What drives sexual selection?

Meiotic drive, stress and mate choice in stalk-eyed flies

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

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ABSTRACT

In many species females have been shown to preferentially mate with males that exhibit the most elaborate sexual ornaments. The handicap hypothesis is a major theory proposed to explain the evolution of such exaggerated traits. It postulates that the ornament is costly and handicaps the bearer such that only high quality males are able to produce the most exaggerated ornamentation.

In this thesis I examined questions about male ornament evolution and the handicap hypothesis in the stalk-eyed fly, *Teleopsis dalmanni*. I examined how meiotic drive, a selfish genetic element that produces female-biased broods, associated with male eyespan (the sexually selected trait in *T. dalmanni*) in natural populations. I demonstrated a link between meiotic drive and ornament size, whereby small eyespan males were more likely to carry the meiotic drive X chromosome. I then examined how meiotic drive affected the condition-dependent expression of male eyespan. I found that although the mean eyespan of meiotic drive males was smaller, the overall degree of condition dependence was unaffected.

Next, I explicitly tested whether there was empirical evidence for the handicap hypothesis in *T. dalmanni*. In wild populations, I found that under high experimental stress, survival was strongly correlated with male eyespan. In contrast, there was no relationship between eyespan and survival when flies were under low experimental stress. These results provide strong support for the handicap hypothesis. Laboratory experiments yielded similar results.

I then explored how environmental quality influenced key components of sexual selection (lek structure and behaviour). I found that under low environmental quality, mean and variance in harem size and the strength of mate choice declined, suggesting reduced sexual selection in poor environments. Finally, I describe for the first time the existence of male mate choice in *T. dalmanni,* a species that had previously been invoked to exemplify a traditional model of female choice.

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General introduction

1.1 OVERVIEW

I begin this introduction with an examination of why the evolution of elaborate male secondary sexual traits has historically been viewed as problematic. I discuss differing historical viewpoints, and how these have influenced our current understanding. I examine the theoretical underpinning of the two major models proposed to account for the evolution of such traits in nature, Fisher's runaway process and the handicap hypothesis. As it has the most relevance to this thesis, I have focussed primarily on the condition-dependent model of the handicap hypothesis. I examine condition dependence in detail and review the evidence for its widespread existence in sexual traits. I briefly examine our current understanding of the impact that environmental factors have on sexually selected traits, in general and on condition-dependent trait expression, in particular. I move to a brief discussion of the evolution of selfish genetic elements, specifically meiotic drive, with particular focus on the interplay between meiotic drive and sexual selection. Then I justify the use, throughout this thesis, of stalk-eyed flies (Diptera: Diopsidae) as model organisms for the study of sexual selection and document their biology and evolution. I conclude the introduction by outlining the aims and content of each subsequent chapter in the dissertation.

1.2 HISTORICAL PERSPECTIVE: THE EVOLUTION OF SECONDARY SEXUAL TRAITS

Exaggerated male secondary sexual traits emerged as one of the great conundrums facing the theory of evolution by natural selection (Darwin, 1859). Extravagant morphology, songs and other behavioural displays, vivid colour patches, horns and weapons were all

observed in the natural world, and yet typically only in males. These traits appeared costly and with no natural selection benefit. They pose problems for explanations that rely on differences in survival between individuals. If such traits conferred a survival advantage they should also be present in females, or if they reduced survival they should be countered by natural selection. The solution to these problems was first proposed by Darwin in *The Origin of Species* (1859) and was subsequently greatly expanded in *The Decent of Man* (Darwin, 1871). Darwin made a key distinction between natural and sexual selection, stating that sexual selection "depends not on a struggle for existence, but on a struggle between males for possession of females; the result is not death to the unsuccessful competitor, but few or no offspring" (Darwin, 1859 p. 88).

Darwin brilliantly elucidated the two major mechanisms of sexual selection, which are still held to this day. He hypothesised that horns and weaponry evolved through competition amongst males for access to females, and that male ornaments evolved through female preference; "in a state of nature female birds, by having long selected the most attractive males, have added to their beauty. No doubt this implies powers of discrimination and taste on the part of the female" (Darwin, 1871 p. 259).

Despite the strength of Darwin's conviction about female preference, he foresaw the looming controversy as he added to his remarks above "…which will at first appear extremely improbable; but I hope hereafter to show that this is not the case" (Darwin, 1871 p. 259). Alas criticism of sexual selection theory and female preference for male ornaments in particular was abundant and led by a number of leading biologists at the time, notably Alfred Russel Wallace, Julian Huxley and T.H. Morgan (reviewed in Pomiankowski, 1988; Andersson, 1994). Wallace argued that although horns and weapons could evolve through competition over mates, this was just selection acting

on male survival, vigour and fighting ability and thus elaborate male weaponry and the associated male-male competition was just an extension of natural selection. There is no simple explanation as to why favoured males would be of superior quality than any other male, and thus Wallace saw no requirement for female preference, invoking natural selection-based concepts such as mimicry and warning colouration to explain elaborate sexual traits (Wallace, 1889). T.H. Morgan was a fierce critic, providing twenty objections to the idea of sexual selection, concluding, "the theory meets with fatal objections at every turn" (Morgan, 1903 p. 221). Such was the criticism, that pro-Darwinian Vernon Kellogg came to state "in the eyes of most biologists, sexual selection is practically discredited" (Kellogg, 1907 p. 86).

R.A Fisher was the first to formalise how female preferences may have evolved (Fisher, 1915). His 1930 monograph, *The Genetical Theory of Natural Selection*, builds on his early work and complement's Darwin's theory with a testable coherent explanation for the evolution of male sexual traits through female preference. He argued that female preferences could evolve and be adaptive if females gained fitness benefits from mating with preferred males (Fisher, 1930). This has become a cornerstone of sexual selection theory and was a major catalyst for what is now a vibrant field of research.

1.3 MODELLING THE EVOLUTION OF SEXUAL ORNAMENTS

1.3.1 Fisher's Process

Although Fisher made many key insights, an especially influential part of his legacy was to provide a cohesive theory for the evolution of exaggerated male traits through female preference (Fisher, 1915, 1930). His theory invoked a two-stage process: natural followed by sexual selection. He assumed that both female preference and male ornamentation exhibited heritable genetic variance. He proposed that female preference would evolve if the preferred male traits confer a natural selection advantage; that is, the offspring of discriminating females would have greater fitness. As a result of non-random mating for these traits, female preference alleles would become associated with alleles for the preferred male trait. The genetic covariance for these traits was predicted by Fisher to cause dramatic evolutionary changes to both female choice and male ornaments, as it would alter the relative mating success of males, and with it the relative advantage of preferring such males as mates. The sons of choosy females possess the desirable trait and thus a mating advantage, which accelerates not only the spread of the desirable trait but also the preference genes that are linked to it. Fisher argued that this genetic covariance drives a feedback loop, a 'runaway process' by which preference and ornament size would continue to increase unless they were checked by strong counter selection to reach equilibrium. This would result in exaggeration of male trait sizes to far exceed the natural selection optima, checked either when the viability cost of increasing ornament size outweighs the mating advantage or when there is no more additive genetic variance in the male trait. Fisher's hypothesis was not fully formalised until the 1980s. Lande (1981) found a stable line of equilibria between ornament and preference, as for each preference value

a stable level of exaggeration was shown where the mating advantage of preferred males was exactly balanced by the reduced survival that results from having a large ornament. Following on from this model, Kirkpatrick (1982) found qualitatively identical results, using a simpler set of genetic assumptions. A caveat to these formalisations is that they predict a failure of Fisher's process if fitness costs are incurred by females as a result of exercising their preference (Kirkpatrick, 1985; Pomiankowski, 1987a). When preferences are costly (for example through time costs associated with mate searching), both ornament exaggeration and preference for those ornaments is predicted to disappear (Pomiankowski *et al.*, 1991). Pomiankowski *et al.* (1991) showed that as long as there is a deleterious mutation bias (mutations are more likely to be deleterious than advantageous) in ornaments, the Fisher process can lead to the stable exaggeration of male traits in spite of costly female choice.

1.3.2 The Handicap Hypothesis and Condition Dependence

Fisher assumed the costs associated with sexual male ornaments were merely a by-product of their exaggeration. Zahavi (1975, 1977), however, postulated that these ornaments evolved precisely because they were costly. Whilst Fisher argued that the advantage of female preference for exaggerated males arose through the production of attractive sons, Zahavi hypothesised that females gained viability benefits from mating with highly ornamented males (Zahavi, 1975, 1977). In line with Maynard Smith (1987 p. 12), I use the term "viability" to mean components of fitness other than mating success (Maynard Smith, 1987). Under this theory, only males of progressively higher viability are better able to bear any extra costs of progressive exaggeration. Assuming heritable genetic variance in viability, females that preferentially mated with these males would produce offspring that had a higher viability and so preference

would be favoured by selection. Zahavi's original hypothesis has since been refined into three different sub-types: Zahavi's handicap, revealing handicaps and condition-dependent handicaps (reviewed in Pomiankowski, 1988; Andersson, 1994). It is beyond the scope of this introduction to examine all three. I shall focus on the condition-dependent handicap hypothesis as this dominates sexual selection theory and has substantial theoretical and empirical support. Under this theory, the degree of exaggeration seen in male ornaments is assumed to be proportional to the overall condition of the male, such that males in good condition will have more exaggerated ornamentation and higher viability (Zahavi, 1977; Kodric-Brown and Brown, 1984; Zeh and Zeh, 1988; Rowe and Houle, 1996; Cotton *et al.*, 2004a; Getty, 2006).

In order to identify the criteria needed for the evolution of condition-dependent handicaps I will examine the quantitative genetics model of Iwasa and Pomiankowski (1994) where ornament size was found to be dependent on three factors in a linear model:

$$s = t + t'v\,.$$

Here, ornament size (s) is equal to the value of genes for the male trait *per se* (t) added to the product of male viability (v) and a condition dependence parameter (t'). The relationship between ornament size (s) and viability (v) is reflected in values of t'. When $t' = 0$, then ornament expression is independent of viability (v), characteristic of purely Fisherian traits. When $t' > 0$, then ornament size (s) is an increasing function of viability (v), characteristic of the handicap hypothesis. Whilst viability (v) is expressed in both sexes, genes for the trait (t) and the condition dependent parameter (t') are sex-limited in males (Iwasa and Pomiankowski, 1994).

In the model described above, Iwasa and Pomiankowski (1994) outlined how male fitness is determined by three key components: female preference, male viability and costs of male ornament size. I shall briefly describe each of these in turn. An increase in male mating success is proportional to the strength of (the average) female preference ($\bar{p}$). When $p = 0$, females have no preference for male ornaments and they mate at random. When $p > 0$, females prefer to mate with males that have above average ornament sizes, so males with large ornaments gain a sexual selection advantage. Male mating success is also dependent directly on the effect of male viability, where fitness increases as a function of viability (v). Finally, male mating success is dependent on the costs associated with ornament size. The survival chances for a given ornament size (s) vary with male viability (v). The 'cost differential', given by k, is used to examine how viability in males (v) affects the costs associated with producing large ornaments. When the cost differential $k = 0$, then male viability has no effect on survival. When $k > 0$, poor quality males with low viability pay a greater cost for having a given ornament size than high quality, high viability males (Iwasa and Pomiankowski, 1994, 1999).

The models of Iwasa and Pomiankowski (1994, 1999) highlight two conditions that are essential for the handicap principle to be a powerful force for the evolution of costly mate choice. Firstly, viability (v) must be subject to a deteriorating force (e.g. biased deleterious mutation) that maintains genetic or environmental variation in fitness (Iwasa *et al.*, 1991), and second, ornaments must be condition-dependent ($t' > 0$). Iwasa and Pomiankowski (1994, 1999) also show that condition dependence only evolves when low quality males pay a higher cost of survival for larger ornament sizes (i.e. when $k > 0$). This means that it is necessary for the costs of ornament exaggeration to encroach on survival for ornaments to become condition-dependent. These

conditions ensure that male ornaments are able to be effectively evaluated by females, who are then able to offset any costs to themselves incurred in exercising their mate choice by gains in heritable fitness benefits for their offspring. When preference is at equilibrium, the costs associated with mate preference are exactly balanced by the benefits accumulated through the increased viability of progeny.

All the initial models of the handicap hypothesis were based on handicaps being successful only in conjunction with Fisher's self-reinforcing process. Grafen (1990) created a game theory model (Maynard Smith, 1982) to examine whether the evolution of exaggerated traits can occur under the handicap hypothesis, without invoking Fisher's runaway mechanism. Grafen (1990) constructed a unique model of a haploid population where variation at a single locus described both sex-limited advertising (males) and preference rules (females). This ensured that Fisher's process was inhibited from operating, as the runaway hypothesis requires independent genetic variation in each of the traits, as well as covariance between them. Similarly to Iwasa and Pomiankowski (1994), Grafen (1990) found that male advertisement was required to be costly and that the cost of that advertisement must vary for males depending on their quality, with a greater cost incurred by males of low quality. When these rules were met, Grafen (1990) found that a signalling equilibrium existed for handicaps as long as females were able to successfully infer male quality from male advertisement. Thus Grafen produced the first successful handicap model that did not invoke Fisher's process, but instead acted as an independent model to explain the evolution of elaborate sexual ornaments.

An assumption of all handicap models is that ornament size is a 'honest' reflection of male (genotypic or phenotypic) quality that is maintained by the differential costs of

ornament production by different quality males. The honesty of the signal ensures that females are able to use the size of the male ornamental trait to infer male quality accurately and reliably. Grafen (1990) assumed that all males employed the same signalling strategy and thus condition dependence accurately reflected male quality equally. This is unlikely to reflect reality however, as the size of the ornament will very likely overestimate male quality in some individuals. Those males that have ornaments overestimating their quality are referred to as 'cheaters' and their frequency in the population determines the effectiveness of the handicap (Johnstone and Grafen, 1993). At high frequency there would be a decline in female preference, as females would continue to pay the cost of mate choice whilst the benefits, in terms of increased offspring viability, would diminish. At low frequency, females would benefit from mate choice discrimination in the majority of cases and thus the exaggerated ornaments only need to be honest "on average" in order to maintain equilibrium (Johnstone and Grafen, 1993). In addition to cheaters, the naturally imperfect evaluation of male ornament size by females would also influence the stability of the handicap hypothesis. Johnstone and Grafen (1992) examined this issue and found that as long as the inferred quality of the male co-varied strongly with the true quality of the male then imperfection in female evaluation is unlikely to disrupt the handicap equilibrium (Johnstone and Grafen, 1992).

1.3.3 Sexual Selection and the Maintenance of Genetic Variation

In the following section I briefly outline how condition dependence can explain one of the greatest puzzles concerning the evolution of sexually selected traits: underlying genetic variation in sexual traits. This is relevant to my thesis, as in chapter 3 I investigate the impact of genetic changes on the condition dependence of sexual traits.

A key pre-requisite of the handicap hypothesis (the assumption that females gain offspring viability benefits from mating with high quality males) is that viability has additive genetic variance. The assumption is that certain genes (the 'good genes') will increase offspring survival independent of other factors, i.e. a high quality male will be a good mate for all females (Colegrave *et al.*, 2002; Neff and Pitcher, 2005). Highly ornamented, high quality males will be preferred by all females and thus there should be strong directional selection on preferred male traits. The directional sexual selection should cause preferred alleles to go to fixation and deplete additive genetic variation in both the preferred male trait and male viability (Tomkins *et al.*, 2004; Kotiaho *et al.*, 2008). This gives rise to the 'lek paradox': why should females continue to exhibit costly mate preference when there is little or no additive genetic variation in viability, as there would be no genetic benefits accrued from choice (Borgia, 1979; Taylor and Williams, 1982; Kirkpatrick and Ryan, 1991)? Initial empirical evidence supported the idea that there was limited genetic variation in viability, as studies found that trait heritability was negatively correlated to fitness contribution such that the heritability of traits closely related to fitness were near to zero (Gustafsson, 1986; Mousseau and Roff, 1987; Roff and Mousseau, 1987). However heritability (the ratio of V_A (additive genetic variance) to V_P (total phenotypic variance)) was found to be a poor estimate of a traits 'evolvability', and thus a more appropriate dimensionless coefficient metric (CV_A) was proposed (Houle, 1992). Life history traits have large and substantial CV_As and male sexual ornaments have been shown to exhibit higher levels of CV_As than non-sexual traits (Pomiankowski and Moller, 1995). How is this genetic variance in traits associated with fitness maintained despite strong directional selection? This field has been extensively reviewed (Falconer and Mackay, 1996; Roff, 1997; Maynard Smith, 1998; Radwan, 2008), and I will touch upon the fundamental cause of genetic

variation (mutation) and briefly discuss the genic capture hypothesis as an explanation for persistent variance in sexual traits. One of the most critical points to note is that the number of genes that affect viability is likely to be extensive and include a large number of loci within the genome (Houle, 1991). The resultant variance in fitness is likely to be maintained through a balance of new variation from mutations at all these loci balanced against the variance removed by selection (Roff, 1997). Whilst the mutation rate (per locus) resulting from, for example, errors in DNA repair is likely to be low, estimates in *Drosophila melanogaster* show that deleterious mutations across the genome may reduce total fitness by almost 20%, suggesting that there is substantial variation in the viability of natural populations (Rice, 1988). Sexually selected traits are subject to strong directional selection and yet, like fitness, exhibit large amounts of genetic variance (Pomiankowski and Moller, 1995). I discussed the condition-dependent expression of sexual traits in the preceding section of this introduction, and its importance is again exemplified here. Rowe and Houle (1996) proposed the genic capture hypothesis; male traits are condition-dependent, so as well as the specific genes for the trait, their expression is reliant on the vast number of potentially mutable loci which underlie overall condition, and it is this large genetic base that gives rise to such high levels of genetic variation. Thus condition is able to maintain genetic variation in sexual traits and provide the basis for genetic benefits to costly female mate choice. The two major assumptions of this hypothesis are that sexual traits are condition-dependent and that there is high genetic variance in condition (Rowe and Houle, 1996).

1.4 FEMALE MATE PREFERENCES AND CONDITION DEPENDENCE

Until this point, the discussions of theory in this thesis have primarily addressed the evolution of male ornaments. It is also true that this focus is reflected in the broader scientific community and thus the fundamental causes of variation in male ornaments are now well understood (Pomiankowski and Moller, 1995; Rowe and Houle, 1996; Cotton *et al.*, 2004a; Tomkins *et al.*, 2004). Variation in female mating preferences, the primary selective force behind the evolution of male sexual traits (Poulin and Vickery, 1996; Rolff, 1998; Cotton *et al.*, 2006a), has in contrast, received relatively little examination (Jennions and Petrie, 1997) and thus in this thesis (chapter 5) I examine the condition-dependent nature of female mating preferences.

Before considering the evidence for condition dependence of female preference it is important (and previously often neglected) to distinguish between female preference and female choice (Heisler *et al.*, 1987; Cotton *et al.*, 2006a). Female preference describes both the behavioural and sensory capacity of females that lead to the differential mating of males based on their phenotype (Heisler *et al.*, 1987). Female choice denotes the action of choosing a certain mate and encompasses not only female preference but is also influenced by other variables such as the costs of mate choice, male-male competition, mate availability and mating coercion (Jennions and Petrie, 1997; Cotton *et al.*, 2006a). Whilst many early studies focussed on describing variation in female preference at the level of a population or group (Jennions and Petrie, 1997; Wagner, 1998), it may be misleading to extrapolate that to individuals, as individual level variation in preference is likely to be high.

The strength of preference is predicted to alter relative to differential costs and benefits, with preferences predicted to be weaker when they are more costly and more intense when there are greater benefits to be gained from discrimination (Pomiankowski, 1987a; Houle and Kondrashov, 2002). The cost to benefit ratio may affect females in different ways if preference varies with female quality. If higher quality females were better able to pay the costs of preference or gain greater benefits from mate discrimination (Cotton *et al.*, 2006a) then preferences should be condition-dependent (Pomiankowski, 1987a; Grafen, 1990; Iwasa *et al.*, 1991; Iwasa and Pomiankowski, 1994).

Under this hypothesis, high quality females in good condition should have more attractive mates, on average, than those of poor quality females. This is because they should have stronger preferences and may be able to invest more time and/or energy on mate searching. This prediction is commonly upheld (Cotton *et al.*, 2006b), for example in stalk-eyed flies, high quality females, with higher fecundity, have been shown to exhibit stronger preferences (Hingle *et al.*, 2001b). Similar to the assumptions of theoretical models of the evolution of male sexual traits, models of condition-dependent female preference require mate preference to be differentially costly with respect to female quality. High quality females should experience lower costs of mate preference than low quality females when making the same mating decision, or alternately high quality females may be more discriminatory for the same cost. Females of the lekking topi *Damaliscus lunatus*, prefer to mate with, and compete aggressively for, central lek males that are larger and have darker facemasks (Bro-Jørgensen, 2002). Females in poor condition (subordinates) pay a higher cost of preferring large males compared to females in good condition (dominants) as they

suffered considerably greater interrupted matings (15% as opposed to 2% of mating bouts, respectively) (Bro-Jørgensen, 2002).

Theory predicts that mating with high quality males will provide either direct benefits (e.g. nuptial gifts, parental care, fertility) or indirect benefits (e.g. 'good genes') to the discriminating female and will result in enhanced fitness of their progeny (Iwasa and Pomiankowski, 1994). It is also true however, that males should benefit from female variation in preference. If female mating preferences are condition-dependent, the most elaborate male ornamentation will attract discerning females that are in better condition. This is advantageous in a number of ways with both potential 'direct' and 'indirect' benefits. Female condition is often correlated with reproductive traits such as fecundity (e.g. Cotton *et al.*, 2010) and thus a key direct benefit would be increased offspring production. Evidence suggests that there is a genetic component to variation in female quality (Roulin *et al.*, 2000), and so the most attractive males may profit from an indirect 'good genes' inheritance of female condition by their offspring. As I discussed previously, Fisher's process relies on linkage disequilibrium between genes for elaborate male ornament and the associated female preference (Fisher, 1930). Through this same process of linkage disequilibrium, condition-dependent preference can result in heightened levels of sexual selection. This is because genes for both female condition and preference become associated, which drives high levels of non-random mating (and hence strong sexual selection) (Tomlinson and O'Donald, 1996).

1.5 THE EVOLUTION OF MEIOTIC DRIVE

Understanding how meiotic drive influences the evolution of sexually selected traits forms the first set of chapters in this thesis. In the section below I introduce meiotic drive and then outline previous work that has been done examining the link between meiotic drive and the evolution of sexually selected traits.

In the natural world, there is a tendency for operational sex ratios to be approximately 1:1. Fisher was the first scientist to provide an evolutionary explanation for why this is so prevalent - the adaptive sex ratio theory (Fisher, 1930). Hamilton (1967) then expanded upon this. Fisher and Hamilton both argued that a 1:1 sex ratio is an evolutionarily stable strategy, reasoning that if one sex in a population were to become increasingly rare, then selection would favour individuals that produced the rarer sex, thereby returning the overall population to a 1:1 sex ratio. A deviation in either direction would ultimately follow this pattern (Fisher, 1930; Hamilton, 1967). These outcomes depend upon a suite of assumptions and violation of any of these assumptions can lead to significant deviations from the 1:1 sex ratio. The assumptions include that there are separate sexes and that zygotes have one mother and one father. In addition there must be Mendelian segregation of alleles that influence the sex ratio and the parental genotype must be able to influence the sex ratio of their offspring. Finally the costs of producing either sex must be equal and mating in the population must be random and with no substructure (Fisher, 1930).

Sex chromosome meiotic drive is a phenomenon that causes a sex ratio bias through violating the assumption of Mendelian segregation. Sex chromosome meiotic drive is caused by a selfish genetic element; a sequence of DNA that promotes its own

transmission disproportionately in the organism's progeny, often at the expense of the rest of the genome. The sex chromosome meiotic drive element is one example of a huge number of independent selfish genetic elements that employ diverse tactics (Hurst and Werren, 2001). The unequal transmission of genes that results from meiotic drive ensures that the laws of Mendelian segregation, which purports that genes are transmitted to 50% of offspring, are violated. Genes that are disproportionally inherited in this manner can spread throughout the population, whether they are good, neutral or even harmful to the organism. A number of key elements allow for potential sex ratio distorting elements to evolve. An XY/XX or ZW/ZZ system is necessary, where one sex is heterogametic (has one of each sex chromosome - XY or ZW). Critically, the sex chromosomes do not recombine and they carry genes that code for the sex of offspring. It is primarily this lack of recombination that makes the evolution of drive loci more likely (Frank, 1991; Hurst and Pomiankowski, 1991; Lyttle, 1991).

Meiotic drive systems require the presence of two loci, tightly linked as so not to be separated by recombination. This is important, as the meiotic drive system would break down if recombination were able to separate the two loci. The two loci consist of a 'drive' or 'killer' locus and a 'target' or 'responder' locus (Lyttle, 1993; Burt and Trivers, 2006; Larracuente and Presgraves, 2012). The most common method by which meiotic drive elements remain in tight linkage is through inversions, where a section of the chromosome (with both loci contained within it) breaks at two points and is then inverted and reinserted into the chromosome. Sex chromosome meiotic drive typically produces female-biased broods (Hamilton, 1967), as the drive complex is usually located on the X chromosome (in species with the XY sex-determination system) and is active in the heterogametic sex (generally males) (Hurst and Pomiankowski, 1991; Lyttle, 1993). The drive complex typically works by causing

differential sperm maturation or survival during spermatogenesis, leading to low survival amongst Y bearing sperm and few male offspring (Lyttle, 1993).

Meiotic drive has been shown in a wide array of species, although the taxonomic distribution is clumped. It has been shown in angiosperms, mammals and insects, although its prevalence is greatest in insects, primarily the Diptera. The consequences of meiotic drive are extensive, with two important contexts being population level extinction and intra-genomic conflict. The presence of meiotic drive in a natural population is expected to skew the operational (sexually active) sex ratio towards the homogametic sex (females in an XY/XX system) and this has been shown to be the case in *Drosophila* (Bryant *et al.*, 1982; James and Jaenike, 1990). When the drive chromosome has a high frequency in the population, which is expected in the absence of counter selection, the number of females would continue to increase, with males becomingly increasingly rare, until the population became extinct (Hamilton, 1967; Hatcher *et al.*, 1999). The continued existence of a population is likely to be heavily influenced by the frequency of the drive chromosome in the population, with small populations being significantly more vulnerable to extinction (Hamilton, 1967; Jaenike, 2001).

The departure from the optimum autosomal sex ratio of 1:1 causes strong counter-selection for suppressors acting against drive, not only on the opposing sex chromosome, but also on the autosomes. This causes intra-genomic conflict, and an evolutionary arms race between the drive loci and the suppressors. Indeed there is strong evidence for very rapid evolution of the drive complex, seen over just a few decades in *Drosophila simulans* (Bastide *et al.*, 2011).

There is also some evidence that meiotic drive could have implications for the evolution of sexually selected traits. A female-biased operational sex ratio could lead to males (as the rarer sex) becoming choosy in their mating behaviour, and thus to the evolution of male mate choice (Randerson *et al.*, 2000) and a reduction in the intensity of sexual selection. Another implication arising from the female-biased sex ratio is that it imposes strong selection on females to be able to maximise the number of sons that are produced, in line with Fisher's adaptive sex ratio theory. One way in which individuals may be able to do this has been seen in stalk-eyed flies. Presgraves *et al.* (1997) first reported the existence of sex chromosome meiotic drive in two stalk-eyed fly species (*Teleopsis dalmanni* and *Teleopsis whitei*). Both species had high levels of female-biased broods (13-17% and 29% of females in the brood respectively). Wilkinson *et al.* (1998a) artificially selected male flies for relatively large and relatively small eyespan (the sexually selected trait in stalk-eyed flies) and reported a genetic association between male eyespan and offspring sex ratios. In one of the pair of replicated small eyespan lines there was a bias towards female-biased broods, whereas both large eyespan lines produced broods with a 1:1 sex ratio (Wilkinson *et al.*, 1998b). The authors predicted that females would benefit by choosing males that are resistant to meiotic drive in order to gain by producing more male offspring (the rarer sex). Johns *et al.* (2005) investigated linkage patterns between microsatellite loci associated with meiotic drive and eyespan. They found a drive chromosome specific haplotype consisting of four X-linked microsatellite markers. The linkage analysis revealed a dramatic reduction in recombination between the drive and the standard X chromosome, indicative that the drive chromosome is located in a region of low recombination (e.g. an inversion). An X-linked QTL, which explained 36% of the variation in male eyespan, was found to be located only 1.3cM from the drive locus on the X chromosome, suggesting a close physical association between a major locus for

eyespan and the locus for drive (Johns *et al.*, 2005). These results suggest that drive may be associated with reduced sexual signalling, and that male eyespan is subject to a form of 'good genes' sexual selection through mate preference for drive resistance (Wilkinson *et al.*, 1998b). However, given that only two artificially selected lines were manipulated, it is plausible that the observed genetic linkage could simply be due to chance. Examining the relationship between meiotic drive and sexually selected traits in populations not subjected to artificial selection forms the basis for chapter 2 in this thesis. I also utilise the relationship between male eyespan and meiotic drive to examine whether sexually selected traits in meiotic drive bearing individuals have a different condition-dependent expression profile (chapter 3).

1.6 STALK-EYED FLIES

Stalk-eyed flies are a proven model system with which to test a range of predictions made by sexual selection theory (Andersson, 1994; Wilkinson and Dodson, 1997; Wilkinson, 2001; Maynard Smith and Harper, 2003; Chapman *et al.*, 2005). In this section I examine the evolution and biology of stalk-eyed flies, focussing specifically on empirical research related to sexual selection in my study species, *Teleopsis dalmanni*.

1.6.1 History and Ecology

Diopsids, a family in the order Diptera, are characterised by hypercephaly, the elongation of the head capsule into long stalks causing the lateral displacement of the eyes and antennae to the end of these stalks (Baker *et al.*, 2001a). Whilst elongated

eyes has arisen in several Dipteran families (Grimaldi and Fenster, 1989; Wilkinson and Dodson, 1997), diopsids are distinctive because all known species (and both sexes) in this family exhibit hypercephaly (Baker *et al.*, 2001a). Discovered by Linnaeus in 1775, there are now over 150 extant species that have been characterised (Feijen, 1989) although others estimate the figure to be nearer 300 (Wilkinson and Dodson, 1997). The majority of stalk-eyed fly species are found in the tropics of South East Asia and Africa. There are species however, from the genus *Sphyracephala,* that are more widespread, having been discovered in both North America and Europe (Feijen, 1989; Papp *et al.*, 1997; Wilkinson and Dodson, 1997).

Stalk-eyed flies are long-lived, atypical amongst fly species, with many species exhibiting longevity of over 6 months in both captive and natural populations (Wilkinson and Reillo, 1994; Wilkinson and Dodson, 1997). Adults in the wild spend the day independently foraging in the forest, and live off fungi, mould and decaying leaf litter (Burkhardt and de la Motte, 1983; Feijen, 1989; Wilkinson and Dodson, 1997). Larvae are saprophagous, and feed on decaying vegetation (de la Motte and Burkhardt, 1983; Feijen, 1989; Wilkinson and Dodson, 1997). When larval food deteriorates, pupation occurs quicker and is triggered at smaller larval sizes (Wilkinson and Dodson, 1997).

1.6.2 Sexual Dimorphism

Eyespan in stalk-eyed flies is defined as the distance between the outermost tips of the eyebulbs. There is substantial variation in eyespan length between different species and in extreme cases the eyespan can exceed the body length (Baker and Wilkinson, 2001). Sexual dimorphism, where males have larger eyespans than females, is evident

in a large number of species (Wilkinson and Dodson, 1997; Baker and Wilkinson, 2001; Baker *et al.*, 2001a) and has evolved independently at least four times (Baker and Wilkinson, 2001). Monomorphic species (no sexual dimorphism in eyespan) are still common however and, although there is some on-going debate (Kotrba and Balke, 2006), they are generally believed to be ancestral in the Diopsidae family (Wilkinson and Dodson, 1997; Baker and Wilkinson, 2001; Baker *et al.*, 2001a).

The initial stage of evolution for hypercephaly is thought to be due to a naturally selected advantage for increased visual acuity. The number of ommatidia (optical components in each compound eye) increases with eyespan size (Burkhardt and de la Motte, 1983) and greater numbers of ommatidia contribute to an increased binocular field of vision (greater than 135°) (Burkhardt and de la Motte, 1983). This, however, does not explain the further evolution of the exaggerated trait seen in sexually dimorphic males. If large eyespan was especially beneficial, the same selection pressure would be expected in females and thus female eyespan should be comparable in size. Sexual dimorphism could technically evolve through natural selection if each sex experienced selection for divergent eyespan size as a result of niche differentiation between the sexes as seen for example in beak size differentiation in the huia (Darwin, 1871; Doflein, 1914; Lande, 1980; Andersson, 1994). However, there is no evidence for niche specialisation driving the differential evolution of eyespan in the sexes, and it is unlikely given that sexual dimorphism has arisen independently on multiple branches in the phylogeny and has also undergone several reductions (Baker and Wilkinson, 2001). The changes needed to support large eyespans are thought to cause a reduction in the ability to quickly process visual signals, which could prevent them from moving quickly (Buschbeck and Hoy, 1998). Stalk-eyed flies have limited aerial ability (Swallow *et al.*, 2000; Ribak and Swallow, 2010) and large eyespan males are

at increased risk of predation, relative to small eyespan males (Worthington and Swallow, 2010). Thus natural selection is unlikely to account for the evolution of exaggerated male eyespan in stalk-eyed flies.

1.6.3 Field Ecology and Behaviour

Behavioural observations and experimental data have shown that sexual selection is responsible for sexually dimorphic eyespan in stalk-eyed flies. In the wild males arrive at the lek sites first, during the initial stage of dusk, and congregate on exposed root hairs overhanging the eroded banks of rainforest streams (Burkhardt and de la Motte, 1985; Wilkinson, 1993; Wilkinson and Dodson, 1997; Cotton *et al.*, 2010). Males aggressively compete with each other for control of these sites and the largest eyespan males typically win these encounters (Burkhardt and de la Motte, 1983; Lorch *et al.*, 1993; Small *et al.*, 2009). Females subsequently arrive during dusk and choose their roosting sites (and therefore mates) from amongst the root hairs where males have established themselves, resulting in a lek style mating system (Cotton *et al.*, 2010). Research in both the field and the laboratory have shown that females prefer to roost and mate with males with larger (absolute and relative) eyespan (Wilkinson and Reillo, 1994; Cotton *et al.*, 2010). Females usually mate at least once each day (Lorch *et al.*, 1993), and a large eyespan male may mate with all of the females in his harem (up to 20 females) (Burkhardt *et al.*, 1994). Male reproductive success in sexually dimorphic stalk-eyed flies is therefore greatly skewed in favour of males with the largest eyespans (Burkhardt *et al.*, 1994). I utilise this previous knowledge of field ecology and behaviour throughout my thesis. For example, in chapter 5 I examine how naturally occurring environmental quality affects lek structure and mating behaviour

and in chapter 6 I examine whether males exhibit mating preferences by preferentially mating with particular females in their harems.

Monomorphic stalk-eyed fly species do not typically exhibit the lekking aggregation and mating behaviour described above. Burkhardt and de la Motte (1985) reported that only one of five monomorphic species formed aggregations, in contrast to all of the dimorphic species. In addition, there is neither male-male competition (Burkhardt and de la Motte, 1985; Panhuis and Wilkinson, 1999) nor female preference on male eyespan in those monomorphic species (Wilkinson and Dodson, 1997; Wilkinson *et al.*, 1998a).

1.6.4 Condition Dependence

One of the key hypotheses arising from the handicap principle is that sexual traits exhibit heightened condition-dependent expression (Zahavi, 1975; Cotton *et al.*, 2004a). In other words, males in good genetic or phenotypic quality signal their superiority through exaggerated trait sizes. Males in poor condition are unable to do so because of the elevated costs that they would incur (Iwasa and Pomiankowski, 1999). Male eyespan in several sexually dimorphic diopsid species has shown heightened condition-dependence relative to non-sexual traits in a number of studies, examining genetic, environmental and gene-by-environment interactions (David *et al.*, 1998; David *et al.*, 2000; Cotton *et al.*, 2004b, c).

In order to examine condition-dependence of *T. dalmanni*, David *et al.* (2000) applied three levels of environmental stress to full- and half-sib families. Certain genotypes proved less susceptible to stress than others, producing large absolute male eyespans

consistently across all environments, whilst others produced eyespan sizes that consistently declined with increasing stress. In contrast, non-sexual traits (female eyespan, male wing length and female wing length) showed genetic variation in condition-dependent expression, but this genetic response was not independent of body size. As in a number of prior studies (Wilkinson, 1993; Wilkinson and Reillo, 1994), this study (David *et al.*, 2000) attempted to account for the effects of eyespan/body size scaling, by dividing the trait value by thorax length. This ratio method is only valid under true isometry, where the relationship between eyespan and thorax is linear and the intercept of the x and y axis is zero (Packard and Boardman, 1999; Cotton *et al.*, 2004a). We know however, that in *T. dalmanni*, eyespan allometries have negative intercepts and relative eyespan values tend to increase with body size. In order to examine condition dependence without this caveat, Cotton *et al.* (2004b) included thorax length as a covariate in their analyses, and confirmed the findings of David *et al.* (2000). Recently condition dependence has been examined through the response of male eyespan to genetic stress. Prokop *et al.* (2010) and Bellamy *et al.* (2013) used inbreeding to induce genetic stress in *T. dalmanni* and *Diasemopsis meigenii* respectively. A prediction of the handicap hypothesis is that sexual traits will be more susceptible to inbreeding than non-sexual traits. *T. dalmanni* showed significantly higher inbreeding depression in male eyespan than the homologous trait in females (Prokop *et al.*, 2010). However, these effects could be entirely explained by changes in body size. By contrast, *D. meigenii* showed a significant decrease in male eyespan relative to other non-sexual traits (including female eyespan) that could not be explained by changes in body size and this study used a more extensive inbreeding regime (Bellamy *et al.*, 2013). Bellamy *et al.* (2013) used crosses between inbred lines to generate crossbred lines and male eyespan showed greater hybrid vigour relative to non-sexual traits and female homologues.

