely (Chapman et al. 2005). Test males were allowed to mate once, defined as copulation lasting ≥30s as durations shorter than this are usually insufficient for sperm transfer (Rogers et al. 2006; Cotton et al. 2015). If the male failed to copulate with the female after 30mins, he was replaced, and if mating still did not occur with the second male within the 15 minute period, the female was discarded. Females were mated either to an SR male followed by an ST male or an ST male followed by an SR male. Post-mating, experimental males (both the first and second male) were immediately removed and stored at -20ºC in 1.5mL Eppendorf tubes filled with 100% ethanol. These males were later measured under a Leica microscope using ImageJ (v1.46; Schneider et al. 2012). Eyespan was defined as the distance between the outer tips of the eyes (Hingle et al. 2001). Thorax length was defined as the distance 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).

Once females had been double mated, the containers were lined with a fresh, moist cotton wool base with 1tsp puréed sweetcorn, which was collected and renewed every 2-3 days for 2 weeks. Females laid their eggs on the base. Given the replacement of the base every 2-3 days, larval density was kept low, maximising survival. Bases removed were placed in Petri dishes and incubated at 25ºC for 14 days to develop into pupae. Each petri dish was then uncovered and placed inside a 1.5L clear plastic container to allow flies to eclose. Emerging adult flies were collected daily until all pupae had hatched, then stored at -20ºC in 1.5mL Eppendorf tubes filled with 100% ethanol. In total, 45 females were successfully mated with two males: 23 to an SR male first and 22 to an ST male first. For ease, each combination of matings was carried out in two batches, one week apart.

3.3.4 Progeny genotyping

SR males from our SR-stock cause complete meiotic drive, and all Y-bearing sperm are dysfunctional (Meade et al. 2019). This means that they only sire female offspring. Consequently, all male offspring in the experiments are derived from the ST male.

In order to assign paternity to female offspring, it was necessary to genotype them using comp16710, an INDEL marker indicative of the SR or ST X chromosome (Meade et al. 2019). The extraction and purification of female offspring DNA was achieved using an adaptation of a standard protocol (Burke et al. 1998). Half thoraxes were dissected from each female, diced, and transferred into a well of a 96-well plate containing 100µL DISGOL solution. The remainder of each fly was returned to its original Eppendorf tube and stored at 20ºC to act as a backup in case of any future re-extraction. 4µL proteinase K was then added to each well, and plates were incubated on a PCR machine at 55ºC for ~16h to break down tissues. The following day, 35µL 4M ammonium acetate was added to each sample to precipitate out proteins, and the plates were chilled on ice for 5mins. The plates were then spun at 4450rpm, 4ºC for

60 min. Next, the DNA was precipitated out by transferring 80µL of the supernatant from each sample to a new plate containing 80µL isopropanol per well. Centrifugation at 4450rpm, 4ºC for 60mins pelleted out the DNA. The supernatant was discarded, and the DNA pellets were washed by adding 100µL 70% ethanol and spinning at 4450rpm for 30min. The ethanol was then removed, and the plates left to air dry for 1hr before adding 30µL TE buffer to each sample and incubating at 37ºC for 30mins to re-dissolve the DNA. Samples were stored at -20ºC prior to PCR analysis.

The X-linked INDEL marker *comp162710* was used to identify offspring of ST and SR fathers, due to its reported accuracy in determining phenotype (>90%; Meade *et al.* 2019). X^{ST} carries a large allele (286 bp), whereas X^{SR} carries a small allele (201 bp). PCR reaction conditions used were the same as those detailed in chapter 2 (see Chapter 2: **SI1**)

3.3.5 Statistical Methods

All tests were carried out in R version 4.1.2 (R core team 2021). First, the proportions of offspring sired by the ST/SR male in the P1/P2 position were compared using Welch's student's t-tests. Proportions were calculated using offspring sex ratios (i.e., the proportion of males), offspring genotypes (genotyping results from the samples of female offspring) and a combination of male offspring and the predicted number of female offspring produced by each genotype.

To test if mating order or genotype affected the number of offspring sired by each male, P1:P2 offspring (the number of offspring sired by P1 relative to the number of offspring sired by the P2 male) or ST:SR offspring (the number of offspring sired by the ST relative to the number of offspring sired by the SR male) were fitted as the response variable in Generalised Linear Models (GLMs) with a binomial error distribution. It is not possible to treat mating order and genotype in a single "global" model as the binomial analysis (y_1, y_2) enters offspring according to mating order (y_1=P1, y_2=P2) or genotype (y_1=ST, y_2=SR). As the GLMs were over-dispersed, a quasi-binomial error distribution was used. The number of larvae collected and the batch in which the matings were performed were assessed as potential confounding variables.

Models of the following form were fitted to assess the impact of male genotype on paternity:
(y_1=P1 offspring, y_2=P2 offspring) ~ P2 male genotype + fixed effects + quasi-binomial error term

And models of the following form were fitted to assess the impact of mating order on paternity:
(y_1=ST offspring, y_2=SR offspring) ~ SR male mating position + fixed effects + quasi-binomial error term

The effect of male thorax length (a proxy for body size) and relative eyespan (the variation in eyespan after controlling for thorax length) were also considered in the analysis. Both traits are strongly condition-dependent and indicators of male genetic and phenotypic quality (David et al. 2000; Cotton et al. 2015; Howie et al. 2019).

Whether these male trait sizes differed between genotypes was tested by fitting thorax length and relative eyespan as the response variable in linear models of the form:
Y ~ male genotype + error term
Where the response variable, Y, is the male trait (thorax length/eyespan/relative eyespan).

In addition, whether mating duration differed by male genotype was tested by fitting mating duration as a response variable in linear models of the same form as above, and by its inclusion as a fixed effect GLMs investigating the effect of male genotype on paternity (outlined above). Full statistical analyses are reported in Chapter 3: Supplementary Information.

3.4 Results

In total, 44 reciprocal matings were performed successfully: 22 SR—ST and 22 ST—SR (labelled P1 – P2). The number of offspring collected per female was mean ± SD = 65.500 ± 42.200 with a range of 3-174. A sample of female offspring from each mating was selected for genotyping to determine paternity. Female offspring (between 3-77 per female parent) were successfully genotyped across the two cross types (16 SR – ST and 21 ST – SR). 36 females had greater than 10 genotyped offspring (16 SR – ST and 15 ST – SR).

Combining male and estimated female offspring sired by each male, there was considerable variation in the proportion of P2 offspring between broods (mean ± SD = 0.316 ± 0.327, Figure 1A). In 9/37 broods, the P2 male gained very little paternity (<0.05), whereas in 2/37 the P2 male gained almost complete paternity (>0.95). Although the distribution was skewed towards first male paternity, which reflects the multiple mating opportunities that male had, the P2 male could nonetheless achieve almost complete dominance in paternity from a single mating. There was likewise considerable variation in the proportion of offspring sired by the SR male between broods (mean ± SD = 0.474 ± 0.376, Figure 1B). In 3/37 broods, the SR male gained very little paternity (<0.05), whereas in 8/37 the SR male gained almost complete paternity (>0.95). This distribution of SR paternity was more even compared to that of P2 male (Figure 3.1).

Using offspring numbers attributable to either male, rather than the proportion of paternity, there was no effect of genotype on the number of offspring sired by each male ($F_{1,35} = 0.030$, $P = 0.862$; Figure 3.3). The total number of offspring was positively associated with the number of P2 offspring ($F_{1,34} = 4.859$, $P = 0.034$); however, its inclusion as a covariate did not alter the lack of a difference in paternity due to genotype ($F_{1,36} = 2.69$, $P = 0.110$). The batch number had no effect on the number of offspring sired ($F_{1,34} = 8.447\times10{-}5$, $P = 0.993$), and its inclusion did not alter the lack of a difference in paternity due to genotype ($P > 0.05$). When adding ST thorax length as a covariate, it did not affect P2 paternity ($F_{1,32} = 0.861$, $P = 0.36$); however, SR thorax length was positively associated with the number of offspring sired ($F_{1,30} = 6.853$, P= 0.014). Neither SR nor ST residual eyespan affected the number of offspring sired by the P2 male when added as covariates (SR males: $F_{1,28} = 1.737$, $P = 0.198$, ST males: $F_{1,32} = 0.861$, $P = 0.360$). Including any male trait size covariate did not alter the lack of difference in paternity due to genotype ($P > 0.05$ in all cases). See **Chapter 3 SI** for all models and effect sizes.

In contrast to genotype, mating position strongly affected paternity; the P1 male sired more offspring than the P2 male (mating position $F_{1,35} = 14.383$, $P < 0.001$; Figure 3.2). Neither the total number of offspring ($F_{1,34} = 4.859$, $P = 0.034$) nor batch number ($F_{1,34} = 8.447 \times 10{-}5$, $P = 0.993$) affected the number of offspring sired by the P2 male. Neither the P1 male thorax length ($F_{1,32} = 2.987$, $P = 0.094$) nor the P2 male thorax length ($F_{1,33} = 1.389$, $P = 0.247$) influenced the number of offspring sired by the P2 male. The relative eyespan of the P1 male had no effect on the number of P2 offspring ($F_{1,28} = 0.730$, $P = 0.400$); however, the relative eyespan of the P2 male did ($F_{1,32} = 7.953$, $P = 0.008$). Including any male trait size as a covariate did not alter the presence of a strong P1 male advantage ($P<0.005$). In addition, though the mating duration of the SR male was longer than the ST males ($F_{1,41} = 6.290$, $P = 0.016$), the mating duration of the P2 male did not alter the paternity gained ($F_{1,33} = 2.949$, $P = 0.095$). See **Chapter 3 SI** for all models and effect sizes.

3.5 Discussion

It is a naïve expectation that drive males perform less well than wildtype males. In Chapter 2, I showed that this expectation does not hold true in *T. dalmanni* under a simple, reciprocal, double mating design, where drive and wildtype males have one mating each. Nor was there evidence of last or first male sperm precedence. A criticism that could be levelled at that experiment is that it does not represent the ecological mating system of stalk-eyed flies, which involves daily multiple matings by males and females over a lifetime of several months (Chapman et al. 2000; Reguera et al. 2004). In order to address this point of view, experiments were performed in which a female was first mated multiple to a single male over a week-long period, and then once to another male with a different genotype. The experiments were reciprocal with the ST and SR males either in the defence (prior multiple mating) or offence (single last mating) roles. Once again, these experiments show no evidence of a genotype effect on paternity – drive and wildtype males are equally successful in the defence and offence roles. However, the experiments do show a strong first male sperm precedence. They also reveal considerable variation in the success of the two males, with the second male sometimes gaining hardly any success and other times garnering almost complete paternity.

Why does the SR genotype do no better or no worse than the ST genotype in these multiple mating assays in contrast to other meiotic drive systems? The clear implication is that despite drive causing the dysfunction of half of a male's sperm, natural selection has acted to rectify this deficit and re-stocked the number of sperm that can be delivered in a single copulation. This is an extraordinary finding but is supported by various observations and experiments. Firstly, SR male testes are grossly exaggerated in size (Meade et al. 2020; Bradshaw et al. 2022). SR males deliver the same number of sperm per copulation as ST males, and this pattern is replicated over several copulations (Meade et al. 2019). SR male sperm is no less able to fertilise eggs and produce offspring than ST sperm (Meade et al. 2020). In double mating competitive copulations, SR males gain as much fertility regardless of whether they are the first or second to mate (Chapter 2). Now, finally, we have shown that SR males gain as much fertility as ST males when mating with females that have already mated many times. All these lines of evidence point in one direction, that there

is no difference in the fertility of drive or wildtype males. Of course, we have not examined all possible permutations of matings, competitiveness, social environments, food conditions etc., and there may be some situations in which drive male fertility can be exposed as inadequate compared to wildtype. The search will go on to address those situations which are likely to typify the environment under which stalk-eyed flies exist in nature. Nevertheless, there is a general conclusion that can be drawn that SR males in *T. dalmanni* stalk-eyed flies have evolved to largely ameliorate the cost of meiotic drive on their fertility. This does not mean that there are no fitness costs of carrying the X^{SR} chromosome. Rather, the major cost of sperm loss has been immensely damped down.

The experiments carried out here show first male sperm precedence. This is not unexpected, as the first male was corralled for a week with a single female. Although we did not explicitly document matings during this period, prior work shows that stalk-eyed flies will mate repeatedly under these conditions (Baker 2001; Chapman et al. 2005). This means the last male to mate would likely have encountered a female whose sperm storage organs were full. Yet this male gained an average of 31.5% of fertility when SR and 35.9% of fertility when ST, and these values did not differ with genotype. This relatively high P2 male reproductive success suggests that sperm mixing is not the norm in stalk-eyed flies, as the P1 male's sperm ought to be more numerous. Furthermore, the success of the last male showed a very high variance, with some cases where he sired all offspring and other cases where he sired almost none (Figure 3.1A). This may reflect variation in the capability of the first male, with some males simply mating less, allowing the last male a greater fraction of fertility. Perhaps in some cases the first male was infertile, though the rate of this is low and all females included in this study were checked for offspring production due to mating with the first male (Meade et al. 2019). Another possibility is that the variation reflects the success of the second male in transferring sperm to females or in displacing sperm that pre-exists in the female sperm storage organs. A final possibility is that there is female control over which sperm are used for fertilisation, which falls under a type of post-copulatory selection known as cryptic female choice (Firman et al. 2017). To date, there is no evidence of this in stalk-eyed flies. All these ideas suggest approaches for the future. In this experiment, we also measured male body size and eyespan of both males, as well as the mating duration of the last male. These will be investigated further in the future.

In summary, this study builds on the work of the previous chapter, providing strong evidence that SR male sperm can compete with ST male sperm under high competition —this is doubtless a factor that contributes to the high prevalence of SR in the wild. A situation such as this has yet to be reported in other Dipteran species. Further research should focus on exploring the extreme variability in P2 male fertility, to elucidate if this arises due to cryptic female choice.

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3.7 Figures

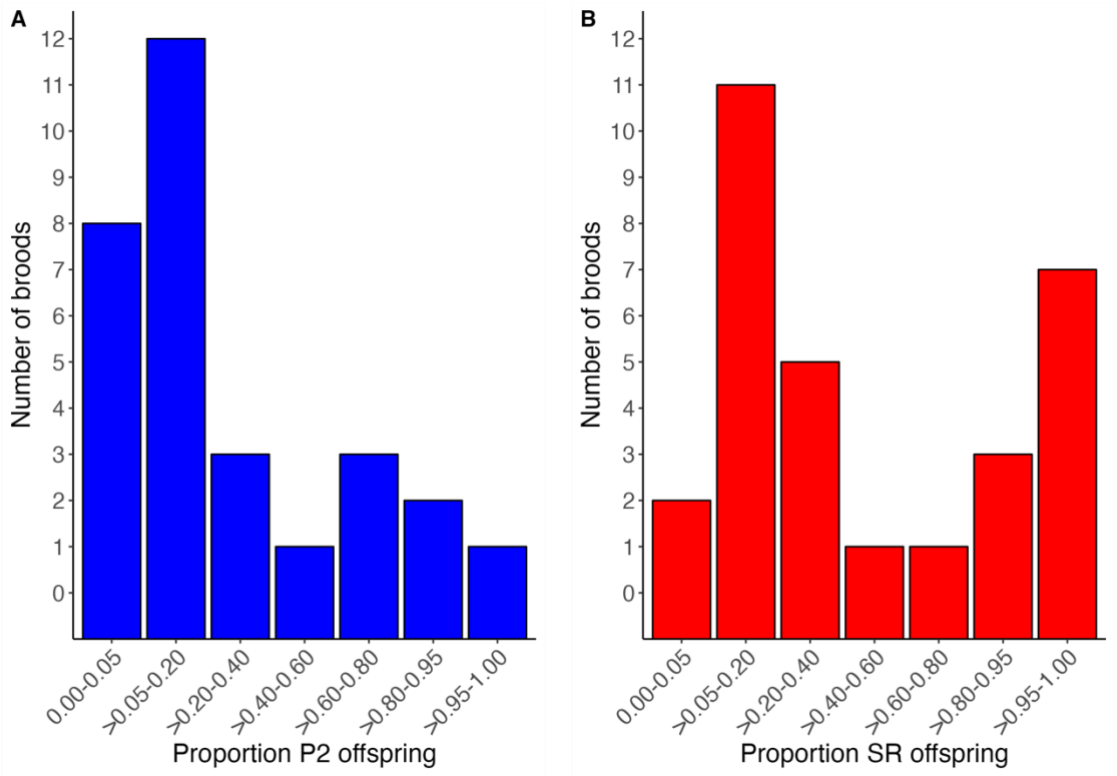

Figure 3.1 A) The distribution of the proportion of offspring sired by the second male, P2, is shown per brood in blue. B) The distribution of the proportion of offspring sired by the SR male is shown per brood in red.

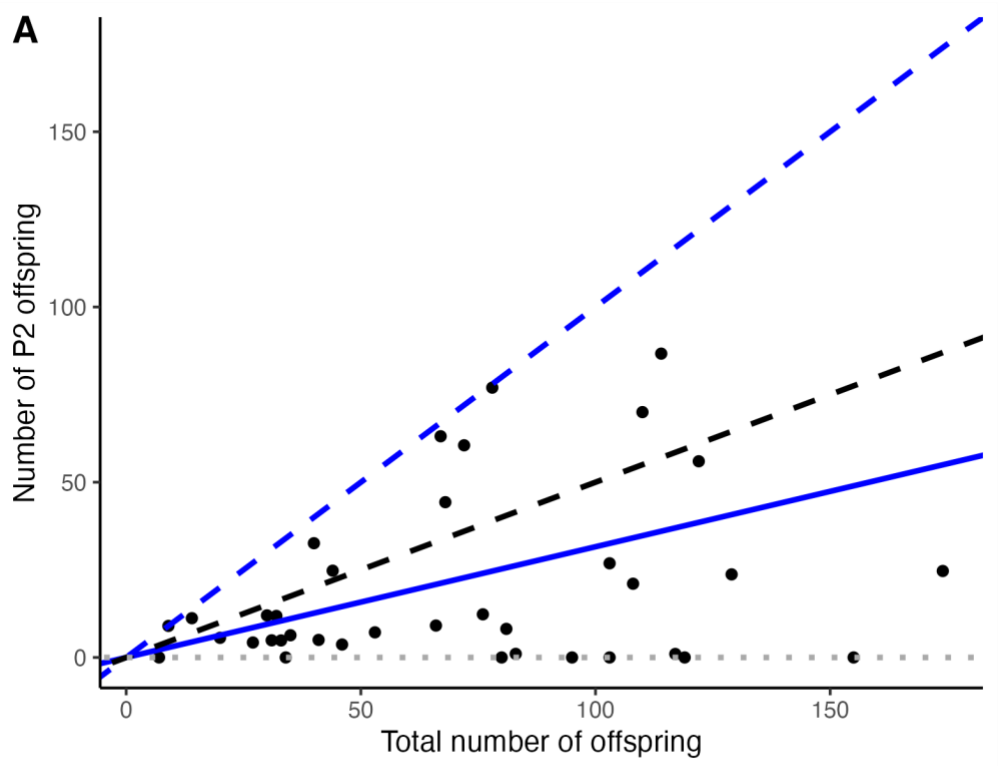

Figure 3.2: points correspond to the overall number of P2 offspring per brood. The solid red line represents the actual proportion of P2 offspring sired per brood (mean ± SD, 0.316 ± 0.327). The blue dashed line represents P2 = 1.000 (all P2 offspring), the black dashed line represents P2 = 0.500 (equal P1 and P2 offspring), and the grey dashed line represents P2 = 0.000 (only P1 offspring). There is a strong P1 male advantage.

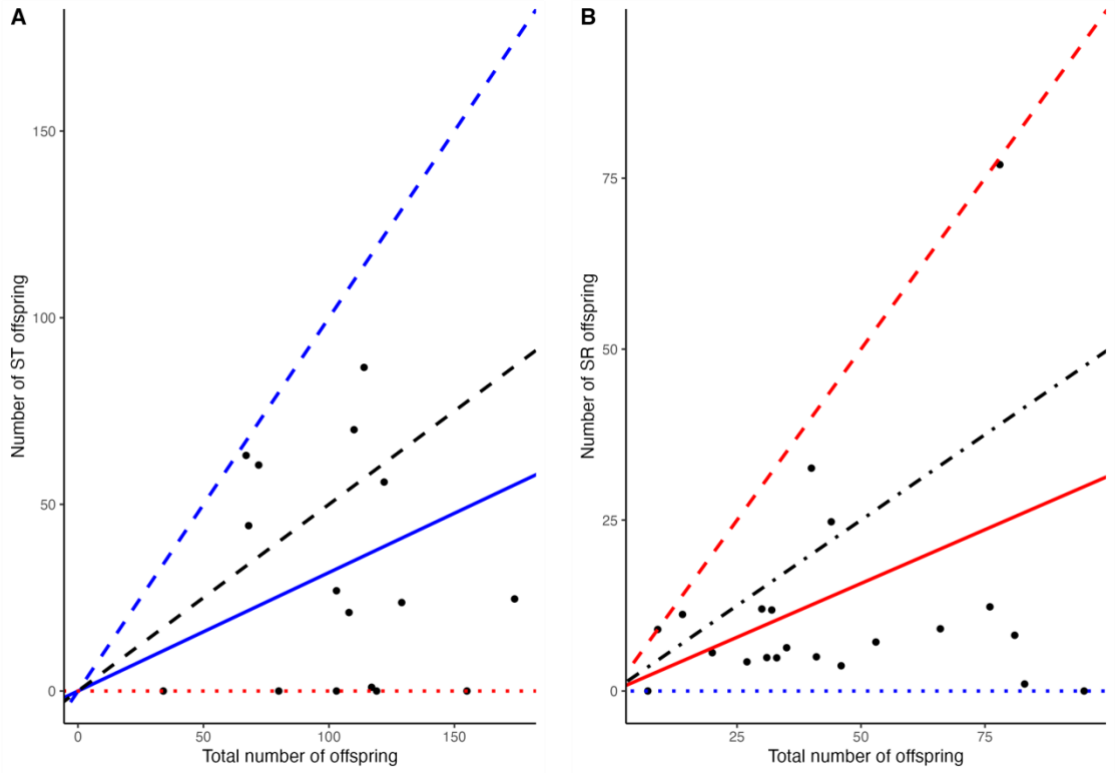

Figure 3.3: the number of offspring sired by each male genotype in the P2 position is shown. Males of each genotype sired similar proportions of offspring. A) points correspond to the number of ST offspring against the total number of offspring per brood. The solid blue line represents the actual proportion of ST offspring sired per brood (mean ± SD, 0.315 ± 0.323). The blue dashed line represents ST = 1.000 (all ST offspring), the black dashed line represents ST = 0.500 (equal SR and ST offspring), and the red dashed dotted represents ST = 0.000 (all SR offspring). B) points correspond to the number of SR offspring against the total number of offspring per brood. The solid red line represents the actual proportion of SR offspring sired per brood (mean ± SD, 0.317± 0.343). The red dashed line represents SR = 1.000 (all SR offspring), the black dashed line represents SR = 0.500 (equal SR and ST offspring), and the blue dotted line represents SR = 0.000 (all ST offspring).

Chapter 4. The consequences of X-linked meiotic drive for female reproductive fitness

4.1 Abstract

In Teleopsis dalmanni, the X-linked driver, Sex Ratio (SR), is maintained at a high frequency of around 20% in wild populations. SR is associated with fitness costs in both sexes, including reduced egg-adult survival. Adverse fitness effects in females are particularly important for stabilising the frequency of SR in wild populations, as they can occur in a dose-dependent manner, causing recessivity and, thus, negative frequency-dependent selection. Much of the study of the effects of SR has focussed on male reproduction, which is understandable, as sperm-killing affects males. However, are other dose-dependent fitness effects associated with SR for female traits? Here, we examine how SR impacts two crucial components of female reproductive fitness: time to sexual maturity and fecundity. We produced females of each genotype (homozygous SR, heterozygous and homozygous non-drive) with a wide range of body sizes, which controlled for any link between body size and time to sexual maturity or fecundity. First, we determined the time taken for females of each genotype to sexually mature. We find that female time to sexual maturity is not affected by genotype, and – unlike in males of this species – there was no interaction with body size or eyespan. Next, we dissected mature females and counted the number of eggs in their ovaries to determine their fecundity. We found fecundity was affected by SR in a dose-dependent manner, with homozygous SR females suffering the greatest reduction in fecundity. This trait was also positively associated with body size and eyespan.

4.2 Introduction

In *Teleopsis dalmanni*, the SR driver reduces female egg-to-adult survival, with a higher survival cost in homozygous females (Finnegan et al. 2019). But what effect does SR have on female reproductive fitness — is it also reduced by SR? In this chapter, I investigate two important components of adult female reproductive fitness, the time to sexual maturity and fecundity. These components are also likely to act in a dose-dependent manner, leading to a decreased fitness of homozygous drive individuals compared to heterozygotes. An important consequence of selection on females is the promotion of a stable frequency of the drive variant in wild populations (Angelard et al. 2008; Lindholm et al. 2016; Finnegan et al. 2019; Larner et al. 2019). It is well established that deleterious consequences in males can retard the invasion of drive but do not lead to stability, as they do not act in a frequency dependent manner (Wilkinson and Fry 2001; Price and Wedell 2008; Price et al. 2008; Verspoor et al. 2020; Winkler and Lindholm 2022). In contrast, deleterious consequences in females that are recessive will create increasing selection against drive as it spreads and limit the probability of drive fixation (Lindholm et al. 2016; Zanders and Unckless 2019).

The time taken to reach sexual maturity is an important component of reproductive fitness, as it influences the number of offspring an organism can produce over its lifespan. Yet, there is not a diverse range of insect studies of variation in this trait within and between species. In part this is because sexual maturity is attained either at or shortly after eclosion of the adult stage in many insects. Studies in *Diptera* have shown that time to sexual maturity can vary within species depending on an individual's condition (both as larvae and as adults), their exposure to the opposite sex and their developmental temperature (Craddock and Boake 1992; Papadopoulos et al. 1998; Gomulski et al. 2012; Revadi et al. 2015). Time to sexual maturity has also been shown to vary between closely related species, such as species in the *Drosophila* group, *Bactrocera carambolae* and *Bactrocera papaya*, and *Anastrepha ludens* and *Anastrepha obliqua* (Wee and Tan 2000; Aluja et al. 2001; Markow and O'Grady 2008). In male *Drosophila,* the interspecies variability of this trait is related to differences in the time taken for gametogenesis, as species with longer sperm have a longer sperm elongation phase (Markow and O'Grady 2008).

In male *T. dalmanni,* time to sexual maturity was investigated by Baker *et al.* (2003) and found to be around 25 days after eclosion. This work focussed on how growth in the size of the accessory glands size but not the testes have a positive association with faster time to sexual maturity in males (Baker et al. 2003). In this study, there was no association of the time to sexual maturity with body size; however, only a restricted range was studied (Baker et al. 2003). Recent work has shown that there is a strong inverse relationship of the time to sexual maturity with body size (large males mature faster) using a wide range of body size (Bradshaw et al. *unpublished*). There was no relationship of the time to sexual maturity with genotype, except that SR males with large body size took longer to reach maturity than ST males, but there was no difference between small-bodied SR and ST males (Bradshaw et al. *unpublished*).

