Sexual Selection and Condition Dependence in the Fruit Fly, *Drosophila melanogaster*

by

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Abstract

While natural selection acts to strengthen nonsexual fitness (i.e., survivorship and fecundity), the capacity for sexual selection to enhance this process is the source of much theoretical and empirical debate. The potential for sexual selection to reinforce natural selection in reducing the mutation load is rooted in condition dependence theory. This theory predicts that females select mates on the basis of condition, as indicated by condition-dependent sexual traits, and in doing so simultaneously sort them by genetic quality. The premise of condition-dependent mate choice is transferrable to non-female-choice components of selection; hence, sexual selection at its multiple stages is predicted to remove mutations deleterious to condition.

Support for the notion that sexual selection in toto can reduce the mutation load is mounting; however, understanding which components of sexual selection are important in the removal of deleterious mutations remains an important challenge. To address this issue, I employed the Drosophila melanogaster model system. I demonstrated support for the capacity of sexual selection to remove deleterious mutations at multiple stages of selection (Chapters 2-4), before and after mating. Furthermore, I showed that the efficacy of sexual selection in eliminating
deleterious mutations is dependent on the stage of selection (Chapter 4), and the source of mutation under selection (Chapters 2-4).

I deviated slightly in research objective for my final thesis study (Chapter 5). There is mounting empirical support for precopulatory male mate choice across insect taxa, and emerging support for choice post-copulation (i.e., sperm and seminal fluid allocation). I manipulated perceived female mating status in D. melanogaster, and I demonstrated precopulatory male preference for females perceived as virgins, which suggests male choice for low ejaculate competition risk. At the postcopulatory level, males mated to perceived virgins outperformed those mated to perceived mated females in several key components of sperm competition defence; however, I did not detect a downstream fitness cost to this differential investment.

Ultimately, my thesis has contributed valuable and novel results on the relative impacts of pre- and postcopulatory sexual selection on genetic quality in D. melanogaster, which collectively suggest that sexual selection can be effective against deleterious mutations.
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Statement of Contributions

Chapter 2 was previously published as a coauthored paper. This paper was coauthored by Nathaniel Sharp, and my supervisors, Locke Rowe and Aneil Agrawal. Experimental design was conducted in collaboration with LR and AA. Experiments were conducted with NS. I analyzed the data and was the primary author of the manuscript.
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Chapter 1
General Introduction

1 General Introduction

Beginning conceptually with Darwin’s (1859, 1871) hypothesis that extravagant ornamental traits, observed mainly in males, were the result of the combined intersexual selection by female mate choice and intrasexual selection by male-male competition over access to females, efforts to understand evolution via sexual selection have endured for more than 150 years (Andersson 1994, Arnarqvist and Rowe 2005), albeit with substantial criticism during the first century (Andersson 1994). In the intervening decades, there have been many models of sexual selection proposed to explain the origins and evolution of mate choice: the Fisher process (also called the runaway process), direct benefits, indirect benefits (also called the good genes or indicator process), sensory exploitation, and sexual conflict (Andersson 1994, Arnarqvist and Rowe 2005, Fuller et al. 2005). It is the evolutionary consequences of these collective processes, rather than their causes, that were the central concern of this thesis.

1.1 Sexual selection and natural selection: relative impacts on fitness

Understanding the relative roles of natural\(^1\) and sexual selection in shaping population mean fitness is fundamentally important to evolutionary biology. Of particular interest is to understand the role of sexual selection in determining nonsexual fitness (defined here as that component of fitness outside the context of mating and fertilisation success, including survivorship and fecundity; Andersson 1994). Though it is generally understood that natural

\(^1\) Sexual selection is often viewed conceptually as a process exclusive to natural selection, though it is most appropriately presented as a component thereof (Andersson 1994, Arnarqvist and Rowe 2005). Following this logic, the other major components of natural selection (viability and fecundity) can be collectively referred to as nonsexual natural selection (or simply nonsexual selection) to distinguish them from sexual selection (Andersson 1994; for convenience, I will follow common practice in the literature [e.g., Rowe and Houle 1996, Fricke and Arnarqvist 2007] and refer to ‘nonsexual selection’ simply as ‘natural selection’).
selection acts to increase nonsexual fitness, whether sexual selection has similar consequences is a question rife with both theoretical and empirical debate. This controversy exists because sexual selection has been predicted to have either positive or negative consequences for population mean fitness, depending on the particular mechanism of sexual selection being considered. For example, ‘good-genes’ sexual selection (Fisher 1930, Zahavi 1975, 1977) is predicted to enhance population mean fitness through the production of high-quality offspring. However, processes such as intralocus (e.g., Rice 1992, Chippindale et al. 2001, Gibson et al. 2002, Pischedda and Chippindale 2006) and interlocus sexual conflict (e.g., Rice 1996, Holland and Rice 1998, Arnvist and Rowe 2002b, a) are predicted to reduce population mean fitness (Trivers 1972, Parker 1979, Rice 1984, Gavrilets et al. 2001, Arnvist and Rowe 2005, Rowe et al. 2005). Moreover, these mechanisms can act jointly. Whether sexual selection in its entirety enhances or degrades population mean fitness thus depends on the relative evolutionary importance of these processes, which is unknown. While investigations into the particular mechanisms by which sexual selection arises and is maintained have received considerable attention over recent decades (reviewed in Kirkpatrick and Ryan 1991, Andersson 1994, Kokko et al. 2003, Arnvist and Rowe 2005, Fuller et al. 2005), the evolutionary consequences of sexual selection on fitness have received relatively little.

Interestingly, good-genes selection can be expected to function regardless of the particular mechanism of sexual selection that is operating (Rowe and Houle 1996, Arnvist and Rowe 2005), as condition dependence is expected to evolve for any sexually selected trait that becomes costly (reviewed in Price et al. 1993, Andersson 1994, Johnstone 1995, Jennions et al. 2001, Cotton et al. 2004); that is, healthy males have high mating success (Whitlock and Agrawal 2009). A prediction can thus be made that regardless of the mechanism of sexual selection that is operating, sexual selection can select for higher condition, or higher genetic quality, individuals.

1.2 Condition dependence and sexual selection

The idea that sexual selection can reinforce natural selection in removing deleterious mutations from a population, and hence facilitate adaptation, is grounded in condition dependence theory. Within this theoretical framework, the ‘genic capture hypothesis’ (Rowe and Houle 1996)
conceptually resolves the ‘lek paradox’ (Borgia 1979), which states that, in the face of persistent directional selection by female choice, variation in fitness associated with a male trait should be quickly eroded, thus negating any further benefit to choice (especially when males provide only gametes, and thus no direct benefits to female mates, as on a lek); and yet, the choice (paradoxically) persists. The genic capture hypothesis provides a conceptual resolution to the lek paradox by ‘capturing’ genetic variation in condition\(^2\) in the expression of male sexually selected traits. As a trait transitions from being subject only to stabilizing natural selection to an opposing combination of directional natural and sexual selection, the cost of trait production increases (Rowe and Houle 1996). As the sexually selected trait increases in cost, condition dependence of trait expression is expected to evolve when the marginal cost of trait investment is lower for high condition individuals (Rowe and Houle 1996, Lorch et al. 2003). Condition summarizes a large portion of the genome (Houle 1991); thus, condition-associated loci likewise collectively represent a large mutational target (Houle et al. 1996). Accordingly, the expression of condition (and, logically, condition dependent sexually selected traits) is affected by deleterious mutations occurring across a large proportion of the genome (Houle 1998). As condition dependence evolves, the proportion of condition-associated loci affecting the expression of the sexually selected trait increases, and hence so does the proportion of genetic variance in condition that is expressed by the sexually selected trait, thereby completing the process of genic capture. Thus, females that choose mates using indicators of conditional quality (i.e., sexually selected traits) simultaneously sort them by genetic quality (i.e., frequency of alleles deleterious to condition; Rowe and Houle 1996), thereby allowing sexual selection to operate in the same direction as natural selection in removing deleterious mutations from the population (Whitlock and Agrawal 2009). This process requires high additive genetic variance in fitness, and condition dependence in traits subject to sexual selection; extensive empirical support exists for both (reviewed in Arnqvist and Rowe 2005). While much theoretical (e.g., Rowe and Houle 1996, Houle and Kondrashov 2002, Lorch et al. 2003) and empirical (reviewed

\(^2\) While the particular meaning of condition remains debated in the literature (see Tomkins et al. 2004), here I adopt the widely-accepted definition used by Rowe and Houle (1996) and subsequent authors (e.g., Lorch et al. 2003, Hunt et al. 2004, Tomkins et al. 2004, Bonduriansky and Rowe 2005) that describes condition as the pool of metabolic resources accumulated through an individual’s life history, which is then allocated to the production or maintenance of traits enhancing fitness. Condition represents that combination of environmentally and genetically determined phenotypic parameters which best predicts individual reproductive success (Bonduriansky and Rowe 2005); thus, condition is a multidimensional trait that accounts for a large portion of fitness.
in Price et al. 1993, Andersson 1994, Johnstone 1995, Jennions et al. 2001, Cotton et al. 2004, Arnqvist and Rowe 2005) attention has been applied to demonstrating the cross-taxonomic condition dependence of morphological sexual traits subject to female choice, the other levels of mating and fertilization success (scramble competition, endurance rivalry, contest competition, mate choice, and sperm/ejaculate competition; Andersson 1994) are likely subject to similar condition dependence (Whitlock and Agrawal 2009), and thus should likewise operate as mechanisms for removal of deleterious mutation by sexual selection (Whitlock and Agrawal 2009).

Theory predicated on the notion of condition dependence suggests that populations evolving under sexual selection can experience elevated fitness benefits relative to those without it. According to these models, sexual selection can increase the spread of beneficial alleles (Proulx 1999, Whitlock 2000, Proulx 2001, 2002) and can purge the genetic load (Whitlock 2000, Agrawal 2001, Siller 2001, Houle and Kondrashov 2002). Additionally, Lorch et al. (2003) demonstrated that the genic capture process can enhance the rate of adaptation in temporally fluctuating environments.

One of the predictions peculiar to the genic capture hypothesis is that mutations at many loci in the genome should affect the expression of sexually selected traits (Houle et al. 1996, Rowe and Houle 1996, Houle 1998), given that condition will be affected by any allele that affects the ability of an individual to obtain and use resources (Andersson 1982, 1986). Recent models of condition-dependent sexual selection operate under the nearly untested assumption that the genetic variance in male quality is maintained through genic capture, whereby male genetic quality is reduced by deleterious mutations at condition-associated loci (e.g., Houle and Kondrashov 2002). Assuming mutations deleterious to condition do contribute to male genetic quality, condition-dependent sexual selection is then predicted to reduce the population mutation load (and thus improve mean fitness) when a positive genetic covariance exists between resource acquisition traits and condition (Whitlock 2000, Agrawal 2001, Siller 2001, Lorch et al. 2003).
1.3 Sexual selection and mutation load: weighing the evidence

Several lines of evidence suggest that sexual selection is effective in removing deleterious mutations from experimental populations, though evidence from some classes of experiments are stronger than others. The strongest empirical support emanates from experiments directly assaying the efficacy of sexual selection in eliminating mutations from populations experimentally enriched with deleterious variance (Whitlock and Agrawal 2009). These experiments can be separated into two classes: those demonstrating sexual selection against individual mutations of presumed (MacLellan et al. 2009, but see Arbuthnott and Rundle 2012) or known deleteriousness to nonsexual fitness (Whitlock and Bourguet 2000, Pischedda and Chippindale 2005, Sharp and Agrawal 2008, Hollis and Agrawal 2009), and those showing increased fitness for experimental populations experiencing sexual selection following mutagenesis (Radwan 2004, Almbro and Simmons 2014) (but see Hollis and Houle 2011, Plesnar et al. 2011, Power and Holman 2015) or during/after mutation accumulation (McGuigan et al. 2011) (but see Radwan et al. 2004). This latter category includes a recent and notably elegant study by Lumley et al. (2015), wherein two regimes of strong and weak sexual selection (governed by sex ratio and degree of polyandry, respectively) were imposed on the beetle *Tribolium castaneum* across 6-7 years (45-54 generations) in a wild-type stock population. Following this, up to 20 generations (unless precluded by line extinction) of full-sib inbreeding were imposed to expose recessive deleterious mutations remaining in the selection lines. Families derived from strong sexual selection histories survived 40% longer than those under weak selection (a result that qualitatively corroborates other recent studies demonstrating benefits of sexual selection to reducing extinction risk; Jarzebowska and Radwan 2010, Plesnar-Bielak et al. 2012), and maintained higher reproductive fitness throughout the intensification of inbreeding.

A second class of experiments indirectly addresses the question of whether sexual selection is effective in eliminating deleterious mutations (and thus bolstering natural selection): experimentally evolving populations have the opportunity for sexual selection artificially imposed (through polygamy) or relaxed (via monogamy) to test its effect on mean fitness. The first general category of these studies produced results suggesting that sexual selection is effective (Partridge 1980, Promislow et al. 1998), has no effect (Radwan et al. 2004), or is costly to mean population fitness (Holland and Rice 1999), collectively suggesting generally equivocal effects of sexual selection on mutation load. However, this ambiguity may be an
artefact of experimental design, wherein the short time-scale of these studies exaggerates the ultimate impact of sexual antagonism on fitness, and thus makes drawing conclusions on its fitness effects relative to sexual selection against deleterious mutations challenging (Whitlock and Agrawal 2009).

The second general category of these indirect studies tests the prediction (Lorch et al. 2003) that sexual selection should facilitate adaptation to a new environment. By transferring an experimental population from an environment in which it has adapted for many generations to one that it has never experienced, this approach is expected to increase the standing genetic variance in fitness. I am aware of several empirical studies that used this approach, producing two general results: a net benefit (Fricke and Arnqvist 2007, Plesnar-Bielak et al. 2012), and a neutral effect (Holland 2002, Rundle et al. 2006, Cabral and Holland 2014) of sexual selection on fitness during adaptation to a novel environment. An inverse prediction to that used in these studies can likewise be tested to determine whether natural and sexual selection are operating congruently: that male mating success should be maximized in the environment to which he is adapted (Dolgin et al. 2006). Three studies have addressed this question, and, parallel to the results for sexual selection facilitating adaptation, produced positive (Dolgin et al. 2006) and neutral (Correia et al. 2010, Arbuthnott and Rundle 2014) effects of adaptation on mating success.

Although there are potential limitations to manipulating genetic condition via inbreeding (Whitlock and Agrawal 2009), it is nonetheless an informative tool for assaying the impact of sexual selection on deleterious mutations. Sexual selection was effective at mitigating the effect of inbreeding on extinction risk in bulb mites (Jarzebowska and Radwan 2010) and flour beetles (Lumley et al. 2015), and female house mice preferred the scent of outbred males (Ilmonen et al. 2009). However, there was no effect of inbreeding on territory defense in D. serrata (White and Rundle 2015).

While there is considerable variation in results showing the efficacy of sexual selection in reducing mutation load, a recent study elegantly demonstrated a potential explanation for the lack of consistency in sexual selection impacts on deleterious mutations across populations and taxa. Long et al. (2012) showed that the potential for sexual selection to reinforce natural selection was proportional to the distance of the experimental D. melanogaster population from its fitness peak (a result which is qualitatively supported elsewhere: e.g., Fricke and Arnqvist
That is, daughters of sexually successful males had lower fecundity than daughters of unsuccessful males for populations residing close to their fitness peak (i.e., maintained under ancestral conditions and probably enriched for sexually antagonistic variance), whereas the inverse was true for populations relatively removed from their fitness peak (through introgression of unconditionally maladaptive variation via crosses between flies maintained under different selection histories). Hence, the potential for sexual selection to benefit population mean fitness through the removal of deleterious variance may depend on the relative influence of sexually antagonistic and unconditionally deleterious variance segregating in the experimental population at the start of the experiment (Whitlock and Agrawal 2009), which in most studies is not explicitly controlled (Long et al. 2012).

Finally, theory predicts that sexual selection will reduce mutation load only when selection is stronger in males than in females (Whitlock and Agrawal 2009). While only a handful of relevant studies exist, they collectively suggest that males do indeed experience stronger relative selection (Pischedda and Chippindale 2005, Sharp and Agrawal 2008, Mallet et al. 2011a, Mallet and Chippindale 2011, Sharp and Agrawal 2013).

1.4 The efficacy of sexual selection against deleterious mutations in the fruit fly, *Drosophila melanogaster*

While the studies reviewed above ask whether sexual selection provides a benefit (or cost; Arnqvist and Rowe 2005) to adaptation relative to enforced monogamy (i.e., artificially minimized sexual selection), a question fundamental to these studies, and condition dependent sexual selection generally, is whether sexual selection has the capacity to select against deleterious mutations via their effects on condition; this was the central thesis of my dissertation (in addition to parsing out which stages of sexual selection mattered in selecting against deleterious alleles). A secondary question basal to my dissertation was whether environmental manipulations of condition produce qualitatively parallel effects to genetic manipulations of condition, as predicted under condition dependence theory (e.g., Rowe and Houle 1996, Lorch et al. 2003). Following this logic, experimental manipulations of environmental condition (e.g., adjustment of juvenile diet quality) can be used as a proxy for deleterious mutations that
negatively impact an individual’s ability to acquire resources (i.e., to build the “condition” resource pool; Whitlock and Agrawal 2009).

While considerable attention has been given to the effects of total sexual selection on deleterious mutations (e.g., Holland 2002, Radwan 2004, Radwan et al. 2004, Rundle et al. 2006, Fricke and Arnqvist 2007, Sharp and Agrawal 2008, Hollis et al. 2009, Hollis and Houle 2011), there has been relatively little attention paid to the capacity for separate components of sexual selection to affect deleterious mutations. Specifically, there is a gap in the literature with respect to how postcopulatory sexual selection (cryptic female choice/ejaculate competition; Eberhard 1996, Birkhead and Moller 1998) affects mutation load. There is growing evidence that morphological traits correlated to sperm competition (e.g., testis weight and sperm length in dung beetles: Simmons and Kotiaho 2002; ejaculate size and storage in *D. melanogaster*: McGraw et al. 2007) are correlated with condition. Furthermore, direct components of postcopulatory sexual selection have demonstrated condition dependence. For instance, in *D. melanogaster* sperm transfer to (and storage by) females, refractory period induction (McGraw et al. 2007), and last-male sperm precedence (Amitin and Pitnick 2007) are dependent on male condition.

While this limited number of sperm competition studies collectively demonstrate that condition affects ejaculate competition both directly and indirectly, there is nonetheless a gap in the literature for studies investigating the effects of condition on components of sexual selection outside of its effect on secondary morphological characteristics subject to female choice (Whitlock and Agrawal 2009). Traditional models of good genes selection for indirect benefits require that variance in fitness be correlated with a display trait subject to female choice (Andersson 1994, Arnqvist and Rowe 2005); however, the effect of sexual selection on mutation load depends only on the amount of genetic variance for fitness that exists (i.e., an intermediate display trait *per se* is not required; Whitlock and Agrawal 2009). Furthermore, components of sexual selection outside of the traditional female choice for a showy morphological trait can operate to select against deleterious mutations, including pre- and postcopulatory intrasexual (e.g., male-male fighting; ejaculate competition) and intersexual (e.g., cryptic female choice) components of sexual selection (Whitlock and Agrawal 2009). There is at present little information in the literature on the broad impacts of the different components of sexual selection on deleterious mutations (Whitlock and Agrawal 2009), particularly at the post-copulatory level.
of sexual selection; hence, the broad objective of my dissertation was to investigate how summary components of sexual selection occurring pre- and postcopulation affect deleterious mutations in various forms.

1.5 Study Organism: *Drosophila melanogaster*

I employed the *Drosophila melanogaster* model system throughout my doctoral research. The *D. melanogaster* system is one of key importance across evolutionary biology, and has been particularly consequential to major empirical advancements of sexual selection, perhaps most notably with experiments in sexual conflict (reviewed in Arnqvist and Rowe 2005, Rice et al. 2006). Moreover, with respect to studies of sexual selection and mutation load, the *D. melanogaster* system has been one of disproportionate focus (Whitlock and Agrawal 2009). While this highlights the need for a move to greater cross-taxonomic study of sexual selection for good genes (see Chapter 6), it likewise emphasizes the rich literature base provided by *D. melanogaster* research for empirically testing questions of the efficacy of sexual selection in eliminating deleterious mutations. Additionally, there has been extensive research on the mechanisms underpinning sexual selection pre- and post-copulation for *D. melanogaster* (Andersson 1994, Birkhead and Moller 1998, Chapman 2001, Simmons 2001, Arnqvist and Rowe 2005, Manier et al. 2010, Sirot et al. 2011), including studies of male mate choice (Bonduriansky 2001, Friberg 2006). Thus, *D. melanogaster* offered a fertile system in which to address unanswered questions of sexual selection, including its relative effects on genetic and environmental condition, female condition, and male mate choice.

1.6 Chapter Synopses

In Chapter 2 (Clark et al. 2012), I began addressing the broad question of whether sexual selection has the capacity to be effective in eliminating deleterious mutations (and thus improving mean condition and fitness; Whitlock and Agrawal 2009) by building on a handful of empirical studies that hitherto asked this question of sexual selection *in toto* (see above). As a logical next step, I ran an experiment to investigate the capacity for particular components of
sexual selection to eliminate deleterious alleles. Specifically, last-male sperm precedence ($P2$) is a strong predictor of male sexually selected fitness in *D. melanogaster* (Boorman and Parker 1976, Harshman and Clark 1998, Morrow et al. 2005), and indeed across insects (Simmons and Siva-Jothy 1998, Simmons 2001); thus my primary objective was to test whether offensive ejaculate competition ($P2$) was effective at selecting against deleterious mutations, when the experimental male was the last to mate with a female. My experimental design also allowed a “no-choice” test of precopulatory female mate choice, and thus I was able to run a corollary assay of whether pre- and postcopulatory components of sexual selection that are known to be important to *D. melanogaster* fitness were parallel in their efficacy at selecting against dominant deleterious marker mutations (each with known viability and/or fecundity effects; Sharp and Agrawal 2008, Wang et al. 2009), a question heretofore unaddressed (at commencement of experimentation) with respect to sexual selection against deleterious mutations. Correctly extrapolating from environmental manipulations of condition to parallel genetic effects requires that a link between the two be established through independent experiments, which is empirically rare (Whitlock and Agrawal 2009). Hence, I likewise employed a diet manipulation to investigate the condition dependence of the aforementioned pre- and postcopulatory components of selection, and to test the prediction that this environmental manipulation of condition should be qualitatively similar to the aforementioned genetic manipulation of condition in affecting fitness.

In Chapter 3, I complemented the experimental design in Chapter 2 by measuring the impact of defensive ejaculate competition on recessive deleterious mutations exposed through inbreeding (hence, the assayed components of sexual selection and form of deleterious variation each differed from Chapter 2). Specifically, I measured the impact of inbreeding on remating latency (refractory period), offspring production during the refractory period, and sperm competition defence ($P1$), traits for which there was some empirical precedence for condition dependence (Amitin and Pitnick 2007, McGraw et al. 2007). As for Chapter 2, I used a diet manipulation to assay condition dependence in ejaculate competition defence.

In Chapter 4, I used a powerful design to comprehensively determine which components of sexual selection are important for purging deleterious mutations in *D. melanogaster*. There is nascent empirical interest in determining the relative contributions of pre- and postcopulatory components of selection to total sexually selected fitness; indeed, recent results suggest that pre-
and postcopulatory sexual selection contribute approximately equally to male sexually selected fitness (Pischedda and Rice 2012). With respect to the impact of sexual selection on deleterious mutations, only one study (published after completion of my research; Mallet et al. 2012) has yet compared the relative influence of pre- and postcopulatory components of sexual selection on mutation load. To address this question, I assessed the relative importance of mating success, ejaculate competition defence (incorporating refractory period induction, offspring production therein, and $P1$), and sperm competition offence ($P2$) in selecting against spontaneously accumulated deleterious mutations. Additionally, while the importance of male condition dependence is well established (see above), there is burgeoning evidence that female condition may be similarly important (Cotton et al. 2006), wherein female condition can drive indirect benefits received by females (Long et al. 2010) and the strength of sexual selection on males (Amitin and Pitnick 2007). Thus, I likewise measured the effect of female condition on selection against deleterious alleles.