Male eyespan, consistent with assumptions made by the handicap hypothesis, is characterised by high levels of additive genetic variance. The additive genetic variance in male eyespan was over twenty times greater than the variance of a non-sexual trait and monomorphic species, *Teleopsis quinqueguttata,* was three times less than that of the sexually dimorphic species (Meier and Baker, 2002). Male eyespan in *T. dalmanni* had treble the additive genetic variance in female eyespan (Wilkinson and Taper, 1999). In this thesis, I build on our knowledge of condition dependence by examining how the meiotic drive X chromosome affects the condition-dependent expression profile of male eyespan.

1.6.5 Female Mate Preferences

Laboratory (Wilkinson and Reillo, 1994; Wilkinson *et al.*, 1998a; Hingle *et al.*, 2001b; Cotton *et al.*, 2006a) and field (Cotton *et al.*, 2010) studies have comprehensively established that females prefer to mate with large eyespan males. Wilkinson and Reillo (1994) artificially selected flies for large or small eyespan, and they demonstrated a genetic association between female choice and male eyespan. After thirteen generations of selection, females were given a choice between either a large or small male. Females from the large eyespan lines and control lines (no selection) chose to roost with large eyespan males. Those females from the small eyespan lines however, chose to roost with small eyespan males. The experimental design in most early studies centred on allowing females a choice of mate - usually between a large and small male within a contest arena. Recently however, studies have moved towards ‘no choice’ tests, where females are sequentially presented with males from a wide range of eyespan sizes and acceptance or rejection behaviours are recorded (Cotton *et al.*,

2006a). This creates a far more accurate assessment as the resolution of each individual preference function increases with the number of levels of male eyespan size presented (Cotton *et al.*, 2006a). In addition, studies that use only two levels of male phenotype are unable to accurately assess different selection profiles, such as patterns of directional or stabilising selection operating upon the male ornament (Gerhardt, 1991; Hunt *et al.*, 2005). Studies using seven levels of male phenotype have produced more detailed information on female preference (Cotton *et al.*, 2006a).

Despite research showing that large eyespan males obtain significantly more matings than small eyespan males (Wilkinson *et al.*, 1998a), females lack the ability to discern small differences in the eyespan of potential mates (Hingle *et al.*, 2001b). Females were only able to detect differences in male eyespan when the difference was large (Hingle *et al.*, 2001b). Interestingly, they also found that female size (and therefore potentially condition) indicated the strength of preference for male eyespan size, with larger females exhibiting a stronger preference (Hingle *et al.*, 2001b). A subsequent experiment confirmed this finding by showing that high quality females had a stronger preference for large eyespan males than poor quality females (Hingle *et al.*, 2001a). Current female condition and preference strength are tightly linked as it was shown that when females on high quality food were put on poor quality food, preference strength reduced and females mated randomly with reference to male eyespan (Hingle *et al.*, 2001a). The reverse transition (from poor to high quality food) was also shown to follow the same pattern (Hingle *et al.*, 2001a). This, and similar studies on related species (Cotton *et al.*, 2006a) demonstrate that the strength of (sexual) selection on male eyespan is dependent upon female condition. Building on our current knowledge, in this thesis I specifically examine how female mating behaviour and preferences are altered by environmental stress.

1.6.6 Reproductive Biology

Sperm limitation in males can result in reduced fertility for females. The need for females to increase their fertility is likely to be a major influence in determining levels of female preference. In *T. dalmanni* only 55% of eggs laid in the wild were fertilised (Cotton *et al.*, 2010), and laboratory experiments have revealed that females must mate multiply in order to maintain their fertility (Baker *et al.*, 2001b). When mating opportunities are withheld from females, fertility significantly declines (Cotton *et al.*, 2010). The size of the transferred spermatophore is small (Kotrba, 1996) and the number of sperm stored in the spermathecae following a single mating is very small, with estimates ranging from ~35 (Wilkinson *et al.*, 2005) to ~142 (Rogers *et al.*, 2006). This could explain recent research, which found that females gain no detectable fertility benefit from a single mating (Harley *et al.*, 2010).

Reproductive organ size is correlated with eyespan size in males, which in turn is determined by male condition (Rogers *et al.*, 2008). Accessory gland size is phenotypically and genetically (Baker *et al.*, 2003; Rogers *et al.*, 2005a) associated with male mating frequency. As male eyespan is a key predictor of male accessory gland length (Rogers *et al.*, 2008), sperm-limited females may well select large eyespan males in order to maximize their fertility. Initial work supported this hypothesis, showing that female *T. dalmanni* mated with large eyespan males had higher fertility than those mated with small eyespan males (Rogers *et al.*, 2008). Recent research however, has found that despite large eyespan males having larger reproductive organs, there is no association between male eyespan size and the size of the spermatophore (or sperm number) transferred in a related stalk-eyed fly species *D. meigenii* (Harley *et al.*, 2013). Given the high level of multiple mating by females to

assure fertility, males should invest heavily in sperm competition. Interestingly, however, there is currently little evidence of consistent patterns of sperm precedence in stalk-eyed flies generally and no evidence at all in *T. dalmanni* (Corley *et al.*, 2006). A greater number of sperm are transferred to larger (and more fecund) females, however, suggesting that there is a selective advantage in males investing heavily in large eyespan females (Harley *et al.*, 2013). This has implications for my thesis because sperm limitation and the ability to distinguish between females of differing fecundity are integral to my investigation into male mating preferences (chapter 6).

1.6.7 Meiotic Drive

In wild populations the issue of sperm-limitation is further complicated by the presence of meiotic drive (Presgraves *et al.*, 1997). As this causes the failure of up to 100% of Y bearing sperm, females mated to males carrying the meiotic drive chromosome suffer from decreased fertility (Wilkinson and Fry, 2001; Wilkinson and Sanchez, 2001; Wilkinson *et al.*, 2006). Fitness effects of the driving X chromosome have been investigated (Wilkinson *et al.*, 2006) although further work is needed to fully examine the fitness consequences that meiotic drive has on stalk-eyed flies. Wilkinson *et al.* (2006) found that females heterozygous for the driving X chromosome produced more offspring than females homozygous for either non-driving, or driving chromosomes. The authors found no difference in the ability of drive and standard males to produce offspring, however they did find that drive males had lower sperm precedence and lower fertility under high levels of multiple mating (mating with eight females in 24 hours) (Wilkinson *et al.*, 2006). They also found evidence for a rapid evolution of the drive complex, with two different microsatellite haplotypes found in the same population over 10 years apart (Wilkinson *et al.*, 2006).

As previously discussed, this is in line with previous findings in *Drosophila simulans* (Bastide *et al.*, 2011). In my thesis, I expand upon our previous knowledge of meiotic drive in stalk-eyed flies by testing for the presence of meiotic drive in wild populations and using wild flies to examine the relationship between male eyespan and meiotic drive.

1.7 THE STRUCTURE OF THE THESIS

The handicap hypothesis is one of the major theories used to explain the evolution of elaborate sexual traits. This theory provided the core theme of my thesis, as I investigated a variety of different influences affecting sexual selection in *T. dalmanni*. In addition to this introductory chapter, the thesis comprises five chapters describing my empirical studies, followed by a general discussion of the findings. Finally there is an appendix with a publication that I co-authored during my PhD, but that did not constitute part of the main body of my thesis. Chapters 2 and 3 examine the effect of meiotic drive on male eyespan, the first focusing on the phenotypic link between eyespan (as a sexually selected trait) and meiotic drive and the second evaluating the effect that the meiotic drive locus has on the condition-dependent expression of male eyespan. Chapter 4 examines the handicap hypothesis directly, investigating how eyespan influences survival under different levels of experimental stress. Chapter 5 examines environmental stress, utilising multiple datasets to ask how differences in quality can affect major components of sexual selection such as lek structure (specifically harem size) and mating behaviour. In chapter 6, I test for the existence of male mate preference and investigate possible cues that males may be using when making mating decisions. Dr Sam Cotton provided raw data from the field (except for

the wild males in chapter 2) and Dr Mihaly Földvári undertook the majority of the genotyping for chapter 2.

Chapter 2

Meiotic drive is a phenomenon with wide-reaching implications in contexts ranging from intra-genomic conflict to population-level extinction. One intriguing possibility that has been proposed is that the meiotic drive chromosome may be linked to male eyespan size, providing females with a reliable signal as to the genetic status of the male and thus a mechanism for the maintenance of female preference as females would gain a fitness advantage for offspring by mating with males without the meiotic drive locus. *T. dalmanni* provides an ideal system to examine this possibility in more detail. In this chapter I examined the basic distribution and prevalence of meiotic drive in natural populations. I then used microsatellite markers to test for associations with meiotic drive. Finally, using two independent datasets I tested for associations between meiotic drive status and male eyespan size. This chapter has been published in *Heredity*: Cotton, A.J., Földvári, M., Cotton, S. and Pomiankowski, A. 2014. Male eyespan size is associated with meiotic drive in wild stalk-eyed flies (*Teleopsis dalmanni*). *Heredity*, **112**: 363-369.

Chapter 3

In stalk-eyed flies, the meiotic drive loci are contained within a large inversion on the X chromosome. Given the lack of recombination in heterozygotes, this inversion is likely to accumulate mildly deleterious mutations. The poor genetic quality of this drive chromosome is predicted by the handicap hypothesis to be highlighted in the condition-dependent expression of eyespan, with drive males having a stronger condition-dependent response to stress. I examined this relationship by rearing males

under three different environmental conditions (low, medium and high food quality) and examined the ensuing eyespan expression profile. Males were then mated to laboratory females, and meiotic drive males were identified as producing a significantly biased offspring sex ratio.

Chapter 4

The handicap hypothesis is one of two key theories used to explain the evolution of exaggerated sexual traits. Whilst theoretical evidence is plentiful, empirical tests have focussed on measuring simple correlations between ornament size and survival. These are fairly uninformative as both positive and negative correlations provide support for the handicap hypothesis. This is because although high-quality males are expected to invest in ornamentation so that they maintain higher survival relative to lower-quality males, it has been pointed out that if larger ornaments result in sufficiently greater mating success, high-quality males may evolve increased ornaments to the point they suffer reduced survival. I used an experimental manipulation approach in *T. dalmanni* to examine the handicap hypothesis. To members of one group I applied a paper tag to the thorax, denoting a severe experimental stress and to members of the other group I applied a dot of varnish, denoting a benign experimental stress. I then monitored survival under the two stress regimes and asked how that related to eyespan size. This was done in both the field and the laboratory. All results are discussed in the context of theoretical predictions made by the handicap hypothesis.

Chapter 5

A key prediction of the handicap hypothesis is that exaggerated secondary sexual traits should exhibit heightened condition dependence, as the cost of producing exaggerated ornamentation is dependent on male quality, with males in poor condition paying a

greater cost of ornamentation. Positive correlations between trait size and condition are common, however the relationship between environmental stress and sexual selection is likely to be complex, with condition affecting both the mean and the variance of both traits and preference. I examined how both experimentally controlled stress (in the laboratory) as well as naturally occurring environmental conditions (in the field) influenced two key aspects of sexual selection; lek structure and mating behaviour. I manipulated diet in the laboratory to create flies that were either in good or poor condition. I then examined both lek structure and mating behaviour in both of these groups. In the field I collected flies at sites that differed in environmental quality and examined how differences in this quality affected the mean and variance of lek structure and harem size within populations.

Chapter 6

Sexual selection theory traditionally views females as the choosy sex, with males portrayed as indiscriminate in their choice of mates. The reality, however, is often more complex, and male mating preferences have been discovered in a number of species. I determined whether male mating preferences exist in *T. dalmanni*, a species often portrayed as having a traditional sexual selection system. I investigated this in both the field as well as the laboratory. Furthermore, in the laboratory I manipulated fecundity (through diet manipulation) and analysed the cues that males might be using in mating decisions by disentangling female eyespan size (a visual proxy for fecundity), from the current fecundity of potential female partners. This chapter has been published in *Behavioral Ecology*: Cotton, A.J., Cotton, S., Small, J. and Pomiankowski, A. 2014. Male mate preference for female eyespan and fecundity in the stalk-eyed fly, *Teleopsis dalmanni*. *Behavioral Ecology*, aru192.

Chapter 7

In this chapter I provide a recapitulation and discussion of the main findings of my thesis. I examine how my research relates to the broader context of sexual selection and suggest possible avenues for future research.

Chapter 8

The section contains a published paper that I have contributed to (and co-authored) during my PhD, but that is omitted from full-length inclusion in the main body of my thesis. Appendix 8.1 is an invited review (Evolution: Sex or Survival), written during the course of my PhD and published in *Current Biology*: Howie, J., Pomiankowski, A. and Cotton, A.J. 2013. Evolution: Sex or Survival. *Current Biology*, **23:** R1041-R1043.

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2

Male eyespan size is associated with meiotic drive in wild stalk-eyed flies (*Teleopsis dalmanni*)

2.1 ABSTRACT

This study provides the first direct evidence from wild stalk-eyed fly populations to support the hypothesis that male eyespan is a signal of meiotic drive. Several stalk-eyed fly species are known to exhibit X-linked meiotic drive. A recent QTL analysis in *Teleopsis dalmanni*, found a potential link between variation in male eyespan, a sexually selected ornamental trait, and the presence of meiotic drive. This was based on laboratory populations subject to artificial selection for male eyespan. In this study I examined the association between microsatellite markers and levels of sex ratio bias (meiotic drive) in 12 wild *T. dalmanni* populations. I collected two data sets: a) brood sex ratios of wild-caught males mated to standard laboratory females, and b) variation in a range of phenotypic traits associated with reproductive success of wild-caught males and females. In each case, I genotyped individuals for eight X-linked microsatellite markers, including several that previously were shown to be associated with male eyespan and meiotic drive. I found that one microsatellite marker was associated with meiotic drive whilst a second showed a weaker association. Using both independent datasets, I also found that meiotic drive was strongly associated with male eyespan, where smaller eyespan males produced more female-biased broods. These results suggest that mate preference for exaggerated male eyespan allows females to avoid mating with males carrying the meiotic drive gene and is thus a potential mechanism for the maintenance and evolution of female mate preference.

2.2 INTRODUCTION

The majority of species have approximately 1:1 offspring sex ratios. The prevalence of this phenomenon has been explained by adaptive sex ratio theory (Fisher, 1930). If one sex were to become increasingly rare in the population, then selection would favour individuals that produced the rarer sex, thereby returning the overall population to a 1:1 sex ratio (Fisher, 1930). A number of forces, including local mate competition and differential payoffs for the sexes against environmental gradients, can lead to well characterised deviations from a balanced sex ratio (Hamilton, 1967).

However, deviations from 1:1 ratios can also be caused by a range of selfish genetic elements (SGEs) that promote their own transmission to the next generation, at the expense of the rest of the genome. SGEs further their interests in ways that result in the distortion of the normal offspring sex ratio. Examples are widespread in eukaryotes, with a range of tactics employed by different types of SGEs (Hurst and Werren, 2001). One common form of SGE is sex chromosome meiotic drive, usually linked to the X chromosome and active in the heterogametic sex in species with the XY sex-determination system (Hurst and Pomiankowski, 1991; Lyttle, 1993). Individuals that possess the driving X chromosome (X^D) produce female-biased offspring sex ratios (Hamilton, 1967). This is typically due to differential sperm maturation or survival during spermatogenesis (Lyttle, 1993). The Y-bearing sperm of a number of species fail to undergo complete spermatid development and individualisation, leading to low survival amongst Y-bearing sperm and consequently few male offspring (*Drosophila melanogaster* (Tokuyasu *et al.*, 1972), *D. simulans* (Montchamp-Moreau and Joly, 1997; Cazemajor *et al.*, 2000) and *Teleopsis whitei* (formerly *Cyrtodiopsis whitei*) (Wilkinson and Sanchez, 2001)).

All studies to date have found that meiotic drive systems require at least two distinct linked loci, a drive and its target or responder (Lyttle, 1993; Larracuente and Presgraves, 2012). Associated inversions limit recombination allowing the drive and responder loci to remain in tight linkage (Wu and Beckenbach, 1983). Only a small number of meiotic drive systems have been studied in detail, the best known being the *t*-complex in mice (Silver, 1993), the segregation distortion (*Sd*) system in *D. melanogaster* (Kusano *et al.*, 2003), and the *sex-ratio* system in *D. simulans* (Cazemajor *et al.*, 2000). Given that the ramifications of meiotic drive can range from intra-genomic conflict to species-level extinction (Jaenike, 2001) there is a great need to study the selective and ecological processes that are involved in the evolution and maintenance of meiotic drive in wild populations. Here I examine the meiotic drive system in the stalk-eyed fly *T. dalmanni*, and relate the pattern of drive to the operation of sexual selection in wild populations.

Stalk-eyed flies display a unique form of hypercephaly whereby the head capsule is elongated in the form of eyestalks, causing the lateral displacement of the eyes to the end of these stalks. Whilst many families in the order Diptera exhibit this type of hypercephaly, the diopsid family is distinctive in that both sexes in all species display this trait (Wilkinson and Dodson, 1997). Many species of this family exhibit sexual dimorphism of eyespan (the distance between the outer most edge of the eyes), with males possessing a significantly larger eyespan, relative to their body size, than females (Burkhardt and de la Motte, 1985). Numerous studies have shown that exaggerated male eyespan has evolved through sexual selection, with the trait used in mate choice (Wilkinson and Reillo, 1994; Cotton *et al.*, 2010) and male antagonistic interactions (Small *et al.*, 2009).

One of the most intensively studied stalk-eyed flies is the Malaysian species, *T. dalmanni*. Both sexes spend their day foraging independently on decaying plant matter, and at dusk they congregate on exposed root hairs overhanging the eroded banks of rainforest streams (Wilkinson, 1993; Wilkinson and Reillo, 1994; Cotton *et al.*, 2010). Females choose their roosting sites (and therefore mates) from amongst the root hairs where males have established themselves, resulting in a 'lek' style mating system (Cotton *et al.*, 2010). Males aggressively compete with each other for control of these sites (Small *et al.*, 2009) and females prefer to roost and mate with males with larger (absolute and relative) eyespan (Wilkinson and Reillo, 1994; Cotton *et al.*, 2010). A variety of laboratory studies have provided key data on reproductive traits in males and females. In the laboratory, male accessory gland size co-varies with male mating frequency, both phenotypically (Rogers *et al.*, 2005a) and genetically (Baker *et al.*, 2003). Accessory glands become depleted with repeated matings (Rogers *et al.*, 2006), and the amount of sperm stored by a female is correlated to the testis size of the male that she mates with (Fry, 2006). A similar pattern of co-variation between male eyespan and the size of the testes and the accessory glands has been found in the wild (Cotton *et al.*, 2010).

Presgraves *et al.* (1997) first reported the existence of sex chromosome meiotic drive in two *Teleopsis* species (*T. dalmanni* and *T. whitei*). In the laboratory, genetic analyses revealed that both species had high levels of female-biased broods (13-17% and 29% respectively) and that the sex ratio bias was caused by spermatid degeneration in X^D males, similar to that seen in a number of *Drosophila* species (e.g. Montchamp-Moreau and Joly, 1997). Wilkinson *et al.* (1998) proposed that females might benefit by choosing males that are resistant to meiotic drive in order to gain by

producing more male offspring. To test this, they artificially selected male flies for relatively large and relatively small eyespan for 22 generations and found an association between eyespan and offspring sex ratios. In one of the pair of replicated small eyespan lines there was a bias towards female-biased broods, whereas both large eyespan lines produced fewer female-biased broods. These results suggest that drive may be associated with reduced sexual signalling, and that male eyespan is subject to a form of 'good genes' sexual selection through mate preference for drive resistance (Wilkinson *et al.*, 1998).

To take this analysis further, Johns *et al.* (2005) investigated linkage patterns between microsatellite loci associated with meiotic drive and eyespan. They crossed two of the artificially selected lines (small × large eyespan) that showed significantly biased sex ratios, genotyped F2 individuals, and found an X^D specific haplotype consisting of four X-linked microsatellite markers (*ms54*, *ms125*, *ms244* and *ms395*). The linkage analysis revealed a dramatic reduction in recombination between the X^D and the standard X chromosome, indicative that X^D is located in a region of low recombination (e.g. an inversion). An X-linked QTL, which explained 36% of the variation in male eyespan, was found to be located only 1.3cM from the drive locus on the X chromosome, suggesting a close physical association between a major locus for eyespan and the locus for drive (Johns *et al.*, 2005). This work again suggests that there is an association between meiotic drive and male eyespan. However, given that only two artificially selected lines were manipulated, it is plausible that the observed genetic linkage could simply be due to chance. A more extensive analysis is needed to establish the strength of the association and the predictive power of the microsatellites investigated.

The work to date investigating meiotic drive in stalk-eyed flies was carried out on laboratory populations. There remains little knowledge of either the frequency or distribution of meiotic drive in natural populations of *T. dalmanni*. In addition, despite the potential importance of the hypothesis linking male signalling with meiotic drive, this association has not been tested against data from populations in the wild. To address this, I analysed whether the microsatellites previously linked with meiotic drive in laboratory studies showed the same pattern in natural populations. Using a large sample of male and female flies from twelve wild populations, I examined natural levels of microsatellite variation. I then looked for associations between male eyespan and meiotic drive directly, as well as with those microsatellite loci that had been putatively linked to meiotic drive. In addition, I tested whether these microsatellites were associated with traits that predict reproductive success in males (testis and accessory gland size) and in females (fecundity).

2.3 MATERIALS AND METHODS

2.3.1 Source of Experimental Flies

Wild flies

All analyses were carried out using flies collected from 12 sites along the Ulu Gombak valley, in Peninsular Malaysia, spanning approximately five kilometres. The sites were: Blair Witch (BW) (3º19'N 101º45'E), Cascade (C) (3º19'N 101º45'E), Kingfisher (K) (3º19'N 101º45'E), Lower Field Centre (LFC) (3º19'N 101º45'E), Mihaly (M) (3º19'N 101º45'E), Poppet (P) (3º19'N 101º45'E), Quarry (Q) (3º18'N 101º44'E), Rubbish (R) (3º18'N 101º44'E), Swamp (S) (3º19'N 101º45'E), Tarantula

(T) (3º19'N 101º45'E), Upper Blair Witch (UBW) (3º19'N 101º45'E) and Upper Lazy Dog (ULD) (3º19'N 101º45'E) (Figure 2.1). These sites are a mix of primary and secondary rainforest, 20-40m in length (along a stream), with rootlets found under hanging stream banks.

Laboratory stock

A large sample of *T. dalmanni* was collected in 2005 (by Sam Cotton and Andrew Pomiankowski), from the Ulu Gombak valley, Peninsular Malaysia (3º19'N 101º45'E). All flies (both laboratory and experimental) were collected at night with small clear plastic bags placed over the rootlet trapping the flies inside. This allowed the gentle removal of the whole 'lek' in clearly labelled individual bags. These were then transferred into pots at the field centre. Since transportation back to the UK, flies have been maintained in cage culture at high density (>200 individuals) with an approximately 1:1 sex ratio to minimize inbreeding. The population was kept at 25ºC, with a 12:12 h dark: light cycle and fed pureed sweetcorn twice weekly.

2.3.2 Wild Males

Male flies (N = 134) were collected from five sites (BW, C, Q, UBW and ULD) in September 2009 (N = 31) and September 2011 (N = 103). They were transported to the UK, individually housed in 400ml pots, fed on pureed sweet corn twice a week and kept in constant temperature rooms at 25ºC on a 12:12 hour light: dark cycle. Three virgin laboratory females were added to each male pot. Flies were allowed to mate freely. The bases of the pots were lined with a moist cotton pad and blue paper to allow for easy egg visualisation. Eggs were collected twice a week for three weeks and kept in Petri dishes lined with a moist cotton pad. Pupae were allowed to eclose into

cage culture, and the resulting flies (offspring) were counted and sexed, and an offspring sex ratio was assigned to each male (see below). Male flies were anaesthetized on ice and stored in 100% ethanol.

2.3.3 Adult Phenotypes

Adult male (N = 226) and female (N = 210) flies were collected from all twelve sites along the Ulu Gombak valley in August 2008. Flies were anaesthetised on ice shortly after capture and digital images taken using a monocular field microscope in order to measure eyespan (the distance between the outer edges of the eye bulbs) and thorax length (the distance from the base of the head to the posterior edge of the thorax and is measured as a proxy for body size) to an accuracy of 0.01mm, using NIH image software (v. 1.55). The reproductive tract of each female was dissected and fecundity was measured as the number of mature eggs in the ovaries. The reproductive tract of each male was dissected into phosphate buffered saline (PBS). The accessory glands and testis were extracted and uncoiled, placed on a staged micrometer and photographed digitally under a monocular field microscope (Baker *et al.*, 2003). The length of both the testis and accessory glands were then measured. All of these flies were stored in 100% ethanol.

The density of flies at each of the 12 sample sites was calculated using an average based on three collections taken at the same sample sites over three years (August 2008, March 2009, September 2010). The density was estimated as the number of flies collected per metre of site sampled.

2.3.4 Genotyping

The initial collection of wild males in September 2009 (N = 31), as well as all flies from the adult phenotypes dataset (N = 436) were genotyped at the NERC Biomolecular Analysis Facility at the University of Sheffield, using previously identified (Wright *et al.*, 2004) and proven (Johns *et al.*, 2005) microsatellite loci. The 8 X-linked loci were *ms71*, *ms125*, *ms244*, *ms395*, *mscrc2*, *ms54*, *ms106* and *ms167*. DNA was extracted by grinding each fly with a pestle and following a set extraction protocol: for each sample 48 µl of squishing buffer (25 mM NaCl, 1 mM EDTA, 10 mM Tris-Cl pH 8.2) and 2 µl Proteinase K (10 mg/ml) was used, and incubated at 56°C for 1.5 hours, then treated with a heat shock at 90°C for 5 minutes (Gloor *et al.*, 1993). PCR reactions were performed on a 2720 Thermal Cycler (Applied Biosystems) in 2 µl volumes, which consisted of 1 µl dried genomic DNA, 1 µl QIAGEN Multiplex PCR Mastermix (QIAGEN) and 1 µl Primer mix, with all primers at a 0.2 µM concentration, and using an oil drop on top to avoid evaporation. Primers for the microsatellites were taken from Wright *et al.* (2004), and had been arranged into multiplexes with the help of Multiplex Manager 1.0 (Holleley and Geerts, 2009). A touchdown PCR method was used. As such, the PCR profile had an initial denaturation stage of 15 min at 95°C, followed by 35 cycles of 94°C for 30s, 63°C for 90s (reducing in temperature by 1°C every cycle to 49°C). This was followed by an elongation step of 30 minutes at 60°C and an indefinite hold at 4°C. Negative and positive controls were used during DNA extraction and PCR to ensure that contamination had not occurred. An ABI3730 Genetic Analyzer (Applied Biosystems) was used to visualise the microsatellites, with a LIZ500 size standard. GENEMAPPER 4.0 was used to assign microsatellite allele sizes. One microsatellite marker (*ms71*) did

not amplify sufficiently in any of my datasets and thus all results were produced using the remaining seven X-linked microsatellites.

2.3.5 Statistical Analysis - Wild Males

All males that contributed fewer than 10 offspring to the next generation were discarded from analyses. This cut off was chosen as the theoretical minimum needed for a chi-squared test is $N = 5$ (the expected number of males and females) in each 2×2 cell (Cochran, 1952). The association of each X-linked microsatellite locus with the offspring sex ratio of each male was examined. The sex ratio was defined as the proportion of males (the number of male offspring divided by the total number of offspring). Each microsatellite locus was tested for association with sex ratio bias in a generalized linear model (GLM), assuming a binomial error structure. This assesses the number of male offspring in each brood after controlling for differences in brood size. Microsatellite size was assessed as a nominal variable, split into groups of 10 base pairs. An additional analysis of microsatellites with significant associations was done, splitting the microsatellite allele sizes into two groups (above and below the mean) and comparing these to meiotic drive. This analysis ensured that approximately equal sample sizes were present in each group. Holm-Bonferroni corrections for multiple comparisons were performed (Holm, 1979). A direct test of the relationship between male eyespan and offspring sex ratio was performed, using the same GLM as above, testing the offspring sex ratio against thorax, absolute eyespan and relative eyespan.

2.3.6 Statistical Analysis – Adult Phenotypes

I examined the relationship between X-linked loci and a number of phenotypic traits. The relationship between trait size and allele size was calculated using a standard least squares GLM. The allele size metric for each microsatellite locus was calculated using the proportion of alleles that each individual possessed that were greater than the population mean. As each female only had a maximum of two alleles for each locus, the assigned values were 0, 0.5 or 1. Male genotype was coded as 0 or 1 depending on whether their single allele was greater than the population mean. This was compared to a number of traits: thorax (a proxy for body size), absolute eyespan, relative eyespan, testis size, accessory gland size and fecundity. Relative eyespan was calculated by including thorax in the model as a covariate to control for body size. Analyses were conducted separately on males and females. As different sites will generally have different allele size frequencies, I used 'site' as a covariate (random effect) to ensure the results reflected true associations with sex ratio bias, and were not an artefact of the general site properties. Holm-Bonferroni was applied (Holm, 1979), with each locus having 5 (4) tests for males (females).

The wild male dataset suggested that *ms395* has a reliable association with sex ratio bias. I analysed whether *ms395* associated with different populations as well as population density. In order to calculate a single genotypic value for *ms395* for each individual, I categorised individuals as either having an allele size greater than 218bp or not. This fitted with results showing that this locus had a bimodal distribution larger and smaller than 218bp. To examine associations of allele size with different populations I compared the allele size metric to 'site' (different populations) using a likelihood ratio test. I also compared absolute allele sizes to population density using a

GLM with population sample size included as a covariate in order to remove effects related to sampling.

All statistical analysis was performed using JMP Version. 10.0.0 (SAS Institute, Cary, NC, USA).

2.4 RESULTS

2.4.1 Wild Males

Amongst the sample of flies taken in 2009, 22.6% produced significantly sex ratio biased broods (7/31). A similar pattern of 25.2% sex ratio distortion was found in 2011 (26/103). Overall, most of the families with significant sex ratio distortion were female-biased (25/134) but a few of which were male-biased (8/134) (Figure 2.2).

Locus *ms395* showed a significant relationship with sex ratio bias ($\chi_1^2 = 44.7948$, $N = 29$, $P < 0.0001$), with large *ms395* alleles being associated with more female-biased broods (Figure 2.3). Locus *ms54* also showed an association with sex ratio bias ($\chi_1^2 = 7.5802$, $N = 25$, $P = 0.0226$), again with large allele sizes being associated with more female-biased broods. None of the other loci showed a significant association with sex ratio bias (*mscrc2* $\chi_1^2 = 3.3655$, $N = 30$, $P = 0.7618$; *ms106* $\chi_1^2 = 0.7672$, $N = 15$, $P = 0.3811$; *ms244* $\chi_1^2 = 2.4851$, $N = 28$, $P = 0.4780$; *ms125* $\chi_1^2 = 3.5526$, $N = 29$, $P = 0.4699$; *ms167* $\chi_1^2 = 0.2596$, $N = 23$, $P = 0.6104$. After applying the Holm-Bonferroni correction, *ms395* remained significant whilst *ms54* was rendered non-significant.

When *ms395* allele sizes were split into two groups (above and below the mean (205bp)) and compared to sex ratio bias, I also found a significant association (χ_1^2 = 23.3450, N = 29, P < 0.0001).

I found no relationship between sex ratio bias and body size (χ_1^2 = 1.3686, N = 130, P = 0.2421) or absolute eyespan (χ_1^2 = 1.2790, N = 130, P = 0.2581). I did, however, find a significant relationship between sex ratio bias and relative (male) eyespan (controlling for body size) with small relative eyespan males producing more female-biased broods (χ_1^2 = 6.9516, N = 130, P = 0.0084).

2.4.2 Adult Phenotypes and Allele Size

There was no relationship between body size and *ms395* allele size in either males ($F_{2,161.5}$ = 0.6089, P = 0.5452) or females ($F_{2,184.7}$ = 1.4770, P = 0.2310). Nor was there any relationship between absolute eyespan and *ms395* allele size in either sex (males: $F_{2,188}$ = 2.0549, P = 0.1310; females: $F_{2,179.7}$ = 1.9029, P = 0.1521). However, there was a significant negative association between male relative eyespan (after controlling for body size) and *ms395* allele size ($F_{2,182.8}$ = 4.6991, P = 0.0102), such that smaller eyespan males had larger *ms395* alleles. There was no equivalent relationship in females ($F_{2,183.1}$ = 1.0540, P = 0.3506). I also looked for associations between reproductive traits and *ms395,* but found none in males with testis size ($F_{2,169.3}$ = 1.0774, P = 0.3428) or accessory gland size ($F_{2,168.9}$ = 0.4284, P = 0.6523), and none in females with fecundity ($F_{2,187.8}$ = 0.0147, P = 0.9854). Holm-Bonferroni corrections did not alter the significance of the relationship between relative eyespan and *ms395* (P < 0.05).

The other six X-linked loci were also examined for associations with the phenotypic traits (Table 2.1). Several loci were again associated with male, but not female, relative eyespan (*ms54, ms244, mscrc2*) and there was an association with accessory gland size (*ms54*) and testis size (*mscrc2*).

From the previous dataset I identified *ms395* as the only locus to show a reliable association with sex ratio bias. In order to investigate this further I examined the frequency of *ms395* in different populations. I found a significant difference between sites in allele size at locus *ms395* (χ^2_4 = 36.7211, N = 390, $P < 0.0001$). When this is viewed graphically (Figure 2.4) it is clear that 6 of the 12 sites contain large *ms395* alleles (>218bp) that are associated with meiotic drive. In addition these sites represent geographically distinct populations along the valley (Figure 2.1). When I compared the population density of each site (flies per metre of sampled site) to the *ms395* alleles found in that site, controlling for sample size, I found a significant positive relationship, such that sites with large populations were associated with a high frequency of large *ms395* alleles ($F_{1,582}$ = 13.2839, P = 0.0003).

2.5 DISCUSSION

I investigated meiotic drive in wild populations of the stalk-eyed fly, *T. dalmanni*. First I examined the relationship between meiotic drive, measured as sex ratio distortion of progeny, and a number of X-linked microsatellite loci (Wright *et al.*, 2004; Johns *et al.*, 2005). Locus *ms395* showed a strong relationship with levels of meiotic drive. Large *ms395* alleles (>218bp) were associated with female-biased broods. In addition,

previous work in a laboratory population of *T. dalmanni* found that large *ms395* alleles were linked with meiotic drive (Johns *et al.*, 2005). It would be interesting to establish whether the specific association of 'large' alleles of *ms395* and sex ratio distortion is due to some process that favours the accumulation of repeats in regions associated with meiotic drive. The same pattern is seen for the *Rsp* locus of SD in *Drosophila melanogaster*, which has high repeat numbers in sensitive alleles (Larracuente and Presgraves, 2012). Typically, meiotic drive systems are found in areas of low recombination (Jaenike, 2001), but how this might predispose repeats to increases in number is unclear (Dion and Wilson, 2009). Locus *ms54* also showed an association with meiotic drive and this locus was also shown to be associated with meiotic drive in previous laboratory studies (Johns *et al.*, 2005). The relationship did not survive however, after the Holm-Bonferroni correction was applied.

The laboratory study of *T. dalmanni* found that two other loci (*ms125* and *ms244*) were predictors of meiotic drive (Johns *et al.*, 2005). However, I found no association with *ms125* or *ms244*. The laboratory and wild populations were both collected from the same river catchment in Malaysia. However, differences could have built up in the laboratory population over time since collection, especially as samples of the laboratory population were subjected to artificial selection (for relative male eyespan) and hence to random genetic drift. It is possible that low frequency microsatellite alleles that happened to be in linkage with the meiotic drive locus in the samples used for artificial selection spread to fixation by chance, and thus were identified as co-varying with meiotic drive. The wild populations used in this study were collected in 2008/9, whereas those that founded the laboratory population were collected in 1989 (Johns *et al.*, 2005). It is possible that the difference in my results in due to a rapid turnover of the drive complex in natural populations which is supported by recent

work that has provided evidence for the rapid evolution of the sex ratio complex, over only a few decades, in *Drosophila simulans* (Bastide *et al.*, 2011).

Previous theoretical (Lande and Wilkinson, 1999) and experimental laboratory work (Wilkinson *et al.*, 1998; Johns *et al.*, 2005) has examined the hypothesis that male eyespan is linked to the presence or absence of the X^D chromosome. I complemented this work by examining wild-caught stalk-eyed flies in two independent datasets. I found that male eyespan correlated with meiotic drive directly in my wild male dataset. In addition I also found that male eyespan was correlated with microsatellite *ms395* size. Males with large allele sizes not only had female-biased sex ratios but also small relative eyespan, in line with the direction of results from my wild male dataset. In contrast, there was no association of *ms395* allele size with the control trait, female eyespan, suggesting that linkage has specifically evolved between male eyespan and drive, which is consistent with previous findings from QTL mapping (Johns *et al.*, 2005). The second microsatellite to correlate (prior to Holm-Bonferroni corrections) with meiotic drive (*ms54*) also associated with male eyespan. These results support the hypothesis that meiotic drive could be a factor in the evolution and maintenance of female mate choice for male eyespan size (Wilkinson *et al.*, 1998). Theory suggests that in order to maintain the linkage between male eyespan and meiotic drive, genes for both traits need to be contained within the same inversion (Lande and Wilkinson, 1999; Pomiankowski and Hurst, 1999). Two other X-linked microsatellites were associated with male eyespan but not with meiotic drive (*ms244* and *mscrc2*). This indicates that male eyespan is likely to be controlled by a number of different genes, an observation in line with previous work examining QTLs for eyespan in this species (Wolfenbarger and Wilkinson, 2001; Johns *et al.*, 2005). The most complete linkage map also places these microsatellites in close proximity (all within 20cM) on the X

chromosome, and thus it is possible that they are in linkage disequilibrium (Baker and Wilkinson, 2010), hence the close relationship with male eyespan.

