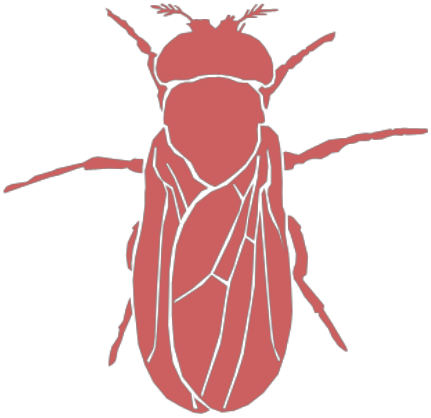
Sex, selfish genes, and the shared genome



Submitted by

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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.

Signature: ……..

Abstract

Sexual conflict can occur whenever the evolutionary interests of males and females differ, and when sexually antagonistic selection acts upon traits shared between the sexes, one or both sexes can be constrained from reaching their phenotypic optima. This intralocus sexual conflict can be characterised by a tug-of-war of allelic replacement until it is resolved, but examples of well-characterised sexually antagonistic loci are rare. This thesis investigates the basis and dynamics of intralocus sexual conflict over insecticide resistance at the *Cyp6g1* locus in *Drosophila melanogaster*, and wing colouration in *Drosophila simulans*.

In *D. melanogaster*, the *Cyp6g1* locus is the site of a series of insecticide resistance alleles, one of which is sexually antagonistic when back-crossed to the old isogenic lab strain *Canton-S*. I investigated the presence of sexual conflict over this same allele in a recently collected and genetically heterogeneous population. I found evidence of balancing selection on resistance (Ch. 2) that could not be explained by overdominance or sex-specific dominance (Ch. 3). However, balancing selection could be explained by resistance conferring increased fecundity to females (Ch. 2-4), and decreased reproductive success to males (Ch. 4). This male cost can in turn be explained by a negative genetic correlation between reproductive success and *Cyp6g1* expression (Ch. 4), possibly influencing levels of reproductive investment (Ch. 2). Additionally, I explored the dynamics of the sex-specific fitness effects of resistance across three *Cyp6g1* alleles back-crossed to a single genetic background. I found no evidence of sexual antagonism, but revealed that the cost of resistance increased with more derived alleles, and that all alleles were more costly to females (Ch. 5). After decades of strong selection imposed by insecticide use an unresolved sexual conflict persists at the *Cyp6g1* locus despite sexual dimorphism in resistance, and it does not appear that more derived *Cyp6g1* alleles are necessarily involved in mediating this conflict.

Wing interference patterns (WIPs) are a newly discovered trait subject to female mate choice in *Drosophila*. I explored the potential for intralocus sexual conflict over WIPs by measuring WIP traits from males and females from populations of *D.*

simulans evolved under relaxed or elevated sexual selection. In response to sexual selection male WIPs evolved to be brighter, higher contrast, and shifted to longer wavelengths of light, but there was no associated response to selection in females (Ch. 6). While WIPs did not appear to be constrained from detectably responding to selection by acute intralocus sexual conflict, male WIPs from the relaxed selection regime were similar to female WIPs, suggesting a cost to sexually selected WIPs that may be indicative of sexually antagonistic selection.

IASC is pervasive and can influence a wide range of fundamental evolutionary processes including sexual selection, speciation, and extinction. The research presented in this thesis adds to a body of evidence that sexual dimorphism does not necessarily resolve IASC, and documents the first evidence that WIPs do not appear to be subject to acute IASC and can evolve in response to sexual selection.

Acknowledgements

There's nothing more exciting than science. You get all the fun of sitting still, being quiet, writing down numbers, paying attention. Science has it all.

***Seymour Skinner**, Bart's Comet, 1995*

When I was a first-year undergraduate I sat down to my first ever meeting with Dave Hosken and told him that I wanted to pursue a PhD in evolutionary biology. At the time he told me he thought that was a good idea, but I don't think he expected to be quite as involved in the process as he ended up being. I hope that the experience hasn't changed his mind. I'm immensely grateful for all the guidance, support, and patience he has shown me over the 8 or so years that I've been his student. I would also like to thank my second supervisor, Nina Wedell, for being an inexhaustible font of optimism, encouragement, and scientific insight over the course of my PhD. To both of my supervisors, thank you for the trust you placed in me by giving me this opportunity.

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Author's declaration

Michael F. Hawkes (MFH) was funded by the Biotechnology and Biological Sciences Research Council, and all research presented within this thesis was conducted under the supervision of Prof. David J. Hosken (DJH) and Prof. Nina Wedell (NW). Each chapter was written by MFH in collaboration with DJH and NW, with additional comments on Chapter 6 given by Jolyon Troscianko (JT) and Jacek Radwan (JR). Additional support and contributions for each chapter are detailed below.

Chapter 2. The data was collected by MFH with the assistance of Celine Gamble (CG) and Elizabeth Turner (ET), for whom a subset of the data was the basis of undergraduate research projects, and Matthew Carey (MC). Florian Bayer provided significant guidance for the gene expression methods. An edited version of this chapter has been published in *Proceedings of the Royal Society: B* with CG, ET, MC, DJH, and NW as co-authors.

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Chapter 6. This chapter was a collaborative project between three research groups. The selection lines were maintained by Eoin Duffy and Richa Joag. Wing dissection and imaging was performed by MFH and AS using hardware apparatus constructed by JT. The *Drosophila* vision model was implemented by JT, who also provided a great deal of guidance and input with regards to issues of vision and sensory ecology.

Chapter 1

General introduction

Sexual conflict is an outcome of the divergent evolutionary interests of males and females that occur because of anisogamy (Parker 1979; Chapman et al. 2003). It is a fundamental evolutionary phenomenon that can be an engine of co-evolutionary change as well as a tether between the sexes preventing independent evolution. Anisogamy has placed males and females on two different evolutionary trajectories that, generally, have selected females to invest more in offspring and males to invest more in securing fertilisations (Bateman 1948; Trivers 1972; Parker 1979). These divergent reproductive interests have seen natural and sexual selection shape the sexes in ways that have allowed them to achieve vastly different life-histories (Archer et al. 2012), morphology (Harano et al. 2010), and behaviour (Maklakov et al. 2008), despite the constraint of a shared genome. Sexual conflict arises in two arenas – between different loci as the result of antagonistic male-female interactions (interlocus sexual conflict) (Chapman et al. 2003; Arnqvist & Rowe 2005), and over the same loci subject to sexually antagonistic selection (intralocus sexual conflict) (Rice & Chippindale 2001; Bonduriansky & Chenoweth 2009). Interlocus sexual conflict has been widely studied, and can result in antagonistic coevolution and the development of arms races between the sexes (Chapman et al. 2003; Arnqvist & Rowe 2005). It is often characterised by males employing coercive or harmful reproductive strategies and females evolving defensive morphology or behaviour to resist male coercion or reduce male-inflicted harm (Chapman et al. 2003; Arnqvist & Rowe 2005). Intralocus sexual conflict, on the other hand, can constrain independent evolution between the sexes until sex-specific genetic architecture evolves, and has received relatively less empirical investigation (Rice & Chippindale 2001; Bonduriansky & Chenoweth 2009).