For female flies, the time taken to reach sexual maturity is dependent on gametogenesis: a female is defined as sexually mature when she has mature eggs. *Drosophila* females have eggs that mature after emergence, and variability in egg maturation times is species-dependent (Markow and O'Grady 2008). Egg

development is classified into discrete stages 1-14 in *Drosophila*, where stage 14 represents a fully matured egg (Cummings and King 1969). The stage of egg maturity at eclosion determines the time remaining to egg maturation, differs by species and determines most of the interspecies variability. For example, *Drosophila melanogaster* females are rapidly maturing, as females have stage 7-8 eggs on emergence, which mature within 2 days, whereas in *Drosophila fulvalinatea,* females emerge with most eggs at stage 1-2, extending the time taken for a female to mature (Kambysellis and Heed 1971; Markow and O'Grady 2008). However, factors such as the presence of males have been shown to increase the rate of oogenesis, suggesting that, as with males, variation in this trait is not exclusively taxonomically determined (Markow and Ankney 1984; Craddock and Boake 1992; Markow and O'Grady 2008).

In contrast to *Drosophila* taxa, the study of factors that influence the time to sexual maturity in female stalk-eyed flies is less developed. There has been a single investigation of female time to sexual maturity in stalk-eyed flies, which formed part of the larger study of time to sexual maturity in both sexes (Baker *et al.* 2003). It reported that females mature slightly in advance of males, around 22 days, with no association with body size (but over limited variation; Baker *et al.*, 2003). As with males, there needs to be further study using a wider range of body sizes to check this relationship. There has been no study of female genotype and the time to sexual maturity. Nonetheless, field observations suggest that SR might cause a delay in female time to sexual maturity. Most females collected from the field site were mature (86% had mature eggs), but this differed with female genotype, with the greatest difference between SR homozygotes and ST homozygotes (X^{ST}/X^{ST} 89%, X^{SR}/X^{ST} 82%, X^{SR}/X^{SR} 79%; Meade et al., *unpublished* field data). If the age distribution of field flies is independent of genotype, this suggests that SR extends the period prior to maturity in females. But the uncontrolled nature of field data means that other explanations are plausible, for instance, if SR-carrying females suffer from lower viability as adults. To complement research on males, this study examines whether female sexual maturity time to sexual maturity scales with body size. We also test whether that the presence of the SR chromosome delays female sexual maturity in a dosage-dependent manner.

Another – and likely more important – component of female reproductive fitness is fecundity. This is typically measured as the number of eggs laid over a particular period or the number of eggs in female ovaries assessed at a particular time point. Insect fecundity has been shown to be affected by numerous factors, including nutrition, the signalling of hormones involved in oogenesis, social condition (i.e., the presence of mature males) and body size (Honěk 1993; Revadi et al. 2015; Santos et al. 2019). Of these, body size is particularly important: larger body size is strongly associated with increased fecundity in a variety of insect species (Briegel 1990; Tammaru et al. 1996; Thurston and MacGregor 2003; Berger et al. 2008).

In stalk-eyed flies, female eyespan (used as a proxy for body size) and adult food quality both have large positive effects on fecundity measured as the number of eggs laid (Cotton et al. 2015). Likewise, among wild-caught flies, female eyespan was positively correlated with fecundity recorded both as the number of eggs laid and in dissected ovaries (Cotton et al. 2010; Meade et al., *unpublished*). The effect of genotype on fecundity was examined as part of a larger study to characterise fitness effects associated with SR in both sexes (Wilkinson et al. 2006). Females of each female genotype were dissected, but there was no association between female

genotype and the number of mature eggs (Wilkinson et al. 2006). However, there was a large effect of age on fecundity and of cross-type (female genotypes were generated by backcrossing both to SR and ST male parents), which may have obscured any association with SR (Wilkinson et al. 2006). Wild-collected flies provide no evidence for an effect of genotype on fecundity (Meade et al., *unpublished*). In addition, the laboratory study of Wilkinson *et al.* (2006) collected offspring produced by females with different genotypes over a 6-week period. This demonstrated an effect of SR, with heterozygous X^{SR}/X^{ST} females producing more offspring than homozygous X^{SR}/X^{SR} females. The authors interpreted their results as indicative of "weak overdominance for female fecundity" as heterozygotes had the highest output, but the evidence for this is weak as X^{SR}/X^{ST} female offspring counts were not significantly different from those of homozygous X^{ST}/X^{ST} females (Wilkinson et al. 2006). A further problem here is that offspring genotype affects egg-adult survival (Finnegan et al. 2019), which in and of itself will alter estimates of fecundity from adult offspring counts. In addition, there was a confounding effect of cross-type; females sired by SR males had reduced offspring production compared to those sired by ST males, independent of female genotype (Wilkinson et al. 2006). These factors complicate the interpretation of the results and suggest that further investigation is needed.

The present study aims to build on the previous work in stalk-eyed flies by using highly controlled experiments to isolate the effects of genotype and body size to establish if SR reduces female fecundity in a dosage-dependent manner. To accomplish this, we dissected mature females of each genotype and counted eggs inside the ovaries to measure fecundity. This avoided the problems associated with measuring fecundity through the number of offspring produced, which is potentially confounded by genotype effects on egg-adult survival and the previously mentioned paternal genotype effects. In addition, preliminary observations found that females lay eggs in a sporadic fashion making estimation unreliable, except over long periods of time, which were untenable.

4.3 Methods

4.3.1 Stocks

Flies for the standard stock (ST-stock) population carry only the wildtype X chromosome (X^{ST}). They were collected (by S. Cotton and A. Pomiankowski) in 2005 from the Ulu Gombak valley, Peninsular Malaysia (3°19′N 101°45′E). They have since been maintained in high-density cages (> 200 individuals) at a 1:1 sex ratio to minimise inbreeding and are regularly monitored to ensure they do not contain the SR driver.

The meiotic drive stock (SR-stock) population is composed of females that are homozygous for a sex-ratio distorting X chromosome (X^{SR}). They were derived from flies collected in 2012 (by A. Cotton and S. Cotton) from the same location as the ST-stock. X^{SR}/Y males produce 100% female offspring due to transmission distortion. The SR female stock is maintained by crossing X^{SR}/X^{SR} (SR-HOM) females with X^{ST}/Y males to produce X^{SR}/Y drive males, who are then mated to the SR-HOM females to generate the next generation of the SR-HOM stock females. Crossing SR-HOM females to ST males from the ST-stock ensures that the two stocks only differ in their X chromosomes and are homogenised for autosomal content.

Both stock populations were kept at 25°C in cage culture (>200 individuals to minimize inbreeding), with a 12:12h dark:light cycle and fed twice weekly with full food, which consisted of 1kg puréed sweetcorn plus 600ml water and 30mL 10% Nipagin preservative (prevents mould growth). Fifteen-minute artificial dawn and dusk periods were created by illumination from a single 60W bulb at the start and end of the light phase.

4.3.2 Experimental flies

To produce female offspring for experiments, mature flies from the stock population were crossed in three combinations to produce females with each genotype (X^{SR}/X^{SR}, X^{SR}/X^{ST}, and X^{ST}/X^{ST}). X^{SR}/X^{SR} females were crossed to X^{SR}/Y males, generating X^{SR}/X^{SR} females only (SR-HOM). X^{SR}/X^{SR} females were crossed to X^{ST}/Y males, generating X^{SR}/X^{ST} females (HET) and X^{SR}/Y males. Finally, X^{ST}/X^{ST} females were crossed to X^{ST}/Y males, generating X^{ST}/X^{ST} females (ST) and X^{ST}/Y males. Male offspring were discarded as they weren't used in the following experiments.

Two culture cages were established for each cross type. Each cage contained around 60 mature adult flies of approximately equal age (3-6 months) in an even sex ratio. Six egglays were placed inside each cage to collect eggs. Egglays consisted of a petri dish lined with a damp cotton round topped with 1 tbsp of larval food, which was made by diluting 225mL 'full food' (the food fed to the stock populations) with 100mL water, equivalent to a 30% dilution of the full food. This reduced larval food was used to produce a wide distribution of adult body sizes, as body size is known to influence female reproductive fitness (Cotton et al. 2010; Finnegan et al. 2021).

Egglays were removed from cages every 3-4 days (twice per week) to be incubated at 25°C and were replaced with fresh egglays. Daily emergence checks were then performed on the incubating egglays, and emerging adults were collected each morning. Females were transferred to large 1000mL clear pots, pooled according to genotype and emergence date. A maximum of 15 flies were kept per pot, to minimise adult stress. Large pots had a damp oval cotton oval base with 1 tbsp of full food, which was replaced twice per week. A sample size of at least 40 females was collected for each genotype.

4.3.3 Adult measurements

14 days after emergence, single females were transferred to small 500mL clear pots with bases consisting of two damp cotton rounds covered with a circle of moist blue tissue paper (to increase egg visibility) and topped with a spatula of full food. The bases of pots were collected and replaced daily. Each base was checked under a Lecia microscope for oviposited mature eggs (eggs that are oval shaped with a diameter >0.7mm). The time to sexual maturity was defined as the number of days taken from eclosion to the appearance of the first egg.

Mature females remained housed in small pots for a further period of 10 days to allow full maturity of their ovaries. On day 11, females were anesthetised by chilling at 14ºC for around 7mins. Thorax length and eyespan were measured under a Leica microscope using ImageJ (v1.46; (Schneider et al. 2012). Eyespan was defined as the distance between the outer tips of the eyes (Hingle et al. 2001). Thorax length was

defined as the distance 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). The head was then removed, and the body was placed on a microscope slide with a drop of PBS. The slide was viewed under a Lecia light microscope and ovaries were extracted from the abdomen by grasping the ovipositor with tweezers and separating the ovaries from the viscera. The extracted ovaries were then tweezered apart to separate eggs for counting. Eggs greater than 0.3mm in length were counted using a cell counter. We made no distinction between mature (eggs over 0.7mm) and immature eggs (eggs between 0.3 and 0.7mm), transparent eggs less than 0.3mm in length were not counted, as these are difficult to distinguish from other tissues.

4.3.4 Statistical analyses

To determine the effect of female genotype on time to sexual maturity, standard linear regression models of the form:
Y ~ genotype + fixed effects + error term, were fitted to the time to sexual maturity data, where the response variable, Y, was female age at sexual maturity in days.
To determine the effect of female genotype on fecundity, general linear models of the form:
Y ~ genotype + Gaussian error term, were fitted to the fecundity data, where the response variable, Y, was the number of eggs counted in the ovaries. A Gaussian model was chosen as egg counts fit this distribution best. Females that died before dissection were removed from the fecundity dataset.

As some studies have shown that body size has an influence on time to sexual maturity and fecundity in *T. dalmanni* (Wilkinson et al. 2006; Cotton et al. 2010), thorax length was included as a fixed effect in all models. Relative eyespan – the variation in eyespan beyond what is expected from variation in thorax length – has also been suggested to indicate female reproductive quality (Cotton et al. 2010). The effect of relative eyespan was analysed separately by including thorax length and eyespan as covariates in all models.

Pairwise comparisons of female genotypes were made using Tukey's post hoc comparison tests. Full models are given in the SI. All statistical analyses were performed using R software version 4.1.2 (R Core Team 2021).

4.4 Results

4.4.1 Female trait size with genotype

Flies were fed a reduced diet during larval development to produce variation in adult morphological traits. Thorax length ranged from 1.709 - 2.583mm, and eyespan ranged from 4.303 - 6.352mm. As expected, there was a strong covariance between thorax length and eyespan ($F_{1,95}$= 226.233, P < 0.001, Figure S4.1).

There was an association between thorax length and genotype ($F_{2,94}$ = 13.454, P < 0.001). SR-HOM females had a shorter thorax length (mean thorax length ± SE mm = 2.082 ± 0.047) than HET females (mean thorax length ± SE mm; 2.264 ± 0.032; Tukey's test, P = 0.001) and ST-HOM females (mean thorax length ± SE mm; 2.317 ± 0.017; Tukey's test, SR-HOM — ST-HOM, P < 0.001, Figure S4.1). HET thorax

length was not different from ST-HOM thorax length (Tukey's comparison HET — ST-HOM, $P = 0.315$, Figure S4.1).

When variation in thorax length was accounted for, there was an association between relative eyespan and genotype ($F_{2,93} = 4.800$, $P = 0.010$, Figures S1 and S2). Relative eyespan did not differ between SR-HOM and HET females (Tukey's test, $P = 0.550$), or SR-HOM and ST-HOM females (Tukey's test, $P = 0.393$, Figure S4.2). However, ST-HOM females were larger than HET females (Tukey's test, $P = 0.009$, Figure S4.2). Owing to this variation and evidence that relative eyespan indicates female condition (Cotton et al. 2010), both thorax length and eyespan were included as covariates in all subsequent models to control for the effect of body size and relative eyespan.

4.4.2 Time to sexual maturity

The time to sexual maturity was measured as the time from eclosion to the date the first egg was laid in days. On average, females reached sexual maturity at mean ± SD = 30 days ± 8 days (N = 101), with a minimum age of 17 days and a maximum age of 55 days. Time to sexual maturity was not dependent on thorax length ($F_{1,95} = 1.174$, $P = 0.678$) or relative eyespan ($F_{1,94} = 2.731$, $P = 0.102$, Figure 4.1). There was no association between genotype and age at sexual maturity ($F_{2,98} = 0.920$, $P = 0.402$) and this outcome was unchanged when thorax length and relative eyespan were added as covariates ($F_{2,92} = 0.245$, $P = 0.783$, Figure 4.2 and S4.2).

4.4.3 Fecundity

We successfully dissected 90 females and counted the number of eggs in their ovaries to measure fecundity. Females had a mean ± SD = 31.478 ± 15.072 eggs in their ovaries on dissection (range = 0 - 79 eggs). There was a positive association between fecundity and thorax length ($F_{1,85} = 12.39$, $P = 0.001$), and relative eyespan ($F_{1,82} = 5.776$, $P = 0.019$). There was also a positive association between genotype and fecundity ($F_{2,87} = 8.039$, $P = 0.001$). ST-HOM females were more fecund than SR-HOM females (mean eggs ± SD; ST-HOM = 36.205 ± 15.237, SR-HOM = 19.875 ± 12.371, Tukey's test, $P < 0.001$). HET females had an intermediate number of eggs (mean eggs ± SD; HET = 30.733 ± 12.846), which was significantly more than SR-HOM females but not different from ST-HOM females (Tukey's comparison = SR-HOM — HET, $P = 0.037$; Tukey's comparison = HET – ST-HOM, $P = 0.230$; Figure 4.3). This relationship persisted after thorax length and relative eyespan were included as covariates ($F_{2,78} = 3.690$, $P = 0.029$, Figure 4.4 and S4.3).

4.5 Discussion

In this study, we have examined the effect of SR on female time to sexual maturity and fecundity across a wide range of body sizes. We had a naïve expectation based on the known fitness costs associated with SR that for females carrying SR, time to maturity would increase and fecundity would decrease, and these effects would be SR-dose dependent. This was based on previous studies that show SR is associated with negative fitness effects, including reduced egg-adult survival, reduced male eyespan and thus male mating opportunities, and a lower remating rate in males (Wilkinson et al. 1998b; Finnegan et al. 2019).

4.5.1 Female time to sexual maturity

We found no evidence of an effect of genotype on time to sexual maturity in females, and no dose effect, which is the same as the situation in males where the presence of SR alone did not alter male time taken to mature relative to ST males (Bradshaw et al. *unpublished*). In males, accessory gland and testis growth are very sensitive to environmental conditions, which are likely to be more important than genotype — these reproductive organs are crucial for the production of the male ejaculate and thus male reproductive success (Baker 2001; Rogers et al. 2008). However, Bradshaw *et al.* (*unpublished*) did find an interaction between size and genotype: in large males, SR males take longer to reach maturity than ST males. Here, as in Baker *et al.* (2001), there was no relationship between female body size and time to sexual maturity in HOM-ST females, However, unlike in Bradshaw *et al.* (2001), there was interaction between size and genotype. Perhaps this is due to body size and relative eyespan being more exaggerated traits in males and very important in attracting female mates (Wilkinson et al. 1998b,a; Hingle et al. 2001; Cotton et al. 2010). SR males must invest in larger testes to compensate for sperm loss, and SR males have smaller body size and smaller relative eyespans than ST males (Meade et al. 2019, 2020; Bradshaw et al. 2022). Hence, large SR males cannot cope with the demands of their rapid accessory gland and testes growth, which is then seen as a longer time to sexual maturity. There is no equivalent of this interaction in females, as SR is not associated with a reduction in female size, nor is female ovary size increased as an adaptive compensation to SR fitness costs (Finnegan et al. 2021).

4.5.2 Fecundity

In contrast to the time to sexual maturity, fecundity showed a large effect of genotype. SR decreased female fecundity, with the ordering of genotypes by increasing fecundity being ST-HOM > HET > HOM-SR. There is also evidence of recessivity as SR-HOM females had significantly reduced fecundity compared with HET females. However, HET female fecundity was insufficiently reduced compared to ST-HOM females to be recorded as significant. As this conclusion is contingent on sample size, female numbers might need to be doubled or further increased to be certain of recessivity or estimate its extent. These relationships are present after controlling for the effect of body size and relative eyespan, both of which are positively associated with fecundity. This result is supported by previous studies that show female body size and eyespan correlate with fecundity and that eyespan is a signal of female quality (Cotton et al. 2010; Meade et al., *unpublished data*).

Our finding that SR is associated with a reduction in fecundity is inconsistent with the findings of Wilkinson *et al.* (2006), where there was no effect of genotype after controlling for the effect of cross-type. Owing to their crossing design, HET females could inherit their X^{SR} chromosomes paternally or maternally, and the fecundity was lower among females sired by SR males (Wilkinson et al. 2006). A side effect of producing heterozygous females via two backcrosses in the Wilkinson et al. (2006) study was the number of HET individuals dissected (N = 234) far outweighed the number of SR-HOM (N = 35) and ST-HOM individuals (N = 29), producing an imbalance in their study design where the effect of cross-type on HET females was magnified. The cause of this effect is unclear and was not fully investigated but may

have obscured the true relationship with genotype (Wilkinson et al. 2006). In our study, the X^{SR} chromosome is inherited paternally in HET females, removing the confounding effect of cross-type, leading to a clear relationship of SR-dose on fecundity. One other factor contributing to our different result is that Wilkinson *et al.* counted mature eggs in female ovaries with ages ranging from 3 - 10 weeks to determine fecundity, whereas all females here were the same age, 11 days past sexual maturity. This removed any confounding effect of time since maturity on the number of eggs present. This might also have contributed to the Wilkinson et al. (2006) study failing to find a genotype effect if there was variance in the age at which different groups of females were dissected (this was not recorded). Overall, we are more able to untangle the effects of female genotype, age and body size in the current study.

Another X-linked Sex Ratio driver (SR) has also been found to reduce female relative fitness in *Drosophila pseudoobscura* (Larner et al. 2019). Like in *T. dalmanni*, homozygous drive females suffer a greater fecundity reduction than heterozygotes. In a previous 2015 study, the authors used a modelling approach to explore the contribution of polyandry (*p*), SR male success under sperm competition (*c*), the strength of drive (i.e., its probability of being transmitted to SR male offspring, *d*) and reduced homozygote female fitness (*h*), to the explore how an equilibrium frequency of SR might be reached (Holman et al. 2015). They found that while polyandry was insufficient to prevent SR fixation, homozygous fitness costs combined with polyandry were able to stabilise SR frequencies. The 2019 study experimentally determines relative female fitness values for each female genotype and incorporates these into a simplified version of their 2015 model. Using this approach, they could predict close to real-world frequencies of the SR allele, reaffirming the potential for homozygous fitness costs to reduce the frequency of SR when it becomes common and polyandry is high (Holman et al. 2015; Larner et al. 2019).

As SR males compensate for sperm loss and can compete with ST males in a singly and maximally mated female, the contribution of female fitness costs to stabilising SR frequency in wild populations is also likely to be magnified. Such a combination of factors is predicted to contribute to negative frequency-dependent selection, allowing SR to stabilise at a high frequency in wild populations (Holman et al. 2015; Dyer and Hall 2019; Finnegan et al. 2019; Larner et al. 2019). Further work is planned to implement a genetic model of the form used by Larner *et al.* (2019), incorporating parameters that have been experimentally derived for *T. dalmanni* in previous work, both in this thesis and by others in the group. These are the average of P1 and P2 success under high polyandry from Chapter 3 (c = 0.474), the strength of drive (d = 0.94, taken from Presgraves et al. 1997) and the relative fecundity of SR-HOM female genotype from the present study (SR-HOM fecundity/ST-HOM fecundity, $h_{fecundity}$ = 0.549), multiplied by the reduced egg-to-adult viability values associated with SR, which were determined by Finnegan *et al.* (2019) ($h_{viability}$ = 0.511). The polyandry parameter values will range from 0%-100%, with the expectation that higher values like those observed in natural populations help stabilise the SR allele at frequencies close to the observed population frequency. Taking this approach will allow us to link the effect sizes observed under experimental conditions with the ecology of wild populations of *T. dalmanni*, improving our understanding of how a reduction in homozygous female fitness might contribute to the frequency of SR in natural populations.

In summary, we have investigated the effect of SR on time to sexual maturity in female *T. dalmanni*, finding no evidence to suggest SR has an effect. We have also shown strong evidence to suggest SR affects fecundity, with evidence that points to a recessivity effect. We also outline future work that will investigate the contribution of this effect to stabilising SR frequency in nature using a population genetic model.

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4.7 Figures

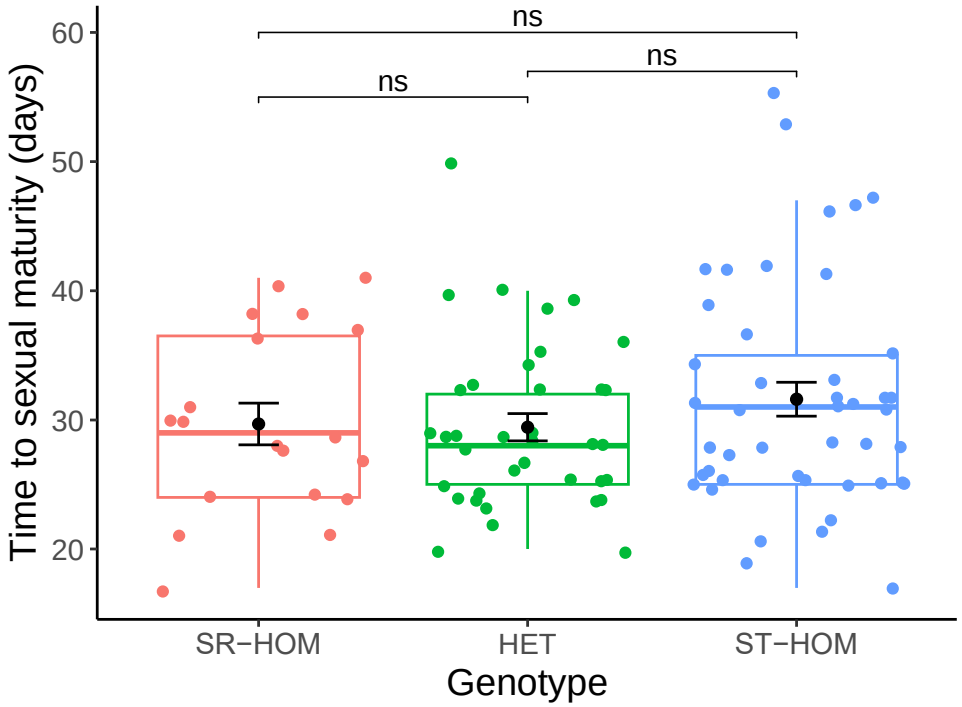

*Figure 4.3: The time to sexual maturity is shown for females of each genotype. Boxplots enclose the first - third quartile range, with median bar and whiskers (1.5 times interquartile range). Inner black circles show the mean number of eggs inside female ovaries per genotype ± SE. Significance values reflect p-values obtained from Tukey's pairwise comparisons between genotypes. Ns = not significant, * P < 0.05, *** P < 0.001.*

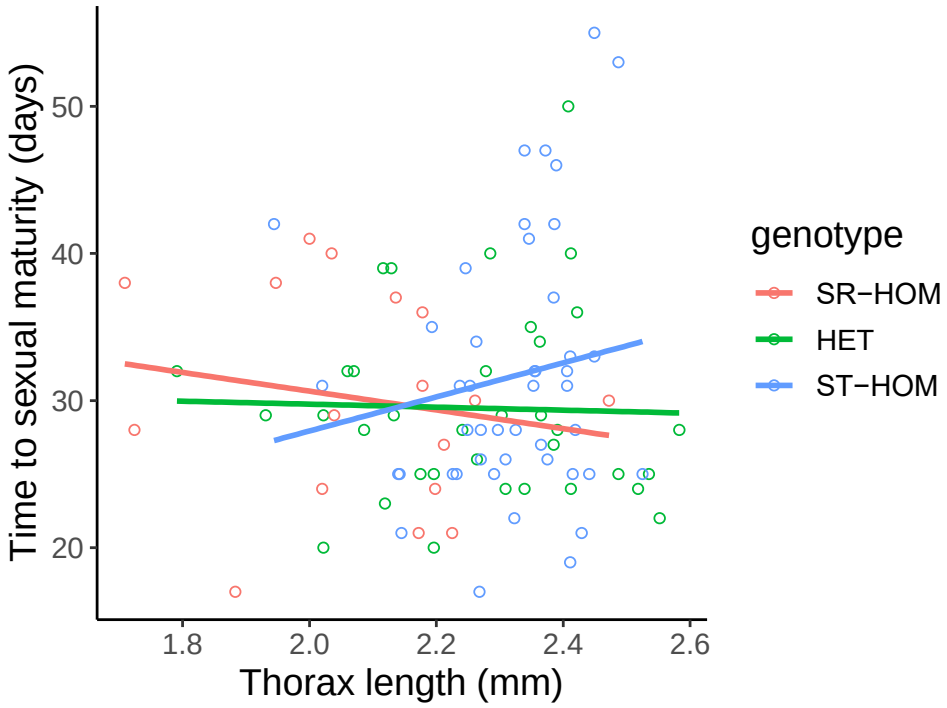

Figure 4.4: The relationship between thorax length and time to sexual maturity is shown for females of each genotype. Shaded areas represent the SE associated with the model for each genotype.