I did not find any associations of *ms395* with male reproductive traits (accessory gland size and testis size), although I did find an association between *ms54* and accessory gland size. Accessory gland size is related to male mating rate (Baker *et al.*, 2003; Rogers *et al.*, 2005a) and thus my results indicate that meiotic drive males may be constrained in their mating rate. This is in agreement with work by Wilkinson *et al.* (2006), who found that drive males produced fewer offspring than standard males and exhibited lower sperm precedence suggesting that there are costs of drive in terms of sperm number or competitive ability (Wilkinson *et al.*, 2006). They found no difference in the number of drive and standard males that produced offspring when mated multiply over a 24-hour period however, suggesting that the relationship between accessory gland size and meiotic drive may not be straightforward. I did not find any association of *ms395* (or any other locus) with female fecundity. In all of the analyses relating to associations between genotype and phenotypic traits, I controlled for general allelic variation between streams by adding stream as a covariate in every model. Due to the lack of detailed information on population structure in the valley, I cannot eliminate a potential role that population structure alone may have had on creating associations.

Prior to this study, there was little evidence for the existence and pattern of meiotic drive in the wild. My results indicate that there is substantial variation in meiotic drive both within and between local wild populations. I found that half of the sites that were sampled from a single river valley did not exhibit alleles associated with meiotic drive (i.e. contained no large *ms395* alleles >218bp; Figure 2.4), while there were varying

degrees of association with alleles associated with meiotic drive in the other sites. Migration between different sites is limited (Sam Cotton, unpublished data), suggesting that stochastic variation may build up at each locality. Differences in population density may explain the observed variation in meiotic drive. I found a significant correlation between density and the level of meiotic drive alleles observed. This was true even after controlling for sample size. There was no meiotic drive alleles in small populations, with levels of drive alleles increasing as population density increased. One possible explanation for this relationship is that if meiotic drive invades a small population, then that population would quickly become strongly female-biased and have a higher chance of going extinct (Hamilton, 1967; Jaenike, 2001). Selection is a weaker force in small populations (Crow and Kimura, 1970), so they are less likely to retain or evolve suppressors and thus less able to counter the spread of sex ratio distorting meiotic drive.

Wilkinson *et al.* (1998) proposed that female mate choice for large male eyespan might have evolved in the stalk-eyed fly as a form of 'good genes' sexual selection. This hypothesis was conceived following the finding in a laboratory experiment that the male sexual character (exaggerated eyespan) in stalk-eyed flies was associated with meiotic drive. This finding has not spurred further examination of the hypothesis, perhaps because the association between meiotic drive and eyespan could easily have arisen by chance, due to the laboratory-breeding regime used. Here, I examined variation in meiotic drive, microsatellite markers and the associated sexual trait in wild populations of stalk-eyed flies. I found that two of the four microsatellite loci previously identified in the laboratory study were associated with meiotic drive, one (*ms395*) very strongly. I further confirmed, using two independent datasets, that there is a strong correlation between male eyespan and the microsatellite locus linked to

drive. My results constitute the first evidence from wild populations that the evolution of female mate choice for male eyespan is plausibly linked to a 'good genes' hypothesis of avoiding prospective mates that harbour an X-linked meiotic drive chromosome.

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Table 2.1. Table showing the relationship between X-linked microsatellite loci and phenotypic traits. *P* values in bold remained significant after Holm-Bonferroni correction ($P < 0.05$), those underlined were not significant after this correction ($P > 0.05$).

		Males						
Trait	***Statistics***	***Locus ms54***	***Locus ms106***	***Locus ms125***	***Locus ms167***	***Locus ms244***	***Locus ms395***	***Locus mscrc2***
Body Size	***F***	3.4844	0.9897	1.0822	0.0984	0.1667	0.6089	2.2662
	DF	1,201	1,73.99	1,210.1	2,61.7	2,33	2,161.50	1,211
	P	0.0634	0.3231	0.2994	0.9064	0.8472	0.5452	0.1337
Absolute Eyespan	***F***	0.0349	0.0556	0.242	0.1612	1.3315	2.0549	0.0753
	DF	1,83.9	1,15.15	1,201.70	2,58.19	2,32	2,188	1,168.20
	P	0.8522	0.8167	0.6233	0.8515	0.2783	0.131	0.7841
Relative Eyespan	***F***	14.9213	3.7209	0.4469	0.0879	6.7569	4.6991	9.1645
	DF	1,195	1,81	1,206.80	2,50.59	2,28.64	2,182.80	1,204
	P	**0.0002**	0.0572	0.5046	0.916	**0.0039**	**0.0102**	**0.0028**
Testis Size	***F***	3.5872	0.0947	0.0201	2.6549	1.5034	1.0774	10.0399
	DF	1,139.70	1,69.18	1,195.40	2,46.84	2,29.22	2,169.30	1,197.50
	P	0.0603	0.7592	0.8874	0.0809	0.2391	0.3428	**0.0018**
Accessory Gland Size	***F***	8.3223	0.5297	0.1169	0.0956	1.0959	0.4284	3.1427
	DF	1,84.72	1,25.26	1,193.30	1,55.16	2,24.39	2,168.90	1,187.80
	P	**0.005**	0.4734	0.8874	0.7584	0.3502	0.6523	0.0779

		Females						
Trait	***Statistics***	***Locus ms54***	***Locus ms106***	***Locus ms125***	***Locus ms167***	***Locus ms244***	***Locus ms395***	***Locus mscrc2***
Body Size	***F***	1.7550	0.6653	0.4045	0.2357	4.3378	1.0581	1.0876
	DF	2,147.9	1,54.22	2,199	2,78.34	2,38	2,189	2,175.10
	P	0.1765	0.4183	0.6679	0.7906	0.0201	0.3492	0.3393
Absolute Eyespan	***F***	3.0286	0.8992	0.8611	0.5474	2.0294	1.0782	3.1723
	DF	2,184.10	1,19.55	2,194.90	2,81	2,37.75	2,188	2,192.40
	P	0.0508	0.3546	0.4243	0.5806	0.1455	0.3423	0.0441
Relative Eyespan	***F***	1.9975	6.5939	1.7193	1.3935	0.1829	0.966	2.525
	DF	2,146.60	1,33.55	2,196.70	2,77.11	2,33.66	2,187	2,158
	P	0.1393	**0.0149**	0.1819	0.2544	0.8336	0.3	0.0833
Fecundity	***F***	0.1518	0.3299	1.1876	1.1244	1.0324	0.0147	0.348
	DF	2,164.20	1,52.81	2,201.70	2,82.53	2,38.3	2,187.80	2,184.30
	P	0.8593	0.5682	0.3071	0.3298	0.3658	0.9854	0.768

Figure 2.1. Map showing the 12 sites used for collections and the University of Malaya Field Studies Centre. All sites represent distinct populations that lie along or near to the small Gombak road, Jalan Gombak, which runs through mountainous rainforest. To the upper left is a major motorway in the valley. In addition to the rainforest, the map also shows the local quarry (bottom centre left). A compass is shown for orientation and the bar on the bottom left indicates a scale of 1000m. Google Earth Image © 2013 DigitalGlobe © 2013 MapIt.

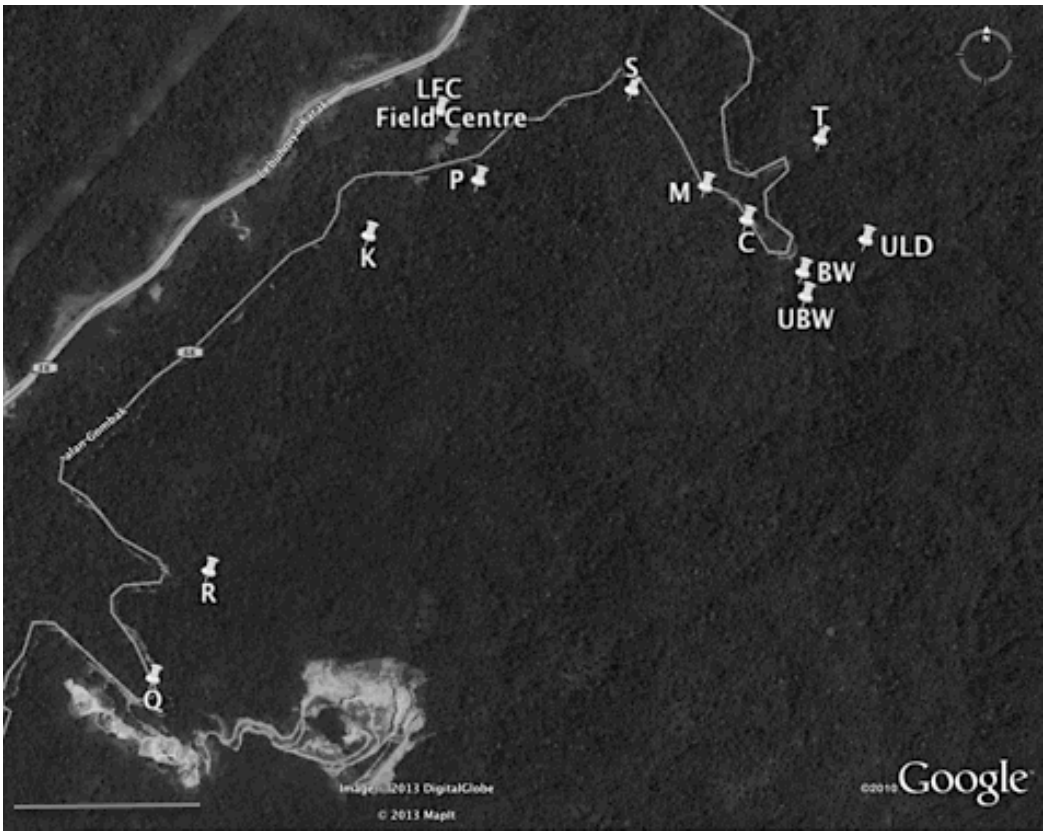


Figure 2.2. Frequency distribution of the proportion of female offspring in each brood for flies collected in 2009 and 2011 ($N = 134$). Dark grey bars indicate sex ratios that differ significantly from 1:1.

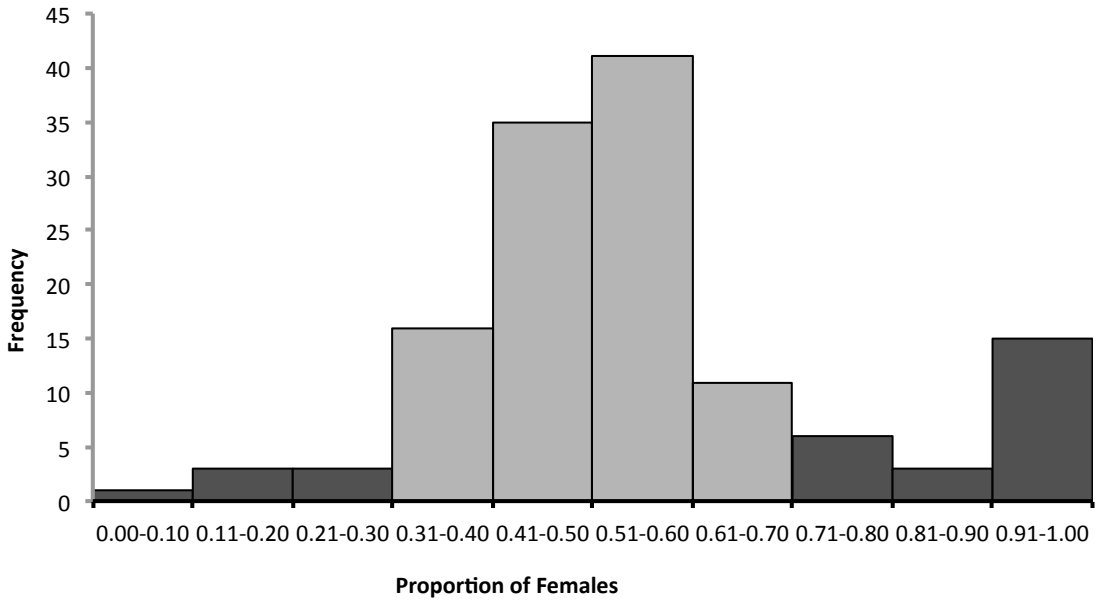


Figure 2.3. Association between sex ratio, given by the proportion of females in the brood, and *ms395* allele size given in 10 bp groupings. The line joins adjacent mean values. A significant relationship was found, with larger *ms395* alleles associated with more female-biased broods.

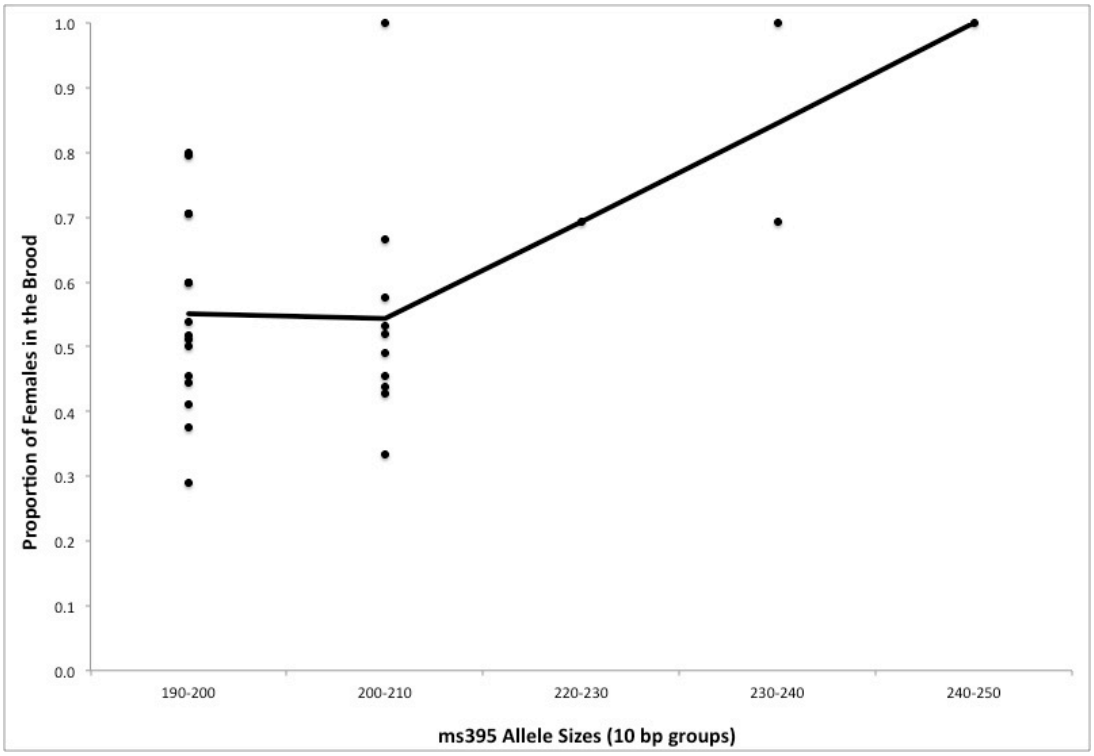
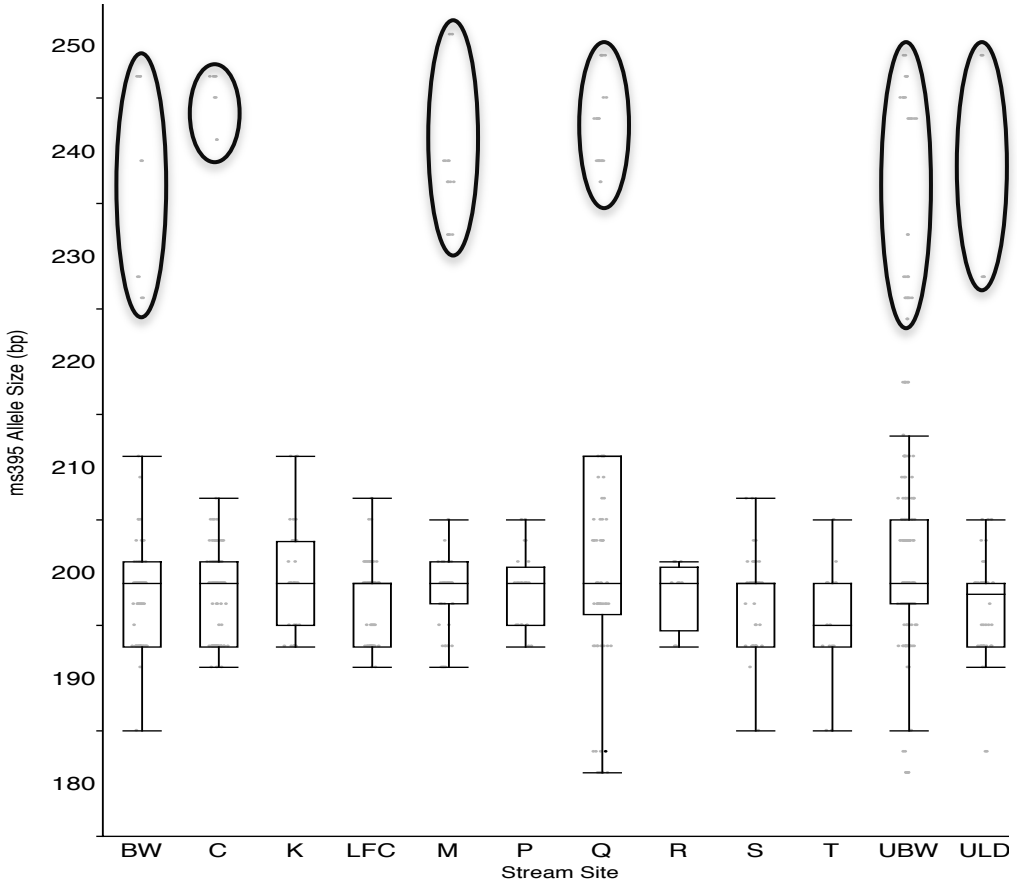


Figure 2.4. Box plot graph depicting the interquartile range of *ms395* allele sizes found at 12 sites along the Gombak valley (see Figure 2.1 for locations). Six of the 12 sites (circled) show the presence of large *ms395* alleles (>218bp), whilst the other six sites show a complete absence of large alleles.



3

Meiotic drive and the condition-dependent expression of a sexual ornament in stalk-eyed flies

3.1 ABSTRACT

A major prediction of the handicap hypothesis is that sexual traits exhibit heightened condition dependence and that females use this honest signalling system to gain genetic benefits from mating with the most well ornamented males. One intriguing example of this is in stalk-eyed flies, where females gain a genetic benefit by mating with males with the largest eyespan as they are less likely to carry the detrimental meiotic drive X chromosome and produce female-biased offspring. The meiotic drive loci are contained within a large inversion and, given the lack of recombination in heterozygotes, this inversion would be expected to accumulate mildly deleterious mutations. The poor genetic quality of this drive chromosome is predicted to be reflected by the condition-dependent expression of eyespan, with drive males having a more sensitive condition-dependent response to stress. In this study, I reared standard and drive males under three different environmental conditions (low, medium and high food quality) and examined the resultant eyespan expression profile. Males were then mated to laboratory females, and meiotic drive males were identified as producing a significantly biased offspring sex ratio. Paradoxically, I found that there was no overall difference in condition dependence between standard and meiotic drive males, although meiotic drive males did have smaller eyespans across the treatments and males producing strong sex ratio biases did have significantly changed coefficients of eyespan variation across treatments. Although I found some evidence to support changes in condition dependence between drive and standard males, overall the results are contrary to predictions and I discuss possible reasons why drive males may not have a stronger condition-dependent response to stress.

3.2 INTRODUCTION

The handicap hypothesis is a key theory used to explain the evolution of exaggerated secondary sexual traits. It posits that the size of the sexual ornament reflects the phenotypic (Bonduriansky and Rowe, 2005) and/or genetic (Hunt *et al.*, 2004) quality of its carrier, with larger ornaments reflecting higher quality. The handicap process works when differential costs maintain honesty in signalling as the cost of producing large ornaments is disproportionately higher for low quality individuals (Zahavi, 1975; Andersson, 1986; Pomiankowski, 1987, 1988; Grafen, 1990; Iwasa *et al.*, 1991; Iwasa and Pomiankowski, 1994, 1999). Some sexual ornaments exhibit heightened condition-dependent expression (Zahavi, 1975; Iwasa and Pomiankowski, 1994; Cotton *et al.*, 2004a), a key prerequisite for the handicap hypothesis as it ensures that females can infer male quality accurately and reliably from the size of the ornamental trait (Cotton *et al.*, 2004a; Bradbury and Vehrencamp, 2011). If sexual signals are not honest, females gain no benefit from using them to discriminate amongst males and the system breaks down (Maynard Smith and Harper, 2003). Although many early studies of condition dependence failed to adequately control for correlations with body size or show *heightened* condition-dependent expression compared to non-sexual traits (Cotton *et al.*, 2004a), there is now good evidence for condition dependence in a wide array of species with exaggerated sexual traits (Cotton *et al.*, 2004b; Bonduriansky and Rowe, 2005; Johns *et al.*, 2014).

The extent of condition-dependent trait expression is expected to alter as a function of environmental context (Candolin, 2000; Cothran and Jeyasingh, 2010). When environmental quality is high, the cost of ornamentation is reduced and thus we would expect most individuals to be able to invest ample resources in ornament displays,

irrespective of their underlying quality, leading to a weakening of the condition-trait size relationship (David *et al.*, 2000; Cotton *et al.*, 2004a; Cothran and Jeyasingh, 2010). Under stressful conditions, the viability cost of an extravagant, exaggerated trait would be expected to be much higher, restricting extreme ornamentation only to males of the highest quality. Thus we would expect stronger condition-dependent trait expression under stressful conditions (Candolin, 2000; Vergara *et al.*, 2012). The manner of these changes has been explored in a number of systems such as stalk-eyed flies (David *et al.*, 2000; Cotton *et al.*, 2004b), crustaceans (Cothran and Jeyasingh, 2010) and red grouse (Vergara *et al.*, 2012). However, there are comparatively few studies examining the genetic basis of condition-dependent trait expression, or gene × environmental interactions (GEI) in relation to condition dependence (Tomkins *et al.*, 2004). Most studies have utilised a quantitative genetic approach, where genetic differences (usually QTL) are inferred using pedigrees, familial breeding experiments or inbred lines (Arnqvist and Thornhill, 1998; Qvarnstrom, 1999; David *et al.*, 2000; Simmons and Kotiaho, 2002; Kemp and Rutowski, 2007; Schielzeth *et al.*, 2012).

The handicap hypothesis postulates that females gain genetic benefits from mating with the most well ornamented males (Zahavi, 1975). One intriguing example of this was examined in chapter 2, whereby females gained a genetic benefit from mating with highly ornamented males, as they were less likely to carry an X-linked meiotic drive chromosome that causes biased offspring sex ratios (Cotton *et al.*, 2014). Meiotic drive is a form of selfish genetic element that causes significant deviations from Mendelian segregation ratios due to differential sperm maturation, survival or fertilization success (Lyttle, 1993). Sex chromosome meiotic drive is associated with deviations of the 1:1 offspring sex ratio predicted by the adaptive sex ratio theory (Fisher, 1930). When linked to the X chromosome it is usually expressed in the

heterogametic sex (Hurst and Pomiankowski, 1991; Lyttle, 1993), and results in female-biased offspring sex ratios (Hamilton, 1967). To date, there are only a small number of meiotic drive systems that have been studied in great detail, primarily the *t*-complex in mice (Silver, 1993), the *sex-ratio* system in *D. simulans* (Cazemajor *et al.*, 2000) and the segregation distortion (*Sd*) system in *D. melanogaster* (Kusano *et al.*, 2003). Research has found that all meiotic drive systems so far require at least two distinct linked loci, a drive locus and its target or responder locus (Lyttle, 1993; Larracuente and Presgraves, 2012). These loci are typically located within inversions, which, when heterozygous, severely limit recombination and allow the drive and responder loci to remain in tight linkage (Wu and Beckenbach, 1983; Kirkpatrick, 2010). Recombination is also responsible for restricting the spread of mildly deleterious mutations (Felsenstein, 1974), and thus without recombination such mutations can build up within inversions, as has been seen in the non-recombining Y chromosome (Charlesworth and Charlesworth, 2000). Meiotic drive systems are also known to have undergone rapid evolution (Bastide *et al.*, 2011; Wilkinson *et al.*, 2014), due to the evolutionary arms race between the meiotic drive system and the evolution of meiotic drive suppressors.

A system that has attracted considerable attention is X-linked meiotic drive in the highly sexually dimorphic stalk-eyed fly, *Teleopsis dalmanni* (Presgraves *et al.*, 1997; Wilkinson *et al.*, 1998; Johns *et al.*, 2005; Wilkinson *et al.*, 2006; Cotton *et al.*, 2014). Stalk-eyed flies, like all members of the diopsid family, display a characteristic elongation of the head capsule into eyestalks, with displacement of the eyes to the end of these stalks (Wilkinson and Dodson, 1997). Eyespan, measured as the distance between the outer edges of the eyes, is sexually dimorphic in many stalk-eyed fly species (Wilkinson and Dodson, 1997). Male *T. dalmanni* have a significantly larger

eyespan, relative to the size of their body, than females (Burkhardt and de la Motte, 1985). Studies both in the laboratory (Wilkinson and Reillo, 1994) and in the field (Cotton *et al.*, 2010) have shown that eyespan has evolved through sexual selection in female choice (Wilkinson and Reillo, 1994; Cotton *et al.*, 2010) as well as male-male competition (Small *et al.*, 2009). *T. dalmanni* is an intensively studied Malaysian species of stalk-eyed fly. During the day, both sexes forage independently on decaying plant matter. At dusk males congregate on exposed root hairs under stream banks and compete for control of single root hairs (Small *et al.*, 2009). Females arrive shortly after and choose which root hair to land and roost on, and therefore which male to mate with for the night. This congregation of males and choosiness by females results in a 'lek' style mating system (Cotton *et al.*, 2010). Females prefer to roost and mate with males that have larger (absolute and relative) eyespan (Wilkinson and Reillo, 1994; Cotton *et al.*, 2010).

Male eyespan in this species exhibits heightened condition dependence compared to non-sexual traits (David *et al.*, 2000; Cotton *et al.*, 2004b, c). In addition, tight genetic linkage was found between male eyespan and meiotic drive, whereby small eyespan males had more female-biased broods and large eyespan males had 50:50 or male-biased broods (Wilkinson *et al.*, 1998). A further QTL analysis of those laboratory flies found that four microsatellite loci associated with meiotic drive in a haplotype (Johns *et al.*, 2005). The authors also discovered that a QTL explaining 36% of variation in the male sexual trait was located only 1.3cM from the meiotic drive locus, which appears to be located within a paracentric inversion with little or no recombination (Johns *et al.*, 2005). In chapter 2, I confirmed the association between meiotic drive and the male sexual trait (eyespan) using two independent datasets of wild stalk-eyed fly populations (Cotton *et al.*, 2014). The paracentric inversion is

inferred to involve a large portion of the X chromosome (Christianson *et al.*, 2011) and this is seen in the scale of genetic differentiation between the standard and the drive X chromosome (Reinhardt *et al.*, 2014). Reinhardt *et al.* (2014) found that a quarter of X-linked genes had at least one fixed difference between the drive and standard copies, In addition, ~500 X-linked transcripts showed differences in gene expression profiles in drive and standard male testes.

In stalk-eyed flies the inversion containing the meiotic drive loci is likely to have accumulated mildly deleterious mutations due to a lack of recombination. In chapter 2 I confirmed that eyespan is related to meiotic drive, with smaller eyespans being related to carriers of the meiotic drive X chromosome. This relationship could be a result of such mutations as deleterious mutations often reduce fitness, which is reflected by male eyespan size. The handicap hypothesis predicts that the poor genetic quality of the meiotic drive carrying males should be highlighted in the expression of eyespan, with drive males having a stronger condition-dependent response to stress. In order to test this prediction, I reared males under three different environmental conditions (low, medium and high stress) and examined the resultant eyespan expression profile. Males were then mated to laboratory females, and meiotic drive males were identified as producing a significantly biased offspring sex ratio.

3.3 MATERIALS AND METHODS

3.3.1 Source of Experimental Flies

Stock population flies

A large sample of *T. dalmanni* was collected in 2005 from the Ulu Gombak valley, Peninsular Malaysia (3º19'N 101º45'E) by Sam Cotton and Andrew Pomiankowski. These flies have been maintained in cage culture at high density (>200 individuals) with an approximately 1:1 sex ratio to minimize inbreeding. This population does not harbour the X-linked meiotic drive system. Flies were fed pureed sweetcorn twice weekly and were kept at 25ºC, with a 12:12 h dark: light cycle.

Meiotic drive flies

Male flies were collected in 2012 from the same location in Malaysia as the stock population. In order to establish and maintain a stock with meiotic drive, a standard protocol has been followed (Presgraves *et al.*, 1997). Males are housed and allowed to mate with three stock females. Their offspring are subsequently collected and sexed. Those males producing female-biased sex ratios (> 80% female, > 10 offspring) are used to establish the next generation. Assuming that these males have genotype X^DY, their F1 female offspring are carriers of the drive chromosome with genotype X^DX. F1 males from this cross are discarded, as they are not carriers of the drive chromosome. The F1 X^DX females are then maintained in high-density cages with stock males, and their offspring are collected and recorded. F2 male offspring are expected to be 50% drive and 50% standard, as they inherit their X chromosome from their mothers. The F2 males are mated to stock females and the process is repeated, whereas F2 female offspring are discarded.

3.3.2 Matings

Virgin females heterozygous for the meiotic drive X chromosome (X^DX) were collected from the meiotic drive population and placed into 1000ml pots with three laboratory males (XY) and allowed to mate freely. Half of the male offspring from these matings are expected to be X^DY drive, and the other half XY standard.

Eggs were collected twice a week for three weeks and reared on one of three stress treatments. The low quality food consisted of 25% corn to 75% sucrose. The medium quality food consisted of 50% corn and 50% sucrose and the high quality food consisted of 75% corn and 25% sucrose. To ensure that all the food had the same viscosity, an indigestible bulking agent, carboxymethycellulose (3% w/v), was added to the sucrose (25% w/v) solution (Rogers *et al.*, 2008). As the drive status of males was not known *a priori*, allocation to stress treatments was random and blind. Each petri dish had a standardised density of 13 eggs per plate. Once pupae had eclosed they were all fed *ad libitum* corn twice a week until sexual maturity.

In order to categorise the drive status of males, they were placed individually in 1000ml pots with three virgin stock females (standard, non-drive females). Flies were allowed to mate freely. The bases of the pots were lined with a moist cotton pad and blue paper to allow for easy egg visualisation. Eggs were collected by removing the moist cotton pad and blue paper twice a week for four weeks and kept in Petri dishes lined with a moist cotton pad. These eggs were reared to eclosion, whereupon the number of male and female offspring in the brood was counted. Only males that produced more than 10 offspring were used. This cut off was chosen as the theoretical minimum needed for a chi-squared test is $N = 5$ (the expected number of males and

females) in each 2 × 2 cell (Cochran, 1952). The focal male flies (N = 209) were anaesthetized on ice and stored in 100% ethanol. They were then measured for eyespan (the distance between the outermost lateral edges of the eye-bulbs) and thorax length (the distance from the base of the head to the posterior edge of the thorax) to an accuracy of 0.01mm, using Image J image software (v. 1.55). Thorax length was used as a proxy for body size.

In addition to males that produced usable offspring sex ratios, males that died before sexual maturity were collected from each stress treatment as well as males that survived to sexual maturity but did not produce a usable sex ratio. A sample of N = 20 per treatment for both of these groups were measured as above. This was done to check whether there was a bias in the eyespan of males that survived and reproduced.

3.3.4 Statistical Analysis

In order to statistically assign males as being either drive (X^DY) or standard (XY), a chi-square value (and associated significance) was generated for each male (brood) using the observed and expected numbers of males and females. In addition, the proportion of female offspring was used as a proxy for the drive chromosome.

The overall relationship was examined using a series of general linear models (GLMs). The first GLM examined how eyespan was affected by stress treatment, drive status (whether the male was a drive (X^DY) or standard (XY) male) and the interaction of the two variables. This was then repeated using the proportion of female offspring in place of drive status. In order to examine how relative eyespan (the non-allometric component of eyespan) was affected by drive status and stress treatment, an additional

variable, stress treatment nested within thorax (a proxy for body size), was added to both models. This was done in order to account for the different eyespan/thorax allometries seen in the three stress treatments.

The condition-dependent response of drive and standard males was then examined separately to see how eyespan changed across the three stress treatments. To do this, Tukey-Kramer HSD *post hoc* tests of multiple comparisons were used to examine between pairs of treatments. Changes in eyespan variance were tested using coefficients of variance (Zar, 1999). I examined whether there was a difference in variance between drive and standard males overall and then this relationship was examined in each of the three stress treatments separately. I then split the drive males into two groups; those with > 90% female offspring (strong drive males) and males that were significantly female-biased, but had > 90% female offspring (weak drive males). This is in line with previous work that has found both intermediate and strong sex ratio biased offspring (Presgraves *et al.*, 1997). I examined whether coefficients of variation changed across treatments in each of the three groups.

Eyespan was compared between those males that produced usable sex ratios (>10 offspring), those that survived to sexual maturity but did not produce useable sex ratios and those that died prior to sexual maturity. A matched pairs analysis was used to examine eyespan differences between each pair and this was examined in all of the stress treatment groups.

All statistical analysis was performed using JMP Version. 11.0.0 (SAS Institute, Cary, NC, USA).

3.4 RESULTS

Of the 209 male broods examined, 77 produced significantly biased brood sex ratios (all female-biased) and were subsequently classified as "drive" males. There was a strong negative relationship between the proportion of female offspring and the total number of offspring collected amongst those males with significantly biased brood sex ratios ($F_{1,74}$ = 12.8980, P = 0.0006; Figure 3.1). The distribution of sex ratios considerably overlapped between standard (range 0.30-0.73) and drive males (range 0.59-1.00) (Figure 3.2). So the proportion of female offspring was used as an alternative means to identify males carrying the meiotic drive chromosome. The number of drive males was distributed equally throughout the three stress treatment groups (χ^2_1 = 0.1540, N = 204, DF = 2, P = 0.9257). I found that 5 males had unusual eyespan to thorax relationships and exerted a large leverage on the results. These individuals were treated as outliers and initially excluded from the models, and then their effects noted.

Condition dependence was evident by a decrease in eyespan between treatment groups ($F_{2,197}$ = 17.5685, P < 0.0001) with high quality food resulting in the largest eyespan and low quality food in the smallest eyespan. There was a significant effect of drive status on eyespan ($F_{1,197}$ = 6.5491, P = 0.0112; Figure 3.3). Introducing a control for body size (adding thorax size as a covariate) revealed that relative eyespan was affected by treatment ($F_{2,194}$ = 6.0919, P = 0.0027) but was marginally non-significantly associated with drive status in the same negative direction ($F_{1,194}$ = 3.2546, P = 0.0728). In neither of these cases was there an interaction between treatment and drive status (absolute eyespan $F_{2,197}$ = 0.6240, P = 0.5368; relative eyespan $F_{2,194}$ = 0.1985, P = 0.8202).

When the analysis was repeated treating the sex ratio as a continuous variable, I found an association of female-biased broods with smaller male eyespan ($F_{1,197} = 10.4643$, $P = 0.0014$) and smaller relative eyespan ($F_{1,194} = 4.3283$, $P = 0.0388$), but there was no interaction between the two variables either for absolute eyespan ($F_{2,197} = 1.5628$, $P = 0.2121$ or relative eyespan ($F_{2,194} = 0.2910$, $P = 0.7478$). When the five outliers were put back into the analysis, there was some change in the exact values of the associations, but they were still all in the same direction.

Treating standard and drive males separately, there was a significant change in eyespan amongst both standard and drive males between low and high quality food (mean difference ± SE in standard males 0.53 ± 0.11, $P < 0.0001$; mean difference ± SE in drive males 0.48 ± 0.15, $P = 0.0044$) and between medium and high quality food (standard males 0.34 ± 0.11, $P = 0.0078$; drive males 0.48 ± 0.15, $P = 0.0046$). There was no difference in eyespan between low and medium quality food (standard males 0.20 ± 0.11, $P = 0.1747$; drive males 0.00 ± 0.14, $P = 0.9998$).

There was no significant difference in the coefficient of eyespan variation between drive and standard males ($\chi^2_1 = 0.1552$, $P = 0.9370$). This was reiterated when low, medium and high treatment groups were examined separately (low $\chi^2_1 = 1.4662$, $P = 0.1582$; medium $\chi^2_1 = 0.0006$, $P = 0.9997$; high $\chi^2_1 = 1.1607$, $P = 0.2072$). When the coefficients of variation were examined within strong drive males, weak drive males and standard males, I found that the coefficient of eyespan variation changed significantly across treatments in strong drive males ($\chi^2_1 = 4.9850$, $P = 0.0414$), with a significant increase in variation as stress increased, but not in weak drive males or

standard males (weak drive males $\chi^2_1 = 0.8669$, $P = 0.3241$; standard males $\chi^2_1 = 0.3223$, $P = 0.4256$; Figure 3.4).

In order to examine any potential bias in the differential survival of flies with differing eyespan I tested for pairwise differences in eyespan between flies that had died prior to sexual maturity (early death), flies that died without producing useable sex ratios (no broods) and flies included in the experiment (broods) in each of the three stress treatments. I found no difference between any of the pairs, in the low quality (early death-no broods, $t = -1.4593$, DF = 14, $P = 0.1666$; no broods-broods, $t = -0.3936$, DF = 14, $P = 0.6998$; early death-broods, $t = 0.9865$, DF = 19, $P = 0.3363$), or the medium (early death-no broods, $t = -0.3914$, DF = 8, $P = 0.7057$; no broods-broods, $t = -0.3810$, DF = 8, $P = 0.7131$; early death-broods, $t = 0.4008$, DF = 19, $P = 0.6931$) or high quality (early death-no broods, $t = -0.6528$, DF = 13, $P = 0.5252$; no broods-broods, $t = 0.4041$, DF = 13, $P = 0.6927$; early death-broods, $t = 0.8749$, DF = 19, $P = 0.3926$) food treatments.