Peripheral to the central question of my thesis, I deviated slightly in research objective for my final data chapter (Chapter 5) to investigate cryptic male mate choice in *D. melanogaster*. A mounting body of evidence suggests precopulatory male mate choice is cross-taxonomically prevalent in insects (Bonduriansky 2001). Furthermore, there is growing empirical support for male mate choice at the postcopulatory stage of sexual selection (Edward and Chapman 2011). Due to trade-offs between the ejaculate and competing reproductive costs, theory predicts prudence in male allocation of sperm according to the social conditions of mating (e.g., perceived level of ejaculate competition; Edward and Chapman 2011), a notion that has garnered cross-taxonomic support (Parker 1998, Wedell et al. 2002). Recently, it has been postulated by theory (e.g., Cameron et al. 2007) and demonstrated empirically (Wigby et al. 2009, Sirot et al. 2011) that the seminal fluid portion of the ejaculate can likewise be strategically allocated to females. Using a manipulation to cause true virgin females to “smell” either virgin or mated to potential mates (Coyne et al. 1994, Friberg 2006), I investigated the immediate and downstream effects on fitness for male ejaculate allocation decisions based on perceived risk of ejaculate competition in female mates (i.e., whether females were perceived as virgin or mated).
Chapter 2
Relative Effectiveness of Mating Success and Sperm Competition at Eliminating Deleterious Mutations in *Drosophila melanogaster*

2 ABSTRACT

Condition-dependence theory predicts that sexual selection will facilitate adaptation by selecting against deleterious mutations that affect the expression of sexually selected traits indirectly via condition. Recent empirical studies have provided support for this prediction; however, their results do not elucidate the relative effects of pre- and postcopulatory sexual selection on deleterious mutations. We used the *Drosophila melanogaster* model system to discern the relative contributions of pre- and postcopulatory processes to selection against deleterious mutations. To assess second-male ejaculate competition success (P2; measured as the proportion of offspring attributable to the experimental male) and mating success, mutant and wild-type male *D. melanogaster* were given the opportunity to mate with females that were previously mated to a standard competitor male. This process was repeated for males subjected to a diet quality manipulation to test for effects of environmentally-manipulated condition on P2 and mating success. While none of the tested mutations affected P2, there was a clear effect of condition. Conversely, several of the mutations affected mating success, while condition showed no effect. Our results suggest that precopulatory selection may be more effective than postcopulatory selection at removing deleterious mutations. The opposite result obtained for our diet manipulation points to an interesting discrepancy between environmental and genetic manipulations of condition, which may be explained by the multidimensionality of condition. Establishing whether the various stages of sexual selection affect deleterious mutations differently, and to what extent, remains an important issue to resolve.
2.1 INTRODUCTION

It is intuitive to think of natural selection as a force operating to remove deleterious mutations from a population, although it is perhaps less conventional to think of sexual selection in the same context. However, if male siring success is condition-dependent, then natural and sexual selection may operate in the same direction on most genes (Rowe and Houle 1996). Accordingly, condition-dependence theory predicts that sexual selection will facilitate adaptation through various mechanisms, including increasing the spread of beneficial alleles (Whitlock 2000), and reducing the genetic load (Whitlock 2000, Agrawal 2001, Whitlock and Agrawal 2009). Likewise, condition-dependent sexual selection is predicted to enhance the rate and extent of adaptation in temporally fluctuating environments (Lorch et al. 2003). Indeed, several recent studies have demonstrated the efficacy of sexual selection against deleterious mutations (Radwan 2004, Sharp and Agrawal 2008, Hollis et al. 2009), and the potential for sexual selection to reduce mutation load through selection on males (Mallet et al. 2011a, Mallet and Chippindale 2011). These effects may have occurred through precopulatory (e.g., mate choice) or postcopulatory (e.g., ejaculate competition) sexual selection, yet these studies did not attempt to partition total sexual selection amongst the components of siring success (in actuality, Mallet et al. 2011b did look at this; however, that citation was missing from the published version of this chapter). Consequently, the relative extent to which selection against deleterious mutations occurs through pre- and postcopulatory processes remains unclear.

In Drosophila melanogaster, postcopulatory sexual selection is generated through differences in sperm number and ejaculate quality. For example, males produce seminal fluid proteins that have substantial effects on sperm transfer, sperm storage, female receptivity, ovulation, and oogenesis (Wolfner 1997). If ejaculate competition is condition-dependent, then most mutations should indirectly affect postcopulatory success via their effects on condition; there is some evidence that this is the case (Amitin and Pitnick 2007, McGraw et al. 2007).

Adaptations to sperm competition can be grouped into offensive and defensive categories. Sperm competition defence occurs when a male is the first to mate with a female (and thus he has to defend against potential future males’ ejaculates), and can include such adaptations as strategic ejaculation of sperm and seminal fluids, mating plugs to retain his and block competitors’ sperm, and behavioural guarding to inhibit females from remating (Simmons
Sperm competition offence occurs when a male copulates with a previously-mated female, and can include adaptations such as sperm displacement and strategic ejaculation (Simmons 2001). For our experiment, we focused on sperm competition offence, which has particular importance to male D. melanogaster sexually selected fitness (Gromko et al. 1984) and can be quantified as the proportion of offspring sired by the second male to mate a female (P2). We first performed a diet manipulation to establish whether P2 success was condition-dependent. We then compared P2 scores for a variety of mutant and wild-type genotypes. Our assay also provided a measure of pre-mating selection against these genotypes, allowing us to compare pre- and postcopulatory selection. Our objective was to determine whether components of pre- and postcopulatory sexual selection, specifically mate choice and sperm competition offence, could produce complementary effects on the mutation load in D. melanogaster. We predicted that mutant males would demonstrate reduced pre- and postcopulatory reproductive success relative to their wild-type competitors.

2.2 METHODS

2.2.1 Study Organisms

Flies for the experiment were derived from a wild-type (+/+ outbred population of D. melanogaster originally collected in 1970 from Dahomey (now Benin), West Africa. The wild-type (+/+) Dahomey stock was maintained in the current laboratory for over six years at a population size of several thousand adults. We obtained six dominant deleterious mutant (M$_i$+/+; where $i$ represents a given dominant mutation) stocks from the Bloomington Drosophila Stock Center (http://flystocks.bio.indiana.edu/), and each mutation was separately introgressed into the Dahomey background through at least ten generations of serial backcrossing. Each of these mutations affected separate autosomal loci, and was located on either the second (Adv, Gla) or third (Dr, Gl, Ly, Sb) chromosome. Mutant alleles had visible phenotypic effects on the eyes (Dr: eyes appear as vertical slits; Gl: eyes appear glossy and slightly reduced; Gla: eyes appear glossy), wings (Adv: wings contain an additional vein, Ly: wings have a reduced, rectangular appearance), or bristles (Sb: bristles are reduced to an abbreviated length); these visible markers allowed for easy identification of mutant individuals when scoring sperm competitor fitness. Four of these mutations (Dr, Gla, Ly, Sb) were chosen due to known viability selection,
fecundity selection, and total sexual selection acting against them from an earlier experiment (Sharp and Agrawal 2008); the other two (Gl, Adv) were chosen based on known viability effects (Wang et al. 2009). All flies were cultured at 25°C under ~60% relative humidity, on a 12L:12D photoperiod.

2.2.2 Mating Trial Design

Sperm competition offence was measured as the siring success of the focal male (either M/i/+ or +/+ ) when he was the second male to mate with a female (i.e., the second-mating position). Focal males (M/i/+ and +/+ ) for each of the six mutations tested were derived from a cross of +/+ females × M/i/+ males. For each of the six mutations used in the experiment, a sample of experimental males was derived by crossing mutant males to outbred wild-type females from a common source population. Each mutation used in the experiment was introgressed into the Dahomey background (see Study Organisms); thus, a cross of mutant males by Dahomey females produced heterozygous mutant offspring and wild-type Dahomey offspring. Wild-type offspring produced from a given mutant cross were compared only with their mutant counterparts from that cross (i.e., the mutant and wild-type flies being contrasted always came from the same cross, and thus experienced identical juvenile competitive conditions). Using the Gla mutation as an example, Gla males were mated to +/+ females, and then Gla and +/+ male offspring from that cross were compared to each other to assess mutant/wild-type relative P2 and mating success for Gla only; thus, +/+ males derived from a cross of +/+ females × M/i/+ males were statistically compared only with mutant males of the same cross. A standard fly stock homozygous for a recessive brown-eye mutation (bw/bw), which had been introgressed onto the Dahomey background, was used as the source of all females and all first mating-position (competitor) males in the experiment.

The design of the sperm competition experiment was standardised across each of the mutations tested. First-male matings were conducted at a rate of approximately 600 per mutation tested, and occurred en masse in shell vials (25x95mm O.D. x height) containing standard yeast-sugar-agar medium by crossing virgin bw/bw males with virgin bw/bw females at a 10M:10F sex ratio for a duration of 2 hours. This time window was chosen because it was sufficient for a single mating in D. melanogaster, and in previous mating experiments using fly lines of the Dahomey
genetic background, only one mating pair was ever found to remate within the prescribed 2 hour observation window (SC pers. comm.). The 2 hour mating window is also conventional in sperm competition research in *D. melanogaster* (e.g., (Clark and Begun 1998)). As the bw/bw mutation was recessive, all offspring of the first male carried the brown-eye phenotypic marker. Following the first-male mating, males were discarded, and females were retained individually in holding vials containing standard medium to ensure they had mated. Mating was confirmed by assaying for the presence of larvae in the holding vials 48 hours post-mating; all females that did not produce larvae were discarded. Three days after the first-male mating, mated females were allowed a three-hour window to remate with either a M/i/+ or +/+ virgin second male (approximately five days old; individual matings were established at a ratio of approximately 3 M/i/+ to 2 +/+ trials, as the likelihood of remating was predicted to be lower for M/i/+ flies.

Specific ratios: *Adv*: 299:199; *Dr*: 300:196; *Gl*: 236:157; *Ly*: 224:152; *Sb*: 286:170; *Gla*: 292:163. Offspring from this second cross were heterozygous with respect to the brown-eye allele; thus, these offspring were phenotypically wild-type with respect to eye colour, which distinguished them from the bw/bw offspring sired by the first male. Following the second-male mating, all males were discarded, and females were transferred into individual laying vials (vial 1) containing standard yeast medium. Females were kept in these vials for 48 hours, and then were transferred to a second set of laying vials (vial 2) for a period of 72 hours, after which females were discarded. Given that second-male copulations were not observed directly, mating was assumed to have taken place if there were wild-type or mutant offspring produced from the second-male mating. This is a reasonably accurate method of determining whether a female mated with the second male, given the strong last-male sperm precedence in this species (Gromko et al. 1984). All data from vials where females did not remate were removed from the dataset prior to P2 analysis; however, these data were useful in providing a measure of precopulatory selection (as described below).

### 2.2.3 Postcopulatory Sexual Selection – Sperm Competition Offence

Sperm competition offence was assayed as the proportion of offspring sired by the second male. This proportion takes the form of \( P2 = N2/(N_{bw/bw} + N2) \), where \( N_{bw/bw} \) is the number of offspring attributed to the bw/bw competitor male, and \( N2 \) is the number of offspring attributed to the focal (M/i/+ or +/+) male (three types of offspring were possible from the M/i/+ crosses: bw/bw, M/i/bw,
and +/-bw; just two types of offspring were possible from the +/- crosses: bw/bw and +/-bw). Offspring from laying vials 1 and 2 were counted and scored based on phenotype (wild-type, brown-eyes, or dominant mutation) on days 13 and 15 of their life cycle (exception: for mutations Gla and Sb, part of dataset was scored on days 12 and 14). To account for viability effects of the M_i alleles, the M_i/bw offspring count was omitted from the P2 calculation. Instead, the number of offspring sired by the mutant male was assessed as 2•(N+/bw) because the expected ratio of M_i/bw to +/-bw offspring is 1:1 under conditions of no viability selection against the M_i allele.

2.2.4 Precopulatory Sexual Selection – Mating Success

While not the primary focus of this experiment, precopulatory sexual selection was approximated for each mutation tested by comparing the proportion of M_i/+ and +/- males that successfully mated with females during the second mating opportunity provided to each female. The second mating was considered successful if mutant and/or wild-type offspring were present among the progeny produced by a given female in vials 1 and 2. If only bw/bw offspring were produced (representing offspring of the first mating), then it was assumed that second males did not mate females. Our experimental design consisted of a “no-choice trial” using non-virgin females, in which each female was assigned a single male (M_i/+ or +/-) as a potential mate; it was not possible for females to simultaneously choose between male types in this experiment.

2.2.5 Condition Dependence

For each of the above assays (P2, mating success), we created high- and low condition males through a diet resource manipulation. Eggs laid by Dahomey females were transferred to vials in groups of 50 to standardise larval density and competition across condition treatments. High condition males were reared on standard medium, while low condition flies were reared on medium containing one-quarter of the standard volume of yeast and sugar. All P2 and mating success assay protocols for high and low condition flies were identical to those aforementioned for M_i/+ and +/- flies. Accordingly, individual matings were established at a ratio of
approximately 3 low condition to 2 high condition trials (specific ratio: 431:281), as it was predicted that, like mutant males, mating success would be lower for low condition males.

2.2.6 Effects of Condition and Mutation on Body Size

The impact on male body size (an index of condition) of each of the six deleterious mutations, and our diet manipulation, was assayed for a group of virgin males separate from those used in the aforementioned fitness assays. Mutant and high/low condition males were produced and housed prior to weighing according to the same protocol as aforementioned for fitness assays. Flies were dried in an oven at 65°C for 25-27 hours, and then were weighed individually on a Sartorius microbalance.

2.2.7 Analysis

All P2 data were analysed in R (v. 2.9.0; R Development Core Team 2009) using general linear models with quasibinomial error structure, independently for each mutation and its paired wild-type competitor. Remating rate and body mass comparisons were assessed in JMP (v. 8.0.1) using a Chi-squared analysis and Student’s t-tests, respectively.

2.3 RESULTS

2.3.1 Sperm Competition Offence

P2 was condition-dependent, but we could not detect an effect of the mutations on P2. Wild-type high condition males produced significantly more offspring than wild-type low condition males in the P2 mating position ($t_{1,83}$=-2.03, $p=0.0452$; Fig. 2.1). However, none of the six mutations tested had a significant depressing effect on P2 ($Adv$: $t_{1,107}=0.339$, $p=0.736$; $Dr$: $t_{1,128}=0.192$, $p=0.848$; $Gl$: $t_{1,39}=-1.22$, $p=0.229$; $Gla$: $t_{1,113}=-0.576$, $p=0.566$; $Ly$: $t_{1,69}=-0.262$, $p=0.794$; $Sb$: $t_{1,153}=-1.62$, $p=0.108$; Fig. 2.2a-f).
2.3.2 Mating Success

Wild-type high condition males performed equally well to wild-type low condition males in obtaining a mating from once-mated females ($\chi^2_{1,655}=0.893$, $p=0.345$; Fig. 2.3). Reduced success of mutant males in obtaining a mating with once-mated females was shown for four mutations ($Dr$: $\chi^2_{1,496}=14.0$, $p=2.0\times10^{-4}$; $Gl$: $\chi^2_{1,393}=17.4$, $p<1.0\times10^{-4}$; $Ly$: $\chi^2_{1,376}=4.64$, $p=0.0313$; $Gla$: $\chi^2_{1,455}=16.4$, $p<1.0\times10^{-4}$; Fig. 2.3). For the remaining two mutations, mutant males were not significantly different than wild-types ($Adv$: $\chi^2_{1,498}=0.023$, $p=0.879$ and $Sb$: $\chi^2_{1,456}=0.123$, $p=0.726$; Fig. 2.3).

2.3.3 Effects of condition and mutation on body size

As expected, high condition males were significantly larger in body mass relative to low condition males (18.3%, $t_{92.9}=-9.04$, $p<0.0001$; Table 2.1). Mutational effects on body mass varied, with half of the mutations significantly decreasing body mass ($Adv$: $t_{83.3}=4.23$, $p<0.0001$; $Gla$: $t_{81.5}=4.41$, $p<0.0001$; $Ly$: $t_{87.5}=6.68$, $p<0.0001$; Table 2.1), and the other half showing no effect ($Dr$: $t_{92.7}=-0.224$, $p=0.823$; $Gl$: $t_{82.3}=1.29$, $p=0.199$; $Sb$: $t_{91.3}=0.319$, $p=0.751$; Table 2.1). Pooling data for mutations that had a significant effect on body mass, wild-type flies were 12.0% larger than mutants ($t_{262.3}=8.35$, $p<0.0001$), an effect size only two-thirds that of condition (Table 2.1).

2.4 DISCUSSION

Work to date probing the effects of sexual selection on female fitness (measured as offspring production) has focused on sexual selection in toto, and has generated each of neutral (Holland 2002), negative (Rundle et al. 2006), and positive (Fricke and Arnqvist 2007) fitness effects. However, studies focusing on total sexual selection on novel deleterious mutations have collectively demonstrated that sexual selection acts to reduce the mutation load (Radwan 2004, Sharp and Agrawal 2008, Hollis et al. 2009). That is, sexual selection on males helps to eliminate alleles that would be bad for either sex. We found that males of several mutations showed reduced mating success, but performed equally well in ejaculate competition relative to their wild-type competitors. For the mutations tested, our results suggest that precopulatory
sexual selection is more effective than postcopulatory sexual selection at reducing the mutation load. This implies that most of the previously demonstrated total sexual selection against several mutations used in our study (Sharp and Agrawal 2008) occurred at the precopulatory phase, as the previous study (Sharp and Agrawal 2008) looked at total sexual selection, and we parsed sexual selection into pre- and postcopulatory components.

Given that our diet manipulation showed reduced P2 for low condition males, we predicted that mutant males would likewise demonstrate lower P2; however, this was not what we observed. There are several possible explanations for this discrepancy. First, “condition” is likely to be multi-dimensional (e.g., Tomkins et al. 2004), and environmental manipulations may alter different aspects of condition than do deleterious mutations. Accordingly, P2 success may be insensitive to those aspects of condition altered by deleterious mutations. Alternatively, the mutations used here may have only weakly affected condition, perhaps making their downstream effects on P2 undetectable. These mutations were known to affect viability and/or fecundity (Sharp and Agrawal 2008, Wang et al. 2009). However, where present, mutational effects on body size tended to be smaller than for diet manipulation (Table 2.1). Furthermore, even the three mutations that did have significant effects on body size (Adv, Gla, Ly) showed no evidence of affecting P2. In contrast to our results, a recent mutation accumulation study reported deleterious effects on both P1 and P2 (Mallet et al. 2011b). Because that study examines the effects of a more representative set of natural mutations, it seems likely that postcopulatory selection does help reduce mutation load. However, our results indicate that the amount of selection occurring through P2 may be very small for some types of mutations.

The precopulatory effect of these mutations was consistent with previous studies of some of them (Sharp and Agrawal 2008). One interpretation of the earlier results (Sharp and Agrawal 2008) was that sexual selection occurred on these mutations via their effects on condition. However, it has been argued that mutations with obvious visible effects, such as those used here, may affect male mating success directly, rather than indirectly through condition (Hollis et al. 2009). In our experiment, precopulatory success was unaffected by our diet-based manipulation of condition, and yet the mutations we tested still seemed to experience selection at the precopulatory stage. These observations suggest two things. First, the “no-choice” measure of precopulatory success used here (see Methods) might have been a rather blunt assay of true mating success, which may be insensitive to condition. Previous studies using very similar diet
manipulations have shown that male mating success is strongly condition-dependent when assessed in competitive mating assays (e.g., Sharp and Agrawal 2009). Second, this observation further supports the notion that these mutations affect mating success directly, rather than indirectly via condition.

Our experiment represents a preliminary attempt to elucidate the relative importance of the different components of sexual selection against mutation load. Our study revealed interesting discrepancies between environmental and genetic manipulations, which may be resolved by using inbreeding or mutation accumulation to manipulate genetic quality (such manipulations are likely to be more representative of segregating mutations). Disentangling the effects of sexual selection on deleterious mutations into pre- and postcopulatory processes remains an unresolved challenge in understanding how and when sexual selection acts on deleterious mutations.
Table 2.1. Effects of condition and mutations on body mass (mean +/- SE). Gene corresponds to the locus of the dominant mutation, whereas mutation corresponds to whether the individual was wild-type or mutant with respect to that locus. Mass is given in milligrams with corresponding standard error. Percent difference in mass for a given gene is expressed as the difference between wild-type (High Condition) and mutant (Low Condition) masses, divided by the mutant (Low Condition) mass. P-values for significant differences are boldfaced.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutation</th>
<th>Mass (mg) ± SE</th>
<th>% Difference</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Condition</td>
<td>Low</td>
<td>0.199 ± 2.91x10^-3</td>
<td>18.3</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>0.236 ± 2.81x10^-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adv</td>
<td>M</td>
<td>0.216 ± 2.82x10^-3</td>
<td>9.73</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td>wt</td>
<td>0.237 ± 4.11x10^-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dr</td>
<td>M</td>
<td>0.236 ± 2.66x10^-3</td>
<td>0.365</td>
<td>p = 0.823</td>
</tr>
<tr>
<td></td>
<td>wt</td>
<td>0.235 ± 2.78x10^-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gl</td>
<td>M</td>
<td>0.234 ± 2.58x10^-3</td>
<td>2.50</td>
<td>p = 0.199</td>
</tr>
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<td></td>
<td>wt</td>
<td>0.239 ± 3.71x10^-3</td>
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<td></td>
</tr>
<tr>
<td>Gla</td>
<td>M</td>
<td>0.220 ± 3.38x10^-3</td>
<td>12.0</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td>wt</td>
<td>0.246 ± 4.96x10^-3</td>
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<td></td>
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<tr>
<td>Ly</td>
<td>M</td>
<td>0.204 ± 3.02x10^-3</td>
<td>15.1</td>
<td>p &lt; 0.0001</td>
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<tr>
<td></td>
<td>wt</td>
<td>0.235 ± 3.48x10^-3</td>
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<td></td>
</tr>
<tr>
<td>Sb</td>
<td>M</td>
<td>0.247 ± 3.61x10^-3</td>
<td>0.688</td>
<td>p = 0.751</td>
</tr>
<tr>
<td></td>
<td>wt</td>
<td>0.249 ± 3.94x10^-3</td>
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</tbody>
</table>
Figure 2.1. Relative offensive sperm competition success, given as second-male paternity (P2; mean +/- SE), for high- and low condition males. Sperm competition success was measured as the proportion of offspring produced by high- or low condition males relative to a standard competitor mated to a given female. High condition males showed significantly higher P2 relative to low condition males.
Figure 2.2. Relative offensive sperm competition success, given as second-male paternity (P2; mean +/- SE), for mutant and wild-type males (a. Adv; b. Dr; c. Gl; d. Gla; e. Ly; f. Sb). Wild-type treatments are listed as wt(Mi), where Mi represents the paired mutant treatment. Sperm competition success was measured as in Fig. 2.1. There were no significant differences between mutants and wild-types for any of the paired comparisons.
Figure 2.3. Relative mating success of mutant and wild-type males. Mating was deemed successful when mutant or wild-type males achieved copulation with a nonvirgin female; unsuccessful matings occurred when males failed to copulate. Black bars represent mutant (or low condition, L) males; white bars depict wild-type (or high condition, H) males. Mutant-wild-type pairs are presented according to mutant genotype. Significant differences are indicated by an asterisk.
Chapter 3
Postcopulatory Sexual Selection Against Males of Low Genetic and Environmental Quality in *Drosophila melanogaster*

3 ABSTRACT

Good genes theory predicts that sexual selection should act to enhance nonsexual fitness by selecting against deleterious mutations. While empirical support for this idea is growing, there is a paucity of data investigating the specific components of sexual selection responsible for affecting mutation load. We used inbreeding to elevate the mutation load in an experimental population of *Drosophila melanogaster* and tested whether our inbreeding manipulation affected three components of postcopulatory fitness: remating latency (refractory period), offspring produced before remating, and sperm competition defence (P1). We found that inbreeding had a significant effect on all three traits; however, a complementary assay using high- and low condition males as a proxy for individuals of high and low genetic quality, respectively, differed qualitatively in results for remating latency and offspring produced before remating (i.e., there was no effect of condition on these traits). Our results suggest that sexual selection is effective at eliminating deleterious mutations at the postcopulatory level, and that environmental condition may not always serve as a good proxy for genetic condition.
3.1 INTRODUCTION

A question receiving considerable interest in recent sexual selection research is whether sexual selection has the ability to reduce the mutation load via selection on condition dependent traits, thereby acting in concert with natural selection to enhance nonsexual fitness. This question is premised on good genes theory, which states that sexual selection can provide indirect fitness benefits to females choosing mates based on their genetic quality, which is reflected phenotypically in condition and condition dependent traits (Proulx 1999, Agrawal 2001, Proulx 2001, 2002, Lorch et al. 2003). While these models tend to be centred on female choice for condition dependent morphological traits, similar effects may occur in other components of sexual selection (e.g., male-male fighting for mates, sperm competition, etc.; Whitlock and Agrawal 2009). As condition encompasses a large portion of the genome, it represents a large mutational target (Houle 1991); hence, good genes models state that females who sort males on the basis of condition likewise sort males on the basis of genetic quality, or good genes, thereby enabling sexual selection to purge deleterious mutations (Rowe and Houle 1996). There is substantial empirical support for the condition dependence of sexually selected traits (Price et al. 1993, Johnstone 1995, Jennions et al. 2001, Cotton et al. 2004).