Intralocus sexual conflict (IASC) occurs when sexually antagonistic (SA) selection acts on a trait that shares the same underlying genetic architecture in both sexes (Chapman et al. 2003; Bonduriansky & Chenoweth 2009). A positive intersexual

genetic correlation (r_{MF}) for traits subject to SA selection then constrains one or both sexes from reaching their sex-specific fitness optima (Bonduriansky & Chenoweth 2009), resulting in a negative intersexual correlation for fitness such that males of a high trait fitness should sire females with a low trait fitness (and vice versa) (Rice & Chippindale 2001). Alleles subject to IASC can be maintained at stable equilibrium frequencies (Kidwell et al. 1977; Turelli 2004; Rostant et al. 2015), but can only reach fixation if there is a net selective advantage when averaged across all offspring (Rice 1984). IASC has many implications for a wide range of evolutionary processes; it can diminish the benefits of sexual selection (Fedorka & Mousseau 2004; Pischedda & Chippindale 2006; Brommer et al. 2007), maintain additive genetic variation across the genome and partially explain the paradox of the lek (Foerster et al. 2007; Prasad et al. 2007; Dean et al. 2012; Rostant et al. 2015), may increase the risk of population extinction (Kokko & Brooks 2003), reinforce speciation (Parker & Partridge 1998), and can facilitate adaptation across fitness valleys (Bonduriansky & Chenoweth 2009).

Bonduriansky & Chenoweth (2009) describe four stages of IASC that progress through (i) pre-IASC, where selection is sexually concordant and r_{MF} for fitness and trait are positive; (ii) acute IASC, were selection is sexually antagonistic and r_{MF} for fitness is negative but r_{MF} for trait is positive; (iii) attenuated IASC, where selection is sexually antagonistic and r_{MF} for fitness and trait are still negative and positive, respectively, but declining in magnitude; and (iv) resolved IASC, where selection is sexually antagonistic and r_{MF} for fitness and trait are both non-negative (fitness at parity and trait values less than or equal to 1). The resolution of IASC requires the genetic architecture underlying traits subject to SA selection to evolve to allow sex-linked gene expression and sexual dimorphism (but see Harano et al. 2010). This can occur via several process including sex-specific posttranscriptional splicing (McIntyrse et al. 2006), sexually antagonistic loci undergoing gene duplication allowing alternative sex-biased expression for the different gene copies (Partridge & Hurst 1998; Rice & Chippindale 2001; Bonduriansky & Chenoweth 2009), and modifier genes evolving elsewhere in the genome that result in sex-biased expression or altered fitness effects of the sexually antagonistic loci in one or both sexes (Rice & Chippindale 2001). While resolution of IASC is possible, networks of

genetic correlation between traits across the genome are likely to maintain some low level of conflict indefinitely (Bonduriansky & Chenoweth 2009; Harano et al. 2010; Hosken 2011).

IASC has been detected across a range of species (Bedhomme & Chippindale 2007) but significant estimates of SA selection in wild populations are rare (Cox & Calsbeek 2009), and in general IASC is expected to be difficult to detect (Bedhomme & Chippindale 2007). Even rarer still are examples of IASC where the precise loci subject to conflict are known, though transcriptomic approaches have begun to propose many candidate genes (Innocenti & Morrow 2010; Griffin et al. 2013) – particularly in the fruit fly *Drosophila melanogaster* where IASC appears pervasive (Innocenti & Morrow 2010; Griffin et al. 2013; Ingleby et al. 2014). One recently characterised example is that of insecticide resistance in *D. melanogaster*, where upregulation of the cytochrome P450 gene *Cyp6g1* appears to be deleterious for males but beneficial for females in certain genetic backgrounds (McCart et al. 2005; Smith et al. 2011; Rostant et al. 2015). Research into insecticide resistance has had great success in elucidating the molecular mechanisms and pleiotropic fitness costs of various forms of resistance, but has until relatively recently largely ignored how sex might complicate these estimates and overlooked the potential for IASC at resistance loci (Coustau et al. 2000; ffrench-Constant & Bass 2017). Insecticide resistance therefore offers unexplored opportunities to investigate IASC in traits where a specific trait can be linked to specific loci (and thus gene expression), and may have sexually antagonistic fitness effects.

Insecticide resistance is a major problem in the management of agricultural pests and disease vectors (Hemingway et al. 2013; Gontijo et al. 2013), and has important social, economic, and human health implications (Grafius 1997; Hemingway & Ranson 2000; Greenwood & Mutabingwa 2002). Resistance typically evolves rapidly under strong directional selection (May 1985; Mallet 1989), and has become prevalent in both pest and non-pest insect populations worldwide as a result of the widespread introduction of organic and synthetic insecticides since the 1950s (Georghiou 1986; Catania et al. 2004). Not only is resistance present in a diverse

range of insect taxa (Mallet 1989), but individual species can evolve both multiple-resistance and cross-resistance to many different types of xenobiotics (Alyokhin et al. 2008). In order to develop dynamic management programmes that remain flexible in response to the constantly evolving resistance profiles of wild insect populations (e.g. Dermauw et al. 2013) we must understand both the direct and indirect fitness effects of resistance. Specifically, understanding the pleiotropic fitness effects of resistance is key to understanding the spread and maintenance of resistance, and critically, can allow us to work towards reducing its prevalence (Brown et al. 2013; Tabashnik et al. 2013).

Theory predicts that resistance should impose costs such that susceptible individuals are more fit than resistant individuals in the absence of insecticide (Crow 1957; Coustau et al. 2000; Hall et al. 2004). This is expected to be the case because wild populations are assumed to be near local fitness optima and any mutant resistance alleles would therefore disrupt a locally adapted and functionally integrated genome (Coustau et al. 2000). As the strong directional selection imposed by insecticides usually acts outside of the standing distribution of susceptible phenotypes, it initially favours single mutations of large effect which are more likely to fall further from any nearby local fitness optima than the wild-type allele (Fisher 1930; Orr 1998; Mccandlish 2013). In functional terms, the expression and maintenance of resistance may be metabolically expensive and trade-off against other fitness components (Carriere et al. 1994), or may reduce the efficiency of some physiological processes by altering their function (Coustau et al. 2000). Such costs associated with resistance could delay or prevent the fixation of resistance alleles, and reduce the frequency of resistance alleles in the absence of insecticide e.g. in resistance management programmes that employ refuges free from insecticide (Brown et al. 2013; Tabashnik et al. 2013).

