Sexual selection in *Drosophila simulans*

Submitted by Manmohan Dev Sharma to the University of Exeter as a thesis for the degree of Doctor of Philosophy in Biological Sciences in October 2010

I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signed: Manmohan Dev Sharma

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Abstract

Over the last 100 years sexual selection has advanced into a vast field of theoretical and empirical research. While Darwin's idea of female preference being an integral mechanism of sexual selection is no longer debated, our understanding of female preference is still very limited. For example, we know little about the genetic variation in female preference, and the costs of preference over and above the costs of mating with particular male phenotypes. Additionally, while costs of mate choice are well documented, the benefits of mate choice and their implications are still debated. For example, controversy exists over the inevitability of good gene benefits and their capability to promote adaptive sexual selection. Furthermore, the adaptiveness of sexual selection itself is debated. Our understanding of the traits involved in mate choice is also far from complete. Here I investigated aspects of sexual selection in *Drosophila simulans*, employing a range of behavioural approaches along with artificial selection and environmental manipulations. The findings presented here indicate that female preference can evolve when directly selected on, and that preference itself is not particularly costly. There was also no conclusive evidence for the good genes benefits of mate choice in *D. simulans*. These benefits are considered crucial in promoting the adaptiveness of sexual selection, and although we found sexual selection to be adaptive under some test conditions it was not adaptive in other conditions. Our investigations into traits involved in mate choice established sex-specific genetic variation in cuticular hydrocarbons and the genetic architecture of this trait was found to sex-specific evolution of cuticular hydrocarbons under natural and sexual selection. Additionally, we found that a secondary sexual character, the sex combs was positively allometric – just like most signalling and weapon traits, and there was no association between trait fluctuating asymmetry and trait size. These findings collectively indicate that sexual selection in *D. simulans* is consistent with classical models of this process.

Acknowledgements

I was once told that the road of knowledge is infinitely long, and it is impossible to traverse it without appropriate guidance. Thus first and foremost I offer my sincere gratitude to Professor David Hosken, who has guided me for the past three years, and showed me how to be a scientist. I am indebted to him for nurturing the unrelenting inquisitiveness within me, and for sharing his wealth of knowledge with me. Those of his students who came before me, have often referred to him as the (tor)mentor with benefits (and we are not talking about mate choice benefits) and the evidence collected by me seems to support this view. Equally, my sincere thanks to Professor Tom Tregenza, for providing much needed guidance and support throughout my research. I have no doubt that I have benefited hugely from the knowledge and experience of both of my supervisors and the limitless insightful support they provided.

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The views presented in this chapter are my own and were developed under the guidance of Prof. David Hosken and Prof. Tom Tregenza.

Chapter Two: Female mate preferences in *Drosophila simulans*: evolution and costs

Prof. David Hosken and Prof. Tom Tregenza provided guidance for planning and structure of all experimental procedures and in preparation of the manuscript. I collected the data, conducted the analysis and am first author on the manuscript. Laboratory assistance was provided by Jack Hollis, Rob Griffin, Connor-Benjamin Parker and Martin Yeo.

Chapter Three: No good genes in *Drosophila simulans*?

Prof. David Hosken and Prof. Tom Tregenza provided guidance for planning and carrying out experimental procedures and in preparation of the manuscript. I collaborated with Jack Hollis and Rob Griffin to collect the data. I conducted the analysis and am first author on the manuscript.

Chapter Four: The genetics of cuticular hydrocarbon profiles in *Drosophila simulans*

Prof. David Hosken, Prof. Tom Tregenza and Dr. John Hunt provided guidance for planning and structure of all experimental procedures and in preparation of the manuscript. Chris Mitchell provided technical support with gas chromatography and mass spectrometry. I collected and analysed the data, and am first author on the manuscript.

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Prof. David Hosken Dr. John Hunt provided guidance for the design and structure of all experimental procedures and in preparation of the manuscript. I collected the data, did the analysis and am first author on the manuscript.

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Prof. David Hosken and Prof. Tom Tregenza provided guidance for development and execution of all experimental procedures and in preparation of the manuscript. I did the data collection and analysis and am the first author on the manuscript.

Chapter Eight: General discussion: Sexual selection in *Drosophila simulans*

The general discussion, conclusions and future prospects presented in this chapter represent my own interpretation of the data presented in the previous chapters, under the guidance of Prof. David Hosken and Prof. Tom Tregenza.

Chapter One

Introduction

It was more than 100 years ago that Charles Darwin formulated his ideas on sexual selection in *The Descent of Man and Selection in Relation to Sex* (Darwin 1871). He noticed that animals frequently possessed exaggerated traits which appeared detrimental to their survival - such as the large and decorative train of a peacock and bright plumage colours in many other birds. He recognised that such traits, despite being non-adaptive, may actually be beneficial if they conferred an advantage in terms of increased mating success to their bearers. He said for example: *"the males have acquired their present structure, not from being better fitted to survive in the struggle for existence, but from having gained an advantage over other males, and from having transmitted this advantage to their male offspring alone. It was the importance of this distinction which led me to designate this form of selection as sexual selection"* (Darwin 1871). Whilst defining sexual selection and distinguishing it from natural selection, Darwin also identified the two essential components of sexual selection. Referring to male-male competition and female choice, he said: *''The sexual struggle is of two kinds; in the one it is between individuals of the same sex, generally the males, in order to drive away or kill their rivals……whilst in the other, the struggle is…. to excite or charm those of the opposite sex, generally the females, which no longer remain passive, but select the more agreeable partners.''* (Darwin 1871).

Although his idea regarding male-male competition was accepted without much debate, the concept of female choice was controversial from the start. Darwin's contemporaries were unwilling to accept that females could have an "aesthetic sense", let alone be capable of higher intelligence to exercise a choice based on their taste (O'Donald 1980; Dewar and Finn 1909; Maynard Smith 2000; reviewed in Gayon 2010). One of the strong arguments against Darwin's notion of female choice was an absence

of any explanation for the origin of preferences (see Morgan 1903). Fisher (1930) was the first person to suggest that "*a sexual preference of a particular kind may confer a selective advantage, and therefore become established in the species"*. He also explained the evolution of female preferences in a coherent theoretical framework – his "runaway process". The rational is that when females mate non-randomly with males bearing exaggerated traits, a genetic correlation can build up between trait and preference if both the male sexual ornament and female preference are heritable (Fisher 1930). For example, consider that males with longer than average tail lengths might have an advantage linked to agility that would help them escape predators quickly. Then also admit that females vary in their tendency to mate with males of different tail lengths. Females choosing to mate with males bearing longer than average tails produce long lived sons (survival benefit). Thus alleles coding for longer traits in males spread, and so do the alleles that make females prefer long-tailed males (Andersson 1994). Once established in a population, mating preference becomes a selective force in its own right, and with increased preference selecting for further trait exaggeration and so on, it can lead to 'runaway' (Fisher 1930). Lande in his (1981) rendition of Fisher's verbal model, demonstrated the potential for runaway selection of male traits and reiterated the importance of genetic variation in female preference and male traits for the process. A renewed interest in sexual selection followed, with several studies documenting female preference (Ryan 1983; Moore and Moore 1988; reviewed in Andersson 1994; Wilkinson and Reillo 1994; Moore and Moore 2006) and models establishing a key role of female preference in sexual selection (e.g. Lande 1981; Kirkpatrick 1982; Iwasa et al. 1991; Pomiankowski et al. 1991; reviewed in Mead and Arnold 2004; Kokko et al. 2006).

Approximately 20 quantitative genetic models of sexual selection were proposed based on Lande's (1981) framework, and most of them included the Fisherian 'runaway' process (Mead and Arnold 2004). Although costs associated with female preference are now considered unavoidable to a large extent (see Bateson 1983), some of the earliest models of mate choice assumed that mate choice was not costly (Lande 1981, 1982; Lande and Arnold 1985; Kirkpatrick et al. 1990). It was only later that models incorporated direct costs to preference and predicted that runaway was unlikely in presence of costs (e.g. Iwasa et al. 1991; Pomiankowski et al. 1991). However, in a review of different quantitative genetics models of sexual selection, Mead and Arnold (2004), pointed out that runaway is more ubiquitous then generally assumed. They suggested that 'runaway' (i.e. evolution of male trait evolving at ever-increasing speed under the influence of ever-increasing preference) can happen perpetually, along an elliptical path in which periods of ornament exaggeration would alternate with periods of diminution (Mead and Arnold 2004). They further argued that the best test for such perpetual evolution would come from examining the variation in ornaments as it may also reflect cyclical dynamics. Nonetheless, there are consequences of costly mating preferences, and these can limit the evolution of both preference and trait in a diversity of ways (Iwasa et al. 1991; Houle and Kondrashov 2002).

Despite the central role of female preference in sexual selection, and the importance of genetic variation in female preference, female preference is still poorly understood, prompting calls for investigations of the genetic variation in female preference (e.g. Heisler 1984; Bakker and Pomiankowski 1995; Wagner 1998; Bakker 1999; Mead and Arnold 2004). Similarly, the costs of female preference have the potential to influence the evolutionary trajectories of various sexual selection models

(Pomiankowski 1987), but investigations of preference costs are rare as it is very difficult to assess costs of preference over and above the costs of mating with particular males (Mead and Arnold 2004; Jones and Ratterman 2009; Maklakov and Arnqvist 2009). As a result, investigations of female preference and potential costs of preference are sorely needed (Maklakov and Arnqvist 2009).

Costs of mating are well documented and mate choice is predicted to decline in presence of such costs (Bateson 1983; Pomiankowski 1987). So how and why is costly choice maintained? The rationale is that although mate choice can be costly, females derive benefits from their choice of mate, and if these benefits are greater than the costs of choice, then costly choice can be maintained (Pomiankowski 1987; Iwasa et al. 1991; Andersson 1994). Females can gain two kinds of benefits from their choice of mate – direct benefits – whereby exercising a non-random mate choice females maximise benefit to themselves (e.g. increased fecundity, paternal care, nupital gifts; Trivers 1972), and indirect benefits where preferred males are also more attractive or are of a high quality and by mating with these males, females produce attractive or high quality offspring (Fisherian and good genes benefits respectively; see Fisher 1930; Lande 1981; Kirkpatrick 1996; Møller and Alatalo 1999; Jennions and Petrie 2000). These indirect benefits are considered to be much smaller than direct benefits (e.g. increased fecundity) (Andersson 1994; Jennions and Petrie 2000). However, if direct costs of mate choice are low, then in the absence of direct benefits, the smaller indirect benefits of female choice may be sufficient to maintain female preference (Kirkpatrick 1996).

Classically, studies investigating indirect benefits of mate choice have treated Fisherian and good genes based benefits separately. The rationale being that Fisherian

benefits are based on attractive males siring attractive sons, whilst the good genes benefits postulate enhanced viability for the offspring. Studies finding relatively weak or no good gene benefits have often concluded that females mostly gain Fisherian benefits from their choice of mate (Jones et al. 1998; Tomkins and Simmons 1999; Head et al. 2005; Hadfield et al. 2006). However, recently there has been a trend to remove this dichotomy. Several authors have controversially considered treating Fisherian and good gene benefits as complementary rather than alternative (e.g. Kokko et al. 2002; Radwan 2002) mechanisms. Equally controversially (Cameron et al. 2003), good gene benefits are also considered inevitable (Jennions & Petrie 2000; Rowe & Houle 1996). Both these stands are problematic, as they ignore the possibility of sexual selection via sexual conflict (e.g. Arnqvist and Rowe 2005). Nevertheless**,** at any given time, either or both the Fisherian process and good gene process may represent indirect benefits of mate choice (Kokko et al. 2003; Andersson and Simmons 2006).

Good genes benefits are considered to be extremely small and are thus hard to detect, although their cumulative effect over extended evolutionary timescales may be substantial (Møller and Alatalo 1999). It has also been suggested that good genes benefits are best detected via daughters as sons can frequently trade-off viability benefits for attractiveness (Pitnick and Markow 1994; Droney 1998; Kokko 2001; Getty 2002). However, contrary to theoretical predictions, there is a growing body of empirical evidence suggesting negative association between sires and daughters fitness (Rice 1984; Norris 1993; Fedorka and Mousseau 2004; Pischedda and Chippindale 2006; Oneal et al. 2007) or finding no support of the viability benefits predicted by good genes (Martin et al. 2004). Additionally, it has recently been suggested that intralocus sexual conflict can negate the good genes benefits of mate choice (Innocenti and Morrow 2010). Nonetheless, our understanding of female

choice would be incomplete without evaluation of all possible benefits of mate choice, and these good genes benefits are especially of interest as it is in the presence of these benefits that the adaptiveness of sexual selection is predicated (Candolin and Heuschele 2008; Hettyey et al. 2010).

According to the good genes model, mating success is positively correlated with genetic quality and thus sexual selection can be adaptive (Jennions et al. 2001; Møller and Alatalo 1999). Theory supports this, showing that sexual selection can facilitate the fixation of beneficial alleles (Whitlock 2000), reduce the mutational load (Whitlock and Agrawal 2009), alleviate some cost of sexual reproduction (Agrawal 2001) and accelerate adaptation (Lorch et al. 2003). Despite the fact that sexual selection was initially postulated to explain the evolution of traits detrimental to survival, Darwin (1871) did consider sexual selection to be inherently beneficial; an "*aid to ordinary selection*". He appreciated that sexual and natural selection can sometimes work hand in hand for "*improvement of the natural breed or species*". The evidence presented above seems to be consistent with some of Darwin's views, however there is evidence against the adaptive nature of sexual selection (e.g. Martin and Hosken 2003; Rundle et al. 2006) and the net benefits or costs of sexual selection are still widely debated (see Candolin and Heuschele 2008 for a review).

It is therefore not clear how evolution is affected by the interactions between natural and sexual selection. There are two contrasting possibilities; first, sexual selection may reinforce natural selection if the two types of selection are concordant. This may happen for example, if the traits beneficial under natural selection are the same as, or are positively genetically correlated with those that are beneficial under sexual selection. Second, natural selection can restrain sexual selection when sexual

traits are detrimental to survival. This has been known since Darwin's initial formulation of sexual selection, but has not been investigated thoroughly as experimental evolution studies often focus on either natural or sexual selection (Maklakov et al. 2010), despite the fact that natural and sexual selection occur simultaneously in nature and are known to interact (Blows 2002). Additionally, the costs and benefits of sexually selected traits are often condition dependent (Emlen and Oring 1977; Edward et al. 2010), and they can also change indirectly during environmental change; for example, by virtue of genotype x environment type interactions (Ingleby et al. 2010). We clearly need more studies to address one of the most fundamental questions regarding sexual selection – is sexual selection adaptive? (Candolin and Heuschele 2008).

Female mate choice decisions are often based on the assessment of multiple male traits which serve as indicators of male quality (Candolin 2003; Hebets and Papaj 2005). Several theoretical models have been proposed to explain the evolution of these traits and their relationship with female preference (Fisher 1930; Zahavi 1975; Kodric-Brown and Brown 1984; Grafen 1990; reviewed in Andersson 1994), and akin to the mandatory requirement of genetic variation in female preference for preference to evolve (see above), genetic variation in male traits is essential for these traits to evolve in response to female choice. Male sexually selected traits can display substantial genetic variation (Pomiankowski and Moller 1995), and a lack of genetic variation in male traits (e.g. in the direction of sexual selection) would have serious consequences for our current understanding of sexual selection. However, despite the importance of genetic variation in male traits, our understanding of these traits is rather limited and more investigations into the genetic variation of sexually selected traits have been called for (Andersson 2004). Similarly, it is also important to assess the genetic

architecture of such traits because of its potential to constrain trait evolution. For example, negative or positive genetic correlations between two different traits under directional selection can impede trait evolution (Lande and Arnold 1983; Barton and Partridge 2000; Blows and Hoffmann 2005), or if we consider traits shared by both sexes then a shared genetic architecture can potentially limit the independent evolution of sexes (Cheverud 1984; Poissant et al. 2010).

A thorough examination of the genetic variance-covariance matrices (G: a measure of genetic constraints on evolution *sensu* Cheverud 1984, 1988) can help us better understand the dynamics of sexually antagonistic selection. Sexes often have different selective optima for shared traits (Fairbairn et al. 2007; Poissant et al. 2010), and it has been argued that the presence of sex-specific genetic variance facilitates the evolution of sexual dimorphism (see Lande 1980; Rice 1984; Lande 1987; Poissant et al. 2010), promoting resolution of sexual conflict (Bonduriansky and Rowe 2005; Fairbairn 2007). Theoretically, the magnitude of r_{MF} between homologous traits and the nature of selection (sexual selection / natural selection) on each sex could affect the short and long-term evolutionary responses of sexually dimorphic traits (Lande 1980). It is plausible that these two forces may independently and/or jointly influence trait evolution. However, while the independent effects of natural and sexual selection on trait evolution have been documented, there is little direct experimental evidence of the combined evolutionary effects of sexual and natural selection on sexual traits (e.g. see Chenoweth et al. 2008). There is an urgent need for empirical studies investigating the joint effects of natural and sexual selection on trait evolution, and the potentially retarding effects natural selection may have on sexual trait evolution (Price et al. 1987).

Another controversial area in sexual selection research has been the allometry of sexually selected traits. Sexually selected traits are generally presumed to show positive allometry (i.e. larger individuals have relatively larger traits). Primary examples of such traits are male weapons and display traits associated with mating success (Green 1992; Petrie 1992; Kodric-Brown et al. 2006), and quite frequently, presence of positive allometry has been interpreted as evidence for directional sexual selection (Petrie 1988; Green 1992; Kodric-Brown et al. 2006). However, the majority of traits display negative static allometry (Cuervo and Møller 2009), and there are sexually selected traits - non-vertebrate genitalia for example, that consistently display negative allometry in contrast to signaling or weapon traits (Eberhard et al. 1998; Bernstein and Bernstein 2002; Hosken et al. 2005; Eberhard 2009). However, in a recent review of trait allometry across various taxa, Bonduriansky (2007) refuted this presumption regarding the ubiquity of positive allometry in sexually selected traits and established that this is misconception was driven by experimental bias. In his critical review, Bonduriansky suggested that any type of allometry is possible depending on the net sexual selection acting on a trait (Bonduriansky and Day 2003; Bonduriansky 2007) and called for investigations of trait allometry based on the functional significance of traits instead of the level of exaggeration.

In addition to the debate surrounding the scaling of sexually selected characters, the relationship between trait symmetry and sexual selection has also been at the focus of many debates (Møller and Swaddle 1997; Tomkins and Simmons 2003). It has been argued that fluctuating asymmetry (FA: small random deviations from perfect symmetry in bilateral traits: Van Valen 1962), is a measure of developmental instability and reflects an individual's genetic quality (Møller and Swaddle 1997). Theory predicts that only large, high quality individuals can afford to bear larger traits

and maintain developmental stability to reduce FA in such traits (Møller and Swaddle 1997). Furthermore, if FA reflects general quality, then FA levels should also be correlated across characters (Møller and Swaddle 1997). However, whilst there is some indication of an association between FA, sexual selection and fitness, this does not seem to be ubiquitous (David et al. 1998; Hunt and Simmons 1998; Martin and Hosken 2002; reviewed in Tomkins and Simmons 2003; Cuervo and Møller 2009).

These are a few of many questions that remain open for investigation under the overarching theme of sexual selection. This thesis is an attempt to investigate the questions outlined above using *Drosophila simulans* as a model organism. *D. simulans* was discovered by Sturtevant (Sturtevant 1919), and is thought to have originated in the Afrotropical region. This cosmopolitan species is believed to have separated from its closest relative – *D. melanogaster* – about 2mya (Powell 1997), and both of these species have been compared with regard to numerous characteristics, ranging from geographic distribution and ecology to their genetics (Capy and Gibert 2004). Although these two species are considered to have shared a common ancestor, they have accumulated many differences (reviewed by Capy and Gibert 2004). *D. melanogaster* is by far the most widely used model organism from this genus however, its popularity has caused a bias in the available sexual selection literature. Recently, Taylor et al. (2009) highlighted the importance of using alternate species such as *D. simulans* in evolutionary research, and documented the differences between *D. melanogaster* and *D. simulans* in relation to sexual selection. *D. simulans* is well suited for laboratory studies as it is easily cultured en masse and has a relatively short generation time. Additionally, it presents many characteristics that make it well suited for studying aspects of sexual selection. For example, costs of mating are relatively low

in *D. simulans* and sexual selection does not appear to be driven by sexual conflict in this species. Overall, sexual selection in *D. simulans* seems to be run by the classical mechanisms, making it an interesting contrast to its counterpart *D. melanogaster* (see Appendix A: Taylor et al. 2009).

As outlined above, multiple aspects of sexual selection in *D. simulans* are investigated in this thesis using behavioural observations, artificial selection, gas chromatography mass spectrometry and environmental manipulations. Female preference plays a central role in sexual selection, but despite this our understanding of female preference is relatively limited. In Chapter Two I investigate the evolution of female preferences in *D. simulans* and ask the following questions: 1) Is there genetic variance in female preference? 2) Can female preference evolve if selected upon? and 3) is female preference costly (over and above the cost of mating with particular male phenotypes)?. In Chapter Three, I investigate the good genes benefits of mate choice in *D. simulans*. These benefits are crucial to our understanding of sexual selection and here I ask the question – are there any good genes benefits of mate choice in *D. simulans*?. Mate choice is often based on multiple traits and genetic variation in sexually selected traits is essential for the evolution of such traits under the influence of sexual selection. Chapters Four and Five focus on investigating cuticular hydrocarbons as a male/female trait involved in mate choice. In Chapter Four, I ask the following questions – 1) Is there genetic variation in the cuticular hydrocarbons of *D. simulans*? 2) What is the genetic architecture of this trait like? – is it sex-specific for example?. Following on from the findings in Chapter Four, Chapter Five utilises experimental evolution to investigate the sexually antagonistic evolution of cuticular hydrocarbons in this species under influence of sexual and natural selection. In Chapter Six I attempt to address one of the most fundamental questions in

evolutionary biology, that still remains debated – Is sexual selection adaptive? This is done by determining the net cost or benefit of sexual selection using an artificial selection approach which was also a part of Chapter Five. In Chapter Seven, I investigate another rather controversial aspect of evolutionary biology – the allometry of sexually selected characters and the relationship between fluctuating asymmetry, fitness and sexual selection. Here I investigated the allometry and fluctuating asymmetry of sex-combs as a secondary sexual character using two distinct populations each from three *Drosophila* species – *D. simulans*, *D. melanogaster* and *D. pseudoobscura*. Chapter Eight is a general discussion which draws together all the findings from Chapters Two to Seven and outlines future directions for research.

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Chapter Two

Female mate preferences in *Drosophila simulans*: evolution and costs

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Female mate preferences in *Drosophila simulans*: evolution and costs

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sexual selection.

Abstract

Female mate preference is central to sexual selection, and all indirect benefit models require that there is genetic variation in female preference. This has rarely been tested however, with relatively few studies documenting heritable variation in female preference and even fewer that have directly selected on mate preference to unequivocally show that it can evolve. Additionally, costs of mate preference are poorly understood even though these have implications for preference evolution. We selected on female preference for *ebony*-males in replicate *Drosophila simulans* lines, and generated a rapid evolutionary response in both replicates, with the proportion of females mating with *ebony*-males increasing from approximately 5% to 30% after five generations of selection. This increase was independent of changes in *ebony*-males as only females were included in our selection regime. We could detect no cost to mate preference itself other than that associated with the fitness consequences of mating with *ebony* males.

Introduction

Sexual selection was proposed by Darwin (1871) to explain the evolution of characters that appeared to be detrimental to survival. Darwin also identified the main mechanisms of sexual selection, female choice and male–male competition. While much of the theory of sexual selection was accepted soon after its publication, female preference was controversial (O'Donald, 1980; Maynard Smith, 2000). A few years after Darwin's (1871) publication for example, Dewar & Finn (1909) suggested that it was 'absurd to credit birds with aesthetic tastes equal, if not superior, to those of the most refined and civilized of human beings'. In spite of this hostility and a long time-lag, female preference for certain male phenotypes has now been documented in many taxa (e.g. Ryan, 1983; Moore & Moore, 1988; Wilkinson & Reillo, 1994; reviewed in Andersson, 1994; Moore & Moore, 2006) and the potential importance of female preference has been verified in a range of sexual selection models (e.g. Lande, 1981; Kirkpatrick, 1982; Iwasa *et al.*, 1991; Pomiankowski *et al.*, 1991; reviewed in Mead & Arnold, 2004; Kokko *et al.*, 2006). Many of these models focus on indirect benefits of female choice, and the genetic correlation between female preference and the male traits targeted by preference is central to these indirect-benefit models. The basic logic is that if some females prefer males with extreme traits, then this nonrandom mating will generate a genetic correlation between trait and preference if there is a heritable component to the male sexual ornament and female mate preference (Fisher, 1930). The strength of this genetic correlation can profoundly influence evolutionary trajectories. For example with strong covariance, runaway can ensue as preference and ornament evolve exponentially in a burst of rapid evolution that ends when natural selection against the male trait becomes sufficiently strong or genetic variation is exhausted (Lande, 1981). Over a wide range of conditions, the genetic correlation approximates to $\beta = aG_PG_T$, where β is the genetic covariance at equilibrium, G_P is the genetic variance in preference, G_T is the genetic variance in the male attractiveness and a is the effectiveness of the male sexual signal and female preference at generating nonrandom mating (Bakker & Pomiankowski, 1995). Obviously, genetic variation in female preference is needed for the genetic correlation to build up, and then, even if there is no direct selection on female preference, preference can evolve via its

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association with male sexual traits, with increased preference selecting for further trait exaggeration and so on (Fisher, 1930; Lande, 1981). However, in spite of the central role female preference plays in sexual selection (Heisler, 1984a; Bakker & Pomiankowski, 1995; Chenoweth & Blows, 2006), and in the importance of genetic variation for preference, preference is still relatively poorly studied. This has prompted repeated calls for more investigations of the genetic variation in female preference (e.g. Heisler, 1984a; Bakker & Pomiankowski, 1995; Wagner, 1998; Bakker, 1999; Mead & Arnold, 2004), as a general lack of variation in preference would require a serious revision of our current understanding of sexual selection (Bakker & Pomiankowski, 1995; Mead & Arnold, 2004).

Direct selection on female preference, either via fecundity or longevity costs, has also been shown to be important in models of sexual selection (Mead & Arnold, 2004), particularly because indirect effects, such as those generated by the correlation between preference and trait, are likely to be small relative to direct effects (Kirkpatrick & Barton, 1997). Furthermore, Lande's (1981) model of runaway assumed no direct selection on female preference, and when direct selection on preferences was initially incorporated into genetic models of sexual selection, single point equilibria frequently resulted, and runaway was therefore considered unlikely (e.g. Iwasa *et al.*, 1991; Pomiankowski *et al.*, 1991). However, more recent investigations suggest that these equilibrium points may be unstable and even with direct selection on female preference, runaway can still occur (e.g. Hall *et al.*, 2000; reviewed in Mead & Arnold, 2004). It has also been suggested that costs of preference are likely to be ubiquitous through pleiotropic effects of preference genes or search costs associated with preference for certain males (Kirkpatrick & Ryan, 1991; Ryan, 1997; Hall *et al.*, 2000), and costs of preference have been documented (e.g. Englehard *et al.*, 1989). Nonetheless, although there are many investigations of costs of mating (e.g. Martin & Hosken, 2004), there is little empirical data on preference costs or direct selection on preference, prompting calls for more effort in estimating them (Wagner, 1998; Hall *et al.*, 2000; Mead & Arnold, 2004). Unfortunately, it is difficult to assess costs of preference beyond those associated with mating with particular males (Maklakov & Arnqvist, 2009), but in spite of this, investigations of potential preference costs are sorely needed (Heisler *et al.*, 1987; Maklakov & Arnqvist, 2009).

One way to unequivocally document genetic variation in female preference is to select on it (Bakker & Pomiankowski, 1995), and this is the approach we use here with *Drosophila simulans*. We have previously investigated the genetics of sexual selection in this species, primarily from a male perspective, to show that male attractiveness is heritable (Taylor *et al.*, 2007) and positively associated with sperm competitiveness (Hosken *et al.*, 2008). Additionally, there does not appear to be any direct selection on female preference because female fecundity is not directly influenced by their choice of mate (Taylor *et al.*, 2008a,b, 2010). However, this inference was based on counts of offspring produced rather than eggs laid, and as such, may partially confound offspring fitness with female fitness (Wolf & Wade, 2001). Here, we first used iso-female lines to test for genetic variation in one measure of female mate preference. We then used artificial selection and experimental evolution to further investigate female preference and potential costs of preference. Although we find that there is variation in preference across isolines and that female preference evolves rapidly when subjected to selection, we could find no costs to preference beyond those associated with the quality of females' mates.

Methods

Fly stocks

The base-line wild-type populations of *D. simulans* used here were derived from twenty iso-female lines supplied by the Centre for Environmental Stress and Adaptation Research, La Trobe University, Australia. These were collected from the wild in 2004. They had been maintained in large population cages (ca. 800–1000 flies/cage) with overlapping generations for 4 years prior to the start of this investigation and have been found to harbour considerable genetic and phenotypic variation (e.g. Taylor *et al.*, 2007, 2008a; Wright *et al.*, 2008). The *ebony* stock population was established using a strain obtained from the Tucson stock centre and was maintained as above for over 50 generations. *Ebony* is a phenotypic body-colour mutant with reduced fitness. *Ebony* flies are partially blind, often have courtship defects (including licking females less and altered courtship song), and males are frequently more aggressive (Søndergaard, 1985). All flies were reared on 'Drosophila quick mix medium' (supplied by Blades Biological, Edenbridge, Kent, UK) at 25 °C and a 12 : 12 h light:dark cycle. Subsequent housing conditions followed this regime unless stated otherwise. Flies to be used in preference selection lines (and subsequent preference cost assays) were initially collected as virgins from stock population laying vials (*ebony* and wild type: housed separately). Briefly, laying pots were left in the population cages overnight and then removed and housed under standard conditions (25 °C: 12 : 12 light). Virgin flies emerging from the laying pots were separated and housed by sex (< 10 flies per vial) with an excess of the culture medium for 3 days before initial pairings to start the selection lines.

Isolines

We used six of our original isolines and collected 60 virgin males and females from each. Again we collected

emerging virgin adults and housed them by sex (within isoline) at densities of no more than 10 flies per vial, with an excess of the culture medium for 3 days prior to experimental pairings. When flies were 3 days old, females from each line were paired with males (i.e. one female was placed with one male) from each line in a fully factorial and balanced design. We measured the time it took for a female to copulate with a male (copulation latency) as our indicator of preference (time from introduction of male to mating – this correlates with time from first courtship to mating but is easier to measure: Taylor *et al.*, 2008a). This is one standard measure of female preference in *Drosophila* (e.g. Speith, 1974; Ritchie *et al.*, 1999; Acebes *et al.*, 2003) and is consistent with preference definitions as it reflects the tendency for females to mate with certain males (Heisler *et al.*, 1987; Jennions & Petri, 1997). Additionally, male *Drosophila* cannot force copulation in nonteneral females (Eberhard, 2002) but use a range of courtship behaviours (e.g. Pitnick, 1991; Droney, 1996; Ritchie *et al.*, 1999; Acebes *et al.*, 2003) that a female interrupts with her own acceptance or rejection signals (Speith, 1974). As such, hardened females primarily determine whether or not copulations occur in *Drosophila* (Markow, 1996). Because females determine when copulation occurs, we reasoned that females should copulate faster with more attractive, preferred males. Additionally, measuring preference this way excludes any potential for male–male competition to interfere with female choice. Mating latency (log transformed) was then analysed using univariate GLM with female and male isolines as random factors. We also note that although it is possible to calculate heritabilities from isolines, this should ideally be done within five generations of line establishment (Hoffmann & Parsons, 1988). Because our lines had been in captivity for much longer than this, we did not calculate preference heritability using these data. Nonetheless, differences across isolines would indicate genetic variation in female preference (Hoffmann & Parsons, 1988).

Selection lines

Here, we selected on *ebony* female preference for *ebony* males in two independent populations (lines) and maintained a single control line at the same average population size as the selection lines. In the selection lines, only *ebony* females that mated with *ebony* males were used to found each subsequent generation, whereas in the control line, females were chosen at random and therefore probably predominantly included females with preference for wild-type males (ca. 95% of stock *ebony* females mated with wild-type males when given a choice). Although this is a highly artificial system, it does demonstrate how preference could evolve and also allows us to investigate potential costs of preference.

On the day of mating, virgin males (collected as virgins from the stock populations each generation of testing) were transferred into the mating vials without any anaesthesia (de Crespigny & Wedell, 2008). We measured female preference by exposing females (100 per line) to pairs of males (one wild-type, one *ebony*) for a maximum of 3 h and scored which male a female mated with. Female preference measured this way is consistent with preference definitions: preference reflects the propensity for females to mate with certain males (Heisler *et al.*, 1987; Jennions & Petri, 1997; and see *Isoline* section above). While this allows for male–male competition, which can potentially confound female mate choice, our selection regime allows us to take this into account. Every generation tester males were derived from our two stock populations (*ebony* and wild-type), and hence there was no opportunity for adaptation to occur in the males (in response to female preference). This also means that if our measure of mate preference was solely because of male–male interactions, or males more generally (i.e. there was no female genetic variation in our measure of preference), then there would be no change in female preference over time. The fact that there was a response indicates that our measure of preference is capturing some component of females' mate preference.

Soon after copulation was complete, males were removed from the vial and in the selection lines, females that mated with *ebony* males were allowed to oviposit for 24 h. Subsequent generations of females in the selection lines were derived from the offspring obtained from these matings and were tested in the same way each successive generation. Females providing offspring for the control line were chosen at random, but the census size of control dams was deliberately kept at the mean selection line number (see Fig. 3).

After five generations of selection, realized heritabilities were calculated using Falconer's (1981) method for threshold traits. With this method, the two phenotypes, mating with *ebony*/not-mating with *ebony*, are assumed to be because of an underlying, continuous trait referred to as liability. Liability is normally distributed and is measured in standard deviation units. Individuals above a certain threshold show a certain phenotype, whereas those below it show another (mating with *ebony*/not-mating with *ebony*) (Falconer, 1981). Because liability is continuous, it is amenable to standard quantitative genetic analyses and trait heritability is then calculated from the cumulative response to selection (e.g. Radwan, 2003). This is the recommended method for calculating preference in binary choice trials such as we use here (Bakker, 1999).

After selecting on preference, documenting a response and estimating its heritability, we randomly (with respect to preference) selected 100 virgin (*ebony*) females from each selection line and housed them with 100 virgin *ebony* and 100 virgin wild-type males in population cages (one for each line). Excess food was provided, and free mate choice and overlapping generations were allowed.

This relaxed the strong directional selection on preference we had previously imposed. After two generations of relaxed selection, *ebony* female preference for *ebony* males was again assessed. This allowed us to estimate the decay of preference and hence preference costs in the absence of direct selection favouring female preference for *ebony*. However, because *ebony* flies have lower general fitness than wild-type flies, we needed to estimate the direct fitness costs of mating with *ebony* males to assess any potential costs associated with the preference itself. To do this, crosses between *ebony* females and *ebony* and wild-type males were conducted. The relative fitness of *ebony* flies was calculated using the number of offspring to emerge within 7 days after first eclosions from vials in which singly mated females had laid for 24 h. This relative fitness measure was then used to predict a rate of preference decay when selection was relaxed. This is easily carried out from generation 1–2 as only *ebony* and wild-type flies are present at generation 1, but in subsequent generations, calculations become complicated by the presence of various heterozygotes and backcrosses. Hence, we only compared preference decay at generation 2. We found that *ebony* × *ebony* crosses had a relative fitness of 0.51 and conservatively assuming the fitness of heterozygotes (*ebony*/wild-type crosses) to be the same as the wild-type females (see Dobzhansky, 1947; Moree & King, 1961), the relative fitness of *ebony* preferring females from generation 1–2 of relaxation was 0.58 (this includes heterozygous females that preferred *ebony* males and *ebony* homozygous females that preferred *ebony* males). In the absence of any additional costs, the decay in female preference for *ebony* once we relaxed selection would be purely because of this fitness deficit of the *ebony*-preferring females (Table 1). Note we ignore all potential indirect effects as they will be small relative to direct effects (Kirkpatrick & Barton, 1997). We note that this aspect of the study is not particularly strong. For example, by estimating cost over only two generation, we could confound costs with epistasis for preference (relaxing selection could lead to recombinational breakdown of more favoured complexes of preference genes and hence rapid decay might not indicate costs *per se*: but see Results), and just because we saw a rapid response to selection we may not see a rapid decay. Nevertheless, we include this component here as an example of how such costs could, *in principle*, be assayed.

Table 1 Expected decay of *ebony* preference in the experimental populations based on an estimated relative fitness of 0.51 for *ebony* preferring females from generation 0 to 1 and of 0.58 from 1 to 2.

Generation	0	1	2
Rep. 1	32.63	16.64	9.65
Rep. 2	28.88	14.73	8.54

Results

Isolines

Analysis of the female preference (latency to mate) across the isolines revealed significant differences across female ($F_{5,324} = 12.26$; $P < 0.001$) (Fig. 1) and male genotypes ($F_{5,324} = 3.98$; $P = 0.002$). The interaction between male and female identity was also significant ($F_{25,324} = 2.93$; $P < 0.001$) (Fig. 2). This indicates that there is genetic variation in this measure of female preference in our population (Fig. 1) that was not simply because of differences in female receptivity, because there were also male identity effects. Additionally, the interaction indicates female responses depended on male identity, and not all female genotypes agreed on the attractiveness of each male genotype.

Selection lines

There was a steady increase in the number of females preferring *ebony*-males as mates. In the base population only about 5% of females mated with *ebony*, and this proportion remained more or less constant in our control line (Fig. 3). In the selection lines however, there was an increase over time to about 31% of females choosing *ebony* by generation 5. The realized heritability of preference averaged across replicates was $h^2 = 0.26 \pm 0.11$ (0.24 and 0.28 in each line).

Based on the relative fitness of the *ebony*–*ebony* cross, 9.7% of females in line 1 were predicted to prefer *ebony* 2

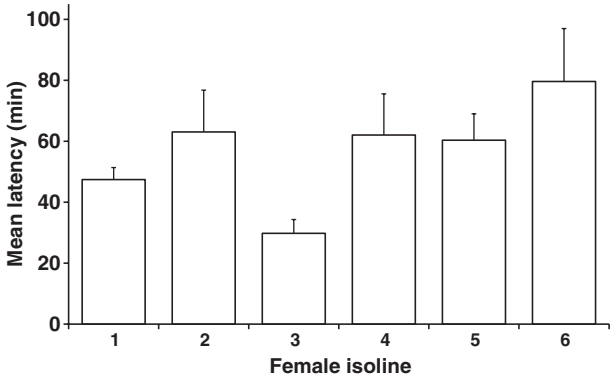

Fig. 1 Mean (±SE) female preference for six *Drosophila simulans* isofemale lines (wild-type). Preference was measured here as mating latency (means for 60 females per line). Shown here are the untransformed data. Significant differences between lines (*post hoc* tests of log-transformed latency – Fisher's PLSD: 3 < 1–6; 1 < 6; 5 < 6; 2 < 6. Tukey–Kramer: all significantly differ) indicated there is genetic variation for this measure of preference, and note that there were also significant effects of male genetic background and an interaction between female and male genetic background (see Fig. 2).

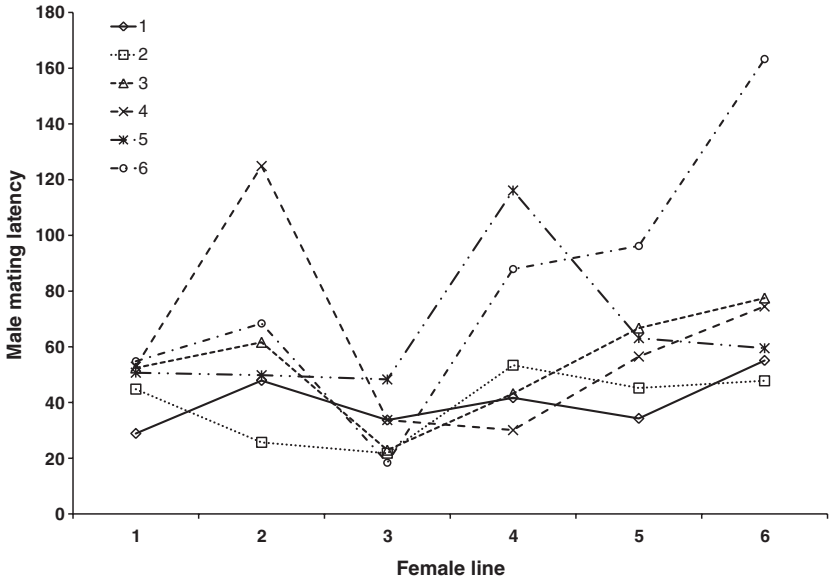

Fig. 2 The interaction between male and female genotype influencing female preference assessed using wild-type isofemale lines. Preference was measured here as mating latency. On the *x*-axis are the same six isolines shown in Fig. 1, with specific responses to each male genotype (the points connected by a line: i.e. all diamonds represent males from isoline 1, open squares males from isoline 2, and so on. See Figure inset). This interaction between male and female genotypes was statistically significant.

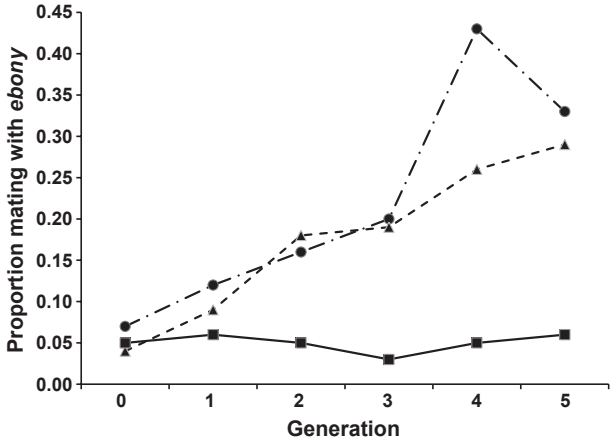

Fig. 3 Changes in the proportion of females preferring to mate with *ebony*-males over five generations of selection. Dashed lines (joining triangles and circular symbols) are the two upward selection replicates, whereas the solid line (connecting the solid squares) is the unselected control. Only females were included in the selection regime (all males came from populations cages every generation) and the control line was maintained at the same census size as the average of the selection lines.

generations after relaxation of selection, whereas for line 2 the corresponding prediction was 8.5%. The observed values were 15% and 12%, respectively. Although the observed values were slightly higher than predicted (i.e. decay was slower than predicted), contingency tests indicated differences were not statistically significant (both $\chi^2 < 1.15$; both $P > 0.28$).

Discussion

Our main finding was that selecting on female preference for *ebony* males generated a steady increase in the proportion of females mating with these males, and our estimate of preference heritability was moderate. We also found that isolines differed in another measure of female preference, the speed at which females mated with a male (latency to mate), which also indicates there is genetic variation in female preference. However, we found no evidence for preference costs over and above the cost of mating with *ebony* males. We discuss each finding and their main consequences in turn.

Female preference can be measured in a number of ways, but it is generally agreed that preference reflects a female's inclination to mate with certain males (Heisler *et al.*, 1987; Jennions & Petri, 1997). In accordance with this, we assessed female preference in two ways and found genetic variation for both measures, including unequivocal evidence that preference can evolve. Genetic variation for female preference has previously been documented in a small number of taxa (e.g. O'Donald & Majerus, 1985; Moore, 1989; Ritchie, 1992; reviewed in Bakker, 1999), including *Drosophila* (e.g. Heisler, 1984b; Scott, 1994; McGuigan *et al.*, 2008). Previous estimates of preference heritability are generally lower than 60% (Bakker, 1999), which is what we find here assuming preference is polygenic, which it seems to be (based on the responses seen). Most models of sexual selection also assume preference is polygenic and consistent with this, there is evidence in *Drosophila* that multiple chromosomal regions contribute to complex behaviours like mating, attractiveness and mate preference (Kawanishi & Watanabe, 1981; Heisler, 1984b; Mackay *et al.*, 2005;

Gleason *et al.*, 2009). It is worth noting that preference was also significantly heritable if we assumed it was determined at a single locus (data not shown).

Genetic variation in preference is essential for models of indirect mate-choice benefits, and the current study provides evidence for genetic variation in female preference in *D. simulans*. We have previously found no evidence of good genes or direct benefits/costs to mate choice in our wild-type population (Taylor *et al.*, 2008a,b, 2010) but have documented genetic variation in male attractiveness (Taylor *et al.*, 2007; Hosken *et al.*, 2008), which indicates that Fisherian benefits are probably the only benefit available to females via their choice of mate in our population. Direct testing of this requires that we identify the precise male characters on which females make their choices. This is something we are currently undertaking, with cuticular hydrocarbons and song likely to be important (e.g. Speith, 1974; Blows & Allan, 1998; Ritchie *et al.*, 1999). However, when there is genetic variation in male attractiveness and female preference (which we find), nonrandom mating (which we also find), and the strength of other selection acting on each trait is relatively weak, it is difficult to see how Fisherian effects can be avoided (Shuster & Wade, 2003; and see Lande, 1981). We nevertheless emphasize that what we document in our study population need not be indicative of natural populations of *D. simulans*, nor does the genetic variation we document in preference indicate preference polymorphism exists in nature (i.e. we are not claiming what we see in the laboratory is necessarily equivalent to the wild). This remains to be investigated. We also note once more that the isoline results indicate genetic variation in female preference (the significant female identity effect), but this was not simply because of receptivity differences between isolines as there were male identity effects and an interaction between female and male genotypes. Thus, female preference depended on male identity, but not all female genotypes agreed on the relative attractiveness of male genotypes. This is precisely what is required to build up a genetic correlation between male attractiveness and female preference.