Several recent experiments provide support for the hypothesis that sexual selection can augment nonsexual fitness by reducing mutation load. The most direct evidence for the ability of sexual selection to eliminate deleterious mutations comes from experiments demonstrating sexual selection against individual alleles of presumed (MacLellan et al. 2009, but see Arbuthnott and Rundle 2012) or known deleterious impact to nonsexual fitness (Whitlock and Bourguet 2000, Pischedda and Chippindale 2005, Sharp and Agrawal 2008, Hollis et al. 2009). Additionally, several experiments show indirect evidence for sexual selection against deleterious mutations via increased fitness for populations evolving under sexual selection during or following experimental elevation of the mutation load using mutagenesis (Radwan 2004, Almbro and Simmons 2014) (but see Hollis and Houle 2011, Plesnar et al. 2011, Power and Holman 2015), mutation accumulation (McGuigan et al. 2011) (but see Radwan et al. 2004), or inbreeding (Jarzebowska and Radwan 2010) (but see White and Rundle 2015). In a particularly elegant and unique design in this class of experiment, Lumley et al. (2015) ran a long-term experimental evolution study using flour beetles (Tribolium castaneum), in which sexual selection was made.
relatively strong or weak using manipulations of sex ratio or degree of polygamy, after which up to 20 generations (or until line extinction) of full-sib inbreeding were employed to expose deleterious recessives remaining in the experimental populations, with the end result that a history of strong sexual selection mitigated extinction risk and enhanced reproductive fitness.

Additional support for the alignment of sexual and natural selection comes from complementary studies showing positive effects of sexual selection on nonsexual fitness during adaptation to a novel environment (Fricke and Arnqvist 2007, Plesnar-Bielak et al. 2012) (but see Holland 2002, Rundle et al. 2006, Cabral and Holland 2014), and inversely benefits of local adaptation to male reproductive success (Dolgin et al. 2006) (but see Correia et al. 2010, Arbuthnott and Rundle 2014). Likewise, recent results suggest that net selection against deleterious mutations is stronger in males than in females (Pischedda and Chippindale 2005, Sharp and Agrawal 2008, Mallet et al. 2011a, Mallet and Chippindale 2011, Sharp and Agrawal 2013, Zikovitz and Agrawal 2013), a result that is required by theory for sexual selection to impact mutation load (Whitlock and Agrawal 2009).

While empirical support for the alignment of sexual and natural selection is mounting, there remains considerable variability in experimental results (e.g., non-positive effects of sexual selection on fitness; see counter-references above). However, a recent study elucidated a likely reason for this variation: the efficacy of sexual selection in positively affecting fitness may be inversely proportional to the proximity of the experimental population to its fitness peak. Long et al. (2012) used crosses between populations of Drosophila melanogaster adapted to different environments to introgress deleterious alleles into an experimental population, thereby removing it from its fitness peak. The authors elegantly demonstrated that these “off-peak” populations showed fitness benefits to sexual selection, while ancestral “peak” populations demonstrated a cost to sexual selection, a result likely attributable to the hypothesized contrasting influence of sexually antagonistic and unconditionally deleterious variance to overall fitness in ancestral versus novel conditions (Whitlock and Agrawal 2009, Long et al. 2012). Data from the literature qualitatively support this argument (e.g., Fricke and Arnqvist 2007).

While there is growing empirical support for the idea that sexual selection can act in concert with natural selection to enhance nonsexual fitness, experiments to date have generally focused on sexual selection in toto (e.g., Holland 2002, Radwan 2004, Radwan et al. 2004, Rundle et al. 2006, Fricke and Arnqvist 2007, Sharp and Agrawal 2008). However, it is necessary to
understand which components of sexual selection are most important for enhancing nonsexual fitness. Several recent studies have begun to address this issue (MacLellan et al. 2009, Pekkala et al. 2009, Clark et al. 2012, Mallet et al. 2012, Almbro and Simmons 2014, Power and Holman 2015).

Although it is desirable to genetically manipulate phenotypic condition (e.g., using mutation accumulation, inbreeding, etc.) to test the hypothesis that sexual selection improves nonsexual fitness (via selection against mutations deleterious to condition), many studies use environmental manipulations of phenotypic condition (e.g., larval diet or density adjustment) as a proxy for genetic manipulations (see Cotton et al. 2004, Whitlock and Agrawal 2009). However, the prediction that sexual selection should be similar in its qualitative effects on mutant and low environmental condition treatment groups (Tomkins et al. 2004, Whitlock and Agrawal 2009) remains nearly untested. The few empirical results that exist suggest that environmental quality may not always serve as a useful surrogate to genetic quality (Bonduriansky et al. 2015, White and Rundle 2015).

Here, we address the impact of several postcopulatory components of sexual selection on deleterious recessive mutations exposed from standing genetic variance through inbreeding in Drosophila melanogaster. Specifically, we predicted that male ability to extend the female refractory period (elapsed time between first and second mating), offspring produced by females during the refractory period, and the proportion of offspring attributable to the first male (P1) to mate with a twice-mated female would each be adversely affected by inbreeding in experimental males. These traits were chosen because of their importance to sexually selected fitness in D. melanogaster (Harshman and Clark 1998), and because there is some evidence for their condition dependence (Amitin and Pitnick 2007, McGraw et al. 2007). Additionally, we tested the hypothesis that genetic and environmental quality are aligned with respect to their effects on the aforementioned sexually selected fitness components. Accordingly, we employed a juvenile diet manipulation to produce low- and high condition adult males to be used in a set of assays identical to those used for the inbreeding treatment, under the prediction that inbred and low condition flies would be qualitatively similar in performing poorly under sexual selection relative to outbred and high condition flies, respectively.
3.2 METHODS

3.2.1 Study Organisms

Flies for the experiment were derived from a wild-type (+/+) parent stock of *D. melanogaster* originating from a field collection in Dahomey (now Benin), West Africa. Standard competitors for the sperm competition experiment were homozygous for a genetically recessive phenotypic marker (bw/bw) for brown eyes that was introgressed into the Dahomey genetic background. Unless otherwise indicated, all line maintenance, experimental matings, and fitness assays were conducted in plastic shell vials (25x95mm O.D.xheight) containing standard yeast-sugar-agar medium supplemented with one pellet of live yeast. All flies were cultured at 25°C under ~60% relative humidity, on a 12L:12D photoperiod.

3.2.2 Inbreeding Depression

An experimental population of inbred flies was established through three generations of full-sib inbreeding. We established the parental (P) generation (f=0) through the formation of 484 monogamous and randomly paired families derived from the Dahomey population. From each P-generation family that produced offspring, one male and one female virgin offspring was randomly collected and mated monogamously to form 393 full-sib families in the F1 generation (f=0.25). This full-sib mating regime was repeated for the F2 generation (f=0.375). In the F3 generation, two male and two female virgin siblings were collected from each of 230 randomly chosen inbred families. Half of these virgins were mated as in the F1 and F2 generations to form full-sib monogamous families (f=0.5); the other half were outbred monogamous crosses, statistically paired with the inbred crosses through the female (i.e., for a given pair of inbred and outbred crosses, the female used for each cross was a full sib; for outbred crosses, males were randomly chosen from the experimental population). Inbreeding coefficients were calculated using the recurrence equation for full-sib mating: \( f(t) = 0.5[f(t-1)] + 0.25[f(t-2)] + 0.25 \) (Lynch and Walsh 1998).
3.2.3 Sperm Competition Assay

To assay sperm competition defence, focal (=inbred or outbred +/-) virgin males were mated to virgin *bw/bw* females. On the evening prior to the experiment, virgin *bw/bw* females were transferred to individual mating vials, each containing a single pellet of live yeast. On the first morning (Day 0) of the experiment, focal males were transferred to empty vials (no food/yeast) approximately one hour prior to mating. Focal males were then transferred from their holding vials into the mating vials that held the *bw/bw* females. Male-female pairs (n=399) were observed continuously for two hours, and mating duration was recorded for each mating pair. Following mating termination, males and females were separated using light CO₂ anaesthesia, and females were retained for remating. All male-female pairs that did not mate (n=95) were discarded from the experiment.

To provide sperm competition against focal males, each *bw/bw* female that mated to a focal male on Day 0 was given the opportunity to remate to a *bw/bw* male on days 1-6 (all offspring produced by *bw/bw* competitor males had brown eyes, which distinguished them from the offspring of focal males, which had phenotypically wild-type eyes). On each of Days 1-6, all *bw/bw* females that had not yet remated were given approximately 2.5 hours to remate with a *bw/bw* competitor male. All *bw/bw* females that remated in a given remating trial were separated from their mates and allowed to lay eggs in ‘paternity vials’ for 48 hours. For all *bw/bw* females that did not remate in a given remating trial, males were removed from the vial and the female was left to lay eggs until the following day, at which time the process of transfer to fresh medium with a new *bw/bw* male for mating observation was repeated. This process continued until day six of the experiment, after which the remating component of the experiment was terminated; thus, any females that did not remate by six days after first mating were not given further opportunity to do so. The physical placement of laying vials inside the growth chamber was randomised daily.

3.2.4 Post-Copulatory Fitness Assay: Offspring Production After First Mating

Focal male offspring production during the refractory period (i.e., after the focal mating and before the competitor mating) was assayed by counting all focal male offspring produced by his
female mate prior to her remating with a competitor male. Offspring from each 24 h laying vial in which a given female laid eggs between her first and second mating were counted and summed across vials on days 13 and 15 of their life cycle. All offspring attributed to these vials were wild-type with respect to eye colour. This offspring count allowed us to estimate the ability of focal males to affect offspring production prior to their engaging in direct sperm competition.

3.2.5 Post-copulatory Fitness Assay: Time to Remating

The aforementioned sperm competition design allowed us to quantify time to remating (i.e., refractory period) for females after mating with focal males. Time to remating was quantified as the number of days between when a female mated with a focal male and when she remated to a competitor male. For females that did not remate, time to remating was right-censored (see Analysis below) as the time between the focal mating and the end of the experiment. Assaying time to remating allowed us to estimate the ability of a focal male to induce refractoriness in females post-mating.

3.2.6 Post-copulatory Fitness Assay: Offspring Production in Direct Sperm Competition

Sperm competition defence was assayed as the proportion of offspring sired by the first (focal) male relative to his brown-eyed competitor. This proportion takes the form of $P_I = N_I/(N_I+N_{bw/bw})$, where $N_I$ is the number of offspring attributed to the focal male, and $N_{bw/bw}$ is the number of offspring attributed to the $bw/bw$ competitor male. Two types of offspring could be produced from this cross: $+/bw$ (offspring from the focal male) and $bw/bw$ (offspring from the competitor male). Offspring from ‘paternity vials’ were scored based on eye phenotype (wild-type or brown-eyes) and were counted and summed across vials on days 13 and 15 of their life cycle.
3.2.7 Condition Dependence

In studies investigating good genes selection, environmental manipulations of condition are often used as a surrogate for genetic quality; therefore, we mirrored our genetic quality (inbreeding) manipulation with an environmental manipulation of focal male condition. For each of the aforementioned assays, we manipulated diet resources to produce high- and low condition focal males. Eggs laid by Dahomey females were transferred to vials in groups of 50 to standardise larval density and competition across condition treatments. High condition males were reared on standard medium, while low condition flies were reared on medium containing one-quarter of the standard volume of yeast and sugar. All above assays were conducted in a separate set of trials for high- and low condition males (after termination of trials for out-/inbred males) following the identical protocol as that used for the inbreeding manipulation, with the exception that the condition experiment ran across five days, while the inbreeding experiment ran for six.

3.2.8 Analysis

Offspring produced between first and second matings and P1 success were assayed using a generalised linear model (GLM) with an exponential error structure. A parametric survival (time-to-failure) analysis was used, with a Weibull distribution and right-censoring for females that did not remate, to assess the impact of inbreeding and condition on time-to-remating (refractory period). For all analyses, data for focal males that had an unsuccessful first mating or zero offspring production from the first mating were excluded prior to analysis (zero offspring production should not occur under our design, as males and females were both virgin for the first mating). For P1 analyses, all unsuccessful second matings were excluded; likewise, all P1 values equal to one were removed prior to analysis, as P1=1 (i.e., no offspring produced by second male) values were not expected if second males mated successfully, given the strong last-male sperm precedence in D. melanogaster (e.g., Boorman and Parker 1976, Harshman and Clark 1998, Morrow et al. 2005). Additionally, all P1=1 values were identified as outliers (=P1 values exceeding 3rd quartile+1.5*interquartile range) for each of inbred, outbred, and low condition treatment groups (P1=1 values were not outliers for high condition treatment group).
Removal of P1=1 values did not affect the results qualitatively. All analyses were conducted using JMP (v.10.0.0).

3.3 RESULTS

3.3.1 Refractoriness and Offspring Production Between Matings

Females mated to outbred males experienced a longer refractory period relative to females mated to inbred males ($X^2_{1}=13.7$, $p=0.0002$; Fig. 3.1a). There was no difference between females mated to high- or low condition males in refractoriness ($X^2_{1}=0.178$, $p=0.673$; Fig. 3.1b). Outbred males produced 39% more offspring between first and second mating relative to inbred males ($X^2_{1}=5.45$, $p=0.0196$; Fig. 3.2a). This effect was explained by a difference between outbred and inbred males in refractory period ($X^2_{1}=78.6$, $p<0.0001$), as the direct effect of inbreeding disappeared when the number of days between matings was included as a covariate in the model ($X^2_{1}=0.0383$, $p=0.845$). There was no difference between high- and low condition males in offspring production between matings ($X^2_{1}=0.221$, $p=0.638$; Fig. 3.2b).

3.3.2 Sperm Competition Defence: P1

The effect of inbreeding alone on P1 success was not statistically significant ($X^2_{1}=3.12$, $p=0.0776$). However, when statistically controlling for differences in refractory period, outbred males performed ~35% higher than their inbred counterparts in P1 success ($X^2_{1}=6.41$, $p=0.0114$; Fig. 3.3a). While focal male condition alone had a significant effect on P1 success ($X^2_{1}=7.86$, $p=0.0051$), adding refractory period as a covariate in the model improved the fit: high condition males achieved ~59% greater P1 success than low condition males ($X^2_{1}=6.41$, $p=0.0113$; Fig. 3.3b) while controlling for refractory period. There was a significant effect of female refractory period on P1 for inbred/outbred males ($X^2_{1}=18.2$, $p<0.0001$; Fig. 3.4a) and for high/low condition males ($X^2_{1}=24.6$, $p<0.0001$; Fig. 3.4b), with the difference in P1 success between inbred/outbred or high/low condition males decreasing with refractory period. The addition of male body mass as a covariate in the model did not produce a significant effect on P1 success.
for inbred/outbred males ($X^2_1=0.842, p=0.359$) or low/high condition males ($X^2_1=0.0733, p=0.787$), and consequently the mass term was dropped from the model in both cases.

### 3.3.3 Effects of Inbreeding and Condition on Body Size

Outbred males were 5.31% larger than inbred males ($F_{1,221}=18.07, p<0.0001$). High condition males were 23.6% larger than low condition males ($F_{1,168}=3.71\times10^2, p<0.0001$).

### 3.4 DISCUSSION

Our results suggest that sexual selection is generally effective at eliminating deleterious mutations exposed through inbreeding in *Drosophila melanogaster*. This was evident at each stage of sexual selection investigated: females mated to inbred males were less likely to manifest a long refractory period, and consequently produced fewer offspring between first and second matings; likewise, inbred males performed poorly relative to their outbred counterparts at direct defensive ejaculate competition (i.e., P1 success). Our diet manipulation of condition (see Methods) was used to emulate the effects of deleterious mutations (via their predicted effects on condition) on the components of sexually selected fitness measured, and we predicted that the qualitative results for condition treatments should mirror those obtained for inbreeding treatments. Our predictions were confirmed for P1 measurements; however, for refractory period and offspring production between first and second mating (which was driven by time to remating), our condition treatments had a null effect, while inbreeding treatments performed as predicted.

Given the intense interest in the evolution of seminal fluid proteins (SFPs; also known in the literature as accessory gland proteins) in *D. melanogaster* with respect to sexual conflict (Arnqvist and Rowe 2005) and ejaculate competition (e.g., Wigby et al. 2009, Sirot et al. 2011), a noteworthy and, to the best of my knowledge, hitherto undemonstrated result emanating from this experiment was the demonstration that females mated to inbred males experienced a refractory period that was significantly shorter in duration than for those mated to outbred
males. Related to this, females mated to inbred males showed decreased offspring production prior to their second mating with a competitor male. Given our comprehensive understanding of the postcopulatory reproductive process in *D. melanogaster* (e.g., Wolfner 1997, Chapman 2001, Wigby et al. 2009, Manier et al. 2010, Lupold et al. 2011, Sirot et al. 2011), these results collectively support the notion that, in this model system, sexual selection is able to eliminate deleterious mutations via their effects on the male ejaculate. Our results are in agreement with the well-documented effects of SFPs on female physiology and behaviour in *D. melanogaster*. Seminal fluid proteins are produced by *D. melanogaster* males, and are transferred to the female with sperm at the time of mating. A myriad of SFPs and associated functions are known to exist in *D. melanogaster* (Wolfner 1997, Chapman 2001, see Arnvist and Rowe 2005 pp. 150-155 for an excellent summary of Acp effects), and they are the source of a large body of sexual conflict research (Arnvist and Rowe 2005). Our refractory period and associated offspring production results correspond with the effects of two SFPs in particular: ovulin and sex peptide. Ovulin acts to increase egg production in virgin females (Herndon and Wolfner 1995, Heifetz et al. 2000), while sex peptide acts to reduce female remating rate (i.e., increase refractory period) and increase egg production, among other influences (Chapman et al. 2003, Liu and Kubli 2003, Carvalho et al. 2006). Of these two SFPs, it is likely that inbreeding was deleterious to the production and/or actions of sex peptide in our experiment, given that time to remating drove offspring production during the refractory period (hence, for the environmental condition manipulation, a lack of effect of condition on offspring production during the refractory period was really a reflection of an absence of any effect of condition on refractory period breadth, rather than a lack of effect directly on offspring production). Given the importance of SFPs to male fitness in *D. melanogaster* (Arnvist and Rowe 2005, Wigby et al. 2009, Sirot et al. 2011), it is noteworthy that our results suggest that they may serve as a trait through which sexual selection can remove deleterious variation from a population.

Alternatively, our remating results may have been produced not by the actions of inbreeding on SFP function, but rather by females choosing to remate sooner after mating to inbred males (i.e., trade-up hypothesis; see Jennions and Petrie 2000); however, this is unlikely, as female remating propensity in *D. melanogaster* is not affected by male genetic quality (Byrne and Rice 2005). Our results suggest that refractory period may be directly driving the number of offspring produced prior to remating for inbred and outbred males, as the inbreeding effect
disappears when statistically controlling for refractory period. This makes intuitive sense, as the longer a female’s refractory period, the more time she has to produce offspring associated with her last mate.

A caveat of associating sexual selection against deleterious mutations with the result demonstrating that females mated to inbred males had a shorter refractory period and produced fewer offspring before remating is that, while there was sexual selection against inbred males in these traits, there was no associated condition dependence. There are several potential reasons for this result. The most obvious is that our diet manipulation failed to elicit an effect on condition. This can be unambiguously ruled out, as there was a major effect of our diet manipulation on male body size (a good indicator of condition; Whitlock and Agrawal 2009), which was similar in effect to a previous diet manipulation on male size for this fly population, using the identical protocol for condition adjustment (Clark et al. 2012). Alternatively, it is possible that our inbreeding treatment directly affected the Acp-related loci associated with inducing the refractory period, instead of acting on their expression via deleterious effects on condition. This result would nonetheless be interesting, as it still suggests that sexual selection can remove deleterious mutations; however, it would mean that sexual selection was not removing mutations via their effect on condition as assumed by the good genes theory (Rowe and Houle 1996, Whitlock and Agrawal 2009). Another possibility is that the traits underlying both refractory period induction and offspring production prior to remating were indeed condition dependent, but that condition is a multidimensional trait, and the aspects of condition affected by our diet manipulation were different from those affecting the traits in question (Bonduriansky et al. 2015). Interestingly, McGraw et al. (2007) found that refractory period was significantly lower for male flies raised on reduced yeast medium (i.e., low condition males); however, the authors did not find an effect of condition on P1, which differs from our results.

For P1 success, both the inbreeding and the condition treatments performed as expected: outbred males outperformed inbred, and high condition males outperformed low condition. There was an interesting effect of refractory period on P1 success for both the inbreeding and condition manipulations, with the difference in P1 success for inbred/outbred and low/high condition males decreasing with increasing refractory period. This makes intuitive sense, as a longer refractory period means females have a longer time to use the first male’s ejaculate; hence, there
will be less ejaculate present in the female reproductive tract to engage in direct sperm competition with a second male.

The congruence of inbreeding and condition P1 results lends strong support to the hypothesis that postcopulatory sexual selection impacted deleterious mutations via their effect on condition, in accordance with good genes predictions (Rowe and Houle 1996). Our results are corroborated by a recent experiment (Mallet et al. 2012) employing mutation accumulation (MA) to generate deleterious variation, in which the authors demonstrated that sexually selected fitness was reduced in MA lines (relative to controls) for total adult fitness, mating success, P1, and P2. Our P1 result contrasts with a recent experiment demonstrating a null effect of offensive sperm competition (P2) on purging dominant deleterious mutations (Clark et al. 2012).