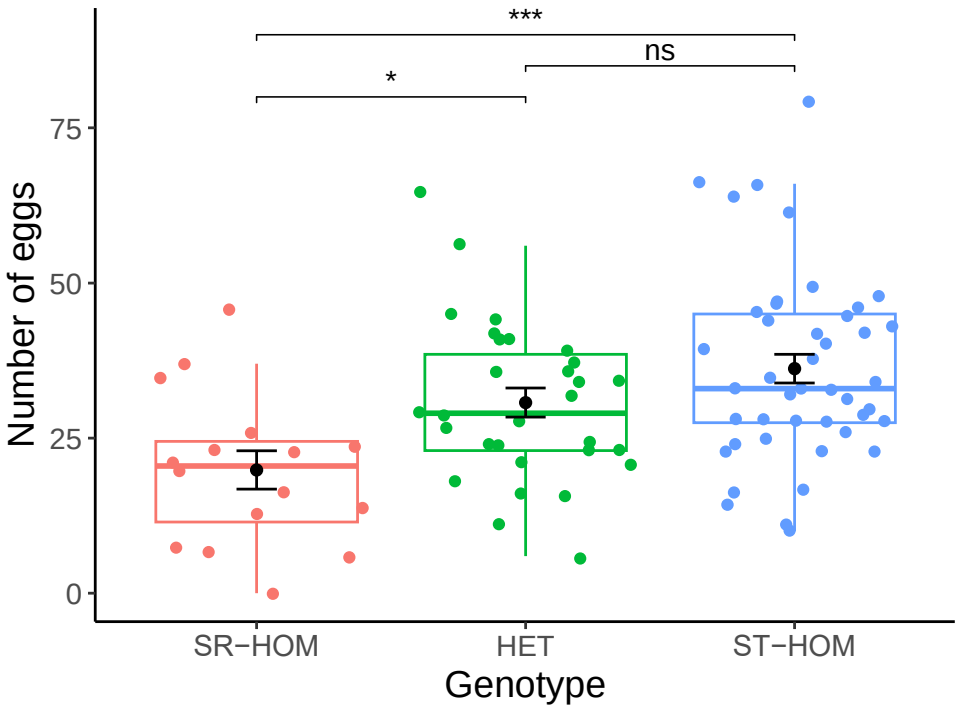

*Figure 4.5: The number of eggs inside female ovaries is shown for females of each genotype. Boxplots enclose the first - third quartile range, with median bar and whiskers (1.5 times interquartile range). Inner black circles show the mean number of eggs inside female ovaries per genotype ± SE. Significance values reflect p-values obtained from Tukey's pairwise comparisons between genotypes. ns = not significant, * P < 0.05, *** P < 0.001.*

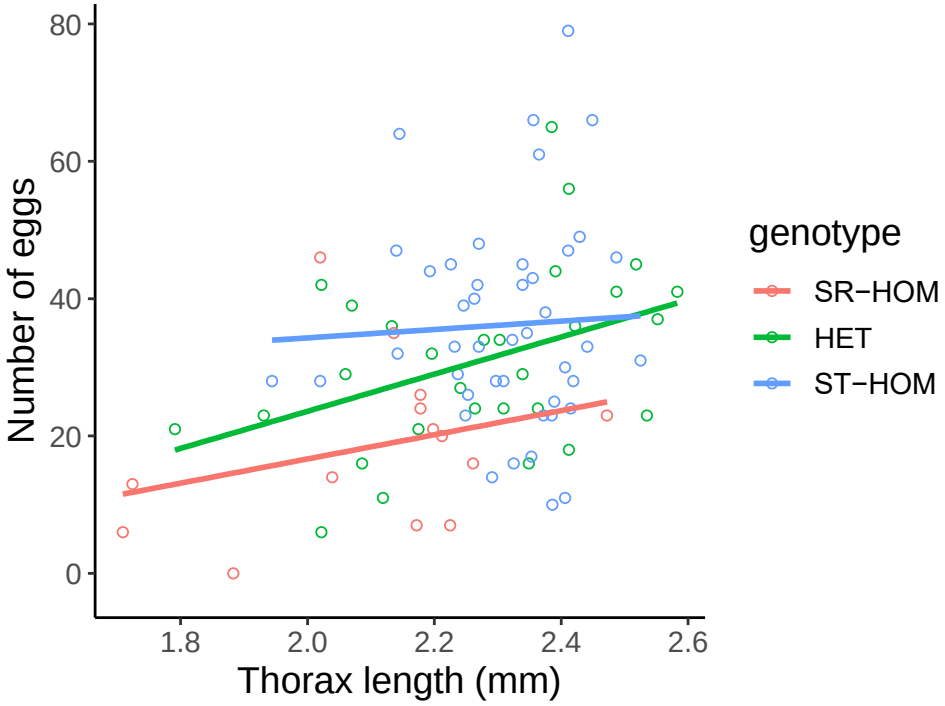

Figure 4.6: The relationship between thorax length and the number of eggs in ovaries of mature females is shown for females of each genotype. Shaded areas represent the SE associated with the model for each genotype.

Chapter 5. OMAnnotation: a novel approach to building an annotated consensus genome sequence

Title Page

Bioinformatics: Genome Analysis
OMAnnotation: a novel approach to building an annotated consensus genome sequence
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Supplementary data are available at Bioinformatics online.

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Author contributions
Concept: CD and YN. SB and YN developed the approach, SB implemented Proof of Principle testing and analysed the results. SB wrote the first draft. SB, YN and CD reviewed and edited the manuscript and approved the final version.

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5.1 Abstract

Motivation
Advances in sequencing technologies have enabled researchers to rapidly sequence whole genomes. However, while genome assembly is improving as a result of these developments, genome annotation (i.e. the identification of protein-coding genes) remains challenging, particularly for eukaryotic genomes: it requires combining several approaches (typically ab initio, transcriptomics, and homology search), each with its own pros and cons. Deciding which gene models to retain in a consensus is far from trivial, and automated approaches tend to lag behind laborious manual curation efforts in accuracy.

Results
Here, we present OMAnnotate, a novel approach to building consensus annotation, by repurposing the OMA algorithm, which elucidates evolutionary relationships among genes from different species and infers ancestral protein repertoires. Each input annotation set is treated as a separate “species”, and the consensus annotation is derived from inferred “ancestral” repertoire. We tested the approach by reannotating the *Drosophila melanogaster* reference genome from the ground up. The consensus annotation inferred by OMAnnotate outperformed each of the three input annotation sets, as well as BREAKER2, a state-of-the-art annotation approach exploiting both transcript alignment and *ab initio* predictions. Furthermore, the approach can, in principle, work with an arbitrary number of input annotation sets.

Conclusion
The successful proof of concept of repurposing an orthology method for genome annotation, seamlessly integrating multiple lines of evidence, opens up new avenues in eukaryotic genome annotation.

5.2 Introduction

With the advances in sequencing technology, it is faster and more affordable than ever to sequence a genome. However, annotating the increasing number of newly sequenced genomes remains labour-intensive (Moghul et al. 2019) and error-prone (Salzberg 2019; Scalzitti et al. 2020). Genome annotation involves identifying the features in a genome sequence, including protein coding genes, inversions and repeats, which is essential for understanding its underlying biology. Here, we focus on the prediction of protein coding genes and describe a new approach to improve the ease and accuracy of this process.

There are three major classes of methods used to predict genes in genome assemblies: *ab initio* gene prediction, transcript alignment and homology alignment (Hoff and Stanke 2015; Mudge and Harrow 2016). The first of these involves using a gene finder algorithm, such as AUGUSTUS (Stanke et al. 2008), to identify genes in a genome assembly based on gene structures in a model species it has been trained on. Transcript alignment involves aligning reads from RNA sequencing to the reference assembly to localise transcripted regions of the genomes. Finally, homology alignment involves searching for regions in the newly sequenced genomes that are similar to coding genes in a closely related species, in order to identify likely homologous genes.

One of the main challenges in genome annotation is combining the genes predicted by different annotation methods into an annotated consensus sequence. A good consensus sequence accurately captures most genes in the genome, i.e., it retains the maximum number of true gene predictions while dropping false gene predictions. Genome annotation pipelines such as BRAKER2 (Brůna et al. 2021) aim to achieve this using RNAseq or protein sequence evidence during the AUGUSTUS (Stanke et al. 2008) iterative training and gene prediction processes, which improves annotation accuracy compared to using AUGUSTUS alone. Nevertheless, combining gene models from multiple evidence sources tends to be needed to remove false predictions from the consensus annotation. BRAKER2 has been reported to encounter issues (lower specificity) when integrating gene precision and protein homology evidence (Brůna et al. 2021; Gabriel et al. 2021). Other approaches, such as EVidence Modeller (EVM) (Haas et al. 2008), produce a consensus annotation by assigning quality weightings to annotation sets produced by different annotation methods. However, this may cause true gene predictions from an overall lower-quality set to be dropped from the consensus.

To address this long-standing issue from a new angle, we sought to build consensus gene sets using an approach developed to model genome evolution. Our tool “OMAnnotation” repurposes OMA (Orthologous MAtrix) standalone (Altenhoff et al., 2019), a state-of-the-art orthology inference software, to reconstruct a consensus annotation set from an arbitrary number of input annotation sets. In essence, the main novelty is that it treats each input annotation set as a different species and uses the ancestral gene repertoire inferred by OMA as the consensus set (Figure 5.1). Bona fide genes missing from any one annotation set appear as gene losses on terminal branches, whereas spurious genes that solely exist in a single input set look like new gene acquisitions on terminal branches.

Furthermore, OMAnnotation also exploits additional evolutionary information to aid in selecting likely true gene models from different annotation methods by including gene models from other species as outgroups. OMA Standalone infers “hierarchical orthologous groups” (Train et al. 2017; Zahn-Zabal et al. 2020) for each ancestral species in the tree relating all input genomes. These “HOGs” correspond to ancestral genes in the usual context of OMA analyses. With respect to the “common ancestor” of the different input annotation sets, the HOGs give us a consensus annotation set. Below, we validate this approach by using it to annotate the latest *Drosophila melanogaster* genome assembly. We compare the OMAnnotation results to those obtained using BRAKER2 (Brůna et al. 2021) to annotate the same genome assembly.

5.3 Methods

5.3.1 Description of the OMAnnotation pipeline

The OMAnnotation pipeline relies on the inference of orthologous groups provided by the OMA Standalone software (Altenhoff *et al.*, 2019) to combine gene predictions from different annotation methods into a consensus annotation. The pipeline takes GFF files resulting from any annotation methods as its main input and uses them to annotate the genome assembly. It is executed in three main steps.

5.3.1.1 Setting up annotation files for OMA

As a prerequisite, it is required to have a local copy of the OMA Standalone software and a file of precomputed orthology relationships between a set of species. This set is selected with the aim to maximise taxonomic diversity and includes species that are closely related to the species being annotated. Such a file can be downloaded from the “Download>Export All-All” section of the OMA Browser (https://omabrowser.org/oma/export/), which downloads an archive of precomputed pairwise comparisons between the selected species and their orthologous sequences. Both the OMA Standalone software and the precomputed orthology relationships are locally stored in what will be hereafter referred to as the ‘OMA folder’.

The first step of the pipeline involves extracting the information needed to run the OMA algorithm from the GFF annotations files and the genomic sequence. Much of this process is automated through the “prepared_data” module of the OMAnnotation software. Briefly, this module takes as input a folder containing any number of GFF annotation files and the genomic sequence to which they correspond. The script extracts all protein sequences described in the GFF file and adds them to the ‘DB’ (database) subfolder in the user’s OMA folder. If the annotation predicts any gene to have multiple isoforms, alternative splicing information in the form of a ‘slice file’ is also added to the DB subfolder. This enables OMA Standalone to select the isoform sequence that is the most like its detected homologs as the single representative of each gene.

5.3.1.2 OMA Standalone

Next, the OMA Standalone pipeline is run as described in Altenhoff *et al.* (2019, 2021). For this purpose, the parameter file is edited to specify the species tree including the various input annotation sets to combine. This is done by adding a branch to the

species tree, which is a polytomy, and whose leaves share the same name as the annotation FASTA file present in the DB folder.

5.3.1.3 Consensus extraction

The last step of the pipeline is the extraction of a consensus annotation from the OMA Standalone. This is done using the “extract_consensus” script of the OMAnnotation software. This script takes as input the aforementioned species tree in Newick format and the HierarchicalGroups.orthoxml file outputted by OMA Standalone. It generates a protein FASTA file and a GFF file corresponding to the consensus annotation.

The software selects as consensus genes any gene that is present in the OMA inferred “ancestral genome” of the different annotation methods. This “ancestral genome” will contain any sequence that is shared by at least two of the combined annotation methods, or those inferred by one of the annotation methods if they have detected orthologs in any of the outgroup species. This allows combining genes inferred by multiple methods but disregarding the ones with low support. The representative sequence for any consensus gene, when multiple annotation methods predict it, is the one with the longest coding sequence.

5.3.2 Proof of principle: Annotating the *Drosophila melanogaster* genome sequence with OMAnnotation

To validate the OMAnnotation approach, we used it to annotate the *D. melanogaster* genome sequence. We downloaded the latest genome assembly (genomic release 6, version 4) without annotations from FlyBase and used 3 annotation methods to predict genes. We then used OMA standalone to combine the resulting annotation sets into a single FASTA and GFF3 sequence file (‘The-Sequence-Ontology/Specifications’, 2022).

5.3.2.1 Ab initio: gene prediction using AUGUSTUS

We ran AUGUSTUS with the unannotated *D. melanogaster* genome as input and the “--species=fly” option to specify *D. melanogaster* gene model parameters. The GFF output was converted into FASTA format for OMAnnotation using the “getAnnoFasta.pl” script.

5.3.2.2 De novo: Transcript alignment using StringTie

17-day *D. melanogaster* adult tissue RNAseq data generated with paired-end Illumina sequencing during a study of the *D. melanogaster* developmental transcriptome (Bryce Daines; 2010) were downloaded from NCBI SRA (accession number SRS065821). Sequences were joined in FASTQ format using the fastq-dump tool from the SRA toolkit v2.10.9, and their quality was checked using FastQC (*Babraham Bioinformatics - FastQC A Quality Control tool for High Throughput Sequence Data*, no date). The adapter sequences were then trimmed using Trimmomatic (Bolger, Lohse and Usadel, 2014) in Paired End mode with the parameters “-phred33 -threads 24 -input< left_reads.fastq> <right_reads.fastq> -output <left_paired_reads.fq.gz> < right_paired_reads.fq.gz > <left_unpaired_reads.fq.gz> <right_unpaired_reads.fq.gz>

ILLUMINACLIP:RNAseq_data/TruSeq2-PE.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36". Next, StringTie was used to align reads to the reference genome, producing a genome alignment file (Pertea et al. 2015). The longest open reading frames were identified using TransDecoder, to produce a GFF3 file of likely peptide sequences.

5.3.2.3 Homology annotation: using GeMoMa

Anopheles gambiae proteome data from the assembly AgamP4 (Sharakhova et al. 2007) was downloaded from UniProt on 25/10/2021. GeMoMa (Keilwagen et al. 2019) was used to infer *D. melanogaster* gene models based on the *A. gambiae* protein sequences.

5.3.2.4 OMAnnotation: using OMA Standalone orthology inference to form a consensus annotation

The annotation sequences from each method were submitted to the OMA DB subfolder in FASTA format as 'species', along with a splice file that was produced using the prepare_data module of the OMAnnotation software. A file of precomputed orthology relationships between the following 25 species was downloaded from OMA Browser as described above: *Saccharomyces cerevisiae (strain ATCC 204508 / S288c), Danio rerio, Mus musculus, Rattus norvegicus, Homo sapiens, Xenopus tropicalis, Xenopus laevis, Asterias rubens, Strongylocentrotus purpuratus, Caenorhabditis elegans, Ixodes scapularis, Strigamia maritima, Daphnia pulex, Bombyx mori, Drosophila grimshawi, Drosophila simulans, Drosophila pseudoobscura pseudoobscura, Aedes aegypti, Apis mellifera, Atta cephalotes, Nasonia vitripennis, Zootermopsis nevadensis, Hypsibius dujardini, Helobdella robusta* and *Octopus bimaculoides*. A species tree of these 25 outgroup species and the 3 annotations clustered on a single branch within the drosophila clade was specified in the OMA parameters file ('parameters.drw') in the OMA folder (see supplementary material for species tree in Newick format used). OMA Standalone was then run as described above, with the parameters specified by the 'parameters.drw' file in the OMA folder. Predicted genes that had orthologs in more than 1 annotation or in an outgroup species were extracted and combined into a consensus annotation set using the extract_consensus module of the OMAnnotation software as described above.

5.3.3 Analysing the quality of annotation sets

A comparison of gene counts between the annotations produced by each method and the *D. melanogaster* reference annotation (genomic release 6, version 4, from FlyBase) was used as a first assessment of the specificity (few false predictions) and sensitivity (few missing predictions) of each method. The completeness of the gene set produced by each method was then analysed with BUSCO (v5.4.2) (Manni *et al.*, 2021) using the *Dipteran* gene set from the odb10 release. Finally, the quality of the gene structure annotations in each annotation set was also compared to the *D. melanogaster* reference annotation using ParsEval (Standage and Brendel, 2012).

Briefly, ParsEval performs pairwise alignments between two annotations and uses interval graphs to define "gene loci", the smallest genomic regions that capture all the

annotations that overlap. In this way, it gives no preference to the reference annotation, and no annotations unique to either set are discarded. ParsEval then compares the gene loci in the reference with the gene loci in the prediction annotation and computes summary statistics to evaluate the closeness of the two annotations. ParsEval analysis was performed with the prediction annotation from each method and the *D. melanogaster* reference annotation. The gene loci statistics — the number of shared gene loci, the number of gene loci unique to the reference, and the number of gene loci unique to the prediction — were assessed to evaluate the sensitivity and specificity of each method. Within gene loci, comparing the number of genes gave a further indication of sensitivity, and the number of matching gene structures gave an indication of the quality of the structural predictions by each method. As the original *D. melanogaster* reference annotation was incompatible with ParsEval due to the presence of a trans-spliced gene, the entries relative to this single gene were removed from the GFF3 annotation before comparison.

5.3.4 Comparison of OMAnnotation with another annotation pipeline

To compare our OMAnnotation approach to other annotation pipelines, we used the BRAKER2 annotation pipeline (Brůna et al. 2021) to annotate the *D. melanogaster* reference sequence with the same RNAseq and *A. gambiae* proteome data. First, we ran a pipeline version with only the RNAseq data added (hereafter referred to as BRAKER2 RNAseq) as evidence for AUGUSTUS training. Next, we reran BRAKER2 with the *A. gambiae* proteome data and RNAseq data (hereafter referred to as BRAKER2) as evidence for AUGUSTUS training. We compared the outputs of each pipeline version to the *D. melanogaster* reference annotation to test if including proteome data improved gene prediction quality or number in the consensus set.

While BRAKER2 has the option to specify GFF3 sequence format as its output, it contains formatting errors that do not conform to the GFF3 standard (Lincoln Stein 2020). Therefore, the gtf_to_gff3 script from the GenomeTools package (Gremme et al. 2013) was used to convert the BRAKER2 GTF output into the correct GFF3 format for downstream analyses. Where necessary, redundant sequences were removed using Awk. These corrected GFF3 files were converted into FASTA format, and a custom script was used to select the longest isoform per gene prior to performing a BUSCO analysis as described above. ParsEval analysis was performed as described above to provide an indication of how closely BRAKER2 was able to reproduce the gold standard reference annotation.

5.4 Results

5.4.1 OMAnnotation produces a high-quality annotation set

We annotated the *D. melanogaster* reference genome assembly (FlyBase, Genomic release 6, version 4) using three primary annotation approaches. Briefly, we performed gene prediction using AUGUSTUS (Stanke et al. 2008), RNAseq transcript alignment using StringTie (Pertea et al. 2015), and homology alignment using GeMoMa (Keilwagen et al. 2019). We then combined the annotations from different methods into a consensus annotation using OMAnnotation. As the *D. melanogaster* reference annotation (genomic release 6, version 4) is an established and high-quality annotation, which has undergone manual curation, we treated it as the gold standard

when assessing the quality of each annotation set in our proof of principle test. An annotation method was deemed to have performed well if it produced an annotation set of comparable quality and with high similarity to the reference set.

Comparing the gene count of each annotation with the reference gives an indication of sensitivity and specificity. The *D. melanogaster* reference gene set contained 13821 genes. Both RNAseq and homology gene sets were missing genes compared with the reference set, containing 7592 and 6092 genes, respectively, indicating a lower sensitivity with these methods. The AUGUSTUS gene set contained 13530 genes, the highest gene number produced by an individual method. The OMA consensus contained the closest gene number to the reference with 13889 genes, indicating that OMAnnotation can combine annotations from different sources to increase predictive power.

Next, we performed a BUSCO analysis with the *Diptera* set to assess the completeness of each annotation (Figure 5.2). Our gold standard reference had 99.9% complete single-copy genes, implying a high level of completeness, with a minimal fraction of duplicated (0.2%), fragmented or missing genes. The RNAseq gene set contained the most duplicated, fragmented and missing genes, which is to be expected as RNAseq annotation is dependent on which genes are expressed in the target tissue at the time of RNA extraction, the handling of multiply spliced transcripts, the quality of the extraction and the filtering of noise. While the homology annotation produced fewer fragmented genes, 14.3% of BUSCO genes were missing from the annotation, highlighting the reliance of this method on access to the proteome of a very closely related species for improved sensitivity. AUGUSTUS had the highest sensitivity of the individual methods but had a significant proportion of fragmented and missing genes. OMAnnotation combined the predictions from individual methods to produce a gene set with 99.1% of the representative BUSCO genes, while maintaining a minimal fraction of duplicated genes (2.5%) and the fewest fragmented (0.1%) genes, a level of completeness comparable to that of the reference set.

Finally, the gene structures of each annotation were compared to the *D. melanogaster* reference annotation (genomic release 6, version 4) using ParsEval analysis. Of the individual methods, the RNAseq and homology methods were less sensitive, with 3209 and 4087 genes missing, respectively, compared to the reference annotation. Conversely, the AUGUSTUS annotation had the highest sensitivity but the most unique predictions, indicating a low specificity. The OMAnnotation consensus had 8129 shared gene loci (96.80%) with the reference annotation, showing that this approach was able to balance the pros and cons of each method to reproduce more of the reference annotation genes. It also had 79.3% matching CDS segments, indicating high conservation of CDS structure features (e.g. identical start and stop codon positions) with the reference annotation. However, the AUGUSTUS and RNAseq annotations contained slightly more CDS segments that matched the reference (80.9% and 80.5%, respectively), suggesting these methods performed better at predicting overall CDS structures of the genes in their shared annotations (Table 5.1).

5.4.2 OMAnnotation is comparable with state-of-the-art annotation pipelines

We annotated the same *D. melanogaster* assembly used for our OMAnnotation of principle test above using BRAKER2, to enable comparison of our new approach with a state-of-the-art annotation pipeline. We ran 2 versions of the BRAKER2 pipeline: one with RNAseq data only, to test its performance using the recommended protocol, and the other with RNAseq and proteome data, to test its performance when combining evidence from different sources. As before, the quality of the annotation sets produced was assessed using gene counts as an approximate measure of sensitivity and specificity, the completeness of the gene sets as determined by BUSCO analysis and the similarity of each annotation to the reference annotation as determined by Parseval.

The BRAKER2 RNAseq annotation set contained 17480 genes, significantly more than the reference annotation (13821 genes), which suggests BRAKER2 is less specific with more false positives in its predicted annotation. The BRAKER2 annotation set (produced with RNAseq and proteome evidence) contained even more genes — 21816 genes, implying this method had trouble integrating predictions from RNAseq and proteome into one consensus. These results were supported by our BUSCO analysis, which showed that whilst the BRAKER2 gene set contained slightly more of the genes in the BUSCO reference set (97.2% complete) compared with BRAKER2 RNAseq (96.9%), it contained more than double the proportion of duplicated genes at 20.2%, compared with 7.8% for BRAKER2 RNAseq (Figure 5.2). This implies that the extra genes predicted in the BRAKER2 set were duplicate predictions resulting from poor integration of evidence from the two different sources. This overprediction of genes by each BRAKER2 pipeline and increasing with including proteome data was also reflected in the results from ParsEval, which was used to evaluate the closeness of each BRAKER2 annotation to the *D. melanogaster* reference annotation.

ParsEval analysis revealed that BRAKER2 RNAseq and BRAKER2 contained 2010 and 1781 gene loci (genomic regions of overlap containing annotations) that were not present in the reference annotation (i.e. false positive predictions). The gene loci in each annotation also contained far more genes than their equivalent gene loci in the reference, and the number of genes increased when proteome data was included (BRAKER2 RNA seq:reference annotation = 1.425:1.151, BRAKER2:reference annotation = 1.832:1.179; Table 5.1). Taken together, this is strong evidence that BRAKER2 overpredicts the number of genes in the *D. melanogaster* assembly. Nevertheless, when comparing CDS structures between loci, both BRAKER2 pipelines had more CDS structure matches with the reference (81.5% BRAKER2 RNAseq, and 81.2% BRAKER2) compared with OMAnnotation (79.3%). However, as ParsEval treats neither sequence as the reference, it does not penalise for a non-match or a duplicate match, meaning that although BRAKER2 predicted too many annotations, more of these predictions shared matching CDS structures with the reference.

5.5 Discussion

5.5.1 OMAnnotation performs better than any individual annotation method

The results from the quality assessment of the annotation sets produced by individual methods are as expected, based on the characteristics of each approach. AUGUSTUS

predicts likely genes using a Hidden Markov Model that is trained with RNAseq and homology data, making it sensitive but prone to false positives. Here, AUGUSTUS was run with the *D. melanogaster* assembly and fly gene model parameters, i.e., a high-quality assembly and a model on which it has been extensively trained — its optimal use case. Whilst it produced a more complete gene set than other individual methods, it also had the most unique predictions compared to the reference. Even with this high sensitivity, the number of missing genes was significantly improved using OMAnnotation. RNAseq data contains what is expressed in the extracted tissue at the time of extraction, meaning genes expressed at different developmental stages can be missed. Detected transcripts are subject to noise and genome mapping errors resulting in an annotation set with the highest number of duplicates and a high number of missing genes relative to other methods. Finally, the success of homology annotation depends on the relatedness between the genome of interest and the reference proteome, and the quality of the reference proteome. In this study, we chose the *A. gambiae* proteome as our reference, to mimic the situation in which the novel genome is from a species with no closely related model organism. This results in a homology annotation set with many missing genes.

Encouragingly, the OMAnnotation consensus sequence contained the highest percentage of complete genes according to BUSCO analysis, more than any individual method and approaching the standard of the reference annotation (Table 5.1). This demonstrates it combines annotation sets from different sources well, retaining the maximum gene predictions without losing accuracy. One caveat to this result is that OMAnnotation uses orthology data from other species to find evidence for including predicted genes in the consensus. It is therefore expected to preferentially select predictions with highly conserved orthologs, and thus, there is some circularity in using BUSCO to assess the quality of the OMAnnotation gene set. However, the gene count and ParsEval analysis results support the BUSCO results, indicating that OMAnnotation produced a consensus closer to the gold standard reference annotation than any other method individually.