Genetic variation in female preference has rarely been documented (Bakker, 1999; Mead & Arnold, 2004) and estimates of preference costs are even rarer (Heisler *et al.*, 1987; Maklakov & Arnqvist, 2009). Costs of preference can come in many forms, but of particular interest are direct costs to females because of differences in their mating preference (Heisler *et al.*, 1987; Maklakov & Arnqvist, 2009). We tried to estimate this latter cost by measuring how quickly preference for *ebony* males was lost from our selection lines once the absolute advantage of *ebony* preference was removed. We found no indication that preference decayed at a rate faster than that predicted based on the relatively lower fitness of females mating with *ebony* males. If anything the declines were slower than predicted (although not significantly so). Thus, although *ebony* preference declined once selection was relaxed, the rate of decline did not indicate that *ebony* preference itself was particularly costly under our experimental set-up. There were potential indirect costs here too as *ebony* sons were not as attractive as wild-type sons in the relaxed-conditions population cages. However, indirect effects ought to have much weaker effects than direct effects (Kirkpatrick & Barton, 1997), so it is perhaps no surprise that we detected nothing that could be interpreted as an indirect cost of *ebony* preference. Similarly, in our population cages, direct costs of being choosy may be minimal as flies are at relatively high density and females are surrounded by males, potentially minimizing all preferences costs. Consequently, the fact that we did not find costs of preference above those because of being *ebony* is perhaps not surprising, and our statistical power was not great. Additionally, as we noted earlier (Methods), this component of the study was not particularly strong for a number of other reasons (e.g. two generations may not be enough to detect decay). Hence, there may be costs to having different mate preferences that we could not detect. However, our failure to detect major costs of preference is at least consistent with previous laboratory studies of this population that did not employ the *ebony* mutant (Taylor *et al.*, 2008a,b, 2010) and supports our supposition that indirect benefits, even small ones, could maintain choice in this system.

In summary, we found unequivocal evidence for genetic variation in female preference but could not detect costs associated with preference. We now need to identify the precise traits influencing male attractiveness in our population, but currently, Fisherian benefits seem adequate to maintain choice in this system.

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Chapter Three

No good genes in *Drosophila simulans*?

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Abstract

Studies investigating indirect benefits of female mate choice frequently find Fisherian benefits to choice, while detecting little or no good genes (viability) benefits. It has been suggested that this could be because sons trade-off viability for increased mating success, and as a result, good genes benefits should be investigated via daughters. However, intralocus sexual conflict could hide sire-daughter associations. Here we investigated potential good genes effects in *Drosophila simulans* using an isofemale line approach. We assessed the attractiveness of males in two different ways and then measured the longevity, and lifetime reproductive success, of their daughters. We were also able to assess potential direct benefits of female mate choice, and assessed son's longevity too. We found no evidence of direct or good genes benefits to mating with attractive males, and failure to find good genes effects via daughters was apparently not due to masking through intralocus sexual conflict. Results are consistent with previous findings in this species, and suggest good genes benefits are at best very small in our study population.

Keywords: sexual selection, mate choice, *Drosophila*, indirect benefits

Introduction

The benefits of female mate choice have the subject of much debate (Andersson 1994; Andersson & Simmons 2006). Direct benefits of choice should be larger than indirect effects (Kirkpatrick 1996; Kirkpatrick & Barton 1997) and meta-analysis seems to confirm this (Jennions et al. 2001; Møller & Alatalo 1999; Møller & Jennions 2001). Nevertheless, females may still gain indirect genetic benefits through their mate choices (Andersson 1994; Jennions & Petrie 2000). Females can derive indirect benefits in two general ways (Andersson 1994; Jennions & Petrie 2000). Firstly, by mating with attractive males, females can produce attractive sons and enjoy fitness benefits via sons' elevated mating success (Fisherian benefits: Fisher 1930; Lande 1981; Kirkpatrick 1985). Secondly, attractive males may be signalling their high genetic quality, and hence by mating with them females could produce high viability offspring (good genes benefits: Andersson 1994; Jennions & Petrie 2000). These indirect benefits, however small, may be enough to maintain female mate preference in absence of any direct costs associated with mate choice (Kirkpatrick 1996).

Good genes benefits of mate choice were initially formulated as viability benefits (Arnold 1983; Lande 1981). However, there has been a tendency to include characters other than viability under a good genes umbrella more recently, with a number of studies finding that mating with attractive males can enhance a range of offspring fitness components (Norris 1993; Petrie 1994; Welch et al. 1998; Boake 1985; Hine et al. 2002; Sheldon et al. 1997; Brooks 2000; Gowaty 2007; Wedell & Tregenza 1999; Evans et al. 2004; Partridge 1980; Møller & Alatalo 1999). It has also been argued that good genes effects are inevitable, as all indirect benefits may ultimately become linked to good genes (Jennions & Petrie 2000; Rowe & Houle 1996). However,

this seems to ignore the inevitability (at least under some conditions) of the Fisher process which can drag sexual traits well beyond their naturally selected optima (Shuster & Wade 2003). Conflict driven sexual selection may also preclude the enhancement of offspring viability (Arnqvist & Rowe 1995).

It has also been suggested that allocation decisions could mask good genes benefits (Getty 2002). For example, sons may trade-off viability with reproductive success (Droney 1998; Kokko 2001; Pitnick & Markow 1994; Getty 2002). This could then mask viability benefits of mate choice in sons, and bias conclusions about good genes benefits (Cameron et al. 2003; Hunt et al. 2004; Kokko et al. 2003; Kokko et al. 2002). As a result, it has been suggested that good-genes benefits should be investigated in daughters (Jennions & Petrie 2000; Hunt et al. 2004). However, there is a growing body of evidence that intralocus sexual conflict could further complicate potential good genes benefits. Intralocus conflict occurs when the genes that make good females make poor males, and *vice versa* (Rice & Chippindale 2001), and negative intersexual fitness associations, the hallmark of this conflict, have been documented in a range of taxa (Norris 1993; Rice 1984; Fedorka & Mousseau 2004; Pischedda & Chippindale 2006; Foerster et al. 2007; Oneal et al. 2007).

Here we investigate potential good genes benefits of mating with attractive males in *Drosophila simulans*. Previous work has documented genetic variation for female mate preference (Sharma et al. 2010), and that male attractiveness is heritable and positively genetically correlated with sperm competitiveness (Taylor et al. 2007a; Hosken et al. 2008). Additionally, females apparently gain no direct benefits from mating with attractive males (Taylor et al. 2008a, b). While good genes benefits have

been reported in several *Drosophila* (e.g. *D. melanogaster* (Partridge 1980; Taylor et al. 1987), *D. montana* (Hoikkala et al. 1998) and *D. serrata* (Hine et al. 2002)), there is currently no evidence for this in our study population of *D. simulans* (Taylor et al. 2010). This is not to say they do not exist, but only that they have not been detected. This may be due to the protocols previously employed, and here we use a new approach in which we reduce variation in female mating behaviour and male attractiveness by using iso-female lines. Each line is essentially a single genotype and by controlling for genetic variation in this way our ability to detect small indirect benefits of mate choice could be enhanced. We employ isolines in two ways. Firstly, we use females from six isolines to judge male attractiveness and calculate an average index of attractiveness for different male genotypes across a range of female genotypes. This provides us with a repeated and potentially accurate measure of male attractiveness than the single female assessments used previously (Tayor et al. 2010). Secondly, we use females from two isolines, expose them to two wild-type males sequentially, and use females' remating decision to assess the relative attractiveness of the two potential mates. In both instances males can be subsequently categorised as attractive or unattractive, and we can then assess the viability of their daughters. The experiments also allow us to assess the direct fitness of mothers mated to attractive or unattractive males, controlling for maternal genotype through the use of isolines. Additionally, we assess daughters' lifetime reproductive success after a single mating to see if any good genes benefits are evident via this route.

Methods

The stock wild-type populations of *D. simulans* used here were derived from twenty iso-female lines supplied by the Centre for Environmental Stress and

Adaptation Research, La Trobe University, Australia. These were collected from a wild population at Tuncurry, Eastern Australia in March, 2004 and had been maintained in large population cages (ca. 800-1000 flies/cage) with overlapping generations or as iso-female lines for 5 years prior to the start of this investigation. All flies were reared on '*Drosophila* quick mix medium' (supplied by Blades Biological, Edenbridge, Kent, U.K) at 25°C and a 12:12 h light:dark cycle. Subsequent housing conditions followed this regime unless stated otherwise. Flies to be used in mating and fitness assays were initially collected as virgins from stock population laying vials or from cleared isoline vials. Briefly, laying pots were left in the population cages overnight and then removed and housed as above (25°C, 12:12 light). Virgin flies emerging from collection vials were separated and housed by sex (< 10 flies per vial) with an excess of the culture medium for 3 days (to ensure sexual maturity) before experiments.

For our initial investigation of potential good genes benefits, individual males from 6 isolines were placed into separate vials and the next morning (between 09:00 & 12:00 = the first three hours of lights on, which corresponds with the period of peak mating activity in natural populations (Gromko & Markow 1993)) one female (from the same 6 isolines) was added to each male vial in a fully factorial manner (= 36 pair combinations). Each combination was replicated 10 times (360 pairs). Pairs were continuously observed for 3 hours, or until the start of mating. Previous work in this population has shown that almost 95% females mate during this time (Taylor et al. 2008a). All further mating assays follow this protocol unless stated otherwise. The time of female introduction and the start of copulation were recorded. We measured the time it took for a female to copulate with a male as our indicator of male attractiveness (mating latency: time from introduction of female to mating - this

correlates with time from first courtship to mating but is easier to measure: (Taylor et al. 2008a)). Male *Drosophila* use a range of courtship behaviors that a female responds to with her own acceptance or rejection signals (Speith, 1974), and males cannot force copulations with non-teneral females (Eberhard, 2002). We therefore reasoned that females should copulate faster with more attractive males, and latency has been widely used as a standard measure of female preference and therefore male attractiveness in *Drosophila* (e.g. Spieth 1974; Ritchie et al. 1999; Acebes et al. 2003). Additionally, measuring attractiveness this way excludes potential for male-male competition to interfere with our assessment of attractiveness. The average mating latencies thus obtained would show how attractive each genotype was judged (on average) by females from all isolines (Fig. 1). Based on this assessment two attractive and two unattractive male lines were identified (Fig. 1), and virgin males from these lines were then used for assessment of daughters viability. Briefly, 50 males from the two most attractive (lines 1 and 2), and two most unattractive (lines 5 and 6) lines were paired with a virgin wild-type female and observed as above (n = 200 males). Mating latency was recorded and we initially assessed congruence between the assessment of wild-type females and the attractiveness score from isoline females. Both the Kruskal-Wallis and Median tests confirmed that latency of males judged to be most attractive by isoline females were also judged to be more attractive by wild-type females (Kruskal-Wallis $\chi^2_1 = 4.09$, $P = 0.04$; Median $\chi^2_1 = 5.78$, $P = 0.024$; Mean Latency Attractive: 73.45 ± 5.74; Mean Latency Unattractive: 94.23 ± 5.74). This suggests that the wild-type females were in agreement with isoline-females' assessment of male attractiveness. Following a single copulation with wild-type females, males were removed and stored for future measurements. Females were left to lay eggs for 24 hours, after which they were transferred to a new egg laying vial for

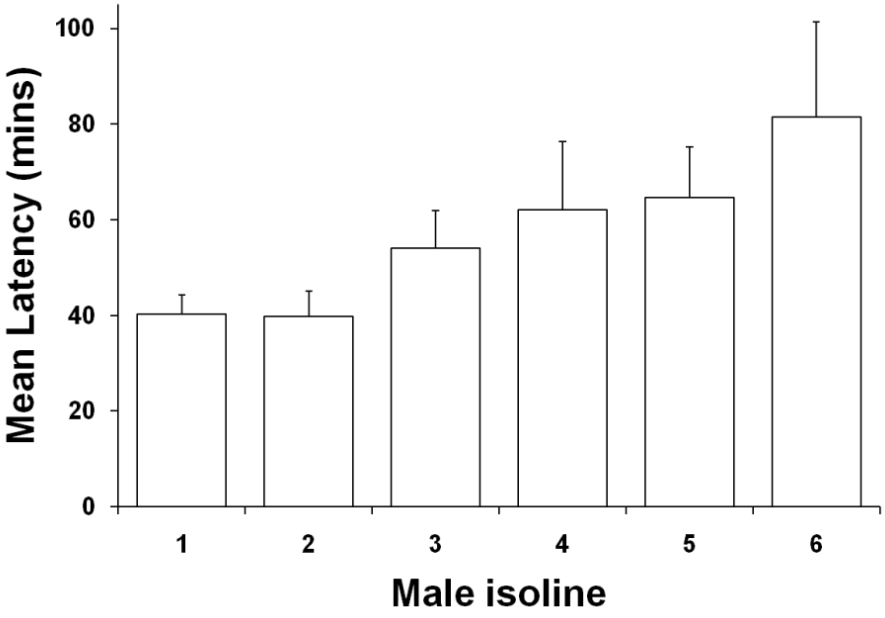

Figure 1. Mean (±SE) male attractiveness for 6 *Drosophila simulans* isofemale lines. Attractiveness here is shown as untransformed mating latency (means for 60 males per line). Lines 1 and 2 were considered as attractive (females mated fast with these males), whilst 5 and 6 were classed as unattractive (females took longer to mate with these males).

another 24 hours. They were then moved into a final vial where they laid eggs for a further 5 days. The lifetime reproductive success (LRS) of each female was subsequently scored as the total number of offspring emerging from these three vials. Offspring from each vial were counted on the 8th day after the first eclosion (*D. simulans* larvae take 8-9 days to develop and eclose, so 7 days eclosing excludes any overlap with possible grandchildren). Taylor et al. (2008a) have previously shown this is a good proxy for lifetime productivity from a single copulation. Two virgin daughters/wild-type dam were collected from these vials, housed alone and transferred to fresh food vials once per week until death to assess their adult longevity. This gave us a total of 400 daughters (= 100 daughters per isoline = 200 daughters of attractive males & 200 daughters of unattractive males). We also collected sons (=100 sons per isoline) and treated them as per daughters to assess their longevity. This enabled us to test for an effect of sire attractiveness on sons' longevity (in spite of the caveats previously mentioned) and to regress mean daughter longevity against mean son longevity to see if any lack of sire-daughter associations were due to negative intersexual correlations (Chippindale et al. 2001).

In our second assessment of potential good genes benefits, we used isoline females to minimise genetic variation for female preference and assessed a male's attractiveness based on a isoline females' remating decision. Our rationale was that when given a choice to remate, females would only remate with a more attractive male (i.e. trade-up), and if there were good genes benefits to mate choice we would be able to detect them in a pairwise comparison of the two males. Female remating decisions have previously been used as a measure of male attractiveness (e.g. Ivy & Sakaluk 2007; Stewart et al. 2008). Whilst both male and female *Drosophila* routinely

copulate with multiple mates and there is no parental care beyond selecting an optimum oviposition site (Powell, 1997) and (virgin) *Drosophila* females once mated are usually reluctant to remate for some time. The duration of this refractory period varies between species and in different strains of the same species (Manning 1962). In *D. simulans* the timing of remating is highly variable, taking between one and eight days, and remating increases LRS, so remating is not costly (Taylor et al. 2008b). By using isoline females we are able to minimise the genetic variance in female preference and together with using a males' capability of inducing a female to remate as an indicator of his attractiveness, we potentially increase our chances of detecting any good genes benefits.

The protocol employed for this is as follows: we paired a three-day old wild-type male with a virgin female from one of two isolines (isolines 7 and 8) and observed them for 3 hours. The mated pairs were the separated into individual vials soon after mating ended. The next day each of the mated females was offered another three day old virgin (wild-type) male and observed for three hours. If the female re-mated, the new mate was classed as attractive and her previous mate was deemed unattractive. The attractive or unattractive males were then mated with new virgin females (from the same isoline as the initial female) and the fitness of subsequent daughters was assessed. This was necessary because we could not differentiate between sires in offspring from the initial matings. However, because we use the same isolines, the genetic background of females is standardised. Briefly, pre-classified males were paired with virgin females and allowed to interact for 48 hours. After 48 hours vials were checked for egg-laying, pairs were then removed and vials incubated. Virgin offspring emerging from these vials were collected as described above. For each sire

we selected multiple daughters at random and assayed them for fitness - approximately half were mated to a 3 day old virgin wild-type male, to measure LRS, and the other half kept as virgins to assess longevity. In total we assayed 466 and 554 daughters for the LRS and longevity assays. Sexually mature daughters were 3-4 days old and paired with a wild-type virgin male, of the same age, for 48 hours. During this period they were transferred to fresh vials with the culture medium every 24 hours (twice), and then to the final egg laying vial for 5 days after removing the males. For the longevity assay, virgin daughters were housed at a density of 3 flies per vial and transferred to fresh vials once per week until death. LRS was scored as described above and virgin females in the longevity assay were checked daily for mortality. Longevity was scored in days since eclosion. We used wing length as a measure of body size (Gilchrist and Partridge 1999) to determine any association between body size and LRS or longevity. Analyses were based on sire family means (n = 46 attractive, 46 unattractive) and comparisons were pair-wise because the attractiveness of males was relative to the other male the initial female was exposed to.

Statistical analysis

Data analysis was conducted using PASW v 18 (formerly called "SPSS"). Raw data were tested for normality using Shapiro-Wilks tests and $\log_{10}$ or square root transformed to improve normality where appropriate. Non-parametric tests were used where normality assumptions of parametric tests could not be met. For the first experiment we used a mixed model univariate analysis of variance (ANOVA), with daughters' (and sons') longevity (dependent), sire attractiveness (fixed effect) and isoline nested within sire attractiveness (random effect), to examine the effect of sire attractiveness on daughters' longevity (NB our conclusions do not change if the

predictors are treated as fixed or random effects). Although, our primary interest was the relationship between sire attractiveness and daughters' longevity, we also examined the direct effects of attractive males on female fitness (i.e. the direct benefits to dams from their mates) by using dams' LRS as the dependent variable and the same predictor variables (sire attractiveness and line within attractiveness). We additionally, regressed mean (per sire family) daughters' longevity against sons' longevity to assess intersexual longevity associations. For the second experiment, our data did not meet assumptions of normality and we could not transform them in such a way to meet parametric model assumptions. We therefore used Wilcoxon matched pairs signed-rank test to assess the differences in the LRS and longevity of daughters sired by attractive or unattractive males (i.e. males were paired based on the common female used to judge their relative attractiveness). Sample sizes vary across some analyses due to missing data.

Results

Our primary aim was to see if females mating with attractive males produced higher quality daughters. Results from the mixed model ANOVA using family means of daughters' longevity (dependent), sire attractiveness and sire line nested within attractiveness (as predictors) revealed no effect of sire attractiveness on daughters' longevity ($F_{1,2}$ = 1.01, P = 0.42). However, we did see a significant line (within attractiveness) effect ($F_{2, 196}$ = 25.67, P < 0.001), which was driven by one attractive line producing long lived daughters (Fig. 2; Table 1). Post-hoc analysis of the line effect indicated that the mean for Line 2 was significantly higher than that of all other lines (all P < 0.001), and none of the other lines differed from each other (all P > 0.9). We also analysed this data by taking line means instead of family means for the daughters'

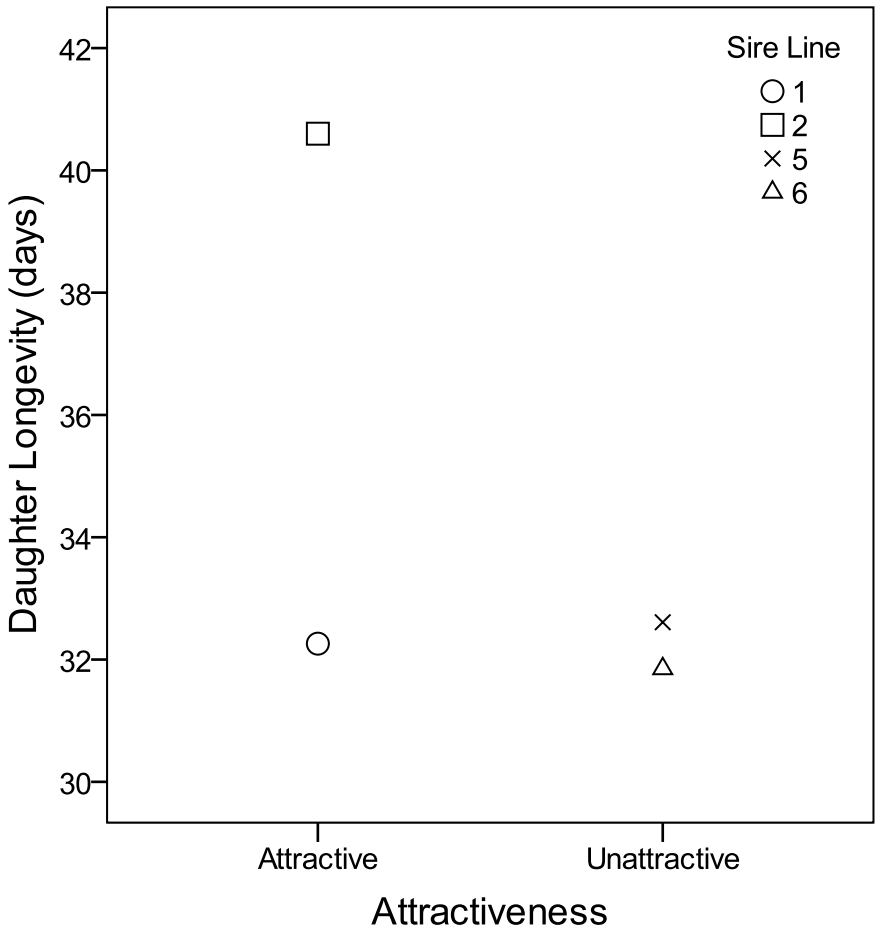

Figure 2. Interaction plot showing a significant isoline effect on daughters' longevity. The mean longevity of one attractive line is between that of the two unattractive lines and daughters of line 2 males had increased longevity. There was no overall effect of male attractiveness. On the X axis is male attractiveness and on the Y axis, longevity of daughters (days). Open circle, square, cross and triangle represent attractive (1, 2) and unattractive (5, 6) lines respectively.

Table 1. Descriptive statistics of offspring longevity and dams' LRS data for attractive (lines 1 and 2) and unattractive lines (lines 5 and 6). *N* is the number of families assessed; Mean ± se values are presented along with standard deviation estimates (SD) for each line. * Note that daughters of line 1 sires have a reduced longevity.

Sire line	Attractiveness	Mean	S.E.	S.D.	N
		Daughters' longevity			
1	Attractive	32.26*	.826	4.549	50
2	Attractive	40.60	.826	7.859	50
5	Unattractive	32.61	.826	4.661	50
6	Unattractive	31.85	.826	5.693	50
		Dams' LRS			
1	Attractive	47.06		13.622	48
2	Attractive	43.82		14.230	49
5	Unattractive	44.30		14.895	47
6	Unattractive	36.14		15.708	43

longevity. This also revealed no difference in the longevity of daughters of attractive or unattractive lines (Kruskal-Wallis $\chi^2_1 = 0.6$, $P = 0.67$). We found similar results when assessing sons' longevity, with no attractiveness effect ($F_{1,2} = 0.19$, $P = 0.70$), but again there was a line (within attractiveness) effect ($F_{2, 196} = 62.88$, $P < 0.001$). This time however post-hoc tests revealed that one attractive line (line 2) significantly elevated sons' longevity relative to all other lines (all $P < 0.001$), but the other attractive line (line 1) produced sons with longevity significantly lower than that of one unattractive line (line 6) ($P = 0.001$). There were no other significant differences.

We also found that family mean daughters' longevity was positively related with family mean sons' longevity ($\beta = 0.52 \pm 0.06$, $F_{1, 198} = 73.42$, $P < 0.001$, $R^2 = 0.27$; see Fig. 3). This relationship remained positive and significant even when we split the data across attractive and unattractive sires ($\beta_{\text{Attractive}} = 0.49 \pm 0.08$, $F_{1, 98} = 39.80$, $P < 0.001$, $R^2 = 0.29$; $\beta_{\text{Unattractive}} = 0.46 \pm 0.11$, $F_{1, 98} = 16.78$, $P < 0.001$, $R^2 = 0.15$).

Assessment of mothers' LRS revealed no direct benefits from mating with attractive males ($F_{1, 2} = 1.45$, $P = 0.35$). However, there was again a significant line effect in the model ($F_{2, 183} = 4.11$, $P = 0.02$; Table 1; Fig. 4) and post-hoc tests revealed this was due to Line 1 having significantly greater LRS than line 6 ($P < 0.01$), but none of the other comparisons differed (all $P > 0.05$).

Results of the second assessment, using two-tailed *P* values from related-samples Wilcoxon signed ranks test, suggested no differences in the LRS ($Z = -0.96$, $P = 0.34$) or longevity ($Z = -0.80$, $P = 0.42$) of daughters sired by attractive or unattractive males. Body size could potentially influence this result (i.e. if daughters of unattractive

Figure 3. Regression of female and male offspring longevity from attractive and unattractive sires. This relationship was significant even when analysed separately for offspring of attractive and unattractive sires. Data shown here are untransformed and include information from offspring of both types of sires.

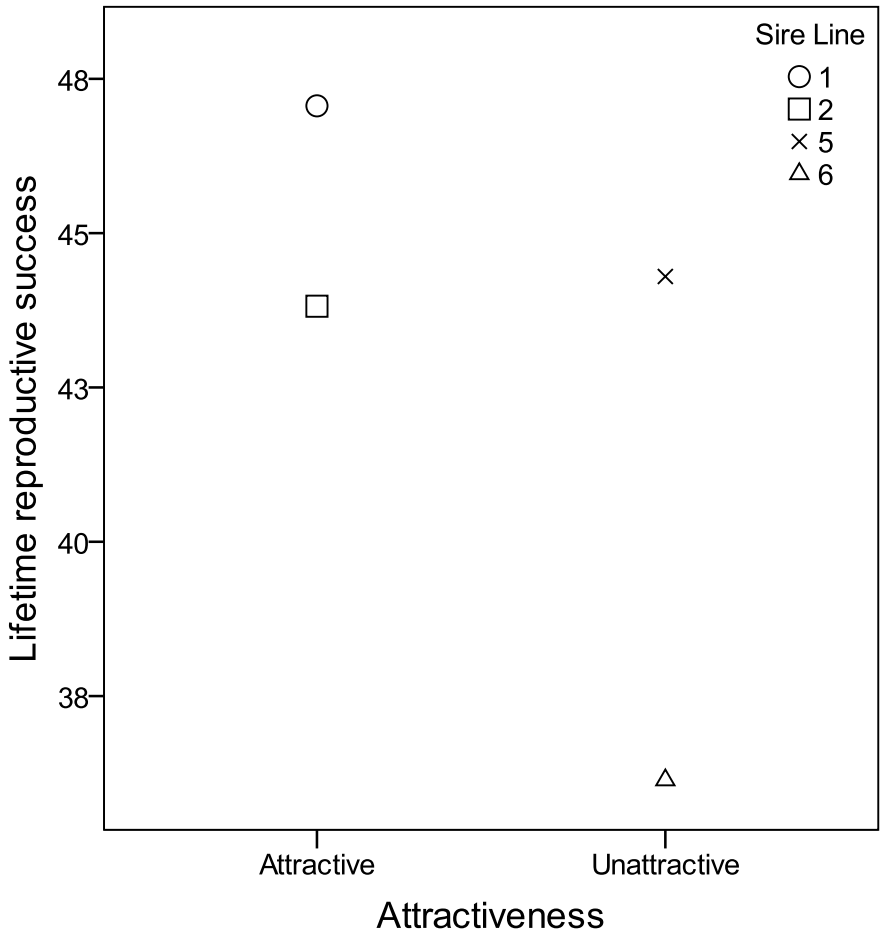

Figure 4. Interaction plot showing a significant isoline effect on dams' lifetime reproductive success. On the X axis is male attractiveness and on the Y axis, lifetime reproductive success of dams mated to attractive or unattractive sires. Open circle, square, cross and triangle represent attractive (1, 2) and unattractive (5, 6) lines respectively.

males were larger than daughters of attractive males), but we could not include size as a covariate in the previous analysis. However, a paired *t*-test of daughters' wing-length found no difference between the body size of daughters from attractive or unattractive sires (N = 38, $t = -0.24$, $P = 0.81$), which suggests the lack of difference in LRS and longevity is not due to size differences in daughters.

Discussion

Our primary aim was to establish whether attractive males produced daughters with enhanced viability, which would be consistent with good genes benefits of mate choice. We assessed daughters' fitness because theory suggests sons may trade viability for mating success, and hence it is possible that good genes effects are only manifest via daughters (Kokko 2001; Getty 2002). We have previously shown that there is genetic and phenotypic variation in female preference (e.g. Taylor et al. 2008a; Sharma et al. 2010). However, while previous studies have reported Fisherian benefits of mate choice in this species (Taylor et al. 2007; Hosken et al. 2008), evidence of good genes effects has not been documented (Taylor et al. 2010), even though these benefits are considered inevitable in sustaining female preferences (Jennions & Petrie 2000).

Here we attempted to increase our chances of detecting (potentially weak) good genes benefits by using two different approaches to assign male attractiveness. Our methods allowed us to account for the inherent variation in female mate-choice while assessing male quality and we also used multiple assessments of male attractiveness rather than one-off estimates. However, in spite using a different approach to previous work (Taylor et al 2010), we did not consistently detect evidence

of good genes benefits of mate choice. We did however find that males from one attractive isoline enhanced the longevity of daughters in one assay. This provides some evidence for a good genes effect, but our second assay, plus the lack of an overall male attractiveness effect, together with previous findings (Taylor et al. 2010) makes us reticent to conclude that attractive males generally confer good genes benefits to female *D. simulans*. Additionally, sons' longevity was not significantly influenced by sire attractiveness, although there are the caveats to this assessment we have previously discussed. It is possible that the single isoline that enhanced daughters' (and sons') longevity was relatively less inbred, or it has undergone more purging, and this accounts for the line effect we found. We have previously documented substantial inbreeding effects in our study population (Wright et al. 2008), which is consistent with this explanation. Alternatively genetic compatibility could be involved (Tregenza & Wedell 2000), but whatever the cause of the line effect, we nevertheless failed to detect a male attractiveness effect (positive or negative) *per se* on daughters' fitness (longevity or LRS). Our results therefore contrast with studies showing fathers' reproductive success negatively affecting their daughters' fitness (Fedorka & Mousseau 2004; Pischedda & Chippindale 2006; Foerster et al. 2007; Oneal et al. 2007), but are consistent with others reporting neutral or weak effects of father's reproductive success on their daughters' fitness (Norris 1993; Jones et al. 1998; Tomkins & Simmons 1999; Rundle et al. 2007; Maklakov & Arnqvist 2009), and with our previous work (Taylor et al. 2010).

It is possible that we failed to detect a sire attractiveness/daughter fitness association because of the standardised and relatively benign laboratory conditions in which assays were conducted (Hunt et al. 2004; Qvarnström & Price 2001; Schmoll et

al. 2005). This is always a possibility, but the same criticism can be made when assays take place under harsh conditions - differences are not detected because severe environments reduce phenotypic variation. Additionally, these are the same experimental conditions under which Fisherian benefits of mate choice were documented (Taylor et al. 2007), and the lack of any detectable good genes benefits is also consistent with previous findings (Taylor et al. 2008a, 2010). In a meta-analysis of good genes benefits of mate choice Møller and Alatalo (1999) found that effects sizes are usually small, and it is possible that our statistical power was simply not great enough, or that the phenotypic space covered by the isolines was small relative to total phenotypic space, obscuring associations. However, the total number of flies assayed here was large (> 200 in assay 1), and assay 2 and previous work employed wild-type males (i.e. sampled a larger proportion of phenotypic space) and also failed to detect any evidence of good genes (Taylor et al. 2010). Hunt et al. (2004) stress that total fitness and breeding values, should both be estimated to assess genetic quality accurately. We used two measures of potential good genes benefits here and previously (Taylor et al. 2010), LRS and longevity (which is the benefit originally envisaged from good genes), and still found no compelling evidence of good genes benefits of mate choice.

We have found that attractive males produce attractive, high quality sons (Taylor et al. 2007; Hosken et al. 2008), but do not produce high quality daughters (Taylor et al. 2010; current study). One mechanism that could generate this outcome is intralocus sexual conflict. Intralocus sexual conflict occurs when the gene combinations that produce a good male, produce a poor female, and negative (genetic) intersexual fitness correlations are considered to be the unmistakable

signature of this conflict (Rice & Chippindale 2001). A large number of studies have now documented evidence of intralocus sexual conflict in *Drosophila* (Rice 1984, 1992, 1998; Rice & Chippindale 2001; Prasad et al. 2007), and Innocenti and Morrow (2010) suggested that intralocus sexual conflict can potentially neutralise any indirect genetic benefits of sexual selection, including good genes effects. However, our data are not consistent with intralocus conflict obscuring good genes because we find that the longevity of sons and daughters were positively correlated, and similar findings have also been found for other insects (e.g. Hosken et al. 2003). Intra-family correlations approximate genetic correlations, especially when the traits are measured in different groups of individuals (Lynch & Walsh 1998), as here, and therefore the positive and significant intersexual association we observe is not consistent with intralocus conflict over longevity. Good genes sexual selection was initially predicated on longevity (Arnold 1983) and this is why we were interested in it, and there is no evidence that our failure to document convincing good genes effects here are due to conflict masking these benefits. Note that we are not suggesting there is no intralocus conflict in *D. simulans*, only that there is little over longevity.

We also found no direct benefits of male attractiveness, but again detected a significant line effect. The former finding is consistent with many previous assays (Taylor et al. 2008a, b) and contrasts with work on *D. melanogaster* (e.g. Pitnick & García-González 2002; Friberg & Arnqvist 2003). The elevation of mothers' LRS when mated to males from one attractive line is interesting because this is not the attractive line that elevated offspring longevity. Again the reasons for this line-specific direct effect are not clear, but could relate to differential inbreeding, which can impact on male fertility (e.g. Gage et al. 2006; Roldan & Gomendio 2009), or genetic compatibility

(Tregenza & Wedell 2000), especially because we only counted offspring and not eggs. In any case, there does appear to be some male genetic variation that directly and indirectly influences female fitness, but this does not seem to be generally related to good genes benefits however, and the most parsimonious explanation is the inbred nature of isolines.

This study was designed specifically to test for good genes benefits of mate choice and we primarily assessed this via daughters' longevity. Our results provide no evidence that females mating with attractive males generally obtain good genes benefits of mate choice, and nor do they generally gain direct benefits. The lack of sire-attractiveness/daughter-fitness association does not appear to be due to intralocus sexual conflict however, because family-level intersexual correlations were positive and significant. However, we did not find that male genetic background had effects on offspring and dam fitness. Nevertheless, current evidence is most consistent with an absence of good-genes benefits of female mate choice in our populations.

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Chapter Four

The genetics of cuticular hydrocarbon profiles in *Drosophila simulans*

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Abstract

Female mate choice is one of the mechanisms of sexual selection and variation in male traits is necessary for female choice to operate. Genetic variation is necessary for these sexually selected traits to evolve. Cuticular hydrocarbons (CHCs) are important in *Drosophila* mate choice, but relatively little is known about the underlying genetic architecture of CHC profiles in *Drosophila simulans*. Here we used gas chromatography-mass spectrometry to investigate patterns of genetic variation in the CHC profiles of male and female *D. simulans* using six iso-female lines. We found substantial genetic variation for CHC profiles and individual CHC components, and individual CHCs were frequently strongly genetically correlated, with a tendency for negative covariance between long and short chain CHCs in males. Intersexual genetic covariances were often weak and frequently differed in sign. This genetic architecture may facilitate sex-specific CHC evolution in *D. simulans*.

Keywords: cuticular hydrocarbons, isolines, genetic variation, heritability, sexual dimorphism.

Introduction

Female preference for certain males has been documented in many taxa (e.g. Ryan 1983; Moore and Moore 1988; Wilkinson and Reillo 1994; reviewed in Andersson 1994). Females base their assessment of male attractiveness on many different character types and frequently assess multiple traits before choosing their preferred mate (Candolin 2003; Hebets and Papaj 2005; Jennions and Petrie 1997; Johnstone 1997; Kodric-Brown and Nicoletto 2001; Patricelli et al. 2003). While morphological or acoustic characters have been the major focus of sexual selection studies (Andersson 1994), chemicals signals provided by insect cuticular hydrocarbons have been the subject of intensive investigation (e.g. Chenoweth and Blows 2003; Blows et al. 2004; Hine et al. 2004).

Cuticular hydrocarbons (CHCs) are found on the exoskeleton of insects. While they are subject to natural selection, influencing desiccation resistance (Lockey 1988; Rouault et al. 2004; Toolson 1982), cold-tolerance (Ohtsu et al. 1998) and starvation resistance (Hoffmann et al. 2001), they are often also key sexual signals (see Howard and Blomquist 2005 for a review), although this role is frequently poorly understood (Johansson and Jones 2007; Johansson et al. 2005). Several recent studies have examined variation in CHCs, and overall these confirm that CHCs are highly sexually dimorphic in many species, with many of the individual components being sex-specific, as one would expect with secondary sexual traits (see Thomas and Simmons 2008 for a review). For example, crickets often have distinct male and female CHC profiles (Warthen and Uebel 1980; Tregenza and Wedell 1997; Mullen et al. 2007). Additionally, even when a CHC occurs in both sexes, the quantities produced by males and females can differ substantially (Thomas and Simmons 2008). Such differences

have been reported in mosquitoes, ticks and fireflies (Anyanwu et al. 2000; Caputo et al. 2005; Estrada-Peña et al. 1996; South et al. 2008), and CHCs are also reported to evolve rapidly and are sexually dimorphic in grasshoppers and crickets (Mullen et al. 2007; Neems and Butlin 1995; Buckley et al. 2003; Thomas and Simmons 2008).

When compared to many other insects, *Drosophila* have a relatively small number of CHCs (< 30 vs. > 100; Howard 1993; Howard and Blomquist 2005), and there is considerable divergence in the chain length, the number or position of double bonds and sexual dimorphism across *Drosophila* (see Ferveur 2005 for a review). Several recent studies have reported substantial genetic variation in *Drosophila* CHCs (e.g. Foley et al. 2007), while others find the majority of this genetic variation is not available for selection due to its orientation relative to the direction of sexual selection (e.g. Blows et al. 2004; Hine et al. 2004; Van Homrigh et al. 2007). More recently McGuigan and Blows (2009) investigated standing genetic variance underlying high and low fitness *D. bunnanda* phenotypes and found substantial genetic variation in low, but not in high fitness males. However, most studies exploring genetic variation and sexual dimorphism in *Drosophila* CHCs are restricted to the Hawaiian radiation (Alves et al. 2010), to *D. melanogaster* (e.g. Foley et al. 2007; Antony and Jallon 1982; Jallon 1984), *D. serrata* (Chenoweth and Blows 2003; Hine et al. 2004) or *D. virilis* (Bartelt et. al. 1986). In contrast, *D. simulans* CHCs remain relatively unexplored in the sexual selection literature, in spite of recent focus on sexual selection in this species (Taylor et al. 2007, 2008a, 2008b, 2009, 2010; Hosken et al. 2008).

Here we use six iso-female lines that have been employed in previous sexual selection investigations to document the genetic architecture of CHC profiles in *D. simulans*.

Taylor et al. (2007) have shown that male attractiveness is heritable in this population and more recently, the presence of genetic variation in female preference and male attractiveness has been established for these isolines (Sharma et al. 2010). Sexual dimorphism has been reported in *D. simulans* CHC peaks, and it has been suggested that CHCs play an important role in determining male attractiveness (Ferveur and Cobb 2010). However little is known about the quantitative genetics of CHCs in *D. simulans*. Here we confirm that CHCs profiles are sexually dimorphic in *D. simulans*, and that there is sex-specific genetic variation in these profiles. We also show that many individual components of the CHC profile are heritable and there is substantial genetic covariation between these components within each sex, with long and short chains tending to negatively genetically covary in males. Moreover, the negative inter-sexual genetic correlations between individual CHCs we document here potentially allows for sex specific evolution of CHC profiles.

Material and Methods

Fly stocks

We used six *Drosophila simulans* isolines, supplied by the Centre for Environmental Stress and Adaptation Research, La Trobe University, Australia. These were collected in 2004 and have since been maintained in multiple large population vials at a density >50 pairs per vial. All flies were reared on '*Drosophila* quick mix medium' (supplied by Blades Biological, Edenbridge, Kent, U.K.) at 25°C and a 12:12 h light:dark cycle. Food was provided in excess so that differential larval competition was minimised. Subsequent housing conditions followed this regime unless stated. We collected 8 virgin males and females from each isoline and housed them individually to prevent social interactions from altering CHC profiles. Visual stimuli are known to trigger CHC

profile changes in *Drosophila*, we therefore isolated housing vials with translucent polypropylene partitions that allow light passage but blur images sufficiently to prevent recognition. Individuals were processed for CHC extraction when they were three days old since adult CHC profiles are completely developed by this time (Antony and Jallon 1981).

Hydrocarbon extraction

To quantify CHCs, individual flies were soaked in 50 ul Hexane containing 100ng of pentadecane as an internal standard. After 4 minutes of soaking, vials were vortexed for 60 seconds to maximize extraction. A 1 ul sample of each fly extract was then injected into a GCMS (Agilent 7890A GC coupled with an Agilent 5975B Mass spectrometer) operating in pulsed split-less mode and fitted with a DB-1ms column (340 °C: 30 m x 250 µm x 0.25 µm) (J&W 122-0132 by J&W Scientific, 91 Blue Ravine Road, Folsom, CA 95630-4714, U.S.A.) using helium as a carrier gas. Extract separation was optimised using a column temperature profile in which the analysis began at a temperature of 70 °C for 1 minute and then rose by 20°C/min to 240°C followed by a 4°C/min rise to 320°C. The inlet and the transfer line from the GC to the MS were set at 250°C. Chromatograms were acquired and analyzed using MSD Chemstation software version E.02.00.493 (Agilent, Foster City, CA).

We analysed extracts derived from 96 flies (8 individual males and females from each of the six iso-lines), along with pentadecane control standards that were loaded at the start and end of each run to check for contamination. CHC peaks were labelled by peak number, which corresponded to their retention times on the GC (see Fig. 1a, b; Table 1). In total, 18 unique CHC peaks were identified and the area under these peaks

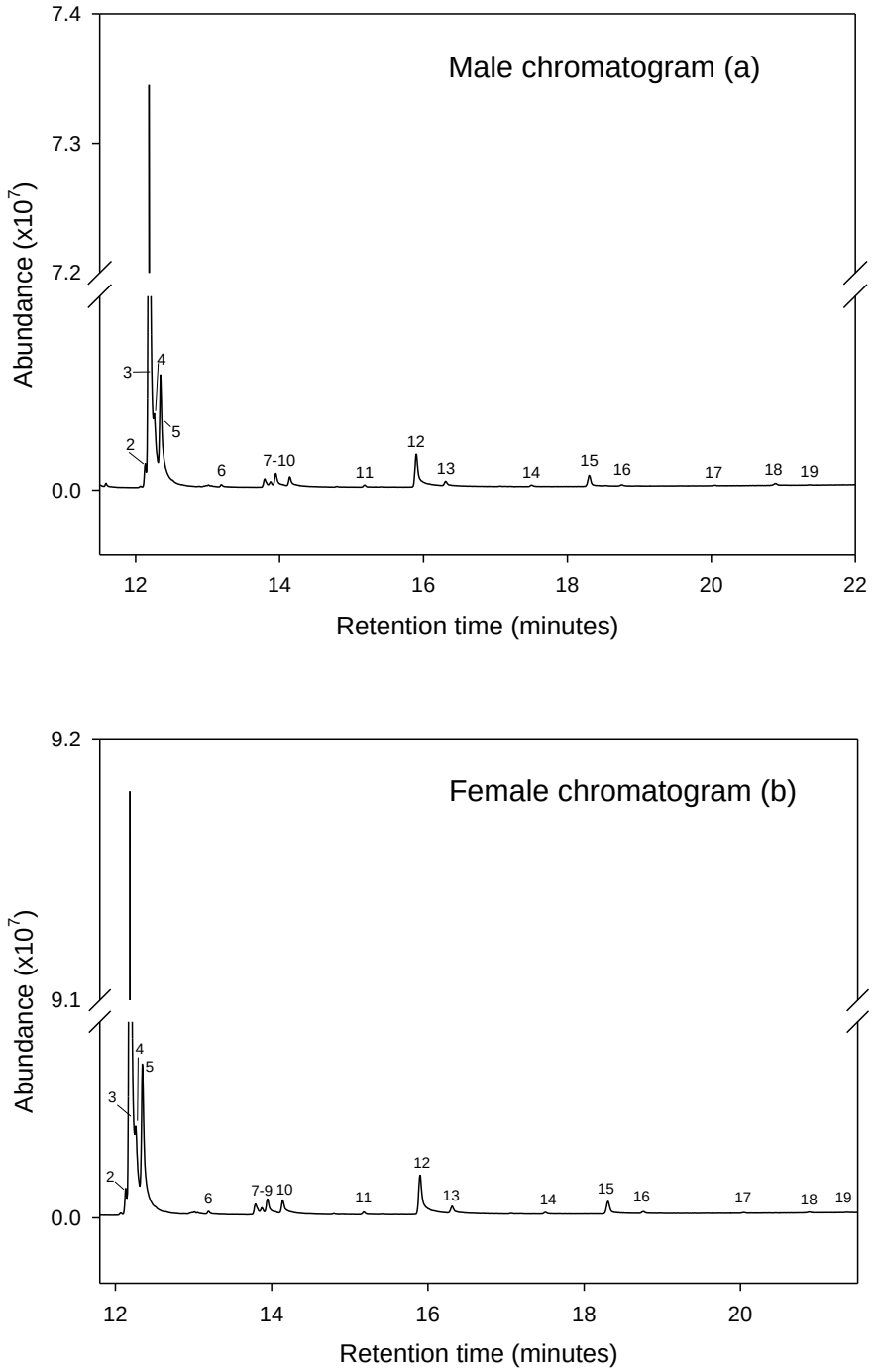

Fig. 1 Typical GC profile for male **(a)** and female **(b)** *Drosophila simulans*. The *x*-axis shows the retention time and the *y*-axis the response from the ionisation detector. Peak numbers are indicated (2-19; see Table 1 for details). Note that peak 1 has been left out to improve visibility of other peaks

Table 1 Mean relative contribution of the 18 cuticular hydrocarbon compounds identified on *Drosophila simulans*, and their retention times, names, formulae and molecular weights

						Male		Female	
Name	Peak	RT		Formula	MW	Mean	SE	Mean	SE
Pentadecane		7.54		C15H32	212				
ISTD	CHC1					-	-	-	-
?-Tricosene	CHC2	12.13		C23H46	322	0.099	0.012	0.092	0.006
7-Tricosene	CHC3	12.19		C23H46	322	4.101	0.394	4.608	0.269
?-Tricosene	CHC4	12.26		C23H46	322	0.483	0.038	0.534	0.025
Tricosane	CHC5	12.35		C23H48	324	1.080	0.084	1.181	0.061
Branched alkane	CHC6	13.20		C24H50	338	0.015	0.001	0.015	0.001
Branched alkane	CHC7	13.79		C25H52	352	0.074	0.011	0.061	0.006
Pentacosene	CHC8	13.88		C25H50	350	0.035	0.004	0.030	0.002
Pentacosene	CHC9	13.95		C25H50	350	0.100	0.010	0.099	0.006
Pentacosane	CHC10	14.14		C25H52	352	0.085	0.009	0.098	0.009
Alkane	CHC11	15.19				0.012	0.001	0.013	0.001
Branched alkane	CHC12	15.91		C27H56	380	0.397	0.045	0.297	0.030
Heptacosane	CHC13	16.31		C27H56	380	0.044	0.011	0.046	0.005
Alkane	CHC14	17.50				0.010	0.001	0.011	0.001
Branched alkane	CHC15	18.30		C29H60	408	0.079	0.008	0.098	0.013
Alkane*	CHC16	18.76		C29H60*	408	0.010	0.001	0.012	0.001
Alkane*	CHC17	20.05		C30H62*	422	0.006	0.001	0.005	0.001
Alkane*	CHC18	20.88		C31H64*	436	0.011	0.003	0.008	0.001
Alkane*	CHC19	21.35		C31H64*	436	0.004	0.000	0.003	0.000

* Trace levels therefore identification is tentative.

were quantified and expressed proportional values by dividing by the pentadecane standard (Peak 1). Use of the internal standard in calculating proportions eliminates the problems of unit-sum-constraints faced when proportions are calculated relative to sum of all peaks. To ensure multivariate normality we $\log_{10}$ transformed our data prior to analysis.