Generally, my results demonstrate a discrepancy between environmental and genetic quality as they affect success in defensive postcopulatory sexual selection (exception: P1 success), wherein manipulations of environmental condition (diet adjustment) did not serve as a reliable proxy for manipulations of genetic condition (degree of inbreeding). This result is intriguing, for two reasons. First, condition dependence theory is predicated on the hypothesis that sexual selection improves nonsexual fitness via selection on traits that reflect mutational frequency in condition (e.g., male success in contest competition, mating, ejaculate competition, etc.; Whitlock and Agrawal 2009), or general health; hence, experimental adjustments of environmental and genetic quality should have qualitatively similar effects on sexually selected fitness components (Tomkins et al. 2004, Whitlock and Agrawal 2009). Second, surprisingly few experiments have explicitly assayed the relative effects of genetic and environmental quality in experiments of sexual selection. I am aware of two outside of my thesis work, and each has shown a disconnect between genetic and environmental quality. White and Rundle (2015) used inbreeding to manipulate genetic quality, and a larval density manipulation to adjust environmental condition in a study assaying territory defence and mating success in *Drosophila serrata*. The authors demonstrated that, while high larval density (low condition) had a significant depressing effect on male territorial defence and mating success, inbreeding did not. Another recent study (Bonduriansky et al. 2015) directly measured the impacts of environmental and genetic quality on trait expression in *D. melanogaster*. Bonduriansky et al. (2015) showed that, while manipulations of genetic and environmental condition had predicted (e.g., Rowe and
Houle 1996) congruent effects on two fitness-related traits (female body size and male wing length) and most morphological traits, there were also sexually selected traits important to fitness (e.g., male sex combs, cuticular hydrocarbons) on which effects of genetic and environmental condition manipulations were incongruent.

There is now a well-established body of empirical studies addressing the question of whether sexual selection should enhance nonsexual fitness through the removal of deleterious mutations. A preliminary requirement is that male mating success be condition dependent, for which there is good evidence (Price et al. 1993, Johnstone 1995, Jennions et al. 2001, Cotton et al. 2004). There is likewise a substantial volume of key results demonstrating that sexual selection acts to reduce mutation load, collected from experiments that directly test whether sexual selection acts to improve nonsexual fitness (for an outstanding and comprehensive review of sexual selection and mutation load, see Whitlock and Agrawal 2009). Our results fit well into this framework.

3.4.1 Conclusion

Our experiment demonstrates that sexual selection can be effective against deleterious mutations at the postcopulatory level in *D. melanogaster*. Our results are critical to understanding the ultimate impact of sexual selection on deleterious mutations, as there are few experiments that investigate the specific components of sexual selection responsible for improving nonsexual fitness. Taken together with recent results from flies and other taxa (summarized in Whitlock and Agrawal 2009), a general pattern of sexual selection facilitating natural selection to enhance nonsexual fitness is emerging. Given results from recent experiments demonstrating components of sexual selection acting against deleterious mutations pre- (MacLellan et al. 2009, Pekkala et al. 2009, Clark et al. 2012, Mallet et al. 2012) (but see Power and Holman 2015, White and Rundle 2015) and post-copulation (Mallet et al. 2012), the next important step is to comprehensively quantify the relative importance of pre- and postcopulatory sexual selection at eliminating deleterious mutations.
Figure 3.1. Remating latency (shown here as the time interval between matings) for experimental females first mated to a) inbred (red line) or outbred (blue line) focal males; or b) low (red line) or high condition (blue line) males, followed by a mating with a standard competitor male 1-6 days (for inbreeding treatment; 1-5 days for condition) after completion of the first mating. Females were offered the opportunity to remate (~2.5 h window) with standard competitors once daily commencing ~24 h after their first mating. Females mated to outbred males were significantly less likely to remate on any given day than those mated to inbred males ($\chi^2_{1} = 13.7; p = 0.0002$); hence, matings with outbred males produced a longer refractory period. Condition had no effect on refractory period ($\chi^2_{1} = 0.178, p = 0.673$).
Figure 3.2. Offspring production between first and second mating for experimental females mated first to a) inbred (I) or outbred (O) males; or b) low (L) or high (H) condition males. Outbred males sired significantly more offspring between matings than did inbred males ($\chi^2_1=5.45$, $p=0.0196$). There was no effect of condition on offspring production between matings ($\chi^2_1=0.221$, $p=0.638$).
Figure 3.3. Proportion (P1) of experimental female’s offspring attributable to the focal (first) male in sperm competition with a standard competitor male for a) inbred (I) or outbred (O) males; or b) low (L) or high (H) condition males. Outbred males were significantly more successful in sperm competition against a standard competitor than inbred males ($X^2_1=6.41$, $p=0.0114$). Likewise, high condition males achieved higher P1 success than low condition males ($X^2_1=6.41$, $p=0.0113$).
Figure 3.4. Regression plots showing effect of female refractory period (shown here as remating interval) on P1 for a) inbred (red line, +) or outbred (blue line, •) focal males; or b) low (blue line, +) or high condition (red line, •) males. For manipulations of inbreeding ($\chi^2 = 18.2$, $p<0.0001$) and condition ($\chi^2 = 24.6$, $p<0.0001$), female refractory period had a significant effect on P1.
4 ABSTRACT

There is building consensus in support of the notion that sexual selection can enhance nonsexual fitness by purging deleterious mutations, an idea rooted in good genes theory. However, there have been only a few attempts to parse sexual selection into its pre- and postcopulatory components to assess their relative importance in reducing mutation load. Furthermore, while it is well established that male condition is an important predictor of the outcome of sexual selection, there is mounting cross-taxonomic evidence that female condition is likewise important. Here, we attempt to determine: i) which components of sexual selection are important for eliminating deleterious mutations in *Drosophila melanogaster*; and ii) whether female condition can influence the outcome of sexual selection against deleterious mutations.

Our experiments highlight two critical aspects of sexual selection for good genes. First, they demonstrate that selection against deleterious mutations can occur through processes acting both before and after copulation, and that the relative importance of these processes varies. Second, while some empirical work has demonstrated the importance of female condition to the outcome of sexual selection, we did not detect any effect of female condition on selection against deleterious mutations.
4.1 INTRODUCTION

There is mounting evidence to support the notion that sexual selection can enhance nonsexual fitness by purging deleterious mutations. This idea is rooted in good genes theory, which states that females can gain indirect fitness benefits by choosing males on the basis of genetic quality (Zahavi 1975, 1977, Iwasa and Pomiankowski 1991, Houle and Kondrashov 2002), which is reflected phenotypically in condition (Rowe and Houle 1996). Condition is influenced by a large portion of the genome, and thus represents a large mutational target (Houle 1991); thus, females selecting mates on the basis of condition simultaneously sort males by genetic quality (Rowe and Houle 1996). It is by this mechanism that sexual selection is predicted to purge deleterious mutations. This process requires substantial additive genetic variance in fitness, and condition dependence of sexually selected traits; there is widespread empirical support for both (reviewed in Arnqvist and Rowe 2005 pp. 22-25, Whitlock and Agrawal 2009).

Direct evidence for the ability of sexual selection to augment natural selection by selecting against deleterious mutations comes from experiments demonstrating that sexual selection can operate against individual alleles of known deleterious influence on nonsexual fitness (Whitlock and Bourguet 2000, Pischedda and Chippindale 2005, Sharp and Agrawal 2008, Hollis et al. 2009, MacLellan et al. 2009) (NB: mutations were presumed deleterious in MacLellan et al. 2009)). Counter to this, Arbuthnott and Rundle (2012) showed that sexual selection either had no effect on, or hindered selection against individual deleterious mutations, though this null/negative result may be explained by the absence of complexity in the testing environment (Singh et al. 2017, Yun et al. 2017). Indirect evidence for sexual selection against deleterious mutations is mixed. First, indirect evidence for the fitness benefits of sexual selection comes from experiments using populations experimentally enriched for deleterious variants—via mutagenesis, mutation accumulation, inbreeding, or a combination thereof—wherein the opportunity for sexual selection was experimentally manipulated (Radwan 2004, Jarzebowska and Radwan 2010, McGuigan et al. 2011, Almbro and Simmons 2014, Lumley et al. 2015) (but see Radwan et al. 2004, Hollis and Houle 2011, Plesnar et al. 2011, Power and Holman 2015, White and Rundle 2015). Additionally, indirect evidence for the capacity of sexual selection to reduce the mutation load comes from a complementary set of studies in which sexual selection provides fitness benefits to populations enriched for variation in fitness while adapting to a novel environment (Fricke and Arnqvist 2007, Plesnar-Bielak et al. 2012) (but see Holland...
2002, Rundle et al. 2006, Cabral and Holland 2014), or, reciprocally, positive effects of local adaptation on male reproductive success (Dolgin et al. 2006) (but see Correia et al. 2010, Arbuthnott and Rundle 2014). There is likewise recent evidence to suggest that net selection is stronger in males than in females (Pischedda and Chippindale 2005, Sharp and Agrawal 2008, Mallet et al. 2011a, Mallet and Chippindale 2011, Sharp and Agrawal 2013, Zikovitz and Agrawal 2013), a result required by theory for sexual selection to affect the mutation load (Whitlock and Agrawal 2009). Finally, the considerable variability in results for the capacity for sexual selection to affect nonsexual fitness may be rooted in variation among studies of the relative influence of sexually antagonistic and unconditionally deleterious variance on fitness, which seems proportional to the distance of a population from its adaptive peak (Long et al. 2012).

There is an emerging interest in the literature for establishing the relative importance of pre- and postcopulatory processes to total sexual selection. It has recently been shown that pre- and postcopulatory sexual selection components contribute approximately equal amounts to total D. melanogaster male sexually selected fitness (Pischedda and Rice 2012). However, there has yet been little attempt to parse sexual selection into its pre- and postcopulatory components to assess their relative importance in affecting mutation load. Experiments thus far have generally focused on sexual selection in toto (e.g., Holland 2002, Radwan 2004, Radwan et al. 2004, Rundle et al. 2006, Fricke and Arnqvist 2007, Sharp and Agrawal 2008, McGuigan et al. 2011, Long et al. 2012, Lumley et al. 2015). Furthermore, while recent experiments have begun to investigate the impacts of individual components of sexual selection on deleterious mutations (e.g., MacLellan et al. 2009, Pekkala et al. 2009, Clark et al. 2012, Almbro and Simmons 2014, Power and Holman 2015, White and Rundle 2015), to our knowledge, only one has yet compared the relative importance of pre- and postcopulatory sexual selection in affecting mutation load, demonstrating substantial fitness declines associated with mutation accumulation in viability, adult fitness, mating success, P1 and P2 (Mallet et al. 2012).

While it is well established that male condition is an important predictor of the outcome of sexual selection (Price et al. 1993, Johnstone 1995, Jennions et al. 2001, Cotton et al. 2004), there is mounting cross-taxonomic evidence that female condition is likewise important (Cotton et al. 2006). Experiments have shown that indirect benefits received by females in D. melanogaster may be condition dependent, with small females experiencing a net benefit to
remating, and large females a net cost (Long et al. 2010). Furthermore, the length of the seminal receptacle (the primary sperm storage organ in *Drosophila*; Pitnick et al. 1999) in the female reproductive tract has been shown to be condition dependent, with high condition females producing longer seminal receptacles (Amitin and Pitnick 2007). This is important, because there is an established pattern of coevolution between seminal receptacle length and sperm length across species of *Drosophila* (Pitnick et al. 1999). Indeed, seminal receptacle length drives the evolution of sperm length (Miller and Pitnick 2002), with males producing long sperm relative to their competitors achieving higher paternity success when mating to females with relatively long seminal receptacles (Pattarini et al. 2006). Thus, seminal receptacle length serves as a form of condition dependent postcopulatory female preference (Amitin and Pitnick 2007). Additionally, condition dependence in female remating rate in *D. melanogaster*, with high condition females remating more often than females of low condition, may intensify competition among male ejaculates in high condition females, thereby intensifying sexual selection (Amitin and Pitnick 2007).

Here, we attempt to determine which components of sexual selection are important for eliminating deleterious mutations in *Drosophila melanogaster*. We did this by measuring mating success, sperm competition defence (P1), refractory period induction, and sperm competition offence (P2) in flies that experienced mutation accumulation across many generations. The strength of selection against deleterious mutations was then assessed for each of these components of sexual selection to determine their relative potential for augmenting nonsexual fitness. We likewise tested whether female condition influenced the outcome of sexual selection against deleterious mutations at each of the aforementioned stages. *D. melanogaster* is an ideal system in which to address these questions, as there is a rich literature base detailing the importance of pre- and post-copulatory mechanisms to male sexually selected fitness (Arnqvist and Rowe 2005), condition dependence of male sexually selected traits before (Sharp and Agrawal 2008) and after copulation (Amitin and Pitnick 2007, McGraw et al. 2007), and, to a lesser extent, female condition dependence of mating biases (Amitin and Pitnick 2007, Long et al. 2010).
4.2 METHODS

4.2.1 Study Organisms

Experimental *D. melanogaster* males were created through a process of mutation accumulation (MA). Initially, genetically identical lines were bottlenecked independently for many generations; this prevented selection against most new mutations, allowing them to fix in the genome (described in Sharp and Agrawal 2012). MA lines could be compared to controls of the same genotype that were not bottlenecked (thus, selection should prevent mutations from accumulating). Experimental males were derived from an outbred population originally collected in 1970 from Dahomey (now Benin) West Africa, and were maintained in the current lab at a population size of several thousand adults for over three years (>75 generations) before generation of experimental lines. The recessive phenotypic marker mutation *bw* (sourced from the Bloomington Drosophila Stock Center, Bloomington, Indiana) was introgressed into the Dahomey background using serial backcrossing. An initially identical copy of chromosome 2 (~40% of the genome; marked with *bw* and designated *bw* *) was shared by all MA and control lines; this was the focal chromosome where new mutations were allowed to accumulate.

Males from each MA line were crossed to a standard stock after 62 generations (~29 months) of MA. Four additional generations of crosses to place replicate *bw* * chromosomes on an isogenic wild-type background (derived from Dahomey population) were employed to produce flies for fitness assays. Chromosomes were tracked and recombination suppressed using standard marker and balancer stocks. The identical crossing protocol was used for replicate lines from each control population; however, control lines were also bottlenecked to a single *bw* * chromosome per line. This procedure eliminated genetic variation within lines (i.e., bottlenecking) and among lines, with the exception of mutations that accumulated (MA lines) or were segregating (control lines) on the focal chromosome and any variation on the unmanipulated fourth chromosome. This procedure was used to produce 30 MA and 32 control lines. Focal chromosomes were kept on balancer chromosomes on an isogenic background for five generations before fitness assay, but were made homozygous for this fitness assay itself. Following this protocol, control lines should carry deleterious alleles on the focal chromosome at a frequency reflective of mutation-selection balance, while MA line focal chromosomes should be enriched for deleterious alleles; otherwise, all lines were genetically identical. We can treat flies from within a line as the same individual (i.e., effective clones), which was
important for comparing selection across pre- and postcopulatory components (described below). All experimental males carried a recessive brown-eye marker (bw/bw).

Standard competitor males (with wild-type eyes, +/-) were derived from the aforementioned wild-type Dahomey population. The wild-type Dahomey stock was maintained in the current laboratory for over six years at the time of experimentation, at a population size of several thousand adults. All experimental females carried the bw/bw marker on a Dahomey genetic background. Unless otherwise indicated, all line maintenance, experimental matings, and fitness assays were conducted in plastic shell vials (25x95 mm O.D. x height) containing standard yeast-sugar-agar medium supplemented with one pellet of live yeast. All flies were cultured at 25ºC under ~60% relative humidity, on a 12L:12D photoperiod.

4.2.2 Manipulation of Female Condition

For each of the assays below, we manipulated larval diet resources to produce high- and low condition experimental females. Eggs laid by Dahomey females were transferred to vials in groups of 50 to standardise larval density and competition across condition treatments. High condition females were reared on standard medium, while low condition females were reared on medium containing one-quarter of the standard volume of yeast and sugar.

4.2.3 Precopulatory Sexual Selection

To assess precopulatory sexual selection, three-day-old virgin experimental males, standard competitor males, and experimental high- or low-condition females were placed into individual holding vials containing standard food supplemented with live yeast 48 h prior to mating (thus, all flies were five days old at the time of mating). Experimental males, standard competitor males, and experimental females were combined without anaesthesia into a single mating vial (containing standard media and live yeast) at a sex ratio of 1Mexp:1Mstd:1F to measure relative mating success of experimental and standard competitor males. Where possible, we ran three replicates per male experimental line (MA or Control) for both high- and low-condition females. All replicates were observed for a two-hour period. Following mating, males were frozen in microtubes at -80ºC, and females were placed in laying vials for 96 h, which were used to assess
the contest winner (as all offspring of each mating pair shared the eye colour of the winning male). Following the laying period, females were transferred to microtubes and frozen at -80°C.

Mating success was assayed by scoring eye colour of the offspring produced from precopulatory assay matings. As all females and experimental males in the assay were homozygous for the recessive bw/bw “brown eye” marker, while standard competitor males were homozygous wild-type (red eyes) with respect to eye colour, all offspring sired by experimental males had brown eyes, while offspring sired by standard competitors had wild-type eyes. Mating success winners were scored only as either wild-type or brown (i.e., offspring were not counted). As matings were directly observed, and males were separated from females immediately following the first mating, females produced offspring from only a single male; thus, all offspring of a given female had the same eye colour (this was verified at the time of scoring by scanning offspring eyes to ensure all were identical in colour). Mating success was analysed using a “contest win score” assigned to each focal male: zero was assigned for a contest loss (to standard competitor male), and one for a contest win.

4.2.4 Postcopulatory Sexual Selection: Sperm Competition Defence

To assess sperm competition defence (P1), three-day-old virgin experimental males and high- or low-condition experimental females were placed into individual holding vials containing standard food supplemented with live yeast 48 h prior to mating (thus, all flies were five days old at the time of mating). For mating observations, experimental males and females were combined without anaesthesia at a sex ratio of 1M:1F in a single vial containing standard media and live yeast. Where possible, we ran three replicates per male experimental line (MA or Control) for both high- and low condition females. Mating observations were conducted for a period of two hours. If mating occurred between a male-female pair during this window, the pair was separated following mating, with females placed in laying vials for 24 h (from which the number of offspring produced prior to second mating was assessed), and males were transferred to microtubes and frozen at -80°C. All females that did not mate were removed from the experiment.

All experimental females that mated successfully to experimental males were allowed the opportunity to remate to a single standard competitor male each day following the experimental
matings up to a maximum of five days (six days for block 2). The mating window allowed each
day was two hours, and all standard competitor males were five days old at the time of mating
(exception: on day five of the remating schedule for block 1 [see below], six-day-old males were
used), with fresh standard competitor males used for each of the potential remating days. If a
female remated to a standard competitor male on any of these days, the male was discarded, and
the female was removed from the observation pool to a 48 h laying vial from which P1 success
(the proportion of offspring attributable to the focal male when he was the first to mate the
experimental female; see below) was assessed. If a female did not remate on a given day, the
male was discarded, and she was transferred to a 24 h laying vial until the following day, on
which she was given another remating opportunity. All females that did not remate on any of the
potential remating days were frozen in microtubes at -80ºC 24 h after the final mating
opportunity.

Three types of fitness measures were attainable from the sperm competition defence assay. The
first was offspring production by an experimental male between a female’s first and second
mating (i.e., during her refractory period). This was measured using vials in which females laid
eggs after their mating with the focal male and prior to remating (females were provided with
fresh laying media every 24 h). The second fitness measure was time-to-remating for
experimental females, which provided an indication of the focal males’ ability to induce a post-
mating refractory period in the female. Time-to-remating was assessed by summing the number
of days between a female’s first and second mating. The final fitness measure was paternity
success for the focal male when he was the first mate in competition with a standard male
competitor (i.e., sperm competition defence, P1). Sperm competition defence was assessed as

\[ P1 = \frac{N_f}{N_f + N_{wt/wt}} \]

where \( N_f \) represents the number of offspring produced by the focal male
(=first male to mate with the female), and \( N_{wt/wt} \) denotes the number of offspring produced by
the standard competitor (=second male to mate with the female). For all offspring count assays,
offspring were counted on, and summed across, days 13 and 15 of their life cycle.

### 4.2.5 Postcopulatory Sexual Selection: Sperm Competition Offence

To assess sperm competition offence (P2), virgin high- or low-condition experimental females
were first mated *en masse* to virgin standard competitor males at a sex ratio of 10M:10F. Flies
were given a two-hour window of opportunity to mate. All males were five days old at the time
of mating, and females were two days old (thus, all females would be five days old for their experimental [second] mating; see below). Following mating, females were separated from males and placed into individual holding vials to ensure they had mated (larval presence in vials after 48 h was used to indicate mating success). Standard competitor males were discarded following mating.

Virgin experimental males were placed into individual holding vials containing standard food supplemented with live yeast 48 h prior to the experimental mating assay; all males were five days old at the time of mating. Experimental matings were conducted by combining experimental males and previously mated experimental females without the use of anaesthesia into vials containing standard media and live yeast. Where possible, we ran three replicates per male experimental line (MA or Control) for both high- and low-condition females. Flies were given a two-hour window of opportunity to mate. If a pair of flies mated, male and female flies were separated following mating. Females were transferred to fresh laying vials containing standard media and live yeast, where they were given the opportunity to lay eggs for 48 h, after which they were transferred without anaesthesia into 72 h laying vials (giving a total of five laying days from which fitness could be assessed), and then they were placed in microtubes and frozen at -80°C. All successful males were transferred to microtubes and frozen at -80°C immediately following mating. All unsuccessful males and females were discarded.

Postcopulatory fitness with respect to sperm competition offence (paternity success for the focal male when he was the last mate in competition with a standard male competitor) was assessed as \( P2 = \frac{N_f}{N_f + N_{wt/wt}} \), where \( N_{wt/wt} \) denotes the number of offspring produced by the standard competitor (=first male to mate with the female), and \( N_f \) represents the number of offspring produced by the focal male (=second male to mate with the female). Offspring production was measured by summing all offspring counted on days 13 and 15 of their life cycles across both the 48 h and 72 h laying vials (see above).

4.2.6 Analysis

Data were analysed for all assays (with the exception of refractory period) using a mixed nested-factorial design (standard least squares ANOVA), with the MA and female condition manipulations and time (block) included as main effects, MA and control lines nested in the MA
treatment as a random effect, and model terms for the interactions between main effects. The Akaike Information Criterion (AICc) was used to simplify models to best fit, to a maximum level of main model effects. For the refractory period analysis, MA and control line means were calculated, and treated as the unit of replication for parametric survival (time-to-failure) analysis, structured with MA and female condition manipulations as main effects, and a model term for the interaction between main effects. This alternative approach (to the aforementioned nested design) was used for refractory period analysis, as the time-to-failure analytical software was not compatible with nested mixed-model design. Data were analysed using JMP (v.10.0.0).

Experiments for this project were reproduced across two blocks. The mating success and sperm competition defence assays were each repeated twice (once per block); the sperm competition offence assay was repeated four times (once in block 1; three times in block 2). There was a significant block effect on the impact of female condition manipulations for mating success and sperm competition offence assays, and on the impact of MA on focal male mass for the sperm competition offence assays. When block effects affected the interpretation of the main effects, we analysed the blocks separately.

Sperm competition defence assays were structured slightly differently between blocks, and thus data were kept separate for analysis.