The empirical evidence for the pleiotropic fitness effects of resistance is mixed, with some studies demonstrating costs (Carrière et al. 2001; Berticat & Boquien 2002; Smith et al. 2011), some demonstrating no costs (Bloch & Wool 1994; Tang et al. 1999; Castañeda et al. 2011), some demonstrating fitness benefits to resistance

(Haubruge & Arnaud 2001; Arnaud & Haubruge 2002; McCart et al. 2005; Mason 2009), and even simultaneous costs in some fitness measures but benefits in others (Brewer & Trumble 1991). There are a range of reasons why studies may fail to detect costs, or why we may not expect to see costs in the first place (Box 1). *Cyp6g1* is just one striking example of variable costs where resistance has been found to incur antagonistic fitness effects between the sexes (Drnevich et al. 2004; McCart et al. 2005; Smith et al. 2011; Rostant et al. 2015).

Upregulation of the cytochrome P450 gene *Cyp6g1* in *D. melanogaster* confers resistance to a range of insecticides including neonicotinoids and the organochloride DDT (dichlorodiphenyltrichloroethane) (Daborn et al. 2002; Daborn et al. 2007). Cytochrome P450 monooxygenases are a group of enzymes that confer insecticide resistance by metabolising xenobiotics through oxidation (Bergé et al. 1998). Upregulation of *Cyp6g1* in *D. melanogaster* is achieved by the presence of cis-regulatory sequences in the long terminal repeat (LTR) of a defective *Accord* transposable element (TE) inserted 291bp upstream of *Cyp6g1* (Daborn et al. 2002; Chung et al. 2007). While *Cyp6g1*-conferred resistance in strains isolated from the wild is monogenic, resistant individuals in some strains experimentally selected for resistance also exhibit reduced cuticular penetration and increased excretion of DDT (Strychartz et al. 2013). The *Cyp6g1*-associated *Accord* insertion is near or at fixation in most populations outside of Eastern Africa, with low levels of microsatellite variation flanking *Cyp6g1* consistent with a recent selective sweep (Catania et al. 2004). The *Accord* insertion has recently been found to be a feature of an allelic series at the *Cyp6g1* locus containing copy number variation and additional nested TE insertions (Schmidt et al. 2010). TE-induced insecticide resistance via novel cis-regulatory sequences, as with *Cyp6g1*, is just one mechanism by which TE insertions can generate resistance. Additionally, TEs may (i) insert into the 3' ends of genes, increasing the stability of mRNA transcripts, (ii) excise a gene during transposition and reinsert it beyond the reach of repressor elements, or (iii) insert into an exon and alter the sequence of resulting transcripts, potentially resulting in novel function (ffrench-Constant et al. 2006). TEs are now recognised as important facilitators of insecticide resistance evolution (Li et al. 2007) with particular

enrichment near xenobiotic-metabolising cytochrome P450 genes (Chen & Li 2007), and specific insertion site preferences (Linheiro & Bergman 2012).

Insecticide resistance at the *Cyp6g1* locus features at least 5 alleles conferring various levels of resistance (Schmidt et al. 2010; Figure 1). The alleles succeed as follows, with each a derivation of the preceding allele; (i) *M*, a susceptible allele with a single copy of *Cyp6g1*, (ii) *AA*, a duplication of *Cyp6g1* into *Cyp6g1-a* and *Cyp6g1-b* following the insertion of an *Accord* TE upstream of *Cyp6g1*, (iii) *BA*, the insertion of an *HMS Beagle* TE within the *Accord* TE upstream of *Cyp6g1-a*, (iv) *BP*, the insertion of a *P*-element TE within the *Accord* TE upstream of *Cyp6g1-b*, and (v) *BP*Δ, a class of alleles with their *P*-elements in various stages of degeneration. Alleles across the series confer increasing levels of total resistance as well as increasing sexual dimorphism in resistance in the more derived alleles (Schmidt et al. 2010). Notably, the level of resistance conferred by *AA* and *BA* is similar, with only the resistance levels of males being significantly different, suggesting cost amelioration via replacement as well as increased resistance may explain evolution through the series (Box 1). Though there is evidence for a recent selective sweep at the *Cyp6g1* locus, there is some evidence that the *Accord* insertion itself may be an old mutation pre-dating the migration of *D. melanogaster* out of Africa (Catania et al. 2004), and previous work has shown that the *BA* allele can be maintained at a low frequency in the absence of insecticide (Rostant et al. 2015). If the *Accord* insertion is indeed an old mutation this has important implications for the expectation of any fitness costs associated with alleles in the series that are of a similar age due to the possibility of cost amelioration having evolved (Box 1).

The pleiotropic fitness effects of the *Cyp6g1* alleles are largely unknown, with previous work only investigating the *BA* allele. When *BA* was introgressed into the old laboratory strain *Canton-S*, which is naturally susceptible to insecticides (homozygous for the *M* allele), resistant males exhibited altered courtship and aggression behaviours, and suffered a fitness cost relative to susceptible males in the form of reduced mating success (Smith et al. 2011). However, Smith et al. (2011) did not find male fitness costs in a second, more recently established genetic

background, suggesting that epistasis has a role in mediating the costs of resistance in this system. In contrast, resistance in females increases fecundity, egg and larval viability of offspring, and decreases larval and pupal development time (McCart et al. 2005). These maternal effects on the offspring are putatively thought to be the result of increased provisioning of *Cyp6g1* mRNA to fertilised eggs (McCart & ffrench-Constant 2008). The mechanism of the male fitness costs is currently unknown. The *BA* allele is thus an exceedingly rare case of IASC where the exact locus under sexually antagonistic selection is known. The presence of this sexual conflict - and the elucidation of its action across the allelic series - represents an important step in understanding the evolution of resistance, as the degree to which the costs of resistance differ between the sexes has been largely overlooked, with many studies preferring to focus on a single sex (e.g. female fecundity) or not explicitly separating the sexes at all (e.g. development time).

The allelic series at the *Cyp6g1* locus in *D. melanogaster* is an important system in which to explore the progression through an adaptive walk of insecticide resistance alleles (both in terms of adapting to novel xenobiotic challenges as well as ameliorating the costs borne as a result of that adaptation) and the dynamics of IASC. It allows us to ask questions about insecticide resistance where some alleles may predate modern insecticides, potentially challenging our expectations of costs associated with resistance assumed to be *de novo* mutations selected by insecticide use. It allows us to explore the relative importance of increased resistance versus cost amelioration by comparing the fitness costs and levels of resistance between alleles that differ in resistance, and between those that do not differ in resistance but may differ in cost. It allows us to elucidate the progression of IASC through a series of allelic replacement from alleles that do not result in sexual dimorphism for resistance to more derived alleles that do, suggesting that more derived alleles may be allowing sex-limited gene expression - perhaps resolving any IASC via cis-regulatory sequences that are sex-limited in their activity (e.g. Abrahamsen et al. 1993). Within the system, many questions also remain to explore the mechanistic basis of the pleiotropic fitness effects across the series.