Statistical analysis

Drosophila hydrocarbons are known to show variation in the overall CHC composition or "bouquet", and also in the absolute amounts of individual components (Jallon 1984; Ferveur and Jallon 1996; Luyten 1982). We therefore analysed and interpreted our data in two ways, firstly by examining the overall CHC composition and then looking at individual components. We used principal component analysis (PCA) to reduce the dimensionality of the CHC profile, because otherwise we would have too many dependent variables and too few degrees of freedom. As PCA summarises a pattern of correlation among variables, it may be possible to interpret the resulting components in terms of functional hypotheses (Moore 1997). Furthermore, PC scores can then be utilised in additional analyses. Note that PC scores describe different and independent aspects of underlying variation as the PCs are orthogonal to each other (Tabachnick and Fidell 1989).

All statistical analyses were performed with PASW (version 18) unless stated otherwise. PCA was performed and the PCs with eigenvalues greater than 1 (Norman and Streiner 2008) were then examined with a multivariate analysis of variance (MANOVA). Isoline and sex were included as main (fixed) effects in the model and the three PCs were entered as dependent variables.

We also estimated the heritability of CHCs for both males and females, as well as the genetic correlations between CHCs both within and across the sexes. These were estimated in two ways. Firstly by using the PC scores to provide estimates for the CHC blends, and then by using the individual $\log_{10}$ CHC proportions. In both instances, heritability was estimated as the coefficient of intraclass correlation (*t*) (Hoffmann and Parsons 1988; David et al. 2005) as:

$$t = \frac{V_b - V_w / n}{V_b + (n-1)V_w / n} = \frac{nV_b - V_w}{nV_b + (n-1)V_w}$$

Here *n* is the number of lines and V_b and V_w are the between line and within line variance components, respectively, estimated directly from an ANOVA including line as the main effect. The standard error of the intraclass correlation (SE (*t*)) was calculated according to Becker (1984) as:

$$SE(t) = \sqrt{\frac{2(1-t)^2[1+(k-1)t]^2}{k(k-1)(n-1)}}$$

Genetic correlations (and their standard errors) for CHC peaks within and between sexes were estimated using the jackknife method of Roff and Preziosi (1994). In short, this procedure first estimates the genetic correlation between two traits using mean estimates for each line (Via 1984; Gibert et al. 1998). A sequence of *N* pseudo-values is then computed by dropping each of the lines in turn and estimating the resulting correlations and using the formula:

$$S_{N,i} = Nr_N - (N-1)r_{N-1,i}$$

where $S_{N,i}$ is the i^{th} pseudo-value, r_N is the genetic correlation estimated using the means of all N inbred lines and $r_{N-1,i}$ is the genetic correlation obtained by dropping the i^{th} inbred line alone (Roff and Preziosi 1994). The jacknife estimate of the genetic correlation (r_j) is then simply the mean of the pseudo-values, and an estimate of the standard error (SE) is given by:

$$SE = \frac{\sum_{i=1}^{i=N} (S_{N,i} - r_j)^2}{N(N-1)}$$

Using simulation models, Roff and Preziosi (1994) showed that this jacknife approach provides better genetic estimates than those based on conventional inbred line means when the number of inbred lines contained in the analysis is small (< 20 lines), as occurs in our study. It is important to note that estimates of genetic (co)variance from inbred lines contain variance due to dominance and/or epistasis and therefore should be considered broad-sense estimates (Falconer and Mackay 1996).

We then examined the male and female genetic correlation matrices (obtained via the PC scores and those obtained from individual CHC components) for information concerning the relatedness of CHC traits (*sensu* Cheverud 1988), looking for highly (genetically) related PCs or CHC peaks, which are likely to co-evolve (Lande 1980; Cheverud 1988). The overall integration of the male and female genetic correlation (r_G) matrices was assessed by using Mantel's randomisation test (Mantel 1967) with ZT (Bonnet and Peer 2002) and was based on 10,000 randomisations. Here the observed matrix correlation is compared to an empirically derived distribution of matrix correlations and the proportion of randomly permuted matrix correlations exceeding

the observed one gives an estimate of the probability of obtaining a matrix correlation greater than the observed one by chance. If the probability is low ($P < 0.05$) then the matrices are more similar than by chance alone. Note that a significant association would indicate that the intra-sexual genetic correlations vary in similar directions and not that the values of any elements are identical in magnitude.

Additionally, we calculated the average absolute values of the correlations in each matrix along with the average disparity between the two matrices. In brief, we summed the absolute values of all off-diagonal correlations and divided by the number of correlation pairs to arrive at the average absolute value of correlation.

$$\bar{X} = \frac{\sum |r_{i,j}|}{n}, \; for \; i \neq j$$

where $r_{i,j}$ refers to the correlation between characters *i* and *j*, and *n* is the number of correlation pairs. The average disparity between corresponding male and female genetic correlation matrices was determined by averaging the absolute values of differences between correlation pairs.

$$D = \frac{\sum | r_{MG,i,j} - r_{FG,i,j} |}{n}, \; for \; i \neq j$$

where $r_{MG,i,j}$ and $r_{FG,i,j}$ refer to male and female genetic correlations between characters *i* and *j*, and *n* is the total number of correlation pairs (Willis et al. 1991; Roff 1995; Waitt and Levin 1998). *D* indicates the overall difference in the magnitude of

association between the matrices, whilst the average, absolute correlation values indicate the overall size of correlation within each matrix.

Results

Analysis of mass spectra and the retention times allowed us to distinguish 18 CHC peaks. These were shared by male and female *D. simulans* (see Fig. 1a, b). No qualitative differences were found between isolines or sexes (i.e. no sex-specific CHC components were detected). However, we did find evidence of quantitative differences both between isolines and sexes (i.e. the same CHC components were expressed to different degrees in the sexes, Table 1).

PCA of the individual CHC components returned three PCs that had eigenvalues greater than 1 and these collectively explained 81% on the variance in CHC composition (Table 2). Correlations between the individual CHC components and the derived PC scores (factor loadings in Table 2) were used to examine the CHC components that contributed the most to each PC. All factor loadings greater than 0.3 were interpreted as biologically important (Tabachnick and Fidell 1989). PC1 was weighted positively by peaks 2-13 (Tricosene, Tricosane, branched alkanes, Pentacosene, Pentacosane and Heptacosane) and negatively by peak 18 (alkane). PC2 was weighted negatively by peak 4 and positively by peaks 6,11, 13-19 (Tricosene, branched alkane, alkane, Heptacosane etc.) and PC3 positively by peaks 13, 15, 18 and negatively by peak 19 (Heptacosane, branched alkane and alkanes). Thus PC1 largely described the content of shorter chain CHCs (plus a trade-off with a single longer chain component), while PC2 largely describes longer chain CHCs (and one trade-off with a Tricosene), and PC3 described trade-offs within the longer chained hydrocarbons.

Table 2 Overall principal component analysis for CHCs. Principal components with an eigenvalue greater than 1 are retained for further analysis. Correlation between CHC peak (log_{10} concentrations) and the three components extracted from the overall principal component analysis are presented as factor loadings. ♂♀ indicates significant heritability in both males and females; ♂ significant heritability in males only; ♀ in females only. Peaks that contribute significantly (loading > 0.3) to the principal components are in bold

		Principal component		
		PC1	**PC2**	**PC3**
Eigenvalue		8.43	4.38	1.73
% variance		46.84	24.35	9.63
Loadings	**Peak**			
?-Tricosene	CHC2 ♂♀	**0.888**	-0.173	0.045
7-Tricosene	CHC3 ♂♀	**0.951**	-0.216	-0.002
?-Tricosene	CHC4 ♂♀	**0.898**	**-0.320**	0.008
Tricosane	CHC5 ♂♀	**0.926**	-0.247	0.084
Branched alkane	CHC6 ♂	**0.684**	**0.539**	-0.005
Branched alkane	CHC7 ♂♀	**0.884**	-0.231	-0.126
Pentacosene	CHC8 ♂♀	**0.851**	-0.169	0.079
Pentacosene	CHC9 ♂♀	**0.922**	-0.089	0.156
Pentacosane	CHC10♂♀	**0.858**	0.249	0.248
Alkane	CHC11	**0.352**	**0.874**	-0.161
Branched alkane	CHC12 ♂♀	**0.870**	-0.182	-0.091
Heptacosane	CHC13 ♀	**0.528**	**0.469**	**0.384**
Alkane	CHC14	0.265	**0.808**	-0.238
Branched alkane	CHC15 ♂♀	-0.047	**0.404**	**0.822**
Alkane*	CHC16 ♀	0.132	**0.838**	0.006
Alkane*	CHC17	0.176	**0.807**	-0.261
Alkane*	CHC18 ♂♀	**-0.473**	**0.378**	**0.660**
Alkane*	CHC19	0.091	**0.608**	**-0.444**

* Trace levels therefore identification is tentative.

Multivariate analysis of the PC scores, using isoline and sex as a fixed factors and the three PC scores as dependent variables indicated isoline (Wilk's $\lambda = 0.224$; $F_{15,227} = 10.87$; $p < 0.001$), sex (Wilk's $\lambda = 0.898$; $F_{3,82} = 3.096$; $p < 0.031$) and their interaction (Wilk's $\lambda = 0.475$; $F_{15,226.767} = 4.68$; $p < 0.001$) all significantly influenced the multivariate combination of PCs. Post-hoc univariate analyses indicated that the isolines effect was driven by PC1 ($F_{5,84} = 8.47$; $p < 0.001$) and PC3 ($F_{5,84} = 15.08$; $p < 0.001$). The same PCs were also responsible for the sex (PC1, $F_{5,84} = 5.3$; $p < 0.02$; PC3, $F_{5,84} = 5.99$; $p < 0.02$) and the interaction effect (PC1, $F_{5,84} = 11.38$; $p < 0.001$; PC3, $F_{5,84} = 2.95$; $p < 0.02$. Fig. 2a, b). PC2 was not significantly influenced by any factor or interaction (all $p > 0.05$).

Sexes differed in the heritability estimates (*t*) calculated for the overall CHC blends (based on PC scores), but both PC1 and PC3 were heritable in males and females, while PC2 was not heritable in either sex (Table 3). Heritability estimates based on the individual CHC components show that peaks 11, 13, 14, 16, 17 and 19 were not heritable in males and peaks 6, 11, 14, 17 and 19 were not heritable in females (Table 4). Genetic correlations for individual hydrocarbon peaks within each sex indicated that the average correlation within the male matrix was 0.177 (Table 5). The overall magnitude of genetic correlation, as measured by the average absolute value of correlations, had a value of 0.740 for males. For females, the genetic correlation matrix had an average correlation of 0.251 (Table 5). In this case the average absolute value of the correlation was 0.92.

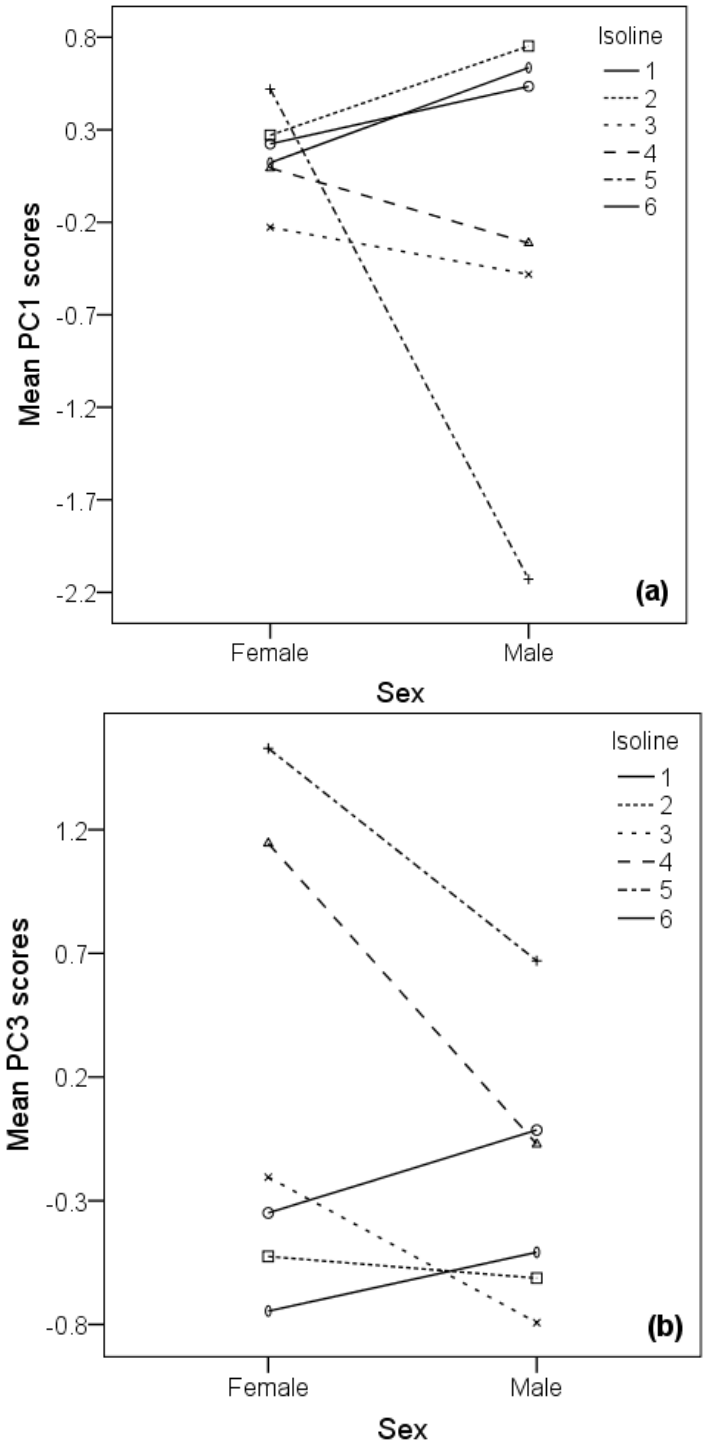

Fig. 2 (a) Graph representing the isoline*sex interaction showing how PC1 varies between isolines and sexes. **(b)** Isoline*sex interaction for PC3. Mean PC1 or PC3 scores for each isoline and each sex are plotted on the respective Y axis

Table 3 Intraclass correlation coefficient (*t*) ± SE for male and female cuticular hydrocarbons in *Drosophila simulans*. These values have been calculated based on the extracted principal components, and are indicative of the "CHC bouquet" heritability

Male *t*			**Female *t***		
PC1	0.915	± 0.053	PC1	0.53	± 0.187
PC2	0.098	± 0.128	PC2	0.166	± 0.152
PC3	0.778	± 0.121	PC3	0.92	± 0.050

Table 4 Intraclass correlation coefficient (*t*) for male and female cuticular hydrocarbons in *Drosophila simulans*

Name	Peak	**Male *t* ± SE**		**Female *t* ± SE**	
?-Tricosene	CHC2	0.938	± 0.040	0.876	± 0.075
7-Tricosene	CHC3	0.945	± 0.035	0.807	± 0.108
?-Tricosene	CHC4	0.927	± 0.046	0.711	± 0.146
Tricosane	CHC5	0.889	± 0.068	0.821	± 0.102
Branched alkane	CHC6	0.540	± 0.186	0.000	± 0.000
Branched alkane	CHC7	0.934	± 0.042	0.902	± 0.061
Pentacosene	CHC8	0.962	± 0.025	0.408	± 0.193
Pentacosene	CHC9	0.925	± 0.047	0.790	± 0.116
Pentacosane	CHC10	0.776	± 0.122	0.765	± 0.126
Alkane	CHC11	0.000	± 0.000	0.000	± 0.000
Branched alkane	CHC12	0.900	± 0.062	0.897	± 0.064
Heptacosane	CHC13	0.000	± 0.000	0.877	± 0.074
Alkane	CHC14	0.000	± 0.000	0.000	± 0.000
Branched alkane	CHC15	0.912	± 0.055	0.923	± 0.048
Alkane*	CHC16	0.000	± 0.000	0.625	± 0.170
Alkane*	CHC17	0.000	± 0.000	0.000	± 0.000
Alkane*	CHC18	0.935	± 0.042	0.955	± 0.029
Alkane*	CHC19	0.000	± 0.000	0.000	± 0.000

* Trace levels therefore identification is tentative.

Table 5 Jacknifed intrasexual genetic correlation (r_G) matrix (male and female CHCs). Male genetic correlations are above the diagonal and females' below the diagonal. Correlation values greater than |0.5| are indicated in bold italics. Note that standard errors are not shown for aesthetic reasons

Peak	CHC 2	CHC 3	CHC 4	CHC 5	CHC 6	CHC 7	CHC 8	CHC 9	CHC 10	CHC 11	CHC 12	CHC 13	CHC 14	CHC 15	CHC 16	CHC 17	CHC 18	CHC 19
CHC 2		**0.737**	**0.877**	**0.922**	**0.676**	**0.958**	**0.949**	**1.011**	**0.892**	0.456	**0.951**	**1.049**	0.013	**-1.107**	-0.154	0.249	**-1.059**	-0.194
CHC 3	**0.921**		**0.994**	**1.009**	0.490	**0.786**	**0.772**	**1.029**	**0.921**	**0.745**	**0.765**	**1.155**	-0.333	**-1.330**	0.196	0.081	**-1.192**	-0.016
CHC 4	**0.762**	**0.942**		**1.012**	0.402	**0.716**	**0.700**	**1.013**	**0.903**	**0.798**	**0.687**	**1.155**	-0.447	**-1.381**	0.319	0.020	**-1.232**	0.066
CHC 5	**0.902**	**0.931**	**0.926**		0.429	**0.762**	**0.743**	**1.000**	**0.869**	**0.763**	**0.727**	**1.153**	-0.418	**-1.391**	0.256	0.047	**-1.186**	0.048
CHC 6	0.321	**0.557**	**0.816**	**0.885**		**0.712**	**0.712**	**0.663**	**0.797**	0.421	**0.790**	**0.569**	0.383	**-0.515**	-0.417	**0.637**	**-0.519**	**-0.777**
CHC 7	**0.575**	**0.741**	**0.692**	**0.467**	-0.033		**0.979**	**0.883**	**0.766**	0.150	**0.981**	**0.870**	0.107	**-0.900**	-0.243	0.429	**-0.836**	-0.257
CHC 8	**0.585**	0.175	-0.018	0.277	-0.349	-0.023		**0.904**	**0.828**	0.132	**0.994**	**0.875**	0.335	**-0.891**	-0.415	0.237	**-0.823**	-0.387
CHC 9	**0.676**	0.316	0.111	**0.573**	0.185	-0.371	**0.667**		**0.959**	**0.542**	**0.906**	**1.021**	0.122	**-1.235**	-0.206	0.027	**-1.068**	-0.346
CHC 10	**0.558**	**0.532**	**0.646**	**0.871**	**0.975**	-0.314	0.176	**0.712**		0.471	**0.850**	**0.938**	0.249	**-0.990**	-0.258	0.132	**-0.897**	**-0.636**
CHC 11	-0.093	-0.123	-0.005	0.239	**0.672**	**-0.866**	-0.180	**0.523**	**0.811**		0.203	**0.649**	-0.186	**-0.553**	0.067	-0.063	**-0.762**	-0.065
CHC 12	0.282	**0.611**	**0.632**	**0.451**	**0.485**	**0.757**	**-0.666**	-0.368	-0.009	-0.370		**0.874**	0.308	**-0.893**	-0.397	0.332	**-0.825**	-0.439
CHC 13	0.242	0.001	0.046	0.371	0.418	**-0.719**	**0.512**	**0.746**	**0.803**	**0.823**	**-0.713**		0.068	**-1.367**	-0.155	-0.121	**-1.290**	-0.186
CHC 14	**0.824**	**0.704**	**0.637**	**0.724**	0.151	**0.617**	**0.766**	0.394	0.293	-0.419	0.105	0.125		0.403	**-1.269**	-0.406	0.347	**-0.725**
CHC 15	**0.632**	**0.716**	**0.884**	**0.963**	**1.133**	0.074	0.262	0.482	**0.977**	**0.571**	0.062	**0.696**	**0.563**		-0.277	-0.197	**1.185**	-0.011
CHC 16	0.328	-0.013	-0.068	0.206	-0.052	**-0.562**	**0.789**	**0.655**	**0.503**	**0.514**	**-0.931**	**0.894**	0.293	**0.516**		0.411	-0.119	**0.678**
CHC 17	**0.609**	**0.594**	0.398	**0.565**	0.349	0.151	-0.263	0.474	0.446	0.338	**0.530**	0.001	-0.074	0.187	-0.191		-0.112	-0.236
CHC 18	0.339	0.157	0.244	**0.520**	**0.561**	**-0.576**	**0.500**	**0.698**	**0.857**	**0.785**	**-0.601**	**0.984**	0.254	**0.815**	**0.871**	-0.015		0.021
CHC 19	-0.447	**-0.518**	**-0.550**	-0.342	-0.091	-0.479	-0.190	0.228	-0.029	0.164	0.144	-0.100	-0.395	-0.448	**-0.487**	0.146	-0.227	

Intersexual genetic correlations (estimated on PC scores) were both positive and negative in sign (Table 6), and a similar trend was seen when genetic correlations were calculated for the individual peaks (Table 7). However, many of the correlations for individual peaks were low (42% were less than 0.5), which indicates the sexual dimorphism for CHCs in a relatively advanced stage (Lande 1980). Furthermore, most of the correlations above 0.5 were negative: in fact, 66% of all the intersexual correlations were negative. Mantel's test indicated that the matrix describing male CHCs significantly differed from the one that described females ($\rho < 0.001$; $P = 0.54$).

Whilst the average absolute value of correlation gives an indication of the overall strength of the individual correlations within a matrix, it can obscure the actual disparity between corresponding individual male and female genetic correlations. A large positive matrix correlation would indicate that male and female correlations vary in similar directions, but it does not provide any information to confirm if the magnitudes of individual correlations are identical. Considering matrix correlations together with average Disparity (*D*) estimates helps resolve this issue. The average disparity between male and female correlation matrices was 0.362, indicating that the individual elements of the male and female matrices are very different from each other.

Table 6 Jacknifed intersexual genetic correlation (r_G) matrix (i.e. male vs. female) of CHC principal components (± SE). Significant correlations are indicated in bold italics

	Male		
Female	PCA1	PCA2	PCA3
PCA1	-0.722 ± 0.799	0.774 ± 0.694	***0.888*** ± 0.056
PCA2	***-0.766*** ± 0.011	***0.759*** ± 0.022	***0.752*** ± 0.025
PCA3	***-0.905*** ± 0.024	***0.898*** ± 0.044	***0.876*** ± 0.049

Table 7 Jacknifed intersexual genetic correlation (r_G) matrix (i.e. male vs. female) of individual CHCs. Heritability (t) estimates form the first column (female) and row (male). Correlation values greater than |0.5| are indicated in bold italics. Note that the values on the diagonal indicate same peak correlations between males and females. Standard errors are not shown

Peak	**Male**	CHC 2	CHC 3	CHC 4	CHC 5	CHC 6	CHC 7	CHC 8	CHC 9	CHC 10	CHC 11	CHC 12	CHC 13	CHC 14	CHC 15	CHC 16	CHC 17	CHC 18	CHC 19
Female	***t***	***0.94***	***0.95***	***0.93***	***0.89***	***0.54***	***0.93***	***0.96***	***0.93***	***0.78***	***0.00***	***0.90***	***0.00***	***0.00***	***0.91***	***0.00***	***0.00***	***0.94***	***0.00***
CHC 2	***0.88***	-0.297	***-0.673***	***-0.784***	***-0.714***	0.009	-0.138	-0.001	-0.313	-0.257	-0.252	-0.032	-0.346	***1.161***	***0.647***	***-1.005***	-0.430	***0.578***	-0.352
CHC 3	***0.81***	-0.419	***-0.911***	***-1.052***	***-0.975***	0.114	-0.146	-0.105	***-0.540***	-0.421	-0.326	-0.100	***-0.591***	***0.832***	***0.755***	***-0.821***	0.059	***0.795***	-0.301
CHC 4	***0.71***	***-0.696***	***-1.183***	***-1.316***	***-1.231***	-0.103	-0.361	-0.382	***-0.842***	***-0.704***	-0.471	-0.373	***-0.889***	***0.519***	***0.935***	***-0.520***	0.235	***1.023***	-0.073
CHC 5	***0.82***	***-0.847***	***-1.287***	***-1.409***	***-1.339***	-0.208	***-0.561***	-0.481	***-0.889***	***-0.662***	-0.494	-0.491	***-0.964***	***0.836***	***0.993***	***-0.750***	-0.188	***1.115***	-0.267
CHC 6	***0.00***	***-1.034***	***-1.202***	***-1.227***	***-1.274***	-0.339	***-0.855***	***-0.862***	***-1.050***	***-0.855***	-0.483	***-0.840***	***-1.050***	0.002	***1.167***	0.088	0.249	***1.279***	-0.072
CHC 7	***0.90***	0.319	-0.194	-0.335	-0.269	0.419	***0.601***	***0.538***	-0.010	-0.067	-0.398	***0.532***	-0.082	0.471	0.057	***-0.614***	***0.545***	0.185	-0.152
CHC 8	***0.41***	-0.024	-0.040	-0.030	-0.017	***-0.565***	-0.089	0.069	0.052	-0.150	-0.216	-0.049	0.155	***0.584***	0.096	***-0.544***	***-0.937***	0.028	0.271
CHC 9	***0.79***	-0.425	-0.399	-0.394	-0.409	-0.259	***-0.507***	-0.275	-0.170	-0.038	-0.047	-0.319	-0.160	***0.833***	0.496	***-0.745***	***-0.837***	0.410	-0.363
CHC 10	***0.77***	***-0.997***	***-1.055***	***-1.055***	***-1.094***	-0.493	***-0.930***	***-0.860***	***-0.990***	***-0.743***	-0.318	***-0.860***	***-1.050***	0.431	***0.967***	-0.280	-0.417	***1.072***	-0.125
CHC 11	***0.00***	***-0.793***	***-0.564***	-0.500	***-0.549***	-0.352	***-0.905***	***-0.865***	***-0.592***	-0.374	0.282	***-0.830***	***-0.571***	-0.113	***0.627***	0.292	-0.327	***0.543***	-0.044
CHC 12	***0.90***	-0.010	-0.467	***-0.605***	***-0.546***	***0.632***	0.323	0.261	-0.201	0.045	-0.422	0.321	-0.405	0.429	0.257	-0.385	***0.951***	0.424	***-0.609***
CHC 13	***0.88***	***-0.754***	***-0.528***	-0.450	-0.490	***-0.738***	***-0.891***	***-0.798***	***-0.574***	***-0.536***	0.089	***-0.831***	-0.472	0.068	***0.637***	0.017	***-0.820***	***0.541***	0.288
CHC 14	***0.00***	-0.218	***-0.554***	***-0.629***	***-0.569***	-0.387	-0.020	0.054	-0.345	-0.444	***-0.640***	-0.034	-0.352	***0.761***	0.491	***-0.712***	-0.454	***0.526***	0.127
CHC 15	***0.92***	***-1.035***	***-1.147***	***-1.168***	***-1.123***	***-0.656***	***-0.876***	***-0.868***	***-1.093***	***-0.949***	-0.500	***-0.876***	***-1.199***	0.342	***1.076***	-0.222	-0.337	***1.022***	0.222
CHC 16	***0.63***	-0.423	-0.205	-0.130	-0.158	***-0.704***	***-0.622***	***-0.531***	-0.281	-0.428	0.317	***-0.586***	-0.073	0.045	0.349	-0.119	***-0.971***	0.200	***0.515***
CHC 17	***0.00***	-0.115	-0.332	-0.423	-0.400	***0.687***	-0.066	0.050	0.003	0.317	0.334	0.129	-0.081	***0.772***	0.393	***-0.804***	0.173	0.328	***-0.872***
CHC 18	***0.96***	***-0.835***	***-0.667***	***-0.600***	***-0.632***	***-0.789***	***-0.924***	***-0.850***	***-0.720***	***-0.686***	-0.046	***-0.884***	***-0.640***	0.085	***0.761***	-0.013	***-0.758***	***0.681***	0.346
CHC 19	***0.00***	0.117	0.283	0.314	0.274	0.263	0.082	0.208	0.391	***0.593***	-0.299	0.199	0.219	0.069	-0.300	0.212	-0.008	-0.273	***-0.623***

Discussion

CHCs seem to play a major role in Dipteran mate choice and have been investigated thoroughly in several *Drosophila* species (e.g. Hine et al. 2004; Chenoweth et al. 2008; Everaerts et al. 2010; Alves et al. 2010; Liimatainen and Jallon 2007). Relatively less attention has been paid to *Drosophila simulans*, despite recent investigations of sexual selection in this species and the differences between it and its more thoroughly studied sister species *D. melanogaster* (Taylor et al. 2007, 2008a, 2008b; Hosken et al. 2008; Sharma et al. 2010). Here we used six isolines to investigate the quantitative genetics of CHCs in male and female *D. simulans*. We found significant genetic variation for CHCs, including heritable variation for some individual CHC components. We also found significant (positive and negative) intra and intersexual genetic correlations for CHCs in our isolines, with the genetic architecture of female and male CHCs differing markedly. Our results also confirm that CHC profiles are sexually dimorphic in this species.

Overall CHC blends and many of the individual CHC components were significantly heritable, and it is this genetic variation that is needed if CHCs are to evolve. Nevertheless, heritability estimates from iso-female lines are best derived within five generations of line establishment from the wild to avoid overestimation of parameter values (Hoffmann and Parsons 1988). Therefore, the exact heritability values presented here should be treated with caution, even though our estimates are based on the intraclass correlation coefficient *t,* which provides more realistic heritability estimates compared with conventional means (David et al. 2005). Heritable variation in CHC profiles has been reported for a range of insects previously (e.g. Chapman et al. 1995; Thomas and Simmons 2009) and our findings suggest that there is sufficient

genetic variation present in this population for CHC profiles to evolve given appropriate selection. Consistent with this we have some evidence that CHC profiles evolve when subject to experimental selection (Sharma et al. unpublished). Our analyses also revealed significant sex by isoline interactions. This indicates the presences of sex-specific genetic variation in CHCs which is reflected in the different heritabilities in the sexes. For example, peak 16 is significantly heritable in females but not males, while the converse is true of peak 6.

In addition to estimating straight heritabilities, we also calculated the genetic variance-covariance matrix (**G**) for *D. simulans* CHCs, both within and across the sexes, and as expected from the differences noted in the variance estimates, the sexes also differed in their covariances. Cheverud (1984, 1988) suggested that **G** can be thought of as a measure of genetic constraints on evolution. Basically, the diagonal elements of **G** measure the short-term readiness of a character to respond to selection, and the off-diagonal elements measure how the evolution of one trait influences the co-evolution of others. Examination of the intrasexual **G** matrices indicates that many individual CHC peaks covary genetically with each other. CHC biosynthesis in *Drosophila* is considered to be a relatively simple system where genetic variants for CHC expression are expected to trade-off expression of one class of compounds for the others (Foley et al. 2007). This is consistent with the many negative genetic correlations we see between CHCs, especially in males between longer and shorter chain CHCs. These negative intra-male genetic correlations suggest that male genotypes that produce more shorter-chained CHCs tend to produce less longer-chained CHCs. In one way this could be interpreted as indicating that genotypes best suited under natural selection - long chain CHCs tend to be involved in desiccation resistance for example - are

genotypes less likely to be favoured under sexual selection where more volatile shorter chains are often more attractive (Ferveur and Cobb 2010). If this is so for *D. simulans*, the **G** for males we estimated may prevent the emergence of a genotype that could excel under both natural and sexual selection. It is interesting however, that comparison of **G** between the sexes indicates males and females do not face the same trade-offs as their intrasexual genetic architecture differs. As one example, females tend to have fewer strong negative correlations than males (10 vs. 28). If selection is ultimately responsible for the shape of **G**, this may reflect the fact that sexual selection is weaker on females, and they are typically viewed as being closer to naturally selected optima than males (Andersson 1994). That is, it is probably easier to optimise **G** for one (naturally selected) task than for two tasks (sexual and natural selection). In any case, the male/female difference can clearly be seen by considering more or less any single CHC. For example, while 7-Tricosene (peak 3) is a major constituent of both male and female CHC profiles in *D. simulans* (see Luyten 1982; Pechine et al.1985; Ferveur and Jallon 1993), females express higher levels of it (Ferveur 1991; and see Table 1). If we compare the **G** matrix values for male peak 3 to those for female peak 3, it is obvious that the intrasexual correlations for each sex vary (see Table 5). Strong intrasexual genetic correlations imply that the individual CHC peaks are not independent of each other, but it does not imply anything about the plasticity of such correlations, as environmental fluctuations may alter their magnitude or sign.

A shared genetic architecture may also constrain the independent evolution of the sexes. This constraint usually manifests as strong intersexual genetic correlations (r_{MF}: Lande 1980; Roff 1997). The magnitude of r_{MF} between homologous traits and the nature of selection on each sex could influence the evolution of sexual dimorphism

(Lande 1980). We found many positive and negative r_{MF} for *D. simulans* CHCs, but the average magnitude of these correlations is weak, suggesting sexual dimorphism in CHCs is at an advanced stage (Lande 1980). Theoretically, the genetic architecture of traits under sexually antagonistic selection should evolve to minimize the genetic constraints on the independent evolution of the sexes, allowing the sexes to meet their sex-specific fitness optima (Lande 1980; Badyaev 2002; Rhen 2000; Rice and Chippandale 2001), and the intersexual covariances we find are largely consistent with this as most of them are below 50%. Nevertheless, the fact that we find correlations at all contrasts with findings from other *Drosophila* species (e.g. Chenoweth and Blows 2003), and with CHC expression studies on mutant *D. melanogaster* (Ferveur and Jallon 1993; Coyne et al. 1999; Dallerac et al. 2000; Wicker-Thomas and Jallon 2001; Fang et al. 2002) which indicate trait expression may be under independent genetic control in the sexes (also see Labeur et al. 2002; Ferveur 2005). However, in a recent meta-analysis, intersexual genetic correlations for homologous traits were predominantly large and positive (Poissant et al. 2010). Our results also contrast with this, for although we do find some covariance between the sexes, associations are for the most part negative. Additionally, **G** for males and females significantly differ, which may facilitate sex specific evolution of CHC profiles even when individual CHCs show strong intersexual covariance. However, our covariance estimates are based on broad-sense estimates of genetic variances because of the isoline approach we have employed, so additive covariances are likely to be even weaker, which may partly reconcile our findings with other *Drosophila* studies (e.g. Chenoweth and Blows 2003). It is important to note that genotype x environment interactions are also expected to influence r_{MF} estimates (Falconer 1989; Lyons et al. 1994; Simmons and Roff 1996). This means CHCs may evolve in a sex specific manner under different environmental

conditions, further underlining the importance of these interactions in sexual selection (Ingleby et al. 2010).

Our results are also consistent with previous work suggesting that *D. simulans* is quantitatively sexually dimorphic in CHC profiles (Cobb and Jallon 1990). Sexual dimorphism is common in sexually selected characters, with sexes often differing in size, shape and degree of sexual trait exaggeration (Darwin 1871; Andersson 1994). Examination of the male and female CHC chromatograms here reveals that all the peaks we detected are shared by the sexes, but that sexes express different quantities for many of the shared peaks (Fig. 1a, b) (and see Ferveur and Jallon 1993; Ferveur and Cobb 2010). Given the multivariate nature of overall CHC profiles, even small differences in CHC production can dramatically alter CHC bouquets and influence behavioural responses during mate choice. Sexually antagonistic selection, where traits shared by males and females have a sex-specific fitness optima (Rice and Chippindale 2001; Bonduriansky and Chenoweth 2009; Hosken et al. 2009), is considered to be an ultimate cause of sexual dimorphism. As stated above, we have some evidence for sex specific CHC changes during experimental evolution, which is consistent with sex-specific fitness optima, but we currently do not know if this is due to sexually antagonistic selection in our experiments or sex differences in **G**.

Overall, our results indicate that *D. simulans* CHC sexual dimorphism is at an advanced stage and therefore the sexes appear to be largely free to evolve their CHC profiles to sex-specific optima. Intersexual differences in the optimal profiles are expected because the sexes invest differentially and differ in their reproductive roles, and hence the direction of sexual and natural selection acting on specific traits should differ

(Johnstone et al. 1996; Bonduriansky 2001). What remains to be determined is precisely how the dynamic interplay between natural and sexual selection actually shapes the evolution of CHCs. For example, does natural selection overwhelm sexual selection or would sexual selection result in trait exaggeration regardless of costs? Answers to these questions are imperative in understanding the interplay between selective bouts that shape trait evolution.

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Chapter Five

Antagonistic responses to sexual and natural selection, and sex-specific evolution of *Drosophila simulans* cuticular hydrocarbons

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Abstract

Natural and sexual selection are classically thought to oppose one another, but direct experimental demonstrations of this are largely lacking. Here we assessed the effects of sexual and natural selection on the evolution of *Drosophila simulans* cuticular hydrocarbons (CHCs), a character subject to both modes of selection. Natural selection and sexual selection were manipulated in a fully factorial design, and after 27 generations of experimental evolution male and female CHC responses were assessed. The effects of natural and sexual selection differed greatly across the sexes. Effects on females were small, but CHC profiles tended to evolve in the direction of natural selection. For males, profiles evolved via sexual and/or natural selection, with some male CHC components only evolving in the naturally selected direction when sexual selection was suspended. These results indicate sex specific responses to selection, and that sexual and natural selection act antagonistically for at least some CHC combinations.

Keywords: CHC, selection, experimental evolution, fly, Diptera

Introduction

Sexual selection is responsible for evolution of many conspicuousness traits and behaviours, and is classically thought to be opposed by natural selection, at least once characters become sufficiently exaggerated (Lande 1981; Arnold 1983; Andersson 1994). This antagonism between sexual and natural selection is built into many evolutionary models of sexual trait evolution (Lande 1981; Pomiankowski *et al.* 1991; Mead & Arnold 2004), and is supported by some iconic studies. For example, sexual selection frequently favours louder or longer calls (e.g. Rand & Ryan 1981; Betsen *et al.* 2006). This may simply be because these call characteristics make males easier to find for females, providing long, loud callers with a mating advantage (Gwynne 2001). However, these same call characteristics can make signallers more conspicuous to non-intended receivers, and there are many examples of predators using sexual signals to locate signalling males (Endler 1980; Tuttle & Ryan 1981; Zuk *et al.* 2006). Nevertheless, while sexual and/or natural selection on specific characters has been documented, there has been little direct experimental investigation of the combined evolutionary effects of natural and sexual selection on sexual traits. This is surprising if only because this fundamental evolutionary tenet has not been investigated as thoroughly as it could, and has prompted calls for experimental investigation of the joint effects of natural and sexual selection on sexual trait evolution, and the potential retarding effects natural selection may have on the evolution of these traits (Price *et al.* 1987; Andersson 1991).

In addition to the antagonism between sexual and natural selection, selection can also be sexually antagonistic, with males and female having different selective optima for shared traits (Rice & Chippindale 2001; Bondiuransky & Chenoweth 2009; Hosken *et*

al. 2009). Sexually antagonistic selection is one ultimate cause of sexual dimorphism, and the widespread occurrence of sexual dimorphism suggests this is common, especially with regard to secondary sexual characters. In non-sex-role reversed species it is males that usually bear elaborate sexual traits because they enhance male mating success. Females usually do not carry exaggerated sexual traits as they are typically under weaker directional sexual selection, and may therefore reside nearer to naturally selected optima when the genetic architecture of the shared traits permits this.

In many *Drosophila* species cuticular hydrocarbons (CHCs) are important determinants of male attractiveness and hence mating success (Cobb & Ferveur 1995; Blows 2002; Wicker-Thomas 2007). CHCs are also subject to natural selection, being important in providing desiccation resistance for many insect species (Hadley 1981; Gibbs & Rajpurohit 2010). Typically longer-chain CHCs are more important in waterproofing, while shorter-chain, more volatile CHCs are involved in sexual signalling over short distances, although longer-chain CHCs can also act as contact pheromones (Hadley 1981; Wicker-Thomas 2007; Ferveur & Cobb 2010). However, while there is population variation and geographic clines for many CHCs, the selection responsible for this is not well understood (Coyne & Elwyn 2006). One of a few exceptions to this generality is *D. serrata*, where sexual selection on CHCs has been extremely well studied (Hine *et al*. 2002; Chenoweth & Blows 2005; Petfield *et al*. 2005; van Homrigh *et al*. 2007). For example, experimental evolution has been used to show that there is an interaction between sexual and natural selection on CHCs in the *D. serrata* complex (Blows 2002), and that female CHCs are under strong stabilising sexual selection (Chenoweth & Blows 2005). Nevertheless, there is a paucity of experimental

investigations of the collective effects of sexual and naturally selection on CHCs (Blows 2002), and in many instances evolution of CHCs via sexual selection is inferred, but has not been demonstrated (Cobb & Ferveur 1995).

Here we use experimental evolution in replicate populations of *Drosophila simulans* to assess the joint effects of natural and sexual selection on male and female CHCs. While these flies are reported to be sexually monomorphic in their CHCs (males and females express the same CHCs), they nevertheless display sexual dimorphism in the relative abundance of particular CHCs (i.e. their CHC bouquets differ) (Cobb & Ferveur 1995). We have previously shown that male attractiveness is heritable (Taylor *et al*. 2007) and CHC profiles are an important determinant of male attractiveness in this species (Ferveur & Cobb 2010). We have also shown that there is genetic variation for female mate preference (Sharma *et al.* 2010), so there is potential for CHCs to evolve via sexual selection. However, there has been no direct demonstration of this, or of natural selection causing CHC evolution. Here we show CHCs evolve via sexual and natural selection, as well as their interaction. This occurs in a sex-specific manner, with evidence for antagonistic sexual and natural selection, especially on male CHC profiles.

Materials & Methods

The flies used in this study were derived from twenty iso-female lines supplied by the Centre for Environmental Stress and Adaptation Research, La Trobe University, Australia. These were collected from a wild population at Tuncurry, Eastern Australia in March, 2004. Stock flies were reared on 'Drosophila quick mix medium' (supplied by Blades Biological, Edenbridge, Kent, U.K.) at 25°C and a 12:12 h light:dark cycle, and

had been maintained in large population cages (ca. 800-1000 flies/cage) with overlapping generations and free mate choice for ca. 4 years prior to the start of this investigation. We have previously shown that this stock harbours substantial genetic and phenotypic variation (e.g. Taylor *et al*. 2007; Hosken *et al.* 2008; Wright *et al.* 2008).