4.2.7 Selection Against Deleterious Mutations

For each component of fitness measured, we quantified selection against deleterious mutations as 

\[ s = \frac{\text{Mean} (\text{Control Line Means}) - \text{Mean} (\text{MA Line Means})}{\text{Mean} (\text{Control Line Means})} \]

using R (v.2.9.0; R Development Core Team 2009). Point estimates of \( s \) were given upper and lower bounds using bootstrapped 95% confidence intervals. Values of \( s \) for which confidence intervals did not overlap with zero were considered significantly different from zero.
4.3 RESULTS

4.3.1 Precopulatory Sexual Selection: Mating Success

MA males achieved lower mating success when competing against a standard competitor relative to control males ($F_{1,52.3}=4.63, p=3.60\times10^{-2}$; Fig. 4.1). The outcome of mating success contests was not affected by female condition ($F_{1,494}=0.117, p=0.733$). There was no effect of block on mating success ($F_{1,512}=1.18, p=0.277$).

4.3.2 Postcopulatory Sexual Selection: Sperm Competition Defence

The two blocks were analysed separately because there was no effect of female condition on total offspring production between matings for block 1 ($F_{1,138}=0.148, p=0.701$; Fig. 4.2a) but there was a significant condition effect for block 2 ($F_{1,162}=8.76, p=3.50\times10^{-3}$; Fig. 4.2b; mating duration was included as a model covariate for blocks 1 and 2). Deleterious mutations had no effect on the total number of offspring produced exclusively by focal males during the female’s refractory period between first and second mating for block 1 ($F_{1,43.5}=0.109, p=0.743$; Fig. 4.2a) or block 2 ($F_{1,46.6}=0.138, p=0.712$; Fig. 4.2b). The interaction between MA and female condition was nonsignificant for block 1 ($F_{1,138}=2.21, p=0.140$) and block 2 ($F_{1,163}=1.56, p=0.214$). When the number of offspring produced by focal males during their mate’s refractory period was expressed as a rate per refractory period day, control males produced ~18% more offspring relative to mutants in block 1 (when mating duration was included as a model covariate; $F_{1,42.9}=5.71, p=2.13\times10^{-2}$; Fig. 4.3a); nonetheless, in block 2, there was no difference ($F_{1,43.4}=0.185, p=0.669$; Fig. 4.3b). Female condition likewise had a significant effect on the rate of offspring production during the refractory period in block 1 ($F_{1,133}=4.89, p=2.87\times10^{-2}$; Fig. 4.3a) and block 2 ($F_{1,169}=7.87, p=5.60\times10^{-3}$; Fig. 4.3b), but the direction of the effect was opposite between blocks. The interaction between MA and female condition was nonsignificant for block 1 ($F_{1,133}=1.40, p=0.239$) and block 2 ($F_{1,169}=1.32, p=0.252$).

The duration of the refractory period was not affected by mutation accumulation for block 1 ($\chi^2=1.01, p=0.314$; Fig. 4.4a) or block 2 ($\chi^2=1.33\times10^{-2}, p=0.908$; Fig. 4.4b). Likewise, there was no significant effect of female condition on refractory period duration for block 1 ($\chi^2=0.602, p=0.438$), or block 2 ($\chi^2=1.98, p=0.159$).
After females mated a second time, we measured the proportion of the subsequent eggs sired by the focal (first) male, P1. There was no effect of mutation accumulation on P1 success for either block 1 \((F_{1,40.7}=7.62 \times 10^{-2}, p=0.784; \text{Fig. 4.5a})\) or block 2 \((F_{1,39.6}=0.568, p=0.456; \text{Fig. 4.5b})\). Female condition showed no effect on P1 success for block 1 \((F_{1,128}=0.238, p=0.627; \text{Fig. 4.5a})\) or block 2 \((F_{1,153}=3.21, p=7.51 \times 10^{-2}; \text{Fig. 4.5b})\). These P1 success results must be interpreted with the caveat that the vast majority of P1 values were zero or nearly zero, and the non-zero values were atypically small.

### 4.3.3 Postcopulatory Sexual Selection: Sperm Competition Offence

Control males outperformed males from the mutation accumulation lines with respect to P2 success \((F_{1,47}=5.24, p=2.66 \times 10^{-2}; \text{Fig. 4.6})\). There was no effect of female condition on P2 success \((F_{1,363}=0.603, p=0.438; \text{Fig. 4.6})\) and no block effect \((F_{1,366}=6.72 \times 10^{-2}, p=0.796)\).

### 4.3.4 Selection Against Deleterious Mutations

Selection against deleterious mutations was strongest for precopulatory sexual selection, showing a decline in fitness for MA males \(~1.3-3.4\) times greater than postcopulatory processes with significant point estimates of selection. MA fitness declines associated with sperm competition defence showed the rate of offspring production during the refractory period by females mated to experimental males was likewise important in selecting against deleterious mutations, but only for block 1. P2 success likewise was important for selecting against deleterious mutations, but at a rate \(~71\%\) lower than for mating success. All point estimates of selection against deleterious mutations in our assays of pre- and postcopulatory sexual selection are summarised in Table 4.1.

### 4.3.5 Effects of Mutation Accumulation and Female Condition on Body Size

Male mass was estimated from males in each of the assays. Using males from the precopulatory mating success assay, we found no effect of MA on focal male body size \((F_{1,46.8}=0.594, p=0.468; \text{Fig. 4.6})\).
In Block 1 of the sperm competition offence assay, there was a significant effect of MA on body size, but in the opposite direction than predicted (Table 4.2). The sperm competition defence assay showed significant effects of mutation accumulation on male body size, although in different directions between blocks (Table 4.2). Our manipulation of larval female diet resources produced low condition females that were ~20-24% smaller in body mass relative to high condition females across all assays (Table 4.3).

4.4 DISCUSSION

Our results clearly suggest that sexual selection contests occurring both before and after copulation are effective at eliminating new deleterious mutations accrued through mutation accumulation in *Drosophila melanogaster*. This effect was manifested at two of the three major stages of sexual selection assayed: mutant males competing against standard competitor males for access to virgin females (where direct male-male competition and female choice were operational) gained fewer matings than control males; and mutant males were likewise outperformed in second male paternity success (P2) by control males when engaged in sperm competition against a standard competitor male. Although we detected a clear impact of deleterious mutations on the outcome of sexual selection contests, our manipulation of female condition did not produce any general effects. Our diet manipulation had a major and repeatable effect on female condition, however, there was no appreciable effect of female condition on the outcome of mating success or sperm competition offence contests. There were some effects of female condition in several sperm competition defence assays, but they were not repeatable, and sometimes were contradictory between blocks.

In their review of the effects of sexual selection on mutation load, Whitlock and Agrawal (2009) argue that very few data exist on the magnitude or importance of sexual selection for selecting against deleterious mutations at all levels of female choice and male intrasexual interactions (e.g., sperm competition), and that such data would be extremely valuable in advancing our understanding of the impacts of sexual selection on deleterious mutations. To our knowledge, there is but a single study to date (Mallet et al. 2012) that has attempted to address this issue in a complementary manner to our present study, decomposing sexual selection into its pre- and postcopulatory components to look at how deleterious mutations affect reproductive success at
each of these stages. Mallet et al. (2012) showed significant declines in fitness in MA males for mating success, P1, and P2; we also found an effect on P2 but not on P1. While selection was ~3.4 times stronger for mating success than P2 in our experiment, selection was similar in strength for both processes for Mallet et al. (2012). Furthermore, strongest selection against deleterious mutations came from P1 success for Mallet et al. (2012), a fitness component for which we found no significant selection against deleterious mutations (but see Chapter 3). Additionally, Clark et al. (2012) conducted as part of their experiments a no-choice mating success assay (differs from present design; see Methods), and found a significant effect of dominant marker mutations on male mating success using a \textit{D. melanogaster} stock of similar ancestry to that used in the present study. As in these previous experiments (Clark et al. 2012, Mallet et al. 2012), reduced mating success in the present study for males enriched for deleterious mutations suggests an impact of new mutations on male attractiveness to females, and/or the ability of MA males to compete intrasexually for access to mates.

Our result demonstrating that MA males were inferior relative to control males at achieving P2 success is likewise in agreement with previously published results (Mallet et al. 2012; but see Clark et al. 2012), and is particularly interesting given the well-documented importance of last-male paternity success to male \textit{D. melanogaster} fitness (Boorman and Parker 1976, Harshman and Clark 1998, Morrow et al. 2005). In \textit{D. melanogaster}, P2 success is driven by a male’s ability to displace competitors’ sperm (Manier et al. 2010); thus, it is probable that the mechanism underlying our P2 result is a reduced ability of MA males to displace competitor sperm, possibly through a reduction in ejaculate size (Manier et al. 2010), or in sperm size (Pattarini et al. 2006, but see Amitin and Pitnick 2007). Furthermore, P2 success is condition dependent in \textit{D. melanogaster} (Amitin and Pitnick 2007, Clark et al. 2012), as is the number of sperm transferred to and stored by females (McGraw et al. 2007); thus, there exists clear potential for these male traits to facilitate selection against deleterious mutations via their effects on condition (Rowe and Houle 1996).

Our sperm competition defence experiments generally showed no effect of deleterious mutations on reproductive success. The only sperm competition defence result for which deleterious mutations affected the outcome was the number of offspring produced per day by focal males prior to female remating: MA males produced offspring at a lower rate relative to controls. However, this result was not reproduced when the experiment was repeated (block 2).
These sperm competition defence results were somewhat surprising, given known condition dependence for postcopulatory sexually selected traits (Amitin and Pitnick 2007, McGraw et al. 2007). Additionally, a recent study (Clark et al. in prep.) investigating the impacts of deleterious mutations exposed through inbreeding on similar metrics of sperm competition defence showed that deleterious mutations significantly affected the number of offspring produced by females mated to experimental males prior to remating, the duration of the refractory period, and P1, with inbred males performing less well relative to controls in all cases. Our results likewise differed from those previously published by Mallet et al. (2012) for P1 success; however, we are limited in the conclusions we can make about our P1 results, as both MA and control males produced atypically small numbers of offspring to those expected based on preliminary P1 experiments using flies of similar ancestry.

Models of good genes selection predict that deleterious mutations are removed by sexual selection via their effects on condition (Rowe and Houle 1996); thus, particularly for assays where we detected effects of MA on the outcome of sexual selection, we should likewise have detected an effect of MA on male condition. While we did not manipulate male condition directly in this experiment (given previous experiments demonstrating clear condition dependent sexual selection both pre- and postcopulation in D. melanogaster)(e.g., Amitin and Pitnick 2007, Clark et al. 2012), we used experimental male body size as an indicator of condition (Whitlock and Agrawal 2009). Thus, it is somewhat perplexing that the assays for which we detected a significant effect of MA on the outcome of sexual selection (mating success and sperm competition offence) were precisely the ones for which there was no general effect of MA on male body size. This may suggest that sexual selection is acting directly on deleterious mutations, rather than indirectly via their predicted effects on condition (Rowe and Houle 1996). However, past experiments in the Dahomey population have shown minimal mutational effects on male body size relative to environmental manipulations of condition (e.g., Clark et al. 2012); accordingly, it is conceivable that mutational effects on body size in our present study were small to the extent that they were immeasurable within the scope of our design. Alternatively, as condition is likely to be a multidimensional trait (e.g., Tomkins et al. 2004), deleterious mutations may affect different aspects of condition than do environmental changes to larval resource availability (Bonduriansky et al. 2015). Thus, body size may not be a good indicator of those dimensions of condition affected by spontaneous mutation accumulation.
Our selection estimates suggest that precopulatory mating success is primarily driving selection against deleterious mutations in our experimental fly population, and postcopulatory selection via sperm competition offence is of secondary (but nonetheless consequential) importance. We are aware of only one other study (Mallet et al. 2012) that has attempted to measure the relative contribution of pre- and postcopulatory sexual selection components to purging deleterious mutations (see above). While our results differ quantitatively from Mallet et al. (2012), the general qualitative similarity is perhaps of greater importance, demonstrating that sperm competition, in addition to precopulatory processes of selection, is important for selecting against deleterious mutations.

Our aim with the present study was to provide a comprehensive overview of the pre- and postcopulatory potential for sexual selection to affect deleterious mutations in *D. melanogaster*. We are obviously limited in the scope of our conclusions by the specific components of sexual selection measured. While we have a powerful design to test our objectives, a possible improvement for future work could be to adopt the very elegant design of Pischedda and Rice (2012), wherein sexual selection was partitioned into mating and fertilisation success, while allowing the focal males to interact ‘naturally’ with competitors and potential mates. A further limitation of our experiment may be that the isogenic male lines (MA and control) we used were not representative of natural populations, or even other lab-adapted populations, and thus neither are our conclusions. We make no claims that our flies are representative of their wild counterparts; however, MA lines represent a very powerful tool for directly testing the prediction of good genes selection that sexual selection can remove deleterious mutations from populations (Rowe and Houle 1996), which makes them an obvious choice for our study.

Our experiments have shed light on two critical aspects of sexual selection for good genes. First, they have established, along with recent empirical work (Mallet et al. 2012), that selection against deleterious mutations can occur through processes acting both before and after copulation, and that the relative importance of these processes varies. Second, while recent experiments have shown importance of female condition to the outcome of sexual selection (Amitin and Pitnick 2007, Long et al. 2010), ours does not, as there was no evidence from our experiments that female condition affected selection against deleterious mutations. Despite this, our results nonetheless provide a valuable contribution to closing the literature gap for sexual selection studies incorporating female condition dependence. There is building consensus that
sexual selection is important for selecting against deleterious mutations (Whitlock and Agrawal 2009), and hence for facilitating natural selection in improving nonsexual fitness. Given this consensus, future work should attempt to establish the sexually selected mechanisms through which deleterious mutations are removed before and after copulation.
Table 4.1. Point estimates for selection against deleterious mutations for each of the pre- and postcopulatory sexual selection assays (mutation accumulation=MA; control=CTL). Selection was quantified as $s = \frac{\text{Mean}(\text{CTL Line Means}) - \text{Mean}(\text{MA Line Means})}{\text{Mean}(\text{CTL Line Means})}$. Point estimates of $s$ were given upper and lower bounds using bootstrapped 95% confidence intervals. Values of $s$ for which confidence intervals did not overlap with zero were considered significantly different from zero (values boldfaced). Mating success was quantified using the “contest win score” (see Methods); refractory period as the mean number of days between first and second mating; Refractory Offspring as the number of offspring produced by focal-male-mated females prior to remating; Refractory Offspring/Day as the number of refractory offspring produced per refractory period day; P1 as the proportion of offspring attributed to the focal male when he was first to mate; P2 as the proportion of offspring attributed to the focal male when he was second to mate.
<table>
<thead>
<tr>
<th>SS Stage</th>
<th>Assay Type</th>
<th>CTL mean</th>
<th>MA mean</th>
<th>s</th>
<th>CI&lt;sub&gt;95%&lt;/sub&gt; Lower</th>
<th>CI&lt;sub&gt;95%&lt;/sub&gt; Upper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precopulatory Success</td>
<td>Mating Success</td>
<td>0.227</td>
<td>0.144</td>
<td>0.363</td>
<td>0.0767</td>
<td>0.596</td>
</tr>
<tr>
<td>Sperm Competition</td>
<td>Refractory Period</td>
<td>2.74</td>
<td>2.38</td>
<td>0.131</td>
<td>-0.0709</td>
<td>0.309</td>
</tr>
<tr>
<td>Defence (block 1)</td>
<td>Refractory Offspring</td>
<td>133.3</td>
<td>121.3</td>
<td>0.0895</td>
<td>-0.149</td>
<td>0.285</td>
</tr>
<tr>
<td></td>
<td>Refractory Offspring/Day</td>
<td>40.3</td>
<td>33.9</td>
<td>0.159</td>
<td>0.0282</td>
<td>0.284</td>
</tr>
<tr>
<td></td>
<td>P1</td>
<td>0.0328</td>
<td>0.0252</td>
<td>0.232</td>
<td>-2.09</td>
<td>0.725</td>
</tr>
<tr>
<td>Sperm Competition</td>
<td>Refractory Period</td>
<td>3.45</td>
<td>3.43</td>
<td>0.00759</td>
<td>-0.166</td>
<td>0.156</td>
</tr>
<tr>
<td>Defence (block 2)</td>
<td>Refractory Offspring</td>
<td>147.2</td>
<td>141.1</td>
<td>0.0409</td>
<td>-0.163</td>
<td>0.214</td>
</tr>
<tr>
<td></td>
<td>Refractory Offspring/Day</td>
<td>41.6</td>
<td>38.98</td>
<td>0.0632</td>
<td>-0.0428</td>
<td>0.165</td>
</tr>
<tr>
<td></td>
<td>P1</td>
<td>0.0401</td>
<td>0.0392</td>
<td>0.0225</td>
<td>-3.53</td>
<td>0.758</td>
</tr>
<tr>
<td>Sperm Competition Offence</td>
<td>P2</td>
<td>0.719</td>
<td>0.642</td>
<td>0.107</td>
<td>0.0165</td>
<td>0.207</td>
</tr>
</tbody>
</table>
Table 4.2. Response of experimental male body mass to the accumulation of deleterious mutations (MA=mutant males; CTL=control males). Male mass (least square means and standard error) are given for control and MA males for each assay. Percent difference is calculated as \(\%\text{Diff}\text{means} = \left(\frac{\text{CTL mass}-\text{MA mass}}{\text{CTL Mass}}\right) \times 100\). Data were pooled across blocks for the mating success assay as there was no block effect \(F_{1,526}=0.304, p=0.582\); however, there were differences among blocks in the effect of MA on male mass for the sperm competition assays; hence, results are presented separately for each block. Significant p-values are boldfaced and highlighted with an asterisk. There has been no correction for multiple tests.
<table>
<thead>
<tr>
<th>SS Stage</th>
<th>CTL Mean Mass (mg)</th>
<th>CTL SE</th>
<th>MA Mean Mass (mg)</th>
<th>MA SE</th>
<th>%Diff means</th>
<th>F-Ratio</th>
<th>df</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precopulatory Mating Success</td>
<td>0.221</td>
<td>3.09x10^-3</td>
<td>0.218</td>
<td>3.13x10^-3</td>
<td>1.54%</td>
<td>0.594</td>
<td>1,46.8</td>
<td>0.445</td>
</tr>
<tr>
<td>Sperm Competition Defence (Block 1)</td>
<td>0.210</td>
<td>3.10x10^-3</td>
<td>0.220</td>
<td>3.38x10^-3</td>
<td>-4.57%</td>
<td>4.37</td>
<td>1,45.7</td>
<td>0.0422*</td>
</tr>
<tr>
<td>Sperm Competition Defence (Block 2)</td>
<td>0.217</td>
<td>4.22x10^-3</td>
<td>0.203</td>
<td>4.57x10^-3</td>
<td>6.27%</td>
<td>4.78</td>
<td>1,44.5</td>
<td>0.034*</td>
</tr>
<tr>
<td>Sperm Competition Offence (Block 1)</td>
<td>0.217</td>
<td>3.02x10^-3</td>
<td>0.233</td>
<td>3.63x10^-3</td>
<td>-7.27%</td>
<td>11.2</td>
<td>1,33.8</td>
<td>0.002*</td>
</tr>
<tr>
<td>Sperm Competition Offence (Block 2)</td>
<td>0.235</td>
<td>3.20x10^-3</td>
<td>0.227</td>
<td>3.43x10^-3</td>
<td>3.43%</td>
<td>2.96</td>
<td>1,42.3</td>
<td>0.0926</td>
</tr>
</tbody>
</table>
Table 4.3. Response of experimental female body mass to larval dietary manipulation of condition. Females in the high condition group were reared on medium containing 100% of standard laboratory quantities of yeast and sugar, while low condition females were fed food reduced to 25% of these quantities (see Methods). Nutrient restriction through a low condition diet had a significant effect on female body mass for each of the precopulatory success, sperm competition defence, and sperm competition offence assays. Female masses (means and standard error) are given for high and low condition females used in each assay. Percent difference is calculated as $\%\text{Diff}_{\text{means}} = ([\text{High mass} - \text{Low mass}] / [\text{High Mass}]) \times 100$. Significant p-values are boldfaced and highlighted with an asterisk. There has been no correction for multiple tests.
<table>
<thead>
<tr>
<th>SS Stage</th>
<th>High Mean Mass (mg)</th>
<th>High SE</th>
<th>Low Mean Mass (mg)</th>
<th>Low SE</th>
<th>%Diffmeans</th>
<th>F-Ratio</th>
<th>df</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precopulatory Mating Success</td>
<td>0.527</td>
<td>2.72x10^{-3}</td>
<td>0.401</td>
<td>2.66x10^{-3}</td>
<td>24.0%</td>
<td>1107.8</td>
<td>1,559</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Sperm Competition Defence (block 1)</td>
<td>0.473</td>
<td>4.70x10^{-3}</td>
<td>0.372</td>
<td>4.25x10^{-3}</td>
<td>21.3%</td>
<td>253.6</td>
<td>1,176</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Sperm Competition Defence (block 2)</td>
<td>0.501</td>
<td>5.70x10^{-3}</td>
<td>0.388</td>
<td>4.83x10^{-3}</td>
<td>22.6%</td>
<td>230.1</td>
<td>1,175</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Sperm Competition Offence</td>
<td>0.510</td>
<td>3.60x10^{-3}</td>
<td>0.410</td>
<td>4.32x10^{-3}</td>
<td>19.5%</td>
<td>311.9</td>
<td>1,364</td>
<td>&lt;0.0001*</td>
</tr>
</tbody>
</table>
Figure 4.1. Mean (±SE) contest win score (see Methods) by control (CTL) or mutant (MA) focal males competing against standard competitor males for mating access to either high (=solid squares) or low (=solid circles) condition virgin females. Control males achieved ~49% higher mating success when competing against a standard competitor relative to mutant males ($F_{1,52.3}=4.63, p=3.60\times10^{-2}$). There was no effect of female condition on the outcome of mating success contests ($F_{1,49.4}=0.117, p=0.733$).
Figure 4.2. Mean (±SE) total offspring production between first and second mating in the sperm competition defence assay for high (=solid squares) and low (=solid circles) condition females, mated first to control (CTL) or mutant (MA) focal males, and then remated to a standard competitor. There was no effect of mutation accumulation on offspring production between matings for (a) block 1 ($F_{1,43.5}=0.109, p=0.743$), or (b) block 2 ($F_{1,46.6}=0.138, p=0.712$). Additionally, there was no effect of female condition on offspring production between matings for block 1 ($F_{1,138}=0.148, p=0.701$), but there was a significant condition effect for block 2 ($F_{1,162}=8.76, p=3.50x10^{-3}$).
Figure 4.3. Mean (±SE) number of offspring produced per laying day between first and second mating in the sperm competition defence assay for high (=solid squares) and low (=solid circles) condition females, mated first to control (CTL) or mutant (MA) focal males, and then remated to a standard competitor. There was a significant effect of mutation accumulation on offspring produced per day between matings for (a) block 1 \((F_{1,42.9}=5.71, p=2.13 \times 10^{-2})\); but not (b) block 2 \((F_{1,43.4}=0.185, p=0.669)\). There was a significant effect of female condition on daily offspring production rate between matings for block 1 \((F_{1,134}=4.89, p=2.87 \times 10^{-2})\) and block 2 \((F_{1,169}=7.87, p=5.60 \times 10^{-3})\), but the direction of the effect was opposite between blocks.
Figure 4.4. Remating latency (shown here as the time interval between matings) for experimental females first mated to control (red line) or MA (blue line) focal males, followed by a mating with a standard competitor male 1-5 days (for block 1; 1-6 days for block 2) after completion of the first mating. Females were offered the opportunity to remate (~2 h window) with standard competitors once daily commencing ~24 h after their first mating. There was no effect of MA on refractory period for (a) block 1 ($\chi^2 = 1.01, p = 0.314$) or (b) block 2 ($\chi^2 = 1.33 \times 10^{-2}, p = 0.908$). Likewise, there was no significant effect of female condition on refractory period duration for block 1 ($\chi^2 = 0.602, p = 0.438$), or block 2 ($\chi^2 = 1.98, p = 0.159$).
Figure 4.5. Mean (±SE) P1 success for control (CTL) or mutant (MA) focal males mated to high (=solid squares) or low (=solid circles) condition virgin females, which were then remated to standard competitor males. a) block 1: there was no effect of mutation accumulation ($F_{1,40.7}=7.62 \times 10^{-2}$, $p=0.784$) or female condition ($F_{1,128}=0.238$, $p=0.627$); b) block 2: there was no effect of mutation accumulation ($F_{1,39.6}=0.568$, $p=0.456$) or female condition ($F_{1,153}=3.21$, $p=7.51 \times 10^{-2}$).
Figure 4.6. Mean (±SE) P2 success for control (CTL) or mutant (MA) focal males mated to high (=solid squares) or low (=solid circles) condition females 72 hours following their first mating with standard competitor males. Control males outperformed mutants at P2 success ($F_{1,47}=5.24$, $p=2.66\times10^{-2}$); however, there was no effect of female condition on P2 success ($F_{1,363}=0.603$, $p=0.438$).
Chapter 5
Impacts of Male Mate Choice for Perceived Female Mating Status Pre- and Post-copulation in *Drosophila melanogaster*

5 ABSTRACT

While a growing body of evidence shows precopulatory male mate choice to be prevalent across insect taxa, there is emerging evidence for male mate choice at the postcopulatory stage of sexual selection. As males trade-off ejaculate expenditure against other processes competing for reproductive energy resources, prudent allocation of male sperm according to social conditions of mating is predicted. Here, we use a special manipulation of perceived female mating status in *Drosophila melanogaster* to establish conditions for pre- and post-copulatory male mate choice, and to determine the potential downstream fitness costs associated with differential investment in matings with perceived virgin and mated females. Our results indicate that males showed a precopulatory preference for perceived virgin females, suggesting explicit choice for females with lower ejaculate competition risk (i.e., virgins). At the post-copulatory level, males mated to perceived virgins outperformed males mated to perceived mated females at several key measures of sperm competition defence, which may suggest differential allocation of seminal fluid proteins affecting oogenesis induction to females of different mating status. However, we detected no downstream fitness costs associated with this differential investment in ejaculate competition, potentially suggesting that males are adjusting the composition of their ejaculate to match female mating status without affecting the overall cost.
5.1 INTRODUCTION

Traditional views on mate choice (Darwin 1871, Bateman 1948) were such that females should be the predominantly choosy sex, and males the sex that indiscriminately competed for access to females (Bonduriansky 2001, Edward and Chapman 2011). Trivers (1972) later postulated that the degree of choosiness in the sexes should be proportional to their degree of parental investment, with males becoming the choosier sex (and females more competitive) when they contribute more than females to parental investment. Furthermore, Dewsbury (1982) demonstrated that the nontrivial costs of ejaculate production should likewise favour male choosiness (Bonduriansky 2001, Wedell et al. 2002). It has since been recognized that in addition to parental investment, mate quality variance, mating investment, and constraints on choosiness are important factors in the evolution of male mate choice (Bonduriansky 2001, Wedell et al. 2002). Indeed, male mate choice occurs in species in which males do not invest substantially in offspring care (Bonduriansky 2001, Edward and Chapman 2011). Male mate choice becomes beneficial when the male capacity to mate is less than female mate availability, when female quality varies, and when the benefit of choosing between females is greater than the male cost of assessing them (Bonduriansky 2001, Edward and Chapman 2011).