5.5.2 OMAnnotation performance is comparable to a state-of-the-art pipeline, BRAKER2

The OMAnnotation set compares favourably with the sets produced by BRAKER2. The BRAKER2 pipeline predicted significantly more genes than BRAKER2 RNAseq, with more duplicates according to BUSCO and more genes than the reference annotation. This is in line with the protocol suggestion to use each source of evidence in separate BRAKER2 runs, then use TBSEBRA to combine sets and filter false positives (Brůna et al. 2021; Gabriel et al. 2021). This is a disadvantage for a user wanting to build a consensus annotation from multiple sources of evidence. Furthermore, there is strong evidence that even the BRAKER2 RNAseq pipeline overpredicted the number of genes in the *D. melanogaster* assembly. The BRAKER2 RNAseq annotation contained 26% more genes than the reference set, a significant number of fragmented and duplicated genes according to BUSCO, and a higher number of genes per loci than the reference according to ParsEval analysis.

One area where BRAKER2 performance is comparable to OMA is the conservation of exon-intron structures of annotations. Despite the overprediction of annotations compared with the reference, the gene structures within both BRAKER2 annotation

sets mapped as closely to the reference as those in the OMAnnotation set. In addition, whilst we focus on protein-coding annotations in this study, BRAKER2 is able to predict non-coding regions – a function not yet available with OMAnnotation. This is an area for future development. Additionally, OMAnnotation does not explicitly model genes or compare gene structure. Therefore, we propose that the use case for OMAnnotation is slightly different than BRAKER2: OMAnnotation is a highly flexible approach for combining annotations from different sources to produce the most complete gene set, while BRAKER2 is a sensitive tool that is better adapted for structural inference and identifying non-coding DNA. Additionally, since OMAnnotation can combine predictions from any GFF, it is not exclusive to any specific combination of annotation methods. Thus, it may also be used to combine annotations from methods that are specially designed to detect correct gene structures (such as BRAKER2) while reducing the prevalence of mispredictions of gene contents in these annotations.

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5.7 Tables

Table 5.1: ParsEval results from pairwise comparisons between the D. melanogaster reference annotation ('reference') and the annotations predicted by each method are shown ('prediction'). The 'ParsEval comparison level' column refers to the level at which the ParsEval features are being compared. 'Gene loci' are determined by ParsEval as the minimal genomic regions containing all overlapping annotations and the 'CDS structure' level is the coding sequence exon-intron structure of each annotation. The 'Comparison' column indicates how the ParsEval feature comparison was computed.

ParsEval comparison level	Comparison	AUGUSTUS ab initio	StringTie RNAseq	GeMoMa homology	OMAnnotation consensus	BRAKER2 RNAseq	BRAKER2
Gene loci	Number shared	8024	6998	6143	8129	9425	9387
	Unique to reference (FN)	282	3209	4087	994	480	459
	Unique to prediction (FP)	1337	24	198	259	2010	1781
	Shared (%)	83.21	68.40	58.91	86.64	79.10	80.73
	Genes per locus reference	1.419	1.344	1.375	1.458	1.151	1.179
	Genes per locus prediction	1.365	0.982	0.756	1.448	1.425	1.832
CDS structure	CDS match (%)	80.3	80.9	58.8	79.3	81.5	81.2

5.8 Figures

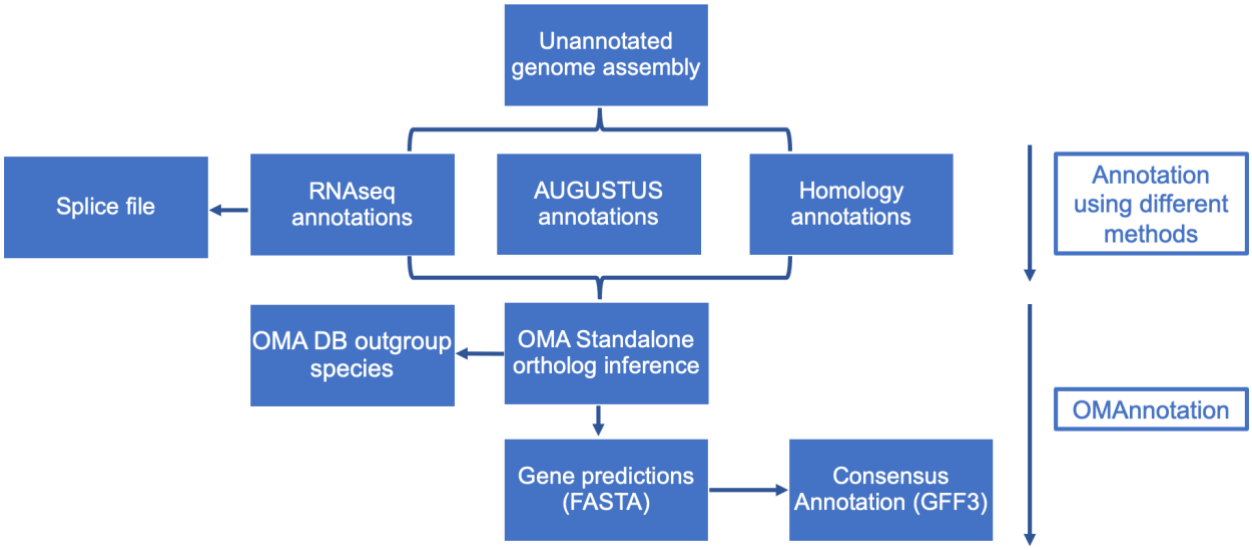

Figure 5.1: an overview of the OMAnnotation workflow. First, a genome assembly is annotated using different methods. FASTA formatted files are prepared from the annotation GFF3s using the custom script 'OMAnnotation.py'. The script also generates a splice file for any annotation containing multiple isoforms per gene. During the OMAnnotation step, OMA infers orthologs at each taxonomic level (HOGs), both between annotations and the user-selected outgroup species. The OMAnnotation.py script is then used to select the longest HOGs and outputs these in FASTA and GFF3 format.

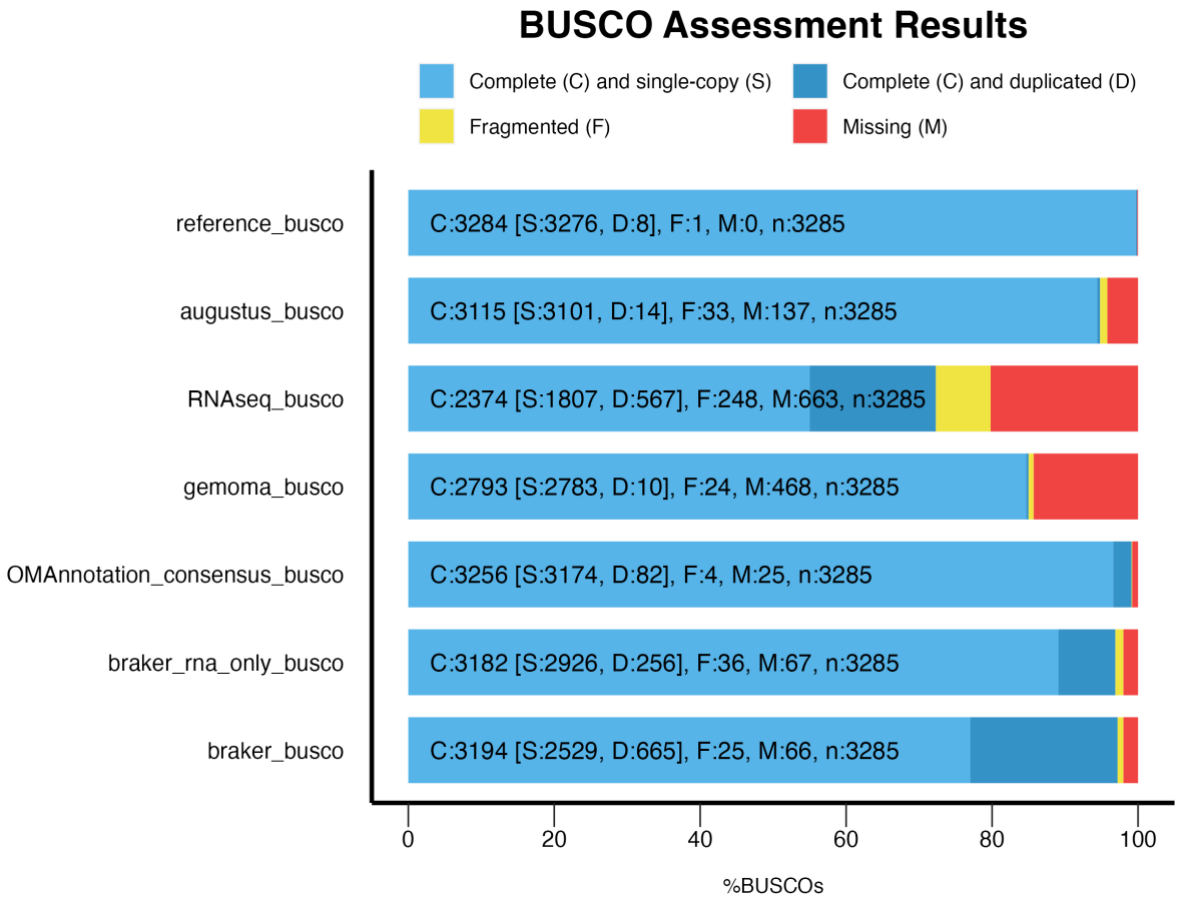

Figure 5.2: BUSCO results for the annotation set produced by each method. The BUSCO results for the D. melanogaster reference annotation are shown at the top ("reference_busco"), for comparison.

Chapter 6. General discussion

6.1 Overview

Sex-linked meiotic drive genes gain a transmission advantage by distorting sex chromosome segregation during meiosis, typically by killing non-drive-carrying gametes (Sandler and Novitski 1957). X-linked drive is particularly common among the *Diptera* and causes the destruction of Y-bearing sperm in males (Courret et al. 2019). In this thesis, I have discussed the fitness effects associated with an X-linked drive system, *Sex Ratio*, in the stalk-eyed fly, *Teleopsis dalmanni*. I discuss its fitness costs for males and females before presenting a novel genome annotation tool developed in collaboration with colleagues at the University of Lausanne, which will be used to annotate the *T. dalmanni* genome assembly.

6.2 Summary of principal findings

6.2.1 Chapter 2: Meiotic drive does not impede success in sperm competition in the stalk-eyed fly, *Teleopsis dalmanni*

The sperm killing caused by X-linked drive is typically associated with fitness costs, resulting in poor success under sperm competition (Sandler and Novitski 1957). X-linked drive is common among *Diptera* and has been widely found to negatively affect male fertility (Newton et al. 1976; Jaenike 2001; Wilkinson and Sanchez 2001; Atlan et al. 2004; Angelard et al. 2008; Courret et al. 2019). I investigated the impact of the X-linked meiotic drive gene *Sex Ratio* (SR) on male fertility in *T. dalmanni.* I performed double mating trials under sperm competition, where females were mated first with an $X^{SR}Y$ (SR) male or $X^{ST}Y$ (ST, non-drive) male, followed by a male of the opposite genotype and then genotyped the progeny to determine paternity.

I reported that SR males sired the same number of offspring as ST males, regardless of their mating position. This finding challenges the assumption that drive males inevitably suffer reduced fertility and contradicts the conclusions reached in a separate study of the fitness effects of SR in this species (Wilkinson et al. 2006; Verspoor et al. 2020). However, though I reached a different conclusion, this does not invalidate the studies by Wilkinson *et al.* (2006), as our experiments have different designs.

The Wilkinson *et al.* (2006) study used a similar double mating design in *T. dalmanni*, but only with SR males in the P2 role and, as in this study, it reported no difference between the number of offspring sired by SR and ST males in mixed paternity broods. However, there were 11 sired solely by the ST male and only 3 sired solely by the SR male (rate 14/40 = 35%). In Chapter 2, I report the opposite pattern: there were 3 broods sired solely by the ST male and 8 sired solely by the SR male (rate 11/51 = 22%). The higher rate of single-parent broods observed by Wilkinson *et al.* (2006) could be explained by another design difference: males were taken from mixed sex cages with no control over prior mating, whereas males were without females for several days to allow their accessory glands to return to full size in my experiments (Rogers et al. 2005). Overall, this suggests a large deficit in SR male single-parent broods is unlikely, which is consistent with previous work that showed no difference in the failure rate of sperm transfer to the spermatheca of females mated once either to ST or SR males (Meade et al. 2019).

In summary, my results fit with previous work showing that the SR and ST males transfer the same number of viable sperm per ejaculate to females during non-competitive single matings (Meade et al. 2019). For the first time, I show that SR sperm performance is not different from ST sperm performance during sperm competition. As such, I provide further evidence that SR males can compensate for sperm loss, likely due to the adaptive evolution of enlarged testes in drive males, and that this adaptation carries over into sperm competition, where SR sperm success is equal to that of ST sperm (Bradshaw et al. 2022).

6.2.2 Chapter 3: Meiotic drive does not impede success under high sperm competition in *Teleopsis dalmanni*

In Chapter 2, I report that SR males perform as well as ST males under sperm competition. However, this was in a singly mated female. Wild T. dalmanni females are sperm-limited, and so mate multiply to maximise their reproductive output. Therefore, in this chapter, I investigated whether drive males can maintain their reproductive success in a multiply mated female — i.e., high competition. Females were maximally mated with a drive or non-drive male prior to receiving a single mating from a male of the opposite genotype, and the paternity of her offspring was determined. Mating male genotypes in both orders assessed the offensive and defensive capabilities of drive and non-drive male sperm.

As expected, based on their higher number of mating opportunities and thus total sperm transferred, males in the first mating position performed best. Importantly, male genotype did not affect reproductive success — drive males are not disadvantaged compared to non-drive males on encountering a female full of rival male sperm. Together with the results I present in Chapter 2, this is compelling evidence that the competitiveness of drive male sperm contributes to the high prevalence of the SR variant in wild populations of *T. dalmanni.*

6.2.3 Chapter 4: the consequences of X-linked meiotic drive for female reproductive fitness

In this chapter, I examine the effects of SR on female reproductive fitness. Adverse fitness effects in females can occur in a dose-dependent manner, causing recessivity and, thus, negative frequency-dependent selection. Theory predicts decreases in fecundity to be particularly important for stabilising drive gene frequency in populations where drive males fare well in sperm competition — which describes the situation in *T. dalmanni*. I examined how SR impacts two crucial components of female reproductive fitness: time to sexual maturity and fecundity. I also checked for any effect of body size and eyespan on both traits by using females with a wide range of sizes in my experiments. I determined that female time to sexual maturity is not affected by genotype, and – unlike in males of this species – there was no interaction with body size or eyespan (Bradshaw et al. *unpublished*). I hypothesise that this might be due to the heightened importance of body size and eyespan for males of this species, as male eyespan is condition dependent and under strong sexual selection. There is a trend in males that SR males mature more quickly when they are small, which might be due to SR males needing to invest in larger testes to compensate for sperm loss

— large SR males take longer to reach sexual maturity as they cannot cope with the demands of rapid testes growth (Meade et al. 2019, 2020; Bradshaw et al. 2022).

I also found that SR affected fecundity – with some evidence of recessivity as homozygous SR females suffered the greatest reduction in fecundity – and that this trait was positively associated with body size and eyespan. Though the effect of body size and eyespan on fecundity is in line with previous studies that indicate these traits are a signal of female quality, the negative effect of SR had not been uncovered before (Cotton et al. 2015, Meade et al., *Unpublished field data*). This contradicts Wilkinson *et al.* (2006), who found no effect of body size or genotype after controlling for female age and cross-type. However, I propose that this difference is due to their crossing design, which led to an overproduction of heterozygous induvial who inherited their X^{SR} chromosomes down maternal and paternal routes, and their dissection of females across a range of ages (Wilkinson and Sanchez 2001). By controlling for these confounding effects, I was able to reveal a body size, eyespan and genotype effect.

6.2.4 Chapter 5: OMAnnotation: a novel approach to building an annotated consensus genome sequence

In this chapter, I present work that was done in collaboration with Prof Dessimoz at the University of Lausanne. I report on a new genome annotation tool and demonstrate its success in proof of principle testing. I also compare its accuracy to state of the art pipelines: BRAKER and BRAKER2.

6.3 Future directions

6.3.1 SR and sperm competition in *Teleopsis dalmanni*

In Chapter 2, I reported that SR males sire the same number of offspring as ST males under sperm competition. However, this study was performed on singly mated females. In the wild, *T. dalmanni* has extremely high remating rates, and females mate up to 10 times per day at dawn to overcome sperm limitation (Baker 2001; Chapman et al. 2005). Therefore, in Chapter 3, I build on the complexity of my early single mating design by carrying out mating trials in a maximally mated female. Once again, I find that SR male sperm perform as well as ST male sperm, with SR males gaining fertility when a female's storage organs are already filled with sperm from a rival male. Combined with the results from Chapter 2, this is strong evidence that the cost of SR for male post-copulatory success is low in *T. dalmanni*. This ability of SR males to compensate for sperm loss is doubtless one of the factors contributing to the high prevalence of SR at around ~20% in wild populations (Wilkinson et al. 2003; Holman et al. 2015). In combination, the results I present in this thesis provide strong evidence that the performance of SR sperm is equal to ST sperm under low and high competition. Yet, it is with the caveat that not all variables have been tested. These include different combinations of mating design besides 1 and 1, and many and 1, male and female condition, ageing, environmental factors, demography etc. There is scope to add further complexity to the mating trial system used here, to recapitulate more of the conditions that SR males encounter in nature.

In the multiple mating assay, the first male sired most of the offspring per brood, the proportion of offspring sired by the second male was relatively high, regardless of male

genotype, at around 30%. Furthermore, this value was extremely variable between broods, with the second male sometimes siring almost none (less than 0.05%) or most of the offspring (more than 0.95%). This implies that sperm mixing is not the sole mechanism by which sperm competition occurs in *T. dalmanni*, a notion which is supported by the variable sperm precedence values reported when females mated once to two males (Chapter 2), and a previous study of sperm precedence in wildtype *T. dalmanni* (Corley et al. 2006). It could be that females of this species can exercise cryptic female choice – are females selectively discarding male sperm (Firman et al. 2017)? Future research should aim to uncover how sperm precedence can vary to such an extent in this species and determine whether there is evidence for cryptic female choice.

6.3.2 The consequences of SR meiotic drive for female time to sexual maturity

In Chapter 4, I reported that SR reduces fecundity in female *T. dalmanni*. However, I found no effect of SR on female time to sexual maturity. This is unlike the trend observed in males, where large SR males mature more slowly, and it has been postulated that this is owing to large SR males being unable to cope with the demands of growing enlarged testes (Bradshaw et al. *unpublished*). As this effect does not exist in females, we observe no effect of SR on time to maturity. Yet, I have not examined the social condition and mating status of females. In my experiment, a female was identified as mature when she laid her first egg. I dissected mature virgin females and counted the eggs inside their ovaries. I chose to do this to prevent my ovary egg counts from varying according to the sporadic nature of egg laying that is typical of this species, where there is a large variation in the number of eggs laid per day (Reguera et al. 2004). However, *T. dalmanni* females have been shown to mature more quickly in the presence of males and lay more eggs when mated than virgins (Reguera et al. 2004). It would be interesting to establish another experiment, focussed solely on time to sexual maturity, where the effect of social and mating conditions of the females was examined. A female could be housed with and without males to reveal if there is any interaction between these factors and genotype acting on time to sexual maturity.

6.3.3 The consequences of SR for female fecundity

In Chapter 4, I show the SR allele is associated with a reduction in female fecundity, with some evidence of recessivity as HOM-SR females had lower fecundity than HET females. I discuss a modelling approach that is adapted from Larner et al. 2019, as a way of further investigating this effect and how it contributes to the stabilisation of SR frequencies in natural populations of *T. dalmanni* (Holman et al. 2015; Larner et al. 2019). This model will be implemented to build on work by Finnegan et al. 2019 – which established that HOM-SR females suffer reduced viability – and will also include average SR success under sperm competition effect determined in Chapter 3. By combining the fitness effects discussed in this thesis with the work of previous researchers, I hope to link fitness effects with the ecology and frequency of SR in natural populations.

6.3.4 Annotating the new *Teleopsis dalmanni* genome assembly

The *T. dalmanni* genome has recently undergone re-sequencing, and a new and improved genome assembly is available. As the annotation tool that I presented in Chapter 5 has proven successful in principle testing, I will use it to annotate the new *T. dalmanni* genome. For completeness, I will also implement re-existing pipelines and compare the annotation sets produced. Little is known about the mechanism behind SR-mediated sperm killing in males or even the precise location of the SR variant. Recently, there have been new discoveries in X-chromosome evolution, which I aim to build on with our new assembly and annotation (Reinhardt et al. 2022). An accurate genome annotation will provide exciting new collaborations and avenues for research.

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Supplementary Information

The following pages contain Supplementary Information from: "Meiotic drive does not impede success in sperm competition in the stalk-eyed fly, *Teleopsis dalmanni*". Supplementary Figures have been renumbered in this document to fit the main thesis text.

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Chapter 2 Supplementary Information 1:

- supplementary methods

Chapter 2 Supplementary Information 2:

- all statistical models and effect sizes for all broods
- supplementary Figures S2.2.1 and S2.2.2

Chapter 2 Supplementary Information 3:

- all statistical models and effect sizes for broods with ≥ 10 genotyped offspring

Chapter 2 Supplementary Information 4:

- resampling

Chapter 2 Supplementary Information 1: supplementary methods

The following protocol was used to extract and purify larval DNA (adapted from standard protocol in Burke *et al.*, 1998)*:*

Larvae within each well were crushed using a micro-pestle prior to incubation for 16hrs at 55ºC, to extract DNA. The following day, 35µL 4M ammonium acetate was added to each sample to precipitate out proteins, and the plates chilled on ice for 5mins. The plates were then spun at 4450rpm, 4ºC for 60min. Next, the DNA was precipitated out by transferring 80µL of the supernatant from each sample to a new plate containing 80µL isopropanol per well. Centrifugation at 4450rpm, 4ºC for 60min pelleted out the DNA. The supernatant was discarded, and the DNA pellets were washed by adding 100µL 70% ethanol and spinning at 4450rpm for 30min. The ethanol was then removed, and the plates left to air dry for 1hr before adding 30µL T10 E0.1 buffer to each sample and incubating at 37ºC for 30mins to re-dissolve the DNA. Samples were stored at -20ºC prior to PCR analysis.

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The following PCR conditions were used for progeny genotyping:

A 2720 Thermal Cycler (Applied Biosystems, Woolston, UK) was used to perform the reactions, which were carried out in 11µL volumes per sample, containing: 0.6µL forward and 0.6µL reverse primers (see Supp. Table 1 for sequences), both at 10µM, 0.12µL Phusion® High-Fidelity DNA Polymerase (New England Biolabs, Herts), 2.4µL Phusion® HF buffer (New England Biolabs, Herts), 6.4µL ddH_20 and either 1µL 10x diluted DNA, 5x diluted or pure DNA (depending on DNA concentration). The PCR programme was a 10min initial denaturation stage at 98ºC, followed by 45 cycles of 10sec denaturation at 98ºC, 30sec

annealing time at 63ºC and 20sec extension at 72ºC. The reaction was completed by a 7min final extension step at 72ºC. The PCR products were analysed via gel electrophoresis on a 3% agarose/TBE gel run at 100V for ~1hr to separate them according to size and the results were visualised using a gel imaging system.

Supplementary Table 1: comp16710 primer sequences

STRAND	Sequence
Forward	CGTGTCCGCATTTATACCAC
Reverse	GGTAGGCTTGTTCTAACGGC

Chapter 2 Supplementary Information 2: all model tables and effect sizes for all broods

Contents

1 Data

This analysis includes all broods with more than 2 offspring genotyped, N = 51. 2 matings were 29secs in duration, however these copulations were still deemed successful they resulted in offspring.

1 ST male failed to mate in the P1 position and was replaced, and 1 SR male failed to mate in the in P2 position and was replaced. 1 female failed to mate with any male and was discarded.

2 Male fertility

2.1 Variation in number of offspring sired with mating position

```
glm(formula = cbind(SR_offspring, ST_offspring) ~ as.factor(mating_position),
    family = quasibinomial, data = by_brood2)
```

Table 1: Analysis of variance table

	Df	Deviance	Resid. Df	Resid. Dev	F	Pr(>F)
NULL			50	554.310		
as.factor(mating_position)	1	12.022	49	542.288	1.307	0.259

Table 2: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.125	0.250	-0.501	0.619
as.factor(mating_position)P2	0.409	0.358	1.140	0.260

The mean proportion of offspring sired by the P2 male was 0.522 ± 0.327 (mean P2 ± sd) and there was no effect of mating position on the number of offspring sired per male.

2.2 Variation in number of offspring sired with male genotype

```
glm(formula = cbind(P2_offspring, P1_offspring) ~ as.factor(male_genotype),
    family = quasibinomial, data = by_brood2)
```

Table 3: Analysis of variance table

	Df	Deviance	Resid. Df	Resid. Dev	F	Pr(>F)
NULL			50	544.092		
as.factor(male_genotype)	1	1.805	49	542.288	0.196	0.66

Table 4: Model coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.284	0.257	1.103	0.275
as.factor(male_genotype)ST	-0.159	0.358	-0.443	0.660

The mean proportion of offspring sired by the SR male was 0.577 ± 0.319 (SR ± sd) and there was no effect of male genotype on the number of offspring sired per male.

2.3 Variation in number of offspring sired with male genotype in the P1 role or P2 role

```
lm(formula = P1_offspring ~ as.factor(male_genotype), data = P1_males_only)
```

Table 5: Analysis of variance table

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
as.factor(male_genotype)	1	0.214	0.214	0.002	0.963
Residuals	49	4916.963	100.346		

Table 6: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	10.296	1.928	5.341	0.000
as.factor(male_genotype)ST	-0.130	2.810	-0.046	0.963

```
lm(formula = P2_offspring ~ as.factor(male_genotype), data = P2_males_only)
```

Table 7: Analysis of variance table

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
as.factor(male_genotype)	1	42.706	42.706	0.434	0.513
Residuals	49	4826.000	98.490		

Table 8: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	13.500	2.026	6.664	0.000
as.factor(male_genotype)ST	-1.833	2.784	-0.658	0.513

Examining the data from P1 males only, there was no effect of genotype on number of offspring sired by the P1 male ($F_{1,49} = 0.002$, $P = 0.963$). The same was true when examining data from P2 males only ($F_{1,49} = 0.434$, $P = 0.513$).

2.4 Number of larvae collected and batch number

The number of larvae collected is a measure of female fecundity, as it is the total number of offspring that were collected including the random sample of offspring from each female weren't genotyped (for logistic reasons). The mean number of larvae collected per female was 48.196 ± 22.735 (mean ± sd), with a range of 6 - 116 offspring.

2.4.1 Variation in P2 number of offspring sired with larvae collected

```
glm(formula = cbind(P2_offspring, P1_offspring) ~ larvae_collected,
    family = quasibinomial, data = by_brood2)
```

Table 9: Analysis of variance table

	Df	Deviance	Resid. Df	Resid. Dev	F	Pr(>F)
NULL			50	544.092		
larvae_collected	1	0.342	49	543.750	0.037	0.848

Table 10: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.287	0.475	0.605	0.548
larvae_collected	-0.001	0.008	-0.193	0.848

2.4.2 Variation in SR number of offspring sired with larvae collected

```
glm(formula = cbind(SR_offspring, ST_offspring) ~ larvae_collected,
    family = quasibinomial, data = by_brood2)
```

Table 11: Analysis of variance table

	Df	Deviance	Resid. Df	Resid. Dev	F	Pr(>F)
NULL			50	554.310		
larvae_collected	1	44.381	49	509.928	5.09	0.029

Table 12: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	1.060	0.481	2.201	0.032
larvae_collected	-0.017	0.008	-2.193	0.033

The number of Larvae collected was not associated with the numbers of P2 or SR offspring.