Experimental populations of flies were propagated under relaxed and elevated sexual and natural selection in a fully factorial design (4 populations per treatment combination = 16 populations in total). The standard rearing temperature of 25^{o}C (to which flies had been exposed for more than 4 years = ca. 140 generations) represented the relaxed natural selection treatment, and constant low-grade temperature stress (a 2^{o}C elevation to 27^{o}C) was used to generate the elevated natural selection treatment. This temperature elevation was chosen because 27^{o}C is very close to the *D. simulans* sterility threshold. Temperature has also been shown to affect life-history traits and the ontogeny of CHCs in *Drosophila* (Murphy *et al*. 1983; Savarit & Ferveur 2002). Sexual selection was relaxed by housing flies in monogamous pairs, and was elevated by housing each female with four males. We had 60 females per population in the elevated sexual selection treatment and 64 females in the non-sexual selection treatment. This difference in female number was to approximately equalize effective population size (Ne) as there were higher numbers of males present in the elevated sexual selection treatment. However, because of female mating behaviour and ca. 80% sperm displacement, we calculated that an additional 4 pairs was sufficient to standardize Ne. Populations were forced to evolve under these experimental conditions for 27 generations before CHCs were measured. This generates a sexual selection differential of 108 male generations between the sexual selection treatments

(this differential is proportional to the number of generations of selection x the number of males competing for a female every generation).

Briefly, our selection protocol was as follows: flies were housed together for 6 days in 'interaction vials' before being placed into new 'egg laying vials' where females were allowed to oviposit for 2 days. After two days of egg laying, adults were discarded and vials incubated until offspring emergence. Offspring emerging from these vials on the peak emergence day (ca. day 9 after laying = stabilising selection on development time) were pooled (within populations) and randomly chosen to start subsequent generations (Figure 1). It is important to note that newly emerged flies were collected and housed by sex (within population) to ensure virginity before individuals were chosen to start each subsequent generation. Food was provided in excess during the experimental evolution so that differential larval competition (e.g. the potential for non-sib competition in the sexual selection lines) was minimised (> 40ml/vial maximises offspring emergence rates: unpublished data).

After 27 generations of selection, a subset of flies from our selection populations were allowed to oviposit for 24 hours and subsequently vials were incubated at 26°C irrespective of the temperature treatment they originally evolved under (25°C or 27°C) until offspring emergence. This standardises the development temperature across all our treatments so that any subsequent differences in CHCs is not simply due to rearing temperature differences during development, which have been shown to alter *Drosophila* CHC profiles (Savarit & Ferveur 2002). Emerging virgin adults were collected and sexed within 4 hours of eclosion as *Drosophila* CHC's have been shown to be identical for both sexes for a short time (3-6 hours) after emergence (Pechine *et*

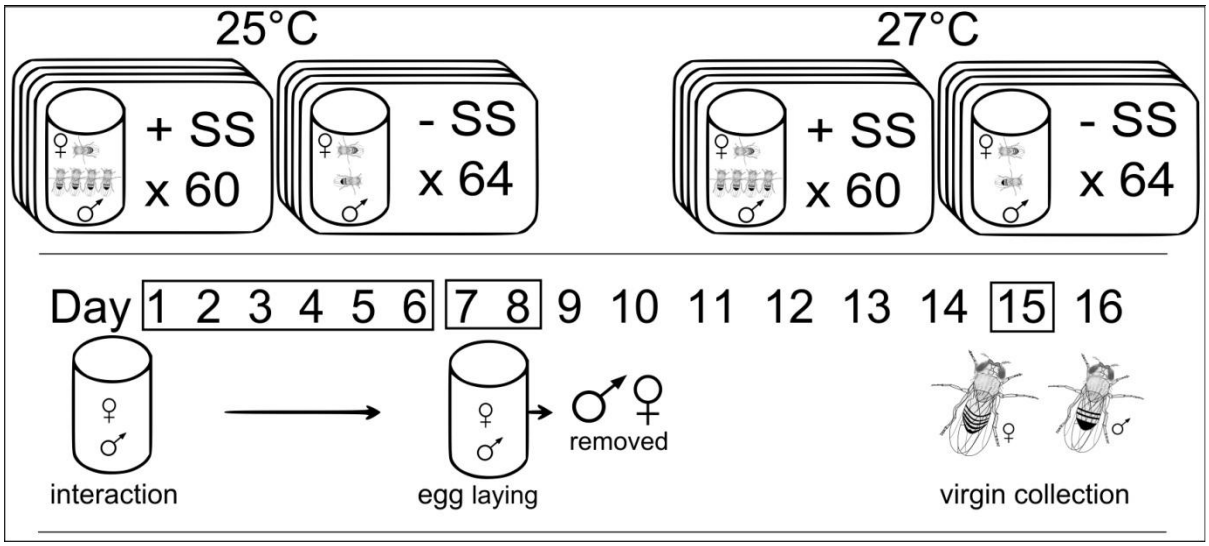

Figure 1. The selection protocol employed. The relaxed natural selection regime populations were maintained at 25°C, which is the temperature flies have been housed at for 5 years. The enhanced natural selection populations were house at 27°C, which is a novel, stressful temperature for these flies (above this temperature males become sterile). The enhanced sexual selection populations were females housed with 4 males, and in the relaxed sexual selection populations, single females were housed with single males. Females and males were housed for 6 days in interaction vials before they were moved to laying vials for two days (days 7 & 8). Adults were then discarded. Eggs from the egg-laying vial were allowed to develop and only individuals emerging from these vials were used to start subsequent generations (virgin collection on day 15). The design is fully factorial with 4 replicate populations per treatment combination. Selection followed these regimes for 27 generations then flies were reared at 26°C for one generation, and CHCs were assayed.

al. 1988). We collected 30 males and 30 females from each population and housed them individually to avoid CHC changes due to social interactions (Kent *et al*. 2008). Visual stimuli are important in *Drosophila* courtship and may lead to individuals altering their CHC profiles. We therefore isolated glass vials visually using translucent plastic partitions that allowed light passage, but would make image recognition difficult. Individuals were processed for CHC extraction when they were three days old, as by this time adult CHC profiles are established (Antony & Jallon 1982; Schaner *et al*. 1989).

To quantify CHCs, individuals were transferred to 1ml glass vials and soaked in 50ul Hexane containing an internal standard of pentadecane at a concentration of 10 ppm, for 5 minutes. The vials were vortexed for the last 60 seconds to maximize extraction. A 1ul sample from each fly extract was then injected into a GCMS (Agilent 7890A GC coupled with an Agilent 5975B Mass spectrometer) operating in pulsed split-less mode and fitted with a DB-1ms column (340 °C: 30 m x 250 µm x 0.25 µm) (J&W 122-0132 by J&W Scientific, 91 Blue Ravine Road, Folsom, CA 95630-4714, U.S.A.) using helium as a carrier gas. Extract separation was optimised using a column temperature profile in which the analysis began at a temperature of 70 °C for 1 minute and then rose by 20°C/min to 180°C followed by a 4°C/min rise to 220°C, and 15°C/min rise to 320°C where it was held for 2 minutes. The transfer line from the GC to the MS was set at 250°C. Chromatograms were acquired and analyzed using MSD Chemstation software version E.02.00.493 (Agilent, Foster City, CA).

CHCs were extracted and analysed from 960 flies (30 individual males and females from each of the 16 populations) along with pentadecane control standards that were

loaded at the start and end of each run. CHC peaks were labelled by peak number (1-25), which corresponded to their retention times on the GC (see Figure 2, Table 1), and proportional values for each peak were calculated by dividing by the pentadecane internal standard. Use of the internal standard in calculating proportions eliminates the problems of unit-sum-constraints faced when proportions are calculated relative to sum of all peaks. Data for each peak was $\log_{10}$ transformed prior to analysis.

Results

We obtained four significant principal components (PCs) for females and five for males, with these collectively explaining 76% and 79% of the variation in CHCs, respectively (Table 2). For both sexes principle component 1 (PC1) described total CHC content, PC2 was largely describing increases in long-chained CHCs, and PC3 represented a trade-off between long and short-chain CHCs. For females, PC4 represented increases in three CHCs (Octadecadience, Hexacosane and peak 21, an unknown alkane). For males, PC4 described a trade-off between one CHC and 4 others (peak 25, an unknown alkane ($C_{30}H_{62}$) vs. Octadecadience, Pentacosadiene, Hexacosane, and peak 21 (an unknown alkane)) and PC5 represents increases in three CHCs (Octadecadience, Docosene & Tricosene).

MANOVA of the male responses revealed natural selection, sexual selection and their interaction all influenced the multivariate combination of male CHC PCs (Table 3). Post-hoc tests revealed the natural selection effect was due to differences in PC1 and PC2, with no other effects significant (Table 3). With elevated natural selection, populations evolved increased PC1 (i.e. flies produced more CHCs) (Figure 3A), and increased PC2 (i.e. flies produced more long chain CHCs) (Figure 3B). This is consistent

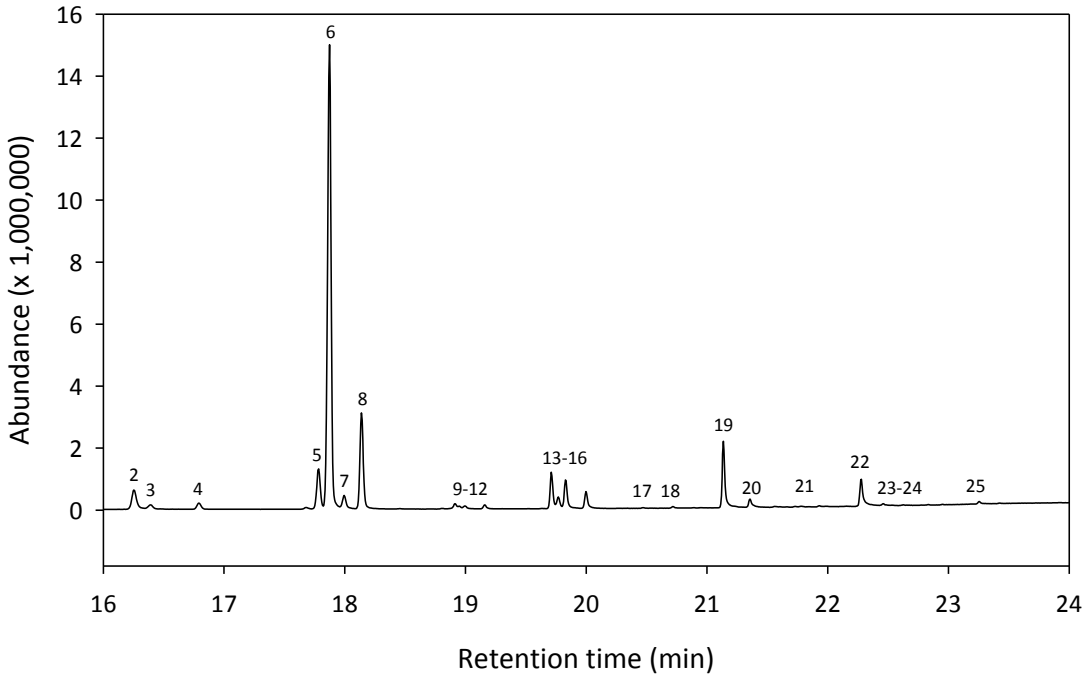

Figure 2. A typical GC profile of a male *Drosophila simulans*. The *x*-axis shows the retention time (in minutes) and the *y*-axis the abundance of each peak (measured as the area under the peak). Note to improve the visualization of the peaks 2-25, the chromatogram does not show the Pentadecane internal standard at a retention time of 7.489 minutes.

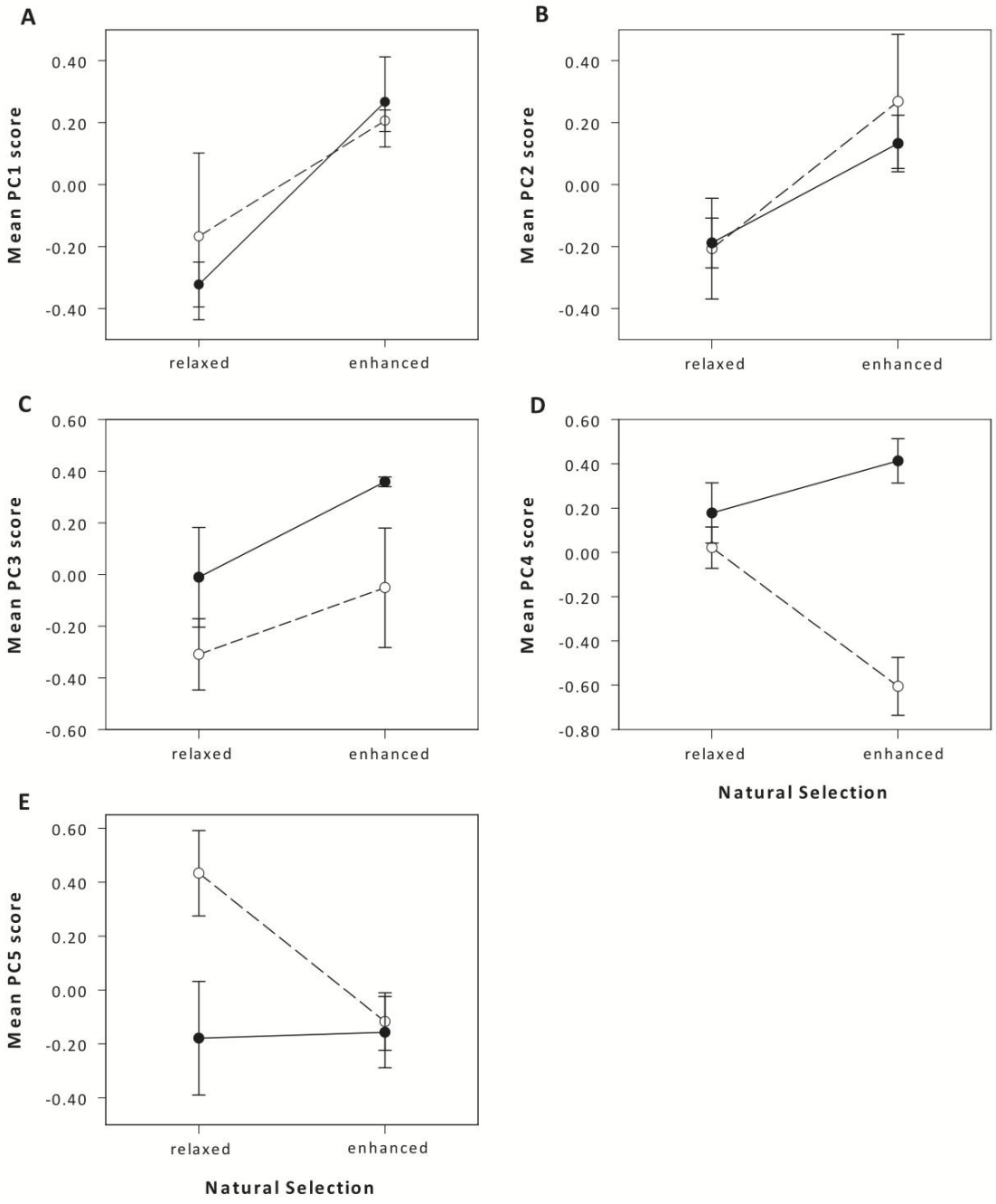

Figure 3. The evolutionary response of male CHCs to natural and sexual selection. Figures A-E represent the mean (±SE) values for each of the 5 principal components, which together explain 79% of the variation in male CHC profiles. In each instance, closed symbols (•) with solid lines represent the enhanced sexual selection treatment, while open symbols (o) with dashed lines represent the relaxed sexual selection treatment.

Table 1. The identification of the 24 cuticular hydrocarbon compounds in male and female *D. simulans* and their relative contribution, expressed as the mean percentage (±SD) of the total abundance of all peaks.

Peak No.	Retention Time	Formula	Molecular Weight	% (±SD) in males	% (±SD) in females	Name
1	7.489	$C_{15}H_{32}$	212			Pentadecane ISTD
2	16.253	$C_{18}H_{38}$	250	0.65 ± 0.46	0.09 ± 0.21	Octadecadiene
3	16.391	$C_{22}H_{44}$	308	0.50 ± 0.12	0.42 ± 0.12	Docosene
4	16.793	$C_{22}H_{46}$	310	1.89 ± 0.62	1.52 ± 0.47	Docosane
5	17.783	$C_{23}H_{48}$	324	3.66 ± 0.71	2.73 ± 0.71	Branched alkane
6	17.873	$C_{23}H_{46}$	322	40.18 ± 4.14	40.05 ± 5.76	7-Tricosene
7	17.990	$C_{23}H_{46}$	322	1.50 ± 0.34	1.43 ± 0.41	Tricosene
8	18.138	$C_{23}H_{48}$	324	22.43 ± 3.21	22.44 ± 3.20	Tricosane
9	18.917	$C_{24}H_{50}$	338	0.39 ± 0.13	0.29 ± 0.10	Branched alkane
10	18.927	$C_{24}H_{50}$	338	0.23 ± 0.07	0.20 ± 0.06	Branched alkane
11	18.996	$C_{24}H_{50}$	338	0.29 ± 0.08	0.24 ± 0.08	Branched alkane
12	19.160	$C_{24}H_{50}$	338	0.93 ± 0.52	0.89 ± 0.26	Tetracosane
13	19.711	$C_{25}H_{48}$	348	1.78 ± 0.92	1.00 ± 0.57	Pentacosadiene
14	19.774	$C_{25}H_{50}$	350	0.96 ± 0.31	1.27 ± 0.58	Pentacosene
15	19.827	$C_{25}H_{50}$	350	2.38 ± 0.50	2.52 ± 0.68	Pentacosene
16	19.997	$C_{25}H_{52}$	352	3.68 ± 1.10	4.83 ± 1.03	Pentacosane
17	20.468	$C_{25}H_{52}$	352	0.14 ± 0.05	0.11 ± 0.05	Branched alkane
18	20.717	$C_{26}H_{54}$	366	0.46 ± 0.77	0.53 ± 0.30	Hexacosane
19	21.135	$C_{27}H_{56}$	380	10.08 ± 3.76	6.31 ± 2.91	Heptacosane
20	21.352	$C_{27}H_{56}$	380	2.15 ± 1.38	4.21 ± 1.70	Branched alkane
21	21.930	Unresolved	Unresolved	0.33 ± 0.50	0.38 ± 0.24	Alkane
22	22.279	$C_{29}H_{60}$	408	4.31 ± 1.65	6.58 ± 2.57	Alkane
23	22.459	$C_{29}H_{60}$	408	0.60 ± 0.49	1.10 ± 0.64	Alkane
24	22.618	Unresolved	Unresolved	0.12 ± 0.07	0.15 ± 0.11	Alkane
25	23.253	$C_{30}H_{62}$	422	0.36 ± 0.19	0.67 ± 0.97	Alkane

Table 2. Principal Component analysis for female and male CHCs, respectively.

Principal components with an eigenvalue over 1 are retained for further analysis and factor loadings over 0.30 (in bold) are interpreted as biologically significant.

	Females					**Males**				
	PC1	**PC2**	**PC3**	**PC4**		**PC1**	**PC2**	**PC3**	**PC4**	**PC5**
Eigenvalue	10.71	4.09	2.08	1.31		9.63	4.70	2.07	1.46	1.02
% variance	44.61	17.05	8.65	5.45		40.14	19.58	8.64	6.09	4.24
Loadings										
Octadecadiene	0.02	**-0.54**	0.15	**0.43**		0.08	-0.25	**0.49**	**0.46**	**0.37**
Docosene	**0.73**	-0.29	**0.37**	-0.08		**0.75**	-0.22	0.08	0.10	**0.36**
Docosane	**0.61**	-0.13	**0.49**	0.12		**0.49**	0.21	**0.73**	-0.15	-0.15
Branched alkane	**0.87**	**-0.31**	-0.11	0.09		**0.91**	-0.24	-0.01	0.13	-0.07
7-Tricosene	**0.83**	**-0.34**	0.21	-0.25		**0.89**	-0.29	-0.04	-0.10	0.19
Tricosene	**0.56**	**-0.36**	**0.52**	**-0.30**		**0.69**	**-0.33**	0.15	-0.22	**0.37**
Tricosane	**0.86**	-0.16	-0.15	-0.15		**0.85**	-0.02	0.16	-0.23	-0.25
Branched alkane	**0.73**	-0.11	**0.39**	0.22		**0.70**	0.22	**0.48**	0.01	0.01
Branched alkane	**0.82**	-0.08	0.25	0.02		**0.78**	0.06	0.16	-0.07	0.10
Branched alkane	**0.70**	-0.18	**0.35**	0.19		**0.75**	-0.10	0.18	0.09	0.15
Tetracosane	**0.73**	**0.36**	0.08	**0.36**		**0.42**	**0.68**	**0.40**	0.14	-0.29
Pentacosadiene	**0.74**	**-0.32**	**-0.46**	0.18		**0.75**	**-0.38**	**-0.30**	**0.34**	-0.16
Pentacosene	**0.86**	-0.01	**-0.35**	-0.01		**0.85**	-0.05	**-0.30**	0.00	-0.16
Pentacosene	**0.87**	-0.08	-0.02	-0.10		**0.88**	0.04	-0.03	-0.12	-0.01
Pentacosane	**0.82**	0.27	**-0.30**	-0.10		**0.65**	**0.57**	0.00	-0.14	-0.26
Branched alkane	**0.55**	**-0.33**	-0.43	0.04		**0.65**	-0.29	-0.26	0.24	-0.24
Hexacosane	**0.36**	**0.75**	0.05	**0.43**		0.06	**0.84**	-0.02	**0.41**	-0.03
Heptacosane	**0.76**	-0.26	**-0.50**	0.15		**0.79**	**-0.32**	**-0.31**	0.29	-0.14
Branched alkane	**0.52**	**0.69**	0.00	-0.23		0.15	**0.87**	0.01	-0.19	0.04
Alkane	0.21	**0.75**	0.11	**0.41**		-0.02	**0.72**	-0.09	**0.54**	0.11
Alkane	**0.79**	0.28	-0.29	-0.15		**0.66**	**0.38**	**-0.44**	-0.09	0.21
Alkane	**0.34**	**0.81**	0.11	-0.05		0.11	**0.80**	-0.23	0.16	0.29
Alkane	**0.33**	**0.39**	0.02	**-0.32**		0.26	0.27	**-0.35**	-0.20	0.07
Alkane	**0.45**	**0.56**	0.12	**-0.32**		**0.33**	**0.52**	-0.25	**-0.44**	0.19

Table 3. Multivariate Analysis of Variance (MANOVA) examining the effect of sexual selection, natural selection and their interaction on the CHC profile of male and female *D. simulans*. To aid the interpretation of the overall multivariate effect, we also provide univariate ANOVAs for each sex.

	Females				**Males**		
	MANOVA						
	Wilks' λ	$F_{4,9}$	*P*		Wilks' λ	$F_{5,8}$	*P*
Sexual Selection (A)	0.528	2.015	0.176		0.249	4.826	0.025
Natural Selection (B)	0.531	1.988	0.180		0.110	12.990	0.001
A x B	0.277	5.887	0.013		0.248	4.864	0.024
	Univariate ANOVAs						
		$F_{1,12}$	*P*			$F_{1,12}$	*P*
Sexual Selection (A)	PC1	1.148	0.305		PC1	0.090	0.769
	PC2	0.397	0.540		PC2	0.155	0.700
	PC3	3.482	0.087		PC3	4.544	0.054
	PC4	0.032	0.862		PC4	25.442	0.000
					PC5	4.319	0.060
Natural Selection (B)	PC1	0.421	0.529		PC1	9.253	0.010
	PC2	0.044	0.837		PC2	7.187	0.020
	PC3	3.238	0.097		PC3	3.567	0.083
	PC4	0.489	0.498		PC4	2.825	0.119
					PC5	2.842	0.118
A x B	PC1	0.766	0.399		PC1	0.468	0.507
	PC2	0.229	0.641		PC2	0.271	0.612
	PC3	6.533	0.025		PC3	0.112	0.743
	PC4	4.426	0.057		PC4	13.685	0.003
					PC5	3.339	0.093

with previous work on other *Drosophila,* which report essentially identical CHC responses to changes in the evaporative environment (Toolson & Kuper-Simbrón 1989; Kwan & Rundle 2010). Post-hoc tests also showed the sexual selection effect was largely driven by difference in PC4 (Table 3), but since this was also the PC responsible for the significant interaction (Table 3), we largely have to interpret the interaction (Figure 3D). This indicates that under relaxed natural selection, populations with and without sexual selection have similar CHC profiles along the PC4 dimension (Figure 3D). However, when natural selection is intensified populations evolve to a new CHC combination (more peak 25, less Octadecadience, Pentacosadiene, Hexacosane, and peak 21) only when sexual selection is relaxed (Figure 3D). This is largely consistent with our understanding of CHC function as longer chains (Savarit & Ferveur 2002) and lower pentacosadiene levels (Toolson & Kuper-Simbrón 1989), reduce evaporative water loss (EWL), and thus in contrast to females (see below), sexual selection overwhelms elevated natural selection on this aspect of male CHC profile. Additionally, the significant sexual selection effect in this model indicates that when evolving with sexual selection males produce more Octadecadience, Pentacosadiene, Hexacosane, and peak 21 (an unknown alkane) and less peak 25 (an unknown alkane), and when sexual selection is relaxed, the reverse is true (Figure 3D).

MANOVA of the female responses revealed a significant interaction between natural and sexual selection treatments that influenced the multivariate combination of CHC PCs (Table 3). Post-hoc tests revealed this was largely driven by changes in PC3 (Figure 4C), as no other comparisons were statistically significant (Table 3). Under relaxed natural selection, the sexual selection and non-sexual selection populations evolved different PC3 CHC blends, with the former populations evolving more longer-chained

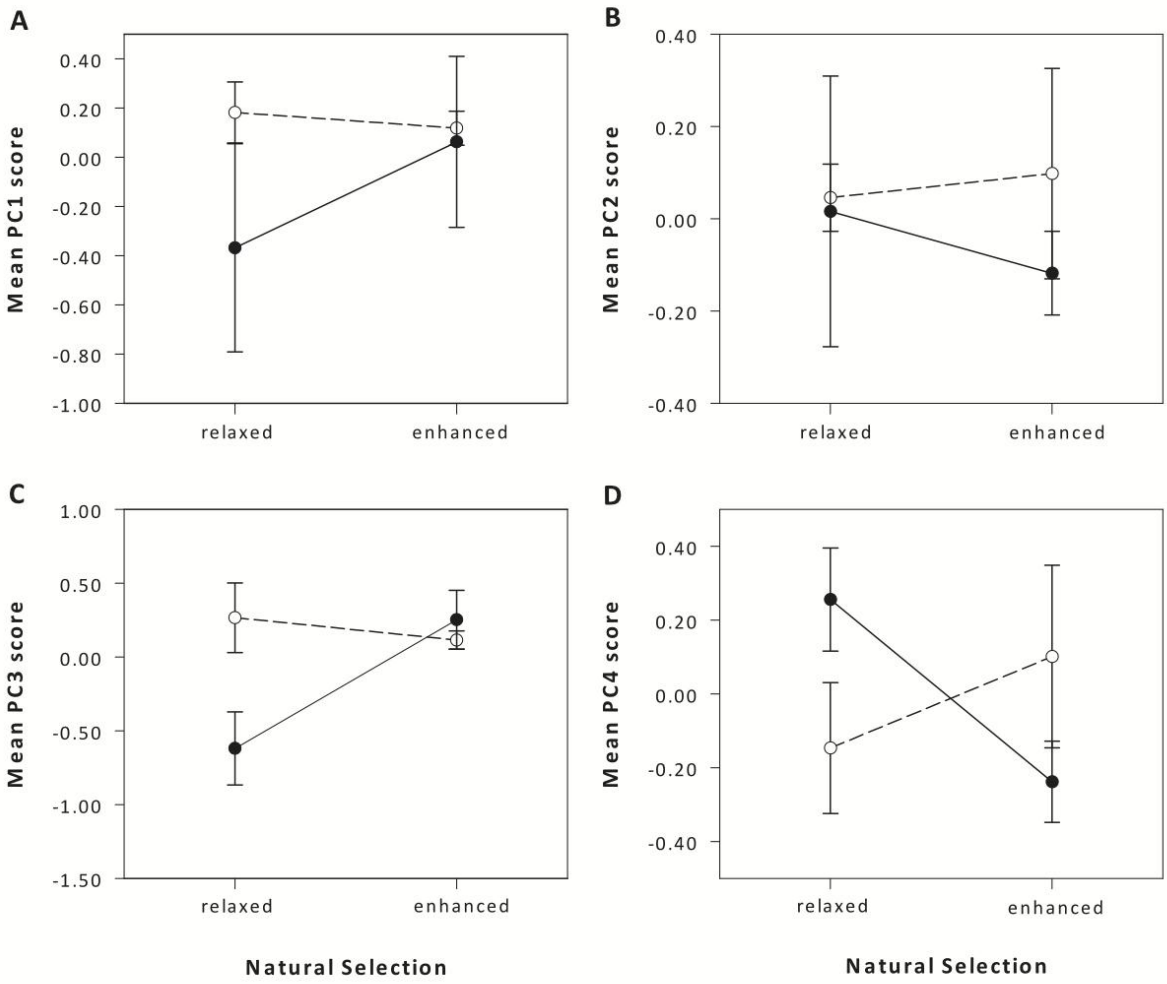

Figure 4. The evolutionary response of female CHCs to natural and sexual selection. Figures A-D represent the mean (±SE) values for the 4 principal components, which together explain 76% of the variation in female CHC profiles. In each instance, closed symbols (•) with solid lines represent the enhanced sexual selection treatment, while open symbols (o) with dashed lines represent the relaxed sexual selection treatment.

and less shorter-chain CHCs (Figure 4C). However, when natural selection was enhanced this difference disappeared as the sexual selection populations converged on the PC3 combination seen in the relaxed sexual selection populations (Figure 4C). Thus natural selection completely overwhelmed sexual selection on this PC when natural selection was increased, but females also evolved in this naturally selected direction when sexual selection was relaxed under ancestral (relaxed) levels of natural selection. At least some of these differences are consistent with predictions based on previous studies of CHC function. For example, pentacosadiene levels were lower at high temperature and low levels of this CHC reduce EWL (Toolson & Kuper-Simbrón 1989).

Discussion

While natural and sexual selection have been implicated in the evolution of sexual traits, there have been very few experimental studies of their joint effects on trait evolution (Andersson 1994). This is remarkable because the accepted dogma is that natural selection brakes the evolution of exaggerate sexual traits, even though this has rarely been demonstrated experimentally. Here we partly redress this paucity using experimental evolution to assess the effects of both sexual and natural selection on the evolution of a sexual trait, the cuticular hydrocarbons (CHCs) of *D. simulans*. The major findings of our study are: 1) responses to both modes of selection were very different for males and females, 2) sexual and natural selection interact to cause CHC evolution, and 3) when sexual and natural selection are elevated, natural selection has a greater effect on female CHC evolution, but sexual selection has a greater effect on males. Additionally, while many aspects of male CHC profiles evolved in a predictable fashion via sexual and/or natural selection, some male CHC combinations were only

able to evolve in the naturally selected direction in the absence of sexual selection. In contrast, there was only limited evolution of female CHC profiles, but there was a significant interaction between sexual and natural selection that influenced female CHC evolution. We discuss each of these findings and their major consequences in turn.

There was significant evolution of many aspects of male CHCs, and natural and sexual selection, together with their interaction, were implicated in this. Elevated natural selection saw males evolve an increase in their total CHC content (PC1) and increase their longer-chain CHCs (PC2). This is presumably because of elevated EWL at higher temperature (= the elevated natural selection treatment) (Gibbs & Rajpurohit 2010), and the evolution of CHCs through natural selection has been documented in *Drosophila* previously, with increased total CHCs in a high EWL environment (Kwan & Rundle 2010), and changes in specific CHCs like pentacosadiene that alter EWL (Toolson & Kuper-Simbrón 1989). Furthermore, sexual size differences - males are smaller than females and thus have higher surface area to volume ratios - could at least partly explain why this evolution was only significant in males (although the pattern for PC1 in females was roughly similar - CHC content tended to increase at higher temperature - see figure 2A). It should also be noted that in the ancestral temperature treatment (relaxed natural selection), males were apparently producing more shorter-chained CHCs than females (PC2) regardless of the sexual selection treatment. Some of these CHCs are apparently needed to stimulate female mating (e.g. 7-Tricosene: Ferveur & Cobb 2010) and the male-specific elevated-natural selection effect for PC2 may be partly because males in the ancestral environment

were further from the increased natural selection (elevated temperature) optima than females.

The interaction between sexual and natural selection influencing male CHC evolution is particularly interesting. When we experimentally elevated natural selection by increasing temperature, male CHCs only evolved along PC4 when sexual selection was relaxed. When sexual selection co-occurred with elevated natural selection, populations did not evolve toward this naturally selected blend at all, and in fact the CHC profile described by PC4 is similar for sexual selection treatments in both the relaxed (ancestral) and elevated natural selection populations. Thus elevated natural selection is only able to drive CHC evolution toward a new naturally selected peak in the absence of sexual selection, and sexual selection can be strong enough to overwhelm natural selection on some aspects of the male CHC profile. This finding is consistent with the conventional interpretation of sexual selection on male sexual traits (Andersson 1994) and additionally implies that sexual selection can be costly for males as it drives/holds males characters from their naturally selected optima. Again, this is consistent with the standard interpretation of net selection on male sexual traits, and similar results have been reported for other *Drosophila*. For example, sexual selection is not adaptive in *D. melanogaster* (Holland 2002), and in fact consistent with our findings, sexual selection opposes viability selection in this species (Wilkinson 1987). However, unlike *D. melanogaster*, there is no evidence for sexual selection via sexual conflict in *D. simulans* (Taylor *et al*. 2008a,b), and hence the results we present are best explained by classical sexual selection. Interactions between natural and sexual selection also influence CHC evolution in other *Drosophila* (Blows 2002), and like here, there is some evidence that male CHC components are costly,

which is generally seen as a prerequisite for honest sexual-signalling (Zahavi 1975; Grafen 1990). Moreover, like here, sexual selection in isolation only acted on male, but not female CHC profiles (Blows 2002). However, under the relaxed, ancestral natural selection conditions males from our sexual and no-sexual selection populations had virtually identical CHC profiles along PC4. This indicates the male blends favoured by sexual selection are not costly under relaxed natural selection (= ancestral conditions). One interpretation of this natural/sexual selection interaction is that given enough time, sexual selection eventually hones in on naturally selected optima, but in the short term, the two types of selection do not align. This outcome is theoretically predicted when there is direct selection on female preference (Kirkpatrick 1985), although there is no evidence for this in *D. simulans* (Taylor *et al*. 2008a,b; Sharma *et al*. 2010). In any case, sexual selection is clearly not always adaptive (Wade 1987). Finally, the populations subjected to sexual selection appeared to be slightly divergent in their CHC profiles (at PC4) in the different environments (elevated/relaxed natural selection). Although far from conclusive, this slight difference could be indicative of some environment-specific sexual selection.

In contrast to males, there was relatively limited female CHC evolution, although the significant interaction is revealing. When sexual selection was relaxed under the ancestral temperature regime (i.e. relaxed natural selection), female CHCs evolved toward a new PC3 profile, and this CHC combination was largely identical to the profile that evolved when natural selection was elevated. This indicates there is some sexual selection on female CHCs, directly or indirectly, but that elevated natural selection overwhelms this. These findings are largely consistent with orthodox interpretation of the relative contributions of sexual and natural selection to female character evolution

- natural selection is generally thought to shape female characters more than sexual selection. For example, females usually have no exaggerated secondary sexual characters because of (presumed) fecundity costs associated with developing and carrying them (Gwynne 2001). This was the only significant microevolutionary consequence of our experimental treatments for female CHC profiles, but largely mirrors work on the *D. serrata* species complex where interactions between natural and sexual selection influenced female CHC evolution in experimental populations (Blows 2002). Whether the sexual selection contribution to this interaction is due to male mate preference for certain female CHC blends, or genetic correlations between male and female CHCs remains to be established, but there are many significant intersexual genetic correlations for CHCs in our populations (unpublished data) which contrasts with at least some other *Drosophila* (Chenoweth & Blows 2003). Thus while intersexual genetic correlations may seem the more likely explanation for our results, the female CHC blend in the relaxed natural selection-enhanced sexual selection treatment includes more longer chain CHCs which are often contact pheromones, and include increases in at least one CHC that induces males courtship behaviour (Pentacosene) (Ferveur & Cobb M 2010). So some male mate-choice causing female CHC evolution cannot be ruled out, especially because male preference for certain female CHC blends has been found in *D. serrata* (Chenoweth & Blows 2005).

Clearly male and female responses to selection on CHC profiles greatly differed. This may be because selection is sexually antagonistic for CHCs, as indicated by sexual dimorphism in CHC profiles, but this sex-specific evolution is also consistent with the genetic architecture (***G***) that exist for CHCs in these flies (unpublished). Sexually antagonistic selection has been documented many times in *Drosophila* (e.g. Rice &

Chippindale 2001; Innocenti & Morrow 2010) and, while inter-sexual genetic correlations for CHCs in other flies are often inconsequential (Chenoweth & Blows 2003), we find many significant correlations across the sexes and the majority of these are negative (unpublished). Additionally, there are significant differences in the **G** matrix of female and male CHCs. It is therefore not surprising that female response to selection did not mirror males, although it is not exactly clear from our work whether **G** or selection is the major cause of this. Nonetheless, similar results have been found in another study where male and female responses to sexual and natural selection also differed greatly (Blows 2002), and male CHC evolution can also be constrained by a lack of genetic variation in the direction of sexual selection even though there is substantial genetic variation in other directions (McGuigan *et al*. 2008).

Overall, our findings indicate that both natural and sexual selection act on *D. simulans* CHCs in a sex specific manner. Furthermore, microevolutionary responses indicate sexual and natural selection act antagonistically on at least some CHC components, so sexual selection is often not adaptive. These findings are largely consistent with conventional views of evolution through sexual selection (Andersson 1994; Gwynne 2001), although these have rarely been demonstrated experimentally. CHCs are only one component of male attractiveness, with variation in CHC profiles explains about 10% of the variation in male mating success in our populations. Whether the CHC profile that make males attractive in one environment is the same that confers attractiveness in others remains to be investigated, but there is some indication this may not be the case. Additionally, how other characters determining male attractiveness are affected by natural and sexual selection is worthy of additional work. Nevertheless, our findings suggest sexual selection shapes male CHC profiles to

a greater extent than female profiles, which tend to evolve in the direction of natural selection. This is consistent with current orthodoxy, and refutes recent claims that sexual selection theory is somehow flawed.

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Chapter Six

Role of sexual selection in adaptation to a novel environment: A study with *Drosophila simulans*

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Abstract

The adaptiveness of sexual selection is much disputed and natural selection is heralded as the adaptive process in most models of evolution. Surprisingly, the adaptiveness of sexual selection to novel environments has rarely been tested as a whole (female choice *and* male-male competition), and available theoretical and empirical evidence does little to clarify the adaptiveness of sexual selection as evidence is conflicting. Here we impose artificial selection on replicate populations of *Drosophila simulans*, evolving under relaxed and elevated natural and sexual selection in a fully factorial design and subsequently compare non-sexual fitness components (i.e. those unrelated to securing mates). Our results were equivocal and did not generally suggest that sexual selection is adaptive. Populations evolving with elevated sexual selection showed higher fitness when assayed under some conditions but not others. Additionally, we also observed a decline in the overall fitness of all our selection populations in the course of 30 generations, but this decline was not due to inbreeding depression and we suggest that presence of *Wolbachia* may explain the fitness decline over time. In summary our results suggest that sexual selection may sometimes be adaptive, but this is not invariably so.

Keywords: natural selection, sexual selection, artificial evolution, non-sexual fitness

Introduction

Darwin (1871) invoked the theory of sexual selection to explain the evolution of characters that were thought to be detrimental for survival. The net benefits or costs of sexual selection are however, still debated (Gage et al. 2002; Cameron et al. 2003; Eberhard and Cordero 2003; Pizzari and Snook 2003; Hosken et al. 2009; and see Candolin and Heuschele 2008 for a review). Until recently, the predominant paradigm was that sexual selection, whilst usually antagonistic to natural selection, was not costly at equilibrium (because the costs of natural selection would be balanced by the benefits of sexual selection). Additionally, sexual selection was generally thought to improve only the sexual component of fitness (e.g. mating success), and this is essentially the Fisherian concept (Fisher 1930). However, Darwin (1871) at times considered sexual selection to be adaptive; an "*aid to ordinary selection*". He recognised that sexual selection and natural selection could work hand in hand to increase the quality of a taxon. For example, in reference to non-sexual fitness components, he says; "*Just as man can improve the breeds of his game-cocks by the selection of those birds which are victorious in the cockpit, so it appears that the strongest and most vigorous males, or those provided with the best weapons, have prevailed under nature, and have led to the improvement of the natural breed or species.*". The notion that sexual selection may be adaptive, has been embraced in some quarters where the benefits of sexual selection are inevitably linked to good-genes (Jennions and Petrie 2000). Theoretical and empirical work also supports this view as sexual selection can enhance the rate of adaptation to novel environments (Proulx 1999; Lorch et al. 2003), promote the fixation of beneficial alleles (Whitlock 2000), accelerate the loss of deleterious mutations (Agrawal 2001; Hollis et al. 2009), reduce the cost of sexual reproduction (Agrawal 2001; Siller 2001), and there is

evidence that non-sexual fitness components can also be enhanced by sexual selection. For example, female choice has been shown to increase offspring survival in organisms as diverse as moths, frogs and lizards (Iyenger and Eisner 1999; Welch et al. 1998; Lancaster et al. 2009).

Natural and sexual selection generally occur simultaneously in nature and often interact (Blows 2002). However, experimental evolution studies frequently focus on either natural selection or sexual selection rarely both (Maklakov et al. 2010). Interactions between natural and sexual selection may also influence the costs or benefits of sexual selection. For example, cuticular hydrocarbons (CHCs), involved in desiccation resistance and mate choice, are influenced by both natural and sexual selection. When natural and sexual selection were permitted to operate in combination, male CHCs became exaggerated to a greater extent than in the presence of sexual selection alone (Blows 2002; and see Chapter 5). These CHCs are costly to produce and such exaggerations are bound to influence the costs or benefits of sexual selection. It is therefore important to conduct experiments where the opportunities for natural and sexual selection are manipulated simultaneously. Furthermore, studies frequently only consider one component of sexual selection (e.g. female choice), and this can be problematic as is exemplified by work on the cockroach *Nauphoeta cinerea* (Moore and Moore 1999), the fly *Drosophila silvestris* (Boake 1989), and the water strider *Aquarius remigis* (Sih et al. 2002). In all these cases, female choice and male-male competition operate antagonistically and consideration of only one aspect of sexual selection would generate a false picture of the study system. Additionally, whilst there is no *a priori* reason to expect both mechanisms of sexual selection to reinforce each other (Moore and Moore 1999), male-male competition and female

mate choice could potentially interact in a diversity of ways and the way in which they combine and interact would alter the strength and form of total sexual selection (Hunt et al. 2009). Furthermore, sexual selection was formulated to explain the evolution of traits disadvantageous to survival (Darwin 1871), and Fisher (1930) noted that evolution of exaggerated sexually selected traits would be opposed by natural selection, suggesting sexual selection as a whole reduces non-sexual fitness. This may be especially true when sexual selection is largely driven by sexual conflict, because then, mate choice involves minimizing costs rather than maximizing sexually selected advantages (Gavrilets et al. 2001). Therefore with sexual conflict or in cases where no net benefits of female choice exist, there may well be no net accumulation of adaptive alleles, but rather populations would carry a selective load that reduces net fitness, which can potentially stop them from reaching fitness optima (Lande 1980; Kirkpatrick 1982), and there is evidence for this (Rice 1996; Pitnick and García-González 2002; Martin and Hosken 2003; Moore et al. 2003). Thus empirical and theoretical arguments suggest selection through sexual conflict makes adaptive sexual selection difficult. As a result, the long-standing debate over the adaptiveness of sexual selection still remains to be resolved.

Unfortunately, there is a paucity of studies investigating the effects of sexual selection as a whole (female choice and male-male competition) on non-sexual fitness components (reviewed in Candolin and Heuschele 2008; and see Hunt et al. 2009). The available evidence comes from a small number of species and is equivocal, making generalisation impossible. Additionally the potential costs and benefits of sexual selection have not been exhaustively assessed to enumerate the net fitness of evolving with or without sexual selection. For example, theoretical work (Lorch et al. 2003)

suggests that sexual selection is most likely to adaptive in novel situations prior to equilibrium. However, once equilibrium is reached it may become less-adaptive as resources are shifted into costly sexual competition, and recent empirical work has supported this idea (Fricke and Arnqvist 2007).

Here we use experimental evolution in replicate populations of *Drosophila simulans* to assess the net effect of sexual selection on adaptation to thermal stress (elevated natural selection). We employed a fully factorial design that allowed us to manipulate the opportunities for natural and sexual selection independently, and used lifetime reproductive success as a measure of non-sexual fitness for each of our 16 experimental evolution populations. Fitness of populations evolving under elevated natural selection was assessed thrice (after 10, 20 and 30 generations of selection), whilst populations evolving under ancestral conditions were only tested after 30 generations of selection. In all cases, selection was relaxed (standardised) for two generations and then populations were tested under evolutionary and standardised conditions (with and without additional larval competition). Additionally we also assessed our populations for evidence of differential inbreeding.