While a growing body of evidence shows precopulatory male mate choice to be prevalent across insect taxa (for comprehensive review, see Bonduriansky 2001), there is emerging evidence for male mate choice at the postcopulatory stage of sexual selection, also known as cryptic male choice (Edward and Chapman 2011). As males tradeoff ejaculate expenditure against other processes competing for reproductive energy resources (e.g., mate searching; Parker 1998), prudent allocation of male sperm according to social conditions of mating is predicted, and has been demonstrated across many taxa (Parker 1998, Wedell et al. 2002). Cryptic male choice can come in the form of male ejaculate allocation to females on the basis of female quality (e.g., fecundity), the differential allocation of parental care, and the potential for ejaculate competition (e.g., female mating status; Edward and Chapman 2011, Lupold et al. 2011).

Recently, attention has turned to whether other ejaculate components (i.e., seminal fluid proteins) can be strategically allocated by males according to the specific social conditions of mating. As with sperm, the production of seminal fluid proteins (SFPs, also known as accessory gland proteins in the literature) is costly, and recent theoretical results suggest that males can exploit the reproductive benefits of competitors’ ejaculates (Hodgson and Hosken 2006,
Cameron et al. 2007, Alonzo and Pizzari 2010). Concomitant with these results, several empirical investigations using the fruit fly *Drosophila melanogaster* have demonstrated that males are able to adjust their allocation of specific SFPs according to the presence of competitors (and hence the potential level of sperm competition; Wigby et al. 2009) and female mating status (Sirot et al. 2011).

As ejaculate production represents a nontrivial mating investment by males (Dewsbury 1982), an interesting question becomes what are the downstream fitness costs to males that mate with females of low perceived risk of ejaculate competition (i.e., virgin females) versus those females of high perceived ejaculate competition risk (i.e., mated females)? Or, alternatively, are ejaculate investments more expensive for matings with virgin or mated females? Our objectives were thus: i) to establish whether our experimental population of *D. melanogaster* demonstrates male mate choice for perceived female mating status, both pre- and post-copulation; ii) given (i), determine the immediate (within experimental mating) and the downstream (sequential post-experimental matings) costs to fitness of male ejaculate allocation to virgin females perceived by males as virgins, and to virgin females perceived by males as mated (in other words, does whether a male mated with a virgin or previously-mated female affect his ability to successfully copulate and fertilize other females as successive mates?); and iii) our design likewise allowed us to assay the immediate fitness consequences to a male making an erroneous ejaculate allocation decision, in this case allocating a “mated” ejaculate dose to a female that was actually virgin. *D. melanogaster* represents an ideal system for pursuing these objectives as: i) there is evidence for ejaculate allocation according to female mating status (see above); ii) there is empirical evidence for effects of perceived female mating status on components of sperm competition defence (Friberg 2006); iii) a body of literature now exists detailing specific fitness effects of SFPs (reviewed in Chapman 2001, Wolfner 2002) and patterns of ejaculate use after transfer to females (Manier et al. 2010).

To address these questions, we employed a technique used to transfer cuticular hydrocarbons (CHCs) from mated *D. melanogaster* females to virgin females (adapted from Friberg 2006), thereby giving them a chemosensory “mated” signal. Female mating in *D. melanogaster* alters the CHC profile (Tompkins and Hall 1981, Tompkins 1984, Everaerts et al. 2010), and so males use it to assess female mating status (Tompkins and Hall 1981, Scott et al. 1988). This technique of CHC transfer between flies was originally developed for various *Drosophilia* spp. by Coyne et
al. (1994), and has been used successfully in various contexts (e.g., Marcillac and Ferveur 2004, Friberg 2006). The CHC manipulation to adjust perceived female mating status (Friberg 2006) is particularly powerful in the context of the present study, as it allows for examination of male-driven effects on sexually selected fitness pre- and post-copulation, as females are perceived to be mated or virgin, but all are actually true virgins (hence, any observed effects can be presumed to be under male control).

5.2 METHODS

5.2.1 Study Organisms

Experimental males and CHC Transfer Females (see below) were derived from a wild-type (+/+ outbred population of *D. melanogaster* originally collected in 1970 from Dahomey (now Benin), West Africa. The +/+ Dahomey stock was maintained in the current laboratory for over seven years at a population size of several thousand adults. A standard fly stock homozygous for a recessive brown-eye mutation (*bw/bw*), which had been introgressed onto the Dahomey background, was used as the source of all competitor males and experimental females for the study. All flies for the study were reared on the same standard yeast-sugar-agar medium, on which they were maintained in the laboratory at 25ºC under a 12L:12D photoperiod.

5.2.2 Transfer of Cuticular Hydrocarbons (CHCs)

We followed the protocol outlined by Friberg (2006) (ultimately derived from Coyne et al. 1994) for CHC transfer from mated to virgin females. Briefly, five-day-old (relative to adult emergence) virgin +/+ males and three-day-old virgin +/+ females were mated at a sex ratio of 10M:10F for a duration of 48 h to create a group of females with a “mated” CHC profile (henceforth: *CHC-+/+* females). These *CHC-+/+* females (now five days old) were then placed in vials with three-day-old virgin *bw/bw* females (thus distinguishable from red-eyed *CHC-+/+* females by eye colour) at a ratio of $60\frac{♀}{♀} CHC-+/+:9\frac{♀}{♀} bw/bw$ (Fig. 5.1a). Females were allowed to interact for 48 h, during which time the *bw/bw* virgin females physically associated with their higher-frequency *CHC-+/+* counterparts such that CHCs were transferred from mated *CHC-+/+* to virgin *bw/bw* females, with the end result that virgin *bw/bw* females would carry a
chemosensory “mated” signal. These \( bw/bw \) females became the “\textit{pseudomated}” (PM) treatment group, and were integral to this ejaculate allocation experiment as they allowed females to be perceived as mated while actually virgin (and thus carrying no sperm or ejaculate products in their reproductive tracts). The CHC transfer process was repeated using virgin \( CHC-+/+ \) females and virgin \( bw/bw \) females to create a “\textit{true-virgin}” (V) control group. Collectively, PM and V females comprised the “experimental” females.

5.2.3 Ejaculate Allocation Assay

\textit{Pseudomated} and \textit{true-virgin} \( bw/bw \) females were separated using light anaesthesia from their \( CHC-+/+ \) counterparts 1.5 h prior to commencement of the ejaculate allocation assay. Experimental +/- males were combined with either \textit{pseudomated} or \textit{true-virgin} \( bw/bw \) females in single pairs (i.e., at a sex ratio of 1M:1F) in vials containing standard media with live yeast. If the pair mated, immediately following mating the \( bw/bw \) female was placed in a vial with two virgin \( bw/bw \) competitor males (with which she remained for the duration of the experiment), and the +/- experimental male was placed in a vial with a new virgin \( bw/bw \) “secondary” female. If the male remated, he was then transferred to a vial with a new “secondary” virgin \( bw/bw \) female. This process was repeated until the male had mated a total of four virgin \( bw/bw \) females (one experimental mating + three secondary matings)(experimental mating protocol summarized in Fig. 5.1b). Following their fourth mating, experimental males were transferred to microtubes and frozen at -80°C. Females were provided with fresh laying vials daily (exception: day 1; see below) for a total of four days, and then were discarded (study total = 4 laying vials/female). We began the assay with approximately 170 experimental males; however, only 101 males mated to the requisite four females (giving a total of 404 vials across 4 matings/exp. male on day 1).

Due to a logistical error in experimental media preparation, during the first 24 h females from the experimental mating were allowed to lay eggs in the mating/resident vial of the standard competitor males, and females from the secondary matings were allowed to lay eggs in their own mating/resident vials, instead of in fresh laying vials. This meant that laying vials for the experimental group on the first experiment day had moderate yeast growth problems that may have affected our ability to assess offspring production (whereas secondary mating laying vials were not yeasty), though they were randomly allocated across PM and V females; hence, there
was no treatment bias. Accordingly, all PM and V flies within the experimental mating group (i.e., first of four matings) were treated identically within day 1, as were all PM and V flies within the secondary mating group (i.e., matings 2-4) on day 1; however, there were potential differences between experimental and secondary mating groups, and generally between media conditions on day 1 and those on days 2-4. Hence, the comparison of PM and V flies for experimental matings on day 1 was not affected; nor was comparison between PM and V flies for secondary matings on day 1. Similarly, there was no issue comparing PM and V flies on days 2-4. However, caution is needed in interpreting the comparison of offspring production of experimental matings to secondary matings because of media issues affecting day 1 of experimental matings, but not secondary matings; all such analyses were run both including and excluding day 1 production, and are reported as such in Results. Likewise, caution is needed in interpreting comparisons of offspring production from experimental matings on day 1 with days 2-4, because yeast issues affected only day 1.

5.2.4 Experimental Mating: Assays of Pre- and Postcopulatory Male Mate Choice

Precopulatory male mate choice was assayed by measuring the time to copulation for each experimental male-female pair (i.e., time-to-copulation was assayed as a component of precopulatory male mate preference). Offspring production by each experimental male with the experimental female was assayed from the set of laying vials for the experimental mating (i.e., mating no. 1) on day 12 of their life cycle. Each of these vials had two potential types of offspring with respect to eye phenotype: wild-type (attributable to +/+ experimental male) and brown-eye (attributable to standard bw/bw competitor males). Offspring production by the experimental male was assessed across each of the four laying days (see above) by counting all individuals with wild-type eyes. The ability of the experimental male to induce a refractory period in the experimental female was assessed by counting the number of days between the commencement of the experiment and the first presence of bw/bw offspring (which indicates that the female remated to a bw/bw competitor male). Offspring production by experimental males prior to remating was assessed by counting all offspring produced during the refractory period, or prior to the end of the experiment if the female did not remate. Three variations of sperm competition defence (the proportion of total offspring produced across days 1-4
attributable to the first male to mate with a female; \( P1 = \frac{N_{focal}}{N_{focal} + N_{bw/bw}} \) were assessed as:

i) \( P1 \) "net defence" (after Friberg 2006 "net defence") = total focal (experimental) male offspring production, including offspring produced prior to remating, and those from females that did not remate (i.e., \( P1 = 1 \)), expressed as a proportion of total experimental female offspring production;

ii) \( P1 \) "net remated" = total focal male offspring production, including offspring produced prior to remating, excluding females that did not remate, expressed as a proportion of total experimental female offspring production; and

iii) \( P1 \) "direct" = focal male offspring produced only under direct ejaculate competition (i.e., after remating), expressed as a proportion of total experimental female offspring production.

5.2.5 Secondary Matings: Effects of Male Mate Choice on Downstream Fitness

Offspring production by secondary females was used to assess the impact of ejaculate allocation decisions by focal males in the experimental mating (based on perceived female mating status) on downstream fitness when males were put under conditions of ejaculate depletion stress (i.e., successive secondary matings). We followed Lefevre and Jonsson (1962) in choosing a total of four matings for each experimental male to induce ejaculate stress. Offspring from these matings were counted on day 12 of their life cycle.

5.2.6 Mating Duration

Mating duration was assayed for the experimental mating, and each of the three secondary matings. Mating duration was measured as the time interval commencing when the male mounted the female and ending when he dismounted. Mating duration has been shown in some \textit{D. melanogaster} studies to be correlated with seminal fluid transfer (e.g., Gilchrist and Partridge 2000, Sirot et al. 2011) and with sperm competition defence fitness (Friberg 2006).
5.2.7 Analysis

Time-to-first-mating data were analysed using parametric survival (time-to-failure) methods assuming a log-normal distribution (chosen using AICc; Klein and Moeschberger 2003; and verified with natural-logarithm transformation of raw distribution) and Type I right-censoring. The impact of perceived female mating status on refractory period was analysed using parametric survival (time-to-failure) methods assuming a log-logistic distribution (chosen using biological rationale; Fox 2001, and AICc; Klein and Moeschberger 2003) and Type I right-censoring. A comparison of offspring produced prior to remating was made using a generalized linear model (GLM) assuming a quasipoisson error distribution (to correct for overdispersion) and adjusted means ± CI95 were back-transformed to original scale using the “effects” library in R (v.3.1.2; R Core Team 2014). All statistical analyses involving sperm competition defence (P1) were done using GLMs assuming a quasibinomial error distribution (to correct for overdispersion), and adjusted means ± CI95 were back-transformed from logits to original probability scale using the “effects” library in R (v.3.1.2; R Core Team 2014). Repeated measures analyses were conducted using hypothesis tests that were either univariate-adjusted (Greenhouse-Geisser [G-G] epsilon and Huynh-Feldt [H-F] epsilon estimates <1; assumption of multisample sphericity assumed violated) (Quinn and Keough 2005) or univariate unadjusted (G-G and H-F epsilon estimates ~1; assumption of multisample sphericity assumed met) (Quinn and Keough 2005). When univariate adjusted methods were used, H-F corrections were employed when epsilon estimates >0.75, and G-G when epsilon estimates <0.75) (Quinn and Keough 2005). While this analytical convention was adhered to, it is important to note that in all cases but one adjusted and unadjusted values were qualitatively identical (the exception was an interaction term for daily focal male offspring production that was significant when unadjusted \[F_{3,207}=2.68, p=4.80\times10^{-2}\], while adjusted terms were not [H-F adjustment reported in results: \(F_{2.55,176}=2.68, p=5.76\times10^{-2}\); however, in that case, G-G and H-F epsilon values were clearly <1, and so the discrepancy was not an issue). Total experimental male offspring production was analysed using a t-test assuming unequal variances; total offspring production with secondary females was analysed using a t-test assuming equal variances.

Time-to-failure analyses (time to first mating and time to remating) and repeated measures analyses (measures of daily offspring production, offspring production by different females) were conducted using JMP (v.11.0.0); statistical analyses for offspring production prior to remating and P1 data were conducted using R (v.3.1.2; R Core Team 2014). Minimal adequate
models were chosen for all general linear models using AICc (Sokal and Rohlf 1995, Quinn and Keough 2005), and for all generalized linear models with quasi-error structures by comparing full and reduced models using the “anova” command with “test= ‘F’” in R (v.3.1.2; R Core Team 2014), which is synonymous with using AICc. Individuals with missing values were excluded from respective analyses (Crawley 2009).

5.3 RESULTS

5.3.1 I. Experimental Mating Investment and Effects of Perceived Mating Status on Sperm Competition Defence

5.3.1.1 Precopulatory Male Mate Choice

Experimental males showed clear precopulatory preference for true virgin over pseudomated females in their time-to-copulation with experimental females (Fig. 5.2): males mating to true virgin females were ~21% faster to mate than with pseudomated females (all males starting the experiment were included in the analysis; Means [SE]: PM=99.6 min [10.7]; V=78.9 min [9.43]; \(X^2_{1}=4.77, p=2.90\times10^{-2}\); Fig. 5.2). A single male-female experimental pair was excluded from analysis because their mating vial was dropped to the floor from the lab bench during mating observations; however, exclusion does not affect the results qualitatively (when vial included in analysis: \(X^2_{1}=3.88, p=4.90\times10^{-2}\)).

5.3.1.2 Postcopulatory Male Mate Choice

5.3.1.2.1 Refractory Period and Offspring Production Prior to Remating

Perceived female mating status did not have a significant effect on the female’s time to remating (i.e., refractory period; \(X^2_{1}=0.839, p=0.360\); Fig. 5.3). However, in an ANCOVA including the covariates refractory period, mating duration, and their interaction term, males mated to true virgin females were ~28% more successful at offspring production prior to experimental female remating than males mated to pseudomated females (\(t_{1,85}=2.61, p=1.08\times10^{-2}\); Fig. 5.4a), and the number of offspring produced prior to remating was proportional to refractory period (\(t_{1,85}=20.2, p<2.00\times10^{-16}\)). Likewise, there was a significant interaction between perceived female mating status and mating duration, whereby males mated to true virgin females produced more
offspring than those mated to pseudomated females following short mating durations, and males from the two treatment groups converged on similar numbers of offspring produced as mating duration increased toward upper extremes of the sample distribution ($t_{1,85}=2.07, p=4.12\times10^{-2}$; Fig. 5.4b).

5.3.1.2.2 Sperm Competition Defence

Focal males mated to true virgin females achieved ~31% higher total $P1_{\text{net defence}}$ than those mated to pseudomated females ($t_{1,83}=2.06, p=4.26\times10^{-2}$; Fig. 5.5a). A second-order polynomial term was fitted to the model because of the curvilinear relationship between $P1$ and refractory period (model covariates included refractory period and its quadratic term; comparison of models with and without quadratic term revealed that quadratic term was appropriate to model: $F_{1,83}=13.5, p=4.23\times10^{-4}$). There was likewise a significant quadratic covariate effect of refractory period on $P1_{\text{net defence}}$ ($t_{1,83}=3.54, p=6.68\times10^{-4}$), with $P1_{\text{net defence}}$ logically operating as an increasing function of refractory period.

When $P1$ Total analysis was restricted to $P1_{\text{net remated}}$, all effects disappeared, though the trend direction remained for both perceived mating status ($t_{1,67}=1.75, p=8.42\times10^{-2}$; Fig. 5.5b) and $P1$ total operating as an increasing function of refractory period (quadratic term: $t_{1,67}=1.97, p=5.26\times10^{-2}$).

When $P1$ Total analysis was restricted to $P1_{\text{direct}}$ (i.e., a measure of $P1$ only when focal males were under direct sperm competition with standard competitor males), while the trend for perceived female mating status remained in the same direction, there was no effect of perceived female mating status on $P1_{\text{direct}}$ ($t_{1,68}=1.33, p=0.190$; Fig. 5.5c). The effect of refractory period on total $P1$ was qualitatively unchanged ($t_{1,68}=3.22, p=1.97\times10^{-3}$).

5.3.1.2.3 Total focal male offspring production from first mating

Focal males mated to true virgin females produced ~28% more offspring (summed across all experimental days) than males mated to pseudomated females in experimental matings (includes focal offspring produced prior to, and after, female remating; $t_{1,84}=2.03, p=4.61\times10^{-2}$; Fig. 5.6a).
There was likewise a significant positive effect of time-to-remating on total focal male first mating offspring production ($t_{1,84}=11.6, p<2.00\times10^{-16}$). This contrasts with total focal male offspring production across secondary females, for which there was no effect of perceived mating status (see below: *Fitness consequences across secondary females*).

5.3.2 II. Downstream Fitness Costs of Experimental (First) Mating Investment

5.3.2.1 Fitness consequences across secondary females

There was no effect of perceived mating status on daily secondary female offspring production ($F_{1,69}=0.291, p=0.641$; Fig. 5.7a), nor were there any significant main or higher-order effects, with the exception of time ($F_{3,207}=38.9, p<0.0001$; Fig. 5.7b). Perceived female mating status had no effect on offspring production summed across secondary females on each experiment day ($F_{1,77}=0, p=0.998$; Fig. 5.8a). However, there was a significant effect of time on offspring production summed across secondary females on each experiment day ($F_{2.49,191}=43.8, p<1.00\times10^{-4}$; Fig. 5.8b). A Tukey’s HSD test revealed that total daily secondary female offspring production on days 1 and 2 was identical, followed by significant increases daily between days 2-4 (Fig. 5.8b). Results remained qualitatively identical when day 1 data were excluded from analysis (see Methods). Perceived female mating status did not affect the total number of offspring produced by focal males with secondary females across all experimental days ($t_{1,77}=2.11\times10^{-3}, p=0.998$; Fig. 5.6b).

5.3.2.2 Fitness consequences across experimental plus secondary females

There was a significant effect of time on daily focal male offspring production (=no. offspring produced by focal males summed across all four females mated, for each of experiment days 1-4; $F_{2.55,176}=37.7, p<0.0001$; Fig. 5.9a,b), and a Tukey’s HSD test revealed that days 1-2 did not differ from each other, while day 3 and day 4, respectively, differed significantly from all other days (Fig. 5.9b). There was no effect of perceived mating status on daily focal male offspring production ($F_{1,69}=0.136, p=0.714$; Fig. 5.9a), nor was there any interaction between mating status and time ($F_{2.55,176}=2.68, p=0.0576$; Fig. 5.9a). All results were qualitatively identical
when data for experimental day 1 were excluded from analysis (see Methods). There was no
effect of perceived mating status on the total number of offspring produced by focal males
across all females summed across all experiment days (Means [SE]: PM=496 [25.0]; V=510
[28.4]; $t_{1,68.2}=0.375, p=0.709$).