While the majority of this thesis explores IASC at the *Cyp6g1* insecticide resistance locus, we have also investigated the potential for IASC in a newly discovered sexual trait in *Drosophila* - wing colouration. The wings of most common *Drosophila* species are seemingly transparent in lab environments and this has led to wing colouration being largely ignored in studies of sexual selection and conflict except for those species that have conspicuous marked wings (e.g. the melanin spotted wings of *Drosophia suzukii*). However, it was recently discovered that *Drosophila* wings actually display striking structural colour patterns when viewed against backgrounds that contrast with the wing (most natural environments) that can be both species and sex-specific (Shevtsova et al. 2011). These patterns, named wing interference patterns (WIPs), are produced via thin-film interference where light striking the wing is refracted and reflected such that the wavelength of the reflected light (i.e. the colour) depends on the thickness of the chitinous membrane of the wing (Shevtsova et al. 2011). This process, along with some other structural features like venation and hair placement, results in vivid colour patterns across the wing. WIPs appear to be heritable and subject to sexual selection via female mate choice in *D. melanogaster*, and may be involved in species recognition (Shevtsova & Hansson 2011), but generally we know very little about the evolutionary forces that shape them. Sexually selected traits are classic cases of traits with a history of IASC (Chapman et al. 2003; Arnqvist & Rowe 2005; Bonduriansky & Chenoweth 2009), and good candidates for traits that may retain some level of conflict (e.g. Harano et al. 2010). For example, sexual selection on male wing thickness as a result of female mate choice for WIPs may be orthogonal to natural selection on wing morphology for other purposes (e.g. flight) in females. Assuming these aspects of wing morphology are governed by the same loci, this sexually antagonistic selection could constrain the response of male WIPs to sexual selection. As human hips must be optimised for locomotion in males but for locomotion and childbirth in females, so too may *Drosophila* WIPs need to be optimised for flight in females but flight and mate choice in males. Given how recently WIPs were discovered, we know next to nothing about their potential role in sexual conflict.

The research presented in this thesis is an investigation into the presence, dynamics, and consequences of intralocus sexual conflict over insecticide resistance

in *Drosophila melanogaster*, and wing colouration in *Drosophila simulans*. The first four data chapters describe work exploring sex-specific fitness, life-history, insecticide resistance, and gene-expression associated with an allelic series at the *Cyp6g1* locus in *D. melanogaster*. The final data chapter is an initial investigation into the potential for sexual conflict over wing interference patterns, a recently discovered sexually selected trait in *D. simulans*. The chapters are presented as intended for publication.

In Chapter 2 we investigate whether the *Cyp6g1-BA* allele is sexually antagonistic in a recently caught and genetically heterogenous population of *D. melanogaster* by estimating fitness components in males and females. Additionally, we estimate the degree of sexual dimorphism in DDT resistance and *Cyp6g1* expression in order to explain our fitness component measures within a functional biological framework. We also use these fitness component measures to derive predictions of allele frequency dynamics from a population genetic mathematical model which we test using experimental evolution. A version of this chapter with some content removed to supplementary material was published in the *Proceedings of the Royal Society: B* (co-authors as stated in the Author's Declarations). In Chapter 3 we explore alternative explanations that may explain balancing selection at the *Cyp6g1* locus including overdominance, sex-specific patterns of dominance for the fitness effects of resistance, and indirect genetic effects from the parental origin of the resistance allele. In Chapter 4 we use isofemale lines to estimate quantitative genetic parameters that describe the intersexual genetic correlation for the fitness effects of the *BA* allele, and the genetic correlation between *Cyp6g1* expression and male fitness components. In Chapter 5 we characterise the dynamics of the sex-specific fitness effects across three *Cyp6g1* alleles back-crossed to a single genetic background to reveal the procession of any sexual conflict across the allelic series. In Chapter 6 we explore the potential for IASC in the wing interference patterns of *D. simulans* by testing whether the wing patterns of males and females are able to respond independently to sexual selection on males.

Box 1

There are three main reasons why studies may fail to detect pleiotropic fitness costs to resistance even if they should exist; (i) experimental design, (ii) cost variability, and (iii) cost amelioration.

(i) Experimental design

The two main approaches to quantifying the fitness effects of resistance are to compare specific fitness components between resistant and susceptible individuals, and to compete resistant and susceptible alleles against one another in populations. A trait-based approach allows us to conclude whether resistance affects those measured characters, but critically, not whether it affects any unmeasured traits. This becomes more problematic when we consider that the fitness effects of resistance can be positive for some fitness measures but negative for others (Brewer & Trumble 1991), meaning that trait-based approaches may result in errors of both degree and sign. Resistance, then, can carry a cost that trait-based approaches may overlook. In comparison, a population approach integrates the fitness effects of resistance across the whole phenotype in both sexes and is a more holistic measure of the composite selection acting on resistance alleles. It cannot, however, demonstrate the differences in phenotype between resistant and susceptible individuals that selection is acting on, nor tell whether these effects are sex-specific. To illustrate the disparity between these two approaches, a recent review of literature regarding the costs of resistance to *Bacillus thurigiensis* (Bt) toxins found costs were detected in 63% of population-based experiments but only 34% of trait-based experiments (Gassmann et al. 2009). Coustau et al. (2000) highlight a valuable distinction between the costs that resistance may impose in any particular trait or fitness measure, and the counterselection that resistance alleles may be under if they result in a net reduction

in fitness in the absence of insecticide. Ultimately, the two approaches are complementary and when used in combination can demonstrate the net fitness effect of resistance alleles as well as the specific phenotypic costs they impose.

(ii) Cost variability

The expectation of costs associated with resistance can vary with the mechanism of resistance (Coustau et al. 2000) as well as the degree of resistance (Gassmann et al. 2009), and epistatic interaction with standing genetic variation may also result in the fitness effects of novel mutations varying in degree and sign depending on the genetic background they appear in (Weinreich et al. 2005). Additionally, costs may vary due to gene-environment interactions whereby costs only become apparent under certain, often stressful, conditions (Martin & Lenormand 2006). This may be due to factors such as host plant type (which includes both nutritional quality and plant defence) (Carrière et al. 2004; Raymond et al. 2005; Raymond et al. 2011), density (Raymond et al. 2005), parasitism (Agnew et al. 2004), or *Wolbachia* infection (Duron et al. 2006). In general, costs can be condition dependent (Janmaat & Myers 2005) and studies that rear experimental animals in ideal conditions may allow for the compensation of these costs (Shikano & Cory 2014).

(iii) Cost amelioration

Fitness costs imposed by resistance are not immutable and can be ameliorated via subsequent adaptation. This can either be through the replacement of resistance alleles with new alleles that confer resistance at a lower cost (e.g. Chevillon et al. 1997), or the evolution of genetic modifiers elsewhere in the genome that can compensate for the costs of any given resistance allele (e.g. McKenzie & Purvis 1984) (Coustau et al. 2000). The potential for cost amelioration means that we may not expect to see costs for old resistance alleles that have had time to coevolve with a particular genetic background. This has far-reaching implications depending on whether resistance is usually conferred by *de novo* mutation or the selection of alleles already segregating at low frequencies.