Material and Methods

Fly stocks

The base-line wild-type populations of *D. simulans* used here were derived from twenty isofemale populations, collected in 2004, and supplied by the Centre for Environmental Stress and Adaptation Research, La Trobe University, Australia. They had been maintained in a large population cage (ca. 800 flies/cage) with overlapping generations and free mate choice for 4 years prior to the start of this investigation and

have been found to harbour considerable genetic and phenotypic variation (e.g. Taylor et al., 2007,2008a; Wright et al., 2008). The *ebony* (a recessive, phenotypic body-colour mutant) stock population, used for larval competition assays, was established using a strain obtained from the Tucson stock centre and was maintained as above for over 50 generations. All flies were reared on '*Drosophila* quick mix medium' (Blades Biological, Edenbridge, Kent, U.K.) at 25°C and a 12:12 h light:dark cycle. Food was provided in excess so that differential larval competition was minimised. Subsequent housing conditions followed this regime unless stated otherwise.

Selection populations

Experimental flies were reared in replicate populations under standard laboratory environmental conditions (as described above) allowing the relaxed natural selection regime to operate (-NS) and in a novel environment that allows elevated natural selection (+NS), with relaxed (-SS) or elevated sexual selection (+SS). Constant low-grade temperature stress (= 2^{o}C elevation to 27^{o}C) was used to generate the novel environment. Slight temperature elevation has been shown to have a negative impact on fly fitness, reducing lifetime fecundity by nearly 50% (Murphy et al. 1983), and 27^{o}C is very close to the sterility threshold of *D. simulans* males (Chakir et al. 2002). This should therefore present a stressful environment to which the fly is not well adapted.

Flies with elevated (+SS) and relaxed (-SS) sexual selection (4 replicate populations of each treatment per environment, 2 environments (-NS, +NS) = 16 populations, with 60 females per population in the +SS treatment and 64 females per population in the -SS treatment) were allowed to evolve in these environments for ca. 30 generations (Figure 1). For the +SS treatment, each female was housed with four males, for the -SS

treatment one female was placed with one randomly chosen male. Males and females were housed together for 6-8 days (see below). Note that housing females with other flies does not alter their reproductive output compared to females housed alone (Taylor et al. 2008b), so competition for resources between adults would not affect outcomes.

Our experimental design allocates a greater number of females (64) in the -SS treatment however, by doing so it equalises the estimated effective population sizes (N_e) between treatments. Classically N_e is measured as $(4n_m n_f)/(n_m + n_f)$, where n_m is male number and n_f is female number (Wright 1931). In the +SS treatment within this experiment, the number of males contributing to population size (that is, siring offspring) would be determined by female mating frequency and sperm precedence patterns. *D. simulans* females will mate on average with two males in a 6-8 day time period (Taylor et al. 2008b), and there is strong last male sperm precedence; males that mate last, sire about 90% of a females subsequent clutch (Hosken et al. 2008 and see Gromko and Gerhart 1984; Champion de Crespigny and Wedell 2006).This means that for every female in the +SS treatment, we have on average 1.14 males. This difference was accounted for by increasing the number of pairs in the -SS treatment by four to give an estimated N_e of 128 in each population of each treatment. As previously stated, each female in the +SS treatment was housed with four males, and each female in the -SS treatment was placed with one randomly chosen male. We reiterate, that although the +SS treatment has a higher number of males, due to female mating patterns (remating frequency) and strong last male sperm precedence, only 1.14 males/female contribute genes to subsequent generations in the +SS treatment. Therefore, by slightly increasing the number of pairs in the -SS treatment,

effective population sizes are equalised across treatments. The same rationale applies when we use a more sophisticated estimator of N_e that includes a predetermined number of matings:

$$(N_e = \frac{4 n_m n_f l}{lN + nm - 1} + \frac{1}{2} + \frac{1}{2N}$$

where all is as previously but l = number of matings and N is census number (Balloux and Lehmann 2003). Note that even if evolution in the +SS treatment alters sperm precedence patterns or female mating rates, effects would have to be very large to generate significant differences in N_e. For example if females in the +SS treatment evolve to mate with only one male on average, N_e would be 120 (using the classical estimator), and if they mated on average with 1.5, N_e would only increase by 16. With this estimated effective population size, inbreeding effects are likely to be minimal: because the fixation index (*F*) depends upon the reciprocal of effective population size, at generation 30, *F* is predicted to be 0.11, assuming F_0 is zero - as it should be because of the large population size of our founder population. *F*-values of this magnitude have no effect on measures of fitness such as egg-adult viability in *D. simulans* (Kosuda 1980). Importantly, even a 20% difference in N_e across the treatments over all *30* generations only means an *F* difference of 0.03. As a result, differential inbreeding between treatments, and inbreeding in general, is predicted to have minimal effect on the final outcomes. Thus our protocol ensures that effective population sizes are standardised across treatments, and even relatively large deviations from equality are unlikely to generate major differences. This is critical as sampling error alone may mean that alleles adaptive for the new environments are present or absent purely due to the effective number of flies (alleles) present. Previous work has largely ignored this problem (but see Hosken et al. 2001). We also note that in the -SS treatment females

can still mate multiply with the single male, so that if mating multiply, rather than mating with multiple males, is beneficial for females, females in both treatments can reap benefits. Finally, after > 30 generations of selection, we tested our experimental populations for differential inbreeding depression (the methodology for this test is described later).

In each population flies were housed together for 6 days (interaction vials) before being placed into a new egg laying vial. This selects on adult survival, as only females surviving the initial period contribute to subsequent generations. After 2 days egg-laying, adults were discarded and vials incubated until offspring emergence. Virgin offspring that emerged on the peak emergence day (ca. day 9 after laying = stabilising selection on development time) from these laying vials were pooled and randomly chosen (within populations) to start subsequent generations (Figure 1). This also means that on average, females that produce more offspring surviving to adulthood will be favoured.

Test Conditions

After evolving under the imposed conditions for 10 & 20 generations, subset of +NS flies (those not chosen to continue the selection populations) were housed under standardised conditions for two generations (Figure 1). Standardising selection ensures that any environmental differences related to the selection regimes are not responsible for any detected effects (Martin and Hosken 2003) and in this instance, females were all housed with two males, rather than with four or one. Subsequent fitness assays were conducted on these flies under the standardised conditions and under the conditions flies evolved under (-SS females with 1 male, +SS females with 4 males). This allowed us to assess adaptation to the novel environments generally, and

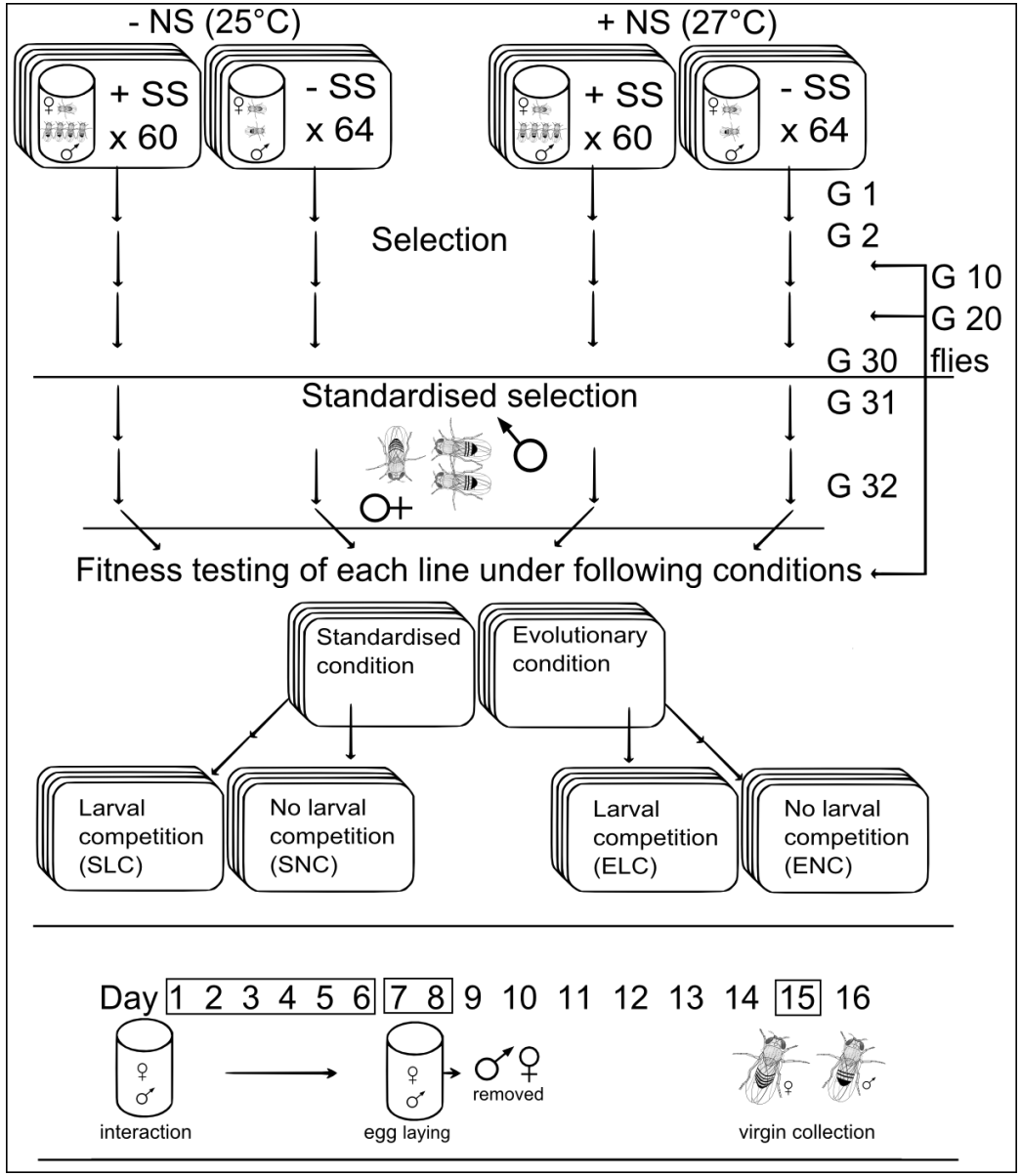

Figure 1. Outline of the basic experimental evolution procedure (see text for details). Flies were assayed after two generations of standardisation, under the standardised conditions and under the conditions in which they evolved (-SS females housed with one male, +SS females housed with 4 males). For the +NS treatment flies were assayed at generation 10 & 20 (plus standardising for 2 generations), as well as at generation 30 (plus standardisation), while the -NS treatment was only assayed after 30 generations (and two generations standardised treatment). Note that all assays were done with and without larval competition. Last part of the illustration depicts the standard rearing protocol, with six days of interaction, two days of egg laying and virgin collection on day 15. Abbreviations - ELC: evolutionary conditions with larval competition, ENC: evolutionary conditions with no larval competition, SLC: standardised conditions with larval competition and SNC: standardised conditions with no larval competition

specifically to the conditions flies evolved under. Finally, after 30 generations of selection, fitness of all 16 populations (i.e. those evolving under the ancestral conditions and those evolving under the novel conditions) was tested. Note that for flies evolving under ancestral conditions (the -NS treatment), fitness was only measured at generation 30, as here we were only interested in sexual-selection load under near equilibrium conditions. This is because sexual selection may be beneficial during adaptation (which is tested with the +NS treatment), but once populations become adapted to their environment, resources may be shifted in costly sexual conflicts. Similar arguments have been made to explain high biodiversity patterns in the tropics: relatively constant environments in the tropics free-up resources for use in competitive interactions (Mittelbach et al. 2007).

Thirty generations of selection should provide enough time for any differences that may arise between treatments to be manifest. Since the sexual selection differential between the two treatments is a function of the product of the number of males competing for the females and the number of generations of selection (Lande 1979; Lande and Arnold 1983; Arnold and Wade 1984a, b), after 30 generations, a difference proportional to approximately 120 male generations of sexual selection separates the two treatments. This should provide sufficient experimental power to detect any potential differences that arise, and it is important to note that previous experimental evolution studies have documented micro-evolutionary change in fewer generations and with less experimental power. For example, Hosken et al. (2001) imposed selection for 10 generations of evolution with 30 male-generations separating treatments and documented large differences between treatments. However, we assessed adaptation to the novel environment (+NS) three times in the current study

because research on bruchid beetles suggests that even over 30 generations, single assays may miss differences in rates of adaptation (Fricke and Arnqvist 2007). That is, if adaptation occurred rapidly, a single assay after 30 generations might not be sufficient to capture the differences in rates of adaptation. Additionally, this allows us to look at the evolutionary trajectory of our populations as they continue to adapt to the novel environment.

Measuring non-sexual fitness in the novel environment

We assessed lifetime reproductive success (LRS: the number offspring surviving to adulthood produced over a female's lifetime), as a measure of non-sexual fitness components (i.e. those not directly related to mating success) for the +NS treatment 3 times during experimental evolution (generation 10, 20 & 30 – Figure 1), under standardised and experimental conditions (see Test Conditions section above). This provides some indication of how general the effects (or lack of effects) of evolving with relaxed or elevated sexual selection are. The nonsexual fitness (LRS) of populations with relaxed and elevated sexual selection was assessed in the new environment (+NS) under the standardised (i.e. housed with two males) and evolutionary conditions (i.e. -SS females housed with one male, +SS females with 4) unless otherwise specified (see Test Conditions section above). Larval competition was also manipulated within these two conditions as difference between treatments may only be manifest with additional stress (Hoffmann and Parsons 1993). In one treatment, overabundant food ensured minimal larval competition and in the second, non-selected *ebony* bodied *D. simulans* competitor larvae were used to create resource competition. This gave us four assay conditions (Figure 1), evolutionary conditions with larval competition (ELC),

evolutionary conditions with no larval competition (ENC), standardised conditions with larval competition (SLC) and standardised conditions with no larval competition (SNC).

LRS Assay: LRS was measured by providing females (10 per population) with new laying substrate every day for the first two days followed by another period of five days and then counting the adult offspring that emerge from these vials. Briefly, on emergence, females were housed with newly emerged males (from the same population as the females), as per the test conditions described earlier. Clutches were then reared under the appropriate experimental conditions (+NS, -NS) and the numbers of emerging offspring were scored. The same procedure was used for competitive LRS assessments, except here competitor flies (one female and two males) carrying the recessive phenotypic marker (*ebony*) were housed in vials for 24 hours prior to the introduction of experimental females and males. When experimental flies were added to a vial, *ebony* flies were removed. While *ebony* adults were therefore never housed with the experimental flies, their larvae provided competition. By carrying the experiment out this way, we provide the *ebony* competitors with a 24 hour opportunity to establish before the introduction of the experimental flies and ensure that the *ebony* competitor females cannot mate with experimental males: the marker is recessive and we would not therefore be able to accurately assign offspring to particular females.

Quantifying the net load or benefit of sexual selection

To quantify the net load or benefit of sexual selection we measured the net fitness (LRS) of the -NS populations evolving with elevated or relaxed sexual selection. Our rationale was that if there is a net benefit to sexual selection under equilibrium conditions (ancestral conditions with -NS) then the LRS of populations evolving with

elevated sexual selection would be greater than that of those evolving with relaxed sexual selection. Here we housed newly eclosed (= virgin) females and males from the same treatments under standardised (females with 2 males) and evolutionary conditions (-SS females with single males, +SS females with 4)(n = 10 pairs per housing regime/population/treatment) in vials with ample food. Females were moved to new egg laying vials daily for the first two days and then, allowed to oviposit for a period of five days in the third vial. Vials were incubated, and eclosing offspring counted on a daily basis for seven days after first eclosion. *D. simulans* larvae take 8-9 days to develop and eclose, so 7 days following the first eclosion allows for almost all the progeny to be accounted for without including any grandchildren. Taylor et al. (2008a) have previously tested the reliability of this short term measure of life time reproductive success and found it to be highly reliable. The LRS was then compared for the +SS and -SS treatments.

Testing for differential inbreeding depression

Although we expected no differential inbreeding depression in our treatments (see above) we nevertheless conducted an assay to test if this was the case or not. We established a factorial design in which a subset from each of our populations (within the elevated and relaxed sexual selection treatments in the novel environment) was either allowed to inbreed or outbreed (see Figure 2). The reproductive outputs of the inbred populations were then compared with the output of the outbred populations. Briefly, a six day old virgin female was paired with two males of the same age, and 20 such pairings were established for each of the combinations shown in Figure 2. The female was then allowed to oviposit for 24 hours in a vial before being moved over to a new vial for another 24 hours of egg laying, followed by a final move to a new vial

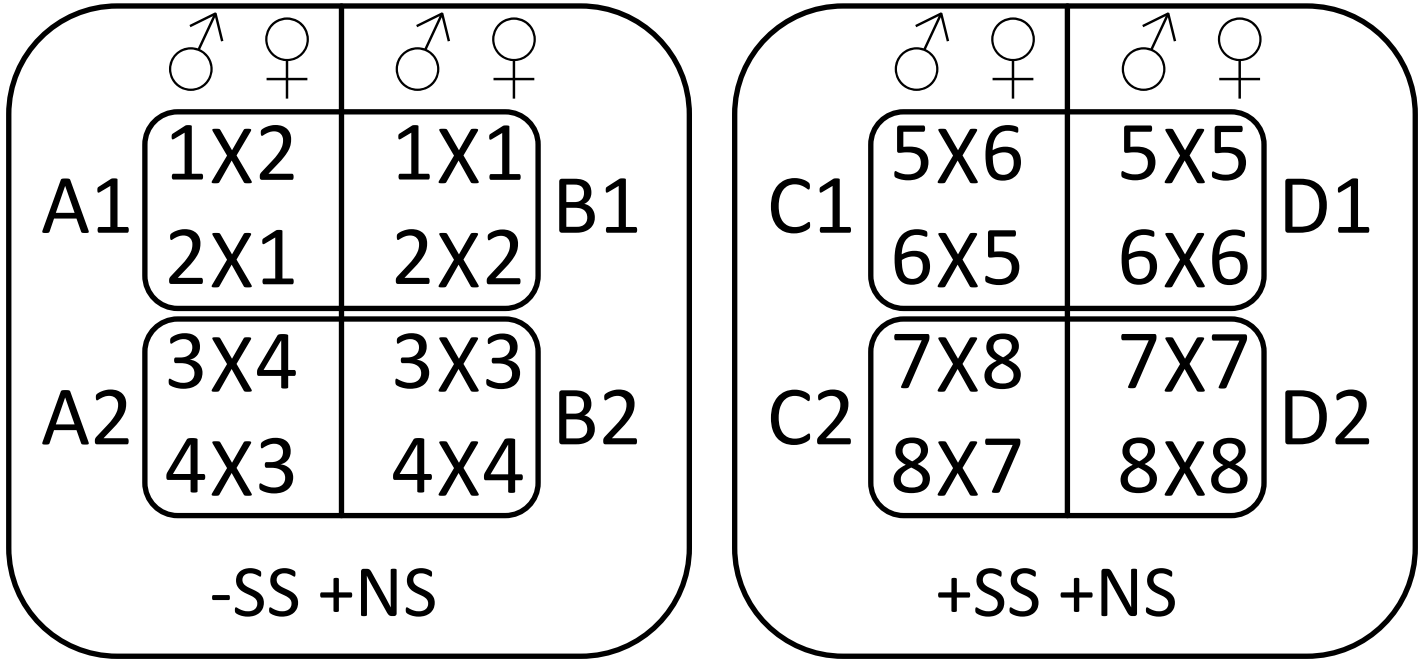

Figure 2. An overview of the mating design used while testing for inbreeding depression after > 30 generations of experimental evolution under elevated natural selection. 8 of our experimental evolution populations which were part of the elevated natural selection treatment (4 replicate populations for the relaxed sexual selection treatment and 4 for the elevated sexual selection treatment) are represented by numbers 1-8 respectively. The life time reproductive success of the inbred and outbred combinations was then tested with a paired *t* test for each treatment.

where she was allowed to oviposit for 5 days. This provides us with a short-term estimate of her LRS. The number of total offspring produced from each mating was counted after 7 days of eclosions by adding up the number of offspring eclosed from each of the three egg laying vials.

Statistical analysis

All statistical analyses were conducted in PASW v 18 (formerly SPSS). To assess the effects of sexual selection on adaptation, we analysed our data with a multivariate analysis of variance (MANOVA). Here sexual selection and natural selection (manipulations of +/-SS or +/-NS) were treated as main (fixed) effects and the measure of LRS from each of our assay conditions (ELC, ENC, SLC, SNC) was used as the dependent variable. We base our main conclusions with regard to the adaptiveness of sexual selection on this model. A slightly different model was used for our assessment of evolution along the three time points where we measured population fitness (generation 10, 20 and 30). Here generation and sexual selection (relaxed or elevated) were used as main (fixed) factors in a MANOVA model with number of offspring produced in each assay condition (as elaborated earlier) as the dependent variables. This model directly tests for changes in fitness (LRS) at three time points under each of our assay conditions. To examine the effects of inbreeding, the total number of offspring produced by the inbred and outbred crosses (see Testing for differential inbreeding depression earlier) were compared with a paired *t* test.

Results

Lifetime reproductive success

Results from the multivariate analysis using sexual selection (+SS/-SS) and natural selection (+NS/-NS) as fixed factors and the LRS scores for each of our four assay conditions as dependent variables reveals a significant effect of sexual selection on the multivariate combination of traits, (Wilks' lambda = 0.226, $F_{(4,9)}$ = 7.69, P = 0.006), but there was no significant effect of natural selection (Wilks' lambda = 0.496, $F_{(4,9)}$ = 2.28, P = 0.14) nor any interaction (Wilks' lambda = 0.398, $F_{(4,9)}$ = 3.41, P = 0.06) between the two selection regimes. Post-hoc univariate analysis suggested that sexual selection had a significant effect on the LRS reported in both our non competitive assays (ENC: $F_{(1,12)}$ = 9.26, P = 0.01; SNC: $F_{(1,12)}$ = 12.84, P = 0.004), and this effect was driven by a higher LRS in the +SS populations compared to -SS populations (ENC: Mean difference = 16.9 ± 5.56, P = 0.01; SNC: Mean difference = 18.81 ± 5.25, P = 0.004) in both cases. Additionally, there was a significant interaction between natural and sexual selection under SNC conditions ($F_{(1,12)}$ = 5.28, P = 0.04), and this was driven by an overall lower fitness of relaxed sexual selection populations in presence of elevated natural selection (see Figure 3). In both the competitive assays however, there was no significant effect of sexual selection, natural selection or any interactions of the two (see Table 1). Overall this suggests that presence or absence of larval competition (i.e. assay conditions) had a significant bearing on the results. We designed this study to include an additional stress as sometimes differences between treatments are only manifest in presence of stress (Hoffmann and Parsons 1993). It seems however, that presence of additional stress in this case negates any adaptive advantages to sexual selection.

Table 1: Results of the univariate analysis testing the effect of natural selection, sexual selection and their interaction on the fitness (number of offspring produced) of 16 experimental evolution populations of *Drosophila simulans*. Fitness measures were derived under four different assay conditions wherein the flies were tested under evolutionary or standardised condition in the absence or presence of larval competition. Significant P values are highlighted in bold. Abbreviations - ELC: evolutionary conditions with larval competition, ENC: evolutionary conditions with no larval competition, SLC: standardised conditions with larval competition and SNC: standardised conditions with no larval competition

Univariate results: Lifetime reproductive success									
	Natural selection			**Sexual selection**			**Interaction**		
Assay condition	***df***	***F***	***P***	***df***	***F***	***P***	***df***	***F***	***P***
ELC	1, 12	.863	.371	1, 12	2.588	.134	1, 12	.009	.927
ENC	1, 12	2.113	.172	1, 12	9.257	**.010**	1, 12	4.029	.068
SLC	1, 12	.322	.581	1, 12	.039	.847	1, 12	.638	.440
SNC	1, 12	3.952	.070	1, 12	12.838	**.004**	1, 12	5.278	**.040**

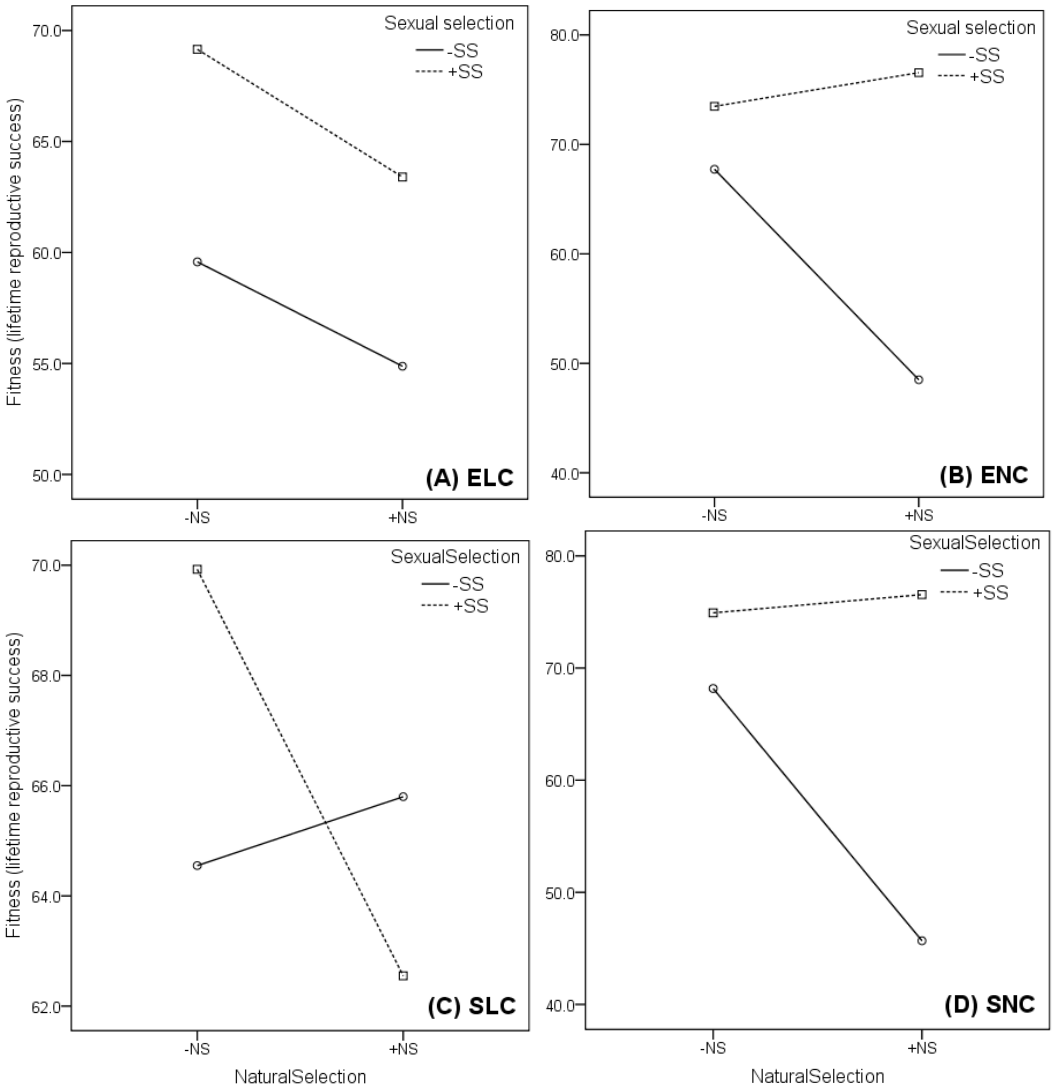

Figure 3: This plot shows the interaction between sexual and natural selection for lifetime reproductive success. Panels A, B, C and D represent the four assay conditions employed for fitness tests (ELC: evolutionary with larval competition, ENC: evolutionary with no larval competition, SLC: standardised with larval competition and SNC: standardised with no larval comp). The manipulations of natural selection are represented on the X axis and the population fitness as measured by lifetime reproductive success is on the Y axis. Note that only the interaction represented by panel D was significant (see Table 1) and here the LRS of sexual selection (+SS) populations appears to be higher than that of the relaxed sexual selection (-SS) populations, and particularly when natural selection was also elevated.

Evolution over time

We also assessed the trajectory of our elevated natural selection (novel environment) populations through time. Results from the multivariate analysis of this dataset indicated that both assay generation (Wilks' lambda = 0.05, $F_{(8,30)} = 13.69$, $P < 0.001$) and sexual selection (Wilks' lambda = 0.25, $F_{(4,15)} = 11.29$, $P < 0.001$) had significant effects on the multivariate combination of dependent variables, and there was a significant interaction between them (Wilks' lambda = 0.29, $F_{(8,30)} = 3.19$, $P = 0.01$). Post-hoc univariate analysis indicated that the effect of this interaction varied between the assays and was significant only for the ELC and SLC assays (ELC: $F_{(2,18)} = 6.58$, $P < 0.01$; ENC: $F_{(2,18)} = 0.24$, $P = 0.8$; SLC: $F_{(2,18)} = 6.77$, $P < 0.01$; SNC: $F_{(2,18)} = 2.91$, $P = 0.08$; and see Figure 4). The effect of sexual selection was significant in the ENC and SNC assays, and it was driven by an overall higher fitness of populations evolving with elevated sexual selection compared to those evolving with relaxed sexual selection (at all three time points – generation 10, 20 and 30).

This suggests that the evolutionary trajectory of our populations along the three time points was dependent on the assay conditions. For example, in the ELC assay relaxed sexual selection populations had a higher fitness than elevated sexual selection populations at generation 10. However, this relationship changed by generation 20 when the fitness of relaxed sexual selection populations was lower than that of the elevated sexual selection populations (see Figure 4). An overall pattern apparent from this analysis is that, both the elevated sexual selection and relaxed sexual selection populations had significant declines in their fitness (LRS) from generation 10 to generation 30. This decline in fitness over time complicates the interpretation of previous work, and is indicative of inbreeding depression.

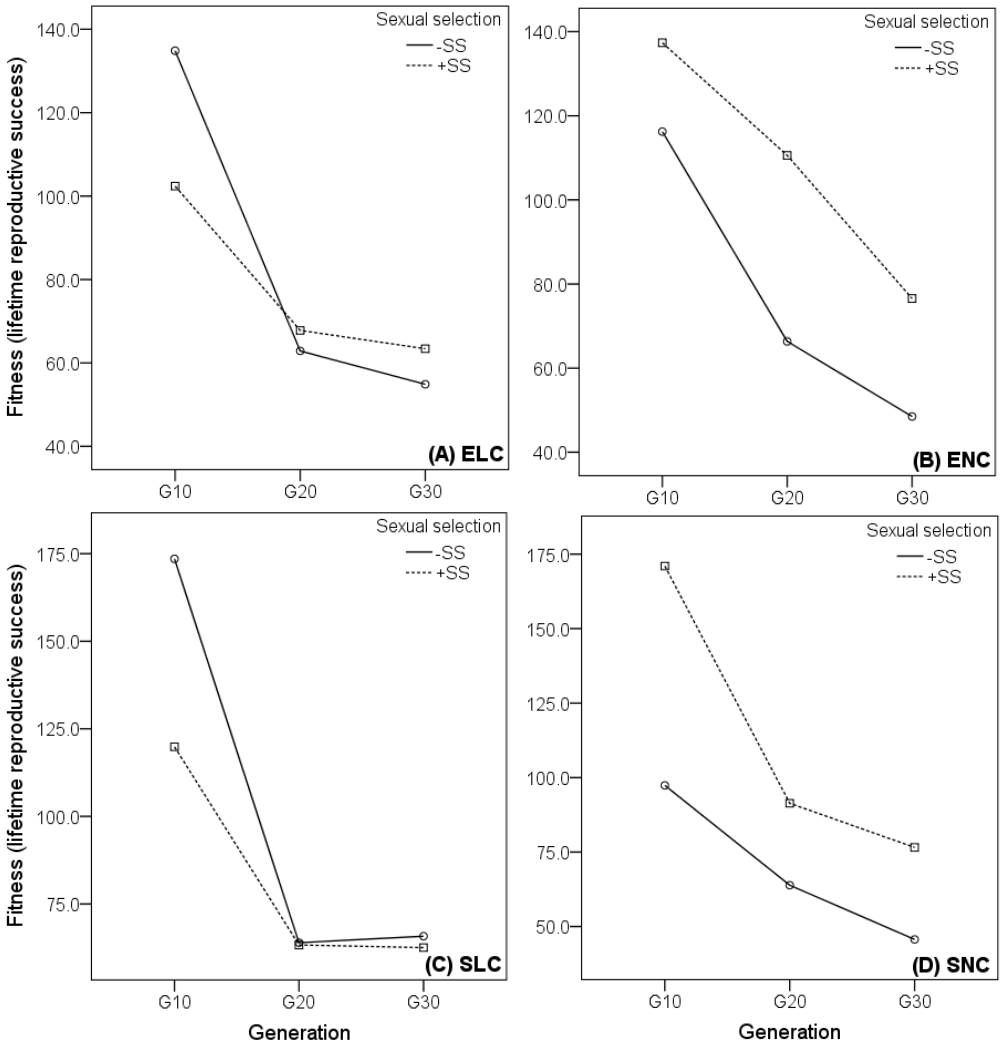

Figure 4: Interaction plots showing the trajectory of our elevated natural selection populations under relaxed or elevated sexual selection. Panels A, B, C and D represent the four assay conditions employed for fitness tests (ELC: evolutionary with larval competition, ENC: evolutionary with no larval competition, SLC: standardised with larval competition and SNC: standardised with no larval comp). On the X axis is the generation when the test was conducted and the Y axis represents fitness as measured by lifetime reproductive success.

Differential inbreeding

Inbreeding depression is caused by increased homozygosity and is known to operate via two genetically distinct mechanisms: increased homozygosity for partially recessive detrimental mutations and increased homozygosity for alleles at loci with heterozygote advantage ('overdominance') (Charlesworth and Willis 2009). Thus by crossing lines we should reduce homozygosity (restore heterozygosity) and see a fitness rebound if inbreeding depression caused the fitness decline over time reported above. Data from our differential inbreeding detection assay was analysed with a paired samples *t*-test. Results indicated that there was no difference in the number of offspring produced by the outbred (Mean= 118.89, SD=2.92) and inbred (Mean=110.66, SD=5.72) crosses when both sexual and natural selection (+SS +NS) were elevated ($t_{(1)}$=4.15, $P_{T<=t}$=0.15). Populations with relaxed sexual selection and elevated natural selection (-SS +NS) also showed no fitness differences between the outbred (Mean=81.27, SD=4.74) and inbred (Mean=82.87, SD=2.11) crosses ($t_{(1)}$=4.15, P=0.15). This evidence suggests that differential inbreeding depression did not cause the decline in fitness of our populations (see Figure 5).

Discussion

The main finding of this study was that sexual selection in *Drosophila simulans* can aid adaption to a novel stressful environment under some conditions. However, under other conditions sexual selection did not facilitate adaptation. While we found sexual selection to be adaptive in the absence of additional stress, the adaptive advantage of sexual selection was lost when fitness was tested in presence of elevated larval competition. Furthermore, the overall fitness of our populations declined over the 30 generations of selection although this was not due to differential inbreeding

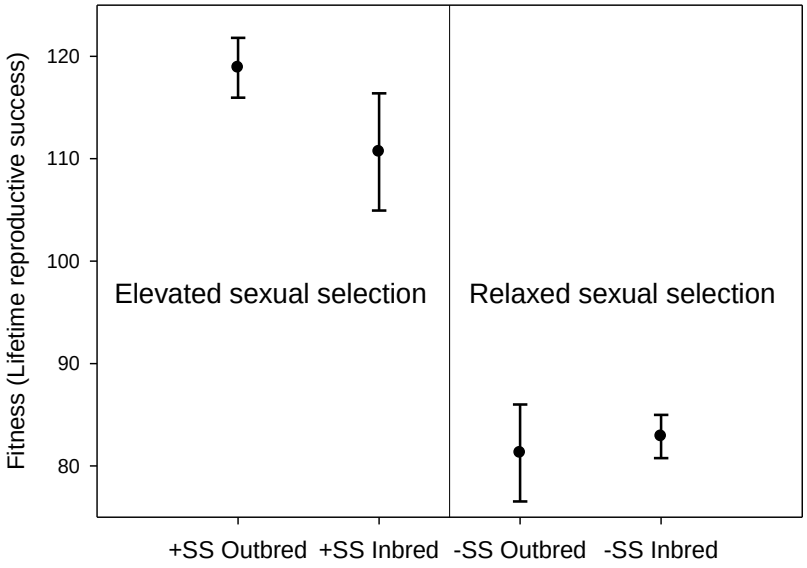

Figure 5: Plot showing the results from the paired *t* test. Left half of the plot shows the lifetime reproductive success from the outbred and inbred combinations within the elevated sexual selection treatment (see text) and the right half represents the relaxed sexual selection treatment. Y axis represents fitness as measured by the lifetime reproductive success and the error bars represent ±SD.

depression. Below, we discuss these results and their implications in light of the existing debate on the adaptive nature of sexual selection.

Results from the multivariate analysis of lifetime reproductive success showed a significant effect of sexual selection on the LRS of our experimental populations. However, post-hoc analysis revealed that the assay conditions had a significant impact on the outcomes. For example, while the adaptive effect of sexual selection was apparent in the two assays without any additional larval competition (ENC and SNC), the same effect was not apparent in the assays with additional larval competition (ELC and SLC). By adding larval competition as an additional stress we intended to increase our chances of detecting any differences between treatments, as it has been suggested that such differences may sometimes only manifest in presence of additional stress (Hoffmann and Parsons 1993). Contrary to our expectations, we found that additional larval competition masked the apparent adaptive nature of sexual selection. While part of our result supports theoretical models that sexual selection can enhance the rate of adaptation to novel environments (Proulx 1999; Whitlock 2000; Lorch et al. 2003), the other part concurs with a majority of experimental evolution studies that find a negative or no impact of sexual selection on adaptation to a novel environment (e.g. Holland 2002; Rundle et al. 2006).

In one of the first attempts to test the benefits of sexual selection, Partridge (1980) used a single generation experiment and manipulated the opportunity for sexual selection in *Drosophila melanogaster* by establishing a set of 'choice' and 'no choice' lab populations (+SS and –SS respectively). Larval viability of these populations was then measured. Results suggested that the larval viability for the offspring of +SS

treatment females was 1-2% higher than that for the –SS treatment females. Our findings from the no larval competition assays are in agreement with Partridge's general conclusion that mate choice can augment components of fitness. Promislow et al. (1998) applied selection for multiple generations and then relaxed selection for a period to eliminate potential confounding effects of nongenetic factors, before testing larval and adult fitness components. Promislow et al. (1998) did find evidence to suggest that sexual selection led to an increase in some adult fitness components, however their experiment showed no significant differences in adult survival under the imposed selective conditions (i.e. less than 30 days of age). Additionally, they found no effect of selection treatments on the larval viability / competitive ability in their test populations and the this lack of an effect was attributed to insufficient levels of larval competition (Promislow et al. 1998). In contrast, we find that additional larval competition actually masks the adaptive effects of sexual selection that we detect in assays without elevated larval competition. Holland and Rice (1999) also employed experimental evolution in populations of *D. melanogaster* with and without sexual selection. After 47 generations they showed that the net reproductive rate of adult females was greater in the absence of sexual selection, and in another experimental evolution study, Holland (2002) subjected two replicate experimental populations of *D. melanogaster* to 36 generations of thermal stress in presence and absence of sexual selection (polyandrous and monogamous populations respectively). Holland (2002) found no differences in the fitness of polyandrous and monogamous populations, consistent with the notion that sexual selection, as a whole, is not adaptive. However, the net reproductive rate of experimental populations was reported to be significantly higher in relation to the controls. Sex in *D. melanogaster* includes a substantial element of conflict (Rice 1996; Pitnick and García-González 2002), thus Holland's

finding was also consistent with the prediction that sexual selection is unlikely to be adaptive in systems with substantial selection through sexual conflict.

In contrast to *D. melanogaster* where the mate choice system is driven by sexual conflict (Rice 1996; Pitnick and García-González 2002; Wigby and Chapman 2004; Orteiza et al. 2005), evidence for *D. serrata* is suggestive of good-genes mate choice (Hine et al. 2002). Rundle et al (2006) used 12 replicate populations of *D. serrata*, in a two-way factorial design where they independently manipulated the opportunities for both sexual and natural selection for 16 generations. Their rationale was that the presence of good-genes mate choice should improve the rate and extent of adaptation to a novel environment (Lorch et al. 2003). Although their experimental design was robust and could estimate the independent and the combined roles of natural and sexual selection during adaptation to a novel larval food resource, they did not find sexual selection to be adaptive.

In species other than *Drosophila*, Martin et al (2004) found that singly mated yellow dung fly females (*Scathophaga stercoraria*) from polyandrous populations had lower fitness than those from monogamous populations. Similar studies on the bulb mite (*Rhizoglyphus robini*) were inconclusive as they found no clear effect of sexual selection on population viability (Radwan et al. 2004; Tilszer et al. 2006). Overall, the role of sexual selection in augmenting natural selection is far from clear and our results seem to provide evidence both for and against an adaptive influence of sexual selection.

Given that costs of mating are reported to be low in *D. simulans*, and sexual selection does not seem to be driven by sexual conflict in this species (Taylor et al. 2009), the most parsimonious explanation for what we observe here in the no larval competition

assays (i.e. +SS populations had higher fitness than –SS populations) should come from the good-genes models of mate choice. These models predict rapid adaptive evolution when sexual selection is elevated (e.g. Lorch et al. 2003). Good-genes benefits are known to be very small at best (Møller and Alatalo 1999) and repeated attempts to quantify these benefits in *D. simulans* have failed to detect any evidence for good-genes benefits in this species (Taylor et al. 2010; Sharma et al. MS). However, both of these studies did not employ multi-generation experimental evolution and it is possible that the adaptive effect of sexual selection we observed in the current study was supported by an accumulation of small good-genes benefits over 30 generations of selection. Furthermore, we tested the fitness of our populations under the standardised and evolutionary conditions to determine if they had adapted more "generally" or "specifically" to the conditions they evolved under. However, in both cases we find that presence of additional larval competition changes the outcomes. Sexual selection is known to be maladaptive under sudden changes (see Candolin and Heuschele 2008), and this may explain why the benefits of sexual selection (higher LRS) were not apparent when populations were tested in presence of additional larval competition. It is also worth considering that by manipulating larval competition we essentially tested the populations in a different environment for which they adapted for many generations, and this could have caused a strong genotype x environment (GxE) type interaction. A 'strong' GxE interaction is characterised by a reduced performance of a genotype in one environment relative to the other (see Ingleby et al. 2010 for a review), and this is essentially what we observe here in the assays with additional larval competition – populations evolving with elevated sexual selection performed no better than those evolving without. In a recent review of experimental evolution studies, Edward et al. (2010) highlighted the need to consider such factors

(e.g. GxE interactions) in concluding whether the difference between populations is because of the selective regime applied and recommended more caution in this respect.

We assessed adaptation to the novel environment three times in this study. Our rationale was that a single assay after 30 generations may miss the differences in rates of adaptation (Fricke and Arnqvist 2007). Furthermore, this allowed us to view the evolutionary trajectory of our populations as they evolved in the novel environment. Results indicated that when fitness was assayed without additional larval competition there was a significant effect of sexual selection over the generations, and the elevated sexual selection populations had higher fitness than the relaxed sexual selection populations. However, when populations were assayed in presence of additional larval competition, the relative fitness of populations evolving with relaxed or elevated sexual selection changed over time. For example, in the ELC assay, relaxed sexual selection populations had a higher fitness at generation 10, but at generation 30 the elevated sexual selection populations had a higher fitness. Furthermore, there was a sharp decline in the number of offspring produced over time under both selective regimes (i.e. elevated or relaxed sexual selection). A possible explanation of such a decline would come from inbreeding depression (Snook 2001; Martin and Hosken 2003; Wigby and Chapman 2004). Wright et al. (2008) reported substantial inbreeding depression in life history traits for *D. simulans* which could explain the declines we saw. However, we found no evidence of inbreeding depression in our populations. This indicates that the effective population sizes we estimated as part of the main experimental design (see material and methods section) were large enough,

and the experimental setup robust enough, to prevent the occurrence of (differential) inbreeding. Thus the decline in fitness over time cannot be explained by inbreeding.

An alternative explanation is the presence of selfish genetic elements or reproductive endosymbionts (Stouthamer et al. 1999; Hurst and Werren 2001) such as *Wolbachia* and *Spiroplasma*. For example, *Wolbachia* are present in many insects (Werren et al. 2008) and are known to decrease population productivity (number of offspring produced each generation), reduce sperm competitive ability (Champion de Crespigny and Wedell 2006), increase extinction risk by reducing genetic diversity and reduce effective population size (reviewd in Charlat et al. 2003). Four distinct reproductive phenotypes of *Wolbachia* are known (Werren et al. 2008), and the cytoplasmic incompatibility (strong reproductive incompatibilities between uninfected females and infected males: CI) inducing phenotype is known to dramatically reduce reproductive output when it is not fixed in a population (Champion de Crespigny and Wedell 2007). The presence of a CI inducing *Wolbachia* in our populations could explain the decline in reproductive output we observe, and if *Wolbachia* infected males had a lower fitness (e.g. lower sperm competitive ability, or lower amounts of fertile sperm) it could potentially explain why sexually selected populations seemed to have a higher fitness (at least under the non competitive assays), in absence of an explanation via good-genes benefits. This remains to be tested.

Conclusions

Overall our results provide mixed evidence for the adaptiveness of sexual selection. On one side, sexual selection appears to be adaptive, and whilst this can be explained by good-genes models of sexual selection, we have no evidence for good-genes benefits in *Drosophila simulans* (Taylor et al. 2010; Sharma et al. MS). However it remains

possible that small good-genes benefits accruing over time may be responsible for the effects we observe here. In contrast, advantages of sexual selection were lost in presence of additional stress. Our results therefore suggest that sexual selection is not always adaptive. Also, the decline observed in the overall fitness of our selection populations over time currently remains unexplained and complicates our interpretation of the adaptiveness of sexual selection. It also raises more questions that warrant further experimental work, for example, if sexual selection was adaptive why did we see a decline in fitness of sexually selected lines over time? A likely explanation for these results is based on the presence of selfish genetic elements like *Wolbachia*, but their presence, and mechanism of action in our populations remains to be determined.