2.4.3 Variation in P2 number of offspring sired with larvae collected and mating order

```
glm(formula = cbind(SR_offspring, ST_offspring) ~ larvae_collected +
    mating_position, family = quasibinomial, data = by_brood2)
```

Table 13: Analysis of variance table (Type II tests)

	Sum Sq	Df	F value	Pr(>F)
larvae_collected	58.453	1	6.745	0.012
mating_position	26.093	1	3.011	0.089
Residuals	415.988	48		

Table 14: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.939	0.488	1.925	0.060
larvae_collected	-0.020	0.008	-2.514	0.015
mating_positionP2	0.634	0.369	1.716	0.093

2.4.4 Variation in SR number of offspring sired with larvae collected and genotype

```
glm(formula = cbind(P2_offspring, P1_offspring) ~ larvae_collected +
    male_genotype, family = quasibinomial, data = by_brood2)
```

Table 15: Analysis of variance table (Type II tests)

	Sum Sq	Df	F value	Pr(>F)
larvae_collected	0.805	1	0.086	0.771
male_genotype	2.268	1	0.242	0.625
Residuals	450.489	48		

Table 16: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.429	0.561	0.765	0.448
larvae_collected	-0.002	0.008	-0.293	0.771
male_genotypeST	-0.182	0.371	-0.491	0.626

When the number of larvae collected was added as a covariate, there was no affect on the trends previously observed: neither mating order nor male genotype had an affect on the number of offspring sired by each male ($P > 0.05$).

2.4.5 Variation in P2 number of offspring sired with mating order and batch number

```
glm(formula = cbind(SR_offspring, ST_offspring) ~ batch + mating_position,
    family = quasibinomial, data = by_brood2)
```

Table 17: Analysis of variance table (Type II tests)

	Sum Sq	Df	F value	Pr(>F)
batch	26.229	1	2.881	0.096
mating_position	12.733	1	1.399	0.243
Residuals	437.002	48		

Table 18: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-1.134	0.652	-1.740	0.088
batch	0.623	0.369	1.687	0.098
mating_positionP2	0.425	0.361	1.179	0.244

2.4.6 Variation in SR number of offspring sired with genotype and batch number

```
glm(formula = cbind(P2_offspring, P1_offspring) ~ batch + male_genotype,
    family = quasibinomial, data = by_brood2)
```

Table 19: Analysis of variance table (Type II tests)

	Sum Sq	Df	F value	Pr(>F)
batch	14.562	1	1.581	0.215
male_genotype	1.954	1	0.212	0.647
Residuals	442.084	48		

Table 20: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.453	0.640	-0.708	0.482
batch	0.462	0.368	1.255	0.216
male_genotypeST	-0.166	0.361	-0.460	0.647

The effects of mating order and genotype remained insignificant ($P > 0.05$) when Batch number (mating trials were carried out in two batches, for ease) was added as a covariate.

2.5 Male fertility and single parent broods

Table 21: Single parent broods

mating position	proportion P2 offspring	male genotype	proportion SR offspring
P1	0.028	ST	0.972
P1	0.000	ST	1.000
P1	0.956	ST	0.044
P1	0.000	ST	1.000
P2	1.000	SR	1.000
P1	0.000	ST	1.000
P2	0.955	SR	0.955
P2	0.958	SR	0.958
P1	0.000	ST	1.000
P1	0.967	ST	0.033
P1	0.000	ST	1.000
P2	0.960	SR	0.960
P2	0.969	SR	0.969
P2	0.034	SR	0.034
P2	1.000	SR	1.000

2.5.1 Variation in number of offspring sired with mating position with only single parent broods

```
glm(formula = cbind(SR_offspring, ST_offspring) ~ as.factor(mating_position),
    family = quasibinomial, data = subset(by_brood2, proportion_SR <=
        0.05 | proportion_SR >= 0.95))
```

Table 22: Analysis of variance table

	Df	Deviance	Resid. Df	Resid. Dev	F	Pr(>F)
NULL			14	325.901		
as.factor(mating_position)	1	24.816	13	301.085	1.188	0.296

Table 23: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.152	0.729	0.209	0.838
as.factor(mating_position)P2	1.226	1.162	1.056	0.310

When only extreme P2 offspring proportions of ≤ 0.05 and ≥ 0.95 are considered, the proportion of offspring sired by the P2 male was not different from 0.5 (proportion P2 = 0.522 ± 0.497), and mating order had no effect on the number of offspring sired.

2.5.2 Variation in number of offspring sired with male genotype with only single parent broods

```
glm(formula = cbind(P2_offspring, P1_offspring) ~ as.factor(male_genotype),
```

```
    family = quasibinomial, data = subset(by_brood2, proportion_P2 <=
        0.05 | proportion_P2 >= 0.95))
```

Table 24: Analysis of variance table

	Df	Deviance	Resid. Df	Resid. Dev	F	Pr(>F)
NULL			14	340.750		
as.factor(male_genotype)	1	39.665	13	301.085	1.898	0.192

Table 25: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	1.378	0.904	1.525	0.151
as.factor(male_genotype)ST	-1.531	1.162	-1.318	0.210

When only extreme SR offspring proportions of ≤ 0.05 and ≥ 0.95 are considered, the proportion of offspring sired by the SR male did not differ from 0.5 (proportion SR $\pm$ sd $= 0.795 \pm 0.393$), and male genotype had no effect on number of offspring sired.

2.5.3 Variation in number of offspring sired with mating position when single parent broods are excluded

```
glm(formula = cbind(SR_offspring, ST_offspring) ~ as.factor(mating_position),
    family = quasibinomial, data = subset(by_brood2, proportion_SR >
        0.05 & proportion_SR < 0.95))
```

Table 26: Analysis of variance table

	Df	Deviance	Resid. Df	Resid. Dev	F	Pr(>F)
NULL			35	188.308		
as.factor(mating_position)	1	1.224	34	187.084	0.243	0.625

Table 27: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.226	0.216	-1.045	0.303
as.factor(mating_position)P2	0.153	0.310	0.493	0.625

When extreme P2 offspring proportions of ≤ 0.05 and ≥ 0.95 are excluded, the proportion of offspring sired by the P2 male was not different from 0.5 (proportion P2 $\pm$ sd $= 0.522 \pm 0.233$) and mating order had no effect on number of offspring sired.

2.5.4 Variation in male number of offspring sired with male genotype when single parent broods are excluded

```
glm(formula = cbind(P2_offspring, P1_offspring) ~ as.factor(male_genotype),
    family = quasibinomial, data = subset(by_brood2, proportion_P2 >=
        0.05 & proportion_P2 <= 0.95))
```

Table 28: Analysis of variance table

	Df	Deviance	Resid. Df	Resid. Dev	F	Pr(>F)
NULL			35	191.796		
as.factor(male_genotype)	1	4.711	34	187.084	0.936	0.34

Table 29: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.073	0.222	-0.331	0.743
as.factor(male_genotype)ST	0.300	0.310	0.966	0.341

When extreme SR offspring proportions of ≤ 0.05 and ≥ 0.95 are excluded, the proportion of offspring sired by the SR male was not different from 0.5 (proportion SR $\pm$ sd $= 0.486 \pm 0.234$) and male genotype had no effect on number of offspring sired.

3 Male trait size and mating duration

3.1 Variation in male traits

Table 30: Mean thorax length per male genotype

male_genotype	N	thorax	sd	se	ci
SR	49	2.190	0.163	0.023	0.047
ST	50	2.297	0.178	0.025	0.051

Table 31: Mean eyespan per male genotype

male_genotype	N	eyespan	sd	se	ci
SR	49	7.304	0.776	0.111	0.223
ST	50	7.897	0.815	0.115	0.232

Table 32: Mean residual eyespan per male genotype

male_genotype	N	residual_eyespan	sd	se	ci
SR	49	-0.112	0.478	0.068	0.137
ST	50	0.092	0.531	0.075	0.151

```
lm(formula = eyespan ~ thorax, data = by_male_id2)
```

Table 33: Analysis of variance table

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
thorax	1	44.406	44.406	167.242	0
Residuals	97	25.756	0.266		

Table 34: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.875	0.658	-1.330	0.187
thorax	3.778	0.292	12.932	0.000

```
lm(formula = thorax ~ male_genotype, data = by_male_id2)
```

Table 35: Analysis of variance table

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
male_genotype	1	0.285	0.285	9.783	0.002
Residuals	97	2.826	0.029		

Table 36: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	2.190	0.024	89.812	0.000
male_genotypeST	0.107	0.034	3.128	0.002

```
lm(formula = eyespan ~ male_genotype, data = by_male_id2)
```

Table 37: Analysis of variance table

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
male_genotype	1	8.720	8.720	13.766	0
Residuals	97	61.442	0.633		

Table 38: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	7.304	0.114	64.239	0
male_genotypeST	0.594	0.160	3.710	0

```
lm(formula = eyespan ~ thorax + male_genotype, data = by_male_id2)
```

Table 39: Analysis of variance table (Type II tests)

	Sum Sq	Df	F value	Pr(>F)
thorax	36.651	1	141.924	0.000
male_genotype	0.964	1	3.734	0.056
Residuals	24.791	96		

Table 40: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.583	0.666	-0.875	0.384
thorax	3.601	0.302	11.913	0.000
male_genotypeST	0.207	0.107	1.932	0.056

Thorax length varies between male genotypes. Eyespan has strong covariance with thorax. Relative eyespan — the variation in eyespan not predicted by thorax length — is not significantly different between genotypes. Therefore, relative eyespan is not added to binomial GLMs for paternity.

3.2 Male fertility with thorax length

```
glm(formula = cbind(ST_offspring, SR_offspring) ~ thorax.P1 +
    thorax.P2 + mating_position, family = quasibinomial, data = by_brood2)
```

Table 41: Analysis of variance table (Type II tests)

	Sum Sq	Df	F value	Pr(>F)
thorax.P1	9.464	1	0.977	0.328
thorax.P2	0.515	1	0.053	0.819
mating_position	11.241	1	1.161	0.287
Residuals	426.130	44		

Table 42: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	3.139	3.542	0.886	0.380
thorax.P1	-1.058	1.076	-0.984	0.330
thorax.P2	-0.291	1.265	-0.230	0.819
mating_positionP2	-0.452	0.421	-1.074	0.289

```
glm(formula = cbind(P1_offspring, P2_offspring) ~ thorax.ST +
    thorax.SR + male_genotype, family = quasibinomial, data = by_brood2)
```

Table 43: Analysis of variance table (Type II tests)

	Sum Sq	Df	F value	Pr(>F)
thorax.ST	0.023	1	0.002	0.961
thorax.SR	7.167	1	0.731	0.397
male_genotype	3.619	1	0.369	0.547
Residuals	431.689	44		

Table 44: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-2.510	3.512	-0.715	0.479
thorax.ST	-0.055	1.127	-0.049	0.961
thorax.SR	1.026	1.204	0.852	0.399
male_genotypeST	0.236	0.388	0.607	0.547

Though thorax length is different between SR and ST males, it does not affect number of offspring sired by each male, nor does it affect number of offspring sired by P2 and SR males.

3.3 Male fertility with mating duration

Mating duration is the observed time taken for a single copulation in seconds.

3.3.1 Variation in mating duration with mating position

```
lm(formula = mating_duration_sec ~ mating_position, data = by_male_id2)
```

Table 45: Analysis of variance table

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
mating_position	1	2306.127	2306.127	0.943	0.334
Residuals	100	244633.451	2446.335		

Table 46: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	63.941	6.926	9.232	0.000
mating_positionP2	9.510	9.795	0.971	0.334

Mating duration did not differ between P1 and P2 males (mean P1 mating duration ± se: 63.94sec ± 3.43sec, mean P2 mating duration ± se: 73.45sec ± 3.43sec; $F_{1,100} = 0.943$, $P = 0.334$).

3.3.2 Variation in mating duration with male genotype

```
lm(formula = mating_duration_sec ~ male_genotype, data = by_male_id2)
```

Table 47: Analysis of variance table

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
male_genotype	1	6321.657	6321.657	2.627	0.108
Residuals	100	240617.922	2406.179		

Table 48: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	76.569	6.869	11.147	0.000
male_genotypeST	-15.745	9.714	-1.621	0.108

Mating duration did not differ between ST and SR males (mean ST mating duration ± se: 60.82sec ± 2.36sec, mean SR mating duration ± se: 76.57sec ± 9.42sec; $F_{1,100} = 2.627$, $P = 0.108$).

3.3.3 The effect of mating duration on the number of offspring sired per mating position

```
glm(formula = cbind(P2_offspring, P1_offspring) ~ mating_duration_sec.P1 +
    mating_duration_sec.P2, family = quasibinomial, data = by_brood2)
```

Table 49: Analysis of variance table (Type II tests)

	Sum Sq	Df	F value	Pr(>F)
mating_duration_sec.P1	35.880	1	4.082	0.049
mating_duration_sec.P2	0.196	1	0.022	0.882
Residuals	421.956	48		

Table 50: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	1.094	0.577	1.895	0.064
mating_duration_sec.P1	-0.015	0.008	-1.913	0.062
mating_duration_sec.P2	0.000	0.003	0.149	0.882

Mating duration did not affect the number of offspring sired by the P2 males ($F_{1,48} = 0.022$, $P = 0.882$), but P1 males with shorter mating durations sired more offspring ($F_{1,48} = 4.082$, $P = 0.049$).

3.3.4 The effect of mating duration on the number of offspring sired per male genotype

```
glm(formula = cbind(ST_offspring, SR_offspring) ~ mating_duration_sec.ST +
    mating_duration_sec.SR, family = quasibinomial, data = by_brood2)
```

Table 51: Analysis of variance table

	Sum Sq	Df	F value	Pr(>F)
mating_duration_sec.ST	30.422	1	3.366	0.073
mating_duration_sec.SR	2.226	1	0.246	0.622
Residuals	433.796	48		

Table 52: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-1.380	0.896	-1.541	0.130
mating_duration_sec.ST	0.024	0.014	1.734	0.089
mating_duration_sec.SR	-0.001	0.003	-0.488	0.628

Mating duration did not affect the number of offspring sired by the SR males ($F_{1,48} = 0.246$, $P = 0.622$). However, it did affect the number of offspring sired by the ST males ($F_{1,48} = 3.366$, $P = 0.073$).

3.3.5 The effect of mating position with mating duration on the number of offspring sired

```
glm(formula = cbind(ST_offspring, SR_offspring) ~ mating_duration_sec.ST +
    mating_duration_sec.SR + mating_position, family = quasibinomial,
    data = by_brood2)
```

Table 53: Analysis of variance table (Type II tests)

	Sum Sq	Df	F value	Pr(>F)
mating_duration_sec.ST	32.549	1	3.565	0.065
mating_duration_sec.SR	0.535	1	0.059	0.810
mating_position	11.025	1	1.208	0.277
Residuals	429.068	47		

Table 54: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-1.269	0.892	-1.423	0.161
mating_duration_sec.ST	0.024	0.013	1.797	0.079
mating_duration_sec.SR	-0.001	0.003	-0.240	0.811
mating_positionP2	-0.407	0.372	-1.096	0.279

Mating position still did not affect number of offspring sired by each male when mating duration was included as a covariate ($F_{1,47} = 1.208$, $P = 0.277$).

3.3.6 The effect of male genotype with mating duration on the number of offspring sired

```
glm(formula = cbind(P1_offspring, P2_offspring) ~ mating_duration_sec.P1 +
    mating_duration_sec.P2 + male_genotype, family = quasibinomial,
    data = by_brood2)
```

Table 55: Analysis of variance table (Type II tests)

	Sum Sq	Df	F value	Pr(>F)
mating_duration_sec.P1	35.442	1	3.950	0.053
mating_duration_sec.P2	0.052	1	0.006	0.940
male_genotype	0.636	1	0.071	0.791
Residuals	421.668	47		

Table 56: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-1.156	0.630	-1.835	0.073
mating_duration_sec.P1	0.015	0.008	1.882	0.066
mating_duration_sec.P2	0.000	0.003	-0.076	0.940
male_genotypeST	0.099	0.372	0.266	0.791

Male genotype type still does not affect the number of offspring sired by each male when mating during is included as a covariate ($F_{1,47} = 0.071$, $P = 0.791$).

4 Supplementary figures

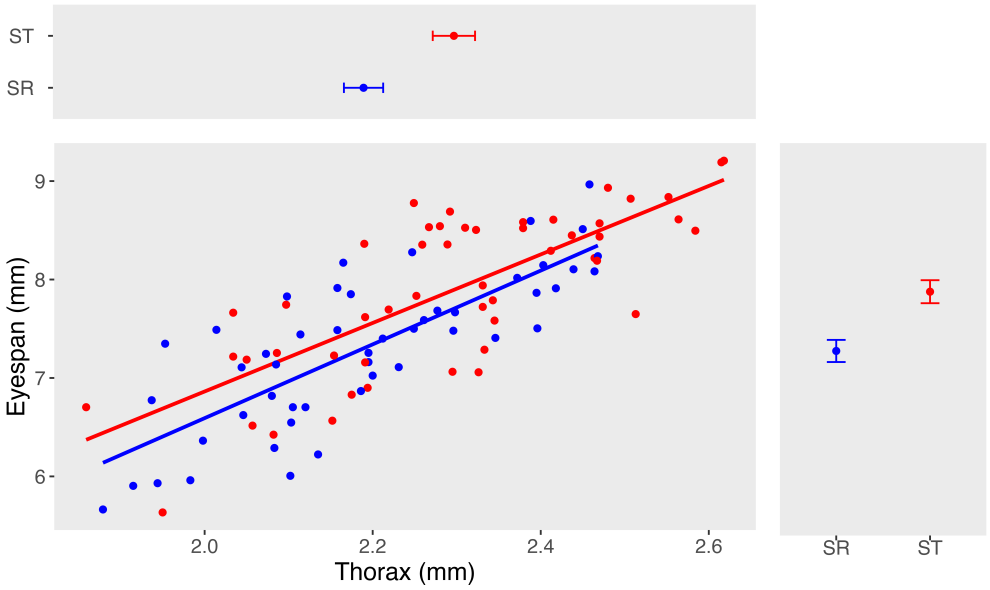

Figure S2.2.1: Variation in thorax length and eyespan with male genotype

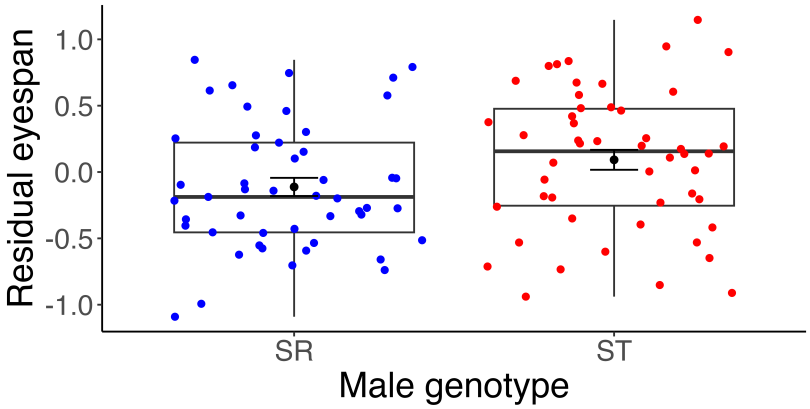

Figure S2.2.2: Variation in relative eyespan with genotype

Chapter 2 Supplementary Information 3: all model tables and effect sizes for broods with 10 or more offspring genotyped

Contents

1 Data

This analysis includes all broods with ≥ 10 offspring genotyped, N = 47. 2 matings were 29secs in duration, however the copulations were still deemed successful as they resulted in offspring.

1 ST male failed to mate in the P1 position and was replaced, and 1 SR male failed to mate in the in P2 position and was replaced. 1 female failed to mate with any male and was discarded.

2 Male fertility

2.1 Variation in number of offspring sired with mating position

```
glm(formula = cbind(SR_offspring, ST_offspring) ~ as.factor(mating_position),
    family = quasibinomial, data = by_brood2)
```

Table 1: Analysis of variance table

	Df	Deviance	Resid. Df	Resid. Dev	F	Pr(>F)
NULL			46	533.470		
as.factor(mating_position)	1	15.801	45	517.669	1.643	0.207

Table 2: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.189	0.26	-0.726	0.472
as.factor(mating_position)P2	0.472	0.37	1.277	0.208

The mean proportion of offspring sired by the P2 male was 0.561 $\pm$ 0.309 (mean P2 $\pm$ sd) and there was no effect of mating position on the number of offspring sired per male.

2.2 Variation in number of offspring sired with male genotype

```
glm(formula = cbind(P2_offspring, P1_offspring) ~ as.factor(male_genotype),
    family = quasibinomial, data = by_brood2)
```

Table 3: Analysis of variance table

	Df	Deviance	Resid. Df	Resid. Dev	F	Pr(>F)
NULL			46	518.302		
as.factor(male_genotype)	1	0.633	45	517.669	0.066	0.799

Table 4: Model coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.284	0.263	1.079	0.286
as.factor(male_genotype)ST	-0.095	0.370	-0.257	0.799

The mean proportion of offspring sired by the SR male was 0.546 ± 0.312 (SR ± sd) and there was no effect of male genotype on the number of offspring sired per male.

2.3 Variation in number of offspring sired with male genotype in the P1 role or P2 role

```
lm(formula = P1_offspring ~ as.factor(male_genotype), data = P1_males_only)
```

Table 5: Analysis of variance table

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
as.factor(male_genotype)	1	15.201	15.201	0.144	0.706
Residuals	45	4754.203	105.649		

Table 6: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	11.304	2.143	5.274	0.000
as.factor(male_genotype)ST	-1.138	2.999	-0.379	0.706

```
lm(formula = P2_offspring ~ as.factor(male_genotype), data = P2_males_only)
```

Table 7: Analysis of variance table

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
as.factor(male_genotype)	1	0.272	0.272	0.003	0.957
Residuals	45	4213.217	93.627		

Table 8: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	13.500	1.975	6.835	0.000
as.factor(male_genotype)ST	0.152	2.823	0.054	0.957

Examining the data from P1 males only, there was no effect of genotype on number of offspring sired by the P1 male ($F_{1,45} = 0.144$, $P = 0.706$). The same was true when examining data from P2 males only ($F_{1,45} = 0.003$, $P = 0.957$).

2.4 Number of larvae collected and batch number

The number of larvae collected is a measure of female fecundity, as it is the total number of offspring that were collected including the random sample of offspring from each female weren't genotyped (for logistic reasons). The mean number of larvae collected per female was 50.957 ± 21.159 (mean ± sd), with a range of 15 - 116 offspring.

2.4.1 Variation in P2 number of offspring sired with larvae collected

```
glm(formula = cbind(P2_offspring, P1_offspring) ~ larvae_collected,
    family = quasibinomial, data = by_brood2)
```

Table 9: Analysis of variance table

	Df	Deviance	Resid. Df	Resid. Dev	F	Pr(>F)
NULL			46	518.302		
larvae_collected	1	2.723	45	515.579	0.284	0.597

Table 10: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.486	0.505	0.962	0.341
larvae_collected	-0.004	0.008	-0.533	0.597

2.4.2 Variation in SR number of offspring sired with larvae collected

```
glm(formula = cbind(SR_offspring, ST_offspring) ~ larvae_collected,
    family = quasibinomial, data = by_brood2)
```

Table 11: Analysis of variance table

	Df	Deviance	Resid. Df	Resid. Dev	F	Pr(>F)
NULL			46	533.470		
larvae_collected	1	35.504	45	497.966	3.826	0.057

Table 12: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.960	0.511	1.881	0.066
larvae_collected	-0.016	0.008	-1.912	0.062

The number of Larvae collected was not associated with the numbers of P2 or SR offspring.

2.4.3 Variation in P2 number of offspring sired with larvae collected and mating order

```
glm(formula = cbind(SR_offspring, ST_offspring) ~ larvae_collected +
    mating_position, family = quasibinomial, data = by_brood2)
```

Table 13: Analysis of variance table (Type II tests)

	Sum Sq	Df	F value	Pr(>F)
larvae_collected	47.848	1	5.202	0.027
mating_position	28.145	1	3.060	0.087
Residuals	404.700	44		

Table 14: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.817	0.519	1.576	0.122
larvae_collected	-0.019	0.008	-2.219	0.032
mating_positionP2	0.658	0.380	1.730	0.091

2.4.4 Variation in SR number of offspring sired with larvae collected and genotype

```
glm(formula = cbind(P2_offspring, P1_offspring) ~ larvae_collected +
    male_genotype, family = quasibinomial, data = by_brood2)
```

Table 15: Analysis of variance table (Type II tests)

	Sum Sq	Df	F value	Pr(>F)
larvae_collected	3.393	1	0.346	0.559
male_genotype	1.302	1	0.133	0.717
Residuals	431.023	44		

Table 16: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.591	0.588	1.005	0.320
larvae_collected	-0.005	0.008	-0.588	0.560
male_genotypeST	-0.139	0.381	-0.364	0.717

When the number of larvae collected was added as a covariate, there was no affect on the trends previously observed: neither mating order nor male genotype had an affect on the number of offspring sired by each male ($P > 0.05$).

2.4.5 Variation in P2 number of offspring sired with mating order and batch number

```
glm(formula = cbind(SR_offspring, ST_offspring) ~ batch + mating_position,
    family = quasibinomial, data = by_brood2)
```

Table 17: Analysis of variance table (Type II tests)

	Sum Sq	Df	F value	Pr(>F)
batch	25.582	1	2.681	0.109
mating_position	16.480	1	1.727	0.196
Residuals	419.803	44		

Table 18: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-1.194	0.675	-1.769	0.084
batch	0.621	0.382	1.627	0.111
mating_positionP2	0.488	0.373	1.309	0.197

2.4.6 Variation in SR number of offspring sired with genotype and batch number

```
glm(formula = cbind(P2_offspring, P1_offspring) ~ batch + male_genotype,
    family = quasibinomial, data = by_brood2)
```

Table 19: Analysis of variance table (Type II tests)

	Sum Sq	Df	F value	Pr(>F)
batch	15.656	1	1.628	0.209
male_genotype	0.693	1	0.072	0.790
Residuals	423.024	44		

Table 20: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.486	0.658	-0.739	0.464
batch	0.483	0.379	1.273	0.210
male_genotypeST	-0.100	0.372	-0.268	0.790

The effects of mating order and genotype remained insignificant ($P > 0.05$) when Batch number (mating trials were carried out in two batches, for ease) was added as a covariate.