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Tables and Figures

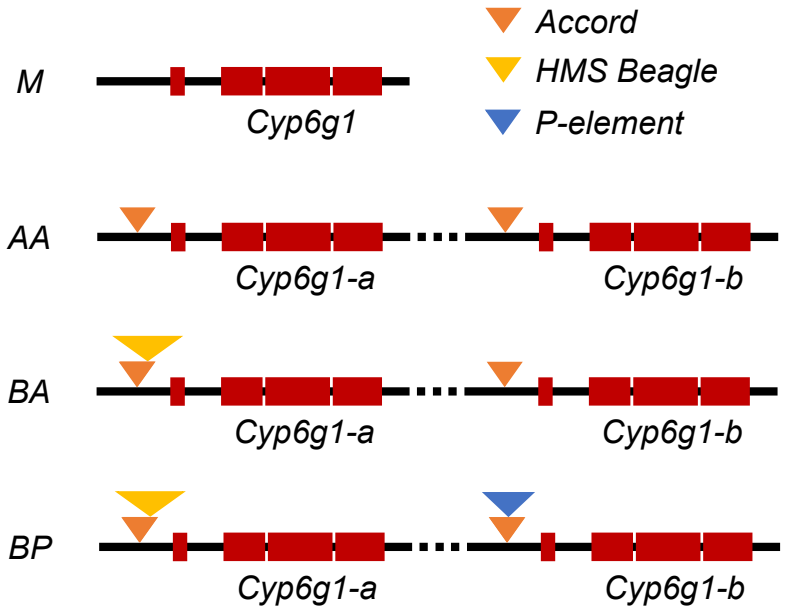

Figure 1 The four primary *Cyp6g1* alleles (adapted from Schmidt et al. 2010). *M* has a single copy of *Cyp6g1*. *AA* has a tandem duplication of the entire gene with *Accord LTR* transposable elements (TEs) inserted upstream of both copies of *Cyp6g1*. *BA* has an *HMS Beagle* TE inserted within the *Accord* upstream of *Cyp6g1-a*. *BP* has a *P*-element inserted within the *Accord* upstream of *Cyp6g1-b*.

Chapter 2
Intralocus sexual conflict and insecticide resistance

Abstract

The *BA* allele of the *Drosophila* cytochrome P450 gene *Cyp6g1* confers resistance to a range of insecticides. It is also subject to intralocus sexual conflict (IASC) when introgressed into the *Canton-S* background, whose collection predates the widespread use of insecticides. In this genetic background the allele confers a pleiotropic fitness benefit to females but a cost to males, and exhibits little sexual dimorphism in conferred insecticide resistance. It is unclear whether these sexually antagonistic effects also exist in current populations that have naturally evolved with insecticides, where genetic modifiers that offset male costs might be expected to evolve. Here, we explore these issues using *D. melanogaster* caught recently from an Australian population in which the *BA* allele naturally segregates. While we find increased fecundity in insecticide-resistant *BA* females and no consistent evidence of fitness costs in males, experimental evolution indicates balancing selection at the locus. We suggest that this apparent discrepancy may be due to reduced investment in reproduction in resistant males. Our results at the population level are consistent with previous work, and suggest that individual-level fitness assays do not always capture sexually antagonistic fitness effects that emerge in a population context.

Introduction

Explaining how genetic variation can be maintained in the face of selection is a major goal of evolutionary biology. One mechanism that can maintain variation is balancing selection through intralocus sexual conflict (IASC). IASC occurs when one or both sexes are constrained from reaching sex-specific phenotypic optima by a shared genetic architecture underlying traits subject to sexually antagonistic selection (Rice & Chippindale 2001; Chapman et al. 2003; Bonduriansky & Chenoweth 2009). When such a constraint occurs, any improvement in the fitness of one sex will be counterbalanced by a fitness reduction in the other (Bonduriansky & Chenoweth 2009). The result is a 'tug-of-war' (Harano et al. 2010) of allelic replacement between males and females driven by sexually antagonistic selection at the same loci, dragging the two sexes' trait values to and fro between their sex-specific phenotypic optima (Rice & Chippindale 2001). Thus, in order to reach fixation, any sexually antagonistic allele must provide a net selective advantage when averaged across both sexes (Rice 1984). When this is not the case, sexually antagonistic alleles can be maintained at stable equilibrium frequencies by balancing selection (Kidwell et al. 1977; Dean et al. 2012; Rostant et al. 2015). Cases in which precise loci currently or previously subject to IASC have been identified are exceedingly rare. One such case is insecticide resistance at the *Cyp6g1* locus in the fruit fly *Drosophila melanogaster* (McCart et al. 2005; Smith et al. 2011; Rostant et al. 2015).

Upregulation of the cytochrome P450 gene *Cyp6g1* in *D. melanogaster* can confer resistance to a broad range of insecticides including neonicotinoids and the organochloride dichlorodiphenyltrichloroethane (DDT) (Bergé et al. 1998; Le Goff et al. 2003; Daborn et al. 2007; Jones et al. 2010). The *Cyp6g1* locus in *D. melanogaster* is the site of a series of at least 4 DDT resistance alleles (and an ancestral susceptible allele) featuring a tandem duplication of the gene and several transposable element (TE) insertions, where progressively higher levels of resistance are conferred across the evolutionary series (Schmidt et al. 2010). At least some of these TEs contain novel cis-regulatory sequences responsible for upregulating *Cyp6g1* expression, namely the long terminal repeat of a degenerate *Accord* TE inserted 291bp

upstream of *Cyp6g1* (Daborn et al. 2002; Chung et al. 2007). The *Cyp6g1*-associated *Accord* insertion is near or at fixation in most populations outside of Eastern Africa, with low levels of microsatellite variation flanking the gene consistent with a recent selective sweep – most likely due to the onset of DDT use in the mid-20th century (Catania et al. 2004).

Recent work has shown that one of the alleles in the series, the *BA* allele, is subject to sexually antagonistic selection when introgressed into the *Canton-S* strain (relative to the susceptible *M* allele) in the absence of insecticide. *Canton-S* is a naturally DDT-susceptible stock (carrying the *M* allele) collected in the early 20th century before the widespread use of DDT began, and is thus naive to selection imposed by DDT. Resistant $BA^{Canton\text{-}S}$ females are more fecund, have higher egg and larval viability, and shorter egg and larval development times than susceptible $M^{Canton\text{-}S}$ females (McCart et al. 2005). In contrast, resistant $BA^{Canton\text{-}S}$ males have lower mating success when in direct competition with susceptible $M^{Canton\text{-}S}$ males (Smith et al. 2011), and insecticide resistance exhibits low levels of sexual dimorphism in *Canton-S* consistent with these sexually antagonistic fitness effects. This sexual conflict is able to explain frequencies of the *BA* allele during its recent evolutionary history, including the maintenance of variation at the locus (Rostant et al. 2015). Thus, the *BA* allele is a rare case of IASC where the precise locus of conflict is known and the allele is segregating in wild populations.