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Chapter Seven

Sex combs, allometry and asymmetry in *Drosophila*

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Abstract

There has been recent debate about the expected allometry of sexually selected traits and although they exhibit a diversity of allometric patterns, signalling characters are frequently positively allometric. In contrast, insect genitalia tend to be negatively allometric, but the allometry of non-genital sexually selected characters in insects is largely unknown. It has also been suggested that there should be a negative association between the asymmetry and size of bilaterally-paired, sexually-selected traits, but this claim is controversial. We assessed the allometry and asymmetry (FA) of a non-genital contact-courtship structure, the sex comb, in replicate populations of three species of *Drosophila* (we also measured wing FA). Sex combs are sexually selected characters used to grasp the females' abdomen and genitalia and to spread their wings prior to and during copulation. While species differed in the size of the sex combs, all combs were positively allometric, and comb allometry did not generally differ significantly between species or populations. Comb and wing asymmetry did vary across species, but not across populations of the same species. However, FA was trait specific and was never negatively associated with trait size.

Keywords Developmental stability, Diptera, *D. melanogaster, D. pseudoobscura*, *D. simulans,* FA, scaling.

Introduction

The study of allometry, changes in trait dimensions relative to changes in overall organismal size, has a long history (Gould, 1966; Huxley, 1932; Huxley, Needham & Lerner, 1941; Huxley & Teissier, 1936). The scaling relationship among individuals of the same species between one trait and total body size, or between two traits at a single developmental stage is called static allometry (Cock, 1966; Gould, 1966). Most traits tend to display negative static allometry (Cuervo & Møller, 2009; Eberhard, 2002) and perfect isometry seems to be rare (Gould, 1966). In contrast to general scaling patterns, many sexually selected traits show positive allometry (Green, 1992; Kodric-Brown, Sibly & Brown, 2006; Petrie, 1988; Simmons & Tomkins, 1996) leading to the suggestions that positive allometry is indicative of (directional) sexual selection (Green, 1992; Kodric-Brown *et al.*, 2006; Petrie, 1988). However, positive allometry can be generated without directional selection (Bonduriansky & Day, 2003). Additionally in spite of claims that sexually selected traits always show positive allometry (Kodric-Brown *et al.*, 2006), a recent review found that many sexually selected characters do not scale in this way (Bonduriansky, 2007), although signalling characters and weapons were generally positively allometric (Bonduriansky, 2007). What is also apparent from Bonduriansky's (2007) review is that the allometry of structures under sexual selection often evolves rapidly and divergently in closely related species (e.g. Baker & Wilkinson, 2001; Emlen, Hunt & Simmons, 2005; Shingleton *et al.*, 2007) and geographically isolated populations of the same species can also differ in trait allometry (Moczek & Nijhout, 2003).

In contrast to characters used in sexual signalling, the male genitalia of insects and spiders, which are also sexually selected (Eberhard, 1985; Hosken & Stockley, 2004),

tend to show negative allometry (Eberhard *et al.*, 1998; Hosken, Minder & Ward, 2005). The low allometric slopes of arthropod genitalia can be explained by mechanical and stimulatory versions of the one-size-fits-all hypothesis (Eberhard, 2009), but while the allometry of insect genitalia has been extensively investigated, fewer studies have looked at the allometry of non-genital characters in insects. This may, partly be because it is not always clear whether particular non-genital characters are sexually selected in insects or not.

In addition to debates about the scaling of sexually selected characters, there have also been disputes about the relationship between trait symmetry and sexual selection (Møller & Swaddle, 1997; Tomkins & Simmons, 2003). Fluctuating asymmetry (FA: small random deviations from perfect symmetry in bilateral traits: Van Valen, 1962), a measure of developmental instability, has been argued to reflect an individual's genetic quality (Møller & Swaddle, 1997). Higher quality individuals are predicted to have lower FA and at the same time also bear larger sexual traits. Essentially, larger sexually selected traits should be more symmetrical since only high quality individuals can pay the costs of the larger traits and maintain developmental stability (Møller & Swaddle, 1997). Furthermore, FA levels should also be correlated across characters because FA reflects general quality (Møller & Swaddle, 1997). However, while FA has been linked to sexual selection and fitness in some taxa, this seems not to be the case in many species (Cuervo & Møller, 2009; David *et al.*, 1998; Hunt & Simmons, 1998; Martin & Hosken, 2002; Tomkins & Simmons, 1995; reviewed in Tomkins and Simmons, 2003).

The sex comb(s) of *Drosophila* are male-specific secondary sexual characters. They consist of a row of stout, modified mechanosensory bristles (comb teeth) on the fore-leg tarsus (Figure 1) and are found in all species groups in *Sophophora* with the exception of the Neotropical *saltans* and *willistoni* groups (Lakovaara & Saura, 1982). They are sexually selected, being involved in male-female tactile interactions during courtship and mating (Cook, 1977), although selection on the combs varies across species. For example, in free-living *D. simulans,* mating success is negatively associated with tooth number and comb size (Markow, Bustoz & Pitnick, 1996), while in *D. bipectinata*, males with larger and more symmetrical sex combs have greater mating success (Polak, Starmer & Wolf, 2004). In contrast, sex comb size in *D. pseudoobscura* does not seem to significantly affect mating success (Markow *et al.*, 1996). Little is known about the allometry of sex combs however, prompting calls for investigations of how they scale with body size (Bonduriansky, 2007). Here we investigate the allometry and asymmetry of sex combs for two distinct geographical populations of each of three closely related *Drosophila* species, *D. melanogaster, D. simulans* and *D. pseudoobscura.*

Methods

We used three *Drosophila* species, with each represented by two populations from distinct geographical areas. *Drosophila simulans* populations came from Tincurry (T) and Denmark (D) in Eastern and Western Australia respectively. The *D. melanogaster* Canton-S (C) population came from the *Drosophila* stock centre and the other was from Walpole (W) in Western Australia. *D. pseudoobscura* populations were collected

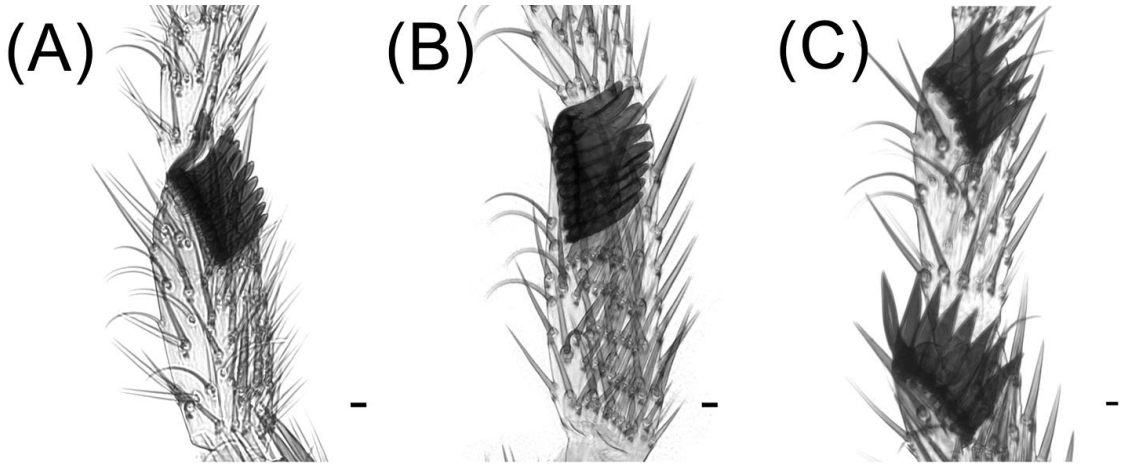

Figure 1: The sex combs of (A) *D. simulans*, (B) *D. melanogaster* and (C) *D. pseudoobscura*. Measurements were taken along the base of the sex combs. Scale bars represent 10µm. *D. pseudoobscura* has two sex combs, one each on the first and second tarsus whilst *D. simulans* and *D. melanogaster* have a single sex comb on the first tarsus.

from Sholow, Arizona (S) - and Lewiston, Montana (L) in the USA. Details of sex comb tooth arrangement in all populations are listed in Table 1. All fly stocks were lab adapted for being housed in population cages for > 50 generations and reared on *Drosophila quick mix medium* (supplied by Blades Biological, UK) at 25°C (±0.1°C) under a 12:12 light/dark cycle. Flies were sexed under light anaesthesia with CO_2 within 8 hours of eclosion. Virgin males were then collected and housed in 45mL vials at a density of no more than 10 males per vial. After three days, males were collected under light CO_2 anaesthesia and preserved in 70% ethanol at -80°C prior to measurement. All flies were treated with 10% KOH solution at 75^{o}C for forty five minutes, and transferred to 80% glycerol-ethanol solution after passing through graded washes (adapted from Atallah *et al.*, 2009). The right and left wings of males along with both pro-thoracic legs were removed from each fly and then mounted on slides using Hoyer's medium, for measurement of sex comb, tarsus and wing dimensions. Measurements were made on at least 100 males of each species, with a minimum of 50 males measured per population. Empirical evidence in *Diptera* suggests that wing length tends to scale negatively whilst hind-tibia length (HTL) scales isometrically (Eberhard, 2002). A preliminary analysis was conducted using both measures and results (not shown) suggested higher positive slopes when we use HTL instead of wing length. Therefore we conservatively used wing length as a measure of body size (Gilchrist & Partridge, 1999) instead of HTL.

Wing (50X) and foreleg images (300X) were acquired using a Leica inverted microscope connected to a PC digital image acquisition system and later analysed manually using NIH Image J. Wing length (WL) was measured as the length of the 1st posterior cell, from anterior cross-vein (the junction of the longitudinal vein III) to distal tip (border of

Table 1: Details of sex comb tooth number in different populations of three species of *Drosophila*.

Species	Population	First tarsomere teeth no.(range)		Second tarsomere teeth no.(range)	
		Right comb	Left comb	Right comb	Left comb
D. simulans	T	9 (7-11)	9 (7-11)	0	0
	D	9 (6-11)	9 (7-11)	0	0
D. melanogaster	C	10 (8-13)	10 (8-12)	0	0
	W	10 (7-12)	10 (8-12)	0	0
D. pseudoobscura	S	7 (5-9)	7 (5-8)	6 (4-6)	5 (5-7)
	L	6 (4-8)	6 (5-8)	5 (4-6)	5 (4-7)

the wing) of vein III. Both wings of each individual were measured and an average value calculated. Comb length was measured as the greatest length subtended by the sex comb (CL) along its base, and sex comb tooth number (TN) was also counted. All traits were measured twice on different days without reference to the previous measurement to allow analysis and partitioning of measurement error (ME) which is essential for subsequent FA analysis (Palmer, 1994). Regression of WL, CL and TN measure one on measure two showed they were significantly associated (WL: r^2 = 0.999, $F_{1,318} = 1.4^{e+8}$, $P < 0.001$; CL: $r^2 = 0.998$, $F_{1,318} = 2.04^{e+6}$, $P < 0.001$; TN: $r^2 = 0.998$, $F_{1,318} = 1.6^{e+6}$, $P < 0.001$). We also assessed the impact of our mounting technique on measurement error and again regression of measure 1 on measure 2 (after remounting specimens) revealed that our techniques were highly repeatable (CL: r^2 = 0.92, $\beta = 0.96$, $F_{1,40} = 4.25^{e+2}$, $P < 0.001$; TN: $r^2 = 1$, $\beta = 1$, $F_{1,160} = 1.57^{e+32}$, $P < 0.001$). Prior to any analyses, data were checked for potential outliers following Palmer (1994) and Palmer and Strobeck (2003). Grubb's test revealed 7 extreme data points which were removed (Palmer & Strobeck, 2003). The filtered dataset was used for all subsequent analyses.

Comb allometry was then quantified by regressing $\log_{10}$ transformed comb lengths against $\log_{10}$ wing lengths and obtaining the regression slope. Several regression techniques were used (Table 2), although Model II regression or structural models are recommended for allometric analyses (Harvey & Pagel, 1991) - model 1 regression (ordinary least squares (OLS) regression) assumes that there is no measurement error in the predictor variable. Model 2 regression, on the other hand, assumes errors in both *x* and *y* directions, while structural models assume errors are uncorrelated and that the value of λ (ratio of the two error variances) is known (Harvey & Pagel, 1991).

Table 2: Details of allometry of (log) comb length (regressed against (log) wing length) as calculated by various methods. P-values are from significance test of OLS, MA and RaMA slopes against zero. The SE of the SMA slope is equal to the standard error of the slope calculated for an OLS model and the SE of the MA and ML slopes are equal.

Allometric relationship of CL (1st tarsus) to WL:								
Species/Popl.	N	Method			Intercept	Slope (±SE)	F(df)	p
Drosophila simulans								
T	49	OLS	r^2	0.230	-0.522	0.749 (0.199)	14.03 (47)	0.000
		MA			-5.359	2.346 (0.626)		
		SMA			-2.983	1.561 (0.199)	NA *	
		RMA			-7.040	2.901 (NA)		
		ML			-4.257	1.982 (NA)		
D	58	OLS	r^2	0.137	-0.902	0.867 (1.265)	8.92 (56)	0.004
		MA			-14.550	5.338 (1.787)		
		SMA			-5.391	2.338 (1.265)	NA *	
		RMA			-16.469	5.967 (NA)		
		ML			-11.150	4.224 (NA)		
Drosophila melanogaster								
C	50	OLS	r^2	0.100	-0.650	0.797 (0.344)	5.36 (48)	0.025
		MA			-19.335	6.833 (2.952)		
		SMA			-5.971	2.516 (0.344)	NA *	
		RMA			-21.447	7.515 (NA)		
		ML			-14.752	5.353 (NA)		
W	58	OLS	r^2	0.127	-0.395	0.712 (0.250)	8.13 (56)	0.006
		MA			-11.928	4.433 (1.554)		
		SMA			-4.384	1.999 (0.250)	NA *	
		RMA			-14.240	5.179 (NA)		
		ML			-8.836	3.435 (NA)		
Drosophila pseudoobscura								
L	56	OLS	r^2	0.357	-1.745	1.095 (0.199)	30.00 (54)	0.000
		MA			-6.355	2.546 (0.465)		
		SMA			-4.088	1.833 (0.199)	NA *	
		RMA			-7.435	2.886 (NA)		
		ML			-5.375	2.237 (NA)		
S	49	OLS	r^2	0.174	-1.596	1.050 (0.333)	9.93 (47)	0.002
		MA			-15.029	5.258 (1.669)		
		SMA			-6.270	2.514 (0.333)	NA *	
		RMA			-16.679	5.775 (NA)		
		ML			-11.931	4.287 (NA)		
Allometric relationship of CL (2st tarsus) to WL:								
Drosophila pseudoobscura								
L	56	OLS	r^2	0.073	0.012	0.517 (0.249)	4.28 (54)	0.043
		MA			-15.178	5.298 (2.561)		
		SMA			-4.408	1.908 (0.249)	NA *	
		RMA			-19.105	6.534 (NA)		
		ML			-10.744	3.902 (NA)		
S	49	OLS	r^2	0.088	-0.796	0.771 (0.363)	4.51 (47)	0.039
		MA			-22.726	7.641 (3.598)		
		SMA			-6.653	2.606 (0.363)	NA *	
		RMA			-25.352	8.464 (NA)		
		ML			-17.412	5.977 (NA)		

While we present regression slopes calculated using all methods (ordinary least squares (OLS), major axis (MA), standardised major axis (SMA = reduced major axis), ranged major axis regressions (RaMA), along with structural relationship (STR/ML) regression (maximum likelihood regression)), we base subsequent analyses on MA slopes and intercepts as MA regression is a preferred method for calculating allometry (Harvey & Pagel, 1991). Regression slopes for OLS, MA, SMA and RaMA methods were computed in "R" using the lmodel2 package (Legendre, 2008), and their statistical significances (> 0) were assessed in JMP (v 8.0) and SPSS (v 15). SMATR (http://web.maths.unsw.edu.au/~dwarton/programs) implemented on the R software platform (R Development Core Team 2007) was used to test MA slopes for isometry (β = 1) using one-sample t-tests, and then to test for a common slope within species and then across species, using Barlett corrected maximum likelihood tests (Warton *et al.*, 2006).

The asymmetry of each trait was measured as the signed (R-L) difference. FA1 is the absolute value of this measure. A two-way analysis of variance (ANOVA) was used to assess whether asymmetry could be distinguished from measurement error (ME) (Palmer, 1994; Palmer & Strobeck, 2003). This is essential as ME can confound FA interpretations. Further analyses were done using a worksheet provided by A.R. Palmer (www.biology.ualberta.ca/palmer/DataFiles/FA_Calc.xls). We calculated FA4a, FA10a, ME1, ME3, ME5 and ME1 as percentage of FA4a (Table 3), following Palmer (1994) and Palmer and Strobeck (2003), however, subsequent analyses are based on FA1 values. *Drosophila simulans* (D) and *D. melanogaster* (W) populations showed significant directional asymmetry (DA) in comb length. As a result and to be conservative, comb length FA was excluded from all further FA analyses. All remaining

Table 3: Descriptors of fluctuating asymmetry and measurement error derived from the results of a mixed model ANOVA of sex-comb length, sex-comb tooth number and wing length, for different populations. FA1, FA4a and FA10a: descriptors of FA. ME1 reports measurement error in the original units, and ME3 represents the mean difference between replicate measurements as a proportion of mean difference between sides (mean squares of the sides x individual interaction). ME5 expresses FA variation as a percent of the total between-sides variation (including ME), and provides a standardized measure of FA repeatability (see Palmer & Strobeck, 2003).

		D. simulans T			*D. melanogaster C*			*D. pseudoobscura L*				
		CL	TN	WL	CL	TN	WL	CL-T1	CL - T2	TN - T1	TN - T2	WL
a) FA1 mean (in µm for CL)		1.5596	0.2653	-2.1976	1.0217	0.18	1.3568	0.8805	0.1706	0.0714	-0.0536	-3.1551
±SE		0.8882	0.1816	1.3669	0.8826	0.1731	1.8236	0.6383	0.6476	0.0982	0.0898	1.6808
b) FA4a = 0.798√MS_{SI} (in µm for CL)		4.9719	1.0045	7.6556	4.9808	0.9765	10.291	3.8526	3.9062	0.5365	0.5318	10.0481
c) FA10a (in µm for CL)		19.3346	0.7845	45.9479	0.3257	19.3926	83.0781	11.5763	11.9042	0.2126	0.2176	79.1937
	df	48	47	48	48	49	49	54	54	49	53	55
d) ME1 = 0.798√MS_{err} (in µm for CL)		0.3093	0.0987	0.2986	0.3321	0	0.3091	0.3149	0.3121	0.1306	0.0754	0.3191
e) ME1 as % FA4a		6.2203	9.83	3.9002	6.6677	0	3.0033	8.1732	7.9908	24.3468	14.1793	3.1759
f) ME3 = MS_{err} as % MS_{SI}		0.3869	0.9663	0.15	0	0.4446	0.09	0.668	0.6385	5.9268	2.0102	0.1
g) repeatability (ME5)		0.7243	0.5112	0.8701	0.6913	1	0.9172	0.5704	0.5815	0.1241	0.3032	0.8984
		D. simulans D			*D. melanogaster W*			*D. pseudoobscura S*				
		CL	TN	WL	CL	TN	WL	CL-T1	CL - T2	TN - T1	TN - T2	WL
a) FA1 mean (in µm for CL)		2.5001	0.069	-0.8191	2.8489	0.2241	0.2971	1.8867	0.9095	0.1224	0	1.0431
±SE		0.6237	0.141	1.3216	0.6982	0.1477	1.8544	0.6874	0.6457	0.1153	0.0922	1.8387
b) FA4a = 0.798√MS_{SI} (in µm for CL)		3.8271	0.8394	8.0453	4.2507	0.8787	11.2606	3.8537	3.6641	0.644	0.5084	10.2666
c) FA10a (in µm for CL)		11.4221	0.5489	50.7418	14.1013	0.602	99.4654	11.5834	10.4559	0.7488	0.1978	82.6739
	df	56	56	57	56	56	57	47	47	49	46	48
d) ME1 = 0.798√MS_{err} (in µm for CL)		0.3154	0.0741	0.3192	0.3305	0.0741	0.3478	0.3137	0.3306	0	0.0806	0.329
e) ME1 as % FA4a		8.2409	8.8264	3.9675	7.7745	8.4313	3.089	8.1393	9.0217	0	15.8538	3.2048
f) ME3 = MS_{err} as % MS_{SI}		0.6791	0.7791	0.16	0.6044	0.7108	0.1	0.6625	0.8139	0	2.5144	0.1
g) repeatability (ME5)		0.5577	0.5233	0.8454	0.5864	0.5463	0.9003	0.6048	0.5543	1	0.2836	0.9085

populations displayed non-significant levels of skew and kurtosis after Bonferroni correction (data not shown). Whilst all these analyses were conducted at the population level, species level analyses also indicated that FA was discernable from ME (data not shown).

Results

We first used multivariate analysis of variance (MANOVA) to see how species and populations differed in the average size (mean of left and right characters) of the traits we measured. In this analysis we were only interested in the sex combs on the first tarsal segment as this trait was shared by all species - only *D. pseudoobscura* has multiple combs. Species and population nested within species were our predictor variables, and wing length, sex-comb length, and comb tooth number were the dependent variables. This analysis revealed a significant effect of species (Wilks Lambda $F_{6,624} = 570$, $P < 0.001$) and population (within species) (Wilks Lambda $F_{9,759.5} = 9.11$, $P < 0.001$) on the multivariate combination of these traits. Univariate post-hoc tests of the species effect revealed that comb length, tooth number, and wing length all varied across species ($F > 169$, $P < 0.001$) (CL: *D. simulans* = *D. pseudoobscura* < *D. melanogaster*. TN: *D. pseudoobscura* < *D. simulans* < *D. melanogaster*. WL: *D. simulans* < *D. melanogaster* < *D. pseudoobscura*). Within species, wing length was found to be significantly different between populations of *D. simulans* ($F_{1,105} = 42.5$, $P < 0.001$: W > T) and *D. pseudoobscura* ($F_{1,103} = 18.2$, $P < 0.001$: S > L), but populations of *D. melanogaster* did not differ significantly in wing length ($F_{1,106} = 2.27$, $P = 0.14$). For comb length and comb tooth number, only *D. pseudoobscura* showed a significant difference between populations (CL: $F_{1,103} = 8.42$, $P = 0.004$; TN: $F_{1,103} = 4.36$, $P = 0.039$:

in both cases S > L); these traits did not differ significantly across population in the other two species ($F < 1.67$, $P > 0.199$).

All the Type II regression slopes and the slope generated from the structural model suggest positive allometry for comb length (Table 2), and it is these models that are recommended for allometric slope estimation (Harvey & Pagel, 1991). We also fitted quadratic equations to the allometry data, but polynomial equations were not significant, even before Bonferroni correction (data not shown). All MA slopes were significantly greater than 1 for all populations (T: $F_{1,47} = 12.9$, $P < 0.001$; D: $F_{1,56} = 59.2$, $P < 0.001$; C: $F_{1,48} = 59.9$, $P < 0.001$; W: $F_{1,56} = 36.0$, $P < 0.001$; S: $F_{1,47} = 63.7$, $P < 0.001$; L: $F_{1,54} = 34.8$, $P < 0.001$; also see Table 2 and Figure 2). We also calculated the allometry of the second sex comb (on the 2nd tarsus) of *D. pseudoobscura* and found this displayed positive allometry in all models (excluding OLS) (Table 2), and again the MA slope was greater than 1 (Population S: $F_{1,47} = 63.6$, $P < 0.001$; Population L: $F_{1,54}$ 27.9, $P < 0.001$).

MA slopes within species were then tested for differences using Barlett–corrected likelihood ratios (lr), and a common MA slope (β_{com}) was determined when possible. We found that populations of *D. simulans* (lr = 3.46, $P = 0.06$, $\beta_{com} = 4.03$) and *D. melanogaster* (lr = 0.61, $P = 0.44$, $\beta_{com} = 5.62$) did not significantly differ in their comb length allometry. However, *D. pseudoobscura* populations had significantly different slopes (lr = 4.58, $P = 0.03$). We then compared allometric slopes across species (for the first tarsal comb). However, as a common slope could not be calculated for *D. pseudoobscura* populations these were compared separately. Comb allometry for *D*.

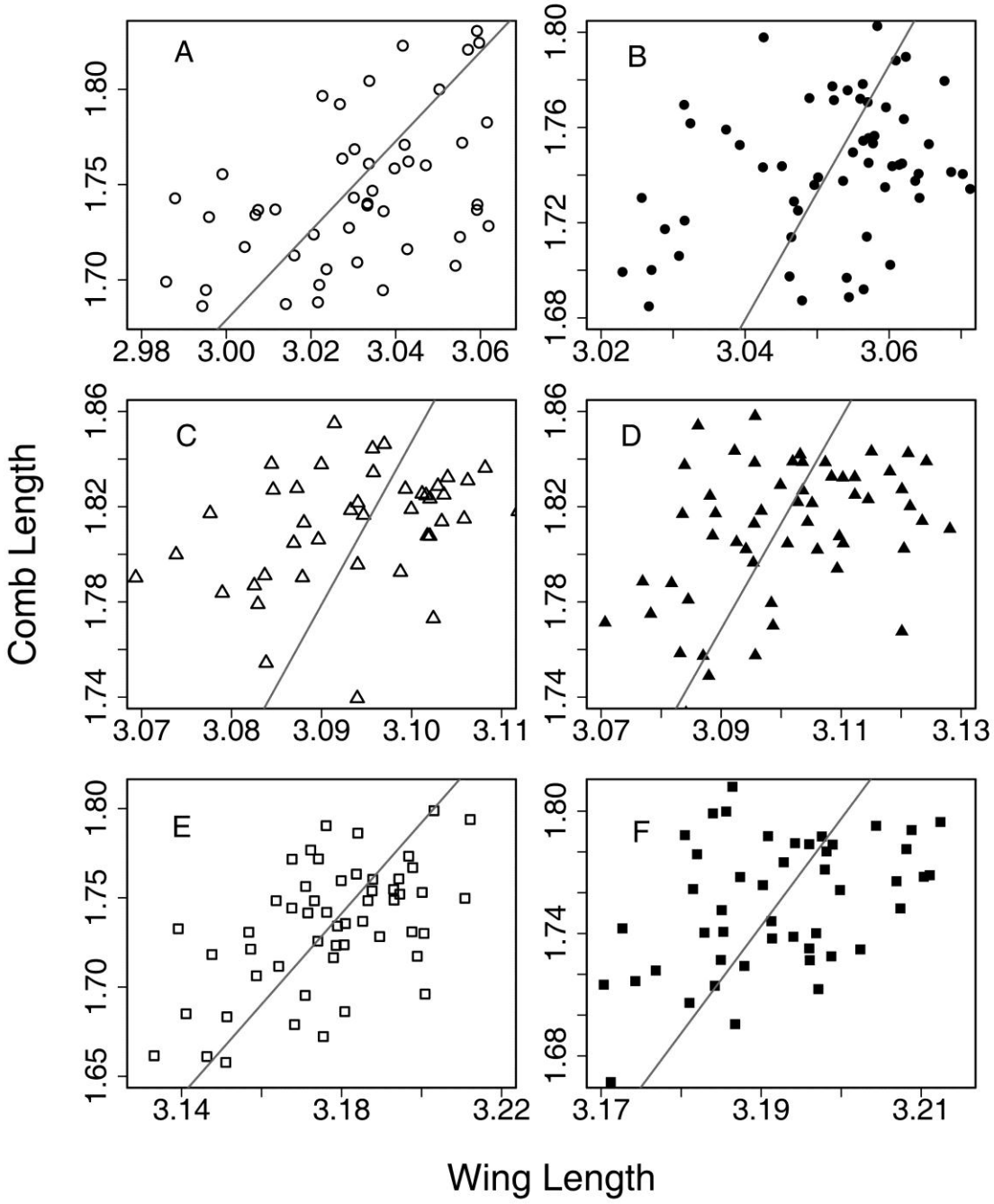

Figure 2: Major axis regression plots showing scaling association between comb length and body size (wing length) for six *Drosophila* populations. (A) *D. simulans* - Tincurry. (B) *D. simulans* - Denmark. (C) *D. melanogaster* - Canton-S. (D) *D. melanogaster* - Walpole. (E) *D. pseudoobscura* - Sholow. (F) *D. pseudoobscura* - Lewiston. Wing length and comb length are represented as log10 values on the X and Y axis. Lines of best fit are shown in each panel. All slopes are positively allometric (Type II regression: see Table 2).

simulans was similar to that of *D. melanogaster* (lr = 3.42, *P* = 0.06, β_{com} = 4.43) and *D. pseudoobscura* (L: lr = 0.034, *P* = 0.85, β_{com} = 2.60; S: lr = 2.70, *P* = 0.10, β_{com} = 4.04). Similarly *D. pseudoobscura* S had slopes that did not differ from *D. melanogaster* (S: lr = 0.03, *P* = 0.87, β_{com} = 5.47), but slopes of *D. pseudoobscura* L did (lr = 5.93, *P* = 0.02). Since *D. pseudoobscura* has two sex combs we also compared them within and across the two populations. Within each *D. pseudoobscura* population the two combs had allometric slopes that did not significantly differ (L: lr = 2.91, *P* = 0.09, β_{com} = 3.08; S: lr = 0.47, *P* = 0.49, β_{com} = 6.19). Furthermore, while comb one slopes were significantly different across the two populations (as noted above: lr = 4.57, *P* = 0.03), those of comb two were similar (lr = 0.27, *P* = 0.60, β_{com} = 6.70).

We again used MANOVA to compare FA levels in wing length (WLFA) and sex comb tooth number (TNFA) across species and populations (with population again nested within species). We included wing length as a covariate in this analysis as levels of FA may vary simply because larger traits may have larger FA, and trait-size differed across populations and species. This analysis revealed that the multivariate combination of these traits was significantly influenced by species (Wilks Lambda $F_{4,624}$ = 3.45, *P* = 0.01) but not population (nested within species) (Wilks Lambda $F_{6,624}$ = 0.689, *P* = 0.66). Body size was not associated with the multivariate combination of FA in WL and TN (Wilks Lambda $F_{2,312}$ = 1.26, *P* = 0.29). Univariate post-hoc tests of the species effect revealed that both WLFA ($F_{2,317}$ = 4.93, *P* = 0.008) and TNFA ($F_{2,317}$ = 13.2, *P* < 0.001) differed significantly between species. Post-hoc Bonferroni tests revealed that WLFA of *D. simulans* was significantly lower than that of *D. melanogaster* (*P* = 0.013) and *D. pseudoobscura* (*P* = 0.034), but *D. melanogaster* and *D. pseudoobscura* did not differ in

WLFA (*P* = 1.00) (*D. simulans* < *D. pseudoobscura* = *D. melanogaster*). Post-hoc Bonferroni tests revealed that TNFA of *D. pseudoobscura* was significantly lower than that of *D. simulans* (*P* < 0.001) and *D. melanogaster* (*P* < 0.001), whereas *D. simulans* and *D. melanogaster* did not differ (*P* = 1.00) (*D. pseudoobscura* < *D. simulans* = *D. melanogaster*)

Individuals were ranked for WLFA and TNFA and Spearman's rank correlations were then used to assess the congruence of within individual FA by regressing WLFA rank against TNFA rank - this was done within population (i.e. individuals were ranked within each population and regression were done for each population), then within species and then across all individuals. None of these analyses revealed a significant association (all |Rho| < 0.26, *P* > 0.2), except there was a weak positive association between the two rank scores in one *D. melanogaster* population (W: Rho = 0.262, *P* = 0.047) which would be non-significant with Bonferroni correction. We also looked at potential associations between mean trait size and trait FA by regressing WLFA vs. WL, TNFA vs. TN and TNFA vs. CL - this was done within populations, then within species and then across all individuals. OLS estimates for all three relationships were close to zero (β<0.1, SE<0.1, *P* > 0.05) in each analysis, except for TNFA vs. TN where we see a significant positive association when all individuals are pooled ($\beta = 0.05 \pm 0.01$, $F_{1,318} = 17.2$, *P* = 0.001).

Discussion

Our major finding was that the sex combs of all species examined display positive allometry. Additionally, comb allometry did not significantly differ across populations, except for the allometry of the first comb in *D. pseudoobscura,* and there were few

statistically significant differences in allometry across species. There were differences between species and populations in trait sizes and asymmetries, but little evidence that FA of single traits reflected overall developmental stability, or that FA was generally associated with trait size. We discuss each of these findings in turn.

Sex combs were positively allometric for all species examined. Thus, sex combs scale more like signalling characters (Alatalo, Höglund & Lundberg, 1988; Baker & Wilkinson, 2001; Petrie, 1988) than insect genitalia (Eberhard *et al.*, 1998; Hosken *et al.*, 2005). This is perhaps surprising as there is evidence in one of our study species that sexual selection favours smaller combs (Markow *et al.*, 1996), while exaggerated signalling traits are usually favoured by sexual selection (Andersson, 1994). Furthermore, like male genitalia, combs are frequently brought into direct contact with females during mating. Both *D. melanogaster* and *D. simulans* males use sex combs for "precision grasping" of extruded female genitalia before mounting, whereas *D. pseudoobscura* males use the sex combs to spread the females' wings during copulation (Cook, 1977; Spieth, 1952). These functions are similar to those of some genital characters like non-intromittent claspers (e.g. Hosken & Ward, 2000), but while genital claspers scale with negative allometry in at least some Diptera (e.g. Hosken *et al.*, 2005), the sex combs do not. Positive allometry has also been reported for the fore-legs of another fly and these are also used to hold onto females' wings during copulation (Eberhard *et al.*, 1998). Why the scaling differences exist when functional differences apparently do not (i.e. claspers vs. sex combs) is not clear, and further investigations are needed to explore selection acting on the sex combs of our experimental populations. Recent work on another species finds that fertilization success during competitive mating is positively associated with sex comb size (Polak & Simmons, 2009), and in one of our *D.*

simulans populations (T) pre- and post-copulatory success are positively associated (Hosken *et al.*, 2008). Since negative associations between mating success and comb size have been reported in this species (Markow *et al.*, 1996), this tentatively suggests comb size could also be negatively associated with sperm competitiveness. Again, this remains to be established.

The allometric relationships of sexually selected traits frequently diverge rapidly (e.g Baker & Wilkinson, 2001; Shingleton *et al.*, 2007), and geographically isolated populations of the same species can also differ substantially in trait allometry (Moczek & Nijhout, 2003). Here however, we did not find significant differences in sex comb allometry for the most part. Whilst populations of *D. pseudoobscura* differ from each other in the scaling of the first comb, they do not differ from each other for the second comb, and one population did not significantly differ from *D. simulans* or *D. melanogaster* (for the first comb), which were also similar to each other. Furthermore, there were no significant differences in comb scaling across populations of *D. simulans* or *D. melanogaster*. These significance levels were assessed with likelihood ratio tests, and when assessed with *F*-tests, comb allometry differed significantly between all populations. However, because the numerator and denominator sums of squares are not independent, testing MS slopes using *F*-tests it is not recommended (Warton *et al.*, 2006). So while there appear to be differences in the scaling of the sex combs, using the appropriate tests, these are not significant and hence we must conclude allometry has not diverged greatly across our samples. Interestingly, the only species with statistically different MA slopes across populations (one of which differed from the other species too) was *D. pseudoobscura*. This is the one species we investigated where there is no direct evidence that sexual selection acts on the sex combs (Markow

et al., 1996). However, since our sample size at the species level is small (n = 3) it is difficult to draw conclusions from this. Additionally, in spite of the general similarity in comb allometry, all species differed in the absolute size of the characters we measured and there were some differences between populations within species too - primarily in wing length. Since flies were all reared under identical environmental conditions, this indicates genetic differences between populations for some traits, and similar findings have been reported across populations of other flies (e.g. Demont *et al.*, 2008).

Across *Drosophila*, there is considerable variation in the number of comb teeth per row, number of rows and in the orientation and position of rows (Kopp & True, 2002). Consistent with this, the total sex comb tooth number and comb length for all three species we investigated were significantly different from each other (and see Figure 1 for apparent orientation differences). Sex combs are like male genitalia in this regard (Eberhard, 1985; Hosken & Stockley, 2004), and even species like *D. simulans* and *D. melanogaster* which are morphologically very similar, clearly differ in these characters. While species differed in comb attributes, populations within species differed far less, a pattern also reported for other sexual traits (e.g. Civetta & Singh, 1998; Karr & Pitnick, 1996). There are many reasons for a lack of within species differentiation, but divergence of sexual trait morphology across species suggests the precise focus of (sexual) selection on the combs varies between species. For example, mating *D. simulans* males have significantly fewer sex-comb teeth than non-copulating males (Markow *et al.*, 1996), which may explain the lower number of teeth in the combs of this species compared with *D. melanogaster*. Similarly with comb length, as *D. simulans* males with larger sex combs have reduced mating success (Markow *et al.*, 1996).

We found no evidence that sex-comb FA was associated with sex-comb size unless we pooled all individuals, but even then the association was positive. Arguably, this is the association we could expect if sexual selection is for smaller comb size, as seems to be the case in *D. simulans* (Markow *et al.*, 1996). However, this association was only apparent across all individuals and not within or across *D. simulans* populations, and in previous work there were also no associations between comb FA and comb size (Markow *et al.*, 1996). Therefore our study, and previous work on sex combs (e.g. Markow *et al.*, 1996; Polak & Taylor, 2007), provides no evidence to support the predicted negative relationship between FA and (sexually-selected) trait-size. This may be because combs are not particularly costly to produce, but this seems highly unlikely given that there is some evidence of condition dependence of sex combs (Polak & Starmer, 2005). However, lack of FA/sexual-trait size associations have been reported for a number of other insects (e.g. David *et al.*, 1998; Tomkins & Simmons, 1995), and overall the evidence for this association is weak at best (Polak, 2008). Additionally, while there is some evidence that FA occasionally influences mating success in two of our species, the reported associations are not always consistent with theory. For example, a positive association between FA and mating success has been reported in *D. pseudoobscura* and a negative association in *D. simulans* - there was no association in a third species - (Markow & Ricker, 1992). However, this previous study did not assess measurement error and subsequent work found no associations between FA and mating success in either *D. simulans* or *D. pseudoobscura* (Markow *et al.*, 1996). Furthermore, there is no association between FA and fecundity in *D. melanogaster* (Woods *et al.*, 2002), which also suggests FA genetic quality associations are at times

weak (also see Martin & Hosken, 2002), a stance further supported by the lack of consistency of within-individual FA that we find here.

For FA to be useful as an indicator of general individual quality, it should at least be consistent across different traits measured on the same individual, even if the correlations are weak (Whitlock, 1996). However, while we found that trait asymmetry differed between species - but not between populations - there were no significant associations between FA in different traits. This supports claims that FA is trait rather than individual specific (e.g Clarke, 1998; Hosken, Blanckenhorn & Ward, 2000; Palmer & Strobeck, 1986) as may be expected if different traits are developmentally buffered to different degrees (e.g. Lüpold, McElligott & Hosken, 2004). However, this lack of congruence may only generally be true when comparisons are across trait classes (e.g. sexual vs. non-sexual) (Polak *et al.*, 2003), as here, and comparisons of differences in congruence across characters (and character classes) may in fact reveal important information about trait developmental integration (Klingenberg, 2003). In any case, there is currently little consensus on how informative FA is from a sexual selection perspective (Møller & Cuervo, 2003; Palmer, 1999; Tomkins & Simmons, 2003; Uetz & Taylor, 2003). It is possible that comb-FA associations were present but undetectable in our populations, and that our null results reflect a lack in statistical power. Furthermore, much emphasis has been put on the problem of distinguishing FA from ME (Palmer, 1994; Palmer & Strobeck, 1986). However, we have followed Palmer's (1999) guidelines whilst performing all our calculations of FA, can discern ME from FA, and our sample sizes exceeded those recommended by Palmer (1999). We did find significant differences in trait FA across species, but not across populations, and differences were not associated with simple

trait-size differences which were controlled for in the analyses. As all flies were reared at the same temperature, which is the temperature they have been reared at since their capture, these FA differences reflect variation in developmental stability under a standard developmental regime. This variation across species is unlikely to simply be the result of differences in captivity duration as the two *D. simulans* populations have been in the laboratory for very different lengths of time and their FA did not differ. It is possible that variation in heterozygosity across the different species has affected FA (Mitton, 1997; Woolf & Markow, 2003), but it would be fortuitous if the different populations of the same species had similar heterozygosity, but different species did not. As a result, we are not sure of the mechanistic basis for the FA differences we see across species, but variation in FA across populations and taxa, including *Drosophila*, has been reported previously (e.g. Civetta & Singh, 1998; Mitton, 1997).

In summary, we find that sex combs are positively allometric in all the populations and species we sampled. So sex-combs appear to scale like sexually selected signalling traits. In spite of some variation in slope estimates, differences across populations and species were mostly non-significant. Finally, there were differences in trait FA across species, but we found no consistent evidence that FA was associated with trait size.

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Chapter Eight

General discussion: Sexual selection in Drosophila simulans

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Darwin (1871) formulated the theory of sexual selection as a corollary to natural selection (Gayon 2010). He observed that animals quite often possessed exaggerated traits such as the large and decorative train of a peacock, or bright plumage colours in many other birds, which were generally detrimental to their survival. He recognised that such traits, despite being non-adaptive, may actually be beneficial if they confer an advantage in terms of increased mating success to their bearers. He also identified two essential components of sexual selection - male-male competition and female choice. While most of Darwin's peers readily accepted the sexual selection theory and its idea of male-male competition, they generally disagreed on the concept of female choice (reviewed in Gayon 2010; and see Dewar and Finn 1909; O'Donald 1980; Maynard Smith 2000). Nevertheless, in the last 50 years or so, female preference for particular male phenotypes has been repeatedly documented (Ryan 1983; Moore and Moore 1988; Wilkinson and Reillo 1994; reviewed in Andersson 1994; Moore and Moore 2006) and its importance in sexual selection models has been highlighted time and again (e.g. Lande 1981; Kirkpatrick 1982; Iwasa et al. 1991; Pomiankowski et al. 1991; reviewed in Mead and Arnold 2004; Kokko et al. 2006). Whilst direct benefit models of sexual selection suggest that females exercise mate choice to maximise benefit to themselves (e.g. greater number of offspring, nuptial gifts or parental care; Trivers 1972), indirect benefit models suggest that preferred males are also the ones with high quality (e.g. those with higher than average viability or those that are more attractive than others), and by mating with these males, females produce high genetic quality offspring (good genes) or produce offspring that are highly attractive (Fisherian benefits) (reviewed in: Kirkpatrick and Ryan 1991; Andersson 1994). Most sexual selection models focussing on indirect benefits of female choice require the presence of genetic variation in

female preference, but it has only been documented in a small number of taxa including *Drosophila* (O'Donald and Majerus 1985; Ritchie 1992; Moore 1989; reviewed in Bakker 1999). Additionally, only a handful of studies have actually selected on female preference to show that it can evolve. Furthermore, despite the fact that costs of female preference can influence the evolution of mate choice, these costs are poorly understood.

Chapter Two presented unequivocal evidence of genetic variation for female preference in the laboratory populations of *D. simulans* tested, and showed that female preference can evolve. It is generally agreed that a female's inclination to mate with a particular male defines her preference (Jennions and Petrie 1997). In accordance with this definition, we assessed female preference in two ways, firstly by using mating latency as an indicator of female preference (e.g. Spieth 1974; Ritchie et al. 1999; Acebes et al. 2003) and then by using female choice (as a yes / no response for choosing to mate with a particular male over another). We found evidence for genetic variation in female preference for both the measures. Additionally, after selecting on female preference for five generations and documenting a response to selection, we obtained estimates of realized heritability similar to those reported previously (Bakker 1999).

It is generally assumed that female preference can be costly (Iwasa et al. 1991; Pomiankowski et al. 1991). However, the words 'preference' and 'choice' are frequently used interchangeably and it is important to highlight the subtle but important difference between the costs of preference and the costs of choice as it is often very difficult to disentangle the two (Jennions and Petrie 1997; Maklakov and Arnqvist 2009). For example a female that prefers a certain male phenotype may carry

a cost of preference simply by virtue of having a 'preference'. Additionally, a very choosy female may find preference to be costly because it can affect her foraging success, absolute mating rate, predator avoidance, or amount of paternal care received (e.g. Arnqvist and Kirkpatrick 2005; Ryan and Rand 1993; Arnqvist 2006; and see Maklakov and Arnqvist 2009). In contrast, a female having exercised a choice based on her preferences may have reduced fecundity or longevity, and these would be the cost of her 'choice'. Such direct selection on female preference (via fecundity or longevity costs) is considered important in models of sexual selection (Mead and Arnold 2004) particularly because such direct effects are considered to be much larger than small indirect effects (e.g. Kirkpatrick and Barton 1997). The earliest models of sexual selection based on female preference, did not have any costs of preference or choice built into them. For example Lande's (1981) rendition of Fisher's runaway process (1930) did not incorporate any costs of female preference, and later models incorporating these costs predicted runaway to be unlikely (e.g Iwasa et al. 1991; Pomiankowski et al. 1991; but see Mead and Arnold 2004). Despite its importance and potential to influence the evolutionary trajectories of various sexual selection models, investigations of preference costs are rare (Maklakov and Arnqvist 2009). In Chapter Two we tried to estimate the cost of preference (over and above the cost of mating with a particular male phenotype) by measuring its decay in our experimental populations (where we had directly selected on female preference and showed that it can evolve), and found that preference itself was not particularly costly in our experimental populations. It is however essential to point out that this particular component of Chapter Two was not very strong. For example, we allowed preference to decay for only two generations expecting it to decay quickly. It is also possible that the decay in preference we observed was not due to decaying preferences *per se* but

instead was caused by epistasis for preference. Epistatic interactions underline variation in many animal and plant characteristics (Wade 2002), and for example, if preferred combinations of preference genes were broken down due to recombination during two generations of relaxed selection, this could have resulted into the decay we observed.