3.5 DISCUSSION

A key prediction of the handicap hypothesis is that sexual traits exhibit heightened condition-dependent expression (compared to non-sexual traits) (Zahavi, 1975; Iwasa and Pomiankowski, 1994; Cotton *et al.*, 2004a). This condition dependence is postulated to be a mechanism for the evolution and maintenance of honest signalling in exaggerated sexual traits (Cotton *et al.*, 2004a; Bradbury and Vehrencamp, 2011). Under the handicap hypothesis, females gain benefits from mating with the most well ornamented males (Zahavi, 1975). I examined this in chapter 2 and found that females

gained a genetic benefit from mating with highly ornamented males in that they were less likely to carry an X-linked meiotic drive chromosome causing biased offspring sex ratios (Cotton *et al.*, 2014). Sex chromosome meiotic drive is associated with deviations of the 1:1 offspring sex ratio predicted by the adaptive sex ratio theory (Fisher, 1930). When linked to the X chromosome it is usually expressed in the heterogametic sex (Hurst and Pomiankowski, 1991; Lyttle, 1993), and results in female-biased offspring sex ratios (Hamilton, 1967). To date, all meiotic drive systems require at least two distinct linked loci, a drive locus and its target or responder locus (Lyttle, 1993; Larracuente and Presgraves, 2012). These loci are typically found within inversions and limit recombination, allowing the drive and responder loci to remain in tight linkage (Wu and Beckenbach, 1983; Kirkpatrick, 2010). Recombination is also responsible for restricting the spread of mildly deleterious mutations (Felsenstein, 1974), and thus without recombination, such mutations can build up within inversions, for example as has been seen in the non-recombining Y chromosome (Charlesworth and Charlesworth, 2000). In stalk-eyed flies the inversion containing the meiotic drive loci is likely to have accumulated mildly deleterious mutations due to a lack of recombination. The handicap hypothesis predicts that the poor genetic quality (i.e. high mutation load) of the males that possess the meiotic drive X chromosome should be highlighted in the expression of eyespan, with drive males having a stronger condition-dependent response to stress. I examined this prediction in male stalk-eyed flies. I confirmed that male eyespan exhibits condition dependence in stalk-eyed flies (David *et al.*, 1998; David *et al.*, 2000; Cotton *et al.*, 2004b). Overall I found that there was no significant difference overall in male eyespan expression between the two groups. Despite this lack of a difference in condition dependence, as previously discovered there was a significant difference in mean eyespan overall, with drive males having significantly smaller eyespans than

standard males (Wilkinson *et al.*, 1998; Johns *et al.*, 2005; Cotton *et al.*, 2014). I also found that extreme drive males (>90% female offspring) had a significantly higher eyespan variation in the high stress treatment.

Whilst some evidence points to the meiotic drive X chromosome causing an elevated condition-dependent response to stress (decrease in mean and increase in variance of eyespan), overall I found no difference in condition-dependent expression of eyespan. In relation to the experiment, the two most likely reasons for this are that the sample size for my groups were too small and/or the categorisation of males was not completely correct. Some previous work has noted that males can produce both strongly female-biased broods and also weakly female-biased broods (Presgraves *et al.*, 1997). In the current study, I found that this was true, with a small number of males producing significantly female-biased broods (60-90% female) and a peak of strong drive males producing 90-100% female-biased broods. My data showed that I should have increased the sample size of flies to allow for splitting them into three groups and gaining stronger results as I found that strong drive males had the increase in variance associated with stress. Relatedly, it is also possible that using sex ratio biased offspring is not the most conclusive measure of whether a male is carrying the meiotic drive X chromosome. This is because of the presence of meiotic drive suppressors that can mask the phenotypic effects of drive in whole populations (Wilkinson *et al.*, 2014). Future studies should endeavour to identify and utilise genetic markers that are in linkage with the meiotic drive chromosome.

It is also possible that there is, in fact, no difference in condition-dependent expression of eyespan between standard and drive males. This would suggest that deleterious mutations are not building up within the meiotic drive inversion, or that the mutations

have built up but they are not having a large effect. Recent work has shown that meiotic drive complexes appear to undergo very rapid evolution, with the evolution of drive suppressors causing the local extinction of meiotic drive in some populations (Rose *et al.*, 2014). This evidence is not restricted to stalk-eyed flies however, as the rapid evolution of the drive complex in other species such *Drosophila simulans* has also been shown (Bastide *et al.*, 2011). It is possible that this rapid evolution limits the accumulation of deleterious mutations within the inversion. In addition, males are hemizygous and thus the X^D chromosome in this sex is visible to selection and deleterious mutations can be selected against. This is unlike the situation in rare autosomal inversions where they are typically heterozygous with the standard chromosome, and so can accumulate deleterious recessives (Kirkpatrick, 2010).

A series of controls were used to ensure there was no systematic bias in the eyespan of the flies that survived and produced useable offspring sex ratios for this study. I did not find any difference in the eyespan of flies that died before sexual maturity as well as those that survived but did not produce offspring compared to my experimental flies. In the future there will hopefully be a reliable genetic marker for meiotic drive (SNP or microsatellite) and offspring sex ratios will not need to be used to identify drive males. This would have allowed me to use all males that eclosed, including those that died early, or which failed to produce large enough broods for sex ratio typing. In the immediate future it would be interesting to genotype these flies with the *ms*395 microsatellite marker. This marker produced a distinct haplotype in different laboratory populations of meiotic drive flies (Johns *et al.*, 2005), but (although correlated) it failed to do so with wild populations of flies in Chapter 2 (Cotton *et al.*, 2014). It would be interesting to see if this has altered after several generations of breeding in the laboratory.

This study investigated whether there was any effect of X-linked meiotic drive on condition-dependent sexual trait expression in stalk-eyed flies. Overall I found that condition dependence was not significantly altered by meiotic drive, although eyespan was significantly smaller in drive males, in line with previous work (Wilkinson *et al.*, 1998; Cotton *et al.*, 2014). There was also a significant increase in eyespan variation with stress in males that produced >90% female offspring. These results suggest that drive does have some effect on the condition dependence of eyespan expression, but that large sample sizes and a differentiation between extreme and weak drive males should be made in future studies.

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Figure 3.1. The relationship between the proportion of female offspring and the total number of offspring with lines indicating a line of best fit. Standard males are shown in blue and drive males are in red, with the shaded area representing the 95% confidence limit.

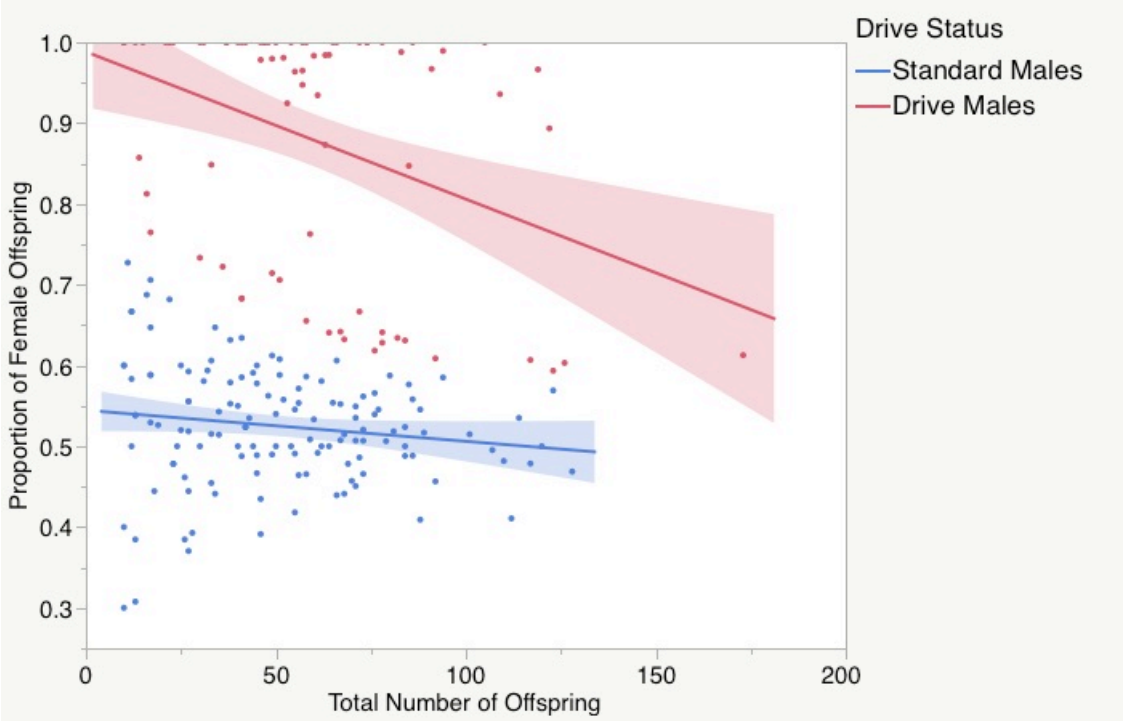


Figure 3.2. Histogram showing the proportion of female offspring produced. The dark grey indicates those that are significantly female-biased and the light grey indicates those that are classified as standard males.

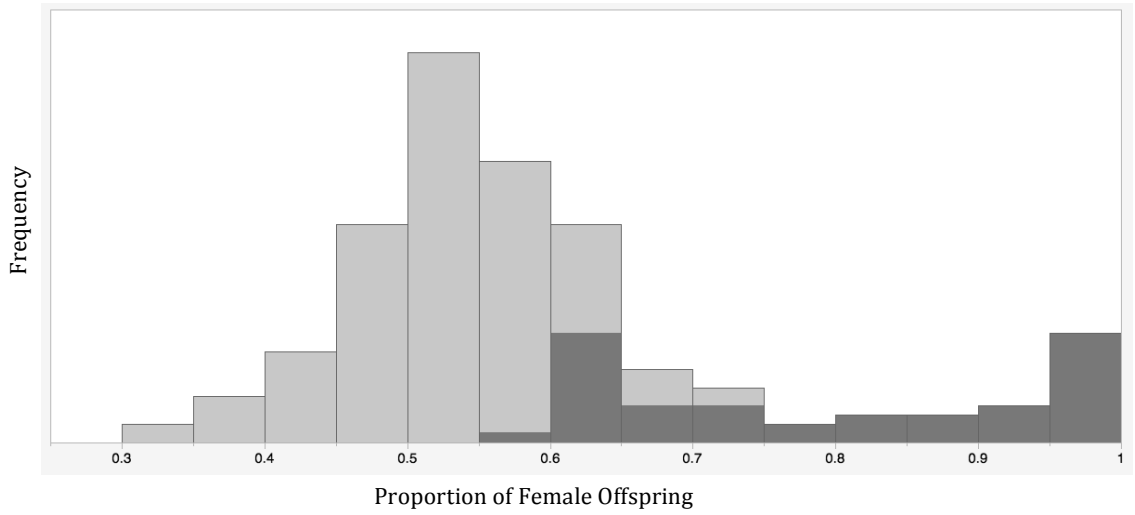


Figure 3.3. The relationship between male eyespan and stress treatment (low, medium and high quality food). Those with the meiotic drive X chromosome are represented by red and standard males are represented by blue.

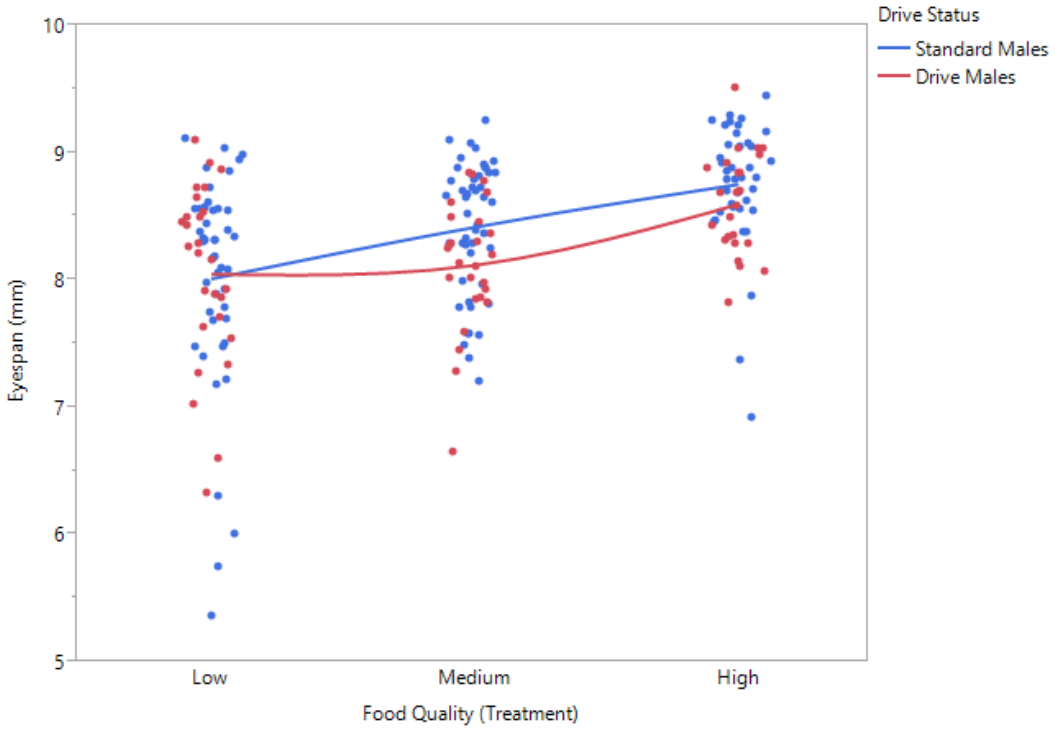
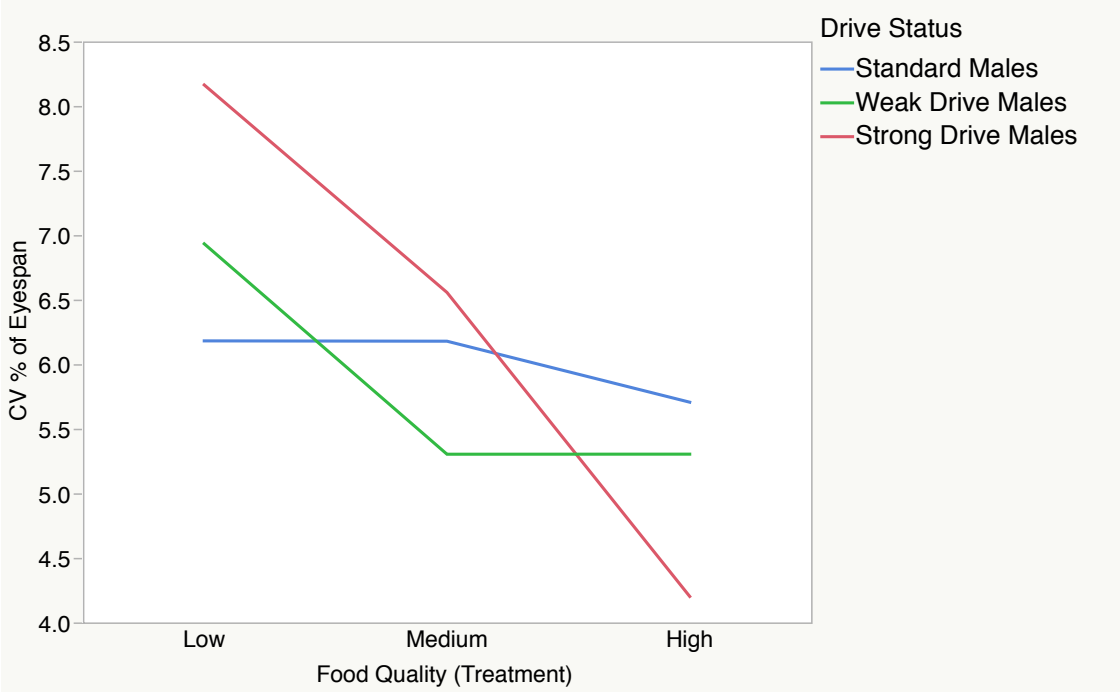


Figure 3.4. The coefficient of variation (%) of eyespan across three stress treatments (low, medium and high quality food) in strong drive males (> 90% female offspring; red), weak drive males (significantly female-biased, but > 90% female offspring; green) and standard males (blue).



4

Do ornaments reflect survival under stress? An experimental test of the handicap hypothesis

4.1 ABSTRACT

Understanding the evolution of exaggerated male secondary sexual traits is a key issue in the study of sexual selection. The handicap hypothesis is one of the primary theories used to explain why such traits evolve. It posits that exaggerated traits are a costly handicap and that only high quality males are able to bear the cost of exaggerated ornamentation. Whilst this has broad theoretical support, direct empirical evidence is limited. Here, using a combination of both field and laboratory experiments I evaluate support for the handicap hypothesis in stalk-eyed flies. In field populations, male ornament size (eyespan) predicted survival under heightened experimental stress, with well-ornamented males surviving longer. In contrast, ornament size was uninformative about survival in males under benign experimental stress. Female eyespan (the unexaggerated homologue of the male ornament) was uninformative under both stress treatments. The field study was complemented by a controlled laboratory study. These results, although somewhat ambiguous, partially mirrored those from the field. The laboratory results are discussed in the context of experimental design. The field results are not compatible with Fisher's runaway process and directly support the hypothesis that ornament size reflects male quality, with only the highest quality males able to bear the dual cost of ornamentation and stress. Future studies are needed to repeat and extend the laboratory experiment in order to provide a conclusive picture.

4.2 INTRODUCTION

Two principal models have been proposed to explain the evolution of costly male ornaments. Under Fisher's runaway process (Fisher, 1930; Lande, 1981) costly ornaments evolve in response to female preference, leading to the exaggeration of both ornament and preference as they become genetically associated. In contrast, the handicap hypothesis proposes that ornaments evolve to signal a male's underlying quality (Pomiankowski, 1987b; Iwasa and Pomiankowski, 1999). The dominant interpretation of this theory, the condition-dependent handicap hypothesis, assumes that males in good condition are better able to bear the fitness costs or reap the fitness benefits of possessing a large ornament (Zahavi, 1977; Kodric-Brown and Brown, 1984; Zeh and Zeh, 1988; Rowe and Houle, 1996; Getty, 1998; Iwasa and Pomiankowski, 1999; Cotton *et al.*, 2004a). This ensures that females are able to effectively evaluate ornaments to glean information about male quality, and offset any costs to themselves from mate choice by gaining direct and/or heritable fitness benefits for their offspring.

The handicap hypothesis has often been claimed to be supported by the presence of positive correlations between ornaments and survival (Jennions *et al.*, 2001). However, simple correlations between ornaments and survival are uninformative, as both positive and negative correlations can arise under the handicap hypothesis. With the standard assumption of male display being condition-dependent, high-quality males are typically expected to invest in ornamentation such that they maintain higher survival relative to lower-quality males (Zahavi, 1975; Iwasa *et al.*, 1991). In contrast, when traits are not condition-dependent, as assumed by Fisher's runaway process, negative correlations between ornament size and survival are expected (Lande, 1981;

Pomiankowski *et al.*, 1991; Kokko *et al.*, 2002). However, this simple dichotomy may not be sufficient to distinguish the handicap and runaway processes. Several authors have pointed out that if increasing ornamentation brings sufficiently high gains in mating success, the handicap hypothesis predicts that high-quality males may evolve increased ornament size to the point that those individuals suffer reduced survival (Höglund and Sheldon, 1998; Eshel *et al.*, 2000; Kokko *et al.*, 2002). This implies that a negative correlation between ornament size and survival could be consistent with either process but a positive correlation of ornament size and survival is predicted by the handicap hypothesis only. While interesting from a theoretical perspective, these authors do not suggest ways to distinguish between the different explanations of ornament evolution.

The true relationship between ornaments and viability may be blurred further by additional, uncontrolled, or unconsidered factors that affect the covariance between ornaments and survival. In particular, exaggerated ornament evolution often leads to developmentally correlated, compensatory changes in morphology and/or physiology. For instance, exaggerated ornaments are typically associated with greater body size, and the latter can also have a positive effect on survival (Blueweiss *et al.*, 1978; Byström *et al.*, 2006; Rossetto *et al.*, 2012). Elongated feathers reduce aerodynamic performance, and this has led to compensatory developmental changes in wing shape and body form (Evans and Thomas, 1992; Balmford *et al.*, 1993; Balmford *et al.*, 1994; Buchanan and Evans, 2000; Painting and Holwell, 2013). In rhinoceros beetles, horn size is males is correlated with increased wing size (McCullough *et al.*, 2012) and across long tailed bird species (after controlling for phylogenetic effects), sexually selected changes in male tail length and shape are correlated with wing dimorphism (Balmford *et al.*, 1994). These relationships mean that ornament size may be positively

correlated with other traits that have a significant or overriding impact upon survival. Hence a true negative effect of ornament size *per se* on survival can be obscured. These considerations point to the need to make experimental interventions, rather than to rely on estimating simple correlations, in order to reveal the underlying relationships.

The main experimental approach used has been the manipulation of ornament size to explore the consequences for survival (Grether, 1997; Saino *et al.*, 1997; Cuervo and de Ayala, 2014) or components of viability (Savalli, 1994; Pryke and Andersson, 2005; Cuervo and de Ayala, 2014). This primarily has been explored in birds such as the widowbird (Pryke and Andersson, 2005) and the swallow (Møller, 1989; Saino *et al.*, 1997; Cuervo and de Ayala, 2014). Tail length is often experimentally shortened and lengthened, with an experimental control (cutting tail feathers off and then gluing them on again to return to the original length) and a non-manipulated control (Andersson, 1982; Møller, 1989; Pryke and Andersson, 2005; Cuervo and de Ayala, 2014). The rationale of these studies is that ornaments should be at an optimum size given an individual's current condition and expectations about future life-history trade-offs. Manipulation of ornament size should then result in a corresponding change in viability as well as attraction. For example, Møller (1989) utilised the ornament manipulation approach, both with addition and reduction of tail length in barn swallows. He found that those individuals with extended tails had impaired foraging efficiency. This resulted in an increase in the number of fault bars and a reduction of tail size after moult, leading to a decrease in mating success the following year (Møller, 1989).

A potential problem with this design is that manipulations not only affect the trait of interest (ornament size), but also simultaneously alters individual quality and thereby the effect of the manipulation on viability. Experimental approaches that utilise ornament manipulation may therefore fail to accurately estimate the overall costs of ornamentation that arise from correlated developmental and life-history constraints (Balmford *et al.*, 1994; Emlen, 2001). For example, a sexually selected trait in beetles, horn size, was found to correlate with other morphological traits such as antennae, eye size and wing size (Emlen, 2001). Moreover, ornament manipulation can alter attractiveness, creating potentially conflicting responses to treatment on survival. Artificially decreasing ornament size could result in reduced mating opportunity and a concomitant reduction in costs of mating and hence an increase in survival prospects. Or the reverse might result, with an increase in risk-taking behaviour in order to secure matings among males that are made less attractive.

An extension of this approach is to apply an experimental stress unrelated to the ornament and consider whether an individual's natural ornament size influences subsequent survival under this manipulation. The handicap hypothesis assumes that high quality individuals suffer less from the costs of ornamentation than low quality individuals (Iwasa and Pomiankowski, 1994). It follows that experimental stress will have a lesser effect on the survival of high quality individuals compared to low quality. If natural ornament size is found to positively correlate with a male's ability to survive experimentally induced increases in stress, this would provide strong support for the handicap hypothesis. A positive correlation is not compatible with Fisher's theory. The runaway process assumes that ornaments are expressed independently of quality so that increases in stress will harm individuals in a way that is independent of ornament size. In this sense, Fisherian sexual traits should be as unresponsive to experimentally

induced stress as are non-sexually dimorphic traits, which can act as control traits in this experimental design (Cotton *et al.*, 2004a). To the best of my knowledge, this approach has not been utilised in either laboratory or field studies of ornament evolution. The handicap process can also predict that high quality males have large ornaments but survive less well than low quality males when the mating success payoff of increasing ornamentation is sufficiently accelerating (Höglund and Sheldon, 1998; Eshel *et al.*, 2000; Kokko *et al.*, 2002). In this case it seems unlikely that an individual's natural ornament size would have a positive effect on its ability to cope with the application of an additional stress.

Relationships between traits are highly sensitive to the environment in which they are measured (Rice, 1988; Ellegren and Sheldon, 2008), so it is crucial to examine sexual signalling of quality in the environment under which ornaments have evolved. Insects are one of the most diverse taxa with regards to sexual ornamentation, yet are the least studied under field conditions. Very little is known about the sexual signalling of quality in wild insects (Grether, 1996; Holzer *et al.*, 2003; Hunt *et al.*, 2004; Cotton *et al.*, 2010; Rodriguez-Munoz *et al.*, 2010), a situation that stands in stark contrast to the knowledge of wild vertebrate populations (Domb and Pagel, 2001; Gonzalez *et al.*, 2001; Peters *et al.*, 2004; Beamonte-Barrientos *et al.*, 2014; Kruuk *et al.*, 2014). It is obvious, however, that validity issues arise from pure field experiments, both logistical (e.g. the inability to control conflicting variables as well as difficulties in obtaining accurate measurements and large sample sizes) and biological (e.g. intervening variables that are unknown and cannot be disentangled). In order to provide a comprehensive examination of questions pertaining to the role of sexual signalling, complementary experiments from both the field and the laboratory (where variables can be minimised and controlled) are necessary (Taylor and Williams, 1982).

In this study I examine the utility of male ornaments as signals of quality under experimentally induced stress in both a wild and laboratory population of the Malaysian stalk-eyed fly, *Teleopsis dalmanni*. Stalk-eyed flies have eyes displaced on the end of stalks projecting laterally from the head in both sexes (Baker and Wilkinson, 2001). Eyespan (the distance between the eyes) is sexually dimorphic in *T. dalmanni*, with males displaying larger eyespans as a result of sexual selection (Wilkinson and Reillo, 1994; Small *et al.*, 2009; Cotton *et al.*, 2010). Male eyespan exhibits heightened condition cdependence relative to the non-sexual female homologue (Cotton *et al.*, 2004b), an essential characteristic of a handicap signal (Cotton *et al.*, 2004a). Extensive laboratory and field studies have found that females preferentially mate with the largest eyespan males (Burkhardt *et al.*, 1994; Wilkinson and Reillo, 1994; Cotton *et al.*, 2010). In natural populations, *T. dalmanni* individuals form nocturnal lekking aggregations on exposed root hairs that hang underneath the eroded banks of rainforest streams (Burkhardt and de la Motte, 1985; Wilkinson and Dodson, 1997; Cotton *et al.*, 2010). Males arrive first in the early dusk period and fight for control of these roosting sites (Wilkinson and Dodson, 1997; Small *et al.*, 2009). Females subsequently arrive and prefer to alight on root hairs controlled by males with large eyespan (Wilkinson and Reillo, 1994; Hingle *et al.*, 2001b, a; Cotton *et al.*, 2010). The vast majority of matings occur in these aggregations during the dusk and following dawn period, when males mate with females in their harem (Burkhardt and de la Motte, 1988; Lorch *et al.*, 1993; Small *et al.*, 2009; Cotton *et al.*, 2010).

The traditional approach to examining the handicap hypothesis involves ornament manipulation. However it isn't possible to manipulate eyespan directly without causing death. Also, there are known morphological correlates of eyespan, such as changes in

wing size, shape and beat frequency (Husak *et al.*, 2011a, b), which cannot be controlled for in the field. Therefore I investigated the relationship between ornaments and survival in *T. dalmanni* using two complimentary studies; a capture-mark-resight study of wild flies and a mark-survival experiment in laboratory flies. Flies were allocated randomly to one of two groups and a different identification tag/stress was used in each group. The first group had a small benign mark placed on the thorax that I hypothesised would have minimal effect on survival, while the second group had a paper tag glued to the thorax that I hypothesised would prove deleterious to survival. I examined correlations between ornament size and survival estimates in these two groups. The relationship between ornaments and survival in marked individuals likely approximates the native correlation seen in un-manipulated individuals. The pattern of correlations involving tagged males is indicative of whether ornament size reflects the ability to survive with an experimentally added burden that creates a deviation from an individual's ornament-survival optimum. Female eyespan was also examined as a non-sexual control, and I predicted that treatment stress would not affect the relationship between eyespan and survival. This is the first experimental investigation to compare a wild and laboratory insect population to establish whether the degree of ornamentation reflects a male's ability to survive when faced with an experimental challenge.

4.3 MATERIALS AND METHODS

4.3.1 Field Study

Capture-mark-resight experiments

Flies were collected after dusk from nocturnal lek aggregation sites located over a ~50m stretch of a tributary of the Gombak river, Peninsular Malaysia (3°19' N, 101°45' E) in September/October 2009. They were anaesthetized on ice and photographed against a known standard with a digital camera attached to a microscope. Eyespan, defined as the distance between the outer tips of the eyes, was determined subsequently from the images with the image analysis program Image J (Version 1.38; National Institute of Health, Bethesda, MD, USA).

Flies were assigned to one of two experimental treatments: marking or tagging. Marked flies had a small spot of coloured nail varnish applied to the dorsal thorax while anaesthetised. It was predicted that these small marks would have relatively little effect on the fly's subsequent behaviour and survival. Tagged flies had a small (~1 × 1mm) paper identity tag bearing 2 symbols (number/letter and vice versa provided ~500 combinations) glued to their dorsal thorax using a small spot of nail varnish. Thus the two experimental groups differed only by the presence or absence of the identity tag. The prediction was that tags would be more deleterious to survival, and hence tagging would constitute an appropriate experimental stress treatment.

Experimental flies were maintained overnight at low density in 500ml pots before being released back at their location of capture at dawn the following day. This ensured that released individuals survived the application of marks or tags by at least

10 hours, and thus that patterns in the resight data likely arose from natural causes rather than from the experimental procedure *per se*. There was very little mortality in this initial period (2-3 flies per treatment) and no difference in survival between the two treatments. Owing to the limited population size at the study site, it was not possible to run both treatments in parallel with sufficient sample sizes. The marking treatment was therefore performed first (N = 39 and 32 males and females, respectively), followed by the tagging treatment 13 days subsequently (N = 53 and 40 males and females, respectively).

After release, intensive searches were performed for marked and tagged individuals along the riverbank and in surrounding vegetation over the 50m stretch of stream twice daily (midday and late evening) for 20 days. More than 1.5 hours was spent looking for flies during each search period. Flies aggregate at lek sites at dusk and show high site fidelity over successive nights (Wilkinson *et al.*, 1998a). Flies do not roost away from riverbanks (*pers. obs*), so resights of experimental flies at night represent accurate censuses of flies at a location. Searches for experimental flies were also conducted during each search period ~100m up and downstream from the study site, both in the surrounding forest, and in adjacent tributaries (~200-500m away). Only a single experimental fly was found outside the sample site (in an adjacent tributary). As a measure to ensure the tags did not affect the attractiveness of the flies, harem size and mating frequency in similar eyespan males both with and without tags was observed. Tags did not appear to affect the attractiveness of the males, as there was no obvious difference (relative to marked or non-experimental males) in either harem size or in mating frequency (Sam Cotton *pers. obs*.).

If resighted individuals possessed a mark, then their eyespan was determined using an established photographic protocol (Small *et al.*, 2009). Eyespan was measured from free-ranging flies by taking standardized digital photographs (Canon EOS 450D) through a 100 mm macro lens set to its minimum focal distance. This creates a fixed distance between the camera and the subject. If the subject is kept perpendicular to the camera by keeping both eye bulbs in focus then eyespan can be deduced, using ImageJ, relative to a known standard, photographed under identical conditions. This method is highly accurate relative to controlled measurements of individuals made under laboratory conditions (Small *et al.*, 2009). If resighted individuals were tagged, then their eyespan was simply obtained by reference to the personal identity code on their tag.

Statistical analysis

The resight data represent repeated sampling of the initial released population of marked or tagged individuals. It was assumed that any observed decline in numbers of resighted individuals is a consequence of mortality. To examine the effect of treatment on mortality, the number of individuals resighted in each sampling period was expressed as a proportion of the number initially released. I used general linear models (GLMs) to evaluate whether the number of resights changed over time, and whether there were any treatment effects on the probability of resight. Data were Box-Cox transformed to normalise errors.

Any consistent change in measured eyespan values of sampled individuals over time would represent a directional shift in the size composition of the experimental population; an increase in the mean eyespan of resighted individuals over time would imply that larger eyespan individuals are more likely to survive and be resighted, and

vice versa. To investigate whether eyespan was associated with the probability of being resighted (i.e. survival) in either of the two treatment groups, I regressed the mean eyespan resighted during each sampling period against time after initial release. I then asked whether any effect of male eyespan on survival was different between treatments (i.e. whether there was a significant treatment × time interaction). Similar models were constructed for female eyespan, as a non-sexual control. To examine whether sexual traits (male eyespan) responded differently to treatment than the non-sexual trait (female eyespan) I tested the significance of the sex × treatment × time interaction in a model containing both male and female data and all lower order interactions and main effects.

By reference to the personal identity code on tagged flies I was also able to calculate individual longevity estimates, defined as the time from release until the last resight. This allowed me to directly test whether any change in mean resighted eyespan over time was due to changes in the probability of observing individuals with different eyespans over time. Data were right skewed, so I applied a Log(Longevity+1) transformation.

4.3.2 Laboratory Study

Mark-survival experiments

The flies used in this study were collected from Ulu Gombak, Peninsular Malaysia (3°19' N, 101°45' E) in 2005 and have since been maintained in laboratory cage culture (>200 individuals to minimize inbreeding) at 25°C on a 12:12 h light: dark cycle. To obtain experimental flies I collected eggs from the cage cultures over a 3-week period by placing Petri dishes with moist cotton wool and sweetcorn in cages and

allowing flies to lay eggs freely. These were removed from cages every 3 days and larvae were reared on variable amounts of pureed sweetcorn to create high variance in eyespan (David *et al.*, 1998; Cotton *et al.*, 2004b).

Upon eclosion, adult flies were housed at medium density in cages (N = 40-50 flies per cage) for the first 10 days on low quality food (20% corn: 80% sucrose) (Rogers *et al.*, 2008). This food type was continued throughout the experiment. When flies were between 10-14 days old, they were anaesthetised on ice and randomly assigned to one of the two stress treatments: heightened stress (tag, N = 300 males and N = 300 females) or benign stress (mark, N = 300 males and N = 300 females). The assigned tag or mark (procedure described above) was placed on the thorax just behind the thoracic spines. All flies that died within 24 hours of the manipulation were excluded from the study to minimise effects due the experimental procedure itself. Due to the large number of individuals used in the experiment, flies were split into two blocks (N = 600 each), with equal sex ratio and numbers in each treatment. The blocks were started one week apart. After the manipulation, flies were placed into 1000ml pots with moist bases at a density of 10 flies per pot. Flies that died during the experiment were replaced with a non-experimental fly of the same sex to maintain a constant density in all pots. The remaining flies were monitored daily for 27 days and fed twice weekly. The day of death was recorded, as was the stress treatment, sex, eyespan and thorax size (the distance from the base of the head to the posterior edge of the thorax) as a proxy for body size. Flies were stored in 100% ethanol upon death. All surviving flies were anaesthetised, had all the above variables measured and were stored in 100% ethanol.

Statistical analysis

Lab data were analysed using Cox's proportional hazards model (CoxPH) in order to examine how eyespan and stress treatment influenced survival (time to death). The dependent variable in my model was the time to death, measured in days since tagging/marking. I used a sequential model, allowing me to evaluate eyespan after thorax (a proxy for body size) had been controlled for as a covariate in the model. The predictive variables were thorax, (relative) eyespan, stress treatment (tagged or marked), as well as the interactions of eyespan x treatment. I examined the data using two methods.

The first method I used involved the traditional censoring of those flies that survived the 27-day experiment (coded 0 = died during the experiment, 1 = alive at the conclusion of the experiment). The model was constructed using the variables described above. In addition to this, I also constructed the same model just examining those flies that had died during the course of the experiment. Due to the uneven death rates in the two treatments (male likelihood-ratio $\chi_1^2 = 60.3094$, $P < 0.0001$; female likelihood-ratio $\chi_1^2 = 58.9837$, $P < 0.0001$) and higher than expected survival rates over 27 days, this statistical approach had low power to detect differences between the treatments.

The second analysis also utilised the CoxPH model with the same variables as described above. But the experiment was terminated when 50% of the flies in each treatment had died (the half-life of each treatment) in order to balance the sample sizes of censored flies across treatments. All flies surviving the 50% threshold were coded as censored (the first 50% of flies to die = 0, the remaining 50% = 1). Males ($N = 579$) and females ($N = 581$) were analysed separately. In addition to the full model, the

effect of relative eyespan on survival was examined separately in each of the treatments and sexes. This analysis allowed me to equalise the number of censored flies in each treatment and therefore had higher power to detect statistical differences in the data.

All statistical analysis was performed using JMP Version. 11.0.0 (SAS Institute, Cary, NC, USA).

4.4 RESULTS

4.4.1 Field Study

Resighting

There was no consistent change in the number of marked flies that were resighted over time ($F_{1,78}$ = 2.1901, P = 0.1429). In contrast, the number of tagged flies declined over time ($F_{1,78}$ = 25.5560, P < 0.0001). Resighting differed between treatments ($F_{1,156}$ = 4.0078, P = 0.0470, tagged mean ± se = 0.04 ± 0.01, marked mean ± se = 0.05 ± 0.01) and through time (treatment × time interaction $F_{1,156}$ = 5.2481, P = 0.0233), indicating that tagged flies died more quickly than marked flies (Figure 4.1). There were no sex differences in the probability of resighting flies over time, either for marked ($F_{1,78}$ = 0.8839, P = 0.3500) or for tagged individuals ($F_{1,48}$ = 2.7447, P = 0.1016).

Eyespan

There was no change in the mean eyespan of marked males over time ($F_{1,25}$ = 0.4864, P = 0.4920). In contrast, I found an increase in the mean resighted eyespan of tagged

males over time ($F_{1,35}$ = 27.3817, P < 0.0001). The mean eyespan of resighted males differed between treatments, being lower for marked males ($F_{1,60}$ = 441.2980, P < 0.0001, tagged mean (mm) ± se = 9.25 ± 0.15, marked mean (mm) ± se = 6.09 ± 0.08) and differed between treatments through time due to the increase of resighted tagged males eyespan through time ($F_{1,60}$ = 21.1388, P < 0.0001).