2.5 Male fertility and single parent broods

Table 21: Single parent broods

mating position	proportion P2 offspring	male genotype	proportion SR offspring
P1	0.028	ST	0.972
P1	0.000	ST	1.000
P1	0.956	ST	0.044
P2	1.000	SR	1.000
P2	0.955	SR	0.955
P2	0.958	SR	0.958
P1	0.967	ST	0.033
P1	0.000	ST	1.000
P2	0.960	SR	0.960
P2	0.969	SR	0.969
P2	0.034	SR	0.034
P2	1.000	SR	1.000

2.5.1 Variation in number of offspring sired with mating position with only single parent broods

```
glm(formula = cbind(SR_offspring, ST_offspring) ~ as.factor(mating_position),
    family = quasibinomial, data = subset(by_brood2, proportion_SR <=
        0.05 | proportion_SR >= 0.95))
```

Table 22: Analysis of variance table

	Df	Deviance	Resid. Df	Resid. Dev	F	Pr(>F)
NULL			11	313.466		
as.factor(mating_position)	1	32.326	10	281.140	1.259	0.288

Table 23: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.042	0.848	-0.050	0.961
as.factor(mating_position)P2	1.420	1.312	1.082	0.305

When only extreme P2 offspring proportions of ≤ 0.05 and ≥ 0.95 are considered, the proportion of offspring sired by the P2 male was not different from 0.5 (proportion P2 = 0.652 ± 0.471), and mating order had no effect on the number of offspring sired.

2.5.2 Variation in number of offspring sired with male genotype with only single parent broods

```
glm(formula = cbind(P2_offspring, P1_offspring) ~ as.factor(male_genotype),
    family = quasibinomial, data = subset(by_brood2, proportion_P2 <=
        0.05 | proportion_P2 >= 0.95))
```

Table 24: Analysis of variance table

	Df	Deviance	Resid. Df	Resid. Dev	F	Pr(>F)
NULL			11	309.558		
as.factor(male_genotype)	1	28.418	10	281.140	1.107	0.317

Table 25: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	1.378	1.002	1.376	0.199
as.factor(male_genotype)ST	-1.336	1.312	-1.018	0.333

When only extreme SR offspring proportions of ≤ 0.05 and ≥ 0.95 are considered, the proportion of offspring sired by the SR male did not differ from 0.5 (proportion SR $\pm$ sd $= 0.744 \pm 0.426$), and male genotype had no effect on number of offspring sired.

2.5.3 Variation in number of offspring sired with mating position when single parent broods are excluded

```
glm(formula = cbind(SR_offspring, ST_offspring) ~ as.factor(mating_position),
    family = quasibinomial, data = subset(by_brood2, proportion_SR >
        0.05 & proportion_SR < 0.95))
```

Table 26: Analysis of variance table

	Df	Deviance	Resid. Df	Resid. Dev	F	Pr(>F)
NULL			34	186.928		
as.factor(mating_position)	1	1.408	33	185.520	0.274	0.604

Table 27: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.238	0.220	-1.081	0.288
as.factor(mating_position)P2	0.164	0.314	0.523	0.604

When extreme P2 offspring proportions of ≤ 0.05 and ≥ 0.95 are excluded, the proportion of offspring sired by the P2 male was not different from 0.5 (proportion P2 $\pm$ sd $= 0.53 \pm 0.232$) and mating order had no effect on number of offspring sired.

2.5.4 Variation in male number of offspring sired with male genotype when single parent broods are excluded

```
glm(formula = cbind(P2_offspring, P1_offspring) ~ as.factor(male_genotype),
    family = quasibinomial, data = subset(by_brood2, proportion_P2 >=
        0.05 & proportion_P2 <= 0.95))
```

Table 28: Analysis of variance table

	Df	Deviance	Resid. Df	Resid. Dev	F	Pr(>F)
NULL			34	190.576		
as.factor(male_genotype)	1	5.056	33	185.520	0.984	0.329

Table 29: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.073	0.224	-0.327	0.746
as.factor(male_genotype)ST	0.311	0.314	0.990	0.329

When extreme SR offspring proportions of ≤ 0.05 and ≥ 0.95 are excluded, the proportion of offspring sired by the SR male was not different from 0.5 (proportion SR $\pm$ sd $= 0.478 \pm 0.233$) and male genotype had no effect on number of offspring sired.

3 Male trait size and mating duration

3.1 Variation in male traits

Table 30: Mean thorax length per male genotype

male_genotype	N	thorax	sd	se	ci
SR	46	2.194	0.167	0.025	0.050
ST	46	2.284	0.177	0.026	0.053

Table 31: Mean eyespan per male genotype

male_genotype	N	eyespan	sd	se	ci
SR	46	7.329	0.788	0.116	0.234
ST	46	7.844	0.828	0.122	0.246

Table 32: Mean residual eyespan per male genotype

male_genotype	N	residual_eyespan	sd	se	ci
SR	46	-0.103	0.485	0.072	0.144
ST	46	0.085	0.542	0.080	0.161

```
lm(formula = eyespan ~ thorax, data = by_male_id2)
```

Table 33: Analysis of variance table

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
thorax	1	40.285	40.285	147.627	0
Residuals	90	24.559	0.273		

Table 34: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.815	0.694	-1.175	0.243
thorax	3.752	0.309	12.150	0.000

```
lm(formula = thorax ~ male_genotype, data = by_male_id2)
```

Table 35: Analysis of variance table

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
male_genotype	1	0.187	0.187	6.275	0.014
Residuals	90	2.676	0.030		

Table 36: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	2.194	0.025	86.316	0.000
male_genotypeST	0.090	0.036	2.505	0.014

```
lm(formula = eyespan ~ male_genotype, data = by_male_id2)
```

Table 37: Analysis of variance table

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
male_genotype	1	6.081	6.081	9.314	0.003
Residuals	90	58.763	0.653		

Table 38: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	7.329	0.119	61.520	0.000
male_genotypeST	0.514	0.168	3.052	0.003

```
lm(formula = eyespan ~ thorax + male_genotype, data = by_male_id2)
```

Table 39: Analysis of variance table (Type II tests)

	Sum Sq	Df	F value	Pr(>F)
thorax	34.969	1	130.795	0.000
male_genotype	0.765	1	2.861	0.094
Residuals	23.795	89		

Table 40: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.604	0.698	-0.865	0.389
thorax	3.615	0.316	11.437	0.000
male_genotypeST	0.189	0.112	1.691	0.094

Thorax length varies between male genotypes. Eyespan has strong covariance with thorax. Relative eyespan — the variation in eyespan not predicted by thorax length — is not significantly different between genotypes. Therefore, relative eyespan is not added to binomial GLMs for paternity.

3.2 Male fertility with thorax length

```
glm(formula = cbind(ST_offspring, SR_offspring) ~ thorax.P1 +
    thorax.P2 + mating_position, family = quasibinomial, data = by_brood2)
```

Table 41: Analysis of variance table (Type II tests)

	Sum Sq	Df	F value	Pr(>F)
thorax.P1	10.918	1	1.084	0.304
thorax.P2	0.007	1	0.001	0.979
mating_position	12.006	1	1.193	0.281
Residuals	412.771	41		

Table 42: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	2.781	3.625	0.767	0.447
thorax.P1	-1.142	1.103	-1.036	0.306
thorax.P2	-0.034	1.311	-0.026	0.979
mating_positionP2	-0.468	0.429	-1.089	0.282

```
glm(formula = cbind(P1_offspring, P2_offspring) ~ thorax.ST +
    thorax.SR + male_genotype, family = quasibinomial, data = by_brood2)
```

Table 43: Analysis of variance table (Type II tests)

	Sum Sq	Df	F value	Pr(>F)
thorax.ST	0.532	1	0.052	0.820
thorax.SR	8.547	1	0.840	0.365
male_genotype	2.133	1	0.210	0.649
Residuals	417.152	41		

Table 44: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-2.241	3.585	-0.625	0.535
thorax.ST	-0.266	1.165	-0.229	0.820
thorax.SR	1.125	1.233	0.913	0.367
male_genotypeST	0.182	0.399	0.458	0.650

Though thorax length is different between SR and ST males, it does not affect number of offspring sired by each male, nor does it affect number of offspring sired by P2 and SR males.

3.3 Male fertility with mating duration

Mating duration is the observed time taken for a single copulation in seconds.

3.3.1 Variation in mating duration with mating position

```
lm(formula = mating_duration_sec ~ mating_position, data = by_male_id2)
```

Table 45: Analysis of variance table

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
mating_position	1	3529.532	3529.532	1.361	0.246
Residuals	92	238504.085	2592.436		

Table 46: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	62.936	7.427	8.474	0.000
mating_positionP2	12.255	10.503	1.167	0.246

Mating duration did not differ between P1 and P2 males (mean P1 mating duration ± se: 62.94sec ± 3.46sec, mean P2 mating duration ± se: 75.19sec ± 3.46sec; $F_{1,92} = 1.361$, $P = 0.246$).

3.3.2 Variation in mating duration with male genotype

```
lm(formula = mating_duration_sec ~ male_genotype, data = by_male_id2)
```

Table 47: Analysis of variance table

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
male_genotype	1	5393.021	5393.021	2.097	0.151
Residuals	92	236640.596	2572.180		

Table 48: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	76.638	7.398	10.360	0.000
male_genotypeST	-15.149	10.462	-1.448	0.151

Mating duration did not differ between ST and SR males (mean ST mating duration ± se: 61.49sec ± 2.51sec, mean SR mating duration ± se: 76.64sec ± 10.16sec; $F_{1,92} = 2.097$, $P = 0.151$).

3.3.3 The effect of mating duration on the number of offspring sired per mating position

```
glm(formula = cbind(P2_offspring, P1_offspring) ~ mating_duration_sec.P1 +
    mating_duration_sec.P2, family = quasibinomial, data = by_brood2)
```

Table 49: Analysis of variance table (Type II tests)

	Sum Sq	Df	F value	Pr(>F)
mating_duration_sec.P1	33.151	1	3.600	0.064
mating_duration_sec.P2	0.075	1	0.008	0.928
Residuals	405.137	44		

Table 50: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	1.111	0.595	1.868	0.068
mating_duration_sec.P1	-0.014	0.008	-1.803	0.078
mating_duration_sec.P2	0.000	0.003	0.090	0.929

Mating duration did not affect the number of offspring sired by the P1 ($F_{1,44} = 3.6$, $P = 0.064$) or P2 males ($F_{1,44} = 0.008$, $P = 0.928$).

3.3.4 The effect of mating duration on the number of offspring sired per male genotype

```
glm(formula = cbind(ST_offspring, SR_offspring) ~ mating_duration_sec.ST +
    mating_duration_sec.SR, family = quasibinomial, data = by_brood2)
```

Table 51: Analysis of variance table

	Sum Sq	Df	F value	Pr(>F)
mating_duration_sec.ST	27.681	1	2.897	0.096
mating_duration_sec.SR	2.373	1	0.248	0.621
Residuals	420.368	44		

Table 52: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-1.289	0.922	-1.397	0.169
mating_duration_sec.ST	0.023	0.014	1.613	0.114
mating_duration_sec.SR	-0.001	0.003	-0.490	0.626

Mating duration did not affect the number of offspring sired by the SR males ($F_{1,44} = 0.248$, $P = 0.621$). However, it did affect the number of offspring sired by the ST males ($F_{1,44} = 2.897$, $P = 0.096$).

3.3.5 The effect of mating position with mating duration on the number of offspring sired

```
glm(formula = cbind(ST_offspring, SR_offspring) ~ mating_duration_sec.ST +
    mating_duration_sec.SR + mating_position, family = quasibinomial,
    data = by_brood2)
```

Table 53: Analysis of variance table (Type II tests)

	Sum Sq	Df	F value	Pr(>F)
mating_duration_sec.ST	29.783	1	3.095	0.086
mating_duration_sec.SR	0.451	1	0.047	0.830
mating_position	14.324	1	1.488	0.229
Residuals	413.839	43		

Table 54: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-1.152	0.916	-1.258	0.215
mating_duration_sec.ST	0.023	0.014	1.681	0.100
mating_duration_sec.SR	-0.001	0.003	-0.215	0.831
mating_positionP2	-0.467	0.385	-1.215	0.231

Mating position still did not affect number of offspring sired by each male when mating duration was included as a covariate ($F_{1,43} = 1.488$, $P = 0.229$).

3.3.6 The effect of male genotype with mating duration on the number of offspring sired

```
glm(formula = cbind(P1_offspring, P2_offspring) ~ mating_duration_sec.P1 +
    mating_duration_sec.P2 + male_genotype, family = quasibinomial,
    data = by_brood2)
```

Table 55: Analysis of variance table (Type II tests)

	Sum Sq	Df	F value	Pr(>F)
mating_duration_sec.P1	32.985	1	3.501	0.068
mating_duration_sec.P2	0.033	1	0.004	0.953
male_genotype	0.113	1	0.012	0.913
Residuals	405.125	43		

Table 56: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-1.137	0.648	-1.754	0.087
mating_duration_sec.P1	0.014	0.008	1.778	0.082
mating_duration_sec.P2	0.000	0.003	-0.059	0.953
male_genotypeST	0.042	0.384	0.110	0.913

Male genotype type still does not affect the number of offspring sired by each male when mating during is included as a covariate ($F_{1,43} = 0.012$, $P = 0.913$).

Chapter 2 Supplementary Information 4: resampling

Contents

1 Building the resampling method

1.1 Setting a seed

Here, a seed was set using the set.seed() function. It is used for all subsequent random number generation functions, including sample(), runif(), rnorm(), etc., unless it is explicitly reset with a different value or by calling set.seed(NULL).

```
set.seed(1234)
```

1.2 Write a function to fit a GLM and extract the whole model coefficient table

In this case we fit the following binomial GLM of the same form that we fitted to our data:

$$(Y_1, Y_2)\tilde{}X$$

We extract the t and p values associated with the explanatory variable, X, which corresponds to either mating position or male genotype.

Mating position: The response variable is the numbers of SR and ST offspring, explained by mating position.

Male genotype: The response variable is the numbers of P2 and P1 offspring, explained by male genotype.

```
# Define the function to compute and extract the model
# coefficients where X is the explanatory variable,
# male_genotypeST or mating_positionP1
compute_coefficients <- function(matrix) {
    # Fit GLM with quasibinomial family
    model <- glm(cbind(y1, y2) ~ as.factor(X), family = quasibinomial,
        data = matrix)

    # Extract the coefficients from the model coefficients
    # table
    coeffs <- summary(model)$coefficients
    # Extract the row index from the model coefficents
    # table
    row_index <- grep("X", rownames(coeffs))
    # Extract the coefficient values and store them in a
    # dataframe
    df <- data.frame(variable = row.names(coeffs)[row_index],
        estimate = coeffs[row_index, "Estimate"], std.error = coeffs[row_index,
            "Std. Error"], t.value = coeffs[row_index, "t value"],
        p.value = coeffs[row_index, "Pr(>|t|)"], row.names = NULL)

    return(df)
}
```

1.3 Example loop x10 repetitions: resample to increase the orginal matrix 2 fold and compute the model coefficents

```r
# Define the number of repetitions
n_repetitions <- 10
# Define the fold change to increase the sample size by -
# this time we double the sample size
fold <- 2

# Extract 10 rows data from the original dataset
original_matrix <- by_brood[1:10, c("SR_offspring", "ST_offspring",
    "mating_position")]
# reset the row numbers for extraction during random
# sampling
row.names(original_matrix) <- NULL
# reset the column names for model fitting
names(original_matrix) <- c("y1", "y2", "X")

# Get the number of rows in the original matrix
n_original <- nrow(original_matrix)

# Create an empty dataframe to store the coefficient values
master_df <- data.frame(variable = character(), coefficient = numeric(),
    std.error = numeric(), t.value = numeric(), p.value = numeric(),
    stringsAsFactors = FALSE)

# Run the loop 10 times
for (i in 1:n_repetitions) {
    # randomly sample 2x 10 from the original 10 rows to
    # generate new dataset where n = 2x
    resampled_matrix <- original_matrix[sample(n_original, n_original *
        2, replace = TRUE), , drop = FALSE]
    # Call the function and get the coefficients for each
    # iteration
    coefficients_df <- compute_coefficients(resampled_matrix)
    # Append the coefficients to the master dataframe
    master_df <- rbind(master_df, coefficients_df)
}

# show the model coefficients table
kable(master_df, caption = "male genotype model coefficients for
      repeats 1:10 of resampling with 2fold sample size increase")
```

Table 1: male genotype model coefficients for repeats 1:10 of resampling with 2fold sample size increase

variable	estimate	std.error	t.value	p.value
as.factor(X)P2	-2.4760600	0.5339495	-4.6372552	0.0002048
as.factor(X)P2	-2.1153677	0.6856259	-3.0853090	0.0063810
as.factor(X)P2	-0.9765096	0.5065651	-1.9277082	0.0698217
as.factor(X)P2	-2.8602662	0.9950266	-2.8745625	0.0100840
as.factor(X)P2	-1.3142481	0.7778277	-1.6896390	0.1083424
as.factor(X)P2	-0.3630761	0.5629462	-0.6449569	0.5270907
as.factor(X)P2	-0.8490216	0.4175205	-2.0334848	0.0570170

variable	estimate	std.error	t.value	p.value
as.factor(X)P2	-0.9892438	0.8265421	-1.1968461	0.2468926
as.factor(X)P2	-1.9793221	0.6842462	-2.8927045	0.0096969
as.factor(X)P2	-0.1110940	0.5953275	-0.1866100	0.8540535

2 Implementing the resampling method for the whole dataset

2.1 Resampling the effect of mating order on paternity

2.1.1 1 fold sample size increase, x1000 model repeats

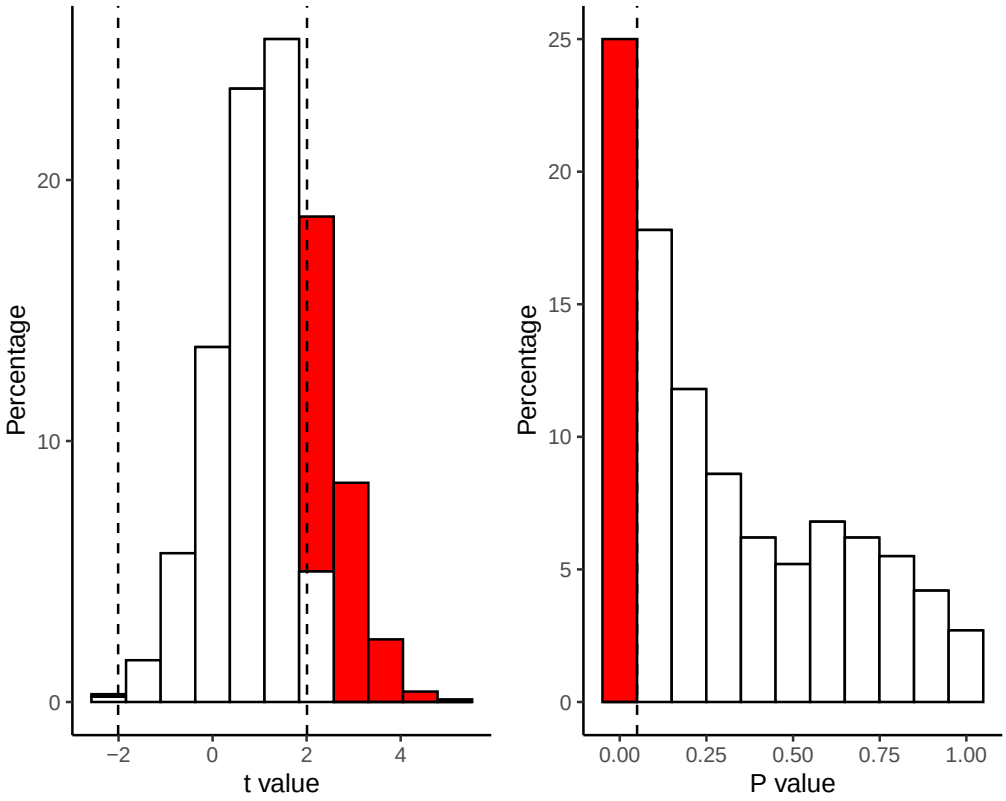

Figure S2.4.1: Percentage distribution of t statitics and associated p values from a 1 fold resampling of the effect of mating position on paternity. 95% confidence interval of the t statistic: -0.982-3.353 . For mating order positive values indicate that P2>P1.

2.1.2 2 fold sample size increase, x1000 model repeats

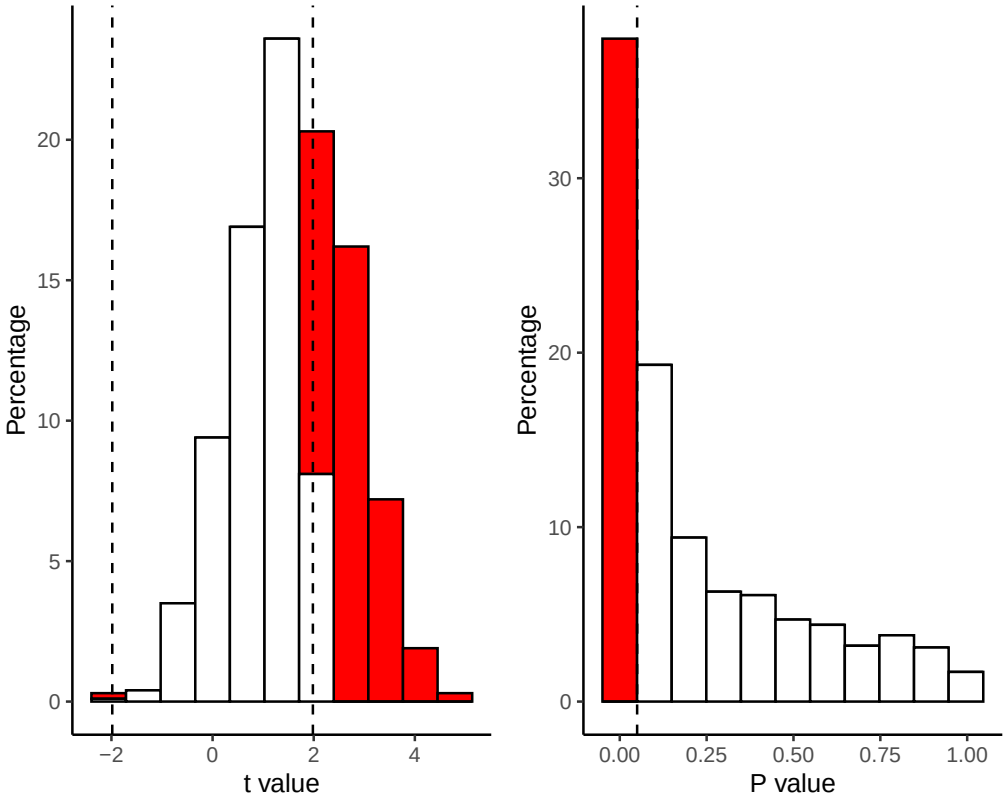

Figure S2.4.2: Percentage distribution of t statitics and associated p values from a 2 fold resampling of the effect of mating position on paternity. 95% confidence interval of the t statistic: -0.598-3.707 . For mating order positive values indicate that P2>P1.

2.1.3 3 fold sample size increase, x1000 model repeats

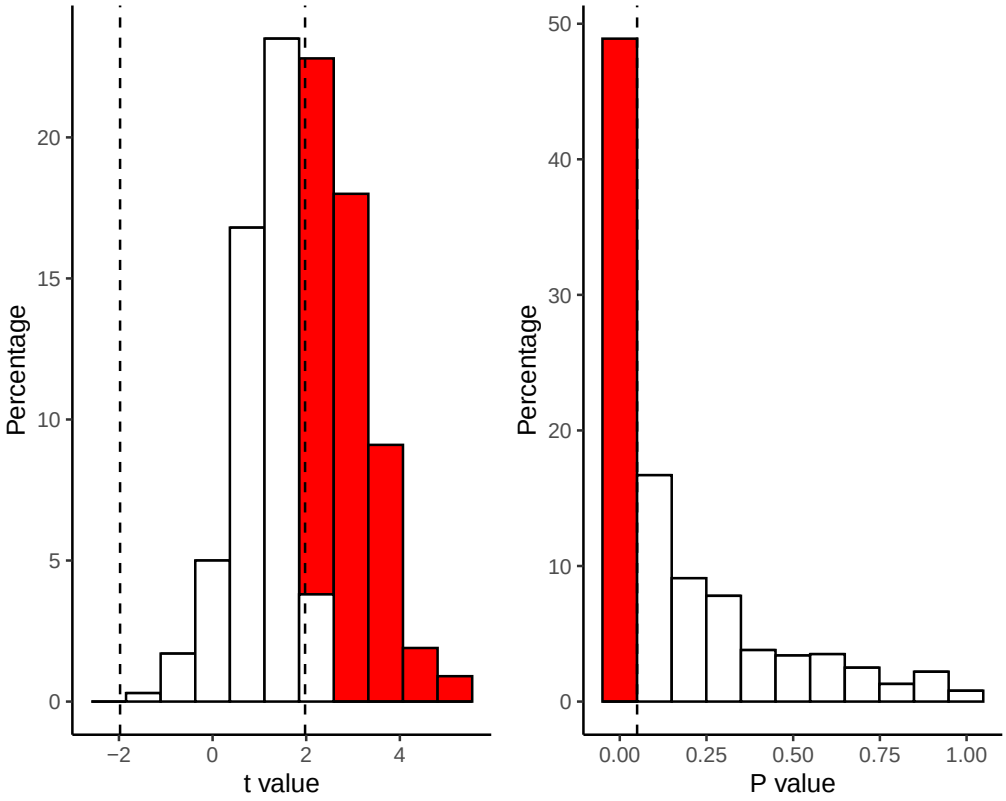

Figure S2.4.3: Percentage distribution of t statitics and associated p values from a 3 fold resampling of the effect of mating position on paternity. 95% confidence interval of the t statistic: -0.187-4.091 . For mating order positive values indicate that P2>P1.