Although, costs of mate choice or preference for particular mates are difficult to demonstrate or to quantify, the general methodology adopted in Chapter Two established a framework for future studies attempting to assess the costs of preference. Additionally, the fact that we found no major costs of preference was consistent with reports from previous studies (Taylor et al. 2008a; Taylor et al. 2008b; Taylor et al. 2010) on *D. simulans*. This finding had implications on our understanding and interpretation of mate choice mechanisms in this species. For example, in absence of direct costs of mate choice, in species where females do not derive any direct benefits (e.g. increased fecundity) by mating with preferred males small indirect benefits (e.g. Fisherian benefits and good-genes viability benefits) of female choice (Andersson 1994; Jennions and Petrie 2000) may be sufficient to maintain female preference (Kirkpatrick 1996). In line with this, previous studies in *D. simulans* have reported no direct benefits/costs of mate choice and now our study has provided further evidence that female preference may not be costly – at least under the conditions we tested for it. So it is possible that small indirect benefits may be enough to maintain mate choice in *D. simulans.*

Previous investigations into indirect benefits of mate choice in *D. simulans* have found evidence for Fisherian mate choice benefits (Taylor et al. 2007; and see Taylor et al. 2009) but convincing evidence for good gene benefits in this species is lacking

(Taylor et al. 2010). So the question is, are there no good genes benefits in *D. simulans*? Several authors have suggested that Fisherian benefits could not be the only mechanism driving the evolution of mate choice (e.g. Andersson 1994; but see Pomiankowski 1987). However, the idea that Fisherian benefits are not the only benefits is conditional. For example, Pomiankowski (1987) in his theoretical review of the costs of choice argued that in species with exaggerated male characters, either female choice is not costly, or if it is costly, then choosy females must get additional benefits over Fisherian benefits. This "costly" choice is a product of the viability costs of mate choice (Pomiankowski 1987). If we agree with this view then there may be little reason to expect good gene benefits in *D. simulans* (given that mate choice does not seem to be costly here), but then theory does also suggest that good genes benefits are inevitable as all indirect benefits may become linked to good genes (Jennions and Petrie 2000; Rowe and Houle 1996). Then there must be some scope of finding these benefits in *D. simulans*.

It is important to make a distinction between the classical and more modern interpretation of good genes (indirect) benefits. Classically, indirect benefits were interpreted as either Fisherian benefits – realised when females produce attractive sons by mating with attractive sires; or good genes benefits – that materialise as viability benefits for offspring. However, this classical interpretation has been subject to debate more recently as researchers argue about the validity of separating good genes and Fisherian benefits (e.g. Kokko et al. 2002). They recommend that empiricists should concentrate on the total fitness instead of just viability. The model generated by Kokko et al. (2002) shows good genes and Fisherian benefits as complementary, rather than alternative mechanisms of sexual selection. This model also suggests that

the relative weights of good genes and Fisherian mate choice benefits can change depending on the costs of mate choice. Regardless of which interpretation of indirect benefits one subscribes to - i.e. treating Fisherian and good genes benefits separately or inclusively – it does not preclude the possibility that either or both of these mechanisms may be distinctly influencing female preferences at any one time (Taylor et al. 2010).

It has also been suggested that males may trade-off viability benefits for increased attractiveness, and thus good genes benefits (per the classical definition) should be investigated via daughters instead of sons (see Taylor et al. 2010). In Chapter Three we made an attempt to uncover good gene benefits via daughters using a different experimental design than previously employed by Taylor et al. (2010). Palmer (2000) recommended such repeat experiments as they increase the chances of detecting small effects. The new design used iso-female lines and incorporated female remating decision as an additional indicator of mating preference (e.g. Ivy and Sakaluk 2007; Stewart et al. 2008). However, despite using a different approach to earlier work (Taylor et al. 2010), we still found no convincing evidence of good genes benefits of mate choice in *D. simulans*. This is in contrast with evidence for offspring viability benefits to female mate choice in other Drosophlids (e.g. Taylor et al. 1987; Partridge 1980; Hoikkala et al. 1998; Hine et al. 2002). We did however find that daughters sired by males from one of our attractive isolines lived longer. Although this could be interpreted as some evidence of good genes benefits, there was no overall effect of sire attractiveness on offspring longevity. This isoline effect could potentially be explained by differential inbreeding depression between the isolines employed in this experiment, or by genetic compatibility (Tregenza and Wedell 2000) but these

potential causative agents were not investigated as part of this experiment. Additionally, we found a positive and significant intersexual correlation for longevity. This suggested that any potential good gene benefits were not being masked by intralocus conflict. Thus results from this chapter concurred with those of Taylor et al. (2010), and with several other studies reporting weak or no effect of sire attractiveness on daughters' fitness (e.g. Norris 1993; Jones et al. 1998; Tomkins and Simmons 1999; Maklakov and Arnqvist 2009), but contrasted with studies detecting a negative effect of sire attractiveness on daughters' fitness (e.g. Fedorka and Mousseau 2004; Oneal et al. 2007; Foerster et al. 2007). So it seems that the good genes benefits were not manifest in our populations.

Although we failed to detect strong evidence supporting good genes benefits in *D. simulans* this does not rule out the possibility that these benefits exist. It is possible for example, that our failure to detect good genes benefits was because they are even smaller than the indirect (Fisherian) benefits previously reported in this species (Taylor et al. 2007). An experimental evolution study utilising multiple generations of selection may allow small benefits to accrue and become detectable. Some of the evidence we gathered in Chapter Six, seems to indicate that good genes benefits may indeed be present in our populations. However, at the same time, this indication was condition dependent – we found evidence that could be interpreted in favour of good genes benefits but this evidence only came from some of our assays where the test conditions were relatively less competitive.

Although the male traits subject to female choice under good genes mechanism are considered to be condition dependent and true indicator of a male's quality, the expression of good genes benefits themselves was not formulated to be

condition dependent (see Andersson 1994). So it is plausible that females in our experimental populations only derive Fisherian benefits of mate choice. However, this possibility needs to be tested empirically, and to do this a precise understanding of male traits that are a target of female preference is required. Similarly, by investigating male traits and testing their value as honest indicators of genetic quality (passed on to offspring) we can gain a better understanding of good genes benefits as well. In line with this we investigated some of the male traits in Chapters Four, Five and Seven.

Female mate choice decisions are often based on an assessment of multiple male traits (Candolin 2003; Hebets and Papaj 2005) and variation in male traits is essential for this choice to operate. Additionally, for these traits to evolve via female preference, genetic variation in these traits is essential. Traits involved in mate choice may also be subject to natural selection (Darwin 1871). For example, cuticular hydrocarbons (CHC's) are involved in mate choice and are also known to provide desiccation resistance (Ferveur and Cobb 2010). Chapter Four and Five represent a sequential effort to investigate CHCs as a trait involved in mate choice.

In Chapter Four we investigated the patterns of genetic variation and the underlying genetic architecture of male and female CHC profiles of *D. simulans*. We used isolines in which we have previously found evidence for variation in female preference (Chapter Two). We found substantial genetic variation for CHC profiles, and this genetic variation was sex-specific as well. This was consistent with reports from a range of insects (Chapman et al. 1995; Thomas and Simmons 2009), and suggested that this population had sufficient genetic variation to allow evolution of CHCs under selection. In line with reports from other Dipterans (Ferveur and Cobb 2010), we also found evidence for sexual dimorphism and heritability of individual CHC components in

our populations. It is generally thought that sexual dimorphism evolves in response to sexually antagonistic selection (SAS) (Darwin 1871; Andersson 1994). For example, theory suggests that the genetic architecture of traits under SAS should evolve to minimise the genetic constraints on independent evolution of the sexes – thus promoting sexual dimorphism (e.g. Lande 1980; Rhen 2000; Rice and Chippandale 2001). We therefore examined the genetic variance-covariance matrix (G) that can be thought of as a measure of genetic constraints on evolution (Cheverud 1984, 1988), and in examining the intrasexual G matrices we found that many individual CHC peaks covaried such that there was an apparent trade-off between long and short chained hydrocarbon production. This is expected as CHCs are costly to produce and trade-offs between different classes of compounds have been documented (Foley et al. 2007). Short chained CHCs are frequently used as cues during courtship displays, and long chained CHCs, which are also less volatile, are generally involved in desiccation resistance. Such trade-offs may play an important role in constraining responses to selection and can be of fundamental importance in the maintenance of genetic variation under selection (Reznick 1992; Roff 1992).

Additionally, comparison of G matrices between the sexes suggested that females did not have the same trade-offs as males, and females had relatively fewer negative correlations as well. This was suggestive of a weaker influence of sexual selection on females, and theory suggests that the females are in general, closer to naturally selected optima than males (Andersson 1994). The intersexual genetic correlations we documented were weak on average, and frequently differed in sign suggesting that sexual dimorphism in this species was at an advanced stage. However, the fact that we found correlations and that many of them were negative, was in

contrast to several studies (reviewed by Poissant et al. 2010). Overall, it seemed that the genetic architecture of CHCs in this species might facilitate sex-specific CHC evolution in *D. simulans.* These findings add to the relatively scant literature available on CHCs *Drosophila simulans* (for other *Drosophila* species see Chenoweth et al. 2008; Ferveur 2005; Alves et al. 2010; Everaerts et al. 2010; and reviewed in Ferveur and Cobb 2010), and suggest that CHCs should evolve in a sex specific manner when subject to natural or sexual selection.

So, if natural and sexual selection can shape the evolution of display traits, and both types of selection can promote sex-specific optima (Heinsohn et al. 2005), then it is important to consider the combined evolutionary effects of natural and sexual selection on sexual traits such as CHCs. It is somewhat surprising that there is a lack of such studies despite the relative importance of this approach (see Chenoweth et al. 2008). Chapter Five was aimed to address this paucity to an extent, whilst testing if *D. simulans* CHCs would indeed evolve in a sex-specific manner as suggested by the findings of Chapter Four. Here we reared experimental populations of flies under relaxed and elevated sexual and natural selection in a fully factorial design (also a part of Chapter Six) and then quantified the CHCs of both males and females.

We found that natural selection, sexual selection and their interaction influenced the evolution of male CHCs. For example, elevated natural selection was associated with males evolving an increase in their total CHC content and increasing their longer-chain CHCs. Long chain CHCs are involved in desiccation resistance (Gibbs and Rajpurohit 2010) and similar findings have been reported in *Drosophila* (Kwan and Rundle 2010; Toolson and Kuper-Simbrón 1989). Additionally, there was an interaction between natural and sexual selection. In line with the classical interpretation of sexual

selection on male traits (Andersson 1994), we found that elevated natural selection was only able to drive CHC evolution toward a new naturally selected peak in the absence of sexual selection, and sexual selection was found to be strong enough to overwhelm natural selection on some aspects of the male CHC profile. This also implied that sexual selection can be costly for males as it generally tends to oppose the naturally selected optima for male traits. Interestingly we also found that males from our sexual and no-sexual selection populations, evolving under relaxed natural conditions, shared some aspects of their CHC profiles, suggesting that male CHCs favoured by sexual selection were not costly under ancestral conditions. Together these two outcomes suggest that sexual and natural selection may be antagonistic in the short term. For example, we know that short-chain CHCs are involved in mate choice and long-chained ones are involved in desiccation resistance. Now, under stressful conditions (for e.g. high temperature), natural selection (let's say – to prevent desiccation) would push towards production of more long chained CHCs. In contrast, sexual selection would promote production of more short-chain CHCs to compensate for increased evaporative losses of the short-chained CHCs at the higher temperature. So initially this will result in a tug-of-war between the two selective regimes. However, in the long run, as natural selection shapes the adaptive landscape and populations come to an equilibrium state, (e.g. the ancestral conditions) sexual selection would hone in on the naturally selected optima (Boughman 2002; and see Candolin and Heuschele 2008 for a review).

In contrast to males, female CHC evolution was very limited, although there was a significant interaction between the influences of natural and sexual selection. For example, when both natural and sexual selection were relaxed, female CHCs

evolved to a profile largely identical to that under elevated natural selection. This suggested that female CHC profiles were generally tuned to the naturally selected optima. Classically female traits are presumed to be influenced more by natural selection and our findings were consistent with this view. However, this does not seem to be a general trend as findings from *D. serrata* (Blows 2002) indicate that female profiles can evolve in a direction opposite to that of natural selection. Nevertheless, every study system is unique in some way, and *D. simulans* is known to exhibit significant differences even from its closest related sister species – *D. melanogaster* (Capy and Gibert 2004; Taylor et al. 2009) thus these contrasting results are perhaps not as surprising.

Theory suggests that whilst natural and sexual selection can be antagonistic, selection itself can be sexually antagonistic as well (Rice and Chippindale 2001; Bonduriansky and Chenoweth 2009; Hosken et al. 2009) and overall the above findings from Chapter Five seem to in agreement with this view. That is, male and female responses to selection were very different. It is important to note however, that such sex specific responses are also consistent with the genetic architecture (*G*) for CHCs documented in Chapter Four. Additionally, in Chapter Four we documented many significant negative intersexual genetic correlations, and also found the G matrices to be significantly different between the sexes. It is currently not exactly clear from our work whether selection or G was responsible for the antagonistic responses we documented in Chapter Five.

Nevertheless, taken collectively, findings from Chapters Four and Five suggest that sexual and natural selection act on *D. simulans* CHCs in a sex-specific manner, and sometimes the influence of sexual and natural selection can be antagonistic. Although

sex-specific selection can theoretically facilitate adaptation, this is not always the case (Connallon et al. 2010), and in agreement with this we found that sexual selection was not always adaptive in this species. Chapters Four and Five only investigated one of the various traits that may be assessed by females during mate choice and collectively these two chapters demonstrated how this single trait harbours significant amount of genetic variation and how it can be shaped by sexual and natural selection during evolution. However, there are many other secondary sexual traits that are subject to mate choice (e.g. sex-combs; and see Andersson 1994) and it is currently unclear how females weigh these traits during mate choice. For example, it is possible that some traits may be used as primary indicators of quality while others may add information to the signal of the primary trait (Andersson 1994). Further investigations into the relative importance of such traits are warranted to gain a better understanding of the selective forces acting on them.

A number of authors have hypothesized that sexual selection (specifically directional sexual selection) almost always leads to the evolution of exaggerated traits (see Bonduriansky 2007). The rationale is that if secondary sexual trait size is influenced by directional sexual selection, then increased relative trait size would in turn yield a higher mating success to the bearer (assuming that female choice is based on this trait and the trait is an honest indicator of male quality). However, viability costs would limit trait expression in small individuals or those of lesser quality. This would result in larger individuals having relatively larger traits in proportion to their body size (positive allometry). An alternative explanation for positive allometry comes from differential sexual selection on body size (Maynard Smith 1977; Fairbairn and Preziosi 1994; Webster 1992; Payne 1984). For example, if sexual selection is stronger

for males, then positive allometry is expected (as is generally the case; Shuster and Wade 2003) regardless of the male size being selected upon (Dale et al. 2007; Székely et al. 2004). In contrast, negative allometry is expected in taxa where sexual selection is stronger for females (Walker and McCormick 2009).

Sexually selected traits are generally thought to show positive allometry (Kodric-Brown et al. 2006), however this claim was recently refuted by Bonduriansky (2007). In his review, Bonduriansky (2007) established that the presumed near-universal appearance of positive allometry in sexually selected traits was a product of sampling bias (see Appendix A). His review brought forward substantial empirical evidence to contradict the positive allometry hypothesis and discussed theoretical reasons (e.g. Bonduriansky and Day 2003) why a diversity of allometric patterns could emerge as a result of net sexual selection acting on a trait. Bonduriansky (2007) also called for allometric studies to base their choice of trait(s) on the functional significance (sexual / non sexual functions) of a trait instead of basing it on trait exaggeration as such.

The sex comb(s) of *Drosophila* are male-specific secondary sexual characters that are important in mating bouts (Cook 1977; Kopp and Chen 2008) and have rarely been investigated for their allometry (Bonduriansky 2007). The allometry of secondary sexual traits often evolves rapidly and divergently in closely related species (e.g. Baker and Wilkinson 2001; Emlen et al. 2005; Shingleton et al. 2007) and geographically isolated populations of the same species are also known to differ in trait allometry (Moczek and Nijhout 2003). Therefore, we decided to assess the allometry and asymmetry of sex combs for two distinct geographical populations in three closely related *Drosophila* species (*D. melanogaster*, *D. simulans* and *D. pseudoobscura*). Thus

Chapter Seven not only attempts to reduce the bias in allometric literature (see Bonduriansky 2007), it also forms part of our impetus towards gathering more information about the traits involved in female mate choice (e.g. Chapters Four and Five).

Results from Chapter Seven suggested that sex combs scale positively for all the three closely related species examined (*D. simulans*, *D. melanogaster* and *D. pseudoobscura*). That is, they are more like signalling characters (Alatalo et al. 1988) (Baker and Wilkinson 2001; Petrie 1988) than genital characters which are also involved in mating but are known to scale negatively with body size (e.g. non-intromittent claspers are involved in holding on to females just like sex combs; Hosken et al. 2005). We also found that for the most part there were no significant differences in the sex comb allometry across the populations we tested. Furthermore, consistent with other empirical evidence (Kopp and True 2002; Civetta and Singh 1998; Karr and Pitnick 1996), we found that there was considerable variation in the number of comb teeth and comb length across species, but far less variation between different populations of the same species. This suggested that the focus of sexual selection may vary between species. Empirical evidence seems to confirm this, for example, *D. simulans* males captured from a natural population were found to have significantly fewer sex comb teeth compared to males not found mating (Markow et al. 1996). In contrast *D. bipectinata* males, under similar natural conditions, had a significantly larger number of comb teeth (Polak et al. 2004). Additionally, we found that trait fluctuating asymmetry (FA) varied across species but we found no evidence to suggest that FA was associated with trait size. Classically, FA was used in studies as a measure of developmental noise and developmental instability (Van Valen 1962; Mather 1953)

and was considered to provide an accurate reflection of a population's state of adaptation and coadaptation (Jones 1987). Some authors have argued that FA reflected an individual's genetic quality, and predicted that higher quality individuals would possess large and symmetrical sexual traits (Møller and Swaddle 1997). However, more recently the reliability of FA as an indicator of genetic quality and its predicted association with sexual selection and fitness has come under criticism despite evidence in support of it (Lens et al. 2002; Bjorksten et al. 2000; Tomkins and Simmons 2003; Uetz and Taylor 2003). Our results were in agreement with a number of studies that find no link between sexual selection and fitness and question the usefulness of FA from a sexual selection perspective (Cuervo and Møller 2009; Hunt and Simmons 1998; Martin and Hosken 2002; reviewed in Tomkins and Simmons 2003).

Findings from this study added data to a field where empirical evidence is relatively scant (allometry of unexaggerated secondary sexual traits), and raised more questions that need answering: Does trait allometry differ between trait types (e.g. sex combs, legs, wings)? For example, sex combs are involved in grasping the female during copulation whereas the forelegs bear the combs; in contrast, midlegs do not have such a role. So, would the trait allometry be different as well? Furthermore, wings are involved in song production and are responsible for flight, thus they are subject to both sexual and natural selection just like the CHCs examined earlier in Chapters Four and Five; how would the evolutionary response of trait allometry change if levels of sexual and natural selection were manipulated? Does the ecological significance of trait allometry change across environments? Such investigations will be

important in advancing our understanding of sexual selection and its impact on species.

Sexual selection is generally presumed to be maladaptive (Andersson 1994; Darwin 1871), but there are some models (e.g. Lorch et al. 2003) that predict sexual selection to be adaptive. Several studies have attempted to resolve the debate surrounding the adaptiveness of sexual selection, however there is still a lack of consensus regarding its true nature (reviewed in Candolin and Heuschele 2008). Keeping the above debate in mind, in Chapter Six, we attempted to address the question: Is sexual selection adaptive? We did so by using a factorial design that allowed us to manipulate the opportunities for sexual and natural selection both individually and together. Populations were forced to evolve in a novel environment and their fitness (lifetime reproductive success) assessed at three time points during the course of evolution. Populations evolving under ancestral conditions were also assessed, but only after 30 generations. Larval competition was also manipulated in our assays, as differences between treatments are sometimes apparent only in the presence of additional stress (Hoffmann and Parsons 1993). We then assessed how these populations had evolved, and if the presence of sexual selection was adaptive in the novel environment. The overall evidence from this experiment was inconclusive as results provided evidence both, for and against the adaptive nature of sexual selection. Evidence supporting the adaptive effects of sexual selection came from the assays where larval competition was not increased artificially. This part of our results was in line with theoretical models predicting the adaptive effect of sexual selection (Proulx 1999; Whitlock 2000; Lorch et al. 2003) and was also in agreement with the few studies that have reported adaptive effects of sexual selection (e.g. Partridge 1980;

Fricke and Arnqvist 2007; and see Discussion in Chapter Six). However, the other part of our results (from the assays where we elevated the larval competition in an attempt to increase the chances of detecting any differences between treatments), concurred with a large number of studies that have established negative or no impact of sexual selection on adaptation (e.g. Promislow et al. 1998; Schaeffer et al. 1984; Holland 2002; Rundle et al. 2006; Martin et al. 2004).

Costs of mating are reported to be low in *D. simulans* and sexual selection does not seem to be driven by sexual conflict (Taylor et al. 2009). In such a case, good gene benefits are a likely candidate to explain the adaptive nature of sexual selection (Jennions and Petrie 2000). Although these benefits are very small (Møller and Alatalo 1999) and repeated attempts to detect good gene benefits in *D. simulans* have failed to gather supportive evidence (Taylor et al. 2010; and see Chapter Three), small good gene benefits accrued over time could be responsible for the adaptive nature of sexual selection we observed in Chapter Six. In contrast, our finding that sexual selection was not adaptive under some assay conditions could arguably be explained by the maladaptive nature of sexual selection under sudden changes (reviewed in Candolin and Heuschele 2008) or genotype by environment (GxE) type interactions (see Ingleby et al. 2010). Although these results taken together were inconclusive like those of Radwan et al. (2004) and Tilszer et al. (2006), they nonetheless suggested that sexual selection was not always adaptive and that test conditions had a major impact on the experimental outcomes (see Chapter Six). Experimental evolution studies like ours should therefore be cautious about interpreting their results and should consider the possibility that the results may be due to the selective regimes or due to the test conditions employed (see Edward et al. 2010).

Additionally, in Chapter Six we found that there was a general decline in the overall fitness (number of offspring produced) of our selection lines over time. We recently established that male attractiveness and fertility are influenced by inbreeding depression (see Appendix B); however inbreeding depression was not the cause of the fitness decline we observed in Chapter Six. We suggested that presence of endosymbionts such as *Wolbachia* or *Spiroplasma* may explain this overall decline we observed. For example, *Wolbachia* are present in almost all insect species, and are known to have a negative impact on population fitness (Werren et al. 2008; Saridaki and Bourtzis 2010). Multiple *Wolbachia* phenotypes are known to occur in nature, and out of these the cytoplasmic incompatibility (CI) causing phenotype is the most common in *Drosophila* (Clark et al. 2005). This phenotype is characterised by strong reproductive incompatibilities between uninfected females and infected males, and is known to severely reduce reproductive output (Champion de Crespigny and Wedell 2007). Additionally, *Wolbachia* infected males are known to have reduced sperm competitive ability (Champion de Crespigny and Wedell 2006) as well. Taken collectively these characteristics of *Wolbachia* make its presence a prime candidate for explaining some of the results from Chapter Six. However, the presence of *Wolbachia* in our populations and its actual phenotype (if present) need to be investigated.

Conclusions and future prospects

This thesis was built up from previous work on *D. simulans* in which the costs and benefits of mate choice were evaluated (2007; 2008b; 2008a; 2009; 2010). Our investigations started with examining female preferences as they are a major component of sexual selection and despite more than 50 years of investigations into

different components of female preference – e.g. its costs, benefits and evolution – there was scope for much work to be done. In Chapter One, we examined female preference, its evolution and costs. Then in Chapter Two we re-examined the obscure good gene benefits of mate choice, which were previously investigated by Taylor et al. (2010). Furthermore, we started examining various male traits that are subject to female choice, and chose to look at two traits – cuticular hydrocarbons and sex combs (Chapters Four, Five and Seven). Further assessment of these (and other) traits is essential, and more attention needs to be paid on how these traits, and the information they carry, change under different environmental conditions (Bro-Jørgensen 2010). Additionally, it may be useful to examine the plasticity of these traits as trait plasticity has recently been implicated in the manipulation of sexual and natural selection during evolution (Price 2006; Fusco and Minelli 2010; reviewed in Pigliucci 2010). Moreover in Chapter Six we investigated the overall adaptiveness of sexual selection that has been debated ever since Darwin first proposed sexual selection. Findings from this chapter have highlighted the importance of assay conditions, genotype x environmental interactions and the potential for endosymbiotic relationships that can influence the net effect of sexual selection, and further studies controlling for such factors are needed. It would also be fruitful to utilise microarray techniques like those used my Innocenti and Morrow (2010), to gain a better understanding of the genetic architecture of both male and female traits under sexual selection and to gain a genetic overview of evolution under natural and sexual selection. From the experiment in Chapter Six we collected photographic evidence (yet to be analysed), that would allow us to investigate the evolution of *Drosophila* wing shape and allometry, just like we examined the evolution of cuticular hydrocarbons in Chapter Five. The *Wolbachia* related observations from this chapter still need

considerable work before any conclusions can be drawn regarding the effect of *Wolbachia* on its *D. simulans* host.

In summary, these findings indicated that our populations of *D. simulans* harboured enough genetic variation to allow female preference to evolve, and that the costs of female preference (over and above the costs of mating with particular males) are minimal. Additionally, good genes benefits of mate choice were not evident in these populations, and thus it is possible that mate choice in *D. simulans* is maintained via Fisherian benefits only. Moreover, investigations of traits involved in mate choice suggested that sexually antagonistic evolution of cuticular hydrocarbons is possible in this species as there is considerable genetic variation for this trait and the genetic architecture of the trait permits such evolution under selection. These investigations also suggested that even unexaggerated sexually selected characters can be positively allometric, but the focus of sexual selection may vary between species. Furthermore, sexual selection in this species seems to be adaptive only under certain conditions, and the exact cause of this adaptiveness is debatable. These results are mostly different to those obtained from studies on *D. melanogaster* – a closely related sister species of *D. simulans* - and are generally consistent with classical interpretations of sexual selection (see Appendix C for a comparative review of sexual selection between *D. simulans* and *D. melanogaster*).

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Appendix A

Additional information for Chapter Seven

Table 1. Static allometry in various taxa: Primary intent of this appendix is to suggest that the allometry of non-genital secondary sexual characters has not been given much attention, especially within model species such as *Drosophila*. Data for this table was initially complied from two major allometry reviews (Bonduriansky, 2007; Kodric-Brown, Sibly & Brown, 2006). It was then supplemented with relevant recent studies as indexed in the ISI Web of Science under the keywords – allometry, genital, non-genital, secondary sexual characters. Note that this is not an all-inclusive list of allometric studies. Here trait type distinguishes sexual ("S"), or nonsexual traits ("N"), and trait functions (signal, weapon, grasping, genital etc.) are indicated where possible. Static allometry is characterized as negative ($b < 1$), isometric ($b \approx 1$), or positive ($b > 1$). References used within the table are appended.

Taxon	Trait	Type	Static allometry		Reference
			Males	Females	
Deer (Family: Cervidae)					
Cervus elaphus	Antler – weapon	S	+ve		(Huxley, 1931)
Odocoileus virginianus	Antler – weapon	S	+ve		(Fuller *et al.*, 1989)
Megaloceros giganteus (20 species)	Antler – weapon	S	+ve		(Gould, 1974)
Antelopes (Family: Bovidae)					
Bovidae (76 species)	Horn – weapon	S	+ve		(Gould, 1974)
Muskrats (Family: Cricetidae)					
Ondatra zibethicus	Baculum –genital	S	+ve		(Tasikas *et al.*, 2009)
	Skull length, skull width, hind foot length	N	-ve		
Birds (Order: Passeriformes)					
67 species	Ornamental feathers (signal)	S	+ve	+ve	(Cuervo & Møller, 2009) This study used reduced major axis regression, to recalculate the ordinary least square slopes from the 2001 study below.
(various families)	Non ornamental wing / tail feathers	N	+ve	+ve	
67 species	Ornamental feathers (signal)	S	-ve	-ve	(Cuervo & Møller, 2001)
(various families)	Non ornamental wing / tail feathers	N	-ve	-ve	
Vidua, *Tyrannus savana, Terpsiphone paradisi*	Ornamental tail	S	+ve		(Alatalo, Höglund & Lundberg, 1988)
Rallidae					
Gallinula chloropos	"Frontal shield" of bill	S	+ve	+ve	(Petrie, 1988)
Salamanders (Order: Caudata)					
Triturus vulgaris	Ornamental Tail crest (Snout-vent length)	S	+ve		(Green, 1992)
Poeciliid fishes (Family: Poeciliidae)					
Xiphophorus nigrenis	Ornamental sword (length)	S	+ve		(Rosenthal, Wagner & Ryan, 2002)
X. helleri	Ornamental sword (length)	S	+ve		(Basolo & Wagner, 2004)

Poecilia petensis (Sailfin molly)	Ornamental dorsal fin	S	+ve		(Hankison *et al.*, 2006)
P. velifer	Ornamental dorsal fin	S	+ve		(Hankison *et al.*, 2006)
Poecilia reticulata (guppy)	Black color area (signal)	S	+ve		(Kelly, Godin & Abdallah, 2000)
	Gonopodium length (genital)	S	-ve		
Brachyrhaphis episcope	Gonopodium length (genital)	S	-ve		(Jennions & Kelly, 2002)
Giant Gamba Prawn (Family: Aristeidae)					
Aristaeomorpha foliacea and Aristeus antennatus	Carapace length and lengths and widths of appendix masculina and appendix interna	S	+ve		(Kapiris, Moraitou-Apostolopoulou & Papaconstantinou, 2002)
Crabs (Order: Decapoda)					
Pachygrapsus transverses Gibbs					(Flores & Negreiros-Fransozo, 1999)
Hippa pacifica Dana					(Torres, 2009)
Chaceon affinis	Multiple characters	S	iso		(Fernandez-Vergaz, Lopez Abellan & Balguerias, 2000)
Seedbugs (Family: Lygaeidae)					
Lygaeus equestris	Genital length	S	+ve		(Higgins, Hosken & Wedell, 2009)
Scarab Beetles (Family: Scarabaeidae)					
Xylotrupes pubescens	Horn (weapon) measured pronotum width	S	+ve		(Rowland, 2003)
X. gideon	Horn (weapon) measured pronotum width	S	+ve		(Rowland, 2003)
Onthophagus taurus	Horn (weapon) measured pronotum width	S	+ve		(Tomkins, Kotiaho & LeBas, 2005)
Onthophagus taurus	Horn (weapon)	S	+ve		(Palestrini, Rolando & Laiolo, 2000)
O. binodis	Horn (weapon) measured pronotum width	S	+ve		(Tomkins *et al.*, 2005)
Odontolabis sp (24 species)	Mandible (weapon)	S	+ve		(Kawano, 2000)
Euoniticellus intermedius	Horn (weapon) ---- small body size	S	+ve		(Pomfret & Knell, 2006)
	---- larger body size	S	iso/-ve		(Pomfret & Knell, 2006)

Leaf Beetles (Family: Chrysomelidae)					
Cerotoma salvini	Elytron length (EL)	S	-ve		(Vencl, 2004)
	Antennal clamp / clasper organ	S	iso		
	Frontal plate	S	+ve		
	Aedagus length	S	-ve		
Soldier Beetle (Family: Cantharidae)					
Chauliognathus scutellaris	Male genetalia	S	-ve		(Bernstein & Bernstein, 2002)
	Scape	S	+ve	iso	
Whirligig Beetle (Family: Gyrinidae)					
Dineutus nigrior Roberts	Accessory glands	S	+ve		(Fairn, Schulte-Hostedde & Alarie, 2007)
	intromittant genitalia (aedeagus),	S	-ve		
	protarsal pads	S	iso		
Crickets (Order: Orthoptera)					
D. connectens (giant weta)		S	iso		(Field & Deans, 2001)
Hemideina sp.		S	+ve		
H. crassidens, H. thoracica, H. femorata		S		iso/ -ve	
H. ricta		S		+ve	
H. maori		S		+ve	
Hemideina crassidens	Head length, width	S	+ve	-ve	(Kelly, 2005)
Acheta domesticus, Gryllus bimaculatus, Gryllus rubens, Teleogryllus oceanicus	Wing area	S	iso		(Moradian & Walker, 2008)

	Harp area	S	-ve		
Water striders (Order: Hemiptera)					
Aquarius remigis	External genitalia (grasping)	S	iso		(Bertin & Fairbairn, 2007)
	Internal genitalia (genital)	S	iso		
	Fore-femur width, mid-femur length	N	+ve		
Gigantometra gigas	Fore-femur length (grasping)	S	iso	iso	(Tseng & Rowe, 1999)
	Mid- and hind-femur length	N	+ve	+ve	
Gerris buenoi	Mid-femur length	N	iso	iso	(Tseng & Rowe, 1999)
	Hind-femur length	N	+ve	+ve	
Leptoscelis tricolor	Hind-femur width (weapon)	S	+ve		(Miller & Emlen, 2010)
Fireflies (Order: Coleoptera)					
Photinus pyralis	Elytron length	S	+ve		(Vencl, 2004)
	Lantern area	S	-ve		
	Aedagus length	S	iso		
P. macdermotti	Elytron length	S	iso		
	Lantern area	S	iso		
	Aedagus length	S	iso		
Earwigs (Order: Dermaptera)					
Euborellia brunneri	armament	S	-ve/iso		(Van Lieshout & Elgar, 2009)
42 *Forficula* species	Forceps	S	+ve		(Simmons & Tomkins, 1996)
Forficula auricularia	Forceps	S	+ve		(Forslund, 2003)
Flies (Order: Diptera)					
Drosophilidae					
Chymomyza mycopelates	Head width (signal)	S	-ve	-ve	(Eberhard, 2002a)
	Head bristle separation (signal)	S	-ve	-ve	
	Fore-femur length (signal and/or weapon)	S	iso	iso	

	Fore-tibia length (signal and/or weapon)	S	-ve	-ve	
	Hind-tibia length	N	iso	iso	
	Wings	N	-ve	-ve	
C. exophthalma	Head width (signal)	S	+ve		(Eberhard, 2002a)
	Head bristle separation (signal)	S	+ve		
	Fore-femur length (signal and/or weapon)	S	-ve		
	Fore-tibia length (signal and/or weapon)	S	+ve		
	Femoral spine length (weapon)	S	+ve		
	Femoral spine number (weapon)	S	iso		
	Hind-tibia length	N	+ve		
	Wings	N	-ve		
Scathophagidae					
13 species	Testis	S	+ve*		(Hosken, Minder & Ward, 2005)
	Genital claspers (genital, grasping)	S	-ve*		* General trend.
	Mandibular palp	N	+ve/iso		
Diopsidae					
Cyrtodiopsis whitei	Eye-stalk width (signal, weapon)	S	+ve	iso	(Burkhardt, de la Motte & Lunau, 1994)
Teleopsis dalmanni	Eye span (signal, weapon)	S	+ve	+ve	(Foldvari *et al.*, 2007)
T. thaii	Eye span (signal, weapon)	S	+ve	-ve	
T. quinqueguttata	Eye span (signal, weapon)	S	-ve	-ve	
T. whitei	Eye span (signal, weapon)	S	+ve	+ve	
T. breviscopium	Eye span (signal, weapon)	S	+ve	-ve	
T. quadriguttata	Eye span (signal, weapon)	S	-ve	-ve	
T. rubicunda	Eye span (signal, weapon)	S	+ve	+ve	
Diasemopsis. comoroensis	Eye span (signal, weapon)	S	+ve	-ve	(Carr *et al.*, 2006)
D. meigenii	Eye span (signal, weapon)	S	+ve	-ve	

Piophilidae					
Prochyliza xanthostoma	Antenna, head length, fore-leg length (signal, weapon)	S	-ve/iso	-ve/iso/+	(Bonduriansky, 2006)
	Mid- and hind-tibia, intersetal width, wing length	N	-ve/iso	-ve/+ve	
Neriidae					
Telostylinus angusticollis	Antenna, head length, fore-leg length (signal, weapon)	S	+ve	-ve	(Bonduriansky, 2006)
	Mid- and hind-tibia, intersetal width, wing length	N	-ve/+ve	-ve/iso	
Tephritidae					
Ceratitis capitata, C.catoirii, C. rosa, Neoceratitis cyanescens	18 measurements of head, antenna, legs, wing	S/N	-ve	-ve	(Briceño, Eberhard & Quilici, 2005)
Phytalima mouldsi, P. alcicornis	Antlers and eye stalks	S	+ve		(Wilkinson & Dodson, 1997)
Sepsidae					
Palaeosepsis dentatiformis	Head width	N	-ve	-ve	(Eberhard, 2002b)
	Hind-tibia length (signal)	S	-ve	iso	
	Mid-tibia length (signal)	S	iso	-ve	
	Fore-tibia length (signal, weapon)	S	-ve	-ve	
	Wing length	N	-ve	-ve	
	Wing-spot length (signal)	S	+ve	+ve	
	Genital traits	S	-ve/+ve	+ve/iso	
Various arthropods and vertebrates					
117 arthropod species, 17 vertebrate species	Genitalia	S	-ve		(Eberhard, 2009)

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Appendix B

Male attractiveness, fertility and susceptibility to oxidative stress are influenced by inbreeding in *Drosophila simulans*

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Abstract

Inbreeding frequently leads to inbreeding depression, a reduction in the trait values of inbred individuals. Inbreeding depression has been documented in sexually selected characters in several taxa, and while there is correlational evidence that male fertility is especially susceptible to inbreeding depression, there have been few direct experimental examinations of this. Here we assessed inbreeding depression in male fertility and a range of other male fitness correlates in *Drosophila simulans*. We found that male fertility and attractiveness were especially susceptible to inbreeding depression. Additionally, levels of testicular oxidative stress were significantly elevated in inbred males, although sperm viability did not differ between inbred and outbred males. Copulation duration, induction of oviposition, and the proportion of eggs hatching did not differ for females mated to inbred or outbred males. Nevertheless, our results clearly show that key male fitness-components are impaired by inbreeding and provide evidence that aspects of male fertility are especially susceptible to inbreeding depression.

Keywords: Diptera, testis, sperm, ROS damage, sperm viability

Introduction

Inbreeding, mating between related individuals, increases homozygosity and this in turn decreases fitness by either exposing deleterious recessive alleles or by reducing the frequency of high fitness heterozygotes (Charlesworth & Charlesworth, 1987; Lynch &Walsh, 1998; Roff, 2002). This reduction in fitness, and in trait values generally, is known as inbreeding depression and depends upon the magnitude of directional dominance in a trait, which itself can be explained by selection (Falconer, 1989; Lynch & Walsh, 1998). For example, an allele having favourable fitness effects should go to fixation, and dominant deleterious alleles should be eliminated by selection (Lynch & Walsh, 1998). However, deleterious recessive alleles will be maintained at low frequencies, but directional dominance for characters weakly associated with fitness (or under stabilising selection) should be low because mutations moving trait values up or down will be selectively equivalent (Lynch & Walsh, 1998). Thus the effect of inbreeding is predicted to vary considerably across trait classes (Roff, 1997; Lynch & Walsh, 1998). For example, life history traits are closely linked to fitness and as a result are predicted to be under strong directional selection (Falconer, 1989). This continued directional selection should leave relatively more directional dominance variance for these characters. By contrast, morphological characters are expected to have relatively less directional dominance (Roff, 1997; Lynch & Walsh, 1998), and there is a considerable body of empirical evidence consistent with these theoretical expectations (e.g. Roff, 1998; DeRose & Roff, 1999; Wright *et al.*, 2008).

Like life history characters, many secondary sexual traits such as courtship calls (Drayton *et al.*, 2007) and ornaments (van Oosterhout *et al.*, 2003) also show severe inbreeding depression. These sexually selected characters are important fitness

determinants and are assumed to have a history of directional selection, at least when traits are exaggerated (Hamilton & Zuk, 1982; Zuk *et al.*, 1990). As a result, many sexually selected characters should also have considerable directional dominance and more inbreeding depression than morphological traits (Cotton *et al.*, 2004). However, relatively few studies have investigated the effects of inbreeding on secondary sexual traits or male attractiveness (Aspi, 2000; Joron & Brakefield, 2003; van Oosterhout *et al.*, 2003; Drayton *et al.*, 2007; Bolund *et al.*, 2010; Prokop *et al*., 2010) and only some of these find the predicted larger inbreeding depression in sexual characters (reviewed in Prokop *et al*., 2010). This has consequences for at least one very general model that explains how genetic variation in sexually selected traits is maintained in the face of strong directional selection (Prokop *et al*., 2010). The genic capture model suggests sexually selected characters (sensu Rowe & Houle, 1996) can be large mutational targets because they depend on condition (which is itself influenced more or less by all resource acquisition, assimilation and expenditure), and therefore sexual traits should exhibit more inbreeding depression than morphology (Prokop *et al*., 2010). As noted, this theoretical expectation is not always met and hence additional investigations of the relative inbreeding depression in these characters are warranted.

Primary sexual traits also appear to be particularly susceptible to inbreeding depression. For example, inbreeding depression has been reported for sperm quantity and *in vitro* measures of sperm quality in several species (Wildt *et al.*, 1983; Roldan *et al.*, 1998; Gomendio *et al.*, 2000; van Eldik *et al.*, 2006; Gage *et al.*, 2006). Inbreeding also negatively impacts sperm motility and normal sperm morphology (Gomendio *et al.*, 2000; van Eldik *et al.*, 2006). Furthermore, the production of abnormal sperm increases with increased homozygosity, suggesting that inbred individuals have lower

quality sperm (Gage *et al.*, 2006; Fitzpatrick & Evans, 2009). Surprisingly, although these studies provide ample correlational evidence that male fertility and sperm attributes are negatively impacted by inbreeding, there have been very few experimental studies that have investigated inbreeding depression in these male fitness components (e.g. Michalczyk *et al.,* 2010). As a result, additional experimental studies are needed to directly assess the impact of inbreeding on ejaculate characters.

As discussed above, the quantitative genetics of inbreeding depression are well understood, at least in principle (Falconer, 1989; Roff, 1997). However, its molecular basis has only recently begun to be investigated. The few studies so far indicate that inbreeding has large effects on gene expression and this translates into differential protein expression in inbred versus outbred populations (Kristensen *et al.,* 2005). Furthermore, many of the genes differentially expressed seem to be involved in general stress response, for example being linked to heat-shock proteins, and there is some evidence that inbred individuals may be under oxidative stress (Kristensen *et al.,* 2005). Metabolically active sites like the testes are likely to be especially prone to stress because of the production of reactive oxygen species (ROS) (Blount *et al.,* 1999; Kristensen *et al.,* 2008). This could ultimately result in increased testis and sperm dysfunction, and therefore inbreeding may especially impact on ejaculate components because of this. While there is some evidence consistent with more ROS damage to sperm in inbred populations (Ruiz-Lopez *et al*., 2010), ROS damage has never been specifically documented.

Here we assess inbreeding depression in a range of male fitness correlates in the fly *Drosophila simulans*. We have previously investigated inbreeding depression for a

range of characters in females and found that life-history traits are impacted to a greater extent that general morphology (Wright *et al.,* 2008), as predicted by theory (Falconer, 1989; Roff, 1997). We have previously found that two key components of male fitness, their attractiveness and sperm competitiveness, are heritable and positively genetically correlated (Taylor *et al.,* 2007; Hosken *et al.,* 2008). Here we assess inbreeding depression for both these traits and also conduct an assessment of male sperm quality by comparing the proportion of live/dead sperm and the levels of oxidative stress in the testes of inbred and outbred males. Additionally, because male ejaculate components influence female reproductive output (Chapman, 2001; Wolfner, 2002; Smith *et al.,* 2009), we assess the number of eggs laid by females in the 24 hours after mating to inbred or outbred males and the proportion of those eggs that hatched. We find evidence consistent with strong directional selection on some of these traits, as predicted, and show that inbreeding is associated with increased ROS damage in the testis.

Methods

The stock population of *D. simulans* was the same as that used by Wright *et al*. (2008) although by the time we conducted the current investigation it had undergone at least 35 generations of outbreeding. Stock and experimental populations were reared throughout on *Drosophila* quick mix medium (Blades Biological, UK) with yeast and water at 25^{o}C and 12/12 h light/dark cycle. Carbon dioxide or ice anaesthesia was used for handling and transferring flies.