Like males, there was no change in the mean eyespan of resighted marked females over time ($F_{1,25}$ = 0.0332, P = 0.8568). But unlike males, there was no temporal change in mean eyespan among females bearing tags ($F_{1,14}$ = 0.7170, P = 0.4114). The mean eyespan of resighted females differed between the treatments, being lower for females with tags ($F_{1,39}$ = 4.1252, P = 0.0491, tagged mean (mm) ± se = 5.69 ± 0.13, marked mean (mm) ± se = 5.39 ± 0.08), however there was no difference between treatments through time ($F_{1,39}$ = 0.5478, P = 0.4637).

When the sexes were joined in a single analysis, the indicator value of male eyespan was revealed again as mean resighted male eyespan increased through time compared to female eyespan when tagged and marked individuals were compared (three-way interaction sex × treatment × time interaction $F_{1,99}$ = 4.7334, P = 0.0320).

Individual-based estimates

Another way to analyse the data is to look at individual-based survival estimates. This allows a more accurate analysis of individual movement and survival. This was only possible for tagged individuals, as marked flies did not have individually identifiable tags. The data similarly shows that there was no difference between males and females in longevity ($F_{1,99}$ = 4.7334, P = 0.0320) and that there was a positive relationship between male eyespan and longevity (r = 0.4312, $F_{1,51}$ = 11.6472, P = 0.0013). The

lack of a significant correlation between female eyespan and longevity was supported by individual-based survival estimates in tagged females ($r = 0.1740$, $F_{1,38} = 1.1858$, $P = 0.2830$).

4.4.2 Laboratory Study

There was no difference between experimental blocks (flies starting the experiment one week apart), for survival in males (likelihood-ratio $\chi^2_1 = 0.8620$, $P = 0.3532$) or females (likelihood-ratio $\chi^2_1 = 1.5388$, $P = 0.2148$). Data were therefore pooled across blocks for all further analyses. There was also no difference in the eyespan size distribution between tagged and marked flies in males ($F_{1,577} = 0.0016$, $P = 0.9677$, tagged mean (mm) ± se = 6.61 ± 0.07, marked mean (mm) = 6.60 ± 0.07) or females ($F_{1,579} = 0.3709$, $P = 0.5427$, tagged mean (mm) ± se = 5.02 ± 0.03, marked mean (mm) = 5.05 ± 0.03).

The overall death rate was 72.33% over the 27 days of the experiment and did not differ by sex (likelihood-ratio $\chi^2_1 = 0.8398$, $P = 0.3594$). Tagged flies had reduced survival compared to marked individuals, both in males (likelihood-ratio $\chi^2_1 = 60.3094$, $P < 0.0001$; 86.6% in tagged and 61.5% in marked fly death rate over 27 days) and females (likelihood-ratio $\chi^2_1 = 58.9837$, $P < 0.0001$; 82.8% in tagged; 58.4% in marked fly death rate over 27 days), with no sex difference in survival between treatments (likelihood-ratio $\chi^2_1 = 0.0567$, $P = 0.8117$; Figure 4.2).

Analysis 1: Censor at 27 days

I examined the effect that eyespan (absolute and relative) had on survival rates under the two treatments. Absolute eyespan influenced survival in marked (likelihood-ratio $\chi^2_1 = 33.0031$, $P < 0.0001$) and tagged (likelihood-ratio $\chi^2_1 = 52.0263$, $P < 0.0001$) males. There was no interaction between treatment and eyespan on male survival (likelihood-ratio $\chi^2_1 = 0.4499$, $P = 0.5024$). Similarly, absolute eyespan influenced survival in marked (likelihood-ratio $\chi^2_1 = 27.7998$, $P < 0.0001$) and tagged (likelihood-ratio $\chi^2_1 = 22.4877$, $P < 0.0001$) females, with no interaction between treatment and eyespan on survival (likelihood-ratio $\chi^2_1 = 0.0136$, $P = 0.9070$). There was no 3-way interaction of sex, treatment and absolute eyespan on survival (likelihood-ratio $\chi^2_1 = 0.0866$, $P = 0.7686$).

Relative eyespan (taking body size into account) did not influence survival in marked (likelihood-ratio $\chi^2_1 = 0.9896$, $P = 0.3198$) males, but did significantly influence survival in tagged (likelihood-ratio $\chi^2_1 = 4.5380$, $P = 0.0332$) males. There was no interaction between treatment and relative eyespan on survival in males (likelihood-ratio $\chi^2_1 = 0.6724$, $P = 0.4122$). Similarly, relative eyespan did not influence survival in marked (likelihood-ratio $\chi^2_1 = 1.0559$, $P = 0.3041$) females, but did in tagged (likelihood-ratio $\chi^2_1 = 3.8520$, $P = 0.0497$) females, with no interaction between treatment and relative eyespan on survival (likelihood-ratio $\chi^2_1 = 0.0157$, $P = 0.9002$). In a 4-way interaction, I found no sex difference in how treatment and relative eyespan interacted to influence survival (likelihood-ratio $\chi^2_1 = 0.0767$, $P = 0.7818$).

When I restricted the analysis to those flies that died during the experiment, I found that absolute eyespan was not a predictor of survival in marked males (likelihood-ratio

χ^2_1 = 1.6995, P = 0.1924), but was in tagged males (likelihood-ratio χ^2_1 = 30.1166, P < 0.0001). There was a significant interaction between treatment and absolute eyespan on survival in males (likelihood-ratio χ^2_1 = 7.4822, P = 0.0062; Figure 4.3) with large eyespan tagged males surviving longer than small eyespan males, whilst there was no relationship between eyespan and treatment in the marked males. Absolute eyespan influenced survival in marked (likelihood-ratio χ^2_1 = 4.4868, P = 0.0342) and tagged (likelihood-ratio χ^2_1 = 6.1091, P = 0.0134) females. Unlike in males, there was no interaction between treatment and female eyespan on survival (likelihood-ratio χ^2_1 = 0.2000, P = 0.6547). I found no sex difference in how treatment and absolute eyespan interacted to influence survival (likelihood-ratio χ^2_1 = 0.4271, P = 0.5134).

When I examined relative eyespan of those flies that died during the experiment, I found that relative eyespan influenced survival only in tagged males (marked male likelihood-ratio χ^2_1 = 0.0890, P = 0.7655; tagged male likelihood-ratio χ^2_1 = 22.2886, P < 0.0001). There was a significant interaction between treatment and relative eyespan on survival in males (likelihood-ratio χ^2_1 = 6.1369, P = 0.0132). Relative eyespan did not influence survival in marked females (likelihood-ratio χ^2_1 = 3.1008, P = 0.0783) or tagged females (likelihood-ratio χ^2_1 = 0.2857, P = 0.5930), and there was no interaction between treatment and female eyespan on survival (likelihood-ratio χ^2_1 = 0.1841, P = 0.6678). I found no sex difference in how the interaction between treatment and relative eyespan influenced survival (likelihood-ratio χ^2_1 = 0.0027, P = 0.9586).

Analysis 2: Censor at 50% death

As in the first analysis I found that the treatment regime influenced survival, as tagged flies died sooner than marked flies (male likelihood-ratio $\chi_1^2 = 29.0296$, $P < 0.0001$; female likelihood-ratio $\chi_1^2 = 37.1660$, $P < 0.0001$)

Absolute eyespan strongly influenced survival in both marked (likelihood-ratio $\chi_1^2 = 25.0678$, $P < 0.0001$) and tagged (likelihood-ratio $\chi_1^2 = 54.3367$, $P < 0.0001$) males. There was a borderline non-significant interaction between treatment and absolute eyespan on survival in males (likelihood-ratio $\chi_1^2 = 3.8347$, $P = 0.0502$). Absolute eyespan influenced survival in marked (likelihood-ratio $\chi_1^2 = 27.4409$, $P < 0.0001$) and tagged (likelihood-ratio $\chi_1^2 = 12.8033$, $P = 0.0003$) females, with no interaction between treatment and eyespan on survival (likelihood-ratio $\chi_1^2 = 0.5061$, $P = 0.4769$). There was no sex difference in how treatment and absolute eyespan interacted to influence survival (likelihood-ratio $\chi_1^2 = 1.6836$, $P = 0.1945$).

I found that relative eyespan influenced survival only in tagged males (marked male likelihood-ratio $\chi_1^2 = 1.1069$, $P = 0.2927$; tagged male likelihood-ratio $\chi_1^2 = 16.3286$, $P < 0.0001$). Crucially there was a significant interaction between treatment and relative eyespan on survival in males (likelihood-ratio $\chi_1^2 = 3.8912$, $P = 0.0485$). Relative eyespan did not influence survival in marked (likelihood-ratio $\chi_1^2 = 2.7779$, $P = 0.0956$) or tagged (likelihood-ratio $\chi_1^2 = 3.7177$, $P = 0.0538$) females, and there was no interaction between treatment and eyespan on survival (likelihood-ratio $\chi_1^2 = 0.4180$, $P = 0.5179$). I found no sex difference in how the interaction between treatment and relative eyespan influenced survival (likelihood-ratio $\chi_1^2 = 2381$, $P = 0.6256$).

4.5 DISCUSSION

More than a century and a half after Darwin first considered sexual selection (Darwin, 1859), our understanding of why exaggerated male sexual traits evolve remains elusive. Two major theories have been put forward to explain these traits: Fisher's (1930) runaway process and the handicap hypothesis (Zahavi, 1975; Pomiankowski, 1987b). Fisher proposed that exaggerated male traits evolve when they became co-inherited with the corresponding genes for female preference (Fisher, 1930). In contrast, the handicap hypothesis proposed that exaggerated male traits evolve as honest and costly signals of quality (Zahavi, 1975; Grafen, 1990). Poor quality males pay a disproportionally higher cost of ornamentation, and thus only good quality males are able to produce the most exaggerated traits. Whilst both theories are theoretically plausible (Fisher, 1930; Lande, 1981; Pomiankowski, 1987b; Grafen, 1990), definitive experimental evidence is limited and often contradictory (Grose, 2011). Experimental data has been obtained primarily by examining how ornament manipulation influences survival (Mappes *et al.*, 1996; Grether, 1997; Cuervo and de Ayala, 2014). This methodology fails however to examine the overall fitness consequences that arise from correlated life history constraints (Balmford *et al.*, 1994; Emlen, 2001).

In this chapter, I employ an alternative approach by applying an experimental stress to ask whether naturally occurring eyespan size influences subsequent survival. Using experiments on a wild population of flies, I found that male ornament size was a predictor of survival amongst males that were subjected to experimentally elevated stress caused by the attachment of a small tag to the thorax. Large eyespan males survived longer than small eyespan males with this treatment. In contrast, there was no association between survival and ornament size when males were subject to a

relatively benign stress caused by paint marking on the thorax. Females provide a useful contrast, as the homologous eyespan trait is much less developed and so was not predicted to associate with survival in either stress treatment. I found this to be true, as there was no dependence of survival on female eyespan in either stress treatment. Negative correlations between ornament size and survival are expected under both Fisher's runaway process (Lande, 1981; Pomiankowski *et al.*, 1991; Kokko *et al.*, 2002) and the handicap hypothesis, when increasing ornamentation brings sufficiently high gains in mating success that males may evolve larger ornaments such that those individuals suffer reduced survival (Höglund and Sheldon, 1998; Eshel *et al.*, 2000; Kokko *et al.*, 2002). A positive correlation between ornament size and survival is only predicted by the handicap hypothesis, and my results are highly congruent with this prediction as I found male eyespan and survival to be positively correlated under experimental stress. This provides empirical evidence that male ornament size provides an honest signal of male quality in stalk-eyed flies.

I repeated the approach under controlled laboratory conditions, and the results did not support the field findings, as there was no relationship between survival and ornamentation in males or females. A confounding factor in this study was primarily statistical as a disproportionate number of flies survived in the benign stress treatment and were therefore censored. This discrepancy meant that the large pool of censored flies were unable to be used to calculate the survival distribution curve, but were taken into account as 'unknown flies' by the model providing no statistical traction for underlying relationships to be highlighted (Mike Bonsall, *pers. comm.*). Censoring is employed to account for random death or escaped individuals, but not for biased numbers at the end of the experiment. This problem resulted from high survival rates

at the end of the monitoring period and future experiments will be able to control for this by running the experiment until 100% mortality is reached.

In order to control for the statistical issue in the current experiment, an alternative analysis was employed that equalised the number of flies in each treatment by statistically terminating the experiment when 50% of flies in each treatment had died. This is not a traditional method of analysis, but it presents no obvious statistical errors or issues (Matthias Ziehm, *pers. comm.*). Using this method I found that the laboratory results mirrored the field results with male eyespan influencing survival in males under heightened but not benign stress. The field experiment is only able to consider absolute eyespan and its effect on survival. Absolute eyespan size correlates strongly with body size (Stern and Emlen, 1999; Cotton *et al.*, 2004a) and so the findings from the field could be a result of large bodied individuals being able to survive longer. Using female eyespan as a homologous control trait provides some evidence that the relationship is between eyespan (not body size) and survival because if body size were the primary driver of survival, I would have expected a correlation between female eyespan and survival as well. Nonetheless I used the laboratory study to test this directly by examining how absolute and relative eyespan (controlling for body size) affect survival under different experimental stress regimes. I found that relative eyespan strongly influenced survival in tagged but not in marked males, (or in females), with large (relative) eyespan males surviving longer when they were subjected to a heightened stress. Taken together with the field experiment, my results provide strong initial support for the hypothesis that exaggerated ornamental traits are sexually selected signals of male quality.

I found that the addition of a tag reduced survival both in the laboratory and the field. The exact reason(s) for this is not clear. The tagging process *per se* is unlikely to explain this result, as the process of capturing and anaesthetising the flies and applying adhesive nail varnish was identical in both tagged and marked groups. In the field, I took the added precaution that only those individuals that survived the inevitable stress involved in anaesthesia, tagging and marking by at least 10 hours were re-released. The same procedure was also applied in the laboratory experiment. Interestingly, the tag-induced death seen in the laboratory indicates that the survival cost incurred by these flies was, at least partially, of an intrinsic nature and not entirely due to the more obvious extrinsic factors in the wild such as increased susceptibility to predation. Despite the reduction in survival, both tagged and marked individuals were frequently observed flying and feeding during the day, as well as lekking, fighting and mating at dusk. So tags did not interfere with the normal range of behaviours performed by individuals.

Although survival is a major component of fitness, trade-offs between survival and other major life history traits are well documented (Stearns, 1992). Stalk-eyed flies have a long lifespan (over six months in the field and eight months in the laboratory) and reach sexual maturity 4-8 weeks after eclosion (Wilkinson and Reillo, 1994; Wilkinson and Dodson, 1997; Reguera *et al.*, 2004). Thus survival is a particularly relevant component of fitness to measure in this species as sexually mature males can mate multiply on a daily basis and the overall fitness of a male is highly likely to correlate positively with lifespan.

Whilst the field study provided critical evaluation of the handicap hypothesis in the natural environment, there were some inevitable restrictions in the interpretation of the

results. The sample size available in the field was limited, with a maximum of fifty flies in each group. This limit reflected the number of flies collected at the experimental site on a particular evening. Whilst this was the most available it limited the statistical power for detecting effects (Hedges and Olkin, 1985). Also, I assumed that the decline in resighted individuals was attributable to mortality. This may not be justified for two reasons. First, un-resighted flies may have migrated away from the study area. This hypothesis is unlikely to be correct, since intensive searches were performed ~100m up and downstream from the study site and in the nearest adjacent tributary (~100m away), and only a single fly was observed outside the study area. This was a marked individual, which runs counter to the expectation that lower resight likelihoods for tagged flies were due to emigration. Second, low resight probabilities may have been due to flies spending less time in visible places, rather than being no longer alive. There is some evidence for this because fewer flies were resighted during the day, when they were dispersed in the forest. However, results were unaffected by the time of search (separate analysis day *versus* night resight data, unreported statistics), so differences in the overall probability of resighting flies are unlikely to bias my conclusions. Thus the most parsimonious explanation for systematic changes in resight frequencies is (differential) mortality.

In the parallel laboratory study, large sample sizes were used (600 flies in each treatment group) and survival was monitored daily in order to gain accurate time of death data and allow survival analysis. After the completion of the experiment at 27 days, it was apparent that there was a severe distortion in survival caused by the stress treatment. Those flies that were still alive after 27 days were censored and a statistical model was used to account for them. Unfortunately because there were so many individuals in the censored group and it was so unbalanced, the power to detect trends

in the data was compromised. These problems could be rectified by the continuation of the experiment until all the flies were dead (see future work below). The reason that 27 days was chosen as the cut-off for the laboratory experiment was that, in addition to survival, I was interested in examining the effects of the treatment (tag or mark) on reproductive organ development (accessory glands, testes and fecundity) at sexual maturity. The results from analyses of these data do not form part of this chapter. A subsequent re-analysis, which statistically terminated the experiment when 50% of flies in each treatment had died, was performed to equalise the number of censored flies in each group. This produced results that agreed strongly with those found in the field and suggests that further laboratory study is warranted. In addition, when I examined only those flies that had died during the experiment, the results also mirrored those in the field, providing further evidence that the underlying trends warrant further investigation.

The field study was unable to examine the effect of thorax (a proxy for body size). I examined this variable in the laboratory and found it to be a predictor of survival, unsurprisingly as body size has been shown to be a predictor of survival in a large number of other species such as Caribbean fruit flies (Sivinski, 1993), rubyspot damselflies (Grether, 1996), auks (Harding *et al.*, 2011) and cane toads (Cabrera-Guzmán *et al.*, 2013). I accounted for this variable in my examination of ornament size, whereby I found eyespan to independently influence survival in my second analysis. This is important, as it shows that the degree of ornament exaggeration *per se,* rather than co-variation with body size, reflects underlying quality.

In the field experiment, marked flies were examined first and after that block had concluded then tagged flies were investigated. This was done because it allowed the

entire experiment to take place in one population, ensuring there was no confounding effects of using multiple populations that differed in factors such as population density, environmental quality or genetic structuring (see chapter 5). Logistically, there would always have needed to be two blocks and if both treatments had been run simultaneously, there would have been no way of ensuring marked flies that were resighted in the second block were not marked from the first block. That was why it was decided to avoid this issue and block by treatment. The limitation of this approach is that time is a potentially confounding issue that was not able to be controlled for, with potential environmental changes, such as rainfall, that may have affected resighting rates.

Future work will rectify the experimental design problems in the laboratory experiment by altering the length of the experiment and monitoring flies until they are all dead. This will reduce the amount of heterogeneous data that needs to be censored. Combining this laboratory study with the field study will constitute a robust study in the empirical examination of the condition-dependent handicap hypothesis. My results nonetheless add to the growing body of evidence that male eyespan in stalk-eyed flies is a condition-dependent indicator of male quality (David *et al.*, 2000; Cotton *et al.*, 2004b; Cotton *et al.*, 2010). It would be interesting to examine the generality of my findings by using similar techniques in other species, using both field and laboratory studies to create ecological validity and experimentally robust conclusions.

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Figure 4.1. Probability of resighting an individual during the field experiment (20 days). The blue line indicates the probability of resighting a marked individual and the red line indicates the probability of resighting a tagged individual. The difference between these is significant (P<0.05).

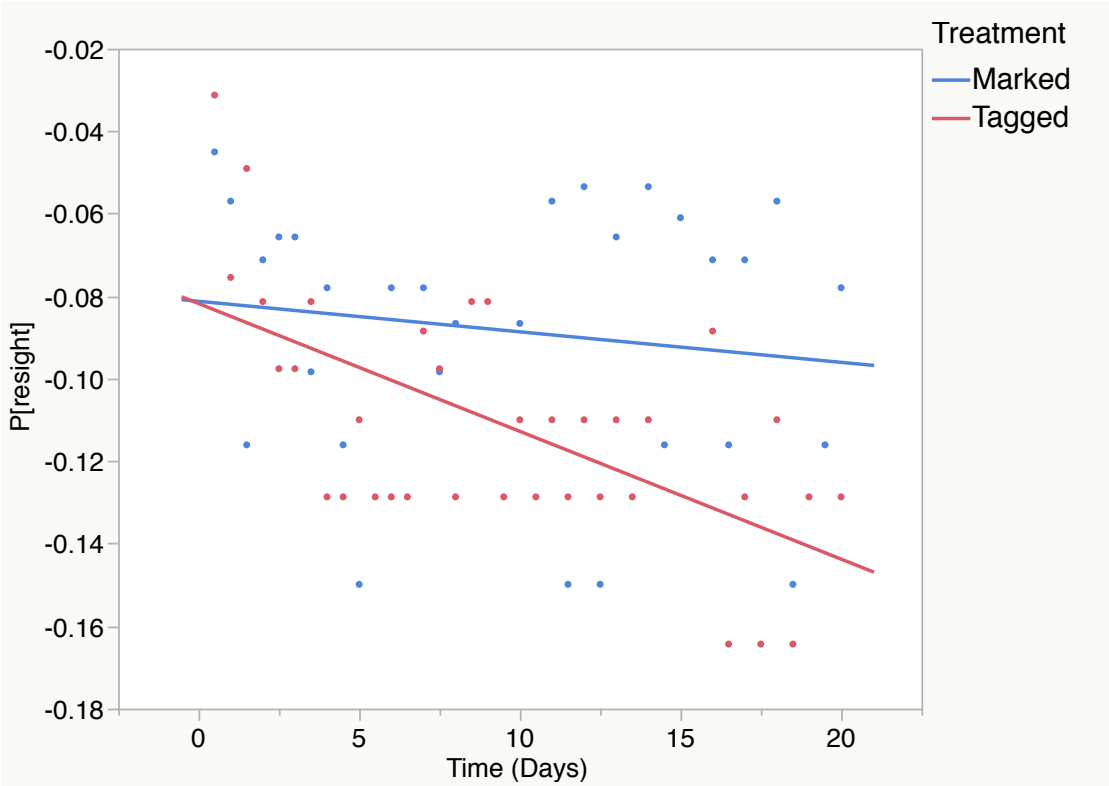


Figure 4.2. Survival curves of laboratory flies under heightened stress (females denoted by the green line, males by the purple line) and under benign stress (females denoted by the blue line, males by the red line).

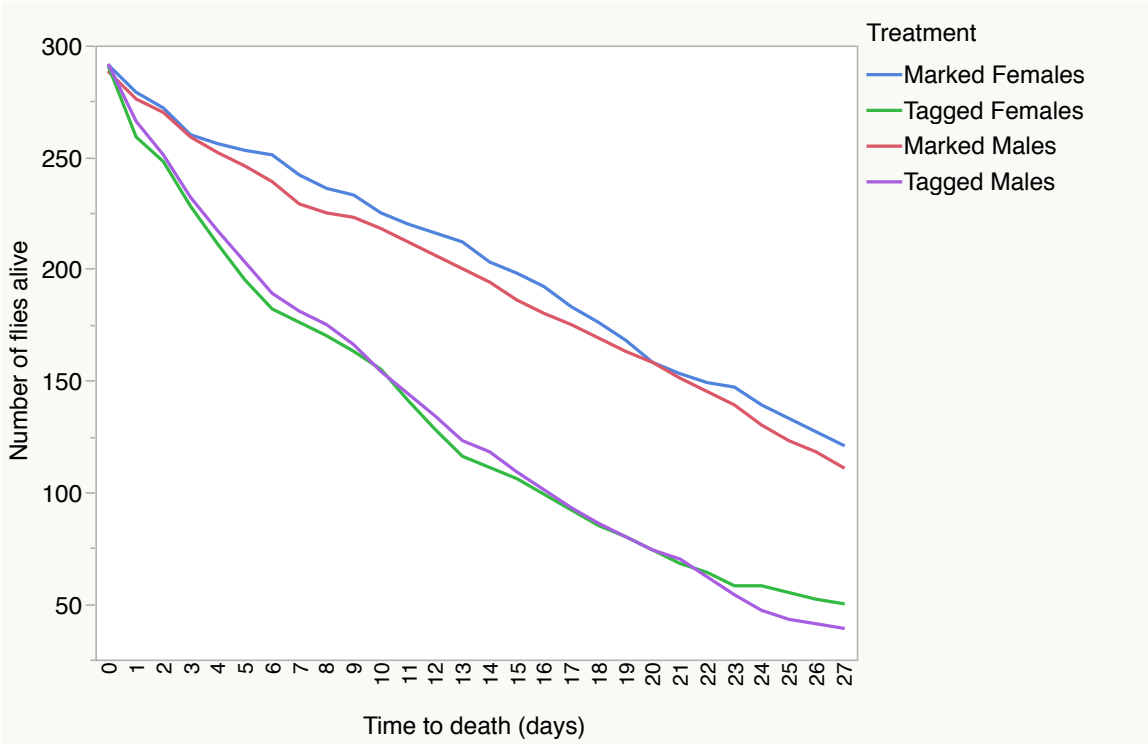
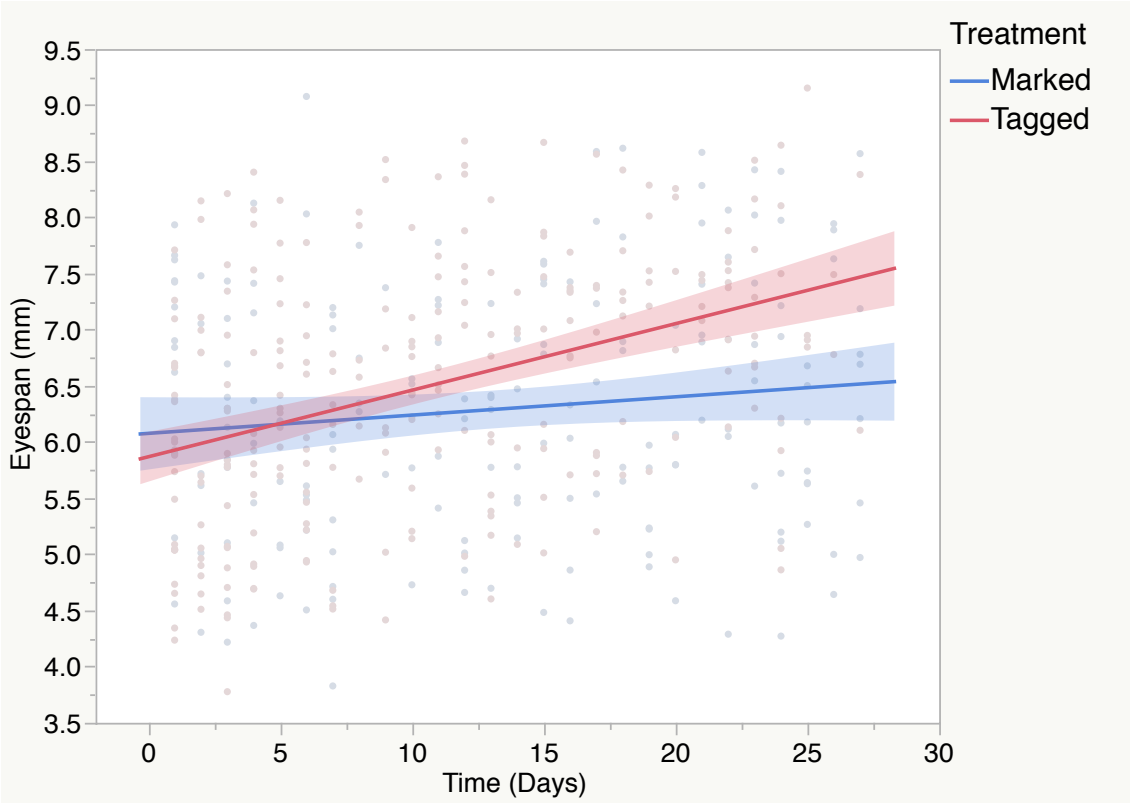


Figure 4.3. The relationship between male ornamentation size (eyespan) and survival for flies that died during the laboratory experiment. The line of best fit for mean eyespan of surviving males under heightened stress due to tags (red line) or benign stress due to marks (blue line) is shown through time. The shaded area represents the 95% confidence interval.



5

The influence of environmental quality on lek structure and behaviour in stalk-eyed flies

5.1 ABSTRACT

The handicap hypothesis predicts that exaggerated secondary sexual traits should exhibit heightened condition dependence, as the cost of ornamentation is greater for those males in poor condition. Whilst positive correlations between trait size and condition are often found, a more complete understanding of how environmental conditions affect ornament evolution and components of sexual selection is needed. In this study I examined how experimentally controlled stress as well as naturally occurring environmental conditions influence lek structure and mating behaviour in stalk-eyed flies. In the laboratory, diet was experimentally manipulated to create high and low condition flies. Mating behaviour and lek structure were then examined in both of these groups. In the field flies were collected at sites that differed in environmental quality and examined this in relation to differences in the mean and variance of lek structure. I found congruence in both the laboratory and the field; less stressful environments resulted in larger lek sizes with a greater number of females per lek. Not only was the mean number of flies per lek affected, but lek size variance (measured as the coefficient of variation) also increased in good quality environments, suggesting an increased likelihood of assortative mating (i.e. stronger sexual selection). This was confirmed as large eyespan males accrued larger leks only when conditions were good. A suite of behavioural traits were also affected, with good environments resulting in increased rejection rates and increased numbers of matings. This provides strong evidence that poor environmental quality can reduce the potential for components of sexual selection in both natural and captive populations.

5.2 INTRODUCTION

A key model of sexual selection, the handicap hypothesis, predicts that exaggerated secondary sexual traits will display heightened condition-dependent expression (Zahavi, 1975; Iwasa and Pomiankowski, 1994; Cotton *et al.*, 2004a). High quality males (those in good condition) are expected to display the largest sexual traits, enabling potential mates or competitors to accurately assess individual condition. The honesty of the signal is maintained by a differential cost of ornamentation, with low quality males incurring a relatively higher cost of ornament production compared to high quality males (Zahavi, 1975; Grafen, 1990; Iwasa and Pomiankowski, 1994).

In addition to ornaments (Cotton *et al.*, 2004b; Johns *et al.*, 2014), mate preferences are also characterised by condition-dependent expression (Cotton *et al.*, 2006a; Holveck *et al.*, 2011). Both these traits exhibit large components of environmental variance (Alatalo *et al.*, 1988; Cuervo and Møller, 2001; Cotton *et al.*, 2006a; Robinson *et al.*, 2012), largely because condition itself has a large component of environmental variance (Price and Schluter, 1991). Condition dependence of sexual traits is evolutionarily important as it enables underlying quality variation in one sex to be sexually selected by the other (Rowe and Houle, 1996; Cotton *et al.*, 2006b).

The relationship between environmental stress and condition-dependent traits such as ornament size and preference is not clear-cut. The majority of research has focussed on this relationship in male sexual ornaments. We know that while environmental stress tends to cause a reduction in mean ornament size, it also tends to cause an increase in the variance of the trait (David *et al.*, 2000; Cotton *et al.*, 2004b). A reduction in mean ornament size may result in diminished sexual selection (Poulin and Vickery, 1996;

Pfennig and Tinsley, 2002) as heritability estimates have been shown to increase in good quality environments (Qvarnstrom, 1999). Simultaneously, however, an increase in the variance of such traits may elevate sexually selectable genetic variation in males, increasing the advantages of sexual selection as females are more easily able to identify males of the highest quality (Rowe and Houle, 1996; David *et al.*, 2000; Cotton *et al.*, 2004b).

While there is some information on how changes to the mean and variance of ornament size caused by environmental stress may affect sexual selection, our knowledge of the relationship between environmental stress and mating preferences is even less clear. Both a female's ability to discriminate between males, i.e. her preference function and the amount of effort she puts into sampling, is likely to be costly (Cotton *et al.,* 2006a). So female preferences should be sensitive to the environment in which they are expressed, with preference phenotypes declining as environmental stress increases. An increasing number of studies have shown that female mating preferences are condition-dependent, with increased stress resulting in lower mean preference and increased variance (Hunt *et al.*, 2005; Cotton *et al.*, 2006a; Hebets *et al.*, 2008; Dakin and Montgomerie, 2014). The result of this is that females in good condition exhibit strong directional selection for highly ornamented males, while females in poor condition tend to mate at random. These changes may affect sexual selection in a number of contrasting ways (Jennions and Petrie, 1997; Syriatowicz and Brooks, 2004; Hunt *et al.*, 2005). Poor quality females that mate randomly with a wide variety of males could reduce the strength of directional selection acting on male ornament size. Alternatively, if female responsiveness to males is diminished and only the most attractive males reach the increased threshold that these females require in order to mate, then this could increase the strength of

sexual selection on male attractiveness. Another interpretation is that high quality individuals exposed to environmental stress, whilst having absolutely lower ornament, preference and fitness values (compared to unstressed individuals), will have proportionately larger ornaments and proportionately stronger preferences *within* their local stressed population. They will therefore tend to mate together and leave disproportionately more offspring *within* their local stressed population. The conclusion is that the net effect of stress is currently both theoretically and empirically unclear.

Environmental effects on sexual selection have been evaluated using contrasts between genetically distinct populations (Grether, 2000). However, selection is expected to act more strongly on individuals, than on groups or populations, so it is important to focus on patterns within, rather than just between populations. A few studies have investigated variation in sexual selection from associated responses of ornaments and preferences to changes in the environmental context of mating (Qvarnström *et al.*, 2000; Gamble *et al.*, 2003; Cotton *et al.*, 2006a). Critically however, many of these studies have assayed only a single trait in isolation e.g. preference or ornaments, and this approach fails to examine how the interaction between preference and ornaments responds to environmental variation. Given that condition dependence of both male and female traits is likely to have evolved in tandem, it is vital to understand how these elements together are affected by changes in the environment.

Moreover, surprisingly few studies have investigated the effects of environmental variation in lekking species. Leks are the locus for sexual selection in many species and represent an arena in which the outcomes of preference and ornaments can potentially be assayed with relative ease (Andersson, 1994). Most studies on lekking

species have focussed on theories of lek positioning (Beehler and Foster, 1988; Balmford *et al.*, 1993a; Alonso *et al.*, 2012; Callander *et al.*, 2012), mate choice (Andersson, 1982; Gibson and Bachman, 1992; Jones *et al.*, 2000; Sardell *et al.*, 2014; Koch *et al.*, 2015) or behaviour (Lanctot and Weatherhead, 1997; Massei and Bowyer, 1999). A small number have examined how factors such as density (Apollonio, 1989; Balmford *et al.*, 1993b), diet (Yuval *et al.*, 1998) or thermal extremes (Llusia *et al.*, 2013) affect lekking, but there are a dearth of studies that have utilised lekking systems to examine how environmental quality variation influences the combined effects of preferences, ornaments and other components of sexual selection. This is surprising given the importance of stress in creating the variance in condition that is signalled by sexual traits (David *et al.*, 2000), and the high prevalence of both stochastic environmental variation (Post *et al.*, 1999; Garant *et al.*, 2004), and deleterious effects (often human-derived) on habitat quality (Hill, 1995; Stratford and Stouffer, 2001).

Stalk-eyed flies, members of the diopsid family, display a form of hypercephaly whereby eyestalks elongate from the head capsule, causing the displacement of the eyes to the end of laterally projecting stalks. Many stalk-eyed fly species exhibit sexual dimorphism of eyespan (the distance between the outer most edge of the eyes), with males possessing significantly bigger eyespan relative to their body size than females (Burkhardt and de la Motte, 1985). A plethora of studies, both experimental and observational, have shown that the exaggerated male trait (eyespan) has evolved through sexual selection, and is used both in male-male antagonistic interactions (Panhuis and Wilkinson, 1999; Small *et al.*, 2009; Egge *et al.*, 2011) as well as in mate choice (Wilkinson and Reillo, 1994; Cotton *et al.*, 2010). *Teleopsis dalmanni* is an intensively studied Malaysian species of stalk-eyed fly. Flies are dispersed in forests during the day, with both sexes foraging independently on decaying plant matter.

Males fly to exposed root hairs overhanging the eroded banks of streams in the early dusk (Wilkinson, 1993; Wilkinson and Reillo, 1994; Cotton *et al.*, 2010). They aggressively compete with other males for control of these rootlets (Small *et al.*, 2009). Females arrive during dusk and choose their roosting sites (and therefore mates) from amongst the root hairs where males have established themselves, resulting in a lek style mating system (Cotton *et al.*, 2010). Extensive field and laboratory experiments have shown that females prefer to roost and mate with males with larger (absolute and relative) eyespan (Wilkinson and Reillo, 1994; Cotton *et al.*, 2010). Males mate frequently (Lorch *et al.*, 1993), and consequently male accessory gland and testis size are major components of male reproductive quality (Baker *et al.*, 2003; Rogers *et al.*, 2005a, b; Fry, 2006; Rogers *et al.*, 2008). Male mating frequency correlates positively with accessory gland size, both phenotypically (Rogers *et al.*, 2006) and genetically (Baker *et al.*, 2003). Accessory gland size, testes size and female fecundity are all highly sensitive to the environment (Hingle *et al.*, 2001a; Baker *et al.*, 2003; Rogers *et al.*, 2008) and show strong associations with ornament size (Cotton *et al.*, 2006a; Rogers *et al.*, 2008) and preference (Hingle *et al.*, 2001a).

Both male ornaments and female preferences exhibit strong condition-dependent expression, with male ornament size and female mating biases (with respect to male ornamentation) declining as flies become stressed (David *et al.*, 1998; David *et al.*, 2000; Hingle *et al.*, 2001a, b). Both male ornaments (David *et al.*, 2000; Cotton *et al.*, 2004b) and female preference are highly variable under stress (preference coefficient of variation 42% in benign and 160% in harsh environments; derived from data published in Hingle *et al.*, 2001a). Female eyespan, an important mechanistic determinant of the strength of female preference in stalk-eyed flies (Hingle *et al.*,

2001b; Cotton *et al.*, 2006a) also exhibits condition-dependent size and variance (Cotton *et al.*, 2004b, c).