2.1.4 4 fold sample size increase, x1000 model repeats

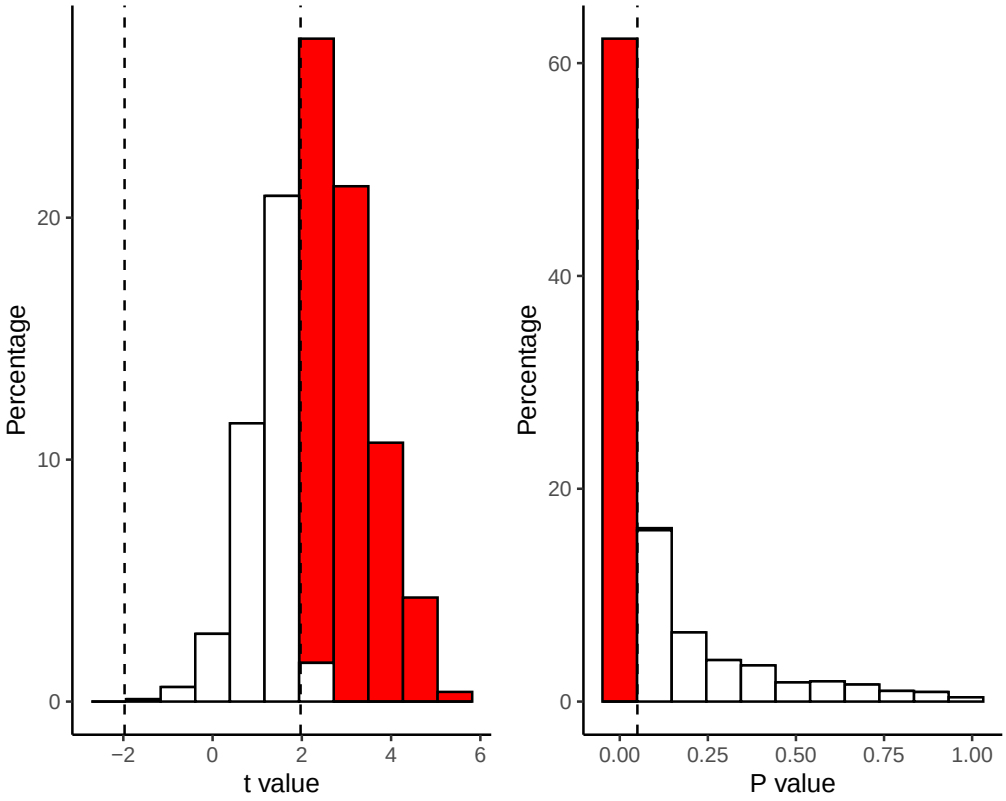

Figure S2.4.4: Percentage distribution of t statitics and associated p values from a 4 fold resampling of the effect of mating position on paternity. 95% confidence interval of the t statistic: 0.216-4.549 . For mating order positive values indicate that P2>P1.

2.1.5 Summary table of the 95% confidence intervals for the t statistic associated with each resampling of mating position

Table 2: Confidence intervals assocatiated with t statistic from resampling the effect of mating position on offspring ratio, where 'fold' is the increase in sample size. Note that the confindence interval no longer spans 0 at a 4 fold increase in sample size.

	2.5%	97.5%
1 fold	-0.982	3.353
2 fold	-0.598	3.707
3 fold	-0.187	4.091
4 fold	0.216	4.549
10 fold	1.484	5.996

2.2 Resampling the effect of male genotype on paternity

2.2.1 1 fold sample size increase, x1000 model repeats

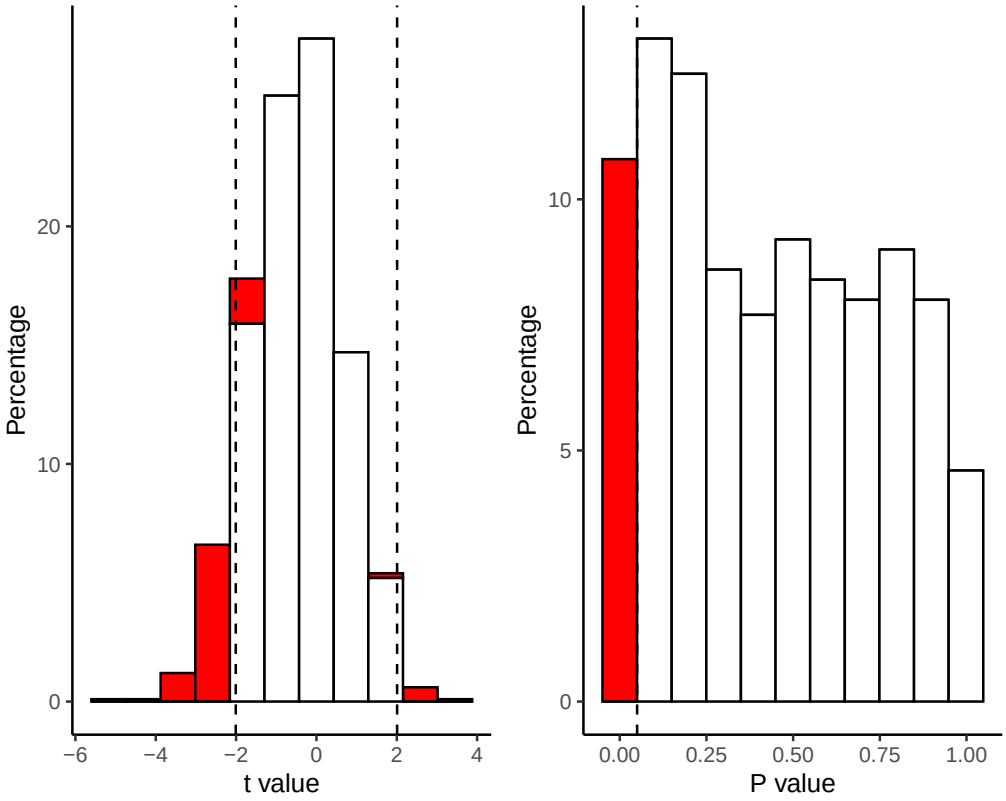

Figure S2.4.5: Percentage distribution of t statitics and associated p values from a 1 fold resampling of the effect of male genotype on paternity. 95% confidence interval of the t statistic: -2.717-1.595 . For genotype negative values indicate that SR>ST.

2.2.2 2 fold sample size increase, x1000 model repeats

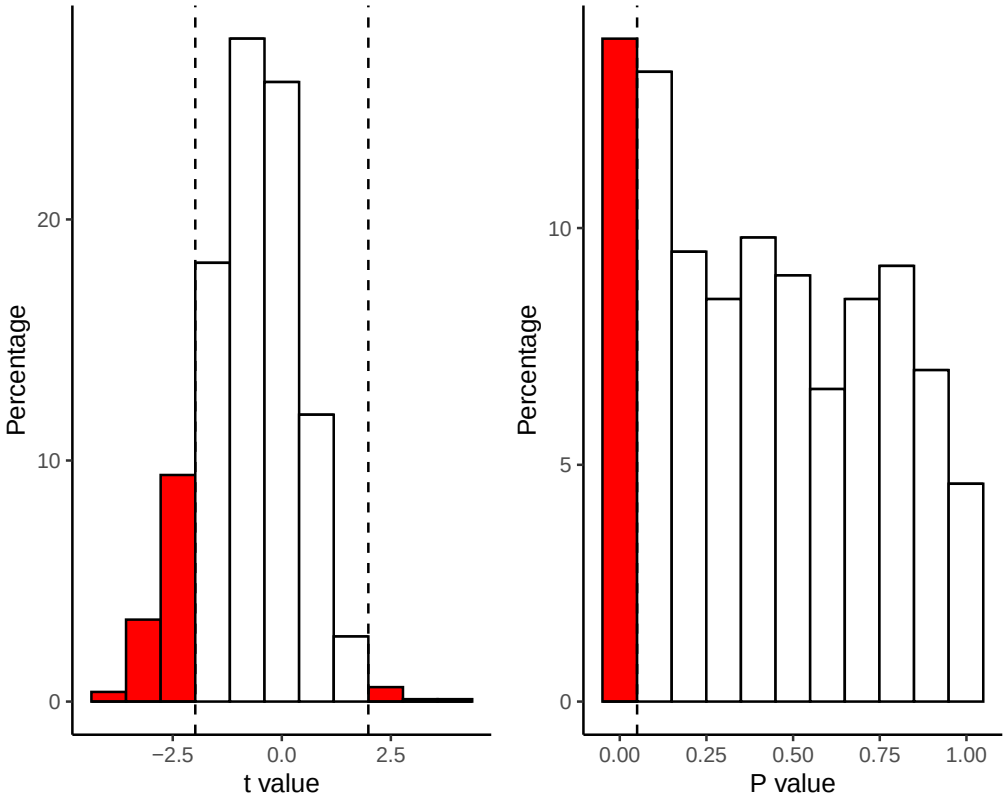

Figure S2.4.6: Percentage distribution of t statitics and associated p values from a 2 fold resampling of the effect of male genotype on paternity. 95% confidence interval of the t statistic: -2.966-1.311 . For genotype negative values indicate that SR>ST.

2.2.3 10 fold sample size increase, x1000 model repeats

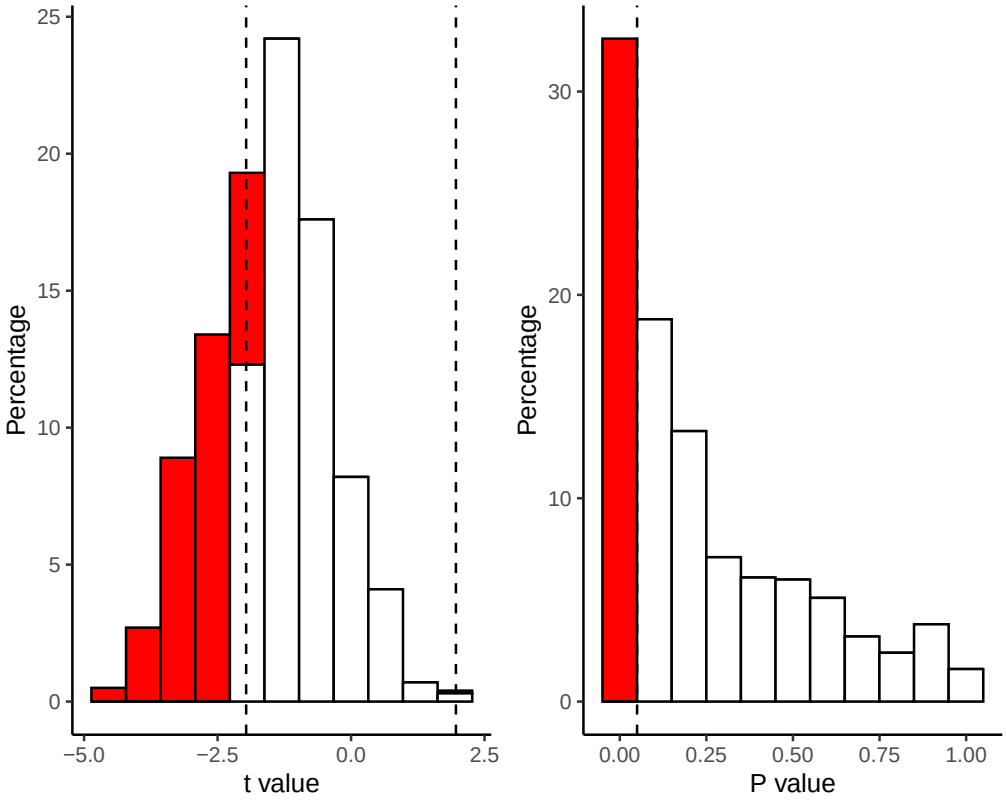

Figure S2.4.7: Percentage distribution of t statitics and associated p values from a 10 fold resampling of the effect of male genotype on paternity. 95% confidence interval of the t statistic: -3.663-0.735 . For genotype negative values indicate that SR>ST.

2.2.4 Summary table of the 95% confidence intervals for the t statistic associated with each resampling of male genotype

Table 3: Confidence intervals assocatiated with t statistic from resampling the effect of male genotype on offspring ratio, where 'fold' is the increase in sample size. Note that even at 10 fold the confidence interval spans zero.

	2.5%	97.5%
1 fold	-2.717	1.595
2 fold	-2.966	1.311
10 fold	-3.663	0.735

Chapter 3 Supplementary information: all model tables and effect sizes

Contents

1 Data

In total, 45 females were mated twice and produced offspring. Of these, 1 females produced no offspring prior to their second mating. One of these females remains in the analysis as she produced offspring from the P1 male, meaning the first male had been successful. The other has been excluded as she produced no offspring from the P1 male.

44 matings are included in the analysis: 22 SR—ST and 22 ST—SR. The mean number of offspring collected per female was 65.523 ± 42.22 (mean ± SD), with a range of 3, 174. A sample of female offspring from each mating was selected for genotyping to determine paternity. Between 3-77 offspring per female parent were genotyped across 37 matings (16 SR—ST and 21 ST—SR). 31 females had greater than 10 genotyped offspring (16 SR—ST and 15 ST—SR).

2 Male trait size

2.1 Variation in trait size with genotype

```
lm(formula = thorax ~ male_genotype, data = by_male_id)
```

Table 1: Analysis of Variance Table

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
male_genotype	1	0.114	0.114	5.38	0.023
Residuals	83	1.767	0.021		

Table 2: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	2.368	0.023	105.19	0.000
male_genotypeST	0.073	0.032	2.32	0.023

```
lm(formula = eyespan ~ male_genotype, data = by_male_id)
```

Table 3: Analysis of Variance Table

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
male_genotype	1	3.85	3.852	13	0.001
Residuals	79	23.46	0.297		

Table 4: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	8.128	0.087	93.1	0.000
male_genotypeST	0.436	0.121	3.6	0.001

Table 5: Analysis of Variance Table

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
thorax	1	19.50	19.502	231	0
male_genotype	1	1.27	1.268	15	0
Residuals	77	6.49	0.084		

Table 6: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.189	0.564	0.335	0.738
thorax	3.318	0.235	14.100	0.000
male_genotypeST	0.258	0.066	3.879	0.000

Relative eyespan varies significantly according to genotype. I have therefore tested relative eyespan as a covariate in the paternity GLMs below.

3 Male fertility

The mean P2 ± sd was 0.316 ± 0.327. In 9/37 broods, the P2 was <0.05. In 2/37 the P2 was >0.95.

The mean SR ± sd was 0.474 ± 0.376. In 3/37 broods, the SR was <0.05. In 8/37 the SR was >0.95. This distribution of SR paternity was more even compared to that of SR male.

3.1 Variation in P2 male fertility with male genotype

```
glm(formula = cbind(total_P2_off, total_P1_off) ~ male_genotype.P2,
    family = "quasibinomial", data = by_brood)
```

Table 7: Analysis of variance table

	Sum Sq	Df	F value	Pr(>F)
male_genotype.P2	1.06	1	0.03	0.862
Residuals	1222.99	35		

Table 8: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-1.013	0.436	-2.323	0.026
male_genotype.P2ST	0.094	0.541	0.174	0.863

The number of offspring sired by each male did not vary according to his genotype ($F_{1,35} = 0.03$, $P = 0.862$).

3.1.1 Variation in P2 male fertility with genotype and total offspring

```
glm(formula = cbind(total_P2_off, total_P1_off) ~ total_offspring_collected +
    male_genotype.P2, family = "quasibinomial", data = by_brood)
```

Table 9: Analysis of variance table

	Sum Sq	Df	F value	Pr(>F)
total_offspring_collected	157.7	1	4.86	0.034
male_genotype.P2	87.4	1	2.69	0.110
Residuals	1103.5	34		

Table 10: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.093	0.661	0.14	0.889
total_offspring_collected	-0.020	0.009	-2.10	0.043
male_genotype.P2ST	1.179	0.738	1.60	0.120

Here, the total offspring collected is added to the model as a covariate, to check if the fecundity of individual females had any impact on the number of offspring sired by the P2 male. There was a positive association between the number of offspring sired by the P2 male and the number of offspring produced by each female ($F_{1,34} = 4.859$, $P = 0.034$). The number of offspring sired by the P2 male remained unaffected by genotype.

3.1.2 Variation in P2 male fertility with genotype and batch number

```
glm(formula = cbind(total_P2_off, total_P1_off) ~ batch + male_genotype.P2,
    family = "quasibinomial", data = by_brood)
```

Table 11: Analysis of variance table

	Sum Sq	Df	F value	Pr(>F)
batch	0.003	1	0.000	0.993
male_genotype.P2	1.024	1	0.028	0.867
Residuals	1222.770	34		

Table 12: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-1.015	0.511	-1.988	0.055
batchB	0.005	0.536	0.009	0.993
male_genotype.P2ST	0.093	0.555	0.168	0.867

Here, batch number is added to the model as a covariate. For logistical reasons, matings were conducted in batches. There was no affect of batch on the number of offspring sired by the P2 male ($F_{1,34} = 8.447 \times 10^{-5}$, $P = 0.993$). The number of offspring sired by the P2 male remained unaffected by genotype.

3.1.3 Variation in P2 male fertility with ST/SR thorax length and genotype

```
glm(formula = cbind(total_P2_off, total_P1_off) ~ thorax.ST +
    male_genotype.P2, family = "quasibinomial", data = by_brood)
```

Table 13: Analysis of variance table

	Sum Sq	Df	F value	Pr(>F)
thorax.ST	9.31	1	0.254	0.618
male_genotype.P2	3.50	1	0.096	0.759
Residuals	1209.54	33		

Table 14: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	1.787	5.515	0.324	0.748
thorax.ST	-1.162	2.325	-0.500	0.620
male_genotype.P2ST	0.198	0.642	0.308	0.760

ST male thorax length did not affect the number of offspring sired by the P2 male ($F_{1,33} = 0.254$, $P = 0.618$) and male genotype had no affect on the number of offspring sired by the P2 male ($F_{1,33} = 0.096$, $P = 0.759$).

```
glm(formula = cbind(total_P2_off, total_P1_off) ~ thorax.SR +
    male_genotype.P2, family = "quasibinomial", data = by_brood)
```

Table 15: Analysis of variance table

	Sum Sq	Df	F value	Pr(>F)
thorax.SR	178.7	1	5.318	0.028
male_genotype.P2	21.7	1	0.647	0.427
Residuals	1075.2	32		

Table 16: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-10.440	4.297	-2.430	0.021
thorax.SR	3.909	1.762	2.218	0.034
male_genotype.P2ST	0.457	0.574	0.795	0.432

While SR male thorax length was positively associated with the number of offspring sired by the P2 male ($F_{1,32} = 5.318$, $P = 0.028$), the effect of male genotype on P2 paternity remained insignificant ($F_{1,32} = 0.647$, $P = 0.427$).

3.1.4 Variation in number of P2 offspring sired with ST/SR relative eyespan and genotype

Here, 'residual_eyespan' corresponds to the variation in eyespan beyond that which is predicted by thorax length (residuals taken from a linear model of the covariance between eyespan and thorax for males of each genotype).

```
glm(formula = cbind(total_P2_off, total_P1_off) ~ residual_eyespan.ST +
    male_genotype.P2, family = "quasibinomial", data = by_brood)
```

Table 17: Analysis of variance table

	Sum Sq	Df	F value	Pr(>F)
residual_eyespan.ST	32.365	1	0.861	0.360
male_genotype.P2	0.247	1	0.007	0.936
Residuals	1202.317	32		

Table 18: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.940	0.475	-1.979	0.056
residual_eyespan.ST	-0.886	0.962	-0.922	0.364
male_genotype.P2ST	-0.048	0.586	-0.081	0.936

When the relative eyespan of the ST male was included as a covariate, it had no effect on P2 male paternity ($F_{1,32} = 0.861$, $P = 0.36$) and the effect of genotype remained insignificant ($F_{1,32} = 0.007$, $P = 0.936$).

```
glm(formula = cbind(total_P2_off, total_P1_off) ~ residual_eyespan.SR +
    male_genotype.P2, family = "quasibinomial", data = by_brood)
```

Table 19: Analysis of variance table

	Sum Sq	Df	F value	Pr(>F)
residual_eyespan.SR	61.0	1	1.737	0.198
male_genotype.P2	15.4	1	0.438	0.514
Residuals	983.0	28		

Table 20: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-1.148	0.485	-2.370	0.025
residual_eyespan.SR	1.875	1.457	1.287	0.209
male_genotype.P2ST	0.452	0.690	0.655	0.518

When the relative eyespan of the SR male was included as a covariate, it had no effect on P2 male paternity ($F_{1,28} = 1.737$, $P = 0.198$) and the effect of genotype remained insignificant ($F_{1,28} = 0.007$, $P = 0.936$).

As male relative eyespan has no affect on the number of offspring sired by either male, it is not included as a covariate in the main models.

3.2 Variation in SR male fertility with mating position

```
glm(formula = cbind(total_SR_off, total_ST_off) ~ mating_position.SR,
    family = "quasibinomial", data = by_brood)
```

Table 21: Analysis of variance table

	Sum Sq	Df	F value	Pr(>F)
mating_position.SR	503	1	14.4	0.001
Residuals	1223	35		

Table 22: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.918	0.320	2.87	0.007
mating_position.SRP2	-1.931	0.541	-3.57	0.001

The number of offspring sired by each male was affected by his mating position, with P1 males siring a greater number of offspring ($F_{1,35} = 14.383$, $P = 5.664 \times 10^{-4}$).

3.3 Variation in SR male fertility with mating position and total offspring

```
glm(formula = cbind(total_SR_off, total_ST_off) ~ total_offspring_collected +
    mating_position.SR, family = "quasibinomial", data = by_brood)
```

Table 23: Analysis of variance table

	Sum Sq	Df	F value	Pr(>F)
total_offspring_collected	57.9	1	1.72	0.198
mating_position.SR	128.0	1	3.81	0.059
Residuals	1142.3	34		

Table 24: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-0.388	1.044	-0.372	0.712
total_offspring_collected	0.012	0.009	1.288	0.207
mating_position.SRP2	-1.324	0.691	-1.917	0.064

Here, the total offspring collected is added to the model as a covariate, to check if the fecundity of individual females had any impact on the number of offspring sired by the SR male. The number of offspring collected did not affect the number of offspring sired by the SR male ($F_{1,34} = 4.859$, $P = 0.034$).

3.4 Variation in SR male fertility with mating position and batch number

```
glm(formula = cbind(total_SR_off, total_ST_off) ~ batch + mating_position.SR,
    family = "quasibinomial", data = by_brood)
```

Table 25: Analysis of variance table

	Sum Sq	Df	F value	Pr(>F)
batch	2.65	1	0.074	0.787
mating_position.SR	482.51	1	13.517	0.001
Residuals	1213.71	34		

Table 26: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.828	0.460	1.800	0.081
batchB	0.145	0.531	0.273	0.787
mating_position.SRP2	-1.911	0.551	-3.470	0.001

Here, batch number is added to the model as a covariate. Like with the P2 male, there was no effect of batch on the number of offspring sired by the SR male ($F_{1,34} = 8.447 \times 10^{-5}$, $P = 0.993$).

3.4.1 Variation in SR male fertility with P1/P2 thorax length and mating position

```
glm(formula = cbind(total_SR_off, total_ST_off) ~ thorax.P1 +
    mating_position.SR, family = "quasibinomial", data = by_brood)
```

Table 27: Analysis of variance table

	Sum Sq	Df	F value	Pr(>F)
thorax.P1	106	1	2.99	0.094
mating_position.SR	358	1	10.06	0.003
Residuals	1138	32		

Table 28: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	7.57	3.984	1.90	0.066
thorax.P1	-2.88	1.699	-1.69	0.100
mating_position.SRP2	-1.74	0.573	-3.04	0.005

When the P1 male thorax length was included as a covariate, it had no effect on the number of offspring sired by the P2 male ($F_{1,32} = 2.987$, $P = 0.094$). The paternity of each male genotype was still determined by mating position ($F_{1,32} = 10.057$, $P = 0.003$).

```
glm(formula = cbind(total_SR_off, total_ST_off) ~ thorax.P2 +
    mating_position.SR, family = "quasibinomial", data = by_brood)
```

Table 29: Analysis of variance table

	Sum Sq	Df	F value	Pr(>F)
thorax.P2	50.6	1	1.39	0.247
mating_position.SR	323.3	1	8.87	0.005
Residuals	1202.3	33		

Table 30: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-6.48	6.407	-1.01	0.319
thorax.P2	2.97	2.575	1.15	0.257
mating_position.SRP2	-1.68	0.586	-2.86	0.007

When the P2 male thorax length was included as a covariate, it had no effect on the number of offspring sired by the P2 male ($F_{1,33} = 1.389$, $P = 0.247$). The paternity of each male genotype was still determined by mating position ($F_{1,33} = 8.873$, $P = 0.005$).

3.4.2 Variation in P2 male fertility with P1/P2 relative eyespan and mating position

As above, 'residual_eyespan' corresponds to the variation in eyespan beyond that which is predicted by thorax length (residuals taken from a linear model of the covariance between eyespan and thorax for males of each genotype).

```
glm(formula = cbind(total_SR_off, total_ST_off) ~ residual_eyespan.P1 +
    mating_position.SR, family = "quasibinomial", data = by_brood)
```

Table 31: Analysis of variance table

	Sum Sq	Df	F value	Pr(>F)
residual_eyespan.P1	27.1	1	0.73	0.400
mating_position.SR	413.2	1	11.14	0.002
Residuals	1038.1	28		

Table 32: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	1.12	0.459	2.441	0.021
residual_eyespan.P1	1.08	1.284	0.841	0.407
mating_position.SRP2	-2.11	0.690	-3.064	0.005

When the P1 male relative eyespan ('residual_eyespan.P1') was included as a covariate, it had no effect on the number of offspring sired by the P2 male ($F_{1,28} = 0.73$, $P = 0.4$). The paternity of each male genotype was still determined by mating position ($F_{1,28} = 11.143$, $P = 0.002$).

```
glm(formula = cbind(total_SR_off, total_ST_off) ~ residual_eyespan.P2 +
    mating_position.SR, family = "quasibinomial", data = by_brood)
```

Table 33: Analysis of variance table

	Sum Sq	Df	F value	Pr(>F)
residual_eyespan.P2	269	1	7.95	0.008
mating_position.SR	651	1	19.23	0.000
Residuals	1083	32		

Table 34: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	1.26	0.382	3.29	0.002
residual_eyespan.P2	2.99	1.148	2.61	0.014
mating_position.SRP2	-2.54	0.658	-3.86	0.001

When the P2 male relative eyespan ('residual_eyespan.P2') was included as a covariate, it had a significant effect on the number of offspring sired by the P2 male ($F_{1,32} = 7.953$, $P = 0.008$). The paternity of each male genotype was still determined by mating position ($F_{1,32} = 19.233$, $P = 1.173 \times 10^{-4}$).

4 Mating duration

4.1 Variation in mating duration with genotype

Table 35: Welch Two Sample t-test: `mating_duration_secs` by `male_genotype.P2` (continued below)

Test statistic	df	P value	Alternative hypothesis	mean in group SR
2.464	25.73	0.02075 *	two.sided	71.86

mean in group ST
58.59

The SR male (SR mean duration = 71.857) had a longer mating duration than the ST male (ST mean duration = 58.591, t = 2.464, $P = 0.021$).

4.2 Variation in P2 male fertility with mating duration

```
glm(formula = cbind(total_SR_off, total_ST_off) ~ mating_duration_secs +
    mating_position.SR, family = "quasibinomial", data = by_brood)
```

Table 37: Analysis of variance table

	Sum Sq	Df	F value	Pr(>F)
mating_duration_secs	97.5	1	2.95	0.095

	Sum Sq	Df	F value	Pr(>F)
mating_position.SR	209.4	1	6.33	0.017
Residuals	1091.3	33		

Table 38: Model Coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	2.899	1.417	2.04	0.049
mating_duration_secs	-0.034	0.024	-1.44	0.160
mating_position.SRP2	-1.441	0.586	-2.46	0.019

The mating duration of the P2 male did not affect his paternity ($F_{1,33} = 2.949$, $P = 0.095$). The effect of mating position on the number of offspring sired is unchanged, more offspring were sired by the P1 male ($F_{1,33} = 6.332$, $P = 0.017$).