Inbreeding design

Replicate inbred and outbred lines were generated using a crossing design that followed Roff (1998; also see Wright *et al.,* 2008) (Figure 1). Eggs were collected from the stock population and, upon adult eclosion, these individuals formed the grandparental generation (P) for the present experiment. Virgin 3-d old flies were randomly taken from the grandparental generation and used to generate male–female (F1) pairs, each housed in separate culture vials. Full-sibling families were obtained from these vials. These full-sib families were then randomly grouped into pairs (i.e. each group consisted of two full-sib families). Virgin 3 day-old flies from each family in each group were crossed as indicated in Figure 1, to generate replicated inbred and outbred F2 flies. Within each group, two inbred lines were produced by brother–sister matings and two outbred lines were produced by reciprocal crosses of a male and female from each parental family in the group. Half the subsequent (F2) progeny lines were therefore products of one generation of full-sib inbreeding (F = 0.25) and the other half were outbred controls. This crossing design was chosen because it should result in an equal representation of alleles, and the same proportions of dominance variance, within each group (Roff, 1998). Sample F2 progeny were collected as virgins from each of the inbred and outbred lines, and used to measure a suite of male characters.

Inbreeding depression assays

Emerging F2 virgins were collected every 12h, separated and housed by sex (within families) with an excess of the culture medium for 3 days (to ensure sexual maturity) before experimental matings. Matings were conducted between 09:00–12:00 (equivalent to the first 3 h of 'daylight' the flies would normally experience and

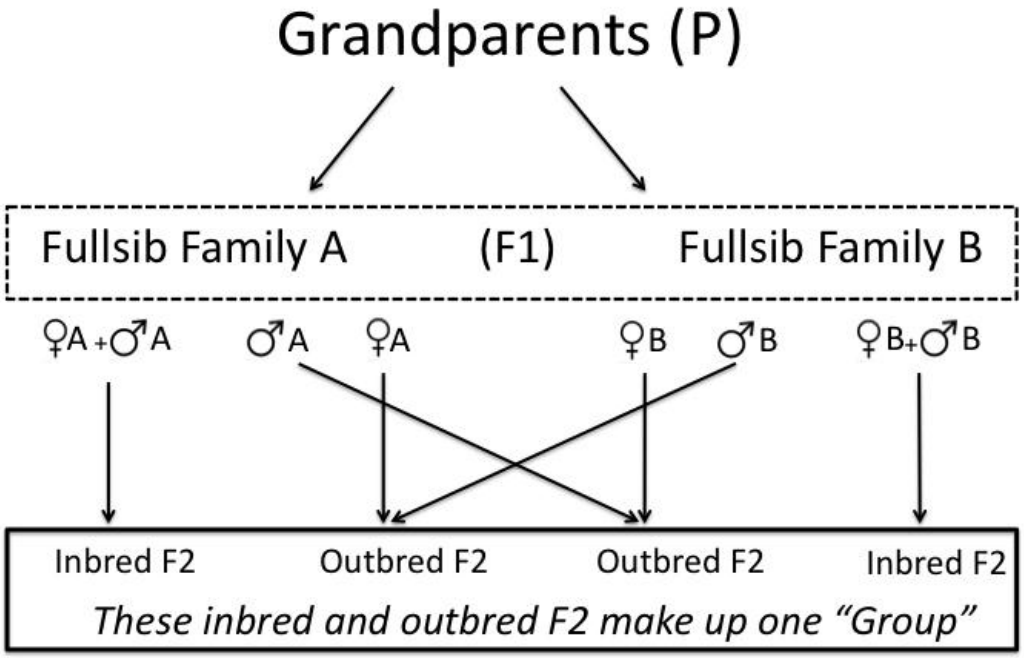

Figure 1. The crossing design used to generate inbred and outbred progeny for a single group. Within each group there are two inbred families, produced by F1 brother–sister matings, and two outbred families, produced by reciprocal crosses between the two F1 families. F2 flies were the experimental individuals.

corresponding with the period of peak mating activity in natural populations (Gromko & Markow, 1993)). The evening before mating, each male was individually aspirated into a mating vial (40 ml) containing culture medium. On the day of mating, one outbred female from another group (i.e. an unrelated female) was added to each vial and continuously observed until copulation ended. Using outbred, unrelated females allows us to assess how inbreeding influences male characters. We measured copulation latency (the time from female introduction to commencement of copulation) as an indicator of male attractiveness: females mate faster with more attractive males (Taylor *et al.,* 2007). This is because copulation only occurs after male courtship (e.g. wing raises, courtship song and genital licking) followed by a female acceptance or rejection signal (Speith, 1974). Copulation latency can therefore be used as a relative measure of female preference for any given male (Barth, 1997; Ritchie *et al.*, 1999; Acebes *et al.*, 2003). Note that measuring latency this way correlates well with the time from first courtship to copulation (Taylor *et al.,* 2008a) but is easier to measure. We subsequently refer to this measure as male attractiveness.

After copulation, males were removed and measured for body size (using wing length as a proxy; Gilchrist & Partridge, 1999; Taylor *et al.,* 2008a). Females were then housed alone to oviposit allowing assessment of male fertility via the reproductive output of their mates. To avoid larval overcrowding, females were transferred to a new vial every 3 days for the first 3 weeks, then every 5 days until offspring eclosion ceased. Male fertility was the total number of offspring emerging from all vials per female. Our sample size for this experiment was 50 groups that contained two inbred and two outbred families (n = 200 males, half inbred, half outbred).

We also counted eggs laid 24 hours after mating and assessed whether the proportion of eggs hatching for inbred and outbred males differed to determine whether sire inbreeding influenced early offspring development (i.e. eggs that did not hatch were deemed to have developmental problems rather than being unfertilized since at this early stage females are unlikely to be sperm limited and not fertilized eggs). To measure these variables we singly mated females (again unrelated to the sires) to either an inbred or outbred male. After copulation, males were removed and females allowed to lay eggs for 24 hours. We then discarded females, counted the number of eggs oviposited, and then 24 hours later counted the number of eggs that had hatched (hatching should take between 18-24 hours at 22^{o}C and will be faster under our housing temperature of 25^{o}C; Ashburner *et al.,* 2005). Hatching proportions were arcsine transformed for subsequent statistical analysis.

Oxidative damage in testes

Levels of testicular malondialdehyde, which is formed by the β-scission of peroxidised fatty acids, were assessed using HPLC with fluorescence detection, as described previously (e.g. Nussey *et al.,* 2009) with some modifications. All chemicals were HPLC grade, and chemical solutions were prepared using ultra pure water (Milli-Q Synthesis; Millipore, Watford, UK). There were 10 inbred and 10 outbred families, with ca. 10 males per family. Testis number per family varied from 7-10 due to dissection difficulties. Testes of males from inbred or outbred families, respectively, were pooled for analyses in order to obtain sufficient material for the assay. Gonads were dissected out and added to 30 µl ultra pure water (Milli-Q) in an Eppendorf tube, then homogenised for one minute using a motorised pestle, before being sonicated in a

water bath at room temperature for 10 mins. Samples were then centrifuged at 13,000 rpm and 4 °C for 1 min. An aliquot (10 µl) of the upper phase or the analytical standard (1,1,3,3-tetraethoxypropane, TEP; see below) was transferred to a clean 2 ml capacity screw-top microcentrifuge tube, and 10 µl butylated hydroxytoluene solution (0.05% w/v in 95 % ethanol), 80 µl phosphoric acid solution (0.44 M), and 20 µl thiobarbituric acid (TBA) solution (42 mM) were added. Samples were capped, vortex mixed for 2 seconds, then heated at 100°C for exactly 1 hour in a dry bath incubator to allow formation of MDA-TBA adducts. Samples were then transferred on ice to a refrigerated centrifuge and spun down (13,000 rpm and 4°C for 1 min), before 50 µl *n*-butanol was added and tubes were vortex mixed for 10 seconds. Tubes were then centrifuged at 13,000 rpm and 4°C for 2 minutes, before a 40 µl aliquot of the epiphase was collected and transferred to an HPLC vial for analysis. Samples (20 µl) were injected into a Dionex HPLC system (Dionex Corporation, California, USA) fitted with a 2 µm pre-column filter and a Hewlett-Packard Hypersil 5µ ODS 100 x 4.6 mm column maintained at 37°C. The mobile phase was methanol-buffer (40:60, v/v), the buffer being a 50mM anhydrous solution of potassium monobasic phosphate at pH 6.8 (adjusted using 5M potassium hydroxide solution), running isocratically over 3.5 min at a flow rate of 1 $ml.min^{-1}$. Data were collected using a fluorescence detector (RF2000; Dionex) set at 515 nm (excitation) and 553 nm (emission). For calibration a standard curve was prepared using a TEP stock solution (5µM in 40% ethanol) serially diluted using 40% ethanol. We were unable to accurately determine the mass of testes, and therefore results are expressed as concentrations of MDA in testes homogenates, and we statistically controlled for variation in testes number by including this as a covariate in the analyses (see below).

Sperm viability

Males from 14 groups (13 groups included 2 inbred and 2 outbred families, and in one group there was 1 inbred and 2 outbred families - Figure 1) were used to assess sperm viability. Sperm viability was assessed using standard sperm LIVE/DEAD (Molecular Probes L-7011) staining procedures for *Drosophila* (Snook & Hosken, 2004; Holman & Snook, 2008; Holman, 2009). We dissected sperm from 4-5 day old virgin male seminal vesicles (where mature sperm are located) in 5ul Beadle solution (Sigma) on a gelatin/chrome alum-coated microscope slide (Snook *et al.,* 1994) using 000 insect pins in pin vices (Starrett, Athol MA USA). Slides were placed in a moist chamber in the dark for 50 minutes after which we added 1ul of Sybr-14 (at 1:50 Sybr-14 (of a 1mM solution): Beadle saline solution). At 60 minutes we then added 1ul Propidium Iodine (of a 2.4 mM solution). These stains cause live sperm to fluoresce green and dead sperm to fluoresce red. We examined sperm fluorescence using a Zeiss LSM 510 confocal microscope using the C-Apochromat 40x/1.2 water corrected lens at a total magnification of 400x. Images were captured using the dual imaging function in which, for each slide, we first scanned using 488nm wavelength (FITC excitation) and then using the 561nm wavelength (rhodamine) before moving to the next slice (each slice representing 1um of the sample). The average stack size was 12um (range: 0 – 43; median = 12). We randomly placed the sample under the microscope and imaged two different places in the sample. After obtaining the two sets of image stacks for each sample, we then counted the number of living and dead sperm in each sample for each male, ensuring that individual sperm were counted only once as some sperm will span multiple slices. A small number of sperm were stained both green and red, and these were ignored. The total number of living and dead sperm for both samples was analyzed. Dissections and counts were done blind with respect to treatment.

Statistical analysis

Inbreeding depression was estimated for each trait by calculating the coefficient of inbreeding depression (σ):

$$\sigma = 1 - \left(\frac{X_I}{X_O} \right)$$

where X_I is the mean inbred trait value and X_o the mean outbred trait value. As inbreeding depression for copulation latency (male attractiveness) and MDA was calculated as the proportional increases, the coefficient of inbreeding depression was estimated as follow:

$$\sigma = \left(\frac{X_I}{X_O} \right) - 1$$

A population mean value of σ was estimated for each trait in two ways. Firstly by comparing the inbred trait value for each inbred family with the outbred population mean, then calculating a population mean σ for each trait by averaging the σ values for all families (subsequently referred to in the text as analysis using 'family' data). Secondly, σ was estimated for each group by comparing mean inbred values from the two inbred families within the group to the population outbred mean, then calculating a mean σ for each trait by averaging all the group values (subsequently referred to in the text as analysis using 'group' data). All subsequent analyses were performed using JMP 6.0 for windows (SAS Institute 2005).

Results

Inbreeding depression varied substantially both among traits and among inbred families (i.e. variances in σ were quite large) (Table 1). Student's *t*-test of the family

level data revealed inbreeding significantly impacted on male attractiveness, with inbred males being less attractive than outbred males, and male fertility (the number of offspring an outbred but unrelated female produced in her lifetime after a single copulation) was also significantly reduced for inbred males. Male body size, copulation duration, the number of eggs oviposited in the 24 hours after mating and egg hatchability did not differ between inbred and outbred males. Paired *t*-tests performed on the group data gave virtually identical results (Table 1). There were also no significant effects of inbreeding on sperm viability either using the family or the group data (Table 1), and this result remained unchanged when we included sperm number or handling times as covariates (data not shown). Thus, we found no evidence of inbreeding depression for sperm viability.

To see if the decline in male fertility was likely to be due to inbred males transferring less sperm to females, we conducted a repeated measures analysis of offspring number produced in the first three vials occupied by the females mated to inbred and outbred males. This represents the first 9 days of eggs laying after mating (3 days per vial), and if inbred males were transferring fewer sperm to females, then the number of offspring produced by their mates should decline faster than that of females mated to outbred males. This is exactly what we saw (Figure 2). The analysis revealed that inbred males had lower fertility, as per the previous analysis ($F_{1,198} = 140.7$; $p = 0.0001$), plus there was a significant decline in fertility over time ($F_{2,396} = 105.5$; $P = 0.0001$; Figure 2) and a significant interaction between fertility and male type ($F_{2,396} = 41.7$; $P = 0.0001$; Figure 2). Thus the difference between in- and out-bred males increased with

Table 1. Inbreeding depression estimates calculated using family and group data (see text for details). Male fertility was assessed by measuring the lifetime productivity of females mated to inbred and outbred males.

Trait	σ×100	Sample size		P value
	Mean ± SE	Inbred	Outbred	
Line data				
Male wing length	-0.18 ± 0.61	100	100	0.97
Male fertility	18.69 ± 1.56	100	100	<0.001
Male attractiveness	59.77 ± 8.25	100	100	<0.001
Copulation duration	-5.91 ± 3.15	100	100	0.644
Egg number	-7.61 ± 6.40	60	60	0.784
Hatching rate	-0.07 ± 2.94	60	60	0.386
Sperm viability	-17.17 ± 10.78	27	28	0.744
Group data				
Male wing length	-0.08 ± 0.58	50	50	0.97
Male fertility	19.10 ± 1.70	50	50	<0.001
Male attractiveness	50.33 ± 7.75	50	50	<0.001
Copulation duration	-3.82 ± 3.20	50	50	0.568
Egg number	-2.62 ± 4.61	30	30	0.718
Hatching rate	0.79 ± 3.10	30	30	0.367
Sperm viability	-8.03 ± 8.31	14	14	0.457

P values were calculated using Student's t-test in line data and Paired t test in group data.

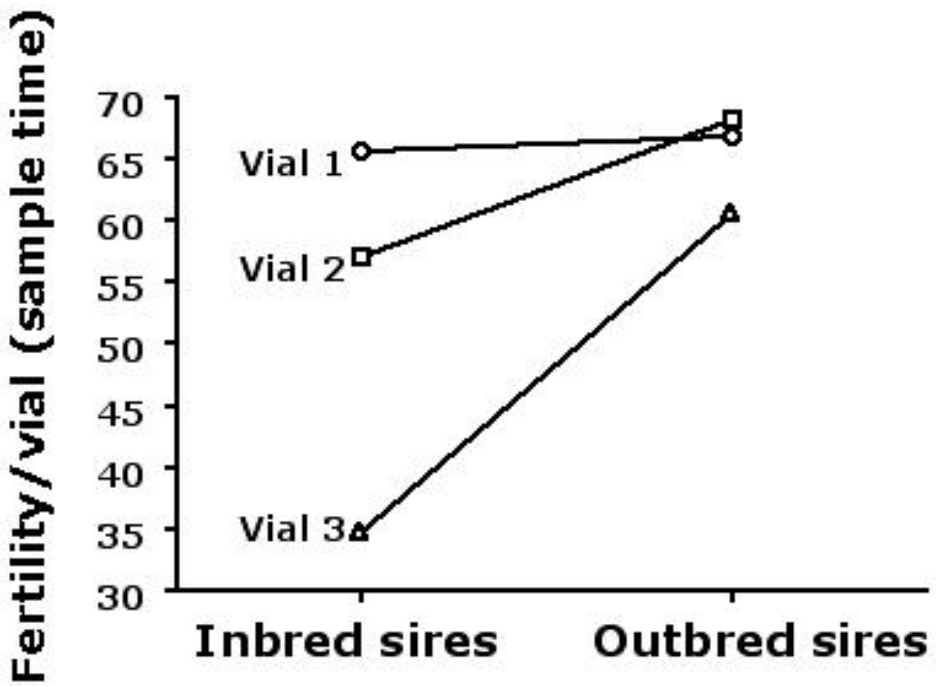

Figure 2. The fertility of inbred and outbred males (as assessed by counting the offspring from single matings to outbred females) over time. While the fertility of the two types of male did not initially differ (Vial 1), the mates of inbred sires produced fewer offspring as time progressed (Vials 2 & 3). Vial 1 represented the first 3 days of offspring production after mating, Vial 2 the next three days and Vial 3 the next three.

time after mating (Inbred: Vial 1 = 65.7±1.2; Vial 2 = 57.1±1.3; Vial 3 = 34.7±1.9. Outbred: Vial 1 = 66.8±1.2; Vial 2 = 68.2.1±1.1; Vial 3 = 60.6±1.2).

The effect of inbreeding on testicular oxidative stress was assessed using ANCOVA because we had to control for testis number, and therefore this analysis was only conducted at the family level. Inbreeding treatment was a fixed effect and testis number the covariate and we used a reduced model that removed non-significant interaction terms from the full model (Grafen & Hails, 2002). The reduced model showed that treatment and testis number had a significant effect on MDA (Treatment: $F_{1,17} = 7.73$; $p = 0.013$. Testis number, $F_{1,17} = 37.71$, $p < 0.001$). The coefficient of inbreeding depression calculated using the relative mean estimated from ANCOVA (inbred line, 0.169 ± 0.007 (relative mean+s.e.); outbred line, 0.140 ± 0.001) was σ = 20.1 (×100). Thus, there was substantial and significant inbreeding depression for susceptibility to oxidative stress in the testes.

Discussion

We found that the lifetime productivity of females who mated with inbred males was significantly reduced compared to outbred males. This decrease occurred even though the level of inbreeding imposed on the flies was a modest but realistic $F = 0.25$. This reduction was apparently not due to early developmental failure of eggs fertilized by inbred males as the proportion of eggs hatching did not differ when sires were in- or out-bred (at least for eggs laid in the 24 hours after mating). Similarly the number of eggs laid by females in the 24 hours after mating, when a large proportion of eggs are laid (Taylor *et al*., 2008b), did not differ for inbred and outbred males. Furthermore,

the proportion of live/dead sperm did not differ between inbred and outbred males. Therefore a likely explanation for the reduction in offspring production for inbred males is that they transfer fewer sperm per mating. This inference is supported by the increasing differences in the fertility of inbred and outbred male over time, and could be related to the elevated levels of oxidative stress detected in the testes of inbred males (as shown by the elevated testis MDA levels). Reactive oxygen species (ROS) are known to cause phenotypic damage, including accelerating rates of ageing, through oxidative stress (reviewed in Dowling & Simmons, 2009), and we found inbreeding caused increased oxidative damage in testes. Dowling and Simmons (2009) suggested ROS damage to sperm decreases motility, fertilization potential, and/or embryonic survival. We did not find any evidence that inbred males sire embryos with lower survival or lower fertilization potential soon after mating (i.e. hatch rates were not lower for eggs laid after 24 hours). However, clearly some aspect of male fertility was impacted by inbreeding, and it seems reasonable to conclude that this was sperm number. If male fertility was reduced as a consequence of lower numbers of sperm transferred per ejaculate, as tentatively indicated by our findings, then inbreeding will probably also affect post-copulatory sexual selection in *D. simulans* because sperm number is an important determinant of sperm competitiveness (Birkhead *et al.,* 2009). Reduced sperm competitiveness of inbred males has been reported for several other species, including *D. melanogaster*, a close relative of *D. simulans*, although this often only occurs at much higher levels of inbreeding than we have imposed here (Hughes, 1997; Konior *et al.,* 2005; Zajitschek *et al.,* 2009; Michalczyk *et al.,* 2010). Other ejaculate parameters such as sperm swimming velocity, percentage motility and viability can also influence sperm competitiveness and fertilization success (e.g. Birkhead *et al.,* 1999; Gage *et al.,* 2004; Garcia-Gonzalez & Simmons, 2005; reviewed in

Snook, 2005). However, no inbreeding depression for sperm viability was found in the present study. A potential limitation in our assay is that sperm viability was assessed once when males were virgin and 5 days old. However, this first batch of sperm has been developing since males were second instar larvae, allowing ample time for inbreeding effects to be manifest - for example for oxidative stress effects to affect sperm. Furthermore, sperm from inbred and outbred males apparently worked equally well in the first 24 hours of egg laying, suggesting viability/fertility differences do not occur at least initially. In this regard our findings contrast somewhat with correlational work in a range of other taxa where it has been reported that inbreeding specifically impacts on sperm quality measures (reviewed in Roldan & Gomendio, 2009). However, it remains possible that differences in sperm mortality in female sperm storage organs occurs to generate the lower fertility of inbred males, but on the basis of the evidence we currently have, differences in the number of sperm transferred seems more a likely explanation to us.

It could be argued that if the reduction in the fertility of inbred males was caused by lower sperm number transferred per ejaculate, as we suggest, then copulation duration should be shorter for these males (see e.g. Simmons *et al.,* 1996). However, sperm transfer in *Drosophila* occurs in the first few minutes of copulation, with the remaining time seemingly spent transferring accessory gland fluid (Gromko *et al.,* 1984; Manier *et al.,* 2010), so a direct association between copulation duration and numbers of sperm passed to females is not expected. Our finding of no difference in the number of eggs females laid in the 24 hours after copulation to inbred or outbred males also indicates there is no inbreeding depression for non-sperm ejaculatory components that influence short-term female productivity (e.g. Wolfner, 2002).

Nonetheless, male fertility was reduced by inbreeding, and this is potentially linked to the increased oxidative stress in the testes of inbred males.

Mean inbreeding depression coefficient estimates for male fertility were about 19%. Thus, male fertility shows high levels of inbreeding depression even at modest levels of inbreeding, indicating substantial directional dominance for this trait and suggesting it may be under strong selection (Roff, 1997; Lynch & Walsh, 1998), as would be expected. This estimate is similar to those reported for *Drosophila pseudoobscura* (22% for fertility: Dobzhansky & Spassky, 1963), and to some estimates for *D. melanogaster* (18% for longevity: Hughes, 1995). In contrast, the one morphological trait we measured (wing length) did not show significant inbreeding depression. This result is consistent with previous work on this population, where the inbreeding depression coefficient for female wing-length was only about 0.1%, although this was statistically significant (Wright *et al.,* 2008). In any case, morphology is often thought to be under relatively weak selection, and hence the directional dominance for morphology is predicted to be lower than for life-history traits, which is why morphology has less inbreeding depression (Falconer, 1989; Roff, 1997; Lynch & Walsh, 1998). The σ estimates for wing length are also similar to those of other *Drosophila* (Lynch and Walsh, 1998). For example, inbreeding depression in wing length for *D. melanogaster* has been reported to be between 1 and 3% (Tantaway, 1957; Tantawy & Reeve, 1956).

Like male fertility, male attractiveness also showed significant inbreeding depression. Mean inbreeding-depression coefficient estimates were very high (about 55%), which suggests male attractiveness is under exceedingly strong directional selection (based

on the arguments presented above; Roff, 1997) in *D. simulans*, and this inference is consistent with previous findings in this population (Taylor *et al.,* 2007, 2010; Hosken *et al.,* 2008). The substantial inbreeding depression for attractiveness is also in accordance with predictions that the secondary sexual traits conferring attractiveness to males should show strong effects of inbreeding (Hamilton & Zuk, 1982; Zuk *et al.,* 1990; Cotton *et al.,* 2004; but see Prokop *et al*., 2010). Our coefficient of inbreeding depression estimates for attractiveness are close to those reported for similar measures in other *Drosophila* (Lynch & Walsh, 1998). For example, inbreeding depression in male mating ability for *D. melanogaster* is between 52 and 76% (Hughes, 1995; Sharp, 1984). Our results also indicate that the effects of inbreeding are important in a mate choice context, and that inbred males are less attractive to females. However, our study does not allow us to pinpoint the mechanism(s) behind this reduction in attractiveness. Aspi (2000) reported relatively high inbreeding depression (about 12-13%) for male courtship-song frequency, one character that influences attractiveness in *Drosophila montana* (also see Ketola & Kotiaho, 2010). However, the levels of inbreeding applied in that study were much higher than we employed here. Nevertheless, while inbreeding may alter specific characteristics of courtship song in *D. simulans*, (and attractiveness is generally associated with characteristics of courtship song in *Drosophila* (Speith, 1974; Ritchie *et al.,* 1999)), our attractiveness measure includes all traits that contribute to male attractiveness (i.e. cuticular hydrocarbon profiles, song, behaviour and so on). The consideration of total attractiveness rather than specific characters that confer attractiveness may be why the inbreeding depression we record is relatively high.

In conclusion, inbreeding had strong negative effects on several male fitness components (fertility, attractiveness and testicular levels of oxidative damage) in male *D. simulans* consistent with selection theory (Lynch & Walsh, 1998). However, sperm viability showed no inbreeding depression in contrast to our *a priori* expectations. Nevertheless, other aspects of sperm quality that we did not assess may have been impacted, and a promising approach for future studies would be to investigate more closely the impact of inbreeding on specific sperm attributes, as well as potential negative synergy between environmental stress and inbreeding on measures of male specific fitness. Elevated ROS damage in the testes of inbred males as we find here, has not been reported previously and may underly the reduce fertility of inbred males. This is certainly worth further investigation. Overall, our findings are consistent with condition dependent sexual selection (Rowe & Houle, 1996), but more work is needed to see if male sexual characters generally show elevated inbreeding depression (Prokop *et al*., 2010).

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Appendix C

Sexual selection in flies: A comparison of *Drosophila simulans* and *D. melanogaster*

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Sexual selection in flies: a comparison of *Drosophila simulans* and *D. melanogaster*

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Abstract

The traditional view of sexual selection via female mate choice is that female preference for certain males either has no net fitness cost or is beneficial to overall female fitness. A more contemporary view is that preferred males can at times reduce female fitness. This view has arisen from the realisation that conflict between the sexes is an inevitable feature of sexual reproduction, as each sex necessarily has a different agenda for maximizing fitness. Despite the hailing of sexual conflict as a paradigm shift and its prevalence in the recent sexual selection literature, compelling evidence that attractive males reduce female fitness remains taxonomically restricted. Here we review the findings of a series of investigations into the fitness consequences of female preference in the fly *Drosophila simulans* and compare them with its sibling species, *D. melanogaster*. We show that there are stark differences in the fitness consequences of mating with preferred males in the two species and discuss this contrast with reference to the current debates in the sexual selection literature.

Keywords

Sexual selection; sexual conflict; female mate choice; fitness

Introduction

It is now more than a century since Darwin first proposed his general theory of sexual selection which he defined as the advantage which certain individuals have over other individuals of the same sex and species purely regarding reproduction (Darwin, 1871). It has taken much of this time for Darwin's ideas regarding female mate choice to become empirically demonstrated and fully accepted. Under this classical view, female preferences for certain males were either neutral with respect to female fitness or generated some net fitness benefit. Under this second premise, preferred males signal their quality using honest indicators of genetic or environmentally determined condition,

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which either the female or her offspring benefit from, or males signal their sexual attractiveness alone, and their sons inherit this attractiveness (Andersson, 1994). While Darwin suggested that sexual selection opposed natural selection, he also suggested that sexual selection might also improve upon or aid natural selection (Darwin, 1871). Lande's models (1987) of runaway selection by necessity also include sexual selection and natural selection coinciding initially, and this adaptive view of sexual selection has been increasingly embraced, resulting in suggestions that preference for viability (good genes) is a virtual inevitability of sexual selection (Jennions and Petrie, 2000). However, because conflict between the sexes is ubiquitous (Parker, 1979; Lessells, 2006; Hosken et al., 2009), a more recent view is that preferred males may often reduce female fitness, and sexual selection is more likely driven by direct costs with little compensation through indirect benefits (Kirkpatrick and Barton, 1997; Rice and Holland, 1997; Holland and Rice, 1998; Gavrilets et al., 2001; reviewed in Arnqvist and Rowe, 2005). Essentially, this has shifted the emphasis from sexual selection being neutral or beneficial to female fitness, to a system driven by direct costs and depressed female fitness. However, experimental evolution has demonstrated that sexual selection can result in positive, neutral and negative net female fitness (Partridge, 1980; Promislow et al., 1998; Holland and Rice, 1999; Holland, 2002; Martin and Hosken, 2003; Martin et al., 2004).

Despite the hailing of sexual conflict as a paradigm shift (Tregenza et al., 2006) and its prevalence in the recent sexual selection literature (Pizzari and Snook, 2003), the circumstances under which sexual conflict is predicted to generate cycles of sexually antagonistic selection, and hence have significant fitness costs, seem rather restrictive (Parker, 1979; Rowe and Day, 2006). Parker (1979) investigated this in models that varied the 'value' (relative fitness payoffs to each sex) and 'power' (the marginal cost of a unit of escalation) of winning a sexual conflict. The interaction of these two parameters may either lead to an escalatory arms race or neutralize the conflict, depending on the specific circumstances of the population in question. In other words, the existence of conflict does not always translate into selection (also see Rowe and Day, 2006), and in fact arms races only occurred in a small proportion of the parameter space investigated. Nevertheless, empirically distinguishing between classical and conflictual models of mate choice is difficult as many of the predictions are the same. For example, 'attractive males siring attractive sons' is a prediction of both Fisher's model of mate choice (Fisher, 1930; Lande, 1981; Kirkpatrick, 1985), and the Holland and Rice (1998) 'chase-away' model of antagonistic seduction. Pizzari and Snook (2003) point out that attempts to test between models based on sexual antagonism as opposed to mutualism also suffer from using poor proxies for fitness. For example, reduced female longevity is not in itself evidence of conflict-based selection if this cost does not reduce reproductive output or rate. The best way to assess the underlying form of selection (classical vs. conflictual) in a system is to document the current sign of direct and indirect selection on female preference (Arnqvist and Rowe, 2005; Hosken et al., 2009).

Major reviews of the benefits of female mate choice conclude that females across a diverse range of taxa do receive both direct and indirect benefits from their mate choices (Møller and Alatalo, 1999; Møller and Jennions, 2001). Reviews also conclude

that many male traits are costly and therefore possibly function as indicator traits, as predicted by traditional benefits models of sexual selection (Jennions et al., 2001). By comparison, one large scale review, examining extra-pair copulatory behaviour across passerine birds, concludes sexual conflict drives extra-pair copulations (Arnqvist and Kirkpatrick, 2005), and across water striders, sexual conflict has also generated significant selection (Rowe and Arnqvist, 2002). More commonly, evidence for selection through sexual conflict is presented from a handful of well-known, often laboratory-based, model systems (e.g. *Drosophila melanogaster*, reviewed in Rice et al., 2006; water striders, reviewed in Arnqvist and Rowe, 2005; *Sepsis cynipsea*, e.g. Ward et al., 1992, Hosken et al., 2003). This suggests that the 'incipient paradigm shift' towards sexual conflict as the assumed driver of sexual selection (Pizzari and Snook, 2003; Tregenza et al., 2006), may be prematurely based on over-generalized evidence from restricted cases (also see Hosken and Snook, 2005). One of the advantages of the *Drosophila* system is that there are many well-studied, closely related species with which comparisons can be drawn. *D. melanogaster* and its sibling species *D. simulans* in particular have been used extensively in comparative studies into speciation events and adaptation (Capy and Gibert, 2004; David et al., 2004). These cosmopolitan species overlap in geographical range, ecology and behaviour and provide an ideal opportunity to test whether the prevalence of sexual conflict documented in *D. melanogaster* also exists in its congener. Here we review the findings from recent investigations of selection on female preference in *D. simulans* and compare them to similar studies in *D. melanogaster*. We find that the fitness consequences of sexual selection in *D. simulans* often contrast with those reported for *D. melanogaster*, and on balance appear to follow a traditional model of sexual selection. We discuss this contrast with reference to the current debate regarding the fitness consequences of and selection on female preference.

Sexual selection in *Drosophila simulans* and *Drosophila melanogaster*

D. simulans is a member of the *melanogaster* species subgroup and is thought to have separated from *D. melanogaster* around 2 million years ago (Powell, 1997). As with its sibling species, *D. simulans* has a polygamous mating system, both males and females routinely copulate with multiple mates, and there is no parental care beyond selecting a suitable oviposition site, usually in decaying and fermenting fruit and vegetation (Markow, 1996). Studies of female preference in *Drosophila* generally follow the definition of Jennions and Petrie (1997) – any sensory or structural properties in females that bias mating or fertilization success towards certain male phenotypes. Female preferences for different males have previously been determined from the copulation latency of pairs of flies placed together under experimental conditions. That is, how long it takes a female to accept a courting male and allow copulation to proceed. This is because females assess males via a stereotypical range of courtship behaviours, e.g. wing raises, 'songs' from wing vibrations and mounting attempts. These are repeated sequentially until the female interrupts the courtship display with her own acceptance

or rejection signals (Speith, 1974). Since copulation only occurs with female cooperation – (there is no forced copulation with sexually mature females but see Markow (2000) for evidence of forced copulations in teneral females) – the latency to copulation can therefore be used as a relative measure of female preference for any given male. This method has shown females to prefer, for example, conspecifics over heterospecifics (Ritchie et al., 1999; Acebes et al., 2003) and preferences for males from similar rearing environments (Barth et al., 1997).

We begin our examination of the fitness consequences of female mate choice in these two *Drosophila* with the simplest potential benefit females can derive from mating with preferred, attractive males – that a female mating with an attractive male can directly increase her fecundity or fertility (Andersson, 1994). This requires direct natural selection on the preference only, and formal modelling predicts that direct selection will produce female preferences for males that benefit females' immediate reproductive success (Kirkpatrick and Barton, 1997). Most of the current evidence suggests that direct benefits are common in nature, but that the overall effect size is small (Møller and Jennions, 2001). In the first of their investigations with *D. simulans*, Taylor et al. (2008a) mated virgin females to a single virgin male and measured the copulation latency (shorter latencies indicated a preferred/attractive male) and resultant fitness of the female (lifetime reproductive success (LRS) and longevity) to assess direct selection on female preference. They also repeated this procedure, exposing females to two males simultaneously to assess female preference under the influence of male-male competition, as these two processes may not always be reinforcing (reviewed in Wong and Candolin, 2005). They found no direct positive or negative effect of mating with attractive males on female LRS (i.e. there was no direct selection on preference). There was a small longevity cost when females mated in the presence of two males, which was not previously apparent when only one male was present, suggesting a negative effect of male-male competition on female lifespan. However, this did not impact on female LRS. These results stand in direct contrast to studies of *D. melanogaster* that show preferred males directly depress female fecundity, fertility and longevity (Pitnick, 1991; Pitnick and García-González, 2002; Friberg and Arnqvist, 2003).

Since females of both species routinely mate multiply in nature (Markow, 1996), the most obvious caveat to Taylor et al.'s (2008a) conclusions is that direct benefits may accrue over several matings, and differences between individual males from a single mating may not be large enough to be detected (Møller and Jennions, 2001). Similarly, any costs of mating may also have a cumulative effect and be phenotype independent. That is, attractive males may not generate higher per mating costs, but rather they cause females to mate too frequently and hence multiple mating may be needed before costs can be seen. To examine this in *D. simulans*, Taylor et al. (2008b) mated virgin females to a single virgin male and then allocated them to one of three treatments: one, two or three matings. LRS and longevity were recorded as in the previous study. Females were found to benefit directly from multiple mating via increased LRS. This result concurs with a widespread finding across insect taxa (Arnqvist and Nilsson, 2000), and contrasts with one interpretation of Bateman's work on *D. melanogaster*, namely that female fecundity is maximized after only one or two matings (Bateman,

1948; and reviewed in Snyder and Gowaty, 2007; also see Brown et al., 2004). Additionally, the number of matings *per se* were also found to have no impact on female longevity in *D. simulans* (Taylor et al., 2008b), which contrasts starkly with the finding that female longevity in *D. melanogaster* is reduced by multiple mating (Chapman et al., 1995; Wigby and Chapman, 2004; Kuijper et al., 2006). Potential fitness consequences of male harassment and multiple mating were also examined in *D. simulans* by housing females with males (Taylor et al., 2008b). Again, virgin females were mated to a single virgin male and then allocated to one of four housing regimes: housed alone; housed with two females; housed with two males; housed alone and exposed to two males, every five days, for a three-hour period. A marked decrease in female longevity resulted from being continuously housed with two males, which is in agreement with other studies of non-mating costs in *D. melanogaster* (Partridge and Fowler, 1990; Chapman and Partridge, 1996; Kuijper et al., 2006). However, the LRS of *D. simulans* females was still higher for females housed with males, so females still enjoyed a net fitness benefit from multiple mating (Taylor et al., 2008b). Again, this contrasts somewhat with findings in *D. melanogaster*, where mating with multiple males generates no fitness benefit for females (Brown et al., 2004). The net result of the *D. simulans* studies discussed to date is that there is no negative (or positive) selection on female preference, and hence selection through sexual conflict does not appear to predominate in this system (Arnqvist & Rowe, 2005).

In the absence of direct selection on preferences, as appears to be the case for *D. simulans*, indirect selection may be sufficient to maintain female mating preferences (Andersson, 1994; Kirkpatrick and Barton, 1997). Taylor et al. (2007) investigate one assumption of indirect benefits models, the heritability of male attractiveness, in *D. simulans* using a full-sib/half-sib mating design. Briefly, sires mated to three dams, and the attractiveness of approximately three sons from each dam (= ca. nine sons per sire) was subsequently assessed. Male attractiveness was significantly heritable with substantial evolvability, suggesting females could benefit via the attractiveness of their sons. Furthermore, the heritability reported falls well within the range of heritabilities reported for other sexually selected traits, including copulation behaviours, which tend to be higher than for life history traits (Pomiankowski and Møller, 1995; Rowe and Houle, 1996; Radwan, 2008). Heritability of male attractiveness is one defining characteristic of Fisherian benefits, which are predicted to evolve where direct selection on preferences is absent - formal modelling of the Fisher process predicts that with direct selection on the preference, lines of equilibrium collapse and females prefer males that benefit their direct fitness (Fisher, 1930; Lande, 1981; Kirkpatrick, 1982; Kirkpatrick, 1985). Taylor et al.'s (2007) finding parallels work on *D. melanogaster* which also concludes attractive males sire attractive sons, although the heritability of attractiveness was not reported (Rundle et al., 2007). However, other studies using *D. melanogaster* provide no evidence for a net fitness benefit via attractive sons, as direct costs to females outweigh this indirect benefit (Orteiza et al., 2005; Pischedda and Chippindale, 2006), as predicted by theory (Kirkpatrick, 1985).

Females mating multiply inevitably leads to sperm competition in these sperm-storing flies, so that sexual selection continues after copulation (Parker, 1970). However,

the relationship between pre- and post-copulatory mating success is generally unclear. Hosken et al. (2008) again employed the full-sib/half-sib approach with *D. simulans*, mating sires to multiple dams and collecting multiple sons from each dam. Sons were then mated to females who had already mated once. Sons' attractiveness and sperm competitiveness were measured - by scoring copulation latency and their share of paternity as the second male to mate. Attractive sons sired a greater proportion of offspring under sperm competition, strongly suggesting that pre- and post- copulatory attractiveness are reinforcing, since males better in the pre-copulatory arena also do better in post-copulatory competition. As with male attractiveness, sperm competitiveness was also significantly heritable (Hosken et al., 2008) and importantly, positively genetically correlated with male attractiveness, so that these traits can potentially evolve in concert. Again, there was no relationship between sperm competitiveness and direct female fitness, so it appears that if females in this *D. simulans* system are benefiting from mating with attractive males, it must be indirectly. Whether this post-copulatory success was attributable to attractive males being intrinsically better sperm competitors or to females selecting their sperm is currently unknown. To our knowledge, there have been no similar studies of the interactive effects of pre- and post- copulatory success in *D. melanogaster* with which to make a direct comparison with these results. However, sperm competitiveness in *D. melanogaster* is non-transitive, making associations between pre- and post-copulatory success unlikely (Clark et al., 2000; Bjork et al., 2007).

Mating preferences based solely on Fisherian mating advantages are predicted to be rare in nature, since female preferences are unlikely to be entirely cost free (Kirkpatrick, 1996; Møller and Alatalo, 1999), although care must be taken in distinguishing between costs of choice *per se* and direct selection on female preference (Heisler et al., 1987). Fisherian benefits themselves are probably initiated via good genes effects, and traits for male attractiveness are expected to become costly and dependent on either environmentally or genetically determined condition (Balmford and Read, 1991; Kirkpatrick and Ryan, 1991; Jennions et al., 2001). Furthermore, heritable attractiveness is also predicted under good genes sexual selection. So females may be receiving indirect benefits through the overall quality of their offspring, but they may 'look' like Fisherian traits if males trade-off investment in sexually selected traits against somatic maintenance (Kokko, 2001). In the absence of detailed information on how males trade-off investment in sexually selected traits and viability, the potential for good genes benefits from attractive males is best evaluated via their daughters (Getty, 2002). To examine this in *D. simulans* Taylor et al. (2009) mated virgin females with a single virgin male and measured the LRS and longevity of their daughters. There was no relationship between the attractiveness of sires and their daughters' fitness. Although this does not rule out the possibility that mothers themselves could have compensated for sexually antagonistic fitness effects of mating with attractive males, it does suggest there are again, no net fitness costs to females mating with attractive *D. simulans* males. This is in direct contrast to evidence from *D. melanogaster* showing that attractive males produce poorer quality offspring (Gibson et al., 2002; Pischedda and Chippindale, 2006). Previous work had linked attractiveness of *D. melanogaster* males with individual

components of offspring fitness, demonstrating the potential for an indirect benefit via offspring quality (Partridge, 1980; Taylor et al., 1987). However, recent work by Priest et al. (2008a, b) suggests these effects are maternal (i.e. environmental) rather than inherited good genes from sires. Again, studies using experimental evolution have provided clear evidence that indirect fitness cannot compensate for the direct costs of harmful males in *D. melanogaster* (Orteiza et al., 2005; Stewart et al., 2005).

Discussion and summary

This brief comparison (summarised in table 1) of the female fitness consequences of mating with attractive males in *D. simulans* with its congener *D. melanogaster* was intended to compare the evidence for alternative models of sexual selection in these species and determine whether findings in *D. melanogaster* apply to *D. simulans*. Much of the evidence for conflict-based selection stems from research in *D. melanogaster*, and this very clearly demonstrates negative selection on female preference as mating with attractive males directly reduces female fitness (e.g. Pitnick and García-González, 2002), and where indirect benefits are found, they are not sufficient to offset these direct costs (Rice et al., 2006), consistent with theory (Kirkpatrick, 1985). Consequently, net female fitness is usually negatively related to male attractiveness, with the natural conclusion that sexual selection is largely conflict driven (Arnqvist and Rowe, 2005). By comparison, the investigations of sexual selection in *D. simulans* reviewed here strongly suggest that female mate choice follows a traditional Fisherian model of sexual selection, and although experimental evolution follow-up of these studies has not been conducted, the available evidence suggests that indirect benefits maintain female preferences in this species – at least in these populations and under the conditions investigated to date. While indirect benefits compensating for direct costs has been the subject of recent debate (Cameron et al., 2003; Cordero and Eberhard, 2003; Hosken and Tregenza, 2005), indirect fitness is always less influential than direct fitness (Kirkpatrick and Barton, 1997), and it is evident that the direct costs to females so prominent in the *D. melanogaster* system seem at present to be the primary difference between it and *D. simulans*.

In a review of antagonistic selection in the *D. melanogaster* system, remating rates appear to be the key female adaptation to direct negative selection on females (Rice et al., 2006). In *D. melanogaster* the number of matings itself is negatively related to female longevity (Chapman et al., 1995; Wigby and Chapman, 2004; Kuijper et al., 2006), whereas in *D. simulans* male harassment, but not multiple copulations, apparently reduces female longevity. A study comparing the amount of sperm stored by wild females caught in the process of re-mating in the field found that female *D. simulans* remate with larger amounts of sperm stored from previous matings than female *D. melanogaster* (Gromko and Markow, 1993). This supports findings of beneficial multiple mating in *D. simulans* and detrimental re-mating in *D. melanogaster*.

Lack of empirical investigation of variation in female preference and preference functions is arguably the major stumbling block to our complete understanding of

Table 1.
Summary of sexual selection in *D. simulans* and *D. melanogaster* from studies reviewed. Effects of sexual selection include measures of fitness outcomes of female choice, along with the overall effect (cost or benefit) concluded by the authors of the associated reference

Effect of Sexual Selection			
Direct effects	*D. simulans*	*D. melanogaster*	*Reference*
Female fecundity/fertility	neutral		Taylor et al. 2008a
		cost	Pitnick 1991
		cost	Pitnick & García-González 2002
		cost	Friberg & Arnqvist 2003
Female longevity	neutral/cost		Taylor et al. 2008a
		cost	Pitnick & García-González 2002
		cost	Friberg & Arnqvist 2003
Indirect effects			
Attractive sons	benefit		Taylor et al. 2007
		benefit	Rundle et al. 2007
Sperm competitiveness in sons	benefit		Hosken et al. 2008
		unlikely	Bjork et al. 2007
Offspring quality	neutral		Taylor et al. 2009
		benefit	Partridge 1980
		benefit	Taylor et al. 1987
		cost	Gibson et al. 2002
		cost	Pischedda & Chippindale 2006
Multiple mating			
Female fecundity/fertility	benefit		Taylor et al. 2008b
		neutral	Brown et al. 2004
Female longevity	neutral		Taylor et al. 2008b
		cost	Chapman et al. 1995
		cost	Wigby & Chapman 2004
		cost	Kuijper et al. 2006

sexual selection (Arnold, 1983; Lande, 1987), but this is an area that is gradually receiving empirical attention (Jennions and Petrie, 1997). The investigation of female preference and its genetic basis, as well as identifying the precise traits on which females are basing their choices should be the focus of future work with *D. simulans* and more generally.

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