Most investigations of the environmental determinants of invertebrate ornaments, preference or mating behaviour have been laboratory-based. Such an approach is justified by the need for simplification and control. But it is important to complement the patterns seen in laboratory environments with studies under natural conditions (Charmantier and Garant, 2005; Charmantier and Sheldon, 2006). The laboratory focus in invertebrates stands in stark contrast to the many intensively studied wild bird and mammal populations (Griffith *et al.*, 1999; Post *et al.*, 1999; Garant *et al.*, 2004; Charmantier and Sheldon, 2006). However, these vertebrate studies lack reciprocal laboratory experiments under controlled environments. Invertebrates provide good systems in which laboratory and field studies can be combined. The lek mating system of *T. dalmanni* allows such dual investigation, in a system exhibiting strong sexual selection (Andersson, 1994). Exaggerated male sexual traits are used in male-male competition as well as in female mating decisions, and the costs associated with female mate choice in the lek mating system are lower (Andersson, 1994). *T. dalmanni* lekking sites in the wild can be observed and manipulated easily, individuals have low dispersal (Sam Cotton, unpublished data), and their high abundance means that (relatively) large sample sizes (both individuals and leks) are obtainable. *T. dalmanni* are long-lived (> 30 days in the field) so individuals can be followed for many days and their sexual behaviour observed.

In this study I examined the effect of variation in environmental quality on lekking behaviour and lek structure in *T. dalmanni.* In wild populations of *T. dalmanni* I examined variation in lek structure and correlated this with natural spatial and

temporal variation in the environment. Additionally, I examined sampling and mating behaviour in wild females. In the laboratory, I subjected flies to high or low environmental stress and examined lek structure as well as a range of behavioural traits such as fighting, lek patrolling, mating and rejection.

5.3 MATERIALS AND METHODS

5.3.1 Field Based Experiments

Flies were collected from 12 populations along the Ulu Gombak valley, in Peninsular Malaysia, spanning approximately five kilometres (see Cotton *et al.*, 2014b for further details). The sites were: Blair Witch (BW) (3º19'N 101º45'E), Cascade (C) (3º19'N 101º45'E), Kingfisher (K) (3º19'N 101º45'E), Lower Field Centre (LFC) (3º19'N 101º45'E), Mihaly (M) (3º19'N 101º45'E), Poppet (P) (3º19'N 101º45'E), Quarry (Q) (3º18'N 101º44'E), Rubbish (R) (3º18'N 101º44'E), Swamp (S) (3º19'N 101º45'E), Tarantula (T) (3º19'N 101º45'E), Upper Blair Witch (UBW) (3º19'N 101º45'E) and Upper Lazy Dog (ULD) (3º19'N 101º45'E). The sites contained a mix of primary and secondary rainforest, with flies found on rootlets hanging under stream banks. The length of stream occupied by flies varied between 20-40 m. Each site represents a discontinuous population of flies with low rates of migration between adjacent populations (Sam Cotton, unpublished data).

All flies in each of the 12 given populations were censused annually over three years (2008-2010). The search for flies was exhaustive and although it is very likely that some flies eluded capture, repeat searches on subsequent evenings rarely yielded

additional flies. Flies were collected at night with small clear plastic bags placed over the rootlet. This allowed the gentle removal of the all the flies on a lek in individual bags. Bagged leks were labelled and returned to the field centre. Fly identity, lek number, site and year were recorded and flies were anaesthetised over ice. A monocular field microscope attached to a digital camera was used in order to measure eyespan (the distance between the outer edges of the eye bulbs) and thorax length (the distance from the base of the head to the posterior edge of the thorax and is measured as a proxy for body size) to an accuracy of 0.01mm, using Image J software (v. 1.55). In addition to eyespan and thorax, the abdomen of each female was dissected into phosphate-buffered saline (PBS) and her fecundity (the number of mature eggs in the ovaries) was recorded. Mature eggs are defined as stages 12–14 using King's standard stages of oogenesis (King, 1970)**.** The reproductive tract of each male was also dissected into PBS. The accessory glands and testis were extracted. The testes were uncoiled and placed on a stage micrometer. A monocular field microscope was then used to take digital photographs (Baker *et al.*, 2003). The length of both the testis and accessory glands were then measured (Rogers *et al.*, 2005a). All of these flies were subsequently stored in 100% ethanol.

In a separate experiment carried out in 2008 at Lazy Dog (LD) (3º19'N 101º45'E**)**, females ($N = 38$) were observed as they entered a lekking population site at dusk. Each observer followed a single female from the initial entry into the lekking area until all mating was over and the flies were immobile. During that time all female behaviour was recorded. Specifically, incidences of abdomen bobbing (vigorous bobbing up and down of the abdomen whilst on the lek), lek walking (repetitively walking up and down the rootlet in a stereotypic manner, often leading to encounters with both male and female conspecifics) and sampling (flying between different males on different lek

rootlets in the same dusk period) were recorded. The number of matings the female received was also documented. Once all activity ceased and females became quiescent, they were collected, anaesthetised over ice and measured for eyespan and thorax length (using the same method, accuracy and software described above). They were then dissected and the fecundity (the number of mature eggs present in the ovaries) of each female was recorded.

5.3.2 Statistical Analysis: Field

In order to quantify variation in environmental stress across the sampled 12 populations, I used a suite of demographic, morphological and life-history traits: local population density at the sampled sites (measured as the number of flies captured per meter of stream sampled), estimates of male and female body size (thorax length), and an estimate of female reproductive output (fecundity – the number of mature eggs in the ovaries). All four of the above variables contained significant site variation (see Results §). For each collection (N = 36; 12 sites × 3 years) I carried out a principal components analysis to create a combined metric of environmental quality. Principal component 1 (PC1) had a strong positive correlation with all variables (see Results) and was used in all subsequent analyses as a quantitative proxy of environmental quality for each collection. PC2 (scaling positively with male thorax and negatively with population density) and PC3 (scaling negatively with female thorax and positively with density) were also calculated. Holm-Bonferroni (correcting for multiple comparisons) was applied to each result.

In a series of general linear mixed models, I examined the effect of these principle components on the number of females per lek (harem size). Given that my primary

focus was to evaluate how environmental stress affects lekking behaviour (utilising both spatial and temporal variation in quality) and each of the 12 sites was sampled over three consecutive years, I needed to statistically control for the geographical non-independence of the data. In order to do this, I included 'site' as a random covariate in each model. As I was interested in how the potential for sexual selection varied with environmental quality, I examined how the number of females per lek and the coefficient of variation co-varied with PC1 scores. Coefficients of variation were used so that variation could be assessed independently of changes in mean values (Zar, 1999); nonetheless, qualitatively identical results were obtained if the variance or range were used (data not shown).

I also constructed a general linear mixed model using the original dataset (not using collection-averaged measures) to examine whether the relationship between male eyespan and the number of females on each lek (i.e. the relationship between ornament size and potential mating success) varied according to differences in environmental quality. 'Site' was again included as a random covariate in all the models. Male thorax is included in my measure of environmental quality (PC1), however it's known that male thorax is highly correlated to male eyespan (Wilkinson and Reillo, 1994), and in this model I am examining the relationship between PC1 and male eyespan. In order to control for this correlation, I created a principal component (PC1*) that was composed of female thorax, fecundity and density (excluding male thorax). In addition to examining how absolute male eyespan size associated with harem size and PC1*, I also examined relative male eyespan size, by including thorax as a covariate in the model.

Spearman's rank tests were used to examine the relationship between fecundity and the four variables of female lekking behaviour (abdomen bobbing, walking, sampling and mating). As well as absolute fecundity I created a categorical variable for whether females were barren or fecund (0 or 1). This was also compared to variables of female behaviour using a chi square test.

5.3.3 Laboratory Based Experiment

A large sample of *T. dalmanni* was collected (by Sam Cotton and Andrew Pomiankowski), from the Ulu Gombak valley, Peninsular Malaysia (3º19'N 101º45'E) in 2005. Since transportation back to the UK, flies have been maintained in cage culture at high density (>200 individuals) with an approximately 1:1 sex ratio to minimize inbreeding. The population was kept at 25ºC, with a 12:12 h dark: light cycle and fed pureed sweetcorn twice weekly. Short (30 minute) artificial dawn and dusk periods were created by illumination from a single 60-W bulb, at the start and end of the light phase. To obtain experimental flies I collected eggs from the stock populations and reared larvae on a variable amount of pureed sweetcorn to maximize eyespan variance (David *et al.*, 1998; Cotton *et al.*, 2004b).

Upon eclosion, flies were allocated randomly to either a low quality diet or a high quality diet treatment. The low quality diet consisted of 20% corn to 80% sucrose, whilst the high quality diet consisted of 80% corn to 20% sucrose. To ensure that all the food had the same viscosity, an indigestible bulking agent, carboxymethycellulose (3% w/v), was added to the sucrose (25% w/v) solution (Rogers *et al.*, 2008). Flies were kept in large cages (30 × 20 × 20cm) at medium density (approximately 40 flies per cage) and fed *ad libitum* twice weekly.

Prior to sexual maturity, flies were anaesthetized on ice and measured for eyespan, defined as the distance between the outermost lateral edges of the eyes (Cotton *et al.*, 2004b). Flies were categorised as being large if they were greater than 0.5 standard deviations from the mean, and small if they were below than the mean by more than 0.5 standard deviations: large males (>8.68mm eyespan), small males (<7.49mm), large females (>6.00mm) and small females (<5.40mm). All intermediate sized flies were removed from the experiment. After categorisation, flies were held in large cages at medium density, separated by sex, eyespan class (large or small) and diet quality (high or low).

All experiments took place after sexual maturity was attained (> 4 weeks post eclosion) (Reguera *et al.*, 2004). Three days before experimentation, all male and female flies were mixed within diet class (i.e. either high or low quality) and allowed to mate freely for 24 hours, before being separated again until the experiment. This was done in order to avoid analysis of virgins, as previous work had shown that virgins have atypically weak mate discrimination (James Howie, unpublished data). In addition, pre-mating simulated the natural state of flies, as sexually mature flies live for several months, and both sexes mate multiply (Reguera *et al.*, 2004; Cotton *et al.*, 2010). For the experiment, a large cage (30cm (L) × 20cm (H) × 20cm (W)) was adapted with lengths of string (10cm long) used to simulate roosting leks. Three strings were hung equidistantly (10cm) from each other along the midline of the cage roof. Flies were placed in this experimental cage in groups of six. One male and two females from each size class (so one large male, one small male, 2 large females and 2 small females) were used to create a 'lekking group' and this was used as the unit of

measure (N = 224 lekking groups, N = 1,344 flies). Half of the lekking groups were composed of high quality diet flies and half of low quality diet flies (N = 112 each).

Just prior to the start of the artificial dusk, a lekking group (all six flies) was placed into an experimental cage. Flies were observed for 30 minutes during the artificial dusk period. Specific behaviours were then recorded: incidences of male-male and female-female fighting, length of total time spent fighting, number of mating attempts, number of rejected mating attempts, incidences of male patrolling (moving up and down the lek repetitively, without pausing), length of total time spent patrolling, copulation frequencies, time spent in copula, and number of rejected mating attempts. Flies were left for an additional 30 minutes after the end of the 30-minute dusk period, to allow for any final roosting site changes and for the flies to become quiescent. The observer then noted the position of all the flies using indirect torchlight so as to avoid disturbing the roosting flies. It was recorded whether flies were located on the lek strings or whether they were outside the lekking arena on the side of the cage.

5.3.4 Statistical Analysis: Laboratory

The behavioural information that I gathered (fighting, patrolling, rejection and mating) was not normally distributed. Consequently, I used Wilcoxon signed-rank tests to evaluate any relationships between those behavioural traits described above and the condition of the flies (good or poor). This allowed me to assess whether there was any effect of diet quality on lekking behaviour(s).

Wilcoxon signed-rank tests were also used to examine the relationship between lek structure and diet quality. A series of lek-structure variables (lek size, whether flies

were on or off leks, the number of males and the number of females on leks) were compared between flies on high and low quality diet. Coefficients of variation between both lek size and the number of females on a rootlet were compared in the two treatments (Roulin *et al.*, 2000). The Wilcoxon signed-rank test was used to examine the effect of eyespan and diet on lek size (the number of flies on a rootlet).

All statistical analysis was performed using JMP Version. 11.0.0 (SAS Institute, Cary, NC, USA).

5.4 RESULTS

5.4.1 Field: Lek Structure

There was significant variation between the 12 different sample sites in female fecundity ($F_{11,535} = 4.4255$, $P < 0.0001$), male thorax size ($F_{11,564} = 4.5549$, $P < 0.0001$), female thorax size ($F_{11,530} = 2.8327$, $P = 0.0013$) and density ($F_{11,848} = 448.0089$, $P < 0.0001$). There was also significant temporal variation in each of my four environmental stress indicators (fecundity $F_{2,544} = 16.3824$, $P < 0.0001$; male thorax size $F_{2,573} = 10.6183$, $P < 0.0001$; female thorax size $F_{2,539} = 16.2842$, $P < 0.0001$; density $F_{2,857} = 15.3179$, $P < 0.0001$).

In order to gather a general measure of environmental quality, I conducted a principal components analysis using these four variables. Each variable included in the principal component analysis (fecundity, male thorax size, female thorax size and density) correlated significantly and positively with PC1 (Table 5.1). Principal component 2

(PC2) correlated positively with male thorax and negatively with density while principal component 3 (PC3) correlated positively with fecundity and negatively with female thorax (Table 5.1) although fecundity was not significant after controlling for multiple testing.

PC1 scores were positively associated with lek size (which includes all males and females) ($F_{1,28.39}$ = 10.7433, P = 0.0028) as well as the number of females per lek ($F_{1,30.17}$ = 4.9378, P = 0.0339; Figure 5.1). PC1 scores also correlated positively with variation in the number of females per lek within a site (coefficient of variation $F_{1,25.69}$ = 26.1769, P < 0.0001).

Across all populations I found that large eyespan males held larger leks ($F_{1,473}$ = 43.6034, P < 0.0001), however I also found that males with larger absolute eyespan held larger harems when PC1 values were high (male eyespan × PC1 interaction, $F_{1,432.2}$ = 12.1753, P = 0.0005). In order to control for the possibility that this relationship was driven by the suite of measurements in PC1, including male thorax size, which strongly covaries with male eyespan (Wilkinson and Reillo, 1994), I derived PC1* that only included female thorax (r^2 = 0.5298, $F_{1,34}$ = 38.3167, P < 0.0001), fecundity (r^2 = 0.5954, $F_{1,34}$ = 50.0289, P < 0.0001) and density (r^2 = 0.5810, $F_{1,34}$ = 47.1377, P < 0.0001). PC1* also showed that males with larger absolute eyespan held larger harems when PC1* values were high (male eyespan × PC1 interaction, $F_{1,444}$ = 13.0540, P = 0.0003) and this relationship held for relative males eyespan (i.e. including male thorax size as a covariate; $F_{1,443.1}$ = 13.1018, P = 0.0003). Thus, the relationship between male eyespan and harem size is not caused by their common relationship with male body size.

PC2 and PC3 did not associate with harem size (PC2 $F_{1,30.25} = 0.1631$, $P = 0.6891$; PC3 $F_{1,33.5} = 0.9258$, $P = 0.3429$) or the coefficient of variation in the number of females per lek within a site (PC2 $F_{1,26.27} = 0.0844$, $P = 0.7737$; PC3 $F_{1,31.34} = 1.5085$, $P = 0.2285$).

5.4.2 Field: Fecundity and Female Lekking Behaviour

Whether a female was fecund or barren did not influence the levels of her abdomen bobbing ($\chi^2_1 = 0.5326$, $N = 33$, $P = 0.4655$) or lek walking ($\chi^2_1 = 1.7964$, $N = 33$, $P = 0.1801$). The lack of a relationship remained when abdomen bobbing was examined against absolute fecundity of the female ($\rho_{31} = 0.1609$, $P = 0.3712$). However there was a positive correlation between fecundity and lek walking ($\rho_{31} = 0.3501$, $P = 0.0458$). Whether a female was fecund or barren influenced the amount of sampling behaviour (moving between leks) a female engaged in, with fecund females exhibiting increased sampling behaviour ($\chi^2_1 = 8.4432$, $N = 33$, $P = 0.0037$). This relationship also held true when examining the absolute fecundity of the female ($\rho_{31} = 0.5421$, $P = 0.0011$; Figure 5.2). Fecund females also mated more frequently (fecund vs. barren, $\chi^2_1 = 8.3412$, $N = 33$, $P = 0.0039$; absolute fecundity, $\rho_{31} = 0.4256$, $P = 0.0135$).

5.4.3 Laboratory: Behaviour and Lek Structure

Fighting

I found no effect of treatment group on the frequency of fighting between males (high quality diet mean ± SE = 0.39 ± 0.08; low quality diet mean ± SE = 0.57 ± 0.12, $\chi^2_1 = 0.4745$, $N = 224$, $P = 0.4909$), or between females (high quality diet mean ± SE = 0.20

± 0.06; low quality diet mean ± SE = 0.09 ± 0.03, χ_1^2 = 2.5961, *N* = 224, *P* = 0.1071). However, fight duration was higher in poor quality diet females (χ_1^2 = 3.9316, *N* = 21, *P* = 0.0474), although there was no difference between the two diet classes of males (χ_1^2 = 0.0344, *N* = 203, *P* = 0.8529).

Patrolling

There was no difference in the frequency of lek patrolling performed by males on the high or low quality diet (large males: high quality diet mean ± SE = 0.96 ± 0.11; low quality diet = 0.68 ± 0.08, χ_1^2 = 2.4637, *N* = 224, *P* = 0.1165; small males: high quality diet = 0.30 ± 0.06; low quality diet mean ± SE = 0.22 ± 0.05, χ_1^2 = 1.6261, *N* = 224, *P* = 0.2022). However, the total time spent patrolling was greater in high quality diet males (high quality diet = 58.22 ± 6.77, low quality diet = 36 ± 5.15, χ_1^2 = 6.5590, *N* = 224, *P* = 0.0104).

Mate rejection

Females in the high diet quality group showed a significantly higher frequency of mate rejection behaviour than females in poor diet quality (χ_1^2 = 7.3974, *N* = 224, *P* = 0.0065; Figure 5.3). Large females in both good and poor diet quality made significantly more rejections than small females (good diet quality χ_1^2 = 8.8058, *N* = 224, *P* = 0.0030; poor diet quality χ_1^2 = 6.0871, *N* = 224, *P* = 0.0136). Mate rejection was non-random with respect to male phenotype among good diet quality females, since small males were rejected as mates significantly more than large males (χ_1^2 = 14.3459, *N* = 224, *P* = 0.0002). Among females in poor diet quality, there was no bias in the rejection of different male phenotypes (χ_1^2 = 1.7156, *N* = 224, *P* = 0.1903).

Mating

The number and duration of matings increased when flies were in good diet quality (number χ_1^2 = 12.9662, N = 224, P = 0.0003; duration χ_1^2 = 6.2908, N = 224, P = 0.0121). The primary difference in mating number was in the large male and large female pairings, with significantly more matings, as well as increased duration, occurring between these size classes when flies were in good diet quality (number χ_1^2 = 11.1456, N = 224, P = 0.0008; duration χ_1^2 = 10.0187, N = 224, P = 0.0015) compared to flies in poor diet quality. Similarly the number (χ_1^2 = 6.5791, N = 224, P = 0.0103) and average duration (χ_1^2 = 5.6890, N = 224, P = 0.0171) of matings between large males and small females also increased significantly when flies were in good diet quality compared to flies in poor diet quality. There was no difference in mating between small males in good or poor diet quality, either in mating frequency (with large females χ_1^2 = 0.2132, N = 224, P = 0.6442; with small females χ_1^2 = 2.4688, N = 224, P = 0.1161) or duration (with large females χ_1^2 = 0.1932, N = 244, P = 0.6602; with small females χ_1^2 = 2.8087, N = 224, P = 0.0938).

Lek structure

I found that poor diet quality flies congregated in smaller leks than those reared on a high diet quality (lek size of poor diet quality flies mean ± SE = 1.3 ± 0.03, lek size of high diet quality flies mean ± SE = 1.50 ± 0.04, χ_1^2 = 18.4289, N = 798, P < 0.0001). Flies were also more likely to roost as solitaries when they were in poor diet quality groups (χ_1^2 = 7.0280, N = 1148, P = 0.0080). These observed changes were primarily the result of changes in female behaviour, since the number of females per lek (harem size) decreased significantly when flies were on the poor quality diet (χ_1^2 = 14.2012, N = 747, P = 0.0002). In contrast the number of males on the lek was not influenced by

diet quality (χ^2_1 = 0.0340, N = 407, P = 0.8538). The coefficient of variation in the number of females per lek increased significantly when flies were raised on the good diet (CV high diet quality = 44.3796%, CV poor diet quality = 32.4046%, Z = 15.4834, $P < 0.0001$).

Large eyespan males were more likely to accrue larger leks (pooled across diets, $F_{1,403}$ = 33.9268, $P < 0.0001$) and large eyespan males were more likely to accrue larger leks under good diet quality (male eyespan × diet interaction, $F_{1,403}$ = 6.4286, P = 0.0116; Figure 5.4). The exact same relationship was seen in females, with larger eyespan females on larger leks ($F_{1,743}$ = 35.2921, $P < 0.0001$), driven by females under good diet quality ($F_{1,743}$ = 11.2883, P = 0.0008).

5.5 DISCUSSION

The handicap hypothesis, a major theory used to explain exaggerated secondary sexual traits, predicts that such traits should exhibit heightened condition dependence (Cotton *et al.*, 2004a). The cost of producing exaggerated ornamentation is not fixed, with males in poor condition suffering a disproportionally larger cost of ornamentation (Zahavi, 1975; Grafen, 1990; Iwasa and Pomiankowski, 1994). Thus the largest males should also be those in the best condition, allowing females to make mating decisions based on honest signals of quality (Zahavi, 1975).

Environmental stress is often found to have opposing effects on the mean (tends to decrease) and variance (tends to increase) of condition-dependent traits (David *et al.*, 2000; Cotton *et al.*, 2004a; Garant *et al.*, 2004; Hunt *et al.*, 2004; Charmantier and

Garant, 2005). Whilst a reduction in the mean of preference and ornamentation would be predicted to result in weakened sexual selection (Poulin and Vickery, 1996; Pfennig and Tinsley, 2002), an increase in variance (both of condition-dependent ornaments (Rowe and Houle, 1996) and preferences (Cotton *et al.*, 2006b)) could heighten the potential for, and advantages of, sexual selection. Whilst our understanding of how environmental variation affects ornament evolution are developing, our knowledge of how the same conditions affect sexual selection more broadly is woefully lacking. In this study I examined how lek structure and mating behaviour were affected by environmental stress, utilising controlled laboratory experiments as well as data from natural populations. My results overwhelmingly suggest that environmental quality can influence components of sexual selection in stalk-eyed flies.

Results from both the field and laboratory were highly congruent, and showed that in poor environments (with PC1 values, a combination of density, fecundity, male and female thorax size in the field; poor diet in the laboratory), the mean number of females per lek (harem size) decreased. In addition to between-population correlations, I also found that within populations there was a significant decrease in (coefficients of) variance in harem size in poor environments, again in field and laboratory. Sexual selection is generated by non-random mating and it is expected that increased variance in mating success between males would result in stronger sexual selection (Andersson, 1994). My results suggest, therefore, a high potential for sexual selection in good environments. Large eyespan males accrued the largest leks, which is in line with previous work in the field (Cotton *et al.*, 2010)**.** Critically however, I found that this relationship was accentuated when environmental quality was high, as mean and variance in harem size increased in good condition. In stalk-eyed flies, harem size and

mating success are highly correlated, with males that accrue large leks mating with significantly more females per day (Sam Cotton, unpublished data).

Much research has been done investigating the potential for fluctuating asymmetry (FA) in sexual traits (such as eyespan) to be a powerful indicator of phenotypic or genetic quality (Arnqvist and Thornhill, 1998; David *et al.,* 1998). FA is where bilateral traits do not exhibit perfect symmetry and is thought to arise as a result of the inability to buffer against environmental changes or disturbances (Van Valen, 1962). Due to the condition-dependent nature of sexual traits, the FA of such traits were often hypothesised to be more sensitive to environmental stress, as compared to non-sexual traits. Evidence for this was inconsistent from the beginning (Arnqvist and Thornhill, 1998; David *et al.,* 1998) and in stalk-eyed flies FA was found to be a poor indicator of developmental stress and genetic quality with zero heritability (Bjorksten *et al.,* 2001). Due to the highly conflicting evidence surrounding FA, I decided it was unlikely that this would be a useful indicator of environmental stress in this study.

The laboratory study allowed me to examine specific behaviours associated with male-male competition and female mate preferences. Large males spent more time patrolling when they were on the good diet. This behaviour appears to be performed by males to increase the likelihood of encountering rivals on the rootlet – and so of guarding females against intruding males. The increase in lek patrolling seen among large, lek-holding males might have been driven by increases in male condition when fed the good diet allowing them more resources for this activity. But it may alternatively have been driven by the associated increase in female reproductive potential on the good diet, as females in good condition are more fecund (Hingle *et al.*, 2001a, b; Cotton *et al.*, 2014a; chapter 6). In addition, condition strongly influenced those behaviours

associated with female mate choice, with increased rejections seen in female flies on the good diet (primarily of small males by large females), as well as an increase in the number and duration of matings (primarily driven by matings between large individuals). These results support previous research that also found that female mate choice exhibited condition-dependent expression (Hunt *et al.*, 2005; Cotton *et al.*, 2006a, b). I did not measure fecundity in laboratory females as a large body of previous work in stalk-eyed flies has shown a strong relationship between female condition and fecundity (Hingle *et al.*, 2001a, b; Cotton *et al.*, 2014a). I found qualitatively similar results in the field compared to the laboratory, showing that mate sampling and mating frequency was significantly higher in more fecund flies. Future work examining female sampling and mating preferences in the field should focus on both within and between site variation and ask how this is correlated with the quality of the site.

Larval diet was manipulated randomly prior to treatment allocation in order to maximise variance in eyespan (Cotton *et al.*, 2004b). Within the adult treatment groups there was an equal contribution of flies raised on the different larval diets i.e. 3 low larval stress and 3 high larval stress flies in each group). Therefore one would expect no biased effects of larval diet manipulation on adult reproductive traits.

In this study I used lek structure and mating behaviour as measurable components of sexual selection. While my current approach is unable to account for other aspects of sexual selection, for example sperm competition (Birkhead and Møller, 1998; Andersson and Simmons, 2006; Smith, 2012) and actual reproductive output (Wade, 1979; Andersson, 1994; Pischedda and Rice, 2012), the measures used in this study are likely to be important determinates of variation in male mating success.

Tributaries of the Gombak river system comprise diverse environments, differing in altitude, aspect, vegetation type, humidity and cover, and all these change through time and are likely to strongly influence the strength of sexual selection (e.g. through food resources, local population density, lek site occupancy). Movement of individuals between adjacent streams is low (Sam Cotton, unpublished data), and thus flies from any given stream are likely to have developed and matured locally. Whilst environmental quality is traditionally determined by recording a series of abiotic factors such as those described above, it is unknown exactly how these factors actually influence the condition of the flies and therefore the important mechanistic linkages are largely conjecture. By using known biological proxies of environmental quality (Hingle *et al.*, 2001a; Rogers *et al.*, 2005a, b; Rogers *et al.*, 2008; Cotton *et al.*, 2014b), I can circumvent this problem, allowing the aspects of individuals phenotypes within a population to reveal the quality of the site. The problems associated with condition indices have been discussed in depth (Tomkins *et al.*, 2004; Cotton *et al.*, 2006b; Lailvaux and Irschick, 2006), with specific issues relating to single indices of condition that are often used such as body mass (Brandt and Greenfield, 2004) or the use of residuals (generally using the regression of body mass on body size) (Kotiaho *et al.*, 2001). Single indices of condition are inadequate because condition itself is a multivariate trait, comprising numerous major life history characteristics, and thus attempting to quantify condition using a single arbitrary trait is an exercise in futility. Therefore multivariate analyses, such as principal components analysis (used in this study), have been touted as a more robust method of assessing individual condition as they consider numerous life history traits and summarise major axes of phenotypic covariance among them (Bussiere *et al.*, 2008). Multivariate approaches therefore

allow complex traits such as condition to be quantitatively estimated with far fewer assumptions that those of single dimension estimates.

There were limitations associated with both elements of this study. In the field study, these arose primarily through the complication of attempting to estimate environmental stress in different populations, as there is no obvious or easily measured variable for this. I chose to utilise a variety of correlated variables (fecundity, population density and male and female thorax size) and one complication of this is that they are not entirely independent from the response variables I was examining. This was exacerbated by the limited sample size, as some populations only had a small number of flies, and thus measures of environmental stress may have been distorted and not provided an accurate view of environmental stress. In the laboratory study, I attempted to combat this issue, by having very clear-cut levels of environmental stress in the form of dietary treatments. I was not, however, able to replicate natural variation in population size and potential lek structure in the laboratory and I chose to use standardised populations comprising 4 females (2 large and 2 small) and 2 males (1 large and 1 small). I did this because it was the smallest combination of eyespan and sex variables in the most biologically realistic scenario – in natural populations, *T. dalmanni* leks will typically be helmed by a single male (with small males behaving as satellites) and have a small number of females (from 1 – 4) (Cotton *et al.,* 2010). By using this number and combination of flies, I was allowing some flexibility in lek structure whilst controlling for variation in population size.

In this study I found that stressful environments were associated with a suite of behavioural and morphological traits that have the potential to decrease the strength of sexual selection. I found that mean and variance in harem size decreased in poor

environments as well as condition-dependence of female mate preferences. The potential implications of this resonate for the field of conservation, where increasing numbers of species are living in highly stochastic (Post *et al.*, 1999; Garant *et al.*, 2004) and increasing human altered (deterministic) habitat (Hill, 1995; Stratford and Stouffer, 2001) where the long term evolutionary consequences of these habitat changes are poorly understood. Further work is needed to examine the potential consequences of major environmental change on other major components of sexual selection, focussing on the reproductive potential and actual reproductive success both within and between different populations.

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Table 5.1. Table showing the relationship between a series of environmental variables (fecundity, male thorax size, female thorax size and population density) and three principal components. *P* values in bold remained significant after Holm-Bonferroni correction ($P < 0.05$), those underlined were not significant after this correction ($P > 0.05$).

		Environmental Variables			
Principal Component	***Statistics***	***Fecundity***	***Male Thorax Size***	***Female Thorax Size***	***Population Density***
PC1	r^2	0.5515	0.3343	0.5233	0.4878
	F	41.8004	17.0771	37.3286	32.3855
	DF	1, 34	1, 34	1, 34	1, 34
	P	**< 0.0001**	**0.0002**	**< 0.0001**	**< 0.0001**
PC2	r^2	0.0440	0.5722	0.0047	0.2248
	F	1.5665	45.4821	0.1606	9.8593
	DF	1, 34	1, 34	1, 34	1, 34
	P	0.2193	**< 0.0001**	0.6911	**0.0035**
PC3	r^2	0.1203	0.0586	0.4613	0.0180
	F	4.6508	2.1151	29.1196	0.6249
	DF	1, 34	1, 34	1, 34	1, 34
	P	0.0382	0.1550	**< 0.0001**	0.4347

Figure 5.1. The relationship between the coefficient of variation (CV) of harem size (number of females per lek) and principal component 1 (PCA1), which act as a representation of environmental quality in the field. Low PCA1 scores correspond to populations of poor quality while high PCA1 scores correspond to high quality populations. The shaded area represents the 95% confidence interval around the line of best fit.

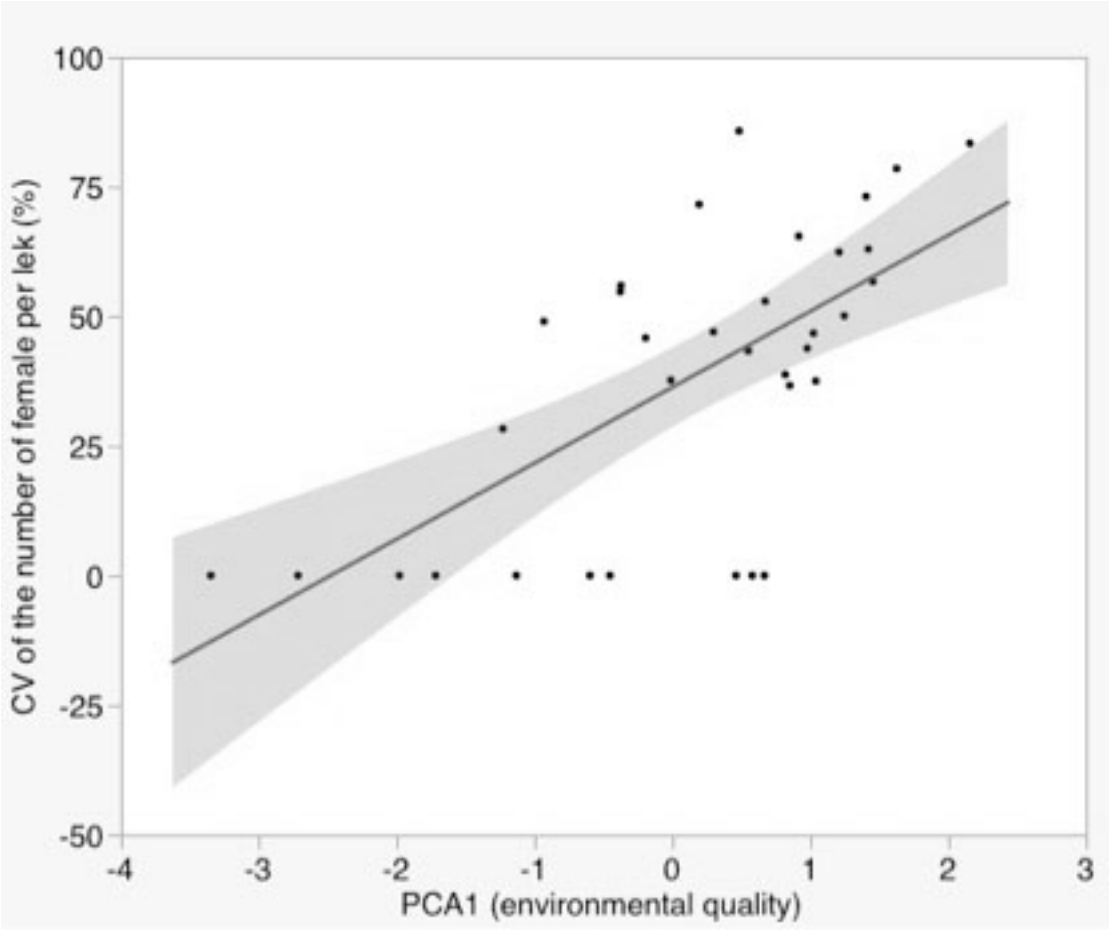

Figure 5.2. Line of best fit showing the relationship between female sampling behavior (a measure of female mate searching) and fecundity. The shaded area represents the 95% confidence interval around the line of best fit.

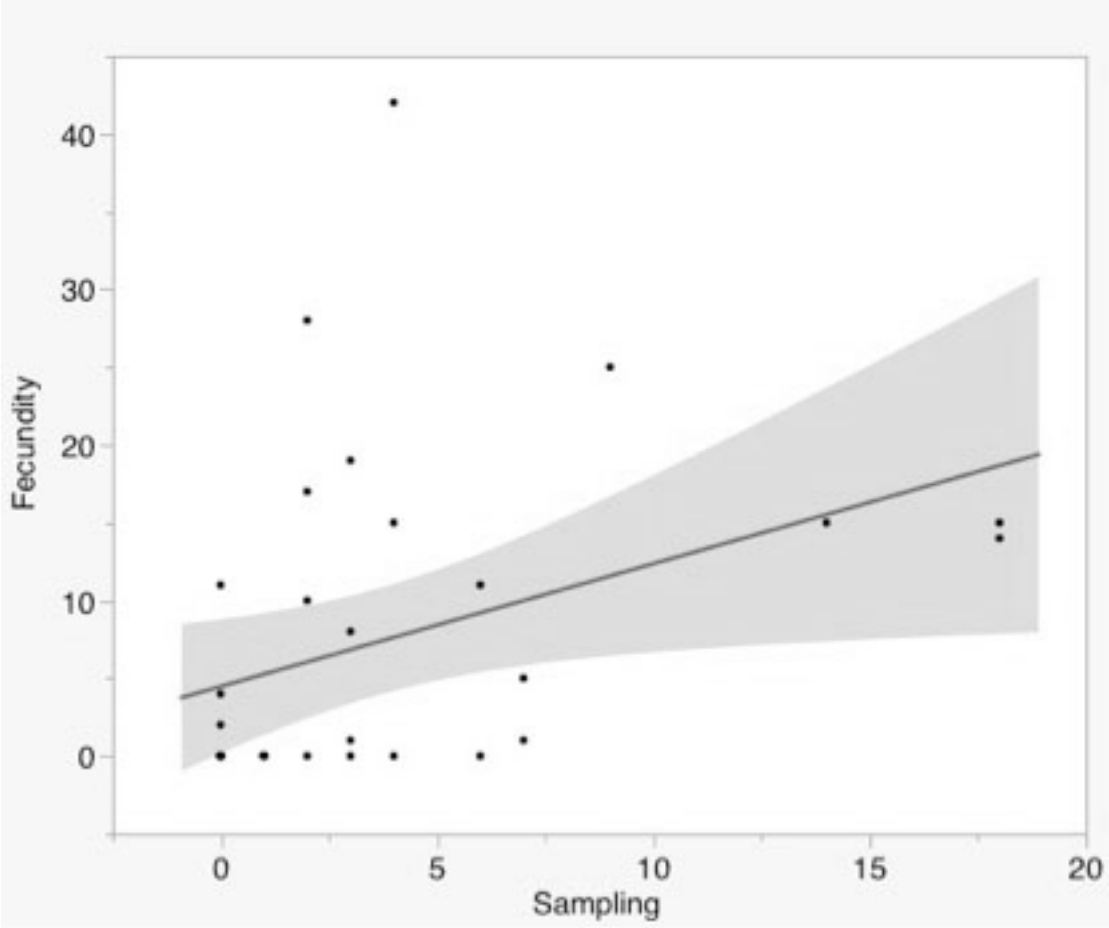

Figure 5.3. Boxplot showing interquartile range of male rejections depending on whether the flies were in good condition or poor condition in the laboratory study. Difference between condition treatments was $P < 0.05$.