### 5.3.2.3 Per female offspring production across experiment

Perceived female mating status had no effect on offspring production across the four female
types (=total offspring production across four days of experiment for each of four females mated
to the experimental male, including progeny of standard competitor males for first female;
$F_{1,69}=0.618, p=0.435$; Fig. 5.10a). However, there was a significant effect of mating order on
total per female offspring production ($F_{3,207}=3.34, p=2.02\times10^{-2}$; Fig. 5.10b). A Tukey’s HSD
test revealed that Female 1 produced significantly fewer offspring relative to Female 2 only, but
that Females 2-4 did not differ statistically in number of offspring produced (Fig. 5.10b).
However, when Day 1 offspring production data were excluded from analysis (see Methods),
the effect of mating order on offspring production disappeared ($F_{3,207}=0.680, p=0.565$), and
Females 1-4 were not significantly different from each other in numbers of offspring produced
across the experiment.

### 5.3.3 III. Mating Duration

Perceived female mating status had no effect on mating duration across the four female types
($F_{1,89}=3.20\times10^{-3}, p=0.955$; Fig. 5.11a). However, there was a significant effect of mating order
on mating duration ($F_{3,267}=26.8, p<1.00\times10^{-4}$; Fig. 5.11b). A Tukey’s HSD test revealed that
mating duration for the first mating was significantly longer than for the other three; mating
durations to secondary females (matings numbers 2-4) were not significantly different from each
other (Fig. 5.11b).
5.4 DISCUSSION

The result from the time-to-first-mating assay for experimental males and females (i.e., focal male mating no. 1) clearly demonstrates precopulatory male preference for true virgin over pseudomated females. This result is predicted by theory (Parker 1998, Bonduriansky 2001, Wedell et al. 2002), wherein virgin females represent reduced risk of sperm competition to the allocating male. This result is likewise in agreement with others from the literature showing evidence for precopulatory male mate choice in *D. melanogaster*. For example, males show precopulatory choice against mated females through reduced levels of courtship (Bastock and Manning 1955, Cook and Cook 1975). Likewise, males preferentially court and mate with large (i.e., fecund) females (Byrne and Rice 2006). Our result is particularly interesting, as the effect is mainly male driven, due to the absence of true differences between female treatment groups, beyond the CHC manipulation (i.e., all females were actual virgins, and so it is unlikely that females would be differentially influencing time-to-first-mating between PM and V groups). Given its clear agreement with the aforementioned ejaculate competition predictions, this precopulatory result confirms that our CHC manipulation to produce PM and V females was effective in deceiving experimental males.

However, at the postcopulatory stage, our results do not necessarily suggest a cryptic male preference for virgins; rather, they may indicate alternate ejaculate strategies (toward PM and V females) to deal with mechanisms of sperm competition peculiar to a given female’s mating history. While males mated to true virgin females in our experiment outperformed males that mated to pseudomated females in major components of sperm competition defence, it is important to note that all experimental females were actually virgins. Thus, the ejaculate allocation to true virgin females presumably matched the physiology of the females receiving the ejaculate, and so in a sense, it is not surprising that males from this group outperformed males mated to pseudomated females (which we assume, based on differential male behaviour pre- and post-copulation toward V and PM females, received ejaculates that were tailored to mated females, and thus may have been physiologically mismatched with respect to pseudomated females’ actual virgin status). This was not a problem with our experimental design; rather, having all females as actual virgins while males perceive them to be either virgin or mated allowed us to make inferences on the mechanisms underlying the effects of male ejaculate allocation without any confounding effects of competitor ejaculates in the female reproductive tract (and thus affecting her physiology). Specifically, recent developments in the
literature on sperm (Lupold et al. 2011) and seminal fluid protein (Wigby et al. 2009, Sirot et al. 2011) allocation in *D. melanogaster*, and mechanisms of sperm use within females after ejaculation (Manier et al. 2010), offer some insight into our sperm competition defence fitness results. First, while male *D. melanogaster* allocate sperm in higher quantities to mated than to virgin females (Lupold et al. 2011), this is most likely a mechanism to facilitate last-male sperm precedence (Gilchrist and Partridge 2000, Manier et al. 2010) using excess numbers of sperm to flush competitor sperm from female reproductive tracts (wherein the size of the second male’s ejaculate is correlated with the amount of resident sperm displaced from storage; Manier et al. 2010), as the numbers of sperm transferred to both virgins and mated females (means from Lupold et al. 2011: ~729 sperm to virgin females; ~836 to mated) are much greater than is necessary to fill female sperm storage organs (wild-type females store a maximum of ~400 sperm in their seminal receptacle and 130 in the paired spermathecae; Manier et al. 2010). Hence, the first-mating differences in offspring production prior to remating between true virgin and pseudomated females in the present study are likely not due to differences in allocated sperm, as each group of females should have received saturating numbers of sperm in their experimental matings (Manier et al. 2010). Rather, interpreting our data in the context of these recent publications, increased offspring production with virgins during the refractory period may be the result of prudent allocation of specific seminal fluid proteins based on their efficacy in virgin and mated females (Sirot et al. 2011). Namely, ovulin is a key SFP responsible for activating oogenesis in virgin females (Chapman et al. 2001), but extra ovulin allocated to previously mated females has no effect on offspring production (Sirot et al. 2011). Concomitant with this, male *D. melanogaster* transfer less ovulin to mated than to virgin females, presumably as a means of conserving reproductive resources (Sirot et al. 2011). In the present study, if males allocated a reduced volume of ovulin to pseudomated females than to true virgin females, the reduced ovulin allocation to pseudomated females could compromise males’ ability to produce offspring with them, given their actual virgin status, thus resulting in reduced offspring production prior to female remating. Increased refractory period offspring production for males mated to V females cannot be attributed to differences in refractory period length between V and PM females, which is driven by sex peptide allocation (=SFP responsible for both maintaining oogenesis at a high level, and inhibition of female sexual receptivity; Chapman et al. 2003), because refractory period duration was not significantly different between PM and V females. Hence, differences in offspring production must be caused by differences in post-first-mating PM and V laying rates, which corresponds to ovulin allocation (Chapman et al. 2001).
The lack of effect of perceived female mating status on time to remating (i.e., refractory period) likewise corresponds well with SFP allocation results from Sirot et al. (2011), wherein there was no difference in the amount of sex peptide transferred to virgin and mated females, as males benefit from inhibition of female remating in both cases.

The interaction between mating duration and perceived mating status that affected the number of offspring produced by the experimental male prior to remating of the experimental female suggests that males are using time in copula to transfer SFPs to females differently based on female mating status, as mating duration is proportional to seminal fluid transfer (but not sperm, as it is in other insects; Simmons 2001) in *D. melanogaster* (Gilchrist and Partridge 2000, Sirot et al. 2011). While ovulin transfer to female *D. melanogaster* is proportional to mating duration (Sirot et al. 2011), in the present study there were no differences in mating duration between V and PM males for either experimental or secondary matings. Thus, it remains unclear why PM refractory period offspring production increases with mating duration while V offspring production does not.

Higher offspring production prior to experimental female remating by males mated to true virgin females than those mated to pseudomated females could likewise demonstrate outright postcopulatory male preference for virgin females, parallel to the aforementioned precopulatory male preference (i.e., virgins preferred by males for decreased risk of ejaculate competition, and so V females were allocated more ejaculate than PM females) (e.g., Parker 1998, Bonduriansky 2001, Wedell et al. 2002, Edward and Chapman 2011). However, this explicit postcopulatory preference for virgins *per se* seems a less likely explanation for our data than the aforementioned maximization of male fitness through differential allocation of seminal fluid proteins based on female mating status (Sirot et al. 2011). Likewise, explicit postcopulatory preference for virgin females should have shown up in downstream fitness cost measurements (see discussion below), and we should have seen differences between PM and V females in refractory period if V females received a relatively larger allocation of sperm+SFPs (which would include the receptivity-inhibiting sex peptide), and there was no such relationship (in fact, though nonsignificant, the trend was in the opposite direction to this; Fig. 5.3).

Collectively with results from offspring production during the refractory period, results from our P1 assay suggest that differences in sperm competition defence between males mated to PM and V females lie mainly in offspring production prior to remating. When all offspring produced by
focal males (before and after remating by focal female, and including females that did not remate) were included in $PI_{\text{net defence}}$ analysis (giving a summary sperm competition defence measure; termed “Net Defence” by Friberg 2006), V males sired a significantly greater proportion of experimental female offspring (when each was under sperm competition with standard competitor males) than did PM males; however, the disappearance of this effect when only remated females were included in analysis (i.e., $PI_{\text{net remated}}$), and likewise when only offspring emanating from direct sperm competition were included (i.e., $PI_{\text{direct}}$), together suggest that two factors were important in determining success in sperm competition defence of males allocating ejaculate according to perceived female mating status: i) offspring produced during the refractory period; and ii) offspring produced by females that did not remate during the time limit imposed by our experimental design (i.e., males that induced a refractory period at least as long as the duration of the experiment). The $PI_{\text{net defence}}$ results likewise collectively suggest that the greater total focal male offspring production from the first mating (summed across experiment) by males mated to V females relative to males mated to PM females is driven by factors (i) and (ii).

In an experiment similar to the experimental mating (i.e., mating no. 1) portion of our study, Friberg (2006) found significant effects of perceived mating status on many similar measures of ejaculate competition defence used here (e.g., total focal male offspring production, net defence), with one remarkable difference: all comparable effects were in the opposite direction to those reported here, whereby males mated to PM females (termed “experimental females” by Friberg 2006) outperformed males mated to V females (termed “control females” by Friberg 2006) in several key measures of ejaculate competition defence. Other than general differences between our experimental fly populations, it is difficult to fathom why our results are opposite. It is notable that Friberg’s (2006) experimental flies copulated for longer than control flies, consistent with the pattern shown in his sperm competition measurements. Friberg (2006) associates differences in copulation duration (and its effect on ejaculate delivery; Gilchrist and Partridge 2000) with differences in sperm competition defence success between males mated to experimental and control females, on the premise that males increased their copulation duration with females they perceived as mated (and thus a high sperm competition risk; Parker 1998), thereby increasing their copulatory investment quantitatively (e.g., increased seminal fluid transfer) to enhance fertilization success. In contrast, in the present study there were no differences in copulation duration between PM and V treatment groups for the experimental
mating (or any of the secondary matings), and, with the exception of a treatment-by-mating duration interaction for offspring produced in the refractory period (discussed above), there were no effects of mating duration on the outcome of sperm competition defence. It is worth noting that results from the literature are ambiguous with respect to the effect of female mating status on copulation duration in *D. melanogaster* (e.g., Singh and Singh 2004, Friberg 2006, Lupold et al. 2011, Sirot et al. 2011). While there are similarities between the present study and Friberg (2006), there are several structural differences of note that are important in the inferences we can draw from our data. First, Friberg (2006) examined the fitness impacts of perceived mating status on sperm competition defence and female fitness within the focal mating only (i.e., there were no measures of downstream impacts of focal mating ejaculate allocations). Second, his design did not permit a measurement of the refractory period, and thus nor did it permit a measure of offspring production within the refractory period.

To ask whether there was a differential cost associated with ejaculate allocation to either PM or V females, we ran a series of three repeated measures secondary matings for each experimental male that competed in an experimental mating (thus, each male had four matings in total; see Methods) on the premise that increased relative ejaculate investment by either PM or V males in the first mating would translate into fitness costs (due to ejaculate depletion) in offspring production from downstream matings. Four matings should have been sufficient to impose the requisite allocation constraints (via depletion of accessory gland reserves; Lefevre and Jonsson 1962, Linklater et al. 2007) to detect any differences in focal mating ejaculate investment between PM and V males. However, we did not detect any differences between V and PM males in downstream costs of ejaculate allocation from the first mating, which would have manifest as a treatment-by-mating-number interaction (indicative of a differential change in offspring production across secondary females between PM and V males), or as a treatment-by-time interaction (indicative of a differential change in offspring production across experiment days between PM and V males). This may suggest that there is no cost differential between the ejaculate strategy for perceived virgin and that for perceived mated females; that is, the composition of sperm and seminal fluid may be differently allocated between PM and V females as alternate male ejaculate strategies to maximize fitness in accordance with the female mating status (i.e., potential level of ejaculate competition), but the overall cost of the summed ejaculate components is identical, and so we do not detect a trade-off in downstream fitness for males mating to females of different perceived mating status. However, it is difficult to conclude
definitively that this is the case, as offspring production increased significantly across experiment days 2-4 (suggesting that females were not in a state of ejaculate limitation), and, while there was a downward trend in offspring production with increasing secondary female number (i.e., mating order position), the decrease was not significant, which suggests (surprisingly; Lefevre and Jonsson 1962, Linklater et al. 2007) that four successive matings may not have been sufficiently physiologically stressful to elicit a reduction in male offspring production potential. Interestingly, mating duration showed a significant drop between the experimental mating and all secondary matings (which were not significantly different from each other in duration), which may indicate ejaculate stress on the part of the experimental male (through reduced SFP allocation; Gilchrist and Partridge 2000, Sirot et al. 2011); however, this did not translate into lost downstream fitness.

Several limitations with our experimental design are worthy of discussion. The first was discussed above in some detail: downstream mating costs imposed by the current design may not have been sufficient to render measurable differences in downstream fitness between PM and V males. However, our design was modeled on past experimental regimes of successive matings that should have rendered males ejaculate-depleted following 3-4 matings (Lefevre and Jonsson 1962). A second limitation of our current design is that we can assess “misallocation” of ejaculate costs only in one direction (i.e., male allocation to virgin female perceived as mated). Assessing fitness impacts of ejaculate allocation to true mated females perceived as virgins would be difficult (though conceivably possible; Friberg 2006), because effects of prior-mated male’s ejaculate would be confounded with experimental male ejaculate effects on fitness. Finally, the aforementioned error in first-day food production limits our ability to comment on offspring production across mating days, though this problem was dealt with statistically (see above).

It has thus far been investigated how the risk of sperm competition (Wigby et al. 2009) and female mating status (Lupold et al. 2011, Sirot et al. 2011) affect sperm (Lupold et al. 2011) and SFP (Sirot et al. 2011) allocation in *D. melanogaster*. It would be interesting in future studies to combine the methodology from these experiments with that of Friberg (2006) and the current study to investigate the immediate and downstream fitness effects of ejaculate allocation according to female mating status, while quantifying sperm and SFP transfer to females of different perceived mating status. This would be a considerable undertaking logistically, but
empirically fruitful in potential. Likewise, it would be useful to look at the condition dependence (Rowe and Houle 1996) of cryptic male choice, as condition dependent cryptic male choice (e.g., high condition males choosy; low condition males not) could lead to assortative fertilization whereby high condition males deliver better ejaculates to preferred females while low condition males do not differentially allocate (akin to assortative mating in precopulatory male choice, for which there is evidence; Bonduriansky 2001, Edward and Chapman 2011). Furthermore, if cryptic male choice is condition dependent, it could serve as a mechanism of sexual selection to reinforce other processes of selection against deleterious alleles (Whitlock and Agrawal 2009).

5.4.1 Conclusions

Our measure of precopulatory male mate choice suggests that male D. melanogaster show preference for true virgin over perceived mated females, which matches predictions from sperm competition theory, and agrees with results from the literature. Sperm competition defence performance from the experimental mating was collectively higher for males mated to true virgin rather than to pseudomated females, and fitness results were in agreement with recently published data on seminal fluid protein allocation (Sirot et al. 2011). Finally, increased ejaculate competition defence performance with true virgin females came without a downstream effect on male fitness, suggesting that costs to males of producing ejaculates for females of different mating status is approximately equal. This suggests that males are adjusting the composition of their ejaculate to maximize fitness according to perceived sperm competition risk, without adjusting the overall cost.
Figure 5.1. Schematic of experimental design for male mate choice experiments. a) Experimental females (♀EXP) were created by allowing brown-eyed virgin females to interact with relatively high numbers of either previously mated (to produce pseudomated group) or virgin (to produce true virgin group) wild-type “CHC Transfer Females” (♀CHC; red eyes) in close proximity for 48 h. These females interacted in a truncated vial space (facilitated by pushing vial plug down into vial; not shown) to increase the potential for physical contact, and thus CHC transfer. b) Experimental Females (brown eyes/circles/arrows) were then mated monogamously to wild-type experimental males (♂EXP; red eyes/circles/arrows). After termination of this mating, the experimental female was transferred to a (re)mating vial containing two virgin standard competitor males (♂COMP; brown eyes). The experimental male was transferred to a (re)mating vial containing a secondary virgin female (♀2°; brown eyes), and after this mating he was transferred in like fashion to two further secondary mating vials, each containing a new virgin female (∴ total of four matings per ♂EXP: 1 experimental+3 secondary). Experimental males were discarded after termination of the 3rd secondary mating, while experimental females remained with competitor males for the duration of the experiment. Experimental females (and competitor males) and mated secondary females were transferred daily for four days to fresh laying medium, and fitness components were assayed from these vials (see schematic and Methods). Vials are displayed in blue, capped with a white plug, and contain brown food (on which sits a single beige pellet of live yeast).
CHC Transfer Females (red eyes)

Interaction Ratio: $60 \frac{\text{CHC}}{\text{EXP}} : 9 \frac{\text{EXP}}{\text{EXP}}$

(Displayed: $20 \frac{\text{CHC}}{\text{EXP}} : 3 \frac{\text{EXP}}{\text{EXP}}$)

Experimental Females (brown eyes)
b) Secondary Matings (x3)
Each mating = ♂️_{EXP} × ♀️_{2⁴}
(Males red eyes, female brown)

Experimental Mating
1♂️_{EXP} × 1♀️_{EXP}
(Males red eyes, females brown)

Downstream Fitness Costs
- Secondary female offspring production

Experimental Mating Fitness Investment
- Precopulatory male choice
- Refractory period induction
- Focal Offspring production
- P1 success

Experimental Female Remating
2♂️_{COMP} × ♀️_{EXP}
(Males and females brown eyes)
Figure 5.2. Effect of perceived female mating status on time to first mating for experimental males. Males were ~21% faster to mate with true virgins than pseudomated females when all males starting the experiment were included in analysis (Means[SE]: PM=99.6 min[10.7]; V=78.9 min [9.43]; $X^2 = 4.77$, $p=2.90 \times 10^{-2}$). True virgin matings are depicted in blue; pseudomated matings in red. Data presented include censored data (i.e., flies that did not mate).
Figure 5.3. Effect of perceived female mating status on time to remating (refractory period). Pseudomated females are represented by the red line, and true virgin females by the blue line. There was no effect of perceived mating status on time-to-remating ($X^2 = 0.839, p = 0.360$). Data presented include censored data (i.e., flies that did not remate).
Figure 5.4. Effect of perceived female mating status (PM=pseudomated; V=true virgin) on offspring production prior to experimental female remating, when controlling for refractory period, mating duration, and their interaction term. Males mating to true virgin females were ~28% more successful at offspring production during the refractory period than males mated to pseudomated females ($t_{1.85}=2.61, p=1.08 \times 10^{-2}$). a) Values are plotted as adjusted means ± CI95, each back-transformed from log (in GLM analysis) to original probability scale. b) There was a significant interaction between mating duration and perceived female mating status in their effect on offspring production prior to remating ($t_{1.85}=2.07, p=4.12 \times 10^{-2}$; PM=solid black line; V=red dashed line).
Figure 5.5. Effect of perceived female mating status on three versions of P1 Total (=ejaculate competition defence), when controlling for refractory period (PM=pseudomated; V=true virgin). a) Males mating to true virgin females were ~31% more successful in $P1^{net \ defence}$ than males mated to pseudomated females, when in contest against a standard ejaculate competitor and all focal male offspring were included in analysis (including those from prior to female remating, and for females that did not remate; $t_{1.83}=2.06, p=4.26\times10^{-2}$); b) when P1 Total analysis was restricted to $P1^{net \ remated}$ (included offspring only from females that remated, but still included their offspring produced prior to remating), all effects disappeared, though the trend direction remained for perceived mating status ($t_{1.67}=1.75, p=8.42\times10^{-2}$); c) when P1 total analysis was restricted to $P1^{direct}$ (included offspring from direct sperm competition only), there was no effect of perceived mating status on P1 total ($t_{1.68}=1.33, p=0.190$). Values are reported as adjusted means ± CI95, each back-transformed from logits (in GLM analysis) to original probability scale.
Figure 5.6. Total focal male offspring production from experimental and secondary matings: a) Focal males mated to true virgin (V) females produced ~28% more offspring (summed across all experimental days) than males mated to pseudomated (PM) females in experimental matings (includes focal offspring produced prior to, and after, female remating; $t_{1,84}=2.03$, $p=4.61 \times 10^{-2}$; presented as marginal means±CI95 back-transformed from log scale in quasipoisson analysis to original scale); b) perceived mating status did not affect the total number of offspring produced (summed across all experimental days) by focal males with secondary females ($t_{1,77}=2.11 \times 10^{-3}$, $p=0.998$; presented as means ±SE).
Figure 5.7. Daily secondary female offspring production (=females from matings 2-4; presented as LS mean ±SE). a) There was no effect of perceived mating status (V=true virgin; PM=pseudomated) on daily secondary female offspring production ($F_{1,69}=0.291, p=0.641$), nor were there any significant main or higher-order effects with the exception of time (see below; female symbols: $♀_{2^0-2}=$solid triangles; $♀_{2^0-3}=$ “x”; $♀_{2^0-4}=$open diamonds; data presented as marginal means ± SE); b) There was a significant effect of time on daily secondary female offspring production ($F_{3,207}=38.9, p<0.0001$), and a Tukey’s HSD test revealed that offspring production on days 1-2 was identical, followed by significant increases between days 2-3, and days 3-4.
Figure 5.8. Offspring production summed across secondary females (=females from matings 2-4) for each of experiment days 1-4 (presented as LS mean ±SE). Perceived female mating status had no effect on daily offspring production summed across secondary females (\(F_{1,77}=0, p=0.998\)); however, there was a significant effect of time (\(F_{2.49,191}=43.8, p<1.00\times10^{-4}\)). A Tukey’s HSD test revealed that secondary female offspring production on days 1 and 2 were identical, followed by significant increases daily between days 2-4. a) Data are separated into pseudomated (open squares) and true virgin (solid circles) treatment groups; b) Data are pooled across treatment groups to show general effect of time. Results remained qualitatively identical when day 1 data were excluded from analysis (see Methods). All results reported in this figure are univariate repeated measures, corrected with Huynh-Feldt correction (as both Greenhouse-Geisser and Huynh-Feldt epsilon estimates were >0.75; Quinn and Keough 2002); however, I ran an unadjusted univariate repeated measures analysis (=partly nested ANOVA; Quinn & Keough 2002) to calculate Tukey’s HSD differences among within-subjects factor levels (i.e., different days of experiment), and to produce means with an estimate of error for this figure.
Figure 5.9. Daily focal male offspring production (= least square mean no. offspring ± SE produced by focal male summed across all four females mated for each of experiment days 1-4). There was a significant effect of time on daily focal male offspring production ($F_{2.55,176}=37.7$, $p<0.0001$), and a Tukey’s HSD test revealed that days 1-2 did not differ from each other, while day 3 and day 4, respectively, differed significantly from all other days. There was no effect of perceived mating status on daily focal male offspring production ($F_{1,69}=0.136$, $p=0.714$), nor was there any interaction between mating status and time ($F_{2.55,176}=2.68$, $p=0.0576$). a) Data are separated into pseudomated (open squares) and true virgin (solid circles) treatment groups; b) Data are pooled across treatment groups to show general effect of time. All results were qualitatively identical when data for experimental day 1 were excluded from analysis. All results reported in this figure are univariate repeated measures, corrected with Huynh-Feldt correction (as both Greenhouse-Geisser and Huynh-Feldt epsilon estimates were >0.75; Quinn and Keough 2002); however, I ran an unadjusted univariate repeated measures analysis (=partly nested ANOVA; Quinn & Keough 2002) to calculate Tukey’s HSD differences among within-subjects factor levels (i.e., different days of experiment), and to produce marginal means with an estimate of error for this figure.
Figure 5.10. Total offspring production (summed across four days of experiment; presented as LS mean ±SE) for each of four females mated to the experimental male (NB: offspring production for first female includes both offspring from focal male, and from standard competitor males). Perceived female mating status had no effect on offspring production across the four female types ($F_{1,69}=0.618, p=0.435$). However, there was a significant effect of mating order (shown as “Female Number” in figure) on total per female offspring production ($F_{3,207}=3.34, p=2.02\times10^{-2}$). A Tukey’s HSD test revealed that Female 1 produced significantly fewer offspring relative to Female 2 only, but that Females 2-4 did not differ statistically in number of offspring produced. a) Data are separated into pseudomated (open squares) and true virgin (solid circles) treatment groups; b) Data are pooled across treatment groups to show general effect of mating order. **NOTE:** when Day 1 offspring production data were excluded from analysis (see Methods), the effect of mating order on offspring production disappears ($F_{3,207}=0.680, p=0.565$), and Females 1-4 are not significantly different from each other in numbers of offspring produced across the experiment. All results reported in this figure are univariate unadjusted repeated measures, (as both Greenhouse-Geisser and Huynh-Feldt epsilon estimates were ~1; Quinn and Keough 2002).
Figure 5.11. Mating durations (LS mean ±SE) for each of four matings (Mating 1=experimental mating; Matings 2-4=secondary matings, ordered chronologically). Perceived female mating status had no effect on mating duration across the four female types ($F_{1,89}=3.20\times10^{-3}$, $p=0.955$). However, there was a significant effect of mating order (shown as “Mating Number” in figure) on mating duration ($F_{3,267}=26.8$, $p<1.00\times10^{-4}$). A Tukey’s HSD test revealed that mating duration for the first mating was significantly longer than for the other three; mating durations to secondary females (mating numbers 2-4) were not significantly different from each other. a) Data are separated into pseudomated (open squares) and true virgin (solid circles) treatment groups; b) Data are pooled across treatment groups to show general effect of mating order. All results reported in this figure are univariate unadjusted repeated measures, (as both Greenhouse-Geisser and Huynh-Feldt epsilon estimates were ~1; Quinn and Keough 2002).
Graph a shows the mating duration (min) for different mating numbers. The data points are represented by circles and squares with error bars indicating variability.