It remains to be seen whether conflict at the *Cyp6g1* locus occurs in contemporary populations of *D. melanogaster* (i.e. those that carry the *BA* allele and have evolved with selection imposed by widespread insecticide use), as theory predicts genetic modifiers should emerge that offset any costs of resistance by allowing sex-specific trait expression (Kulathinal et al. 2004; Labbé et al. 2009; Assogba et al. 2015). However, the strong selection imposed by insecticides may mask sexual antagonism and weaken selection for these modifiers. Indeed, Smith et al. [10] found that the *BA* allele did not carry a cost to males when introgressed into a recently-caught isogenic line, demonstrating that the pleiotropic effects of the *BA* allele can be mediated by epistasis. Additionally, as previous work has investigated the *BA* allele in

isogenic backgrounds we do not know how generalizable this sexual antagonism is. It may be the case that the particular genetic variation fixed in the *Canton-S* strain generates sexual antagonism that would not be seen in more genetically heterogeneous populations.

Here, using homozygous resistant and susceptible populations of *D. melanogaster* produced from multiple isofemale lines caught recently from the wild, we assay fitness in both sexes and show that the previously detected female fitness benefit of the *BA* allele exists in a contemporary population, but the effect on males is less clear. Additionally, because IASC may be resolved by sex-limited gene expression, we quantify sex-specific relative *Cyp6g1* expression as well as resistance to DDT and find significant sexual dimorphism in both alleles. Nevertheless, when estimating the dynamics of the *BA* and *M* alleles in populations allowed to evolve experimentally, we find evidence of balancing selection that is not predicted by a mathematical model of IASC parameterized with fitness estimates we obtain in more simplistic settings. We suggest that this is due to emergent effects at the population level not being reflected by standard fitness assays, and present a hypothesis for a possible cause of this apparent discrepancy.

Materials and methods

Isofemale lines and populations

Genetically heterogeneous populations homozygous for either the susceptible *M* allele or resistant *BA* allele were created from *D. melanogaster* isofemale lines (hereafter isolines, David et al. 2005) (n=15). These isolines were collected from Margaret River, Australia, in 2013 and were polymorphic at the *Cyp6g1* locus with both the susceptible *M* and resistant *BA* allele segregating within them (diagnostic PCR as per Schmidt et al. 2010). Virgin adults were crossed within each isoline and genotyped, with offspring from homozygote crosses retained to produce a homozygous *M* and homozygous *BA* version of each isoline. 20 males and females from each resistant and susceptible isoline were then used to found homozygous *BA* and *M* populations, respectively. Isolines capture and maintain snapshots of the genetic variation and covariation segregating within a source population, and this procedure

maximises the genetic variation within our populations while minimising the genetic variation between them. While it is unlikely for *Cyp6g1* to be the only locus to differ between the populations, this procedure affords a greater level of control over the genetic background than would using different isolines to establish each population. However, we cannot definitively rule out any effects we report being due to the contribution of segregating alleles in linkage with either *Cyp6g1* allele. As the starting N_e was equal between the populations we do not expect differential inbreeding to influence the results presented here.

Populations were maintained in 30 x 30 x 30 cm cages (Bioquip, Knutsford, UK) at densities of approximately 1000 individuals with overlapping generations. All flies were maintained on a cornmeal based medium (standard Jazz mix, Applied Scientific) and housed in incubators at 25°C on a 12:12h light:dark cycle unless otherwise specified.

Experimental animals

Focal experimental animals were collected from the homozygous *BA* or *M* populations described above as first instar larvae, and were all 3-5 day old virgin adults during experimentation. Where specified, *sepia* competitors and mates were collected from a population of *D. melanogaster* homozygous for the recessive *sepia* mutation (obtained from the Bloomington Drosophila Stock Center).

Flies were not exposed to CO_2 within 24 hours of any behavioural assays as recent exposure to CO_2 is known to alter mating behaviour (Barron 2000). Wing size was measured as a proxy for body size using ImageJ (Schneider et al. 2012). In all assays females were housed in experimental vials for 24 hours before experimentation to allow them to habituate.

Male fitness assays

Pre-copulatory competitive ability

To test for effects of the *BA* allele on pre-copulatory male fitness we estimated the mating success of *BA* and *M* males in direct competition. One resistant and one susceptible male were simultaneously introduced into a vial

containing one *sepia* female. Males were marked with pink or blue powder paint to allow identification, with half of the males of each genotype marked with each colour (De Crespigny & Wedell 2007; Smith et al. 2011). Once either male successfully initiated copulation the unsuccessful male was removed from the vial. We recorded the latency to mate, copulation duration, and genotype of the successful male. Assaying mating success in a competitive trial integrates both success in male-male competition and female mate preference (Dougherty & Shuker 2014). Females were subsequently left to oviposit for 5 days. 17 days after the start of oviposition we counted the number of adult offspring that had eclosed. Vials in which neither male secured a mating were discarded (47% of trials, final n=160 in two experimental blocks).

Post-copulatory competitive ability

To test for effects of the *BA* allele on post-copulatory male fitness we estimated the fertilisation success of *BA* and *M* males in competition with a standardised *sepia* competitor. Sperm competition (Parker 1970; Hodgson & Hosken 2006) assays were performed for both sperm defence (P1) and sperm offence (P2). In the P1 assay, resistant and susceptible males were mated to *sepia* females as per the pre-copulatory competition assay with the exception that only one male was included in each trial (total n=70). Mated *sepia* females were then given the opportunity to remate with a virgin *sepia* male for 6 hours every 24 hours until remating occurred. Once remated, females were left to oviposit for 5 days as per the pre-copulatory competition assay. We assigned offspring to their sire based on phenotype (*sepia* or wild-type). We recorded copulation duration for both the initial mating and remating, as well as relative male size and the number of offspring produced before remating. The P2 assay was conducted in the same manner other than the order of *sepia* and focal males was reversed (total n=66).

Female fitness assays

Offspring production

To test for effects of the *BA* allele on female fitness we estimated the fecundity of both *BA* and *M* females. Half of each female genotype was mated

to resistant males and the other half to susceptible males in a fully factorial design to examine any effect of male genotype on offspring production. Females were allowed to oviposit for 5 days following a single mating, with offspring counted 17 days after the start of oviposition in each vial. This measure integrates egg viability, larval viability, and egg production. Females that produced no offspring were removed from analyses (n=25) as it was not possible to determine the source of the mating failure (Greenway et al. 2015), leaving a total sample size of n=147 in two experimental blocks.

Egg production and offspring viability

In order to determine whether differences in offspring production could be attributed directly to maternal fitness rather than indirectly through offspring fitness we estimated egg production, egg-larvae viability, and larvae-adult viability. Resistant and susceptible females were housed with either resistant or susceptible males and allowed to oviposit freely for 18 hours (before eggs would begin to hatch; Markow et al. 2009). Eggs were then counted, and a maximum of 10 eggs per female were haphazardly selected and transferred to fresh media at a density of 5 eggs per vial (2 females did not lay any eggs and were excluded from analyses). 24 hours later the number of unhatched eggs were counted, and 17 days after the start of oviposition the number of successfully eclosed offspring were counted. Females that laid fewer than 5 eggs were excluded from egg to adult viability calculations (3% of females, final n=95).