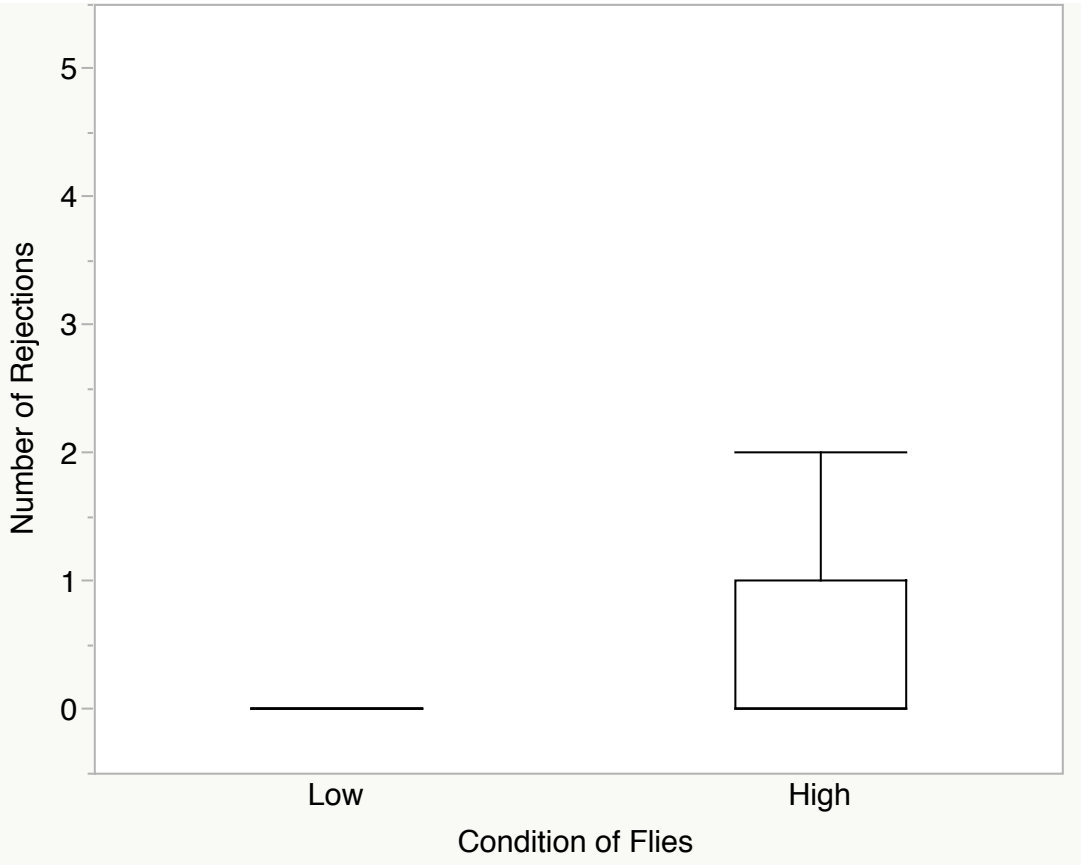

Figure 5.4. Box plot graph depicting the interquartile range of lek sizes held by large and small eyespan males in the laboratory study. Blue plots represent high quality flies while red plots represent poor quality flies.

6

Male mate preference for female eyespan and fecundity in the stalk-eyed fly, *Teleopsis dalmanni*

6.1 ABSTRACT

Traditional views of sexual selection assume males to be the indiscriminate sex, competing for access to choosy females. It is increasingly recognized that mating can also be costly for males, and they are therefore likely to exhibit choice in order to maximize their reproductive success. Stalk-eyed flies are a model species in sexual selection studies. Males are sperm-limited and constrained in the number of matings they are able to partake in. In addition, variation in female fecundity has been shown to correlate positively with female eyespan, so female eyespan could provide males with a reliable signal of female reproductive value. I examined male mate preference in the wild in the stalk-eyed fly, *Teleopsis dalmanni*. In addition, I set-up experiments in the laboratory allowing males a choice between females that varied in either 1) eyespan (a proxy for fecundity) and/or 2) fecundity (manipulated through diet). I found that males exhibited preference for large eyespan females, both in the wild and laboratory studies. As well as using female eyespan as a mating cue, males were also able to assess female fecundity directly. Changes in fecundity among large eyespan females caused corresponding changes in male mate preference, while changes in the fecundity of small eyespan females had limited effect on their attractiveness. These results show that male mate preferences are a prevalent feature of a canonical example of female mate choice sexual selection, and that males use multiple cues when they assess females as potential mates.

6.2 INTRODUCTION

Sexual selection has traditionally been viewed as competition among undiscriminating males for access to choosy females (Darwin, 1871; Bateman, 1948; Trivers, 1972). However, it is increasingly recognized that this perspective is too simplistic, and that mating can be a constrained or costly for males (Dewsbury, 1982; Bonduriansky, 2001; Webberley and Hurst, 2002; Wedell *et al.*, 2002; Andrade, 2003). Males should therefore allocate their matings prudently so as to maximize their reproductive success. This leads to the prediction that males, as well as females, should discriminate among individuals when choosing a mate (Bonduriansky, 2001).

Parker (1983) noted that choosiness is favored when high variance in quality exists in the opposite sex; if there is little variation in mate quality then there will be few advantages accruing from mate preference (Parker, 1983; Gwynne, 1991). In promiscuous species, males are expected to select females on the basis of fecundity (Bonduriansky, 2001). Nonetheless, directional male mate preference is expected to evolve for traits that reflect female reproductive value even when signaling compromises female viability and male mate preference is costly (Servedio and Lande, 2006; Nakahashi, 2008). It has been suggested that males may use female body size as a proxy for fecundity, as size and fecundity tend to correlate (Honěk, 1993). In several polygynous species, females display ornament-like traits that may have initially evolved as a correlated response to sexual selection on homologous male ornaments (Lande and Arnold, 1985). These may subsequently have been co-opted as targets of male mate preference, given that such traits in females are conspicuous, easy to evaluate and often reflect aspects of female quality linked to fecundity (Amundsen, 2000). Male mating preference for female ornaments has been shown in several insect

species where the ornament is thought to be an indicator of fecundity, like dance flies (Funk and Tallamy, 2000; LaBas *et al.*, 2003) and the mosquito *Sabethes cyaneus* (South and Arnqvist, 2011).

The evolution of male mate preference is also likely to be influenced by the constraints and costs arising from mating (Bonduriansky, 2001). If males are able to mate cost-free with every female they encounter, there is little reason for discrimination to evolve. For preference to be favored, male mating investment must be limited. It is increasingly recognised that sperm and ejaculates become depleted by repeated mating. This sets limits to the mating rate (Dewsbury, 1982; Wedell *et al.*, 2002) and to investment in subsequent matings (Preston *et al.*, 2001; Wedell *et al.*, 2002). Mating preference for particular female phenotypes implies lower male mating success (Servedio and Lande, 2006; Nakahashi, 2008). As increasing numbers of males court or mate with attractive females, so each has a smaller chance of success (i.e. of mating and of paternity). On the other hand, the costs associated with finding and assessing females must not be prohibitive (Nakahashi, 2008). Selection will favor the evolution of male mate preference when the costs of mate searching and sampling are not high, for example, when the distribution of females is clumped and males are able to assess them easily (Forsberg, 1987).

The Malaysian stalk-eyed fly *Teleopsis dalmanni* (Diopsidae; Diptera) is an important model species for studies of sexual selection (Wilkinson and Dodson, 1997; Maynard Smith and Harper, 2003). Stalk-eyed flies are characterized by having their eyes displaced laterally from the head on elongate 'eye-stalks', in both sexes. Eyespan (the distance between the eyes) is sexually dimorphic in *T. dalmanni*, being much enlarged in males as a result of sexual selection. In natural populations, *T. dalmanni* form

nocturnal lekking aggregations on root hairs that hang underneath the eroded banks of rainforest streams (Burkhardt and de la Motte, 1985; Wilkinson and Dodson, 1997; Cotton *et al.*, 2010). Males fight for control of these roosting sites (Wilkinson and Dodson, 1997; Small *et al.*, 2009), and females prefer to alight on root hairs controlled by males with large eyespan (Wilkinson and Reillo, 1994; Hingle *et al.*, 2001a, b; Cotton *et al.*, 2010). The vast majority of matings occur in these aggregations during the dawn and dusk period, when males attempt to mate with females in their harem (Burkhardt and de la Motte, 1988; Lorch *et al.*, 1993; Small *et al.*, 2009; Cotton *et al.*, 2010). *T. dalmanni* is therefore a textbook example of harem-based polygyny, with choosy females and competitive males.

However, evidence suggests that there is a high potential for male mate preference in *T. dalmanni*. Laboratory experiments have shown that female fecundity is sensitive to environmental (dietary) stress (Hingle *et al.*, 2001a), suggesting that variance in female quality is high. This is borne out in the wild, where female fecundity is highly variable (Cotton *et al.*, 2010). Moreover, female eyespan is an accurate and reliable indicator of female fecundity in wild females, even after controlling for its co-variation with body size (Cotton *et al.*, 2010). Female eyespan in *T. dalmanni*, like the ornamental homologue in males, is prominent and easily assessed suggesting that it could serve as a useful cue for males to use in mating decisions. In the related African stalk-eyed fly species *Diasemopsis meigenii*, males mate for longer and transfer more sperm to females with larger eyespan (Harley *et al.*, 2013). As female eyespan is positively correlated with fecundity in *D. meigenii*, males could gain a selective advantage by investing more in large eyespan females.

In addition, there is evidence that male stalk-eyed flies suffer constraints on multiple

mating. In *T. dalmanni*, male mating frequency is correlated both phenotypically and genetically with the size of the accessory glands, the paired internal organs involved in spermatophore production (Baker *et al.*, 2003; Rogers *et al.*, 2005a). Accessory glands become depleted with successive matings (Rogers *et al.*, 2005b). When males with larger accessory glands are allowed to mate with multiple females over a short period of time, they confer higher fertility on females, probably because they mate at a higher rate (Rogers *et al.*, 2008). In *Diasemopsis meigenii*, males with larger eyespan (and hence larger accessory glands and testes) show smaller reductions in spermatophore size and number of sperm transferred over successive matings, relative to the performance of small eyespan males (Liz Harley, unpublished data). Taken together these experimental results suggest that there are limitations on the mating rate of males that have attracted many females to their lek sites. In the dawn period, when most mating occurs, males have about 20-30 minutes in which to mate with females in their harem. Yet typically, some females are observed to disperse from lek sites before mating with the dominant male. Under such constraints there is the opportunity for males to direct their mating attempts towards those females that have the highest reproductive value.

Given these features of the mating system in *T. dalmanni*, and the fact that the harem-based mating system in this species allows males to choose among groups of females with relatively low costs of search and assessment, I hypothesized that males should discriminate between females on the basis of their fecundity. I investigated the potential for male mate preference in the flies' native habitat in the Malaysian rainforest and also under laboratory conditions. This combined approach allowed me to examine male mate choice both under biologically realistic conditions as well as under controlled, experimentally manipulated conditions. In the latter case, I

manipulated the eyespan of experimental females in order to assess whether this trait is used in male choice, as well as altering the adult diet of females in order to vary female fecundity in a controlled manner. I then used a series of mate choice tests to examine whether males were primarily using female eyespan as an indicator of fecundity or were able to assess fecundity directly.

6.3 MATERIALS AND METHODS

6.3.1 Male Mate Preference in Field Conditions

Field data were collected during two phases of fieldwork carried out on a *T. dalmanni* population at Ulu Gombak, Peninsular Malaysia (3°19' N, 101°45' E) during July/August in 2006 and 2007. Lek sites (exposed root hairs) on the banks of a tributary of the Gombak river were identified after dusk. Observations of male mating behavior at focal harems was conducted during the following dawn period, starting at approximately 06:55 when flies were still quiescent, and ending at approximately 07:45 when flies had usually dispersed into the forest. In a few instances ($N = 3$) more than one male was present on the root hair. In order to obtain information from a single focal male per harem, the additional males were carefully removed without disturbing the other individuals (the most easily removed male was chosen). The harem size was noted (number of females present) and the frequency of successful matings with each female, defined as a copulation ≥ 30 s, as shorter copulations do not usually result in transfer of a spermatophore (Lorch *et al.*, 1993; Rogers *et al.*, 2006).

Females within each harem were categorized by inspection into large and small eyespan classes. Observers (Sam Cotton and Jen Small) were experienced in judging fly size from prior experience with field populations of *T. dalmanni*. A relative measure was used to categorize females within a lek. Medium sized eyespan females were classified as small when the lek contained larger females, and large when there were no larger eyespan females. Data from harems in which there were no observable differences in female size were excluded from the analysis. Eyespan of the focal male was measured *in situ* non-invasively using standardized digital photographs. Images were taken with a digital SLR camera (Canon EOS 350D) through a 180 mm macro lens set to its minimum focal distance, which creates a fixed distance between the camera and the subject. The focal male was kept perpendicular to the camera by keeping both eye bulbs in focus. Eyespan was then estimated from the size of the resultant image relative to a known standard, photographed under identical conditions. This method is highly accurate compared to controlled measurements of the same individuals (repeatability > 0.93, (Lessells and Boag, 1987; Small *et al.*, 2009)).

6.3.2 Laboratory Studies – Origin of Flies and Generation of Flies for Experiments

The flies used were from a population collected in Ulu Gombak, Peninsular Malaysia (3°19’ N, 101°45’ E) in 2005 (Sam Cotton and Andrew Pomiankowski). They have since been maintained in the laboratory in cage culture (>200 individuals to minimize inbreeding) at 25°C on a 12:12 h light: dark cycle, and fed pureed sweetcorn twice weekly. Fifteen-minute artificial dawn and dusk periods were created by illumination from a single 60 W bulb, at the start and end of the light phase.

To obtain experimental flies I collected eggs from the cage cultures over a 3-week period and reared larvae on a variable amount of pureed sweetcorn to ensure high variance in eyespan (David *et al.*, 1998; Cotton *et al.*, 2004). Upon eclosion, flies were anaesthetized on ice and measured for their eyespan, defined as the distance between the outermost lateral edges of the eye-bulbs (Cotton *et al.*, 2004). Following Rogers *et al.* (2006), females were separated into large and small size classes, defined as having eyespans >5.8 mm or <5.4 mm respectively. Intermediate size females were discarded. To control for the well-documented effects of male eyespan on female mating behavior (Wilkinson and Reillo, 1994; Hingle *et al.*, 2001a, b), I used only large eyespan males (> 8.5 mm) with a low sample variance (N = 36, mean ± SD = 8.94 ± 0.31 mm) in the subsequent assays of preference. In addition, previous work has shown that small eyespan females are less able to discriminate amongst variation in male eyespan (Hingle *et al.*, 2001a), and this effect may extend to male ability to discriminate. Finally, because small eyespan males have fewer opportunities to mate under field conditions (Cotton *et al.*, 2010), their mate preferences may differ compared to large eyespan males. Though this is a topic of interest, it is beyond the scope of the current study.

6.3.3 Male Mate Preference for Female Eyespan

Male mate preference under laboratory conditions was examined using a specially designed cage. This comprised two 500ml transparent plastic pots, one inverted on top of the other, and separated by a pair of opaque removable partitions (Figure 6.1). A roosting string hung from the ceiling of the upper pot extending down to near the base of the lower pot. The base of the test cage contained moist cotton wool to maintain high humidity. The focal male and the pair of tester females, one large and one small,

were introduced during late afternoon on the day prior to the assay. The male was placed in the upper half of the cage and the females in the lower half of the cage, and the partitions were inserted to keep the sexes segregated (Figure 6.1).

At the beginning of the dawn period on the following morning the partitions were removed allowing the flies to interact. Male mating behavior was observed for 30 minutes. A mating was defined as a copulation lasting ≥ 30 s (as in the field study). A sample of $N = 36$ males were assayed for mate preference. Large and small females were drawn at random from a population of each type of tester female ($N > 25$ for each type). Tester females were therefore used more than once in the trials. However, they were never used more than once in any 48-hour period.

All individuals used in the experiment were non-virgins, having been kept in mixed sex groups prior to the mate preference experiment. They were collected over a short period of time (3 weeks) and so were of similar age when used in the experiment (~8+ weeks). Female *T. dalmanni* are highly promiscuous and mate at high frequencies (Wilkinson *et al.*, 1998; Rogers *et al.*, 2005a). Female reproductive lifespan is in the order of months (Wilkinson and Reillo, 1994; Reguera *et al.*, 2004), so the incidence of female virgins under natural conditions is rare, a feature that I aimed to mimic in this design. Females lay eggs continually after reaching maturity, irrespective of whether they have mated (Baker *et al.*, 2001; Reguera *et al.*, 2004). Moreover, the frequency of matings among mated females has no detectable effect on egg output (Baker *et al.*, 2001). So I consider details of female mating history prior to the experiment will have had a minimal influence on the egg-laying rate. Likewise male virgins are also rare in nature, as they also mate promiscuously and live for months (Wilkinson and Reillo, 1994; Reguera *et al.*, 2004). Therefore, I also housed the

experimental males in mixed-sex groups. This procedure further allowed comparison between field and laboratory experiments.

To examine male fitness benefits of mating with large and small females, I measured the fecundity of a sample of females using a previously developed protocol (Cotton *et al.*, 2006a), after the mate preference assays were complete. Tester females (N = 23 large and N = 22 small females) were housed individually in 500ml pots with a roosting string hanging from the top and moist tissue paper and a food tray at the base. The tissue and food were removed from the containers every 2-3 days and all the eggs on both substrates were counted. Females were allowed to acclimatize in their new pots for 3 days, and their fecundity was measured over the subsequent 11 days (N = 5 collections per female).

6.3.4 Male Mate Preference for Female Eyespan and Fecundity

I examined the relative importance of female eyespan and fecundity in determining male mate preference under laboratory conditions. Large and small female eyespan classes were set up as defined above. Fecundity was experimentally manipulated by placing females on a reduced quality diet for two weeks prior to the start of the mate preference assays and then throughout the remainder of the experiment (N = 50 for each eyespan class). The reduced diet consisted of 20% corn to 80% sucrose. The effect of this diet on fecundity was compared to that for females on a high quality diet of 80% corn to 20% sucrose. To ensure that all the food had the same viscosity, an indigestible bulking agent, carboxymethycellulose (3% w/v), was added to the sucrose (25% w/v) solution (Rogers *et al.*, 2008). Half of the females from each eyespan class were placed on each diet. To characterize the effect of the diet manipulation on egg

production, I assessed female fecundity after the completion of the mate preference assays. Eggs were collected (as above) from pots (N = 15) each containing 10 females (in each pot all females were either on the high or reduced quality diet). Eggs were collected every 2-4 days over a 15-day period (N = 6 collections per female).

I examined male mate preference across five different treatment groups (Table 6.1) using the same mate preference assays described above. Treatments 1 and 2 were control treatments for high and reduced fecundity, respectively, while varying eyespan. Treatments 3 and 4 controlled for large and small eyespan, respectively, while varying fecundity. Treatment 5 manipulated both fecundity and eyespan, by giving each focal male the choice between a large female with reduced fecundity and a small female with high fecundity. A sample size of N = 28 males was set up for each treatment.

6.3.5 Statistical Analysis

Male mate preference was assessed using an index based on the difference between the observed and expected numbers of copulations with large females. In the field study, P_{Field} was calculated for individual males and allowed for multiple copulations and variable numbers of large and small females on the lek,

$$P_{Field} = \left(c_L - \sum_{i=1}^{t} \frac{n_{Li}}{N_i} \right) \frac{1}{c_L + c_S} \ ,$$

where c_L is the number of copulations with large eyespan females and c_S is the number of copulations with small eyespan females, n_{Li} is the number of large eyespan females in the harem at the time of mating i, and N_i is the total number of females in the harem

at the time of mating *i*. This index takes into account the changing composition of leks through time, as females occasionally flew away between matings by the focal male. P_{Field} is zero under random mating, $P_{Field} > 0$ for males showing mating preference for large eyespan females, and $P_{Field} < 0$ for males showing preferences for small eyespan males. The minimum/maximum values of P_{Field} always differ by one, but are not necessarily symmetric about zero due to the distribution of large and small females on the lek through time.

P_{Field} values were not normally distributed, so I tested whether the mean of the distribution of individual male P_{Field} values was different from zero using a Wilcoxon signed-rank test. In harems in which more than one mating was observed, I tested whether the observed probability of a mating with a large eyespan female in the *i*th mating attempt was more likely than expected by chance, calculating the expected mating probability n_{Li}/N_i for that mating. I used a repeated measures approach to evaluate variation in preference across matings by testing whether the mean $P_{Field(\text{mating } i)} - P_{Field(\text{mating } i+1)}$ value was significantly different from zero using a Wilcoxon signed-rank test.

In the studies of male mate preference under laboratory conditions I used a similar index of male mate preference. This was simpler as females could not depart from the test cage, and there was always one large and one small female per pot,

$$P_{Lab} = \frac{c_L - c_S}{2\left(c_L + c_S\right)} \quad .$$

As for the field index, P_{Lab} equals zero under random mating, $P_{Lab} > 0$ for preference for large females and $P_{Lab} < 0$ for preference for small females. But in this case, the

minimum/maximum values of P_{Lab} are ±0.5 and are symmetric about zero. I used the same index when the two females differed in fecundity, substituting the copulation rate of females with high (c_H) or reduced (c_R) fecundity for those of large (c_L) and small (c_S) females. As before, I tested whether the distribution of individual P_{Lab} scores had a mean that was significantly different from zero using Wilcoxon signed-rank tests, and used similar procedures to investigate whether there was change in preference across subsequent matings.

In the second laboratory study in which females differed in fecundity and eyespan, I examined the ability of males to distinguish high fecundity females, using female eyespan as a covariate. Pairs of treatments in which fecundity differed but the eyespan of both females was large (treatment three) or small (treatment four) were combined, after separate analysis of each treatment.

In the first laboratory experiment in which females differed in eyespan alone, I examined potential fecundity differences between large and small females by performing a general linear model (GLM) on the number of eggs laid per female (5 repeated measures), nesting female identity within the eyespan variable (large or small). In the second laboratory experiment, I evaluated the effect of the diet manipulation by examining fecundity of females on the two diet treatments. To do this I performed a GLM on each the number of eggs laid per pot (6 repeated measures), nesting pot identity within diet manipulation.

All statistical analysis was performed using JMP Version. 10.0.0 (SAS Institute, Cary, NC, USA).

6.4 RESULTS

6.4.1 Male Mate Preference in Field Conditions

Just over half of observed males mated multiply under field conditions at dawn (13/25) with a mating frequency mean ± SD = 1.52 ± 0.51 (range 1-2). Males preferred to mate with large eyespan females (P_{Field} mean ± SE = +0.24 ± 0.08, Wilcoxon signed-rank = 94.00, DF = 24, P = 0.0074; Figure 6.2).

In harems in which the focal male mated twice I compared the eyespan of females across the two matings. In the first (Wilcoxon signed-rank = 42.50, DF = 12, P = 0.0012) and second (Wilcoxon signed-rank = 30.50, DF = 12, P = 0.0303) mating, males copulated more often with large eyespan females than expected given their frequency in the harem. There was no significant difference in the strength of mate preference across the two mating attempts (Wilcoxon signed-rank = -7.50, DF = 12, P = 0.2500).

I found no relationship between male eyespan and P_{Field} ($F_{1,15}$ = 0.0023, P = 0.9622), suggesting that my results are not confounded by any effect of the focal male's eyespan on male or female behavior. The number or types of female in a focal male's harem could have influenced his ability to express mate preference. However, I found no relationship between the proportion of large eyespan females in the harem and P_{Field} ($F_{1,15}$ = 1.5589, P = 0.2310), or between harem size and P_{Field} ($F_{1,23}$ = 0.6375, P = 0.4328).

6.4.2 Male Mate Preference for Female Eyespan

Within the half hour period allowed, most males (33/36) mated multiply (mating frequency mean ± SD = 3.97 ± 1.63, range 1-7), well in excess of what was typical under natural conditions. Males preferred to mate with large eyespan females (P_{Lab} mean ± SE = +0.18 ± 0.05, Wilcoxon signed-rank = 155.50, DF = 35, P = 0.0010; Figure 6.3). This result was not contingent on singly mated males, as there was still preference for large eyespan females when singly mated males were excluded (P_{Lab} = +0.19 ± 0.05, Wilcoxon signed-rank = 137.50, DF = 32, P = 0.0006). There was no relationship between male eyespan and P_{Lab} ($F_{1,30}$ = 0.4871, P = 0.4906), suggesting that by only using large eyespan males I had removed any potential confounding effect of variation in eyespan of the focal male.

When preference was examined in sequential matings, I found preference for large eyespan females in the first (P_{Lab} = +0.17 ± 0.08, N = 36, Wilcoxon signed-rank = 111.00, P = 0.0438) and second (P_{Lab} = +0.20 ± 0.08**,** N = 33, Wilcoxon signed-rank = 110.50, P = 0.0212) matings. But the patterns of the third (P_{Lab} = +0.16 ± 0.09, N = 29, Wilcoxon signed-rank = 67.50, P = 0.0951) and subsequent matings (Wilcoxon signed-rank = 34.50, P = 0.2080) were not significantly different than expected under random mating. This may partly have been due to the reduced sample size of males that mated more often. But it could have reflected a decline in preference amongst males that mated more often. When this was explicitly tested however I found no significant association between preference and the total number of matings a male engaged in ($F_{1,34}$ = 3.0292, P = 0.0908).

Females from the large eyespan group laid 36% more eggs per day than those from the small eyespan group ($F_{1,180}$ = 9.4249, P = 0.0025; mean ± SE daily egg output, large = 4.76 ± 0.49, N = 23; small = 3.49 ± 0.50, N = 22).

6.4.3 Male Mate Preference for Female Fecundity and Eyespan

I manipulated fecundity through diet, using either a reduced (20% corn) or high quality diet (80% corn). Females on the reduced diet manipulation laid fewer eggs than those on the high quality diet ($F_{1,75}$ = 111.7045, P < 0.0001; mean ± SE daily egg output, reduced = 0.2640 ± 0.02, N = 7, high quality = 1.99 ± 0.10, N = 8).

In line with the first laboratory study of male mate preference (see above), most males mated multiply (128/138), but at an even higher rate (mating frequency mean ± SD = 6.33 ± 3.12, range 1-16). In the control treatments where diet was standardized but eyespan varied (treatments 1 and 2, Table 6.1), there was a significant preference for large eyespan females (P_{Lab} mean ± SE = +0.25 ± 0.08, Wilcoxon signed-rank = 307.50, DF = 55, P = 0.0029). I found a significant difference between the two diet treatments, with stronger male mate preference for large eyespan females when females were on the high quality diet (P_{Lab} (high quality diet) = +0.41 ± 0.10, P_{Lab} (reduced diet) = +0.09 ± 0.11, χ^2_1 = 4.4544, N = 56, P = 0.0348; Figure 6.4).

I further investigated male response to variation in female fecundity by varying diet and standardizing eyespan (treatments 3 and 4, Table 6.1). Overall there was a significant preference for fecund females in the absence of eyespan variation (P_{Lab} = +0.21 ± 0.09, Wilcoxon signed-rank = 217.50, DF = 54, P = 0.0275). There was no

difference in preference when males were presented with either two large or two small eyespan females (χ^2_1 = 1.4418, N = 55, P = 0.2298).

Finally, I studied how changes in fecundity affected preference for large eyespan females. Comparing male mate preference when only large eyespan females differed in diet (treatments 1 and 5, Table 6.1), I found overall preference was for large eyespan females (P_{Lab} = +0.24 ± 0.08, Wilcoxon signed-rank = 252.50, DF = 54, P = 0.0031), and this was stronger when the large eyespan female had high fecundity (P_{Lab} (treatment 1) = +0.41 ± 0.10, P_{Lab} (treatment 5) = +0.06 ± 0.11, χ^2_1 = 4.9108, N = 55, P = 0.0267). In contrast, comparing male mate preference when only small eyespan females differed in diet (treatments 2 and 5, Table 6.1), I found no overall preference for large eyespan females (P_{Lab} = +0.07 ± 0.08, Wilcoxon signed-rank = 87.50, DF = 54, P = 0.3743), nor any difference in preference between the treatments when the small eyespan female had high or low fecundity (χ^2_1 = 0.0410, N = 55, P = 0.8396).

6.5 DISCUSSION

Male mate preference is predicted when there is exploitable variation in female quality, limited male mating capacity, and low costs to finding and assessing mates (Bonduriansky, 2001). These conditions are met in the stalk-eyed fly *T. dalmanni*: females vary considerably in fecundity (Cotton *et al.*, 2010), males have limited ability to mate multiply over short periods of time (Rogers *et al.*, 2005a, b), and the lek mating system congregates females closely with males who can choose mating order. These flies are well known for female mate preference for males with large eyespans (Wilkinson and Reillo, 1994; Cotton *et al.*, 2010), therefore it might be expected that

males might use variation in eyespan to assess females. This is logical as female eyespan has several of the properties usually associated with sexual ornaments. Firstly female eyespan in sexually dimorphic stalk-eyed fly species is an exaggerated trait, in comparison to sexually monomorphic species (Baker and Wilkinson, 2001). Secondly female eyespan is sensitive to stress compared to non-sexual traits (e.g. wing size), even after controlling for body size (Cotton *et al.*, 2004). In addition, female eyespan is a reliable indicator of fecundity even after controlling for the influence of body size (Cotton *et al.*, 2010).

Male mate preference in *T. dalmanni* was investigated both in the wild and under controlled laboratory experiments. The vast majority of previous studies examining male mate preferences, especially in *Drosophila*, have been performed in the laboratory (e.g. Bryne and Rice, 2006; Edward and Chapman, 2013), and thus we are largely ignorant on the importance of male mate preference under natural conditions. Indeed, some authors have noted that laboratory studies involving simultaneous choice tests may result in inflated preference estimates as the abundance of mates they encounter in the lab far exceeds that found in the wild (Barry and Kokko, 2010). Thus my study provides important data on male mating preferences under natural conditions. In the wild, males arrive at lek sites at early dusk. Males fight to be the sole lek holder, with the largest male typically being successful (Small *et al.*, 2009). Females then arrive and choose which lek to join and roost on, with large eyespan males attracting more females (Cotton *et al.*, 2010). Males defend their harem during dusk from intrusions and mating attempts by other non-lek holding males. The majority of the matings occur the following morning before flies disperse (Burkhardt and de la Motte, 1988; Lorch *et al.*, 1993). In wild leks, I found that males with multi-female harems mated more frequently with the largest eyespan females in the harem.

This effect was independent of harem size. I defined large female eyespan as the largest eyespan available for the focal male to mate with, on the basis that his assessment would be among those females in his harem. In parallel, under experimentally controlled laboratory conditions, I confirmed male mate preference for large female eyespan. In these experiments I gave males limited choices between pairs of females, one with large and the other with small eyespan, and restricted the dispersal of females. A large proportion of copulations in the wild occur within 20-30 minutes of dawn (Lorch *et al.*, 1993), and my laboratory experiments mirrored this, considering only a 30-minute window. I also constrained male eyespan in the laboratory experiment, only using males that had large eyespan. So male preference may be different in small eyespan males, though my data from the field (where male eyespan was unconstrained) did not reveal any variation in preference with male eyespan.

Given that female eyespan co-varies positively with body size (David *et al.*, 1998) I cannot discount that my observation of male mate preference for female eyespan arose indirectly from male mate preference for large-bodied females. However, two lines of evidence suggest that eyespan, rather than body size, is likely to be the main cue that males use in their choice of mate. First, flies assess each other face-on, meaning that the laterally elongated eyestalks are more readily assessed than body size, which would require flies to be oriented perpendicular to each other. Second, female eyespan is a condition-dependent trait (Cotton *et al.*, 2004), and is a more accurate signal of fecundity than body size alone (Cotton *et al.*, 2010). It is also possible that cues other than eyespan, such as subtle behavioral cues or chemical signals (Thomas, 2011), influence mating behavior and might allow females to indicate their reproductive value to males. The disentanglement of such highly correlated traits is a general problem

faced by researchers of mate preference in sexually dimorphic species (Hedrick and Temeles, 1989).

Previous work has shown that males sire more offspring (fertile eggs) following a single mating with a large eyespan female (Rogers *et al.*, 2006). Similarly, in wild-caught *T. dalmanni* (Cotton *et al.*, 2010) and in this laboratory experiment, female eyespan was a good indicator of fecundity, and this is also the case in a related stalk-eyed fly species, *D. meigenii* (Harley *et al.*, 2013). These findings echo other studies that have demonstrated male mate preference for females with large body size, or for female ornamental trait values that are good predictors of female fecundity (Amundsen, 2000; Amundsen and Forsgren, 2001; Bonduriansky, 2001; Doutrelant *et al.*, 2008; Baldauf *et al.*, 2011; Potti *et al.*, 2013). My own and these other studies suggest that males with mating preference for large ornaments will, all things being equal, sire more offspring compared to males who mate at random. However, fecund females are likely to attract more matings by males, thereby increasing the potential for sperm competition and diluting the gain in paternity stemming from any particular male or particular mating. This has been the subject of some theoretical consideration (Servedio and Lande, 2006; Nakahashi, 2008), and my experiments do not directly address the range of fitness benefits and costs that follow from male mate preference. For instance, males choosing larger females may have a reduced likelihood of fertilizing any particular egg, but this may be compensated by the fact that larger females lay more eggs. The exact relationship between female ornaments, female quality and fitness needs to be elucidated through experimentation in which the paternity gain to a male mating with an attractive, large eyespan female is compared to a mating with a less attractive small eyespan female. A full analysis will also need to consider the genetic and environmental inputs to female fecundity and any interaction

between these two factors. This will require further analysis both in the field as well as using manipulative laboratory experiments.

In a further experiment, I altered diet as a way of controlling fecundity independently of female eyespan (Hingle *et al.*, 2001a, b). Flies on reduced quality food had relatively low fecundity. By constraining female eyespan whilst manipulating diet I was able to show male mate preference for females with higher fecundity *per se*. These results lend support to the idea that males are using multiple cues when assessing females. Perhaps males detect the distension of the female abdomen that occurs when it harbors many mature eggs. Another possibility is that females signal their fecundity through scent or other sensory modalities as has been shown in other insects (Peeters *et al.*, 1999; Mitra and Gadagkar, 2012). The use of multiple cues in mate preference decisions, such as visual, chemical and behavioral signals, has been the focus of much interest with a key question being what information they signal (Candolin, 2003; Bro-Jørgensen, 2010).

These dietary manipulations also showed that the strength of preference for fecundity differences induced by diet did not differ when both tester females had large eyespan, or both had small eyespan. However, there were interactions between female eyespan and fecundity. Male mate preference was weakened when the large eyespan female was put on a reduced quality diet, but there was no effect on preference of moving the small eyespan female between diets. These results imply that fecundity differences have a greater effect on the attractiveness of large eyespan females than on that of small eyespan females. However, this needs to be verified by further investigation, involving direct measures of individual fecundity. In this context it is vital to further investigate how fecundity differences alter preferences amongst males in the wild.

It could be argued that the distribution of observed copulations results from female behavior rather than male mate preference, for example, if large females are more eager to mate. Indeed, large females do mate more frequently than small females, although this has been interpreted as a reflection of their higher fecundity and hence their need for more copulations to offset the chronic sperm-limitation typical of this species (Baker *et al.*, 2001; Rogers *et al.*, 2005a; Cotton *et al.*, 2010). However, several lines of evidence suggest that effects of female behavior cannot account entirely for the mating biases reported here. If females compete among themselves for access to a male, then one might expect that females with the largest eyespans would prevail, and biased mating distributions would result from intra-sexual competition rather than male mate preference. However, there is no evidence that female eyespan influences contest outcome in female *T. dalmanni* (Al-khairulla *et al.*, 2003). In addition, observations of lek sites reveal no obvious evidence that females compete for access to males on the lek and it is indeed males who exhibit patrolling behavior (*pers. obs.)*. Likewise, I found no evidence that harem size or the proportion of large females in the harem correlated with preference in my wild experiment. In addition, in my laboratory experiments, male eyespan was controlled to avoid strong female mate preference influencing the outcome. Although it is not possible to eliminate female effects, it seems likely that the patterns in my data result primarily from male-controlled biases in mating.

I have shown, using a combination of field studies and controlled laboratory experiments, that males from a well-known model species of harem-based polygyny exhibit strong preference for female traits that indicate fecundity. I also provide evidence that males can directly assess fecundity when variation in morphological

traits associated with male mate preference is controlled for. Males use multiple cues in their mate assessment. Future work should capitalize on these initial findings and seek to explain the variation that exists in male mate preference and estimate how this affects the strength of sexual selection on male sexual ornaments. The effect of male eyespan and condition on male mate preferences (i.e. whether small eyespan males exhibit a difference in preference) should also be examined as condition-dependent male mate preferences could occur. Future work should also endeavor to understand the cues used by females to attract male mating to provide a more complete picture of how sexual selection operates in this species.

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Table 6.1. Examination of multiple signals used in mate choice. Attributes of paired females presented to focal males in each treatment group. Females potentially differed in eyespan (large or small) and/or fecundity (high or reduced).

Treatment	Female Eyespan	Female Fecundity
1	large or small	both high
2	large or small	both reduced
3	both large	high or reduced
4	both small	high or reduced
5	large or small	reduced (large eyespan) or high (small eyespan)

Figure 6.1. Apparatus used for male mate preference assays in laboratory experiments. A focal male was placed in the upper section and two tester females in the lower section. The sexes were separated by removable partitions (cardboard) until testing commenced. A single string resembling a rootlet runs the whole length of the cage, providing a suitable roosting site.