Graph b also illustrates mating duration (min) across mating numbers. The representation includes circles with error bars to depict the variability in the data.
6 General Conclusions

The overarching objective of my doctoral research programme was to understand the potential for sexual selection to shape nonsexual fitness (i.e., fitness outside the context of mating and fertilisation success; Andersson 1994) via its impact on deleterious mutations in an experimental population of the fruit fly model system, *Drosophila melanogaster*. This objective is grounded in condition-dependence theory, which operates under the assumption that genetic variation in male mate quality is maintained through genic capture (Rowe and Houle 1996), whereby mutations occur at many loci throughout the genome that affect condition, which in turn affects the expression of sexually selected traits as they become costly and evolve condition-dependence. Accordingly, condition-dependence theory fundamentally predicts that sexual selection will facilitate adaptation through the removal of deleterious mutations that indirectly affect the expression of sexually selected traits via condition (Rowe and Houle 1996, Lorch et al. 2003, reviewed in Whitlock and Agrawal 2009). My thesis rigorously tested this prediction.

My first two studies (Chapters 2-3) complemented each other in focusing on the impact of different stages of postcopulatory sexual selection on several forms of deleterious mutations. My third study (Chapter 4) built on proceedings from the first two, and investigated the relative contribution of pre- and postcopulatory processes to selection against spontaneously accumulated deleterious mutations. In my fourth study (Chapter 5), I deviated slightly from the basic motivation of earlier chapters, and examined male mate choice for female mating status at the pre- and postcopulatory level.

6.1 Major Findings

6.1.1 Sexual selection and deleterious mutations

The most salient conclusion emanating from my dissertation research is that sexual selection is a force of consequence against deleterious mutations. In all three of my studies investigating the impact of sexual selection on genetic quality (Chapters 2-4), there were significant effects of
sexual selection against deleterious mutations for precopulatory mate choice (Chapters 2,4), ejaculate competition defence (Chapter 3), and ejaculate competition offence (Chapter 4). These results collectively contribute to a growing body of empirical literature supporting the idea that sexual selection can enhance nonsexual fitness (Partridge 1980, Promislow et al. 1998, Fricke and Arnqvist 2007) (but see Holland 2002, Radwan et al. 2004, Rundle et al. 2006) via the removal of unconditionally deleterious mutations (Whitlock and Bourguet 2000, Radwan 2004, Sharp and Agrawal 2008, Hollis et al. 2009, but see Hollis and Houle 2011), and are likewise in agreement with theory predicting benefits to mean fitness via removal of deleterious mutations by sexual selection (reviewed in Whitlock and Agrawal 2009).

The second general conclusion germane to my dissertation is that, while it is clear that sexual selection is effective in removing deleterious mutations from my experimental populations of *D. melanogaster*, it is likewise evident that the type of deleterious variation matters for how sexual selection affects it. I used three different forms of deleterious mutations for my dissertation. For example, in Chapter 2, I used dominant deleterious marker mutations to test whether sperm competition offence (last male sperm precedence, or *P2*, a trait highly important to male *D. melanogaster* fitness; Boorman and Parker 1976, Harshman and Clark 1998, Morrow et al. 2005) was effective in selecting against deleterious mutations, whereas I performed a similar assay in Chapter 4 using spontaneously accumulated deleterious mutations, and found conflicting results (null effect of last male sperm precedence on dominant mutations; predicted negative effect on spontaneous mutations). Likewise, there were discrepancies between the results emanating from the deleterious variance exposed through inbreeding (Chapter 3) and those from the spontaneously accumulated mutations (Chapter 4), wherein sperm competition defence (*P1*) showed a strong effect of inbreeding depression, but no perceptible effect for spontaneous mutations. There is general precedence for these discrepancies within the literature investigating the impacts of sexual selection *in toto* on deleterious mutations. Using an elegant design, Hollis et al. (2009) demonstrated that sexual selection was effective in accelerating the elimination of the deleterious allele alcohol dehydrogenase (*Adh-*) from an experimentally evolving population of *D. melanogaster*; however, in a later study the same base population of flies was mutagenized with ethyl methanesulphonate (EMS) and then allowed to evolve under conditions of experimentally imposed regimes of sustained (polygamy) or relaxed (monogamy) sexual selection for 60 generations, yet showed no benefits of sexual selection in purging the
artificially inflated mutation load, and, contrastingly, showed a net fitness cost to sexual selection (Hollis and Houle 2011). In another example, Radwan (2004) demonstrated a net fitness benefit to sexual selection in bulb mites (Rhizoglyphys robini) mutagenized using ionizing radiation, whereas Radwan et al. (2004) detected no benefit to sexual selection in preventing fitness decline under relaxed natural selection in the same (nonmutagenized) bulb mite population. Thus, taken together with these results from the literature, my thesis results suggest that the efficacy of sexual selection in eliminating deleterious mutations is dependent upon the nature of the deleterious variance under selection.

A third result of general importance proceeding from my thesis is that environmental manipulations of condition (used as a proxy for genetic manipulations of condition for many studies of indirect benefits of sexual selection; see Cotton et al. 2004, Whitlock and Agrawal 2009) do not necessarily produce parallel results to genetic manipulations (using deleterious mutations) of condition. This is potentially problematic because good genes selection is predicated on the idea that sexual selection improves nonsexual fitness by selecting on male genetic quality (reflecting deleterious mutations) as indicated by male condition, or by condition dependent traits (Rowe and Houle 1996). By this logic, mutant and low-condition treatment groups should produce qualitatively similar results (Tomkins et al. 2004, Whitlock and Agrawal 2009); however, in Chapters 2-3, I manipulated environmental condition alongside genetic quality, and in both cases mutational (genetic) manipulations did not produce fitness results that mirrored those for nutritional (environmental) manipulations. In fact, in nearly every case when genetic quality manipulations produced a significant (and predicted) effect, the condition manipulation produced a null effect (and vice versa; exception: for Chapter 3, there were parallel and predicted results for genetic and environmental manipulations of condition for P1 assays). Furthermore, in Chapter 4, though condition was not experimentally manipulated, male body size was measured as an indicator of condition (a useful index thereof; Whitlock and Agrawal 2009); however, the assays for which there were significant effects of mutation accumulation on fitness demonstrated no mutational effect on body size. These disconnects between genetic and environmental effects on phenotypic condition have potentially important consequences, as many studies of indirect benefits of sexual selection manipulate environmental condition (e.g., via diet adjustments), and assume that this manipulation parallels the effects of deleterious mutations on condition, but do not confirm this assumption (see Cotton et al. 2004, Whitlock and Agrawal 2009). An empirical disconnect between environmental and genetic
quality may suggest that sexual selection is acting on deleterious mutations directly (via a pathway not involving condition), or that the aspects of phenotypic condition that I manipulated environmentally were different from those that were affected by deleterious mutations. Alternatively, Bonduriansky et al. (2015) recently suggested that differential effects of environmental and genetic quality may reflect traits whose expression is subject to multiple forms of condition dependence. Bonduriansky et al. (2015) directly assayed the relative effects of genetic and environmental quality on trait expression in *D. melanogaster*, and demonstrated an interesting disconnect between the two: while two fitness-related traits (female body size and male wing length), and most morphological traits, responded in the same direction to genetic and environmental manipulations of condition (as predicted by condition dependence theory; e.g., Rowe and Houle 1996), there were likewise sexually selected traits important to fitness that did not (e.g., male sex combs, cuticular hydrocarbons). My research from Chapters 2-3 complements these results.

Perhaps the most intriguing set of results yielded by my doctoral research suggest that, in addition to precopulatory mate choice (which is nearly exclusively the focus of good genes models of selection; e.g., Houle and Kondrashov 2002, Lorch et al. 2003) (but see Yasui 1997), postcopulatory sexual selection is a powerful force in eliminating deleterious mutations. To the best of my knowledge, at the time of commencement of my doctoral research programme, all published empirical results on the efficacy of sexual selection in bolstering nonsexual fitness focused on sexual selection *in toto* (e.g., Radwan 2004, Fricke and Arnqvist 2007, Hollis et al. 2009). Consequently, there was a dearth of empirical data attempting to parse the relative contributions of the different stages of inter- and intrasexual selection to reducing mutation load, or enhancing nonsexual fitness (data which are empirically valuable; Whitlock and Agrawal 2009). Thus, I addressed this literature gap using a rigorous experimental design (Chapter 4) to assay the relative contribution of pre- and postcopulatory sexual selection components to eliminating spontaneously accumulated deleterious mutations. In addressing this novel question, I found that sexual selection contests occurring both pre- and post-copulation were effective in selecting against deleterious mutations: as predicted, mutant males achieved lower mating success and reduced last-male sperm precedence (*P*2) relative to control males. To my knowledge, there is only a single study (Mallet et al. 2012; published after completion of my experiments) that has used a design complementary to mine to decompose sexual selection into its intrinsic components pre- and post-copulation to understand the relative importance of each
in selecting against deleterious mutations. My results are generally complementary to those of Mallet et al. (2012): in both studies, mutant males showed reduced mating and $P_2$ success (my results differed for $P_1$ success; however, both MA and Control males produced unusually low numbers of offspring for this component of my study; see Chapter 4 Discussion). I likewise measured selection against deleterious mutations for each component of sexual selection assayed (a previously unaddressed problem at the time of experimentation), and produced one of the most stimulating results of my thesis: precopulatory mating success was the primary driver of selection against deleterious mutations, and was ~3.4 times stronger than postcopulatory selection through sperm competition offence. My selection results generally agreed with those of Mallet et al. (2012), wherein there was significant selection against mutant males for mating success, $P_1$, and $P_2$ ($P_1$ did not demonstrate significant selection estimates in my experiment). While precopulatory selection was stronger than sperm competition offence in my experiments, both processes were similar in selection strength for Mallet et al. (2012). Furthermore, $P_1$ success showed strongest selection against deleterious mutations for Mallet et al. (2012). My novel results from this experiment (collectively with Mallet et al. 2012) provide the empirical foundations toward a solution for a hitherto unanswered problem in sexual selection research: pre- and postcopulatory processes are each important forces of selection against deleterious mutations in *D. melanogaster*. Furthermore, my results contributed to answering a broader question that has received little empirical attention in evolutionary biology: which is more important to total sexually selected fitness, pre- or postcopulatory selection? With respect to deleterious mutations, my results suggest that precopulatory processes are more important. However, recent results directly addressing the broader question suggest that the contribution of pre- and postcopulatory processes to total male sexually selected fitness in *D. melanogaster* is approximately equal (Pischedda and Rice 2012).

6.1.2 Male mate choice in *D. melanogaster*

My thesis demonstrated clear pre- and postcopulatory male mate choice (Chapter 5) in *D. melanogaster*, which contributes generally to a growing body of literature demonstrating male choice in sexual selection (Bonduriansky 2001, Edward and Chapman 2011). I showed a clear precopulatory male preference for virgin females, which matches theoretical predictions for ejaculate allocation toward low risk of sperm competition (Parker 1998, Edward and Chapman
Males were also cryptically choosy, showing differential performance in major components of ejaculate competition defence with true virgins and with virgins perceived as mated (incidentally, my results were reciprocally opposite to a recent study of similar design; Friberg 2006). This difference in sperm competition defence performance did not translate into a difference in male offspring production with successive mates, which suggests that, while males adjust their ejaculate to accommodate perceived mating status, there may not be a meaningful difference in cost between the strategies.

6.2 Implications and Contributions

6.2.1 Sexual selection and deleterious mutations

What is the predominant effect of sexual selection on deleterious mutations in *D. melanogaster*? Taken together, results from my thesis (Chapters 2-4) corroborate the idea that sexual selection can be a powerful and effective force in selecting against mutations deleterious to nonsexual fitness. Furthermore, my thesis contributes novel and rigorous support for the idea that sexual selection against deleterious mutations can occur at multiple stages of selection, not simply at the level of precopulatory mate choice for morphological sex traits, which is the focus of traditional theoretical and empirical attention (reviewed in Andersson 1994, Moller and Alatalo 1999, Arnaqvist and Rowe 2005). Indeed, the evidence I have presented herein indicates that the effectiveness of the sexual selection process in eliminating deleterious mutations varies with the stage of selection in question, and with the form of mutational variance.

For much of the history of sexual selection research, the field generally has been focused on the evolutionary causes of sexual selection (e.g., the Fisher process, good genes, sexual conflict, etc.), with particular emphasis on the evolution of female mate choice for showy male morphological traits (reviewed in Andersson 1994, Arnaqvist and Rowe 2005). Thanks to a seminal review by Parker (1970), the conceptual framework for the evolution of sexual selection expanded to include processes occurring after the termination of copulation (e.g., ejaculate competition, cryptic male choice, cryptic female choice, etc.; reviewed in Eberhard 1996, Birkhead and Moller 1998, Bonduriansky 2001, Simmons 2001, Edward and Chapman 2011). Within this body of work, condition dependence of secondary sex traits and mating success was shown to be taxonomically widespread (reviewed in Andersson 1994, Cotton et al. 2004), and
led to the pivotal idea that sexual selection could improve nonsexual fitness by removing mutations deleterious to condition (i.e., selection for good genes) via selection on condition dependent sexual traits (Rowe and Houle 1996, Lorch et al. 2003). Furthermore, Rowe and Houle’s (1996) seminal model predicted that good genes selection should become relevant to the expression of traits under sexual selection whenever those traits evolve condition dependence, and regardless of the mechanisms by which sexual selection for those traits arises. That is, while female preference can evolve by multiple mechanisms (e.g., Fisher process, good genes, sexual conflict), good genes selection should operate in the latter stages of coevolution of trait and preference for each of these mechanisms (Rowe and Houle 1996).

By demonstrating that all mechanisms potentially underlying the evolution of sexual selection can eventually lead to selection for good genes, Rowe and Houle (1996) provided the conceptual groundwork for studying the evolutionary consequences of sexual selection. That is, if healthy males have relatively high mating success, can sexual selection impact mutation load? Theory underlying this problem led to two key empirical questions: i) can sexual selection effectively select against unconditionally deleterious mutations?; and ii) is selection stronger in males than in females (a requirement for [i]). The latter question has received limited empirical attention (e.g., Pischedda and Chippindale 2005, Sharp and Agrawal 2008, Mallet et al. 2011a, Mallet and Chippindale 2011), but is outside the scope of this thesis. Empirical work on the former question (e.g., Partridge 1980, Promislow et al. 1998, Holland 2002, Rundle et al. 2006, Fricke and Arnqvist 2007) has produced mixed results. Furthermore, at the time of commencement of my dissertation research, there was a paucity of data on the relative impacts of sexual selection on deleterious mutations at the different stages of selection, and on the relative fitness effects of genetic and environmental quality (Whitlock and Agrawal 2009). My research from Chapters 2-4 makes novel contributions to filling these identified gaps in the literature, and more generally to the research framework probing the ultimate consequences of sexual selection to fitness.

6.2.2 Sexual selection and condition dependence

Environmental manipulations of condition (e.g., imposing low juvenile diet quality) can be used as a proxy for deleterious mutations, such that individuals subject to low condition treatments simulate those carrying deleterious alleles that impede the acquisition of resources, and/or
converting them into fitness (Whitlock and Agrawal 2009). It has been well established in the sexual selection literature that morphological sexual traits subject to female choice are condition dependent (e.g., Cotton et al. 2004). However, at commencement of my doctoral research it was relatively unclear how condition affected other components of male mating success (e.g., mate search time, female encounter rate, courtship intensity, sperm competition, etc; Whitlock and Agrawal 2009). My thesis contributes to filling this literature gap, providing data on the condition dependence of components of sexual selection outside secondary sex traits subject to female choice (Chapters 2-3). My thesis likewise contributes in small part to a body of literature for which there are even fewer data: female condition dependence (Chapter 4).

6.3 Directions for Future Research

6.3.1 Sexual selection and mutation load

I have devoted the majority of my thesis to decomposing the relatively well-studied effects of sexual selection in toto on mutation load (reviewed in Whitlock and Agrawal 2009) into the relatively little-studied effects of major components of sexual selection on deleterious mutations. The next logical step is to go a degree further in examination to establish the specific mechanisms by which sexual selection removes deleterious mutations. While I have speculated on likely mechanisms for mutation removal throughout my thesis chapters, direct testing would provide valuable insight into how sexual selection affects mutation load. D. melanogaster is an ideal system to employ such a research programme, given the extremely well-studied targets of sexual selection in this system. A potentially fruitful starting point is an experiment testing the effects of mutation accumulation on the expression of male cuticular hydrocarbons (Rybak et al. 2002) and wing shape/song (Rybak et al. 2002, Menezes et al. 2013), and the corresponding effects on mating success. At the postcopulatory level, ejaculate size (Manier et al. 2010), sperm size (Pattarini et al. 2006), and accessory gland protein production (Wolfner 1997, Chapman 2001, Wolfner 2002) are likewise known targets of sexual selection, and so would also be useful starting points.

It has now been reasonably well established that sexual selection generally has a negative effect on deleterious mutations (Whitlock and Agrawal 2009), and that sexual selection has either a positive or neutral (but not negative) effect on fitness during adaptation (Holland 2002, Rundle
et al. 2006, Fricke and Arnqvist 2007); however, we still lack a clear picture of precisely when sexual selection has the potential to bolster fitness during the adaptation process. That is, for a population enriched for deleterious variance (e.g., adapting to a new environment), when are the fitness benefits of sexual selection accrued? At what point does sexual selection become costly with respect to sexual conflict, and at what point do the benefits and costs balance? Does sexual selection enhance the rate of adaptation to new environments, as predicted by genic capture theory (Lorch et al. 2003)? The empirical fitness measurements that exist tend to be point measurements after many generations of experimental evolution, toward the end of the experiment, which do not allow the measurement of adaptational rate per se. An improvement on this design would be to run a baseline fitness assay at the beginning of the experiment, and then at regular intervals as the populations evolve. This would allow a rate to be measured, and the central prediction of Lorch et al. (2003) to be tested directly. These questions are logistically challenging to address, given the laborious nature of measuring fitness in experimentally evolving populations. Nonetheless, the *D. melanogaster* system is well suited for empirical testing of this problem, with well-established protocols for mutagenesis (e.g., Hollis and Houle 2011), and single deleterious mutations of known impact (e.g., *Adh*; Hollis et al. 2009) in experimental evolution studies.

The focus of empirical work investigating the impacts of sexual selection on mutation load has been almost exclusively on *D. melanogaster*, and for good reason: as justified throughout my thesis, the *D. melanogaster* system has in place the historical and logistical machinery necessary to address this problem effectively. However, to assess the general importance of sexual selection in reducing the mutation load, a broader taxonomic base for experimentation is needed. Some efforts have already been made in this vein (e.g., Radwan 2004, Radwan et al. 2004, Rundle et al. 2006, Fricke and Arnqvist 2007); however, more experimentation in other empirically-relevant systems, particularly across insects, would serve to bolster the principle that sexual selection is effective in eliminating unconditionally deleterious mutations. A good first-step in garnering cross-taxonomic support for this idea would be to run a comparative study within the *Drosophila* clade on how sexual selection impacts mean fitness in mutagenized fly populations with imposed polygamy or monogamy (after Hollis and Houle 2011). Again, this study is logistically challenging, but the results would be invaluable.
6.3.2 Male mate choice in D. melanogaster

Recent work in *D. melanogaster* cryptic male mate choice has been put forth to identify fluorescently-labeled sperm from multiple males within the female reproductive tract (Manier et al. 2010), and has been used to investigate how males allocate sperm to females according to perceived sperm competition risk (Lupold et al. 2011). Likewise, a novel method of tracking the allocation of key seminal fluid proteins (SFPs) in the male ejaculate (Sirot et al. 2009) has been used to demonstrate ejaculate adjustments of specific SFPs according to female mating status (Sirot et al. 2011) and perceived level of sperm competition (Wigby et al. 2009). A potentially rich vein of research would be to combine these methods of tracking allocated SFPs and sperm with the fitness assays of Friberg (2006) and my thesis Chapter 5, to produce a set of experiments that unambiguously tie the fitness effects of male mate choice for female mating status to the underlying mechanisms in ejaculate modulation. To the best of my knowledge, this has not yet been attempted. Cryptic mate choice for female mating status has received much recent attention (reviewed in Bonduriansky 2001, Edward and Chapman 2011), and so answering this question comprehensively would be a valuable contribution.

6.4 Summary Remarks

Taken together, my doctoral research provides novel contributions to the sexual selection literature. My work has filled important empirical gaps in resolving the effects of sexual selection on deleterious mutations and condition. I have produced results that, along with recent proceedings from the literature, support the notion that sexual selection is effective in facilitating natural selection in the elimination of deleterious mutations. Most notably, my research has contributed valuable and novel results on the relative importance of pre- and postcopulatory sexual selection to the removal of deleterious mutations, and more broadly to male sexually selected fitness. The results I produced herein serve as a foundation for multiple channels of future research, which offer much potential for building broad consensus for how and why sexual selection affects mutation load. Ultimately, my thesis suggests that sexual selection, including its postcopulatory stages, is a viable force in selecting against deleterious mutations, an idea with potentially broad consequences for evolutionary biology.
Bibliography


Cabral, L. G., and B. Holland. 2014. Courtship song does not increase the rate of adaptation to a thermally stressful environment in a *Drosophila melanogaster* laboratory population. PLoS ONE **9**.