5 Supplementary figures

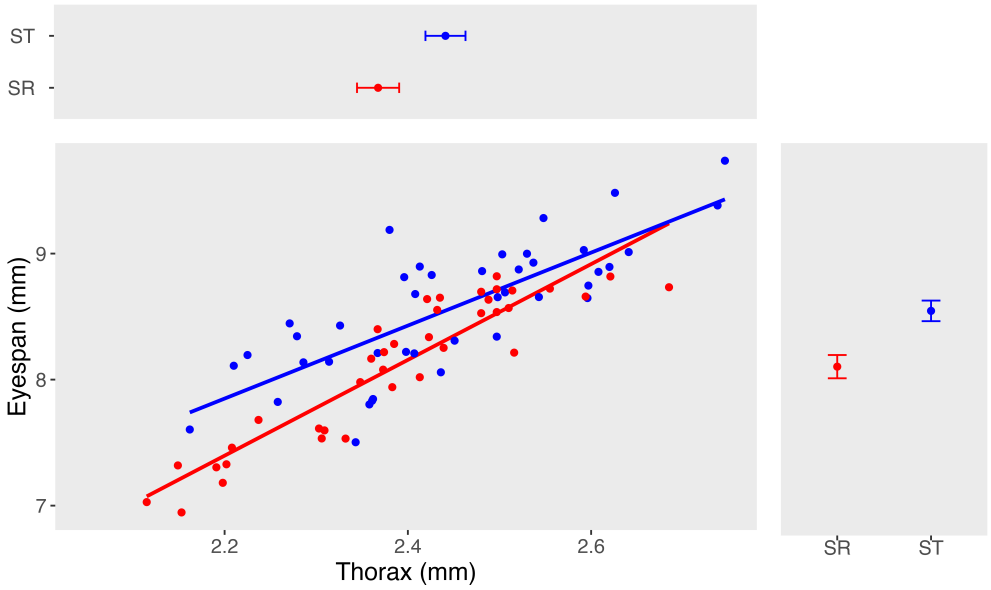

Figure S3.1: Variation in thorax length and eyespan with male genotype

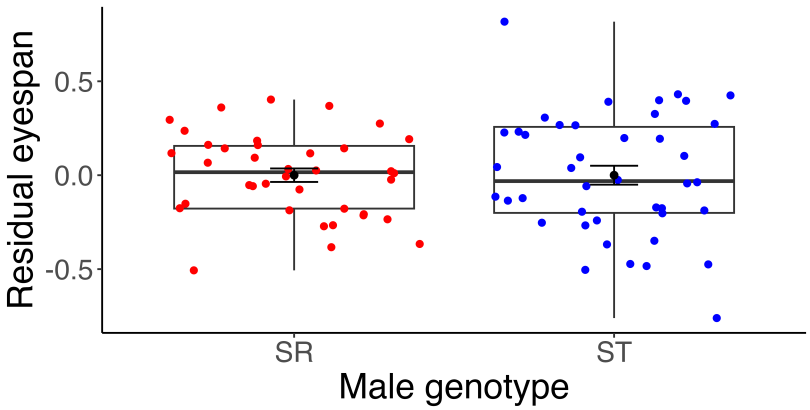

Figure S3.2: Variation in residual eyespan with genotype

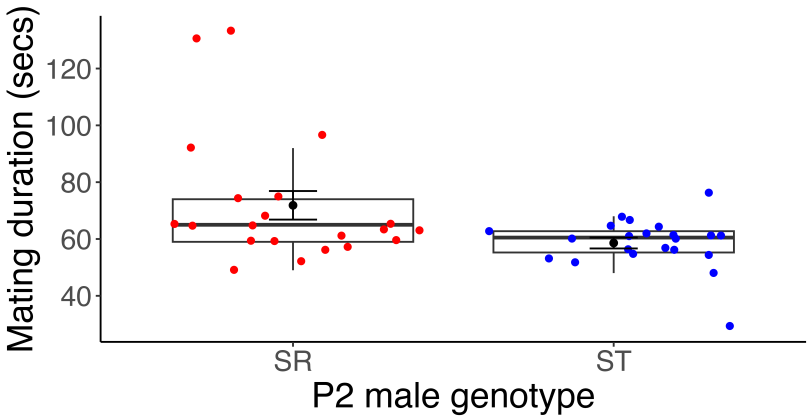

Figure S3.3: Variation in P2 mating duration with genotype

Chapter 4 Supplementary Information: all model tables and effect sizes

Contents

1 Trait size

1.1 Data

A larval diet of 70% food (full food diluted with water by 30%) was used to produce adult females with a range of sizes. Thorax length had a range of: 1.709-2.583mm and eyespan had a range of: 4.303-6.352mm.

Table 1: Mean thorax length (mm) per genotype

genotype	N	thorax	sd	se	ci
SR-HOM	17	2.08	0.194	0.047	0.100
HET	35	2.26	0.187	0.032	0.064
ST-HOM	45	2.32	0.117	0.017	0.035

Table 2: Mean eyespan (mm) per genotype

genotype	N	eyespan	sd	se	ci
SR-HOM	17	5.29	0.502	0.122	0.258
HET	37	5.54	0.422	0.069	0.141
ST-HOM	45	5.83	0.277	0.041	0.083

Table 3: Mean relative eyespan (residuals) per genotype

genotype	N	relative_eyespan	sd	se	ci
SR-HOM	17	0	0.223	0.054	0.115
HET	35	0	0.233	0.039	0.080
ST-HOM	45	0	0.188	0.028	0.056

1.2 Trait size with genotype

There was a strong covariance between eyespan and thorax length. There was an association between thorax length and genotype. Eyespan also varied according to genotype (expected as eyespan covaries with body size). Relative eyespan, the variation in eyespan not determined by thorax length, varied between genotypes ($P = 0.01$), however, a post hoc Tukey test indicates this is largely due to the difference between hets and st. The relationship between eyespan and thorax does not change (i.e. intercept changes not slope). Females with HOM and HET genotypes were smaller than females with ST genotypes.

```
lm(formula = eyespan ~ thorax, data = ttsm_fecundity)
```

Table 4: Analysis of variance table (Type II tests)

	Sum Sq	Df	F value	Pr(>F)
thorax	11.48	1	226	0
Residuals	4.82	95		

Table 5: Model coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	1.28	0.291	4.42	0
thorax	1.93	0.128	15.04	0

```
lm(formula = thorax ~ genotype, data = ttsm_fecundity)
```

Table 6: Analysis of variance table (Type II tests)

	Sum Sq	Df	F value	Pr(>F)
genotype	0.684	2	13.5	0
Residuals	2.391	94		

Table 7: Model coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	2.082	0.039	53.81	0
genotypeHET	0.182	0.047	3.87	0
genotypeST-HOM	0.235	0.045	5.17	0

```
lm(formula = eyespan ~ genotype, data = ttsm_fecundity)
```

Table 8: Analysis of variance table (Type II tests)

	Sum Sq	Df	F value	Pr(>F)
genotype	3.99	2	13.9	0
Residuals	13.81	96		

Table 9: Model coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	5.292	0.092	57.52	0.000
genotypeHET	0.246	0.111	2.22	0.029
genotypeST-HOM	0.535	0.108	4.95	0.000

```
lm(formula = eyespan ~ thorax + genotype, data = ttsm_fecundity)
```

Table 10: Analysis of variance table (Type II tests)

	Sum Sq	Df	F value	Pr(>F)
thorax	8.163	1	173.8	0.00
genotype	0.451	2	4.8	0.01
Residuals	4.368	93		

Table 11: Model coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	1.445	0.296	4.875	0.000
thorax	1.848	0.140	13.183	0.000
genotypeHET	-0.052	0.069	-0.749	0.456
genotypeST-HOM	0.101	0.070	1.438	0.154

Table 12: Tukey's pairwise comparisons of thorax length

term	group1	group2	null.value	estimate	conf.low	conf.high	p.adj	p.adj.signif
genotype	SR-HOM	HET	0	0.182	0.070	0.295	0.001	***
genotype	SR-HOM	ST-HOM	0	0.235	0.127	0.343	0.000	****
genotype	HET	ST-HOM	0	0.052	-0.033	0.138	0.315	ns

Table 13: Tukey's pairwise comparisons of eyespan

term	group1	group2	null.value	estimate	conf.low	conf.high	p.adj	p.adj.signif
genotype	SR-HOM	HET	0	0.246	-0.018	0.511	0.074	ns
genotype	SR-HOM	ST-HOM	0	0.535	0.278	0.792	0.000	****
genotype	HET	ST-HOM	0	0.288	0.088	0.489	0.003	**

Table 14: Tukey's pairwise comparisons of relative eyespan

term	group1	group2	null.value	estimate	conf.low	conf.high	p.adj	p.adj.signif
genotype	SR-HOM	HET	0	-0.067	-0.220	0.086	0.550	ns
genotype	SR-HOM	ST-HOM	0	0.081	-0.066	0.228	0.393	ns
genotype	HET	ST-HOM	0	0.148	0.032	0.264	0.009	**

2 TTSM

2.1 Data

maturity_date = date of first egg
age = days to sexual maturity (date of first egg - emergence date)

All females (N = 101) reached sexual maturity between 17 and 55 days. The mean age at maturity was 30 ± 8 days (mean ± SD).

2.2 TTSM with size

Age at sexual maturity was not dependent on thorax length ($F_{1,95} = 0.174$, $P = 0.678$) or relative eyespan ($F_{1,94} = 2.731$, $P = 0.102$).

```
lm(formula = as.numeric(age) ~ thorax, data = ttsm_fecundity)
```

Table 15: Analysis of variance table (Type II tests)

	Sum Sq	Df	F value	Pr(>F)
thorax	10.6	1	0.174	0.678
Residuals	5807.1	95		

Table 16: Model coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	26.39	10.09	2.615	0.010
thorax	1.86	4.46	0.417	0.678

```
lm(formula = as.numeric(age) ~ thorax + eyespan, data = ttsm_fecundity)
```

Table 17: Analysis of variance table (Type II tests)

	Sum Sq	Df	F value	Pr(>F)
thorax	80.5	1	1.34	0.250
eyespan	163.9	1	2.73	0.102
Residuals	5643.1	94		

Table 18: Model coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	18.90	10.98	1.72	0.088
thorax	-9.41	8.12	-1.16	0.250
eyespan	5.83	3.53	1.65	0.102

2.3 TTSM with genotype

There was no affect of genotype on age at sexual maturity ($F_{2,98} = 0.920$, $P = 0.402$).

```
lm(formula = as.numeric(age) ~ genotype, data = ttsm_fecundity)
```

Table 19: Analysis of Variance Table

	Sum Sq	Df	F value	Pr(>F)
genotype	109	2	0.92	0.402
Residuals	5806	98		

Table 20: Model coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	29.684	1.77	16.810	0.000
genotypeHET	-0.252	2.17	-0.116	0.908
genotypeST-HOM	1.916	2.11	0.910	0.365

2.4 TTSM with genotype and size

There was no effect of genotype on age at sexual maturity ($F_{2,92} = 0.245$, $P = 0.783$) when thorax and relative eyespan were controlled for.

```
lm(formula = as.numeric(age) ~ thorax + eyespan + genotype, data = ttsm_fecundity)
```

Table 21: Analysis of variance table (Type II tests)

	Sum Sq	Df	F value	Pr(>F)
thorax	60.5	1	0.991	0.322
eyespan	110.6	1	1.813	0.181
genotype	29.9	2	0.245	0.783
Residuals	5613.2	92		

Table 22: Model coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	21.215	11.97	1.772	0.080
thorax	-8.517	8.56	-0.995	0.322
eyespan	5.033	3.74	1.347	0.181
genotypeHET	-0.515	2.49	-0.206	0.837
genotypeST-HOM	0.792	2.55	0.311	0.757

3 Fecundity

3.1 Data

laying_duration_days = duration of egg laying before dissection and counting
ovary_eggs = number of eggs present in ovary at dissection

90 females were successfully dissected. Females had 31.478 ± 15.072 eggs (mean ± SD) in their ovaries on dissection, with a minimum of 0 eggs and a maximum of 79 eggs.

As the number of eggs laid is large and follows a normal distribution (W = 0.97251, $P = 0.05322$), standard linear models were fitted to the fecundity data.

Note: I begin by looking at the numbers of eggs after dissection but could look at the total number of eggs, including the eggs on the egglays as well.

```
[1] 90
```

3.2 Fecundity and size

There was a positive association between thorax length and fecundity ($F_{1,85} = 12.39$, $P = 0.001$). There was a positive association between relative eyespan (variation in eyespan when thorax length was controlled for) and fecundity ($F_{1,84} = 7.136$, $P = 0.009$).

```
glm(formula = ovary_eggs ~ thorax, family = "gaussian", data = ttsm_fecundity)
```

Table 23: Analysis of Deviance Table

	Df	Deviance	Resid. Df	Resid. Dev	F	Pr(>F)
NULL			86	20104		
thorax	1	2558	85	17546	12.4	0.001

Table 24: Model coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-37.5	19.68	-1.91	0.060
thorax	30.5	8.67	3.52	0.001

```
glm(formula = ovary_eggs ~ thorax + eyespan, family = "gaussian",
    data = ttsm_fecundity)
```

Table 25: Analysis of variance table (Type II tests)

	Sum Sq	Df	F value	Pr(>F)
thorax	6.6	1	0.034	0.854
eyespan	1373.9	1	7.136	0.009
Residuals	16172.0	84		

Table 26: Model coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-59.68	20.74	-2.878	0.005
thorax	-2.78	15.01	-0.185	0.854
eyespan	17.27	6.46	2.671	0.009

3.3 Fecundity and genotype

Table 27: Mean fecundity of females per genotype.

genotype	N	ovary_eggs	sd	se	ci
SR-HOM	16	19.9	12.4	3.09	6.59
HET	30	30.7	12.8	2.35	4.80
ST-HOM	44	36.2	15.2	2.30	4.63

There was a significant effect of genotype on fecundity ($F_{2,87} = 8.039$, $P = 0.001$).

```
glm(formula = ovary_eggs ~ genotype, family = "gaussian", data = ttsm_fecundity)
```

Table 28: Analysis of Variance Table

	Df	Deviance	Resid. Df	Resid. Dev	F	Pr(>F)
NULL			89	20218		
genotype	2	3154	87	17065	8.04	0.001

Table 29: Model coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	19.9	3.50	5.68	0.000
genotypeHET	10.9	4.34	2.50	0.014
genotypeST-HOM	16.3	4.09	3.99	0.000

Table 30: Tukey multiple comparisons of means

group1	group2	estimate	conf.low	conf.high	p.adj	p.adj.signif
SR-HOM	HET	10.86	0.52	21.2	0.037	*
SR-HOM	ST-HOM	16.33	6.58	26.1	0.000	***
HET	ST-HOM	5.47	-2.44	13.4	0.230	ns

3.4 Fecundity and genotype with size

The effect of genotype on fecundity was still significant when thorax length and relative eyespan were controlled for ($F_2 = 3.69$, $P = 0.029$). The relationship between fecundity and thorax length or relative

eyespan did not change depending on genotype. The number of eggs was not affected by an interaction between thorax length and genotype ($F_{2,78}$ = 1.377, P = 0.258) or relative eyespan and genotype ($F_{2,78}$ = NA, P = NA). ST females were bigger and most fecund overall, then HET females, followed by HOM females.

```
glm(formula = ovary_eggs ~ thorax + eyespan + genotype, family = "gaussian",
    data = ttsm_fecundity)
```

Table 31: Analysis of variance table (Type II tests)

	Sum Sq	Df	F value	Pr(>F)
thorax	90	1	0.497	0.483
eyespan	1045	1	5.776	0.019
genotype	1335	2	3.690	0.029
Residuals	14837	82		

Table 32: Model coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-45.3	21.42	-2.116	0.037
thorax	-10.9	15.40	-0.705	0.483
eyespan	16.2	6.76	2.403	0.019
genotypeHET	10.8	4.67	2.315	0.023
genotypeST-HOM	12.2	4.59	2.649	0.010

```
glm(formula = ovary_eggs ~ (thorax + eyespan) * genotype, family = "gaussian",
    data = ttsm_fecundity)
```

Table 33: Analysis of variance table (Type II tests)

	Sum Sq	Df	F value	Pr(>F)
thorax	90.8	1	0.496	0.483
eyespan	1125.4	1	6.154	0.015
genotype	1335.3	2	3.651	0.030
thorax:genotype	503.8	2	1.377	0.258
eyespan:genotype	323.7	2	0.885	0.417
Residuals	14263.3	78		

Table 34: Model coefficients

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-24.98	38.3	-0.653	0.516
thorax	-18.36	40.3	-0.456	0.650
eyespan	15.38	15.4	0.996	0.322
genotypeHET	-17.96	51.8	-0.347	0.730
genotypeST-HOM	-4.24	59.7	-0.071	0.943
thorax:genotypeHET	33.27	46.2	0.720	0.474

	Estimate	Std. Error	t value	Pr(>|t|)
thorax:genotypeST-HOM	-23.33	47.8	-0.488	0.627
eyespan:genotypeHET	-8.17	19.0	-0.429	0.669
eyespan:genotypeST-HOM	12.44	18.9	0.658	0.512

4 Supplementary figures

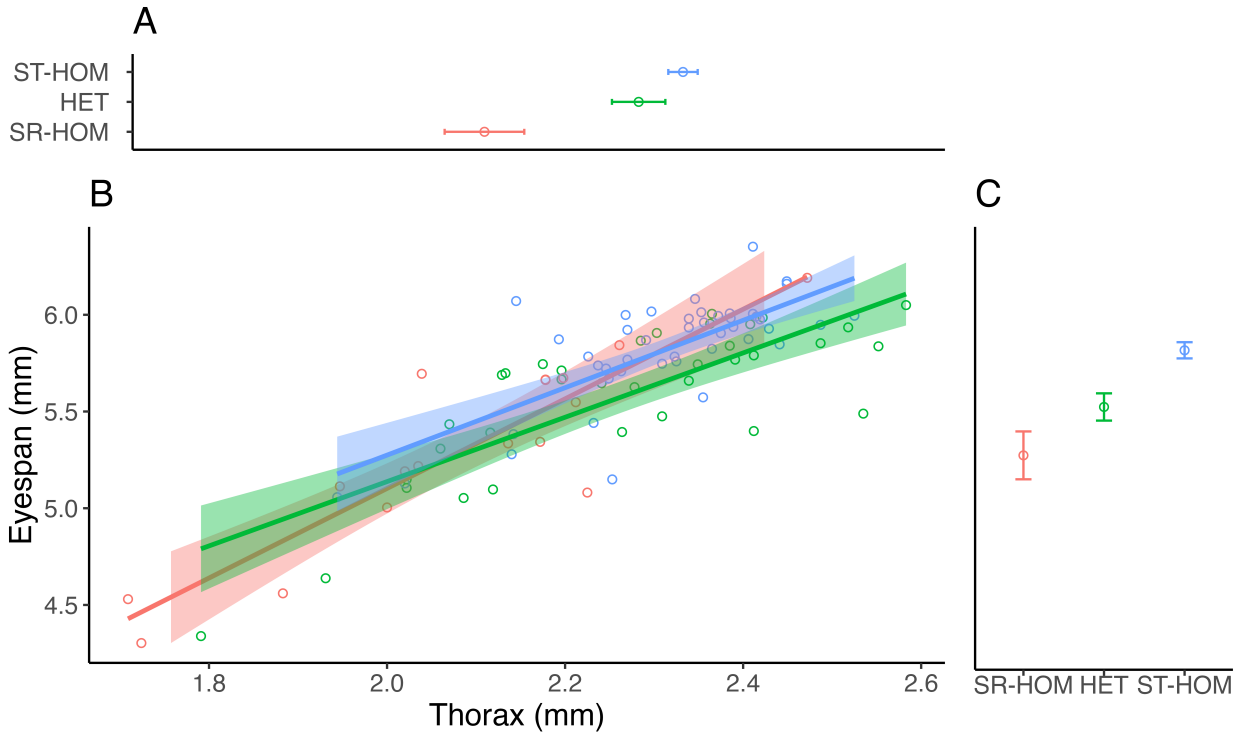

Figure S4.1: The relationship between trait size and genotype is shown for females of each genotype. Measurements of females with the SR-HOM genotype are shown in red, SR-HOM in green and ST-HOM in blue. Mean thorax length (mm) ± SE is shown in panel C and mean eyespan (mm) ± SE is shown in panel A. The correlation between the two is shown in panel B, where residuals of the slope represent Relative Eyespan; the variation in eyespan that is not explained by thorax length.

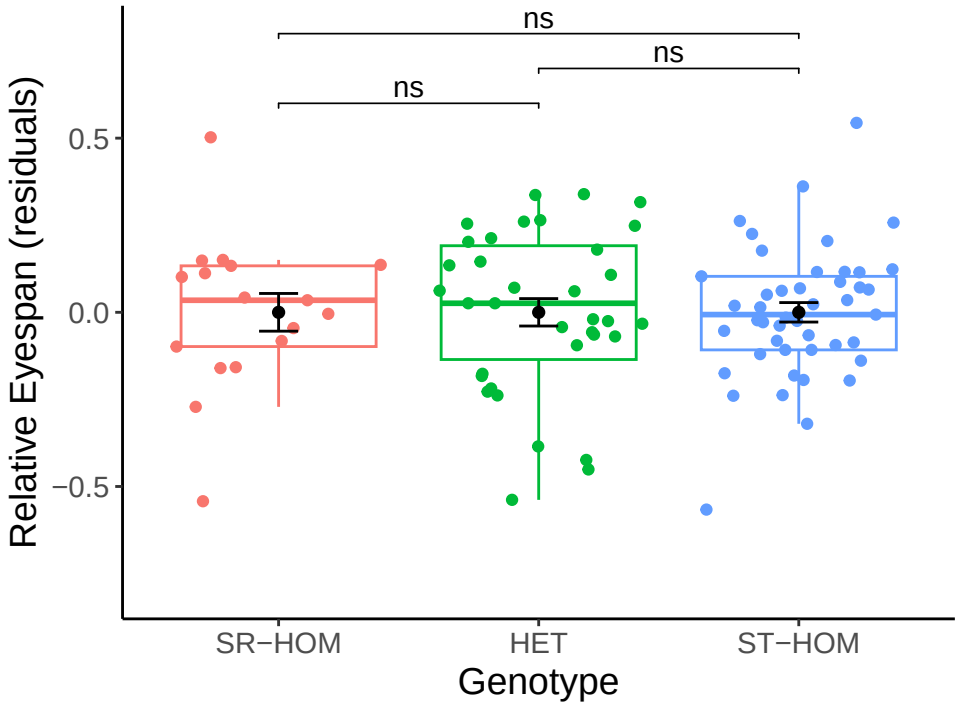

Figure S4.2: Variation in relative eyespan with genotype.

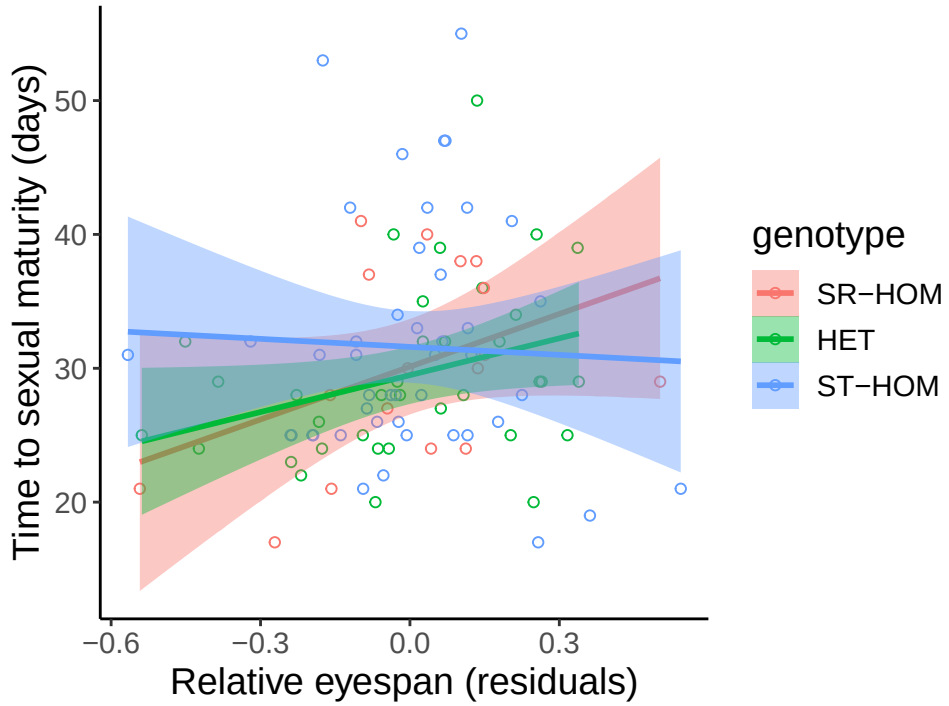

Figure S4.3: The relationship between relative eyespan and time to sexual maturity is shown for females of each genotype. Shaded areas represent the SE associated with the model for each genotype.

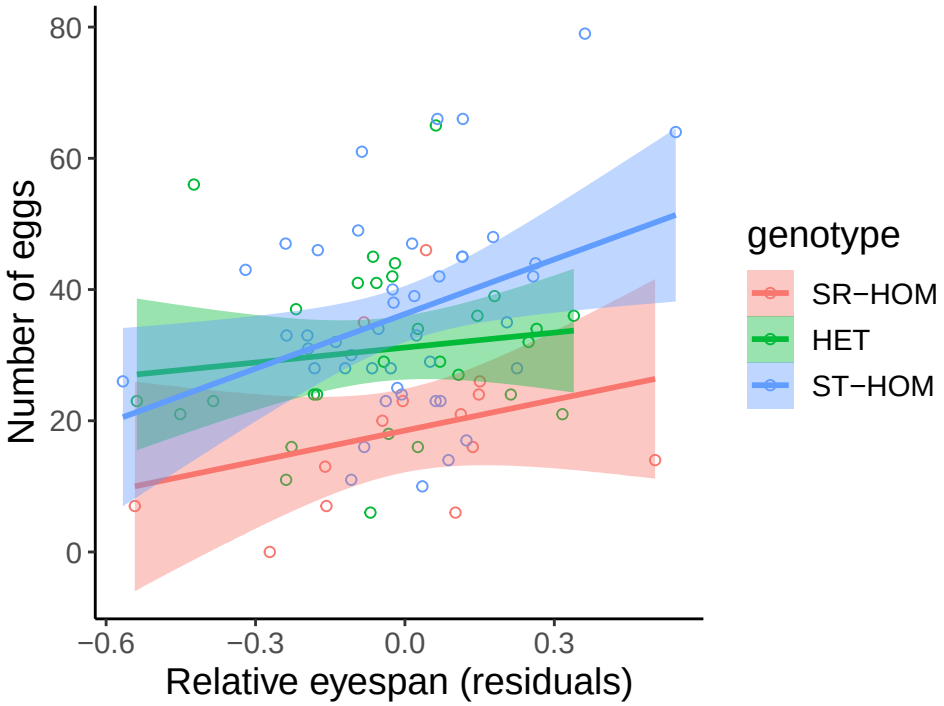

Figure S4.4: The relationship between relative eyespan and the number of eggs in ovaries of mature females is shown for females of each genotype. Shaded areas represent the SE associated with the model for each genotype.