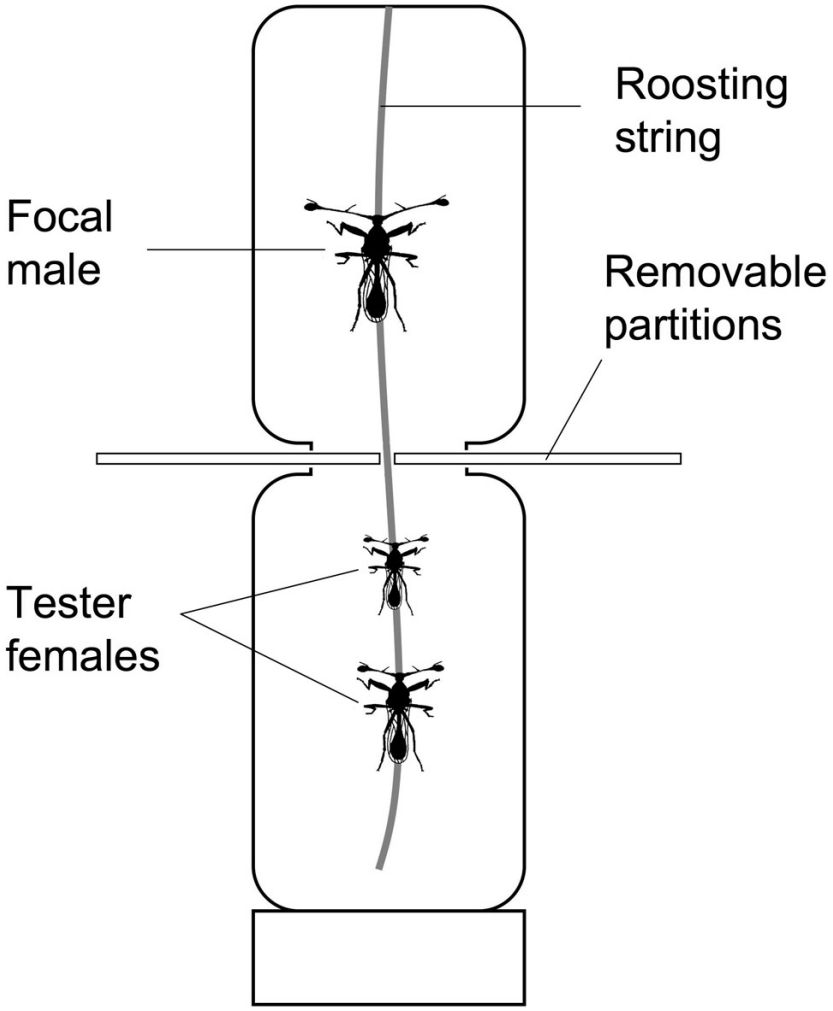


Figure 6.2. Frequency distribution of P_{Field}, the preference function of wild males. P_{Field} accounts for the harem size, the number of large and small females available and the dynamic changes in these variables between matings. $P_{Field} = 0$ indicates no preference, $P_{Field} < 0$ indicates a preference for small eyespan females and $P_{Field} > 0$ indicates a preference for large eyespan females.

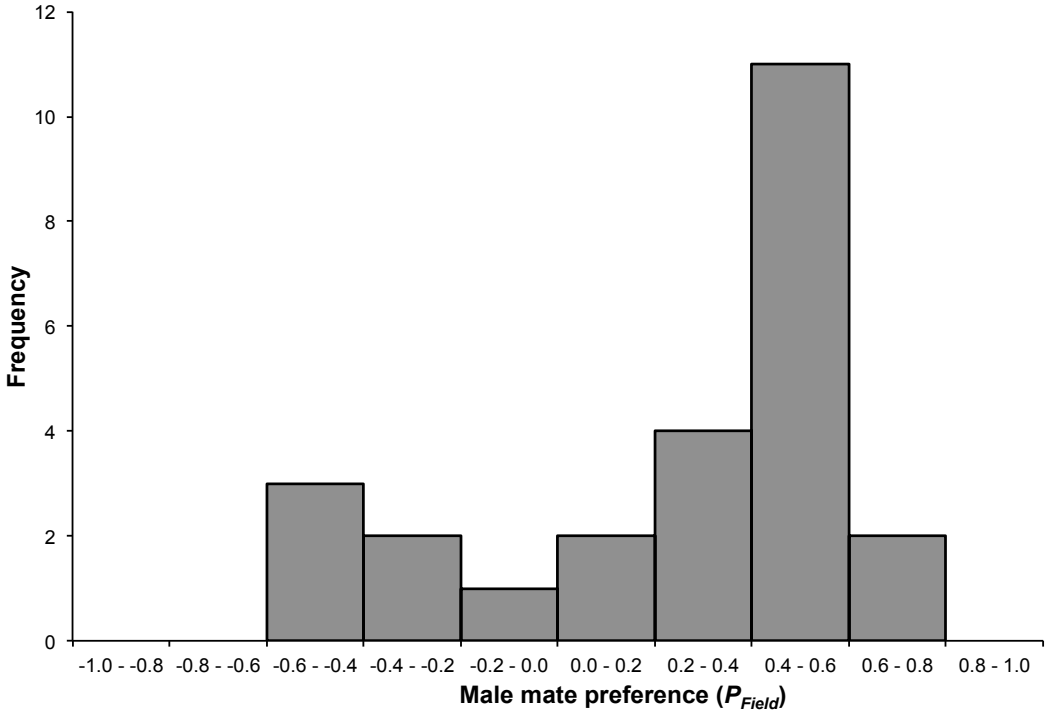


Figure 6.3. Frequency distribution of P_{Lab}, the preference function of laboratory males, when given the choice of mating with either a large or small female. In the laboratory assays there is no dynamic change in the number of females available since females cannot leave the test arena. For P_{Lab}, $P = 0$ indicates no preference, $P_{Lab} < 0$ indicates a preference for small eyespan females and $P_{Lab} > 0$ indicates a preference for large eyespan females.

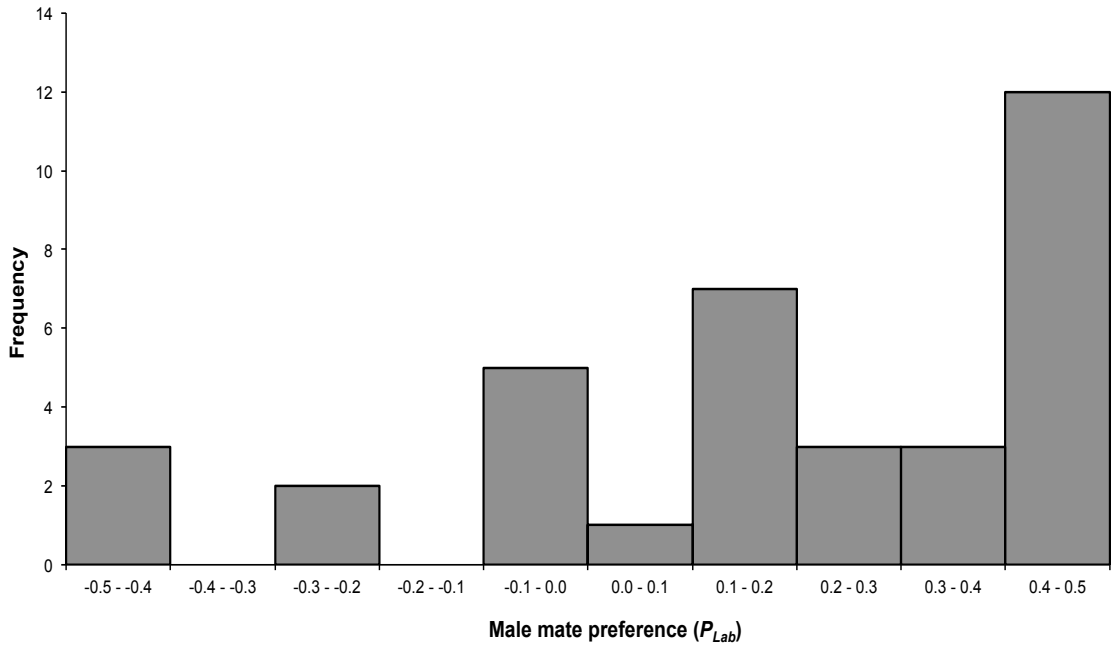
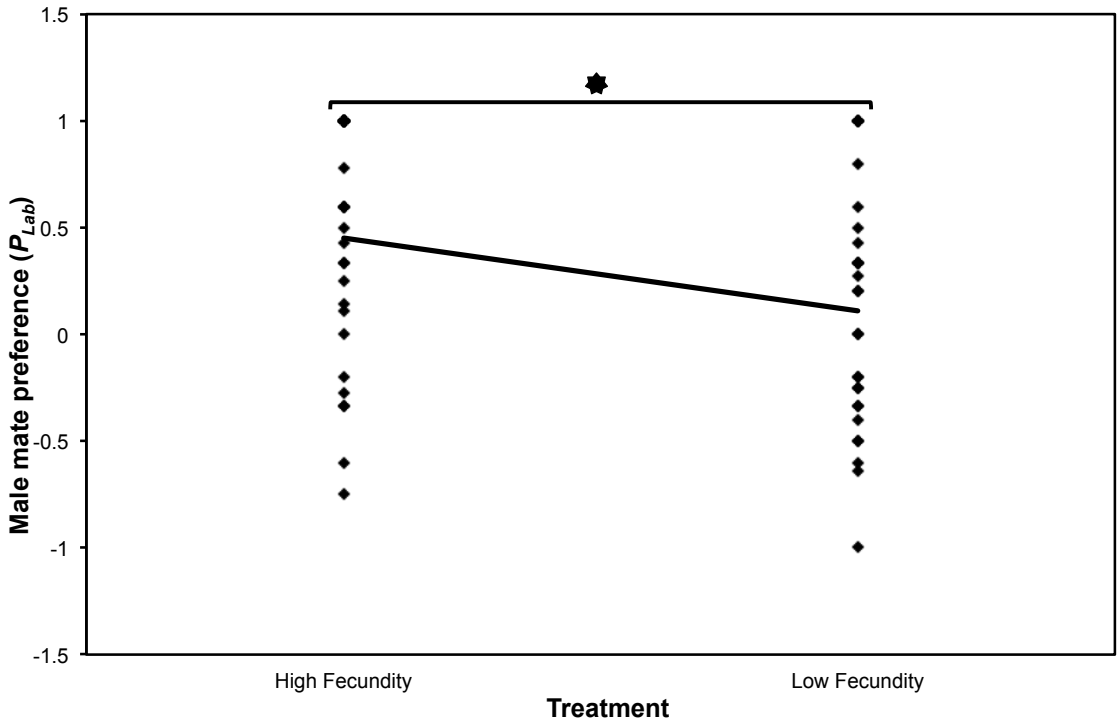


Figure 6.4. The effect of fecundity on male mate preference (independent of eyespan). Male mate preference for large eyespan females (P_{Lab}), when females were fed a high quality diet and had high fecundity (treatment one) or a reduced diet and had low fecundity (treatment two). There was stronger male mate preference when females were on the high quality diet. The line represents the mean preference of the two diet treatments. * indicates $P < 0.05$.



7

General discussion

7.1 OVERVIEW

It has been shown that females of many species preferentially mate with males that exhibit the most elaborate sexual ornaments (Andersson, 1994). The handicap model of sexual selection suggests that ornament size reflects male genetic (and/or phenotypic) quality, so female choice for highly elaborate ornaments results in fitness benefits for their offspring (Andersson, 1986; Pomiankowski, 1987, 1988; Grafen, 1990; Iwasa *et al.*, 1991; Iwasa and Pomiankowski, 1994). In order for females to accurately assess the quality of an individual male, sexual ornaments must exhibit heightened condition dependence (Zahavi, 1977; Kodric-Brown and Brown, 1984; Rowe and Houle, 1996; Cotton *et al.*, 2004a). This is maintained by a differential cost of ornamentation, with low quality males incurring a relatively higher cost for the production of any given ornament size compared to high quality males (Zahavi, 1975; Grafen, 1990; Iwasa and Pomiankowski, 1994).

Exploration of the handicap hypothesis in *Teleopsis dalmanni* was the underlying theme throughout this thesis. In the following section I summarise the main findings for each chapter. Then I highlight a number of key areas arising from this thesis that warrant future investigation.

7.2 SUMMARY OF FINDINGS

7.2.1 Male Eyespan Size is Associated with Meiotic Drive in Wild Stalk-Eyed Flies (*Teleopsis dalmanni*)

Although a 1:1 sex ratio is highly prevalent in species with sex chromosomes (Fisher, 1930), several phenomena have been known to cause deviations from balanced sex ratios (Hamilton, 1967). One such phenomenon is sex chromosome meiotic drive, a selfish genetic element located on the X chromosome in Dipteran flies that causes female-biased sex ratios (Hamilton, 1967; Lyttle, 1993). Several stalk-eyed fly species are known to exhibit X-linked meiotic drive (Presgraves *et al.*, 1997). A previous laboratory study using artificially selected lines found a possible association between the presence of meiotic drive and the size of the sexually selected trait in stalk-eyed flies, male eyespan (Wilkinson *et al.*, 1998). Further to this study, a QTL analysis in *T. dalmanni* found a close genetic association between a major QTL marker for male eyespan and the inversion containing the meiotic drive locus (Johns *et al.*, 2005). I examined the association between microsatellite markers and levels of meiotic drive in 12 wild *T. dalmanni* populations as well as the association between male eyespan size and meiotic drive. I used two data sets: a) brood sex ratios of wild-caught males mated to standard laboratory females, and b) values of a suite of phenotypic traits associated with reproductive success of wild-caught males and females. Each individual was typed for 8 X-linked microsatellite markers, including several that were previously reported to be associated with male eyespan and meiotic drive. I found that one microsatellite marker (*ms*395) showed a very strong association with meiotic drive, in agreement with that reported in Johns *et al.* (2005). I also found that meiotic drive was strongly associated with male eyespan, with smaller eyespan males siring more

female-biased broods, while well-ornamented males had broods with equal sex ratios, suggesting that they lacked the meiotic drive X chromosome. Again, these results concur with previous laboratory findings (Wilkinson *et al.*, 1998; Johns *et al.*, 2005), and constitute the first evidence from wild populations that the evolution of female mate choice for male eyespan is plausibly linked to the genetic qualities signalled by ornament size. Females would benefit from mating with large eyespan males, as they would avoid prospective mates that harbour an X-linked meiotic drive chromosome, which is detrimental to offspring fitness.

7.2.2 Meiotic Drive and the Condition-Dependent Expression of a Sexual Ornament in Stalk-Eyed Flies

The handicap hypothesis postulates that sexual traits exhibit heightened condition dependence, providing a mechanism for the maintenance of sexual traits as honest signals of male quality. A potential signal of male quality was examined in chapter 2, where I found that females gain a genetic benefit by mating with males with the largest eyespan as they are less likely to carry the meiotic drive X chromosome and produce female-biased offspring (Cotton *et al.*, 2014). The meiotic drive loci are contained within a large inversion on the X chromosome with limited recombination (Kirkpatrick, 2010). This lack of recombination is likely to result in the accumulation of mildly deleterious mutations. The poor genetic quality of this drive chromosome is predicted to be highlighted in the condition-dependent expression of eyespan, with drive males having a stronger condition-dependent response to stress. In this chapter I studied this relationship by rearing males under three different environmental conditions (low, medium and high food quality) and examined the ensuing eyespan expression profile. Males were then mated to laboratory females, and meiotic drive

males were identified as producing a significantly biased offspring sex ratio. I found that there was no overall difference in condition dependence between standard and meiotic drive males, although meiotic drive males did have smaller eyespans across the treatments and males producing strong sex ratio biases did have significant changes in the coefficient of variation of eyespan across treatments. There are a number of explanations as to why I did not see an obvious change in eyespan expression profile between meiotic drive and standard males. The first is that the experimental design failed to produce a large enough sample of extreme (>90% female offspring) and weak (<90% female offspring but still significantly female-biased) drive males. In addition, the use of offspring sex ratios to determine the presence or absence of the meiotic drive X chromosome may have resulted in some males being incorrectly categorised. This is because meiotic drive suppressors can mask the phenotypic effects of drive (Wilkinson *et al.*, 2014) resulting in flies that possess the meiotic drive X chromosome producing 1:1 sex ratios. Future studies should endeavour to identify and utilise genetic markers that are haplotypic with the meiotic drive chromosome. It could also be the case that there truly are no differences between the eyespan expression profiles of the two groups. This suggests that deleterious mutations are not building up in the meiotic drive X chromosome, potentially due to the rapid evolution of the drive complex (Bastide *et al.*, 2011; Rose *et al.*, 2014) or because such mutations are visible to selection in hemizygous males.

7.2.3 Do Ornaments Reflect Survival Under Stress? An Experimental Test of the Handicap Hypothesis

Despite over 150 years of research, a definitive understanding of how and why elaborate sexual traits have evolved remains elusive. Along with Fisher's (1930)

runaway process, the handicap hypothesis is one of the principal theories used to explain the evolution of sexual traits that have evolved beyond their natural selection optima. The handicap hypothesis posits that exaggerated traits are a costly handicap that signals the genetic and/or phenotypic quality of the bearer (Pomiankowski, 1987; Iwasa and Pomiankowski, 1999). This honest signal of quality is maintained by the differential cost of ornament production, with poor quality males paying a higher (survival) cost of ornamentation (Iwasa and Pomiankowski, 1994). Whilst this has broad theoretical support (Grafen, 1990; Iwasa *et al.*, 1991; Iwasa and Pomiankowski, 1994, 1999), direct empirical evidence is limited and often contradictory (Grose, 2011). Empirical tests of the handicap hypothesis primarily investigate how ornament manipulation influences survival (Mappes *et al.*, 1996; Grether, 1997; Cuervo and de Ayala, 2014). Ornament manipulation fails however to take into account the influence of changes correlated with ornament exaggeration in other morphological and life history traits (Balmford *et al.*, 1994; Emlen, 2001). I used both field and laboratory experiments to examine the handicap hypothesis in stalk-eyed flies. In both experiments, I subjected flies to one of two stress treatments; either a paper tag attached to the thorax (high stress) or a mark (benign stress). I then monitored subsequent survival. In wild flies, male eyespan predicted survival under the high stress treatment, with large eyespan males surviving longer than small eyespan males. This is in contrast to flies under benign stress, where eyespan was uninformative about survival. Female eyespan (the unexaggerated homologue of the male ornament) was unrelated to survival under both stress treatments. The experimental design in my laboratory study involved the termination of the experiment after only 27 days, creating a large pool of censored flies that were still alive and that could not be correctly controlled for (Mike Bonsall, *pers. comm.*). This constrained simple comparisons of the laboratory and field findings showing no relationship between

survival and ornamentation in males or females. To account for differences in the number of censored flies for each treatment, I performed an alternative analysis that equalised the number of censored flies in each treatment. Under this method I found that the laboratory results were highly congruent with the results from wild flies, showing that male eyespan influenced survival in males under heightened but not benign stress. The relationships shown in the field were not compatible with Fisher's runaway process, as this theory one predicts a negative relationship between male quality and survival (Fisher, 1930). Instead the results uniquely support the hypothesis that ornament size reflects male quality, as only the highest quality males were able to bear the dual cost of ornamentation and stress. Future studies should redo the laboratory experiment, extending the duration beyond 27 days to enable appropriate statistical analysis of treatment-dependent survival.

7.2.4 The Influence of Environmental Quality on Lek Structure and Behaviour in Stalk-Eyed Flies

Condition dependence is a key mechanism explaining how the handicap hypothesis explains the evolution of sexual traits (Iwasa *et al.*, 1991; Iwasa and Pomiankowski, 1994; Cotton *et al.*, 2004a). A number of studies have shown that positive correlations exist between trait size and condition (Andersson, 1994; Cotton *et al.*, 2004b). Given the effect that environmental variance can have on condition dependence of both ornament size (David *et al.*, 2000; Cotton *et al.*, 2004b; Johns *et al.*, 2014) and preference (Hunt *et al.*, 2005; Cotton *et al.*, 2006a; Cotton *et al.*, 2006b), a more complete understanding of how these factors affect key components of sexual selection was needed. I used a dual approach to investigate how environmental stress influenced major aspects of sexual selection, lek structure and mating behaviour, in stalk-eyed

flies. In the laboratory, I experimentally manipulated adult diet to create flies that were in either good or poor condition and then I examined a range of behaviours pertaining to both male-male competition and female mate choice as well as examining aggregation behaviour and lek structure. In the field, flies were collected from 12 different sites at 3 different time points. The environmental quality of collection sites was assessed using principal component analysis of four proxies for quality that exhibited significant special and temporal variation: fecundity, male and female thorax size and local population density. I then examined how quality, as defined by principal component 1 (which correlated positively with male and female thorax size, female fecundity and density), correlated with lek structure, specifically harem size. Results from both the laboratory and the field were highly congruent and showed that less stressful environments resulted in a greater number of females per lek (larger harem size). I found that environmental stress affected not only the mean, but also the variance of harem size, as the coefficient of variation also increased in good quality environments. Sexual selection is characterised by assortative mating, that skews reproductive success in favour of a select few individuals (Andersson, 1994). The increase in variance seen when conditions were good indicates high levels of assortative mating and additionally I found that large eyespan males accrued larger leks only when conditions were good. Aspects of female mate choice were also strongly correlated with environmental quality, with an increase in rejection rates as well as an increased numbers of matings under good conditions. My results provide evidence that key components of sexual selection can be influenced by environmental quality, both in the wild and in laboratory populations.

7.2.5 Male Mate Preference for Female Eyespan and Fecundity in the Stalk-Eyed Fly, *Teleopsis dalmanni*

Stalk-eyed flies have traditionally represented a classic model of sexual selection, with male-male competition for access to females (Small *et al.*, 2009) and female mate choice for large eyespan males (Wilkinson and Reillo, 1994; Cotton *et al.*, 2010). For male mate preferences to evolve, a number of pre-requisites are required in the system. There must be variation in female quality that males are able to assess, males must be limited in their mating capacity and the costs of finding and assessing mates must not be prohibitive (Bonduriansky, 2001). In my study species, *T. dalmanni* we find that these criteria are all met; there is substantial variation in fecundity (Cotton *et al.*, 2010), males are limited in their ability to mate multiply over short periods of time (Rogers *et al.*, 2005a, b), and the lek mating system means that mate searching and assessment costs. Male mate preferences had never been explored in *T. dalmanni* previously and in this chapter I tested for the presence of such preferences in wild and laboratory populations as well as examining more closely how males assessed variation in female fecundity. In addition to examining how wild males chose mates I set up experiments in the laboratory allowing males a choice between females that varied in either 1) eyespan (a proxy for fecundity) and/or 2) fecundity (manipulated through diet). Results from field and laboratory studies were qualitatively identical, with males exhibiting a strong preference for large eyespan females. It has been shown previously that males sire more offspring following a single mating with a large eyespan female than they do from mating with a small female (Rogers *et al.*, 2006). In both *T. dalmanni* (Cotton *et al.*, 2010) and a related stalk-eyed fly species, *Diasemopsis meigenii* (Harley *et al.*, 2013), female eyespan has been shown as a good indicator of fecundity. These results are in line with other studies that have established

male mate preference for females with large body size or for female ornamental trait values that are good predictors of female fecundity (Amundsen, 2000; Amundsen and Forsgren, 2001; Bonduriansky, 2001; Doutrelant *et al.*, 2008; Baldauf *et al.*, 2011; Potti *et al.*, 2013). In addition to using female eyespan as a mating cue, males were also able to assess female fecundity directly. These results suggest that males are assessing females using multiple cues. The exact mechanism for this remains unclear. It is possible that males are able to physically detect changes in female abdomen size related to the number of mature eggs. A further possibility is that fecundity is directly signaled through scent (Peeters *et al.*, 1999; Mitra and Gadagkar, 2012) or other sensory systems. The use of visual, chemical and behavioral cues in mate preference decisions is an active area of research particularly in relation to understanding the information that is conveyed through the different signals (Candolin, 2003; Bro-Jørgensen, 2010). In addition to understanding more about the mechanisms of mate preference signals, future work should also seek to examine how the variation in male mate preferences affects the strength of sexual selection in relation to male sexual traits.

7.3 FUTURE DIRECTIONS

This PhD thesis has explored a number of key areas of sexual selection in stalk-eyed flies. I have identified three key areas arising from my research that I believe would benefit from future study.

7.3.1 The Ecological and Evolutionary Implications of Meiotic Drive

Despite the acknowledged ecological and evolutionary implications of meiotic drive (Jaenike, 2001) there is virtually no information on the impact of meiotic drive in wild populations.

Sex ratios

An obvious assumption arising from wild populations where meiotic drive is known to exist (Cotton *et al.*, 2014) would be that the operational sex ratio in localised stream populations would be sex ratio biased in proportion to the amount of drive present (5-30% in Gombak populations). Using data arising from chapter 2 and chapter 5 I found that none of the streams, either overall or at any time point in the duration of the study, had an adult (lekking) sex ratio that differed from 1:1 (Alison Cotton, unpublished data). In order to investigate this further I collected a small number of females from the wild and examined resultant primary sex ratios. My results showed that the primary sex ratio in these streams is female-biased at eclosion. This suggests that F1 females from drive males are reduced in number between eclosion and sexual maturity. A possible reason for this is that they are dying soon after eclosion at a higher rate than standard, non driving, flies, perhaps due to the action of deleterious mutations in the X chromosome inversion (Kirkpatrick, 2010). For chapter 2, during the collection of offspring sex ratios from wild males, I observed a highly significant difference in the survival to sexual maturity between the offspring of drive and standard males (not reported in the chapter). Offspring from males producing female-biased broods died significantly more than those from 1:1 sex ratios. These initial findings form the platform for future investigation into why and how wild populations containing up to 30% drive have an operational sex ratio of 1:1. Previously (due to time constraints) I

was only able to collect a small number of wild females to examine primary sex ratios, and in the future it will be vital to obtain a larger sample size from a number of different streams with known levels of drive. With this in mind, in the Gombak populations, flies should be collected from Quarry, Upper Blair Witch, Cascade, Mihaly, Kingfisher, Lower Field Centre and Swamp. In addition to field collections it would also be important to undertake breeding experiments in the laboratory in order to understand why offspring from drive males have a relatively heightened post-eclosion death rate prior to sexual maturity. This will require the identification of female offspring's drive genotype (XX, X^DX or X^DX^D). The methods for this have been previously established and can be accomplished using a combination of breeding experiments and microsatellite genotyping (or ideally SNP markers - see section 7.3.2) of the X chromosome (Wilkinson *et al.*, 2006).

Polyandry

Theory predicts that polyandry should be higher in populations that contain meiotic drive as sperm from drive males performs badly during sperm competition (Wilkinson *et al.*, 2006), and thus increased levels of polyandry would increase the chances of a female producing an unbiased brood sex ratio. Polyandry may also affect the ability of rare meiotic drive strains to invade a population (Holman *et al.*, 2015). In addition, female stalk-eyed fly flies are sperm-limited (Baker *et al.*, 2001; Wilkinson *et al.*, 2005; Rogers *et al.*, 2006) so increased levels of multiple mating would be beneficial by compensating for having poor quality (drive) males in the population. Experimental data on fruit flies indeed suggests that such a relationship exists, both in natural and laboratory populations (Price *et al.*, 2008; Price *et al.*, 2014). Examination of whether polyandry is greater in drive populations (comparing high drive streams such as Quarry, Upper Blair Witch and Cascade with non-drive streams such as Rubbish,

Swamp and Lower Field Centre) would be best investigated using both genetic and behavioural analysis. Observational behavioural data of individual female re-mating rates in the wild would provide information on actual copulation number (see data collected for chapter 5 on wild females) and subsequent paternity analysis using microsatellite or SNP data from the offspring of those wild caught females would assess the number of different fathers from those matings.

Sperm transfer

One of the main conclusions of chapter 2 was that male eyespan and drive are genetically and phenotypically linked. Sperm competition theory predicts that the number of sperm a male transfers to a female is the primary determinant of fertilisation success (Parker, 1970; Wedell *et al.*, 2002; Pizzari and Parker, 2009). We know, however, that during sperm competition seminal fluid from 'standard' males incapacitates sperm from drive males, influencing the number of offspring sired by drive males (Fry and Wilkinson, 2004). This was further illustrated when Wilkinson *et al.* (2006) undertook an initial analysis of the fitness effects of meiotic drive males. They found that drive males exhibited lower sperm precedence and lower fertility than standard males under conditions of sperm competition (Wilkinson *et al.*, 2006). It would be interesting to extend this analysis and examine differences in performance between drive and standard males, both in the number of sperm transferred as well as the overall spermatophore size (including accessory fluids). It would be important to match flies on the basis of their body size as well as developmental and nutritional history (e.g. the amount of larval food received). While initially focussing on the potential differences in ejaculate investment in a single mating, it would be interesting to examine how ejaculate investment varies across multiple matings. Do drive males invest heavily in their first mating and then scale back their investment in subsequent

matings or do they invest similarly to standard males in multiple matings? It would also be interesting to see whether drive males' ability to transfer sufficient quantities of sperm during multiple matings is influenced by the levels of polyandry in the population. Finally it would be important to see if ejaculate investment by drive males is affected by the size of the recipient female in the same way as normal males (Harley *et al.*, 2013). Suitable methods would be similar to those used to test whether male ejaculation allocation was influenced by female size and male eyespan in a closely related stalk-eyed fly species, *Diasemopsis meigenii*, where the size of the transferred spermatophore was measured immediately following a single controlled mating (Harley *et al.*, 2013).

7.3.2 Developing Single Nucleotide Polymorphism (SNP) Markers

Until very recently, the primary method of examining the genomic basis of many traits related to sexual selection in stalk-eyed flies, has been through the use of microsatellites (e.g. Johns *et al.*, 2005; Cotton *et al.*, 2014). Prior to and during the course of my PhD, it became apparent that, in stalk-eyed flies, the microsatellites were of limited use for my questions of interest. This was apparent in the poor amplification as well as the monomorphic nature of many markers resulting in insufficient useable markers. In addition, there were severe problems with null alleles (alleles that do not amplify due to mutations in the flanking regions) making paternity analysis completely impossible. Recently, there have been advances in the successful adoption of other genomic techniques, with RNAseq used to examine the differential expression of transcripts between drive males (carrying the X^D chromosome) and standard males (Reinhardt *et al.*, 2014). The next first step would be to create a genomic SNP map in order to better understand the genetic architecture of meiotic drive in this species.

Several interesting projects would become feasible once such a map was completed. One could examine the population genetics of the Gombak valley. I have over 1000 flies that were collected prior to, and during, my PhD from the Gombak valley populations, as well as from populations in 3 surrounding valleys and an outgroup population. It would be very interesting to examine the genetic structuring along the valley as well as in the surrounding areas as it will provide key data on migration and gene flow, which is currently lacking. This has important implications for many areas of interest; for example, the movement of individuals between populations could dramatically influence the spread of both meiotic drive and the associated suppressors.

7.3.3 The Effect of Environmental Stress on Sexual Selection and Reproductive Success in Natural Populations

In chapter 5, I examined the effect that environmental stress had on components of sexual selection. This is an important study as it has potential implications for the field of conservation, where both stochastic (Post *et al.*, 1999; Garant *et al.*, 2004) and human altered habitats (Hill, 1995; Stratford and Stouffer, 2001) are increasingly common, and our understanding of the evolutionary consequences of such changes are not well understood.

There were some limitations to my study however, particularly pertaining to the field, and I believe further investigation is merited. Environmental stress is known to have contrasting effects on trait sizes, as it tends to cause a decrease in the mean but a corresponding increase in the variance (David *et al.*, 2000; Cotton *et al.*, 2004b; Hunt *et al.*, 2004; Charmantier and Garant, 2005). My work could be expanded to properly examine how these opposing changes may affect sexual selection, and I believe that

more data per population would yield tractable patterns. I focussed on evaluating lek structure as a key component of sexual selection. However to fully investigate how stress affects sexual selection, one should collect further information from the field on mating behaviour and crucially also of variation in reproductive success (Andersson, 1994). One method would be to collect all the males and females in each field population (noting lek sizes and the identity of the male lekholders) and then to collect all the offspring of each individual female. All flies (males, females and their offspring) would then need to be genotyped (ideally this would be using SNPs). The known maternal and offspring genotypes would then be analysed against all the collected males in order to assign paternity (Lynch and Ritland, 1999). Then morphological and lek size characteristics could be correlated with the reproductive success of each male collected.

There is a wealth of information providing indirect evidence of variation in reproductive success, such that well-ornamented males have larger accessory glands and higher fertility compared to small eyespan males (Rogers *et al.,* 2008). Accessory gland size correlates strongly with reproductive success as it covaries both phenotypically and genetically with male mating frequency (Rogers *et al.,* 2005a, b). Accessory glands become depleted with each additional mating (Rogers *et al.,* 2005a) and this provides a physiological limit on male mating rate, and hence the number of females that males can mate with in their harem during dawn and dusk. Despite this information, there is only limited data directly examining variation in male reproductive success. Corley *et al.* (2006) examined sperm competition in *T. dalmanni* (controlling for male eyespan) and found that paternity patterns were highly variable – although results indicated values associated with random sperm mixing as opposed to sperm precedence. It has also been shown that males possessing the meiotic drive X

chromosome produce fewer sperm and sire fewer progeny than would be expected given the number of sperm transferred (Wilkinson and Fry, 2001). The latter provides key information for this thesis in relation to chapters 2 and 3, however the lack of good direct evidence linking male eyespan with variance in reproductive success is a major area that needs to be addressed and would provide the basis for many of the assumptions in this thesis and especially for the future work described here.

In order to further understand how environmental quality influences aspects of sexual selection, it would be interesting to examine whether larval conditions (i.e. stress in early life) affect lekking behaviour and structure of adult flies in the laboratory. A straightforward way to examine this would be to raise two groups of flies on either high or low quality food, and then provide them all with the same adult diet (whilst keeping the groups separate). In essence the mating laboratory experiment from chapter 5 could then be repeated to see if lekking behaviour was different between the groups. From this experiment, we could comment on whether stress in early life stages influences important behavioural characteristics during adulthood.

7.3.4 The Effect of Mating Strategies and Eyespan on Reproductive Success

There has been a great deal of interest in alternative mating strategies, with evidence that both males (Shuster and Wade, 1991; Taborsky, 1994) and females (Johnson and Brockmann, 2012) attempt to maximise reproductive success by employing alternative reproductive tactics (ARTs). ART polymorphisms can be behavioural and/or morphological (Gross, 1996; Taborsky and Brockmann, 2010). Strategies have long been thought of in the context of game theory and evolutionarily stable strategies (ESS) models, which assess the coexistence of alternative strategies in a population in

relation to costs and benefits (Maynard Smith and Price, 1973; Parker, 1984; Charnov, 1993).

At first glance, *T. dalmanni* does not appear to constitute a classic case of ARTs. Male eyespan is a continuous trait, which is in contrast to the many examples of discontinuous trait evolution in ARTs (Taborsky and Brockmann, 2010). Female mating and roosting preferences for large eyespan males are strong and male mating success was always assumed to be linear based on this preference. There is no published research to suggest that male *T. dalmanni* are employing any alternative strategies. However, in the field I have made some tantalising relevant observations about mating behaviour in this species. First, the variation in eyespan is much greater in wild flies than in laboratory flies, no matter how variable the larval diet is that laboratory flies receive (Alison Cotton, *pers. obs.*). Second, small males in the field are almost indistinguishable from females, and primarily are found in leks of large males that have a large number of females. This was noted in initial field observations of stalk-eyed flies (Burkhardt and de la Motte, 1985). Medium sized males seem to be unable to do this because the dominant male immediately fights them off. (Alison Cotton, *pers. obs.*) Third, small males often obtain some degree of mating success on these leks, as large males seem unable to distinguish them from other females. Once copulation is underway, large males will often interrupt and chase off the small male - but only if the latter is seen (Alison Cotton, *pers. obs.*).

On the basis of these observations, I believe there is cause to investigate whether there is an alternative mating strategy employed by small eyespan males and whether this influences their reproductive success. Initial data would need to identify the eyespan size range that large eyespan males are able to perceive for small eyespan intruders on

their leks and that triggers efforts to remove small eyespan males. Do only very small eyespan males remain undetected on leks held by large eyespan males? In addition behavioural data on successful and interrupted matings would need to be collected and following on from that, future work would need to establish the relative reproductive success of males, both in terms of eyespan size and also reproductive strategy (dominant or sneak).

Finally, I believe it would be interesting to examine the possibility that mating strategies play a role in the size of male eyespan. It has long been observed that male eyespan variation in the wild in much greater than in the laboratory, even when flies have been subjected to extreme variation in larval food quality. Not only do wild males have much larger eyespans, they also have much smaller eyespans as well. I am interested in the idea that males of naturally small eyespan do not maximise their potential size because if they were the size of small eyespan males in the laboratory then large 'lek holding' males would still be able to recognise them as male and fight them off the leks, thus greatly reducing mating potential. Small males that 'sneak' matings in the field often have very small eyespans, and, even to a trained observer's eye, can be very difficult to distinguish from females. This could be investigated by examining the allometry between body size and eyespan, because if small males are phenotypically 'female', then allometry in these flies should be non-linear, i.e. in small flies the slope is shallow and in larger flies it becomes linear. This could be contrasted with the allometry of laboratory flies where eyespan variance is created by larval diet. A difference in the allometric slope between laboratory and field flies would provide initial evidence that there is a change in the body size – eyespan relationship. Reproductive organs (accessory glands and testes) would need to be measured, as I

would predict that these are comparatively larger than the eyespan size suggests and instead correlate strongly with body size.

7.4 FINAL THOUGHTS

In this thesis I highlight the importance of using multiple complementary methods to address questions regarding ornament evolution. Using the stalk-eyed fly, *T. dalmanni*, I examined the conditions under which ornaments and associated preferences have evolved, in both the ecologically relevant environment of the field and the controlled conditions of the laboratory. Within this two-pronged approach, I used a diverse array of techniques including phenotypic manipulations, genetic analyses, behavioural observations and temporal and spatial studies of populations, to gain a comprehensive overview of sexual selection. I hope that the work contained within this thesis will encourage future researchers to adopt a more diverse suite of approaches in their studies of sexual selection. Only then, will we develop a holistic and integrated understanding of how ornaments, and preferences for them, have evolved.

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Appendix