Mathematical model and experimental evolution

To estimate the evolutionary dynamics of the *BA* and *M* alleles in a more realistic context, replicate populations were founded with the *BA* allele in competition with the *M* allele at either a low initial frequency (0.1, n=3) or a high initial frequency (0.9, n=3) and allowed to evolve for 7 non-overlapping generations. Populations were founded with 200 flies at an equal sex ratio within each genotype. Each generation, populations were given access to food for 72 hours to allow oviposition of the next generation. 96 individuals were haphazardly selected and genotyped to score allele frequencies in each generation (diagnostic PCR as per Schmidt et al. 2010). The next generation

was then founded by all offspring collected across 5 days from the date offspring began eclosing. To generate predictions at the population level from our individual-level fitness estimates, a single-locus non-linear recursion model of IASC presented in Rostant et al. (2015) was parameterized with data from the male and female fitness assays and used to predict allele frequencies which were then compared with the empirical allele frequency data. The model of Rostant et al. (2015) is a single-locus, two allele, nonlinear recursive model that calculates allele frequencies in each subsequent generation by taking the proportions of males and females of each genotype in the current generation, and then calculating the frequencies of each mating combination (RR x RR, RR x RS/SR, RS/SR x SS, SS x SS, where R is the resistant allele, and S is the susceptible allele). Here follows a brief summary of the model, but full details are available in Rostant et al. (2015).

In each generation the proportion of sires of each genotype, *y*, is given by equations 1-3, where *x* is the frequency of the genotype, and *m* is the relative mating success of resistant males.

$$y_{RR} = \frac{mx_{RR}}{m\,(x_{RR} + x_{RS}) + x_{ss}} \quad (1)$$

$$y_{RS} = \frac{mx_{RS}}{m\,(x_{RR} + x_{RS}) + x_{ss}} \quad (2)$$

$$y_{SS} = 1 - y_{RR} + y_{RS} \quad (3)$$

The relative mating frequencies of each genotype combination, *λ*, can then be calculated from the genotype frequencies, *x*, and the proportion of sires of each genotype, *y*, where *i* and *j* represent the male and female genotypes (equation 4).

$$\lambda_{ij} = x_i y_j \quad (4)$$

The relative numbers of each genotype in the next generation, *n*, can then be calculated using equations 5-7, where *F* is the relative fecundity of resistant females including offspring viability effects, and *P* is the relative pupal viability of resistant offspring.

$$n_{RR} = FP\lambda_{RRRR} + {}^{1}/_{2}\,\lambda_{RRRS} + {}^{1}/_{2}\,\lambda_{RSRR} + {}^{1}/_{4}\,\lambda_{RSRS} \quad (5)$$

$$n_{RS} = FP\lambda_{RRSS} + {}^{1}/_{2}\,\lambda_{RRRS} + {}^{1}/_{2}\,\lambda_{RSRR} + {}^{1}/_{2}\,\lambda_{RSRS} + {}^{1}/_{2}\,\lambda_{RSSS} + P\lambda_{SSRR} + {}^{1}/_{2}\,\lambda_{SSRS} \quad (6)$$

$$n_{SS} = F\,{}^{1}/_{4}\,\lambda_{RSRS} + {}^{1}/_{2}\,\lambda_{RSSS} + {}^{1}/_{2}\,\lambda_{SSRS} + \lambda_{SSSS} \quad (7)$$

Genotype frequencies for the next generation can then be calculated using equation 8 where *k* is genotype.

$$x'_k = \frac{n_k}{\Sigma n} \quad (8)$$

DDT resistance

To test for the presence of sexual dimorphism in insecticide resistance we estimated the sex-specific LD_{50} (the concentration at which, on average, 50% of individuals die) of DDT for both alleles. The inside of glass vials was laced with DDT by pipetting 500µl of acetone-DDT solution into the vial and rolling the vial until all acetone had evaporated (vials were left for a further 24 hours to remove any residual acetone vapours). Adult flies were introduced to the DDT-laced vials (10 per vial) and mortality was scored after 24 hours. Each genotype and sex combination was exposed to a range of 12 concentrations of DDT (each replicated 3 times) that spanned the LD_{50} value, the extremes of which were as close to zero and total mortality as practical, and a pure acetone control.

***Cyp6g1* expression**

To test for the presence of sexual dimorphism in *Cyp6g1* expression we estimated sex-specific relative *Cyp6g1* transcript abundance for both alleles using qRT-PCR. RNA was extracted from pooled samples of 10 whole adult

individuals for both genotypes of both sexes homogenized in RNAlater (Fisher) using the Purelink RNA Mini Kit (Ambion) standard protocol (3 biological replicates per genotype-sex combination). qRT-PCR was performed using Brilliant III Ultra-Fast SYBR kit (Agilent) on a Stratagene Mx3000P in technical triplicates. *Cyp6g1* transcript abundance was quantified relative to a pooled master sample of all samples using the efficiency-calibrated method of Pfaffl [34], normalised by the reference gene *Rpl32*. Baseline fluorescence-corrected efficiencies and Cq values were estimated using LinRegPCR version 2016.0 (Ruijter et al. 2009) (mean efficiencies±SD; *Cyp6g1* 1.97±0.09, *Rpl32* 1.91±0.08). Relative *Cyp6g1* expression was performed using the primers in Table 1, and the thermal profile in Table 2.

Statistical analyses

All statistical analyses were performed in R version 3.1.2 (R Core Team 2012). General/Generalized Linear Mixed Models (GLMMs) were implemented in *lme4* (Bates et al. 2015). Overdispersed models were fit with quasi-error structures in the case of General/Generalized Linear Models (GLMs) or fit with an observation level random effect in the case of GLMMs (Harrison 2014). Maximal models were fit with all explanatory variables and their interactions, and then simplified via stepwise term deletion (for brevity we report only significant interactions). *Cyp6g1* genotype was always retained as a main effect as it is the primary variable under investigation.

The proportion of trials won by resistant males was compared to 0.5 (the expected proportion under equal competitiveness) using a two-tailed Fisher's exact binomial test. To examine the effect of male genotype on the remaining fitness measurements we implemented a multivariate analysis of covariance (MANCOVA) with log copulation latency, copulation duration, and number of offspring as response variables; successful male genotype as an explanatory variable; and relative male size and female size as covariates. 48 individuals were excluded from this analysis due to incomplete data (final n=112).

Male P1 and P2 were compared between genotypes using quasi-binomial GLMs with male genotype, relative copulation duration, and the number of offspring produced before remating as explanatory variables.

Female offspring production was compared between genotypes using a Poisson GLMM. Egg production, egg-larvae viability, and larvae-adult viability were compared between genotypes using GLMs fit with quasi-Poisson (egg production) and quasi-binomial (both viability measures) error structures. Full models were fit with female genotype, male genotype, female size, with block and unique ID as random effects for offspring production. Interactions were omitted from the offspring production GLMM as their inclusion prevented model convergence.

Changes in the frequency of the *BA* allele in the experimental evolution populations were assessed using GLMMs with *BA* frequency as the response variable, generation as a fixed effect, and replicate population as a random effect.

Sex-specific LD_{50} values and associated 95% confidence intervals (CI) were estimated using *drc* (Ritz & Streibig 2005). LD_{50} values were considered significantly different when their 95% CIs did not overlap (Schenker & Gentleman 2001).

Cyp6g1 expression was compared between genotypes and sexes using a GLM with log relative fold change (as per Pfaffl [34]) as the response variable with sex and genotype as explanatory variables.

Results

Male fitness assays

Precopulatory competitive ability

Overall, resistant and susceptible males did not differ in their proportion of obtained matings (exact binomial test, $Bin_{0.5}$, number of resistant matings 86 of 160 trials, p=0.38, Fig. 1). However, there was variation between the two experimental blocks with resistant males securing a significantly lower

proportion of matings than susceptible males in block 1 (exact binomial test, $Bin_{0.5}$, number of resistant matings 13 of 43 trials, p=0.01, Fig. 1), but a significantly higher proportion of matings in block 2 (exact binomial test, $Bin_{0.5}$, number of resistant matings 73 of 117 trials, p=0.009, Fig. 1).

Female size had a significant effect on the multivariate combination of log copulation latency, copulation duration, and offspring number because of a large and positive significant univariate effect on copulation duration (Table 3). Univariate tests revealed that copulation duration was significantly shorter for resistant males than susceptible males, but this was not great enough to drive a significant effect in the multivariate test (Table 3).

Sperm competitive ability

P1 was not significantly influenced by male genotype (GLM, $F_{1,67}$=0.08, p=0.77) or relative copulation duration (GLM, $F_{1,66}$=3.54, p=0.06), but decreased significantly as the number of offspring produced before remating increased (GLM, $F_{1,68}$=4.89, p=0.03). P2 was not significantly influenced by male genotype (GLM, $F_{1,63}$=0.15, p=0.7), relative copulation duration (GLM, $F_{1,64}$=0.75, p=0.39), or the number of offspring produced before remating (GLM, $F_{1,62}$=0.22, p=0.64).

Female fitness assays

Offspring production

Resistant females produced significantly more offspring than susceptible females (GLMM, χ^2_5=6.50, p=0.01) (Fig. 2). Larger females also produced significantly more offspring (GLMM, χ^2_5=7.97, p<0.01). Male genotype had no significant effect on offspring production (χ^2_6=0.88, p=0.35).

Egg production and offspring viability

Resistant females produced significantly more eggs than susceptible females (GLM, $F_{1,89}$=10.88, p<0.01) (Fig. 2), whereas male genotype (GLM, $F_{1,88}$=0.51, p=0.48) and female size (GLM, $F_{1,87}$=0.22, p=0.63) had no effect. Egg-larvae viability was not significantly influenced by female genotype (GLM,

$F_{1,88}$=0.16, p=0.69), male genotype (GLM, $F_{1,87}$=2.89, p=0.09), or female size (GLM, $F_{1,86}$=0.02, p=0.89) (Fig. 2). Larval-adult viability was not significantly influenced by female genotype (GLM, $F_{1,86}$=0.01, p=0.34), male genotype (GLM, $F_{1,84}$=0.04, p=0.85), or female size (GLM, $F_{1,85}$=0.21, p=0.65) (Fig. 2).

Mathematical model and experimental evolution

The IASC model of Rostant et al. (2015) parameterized with our data (relative resistant male mating success, m = 1; relative resistant female fecundity, F = 1.56) predicted that across 7 generations *BA* frequency should increase marginally to 0.908 from an initial frequency of 0.9, and increase to 0.26 in the from an initial frequency of 0.1 (Fig. 3). These predictions were not met in the experimental populations. The frequency of the *BA* allele decreased significantly across generations in populations where the allele was at the high initial frequency (GLMM, $F_{1,3}$=18.56, p<0.001) and increased significantly across generations from the low initial frequency (GLMM, $F_{1,3}$=17.68, p<0.001), both converging towards an intermediate frequency (Fig. 3).

DDT resistance

Resistant males had 8.2-fold higher DDT resistance than susceptible males and resistant females had 14.4-fold higher resistance than susceptible females (Fig. 4). Additionally, susceptible females had 5.5-fold higher resistance than susceptible males, and resistant females had 9.7-fold higher resistance than resistant males (Fig. 4). All differences were considered statistically significant as there were no CI overlaps (Bonferroni corrected *t*-tests gave identical results).

***Cyp6g1* expression**

The relative expression of *Cyp6g1* was significantly higher in females than males (GLM, $F_{1,9}$=229.48, p<0.001), and significantly higher in resistant flies than susceptible flies (GLM, $F_{1,9}$=186.99, p<0.001) (Fig. 5).

Discussion

Recent work has shown that the DDT-resistant *BA* allele of the *D. melanogaster Cyp6g1* gene is subject to sexually antagonistic selection in the absence of insecticide when introgressed into the *Canton-S* background (McCart et al. 2005; Smith et al. 2011; Rostant et al. 2015). Here, we find that the previously detected female benefit of the *BA* allele (McCart et al. 2005) exists in a population recently sampled from the wild. In contrast, we find no overall cost of the *BA* allele in males in the relatively simple individual assay environment, but some evidence of variation in the *BA* allele effect on male fitness, as well as reduced reproductive investment by *BA* males. Additionally, we find sexual dimorphism in DDT resistance and *Cyp6g1* expression that help explain the lack of a detectable male mating cost in individual-level assays. A mathematical model of IASC parameterized with our empirical fitness estimates predicted that the *BA* allele should go to fixation. However, using experimental evolution we find evidence of balancing selection as the *BA* allele was maintained at intermediate frequencies, which matches previous findings (Rostant et al. 2015).

In order to assay the pleiotropic fitness effects of the *BA* allele, both McCart et al. (2005) and Smith et al. (2011) introgressed the allele into the naturally susceptible *Canton-S* strain. While introgressing a resistance allele into a common genetic background is a powerful approach to quantify its fitness effects relative to a susceptible allele, we know that epistatic interactions can influence the pleiotropic fitness effects of resistance (Smith et al. 2011). Indeed, intralocus sexual conflict is itself the result of sex-specific epistasis. As the experimental populations used in the present study were founded by 15 isolines, each with higher between-line genetic variation than within-line genetic variation, our fitness measures include the contribution of within-population epistatic interactions (albeit a subset of those that would be present in the wild) and are arguably more generalizable than assaying in any single genetic background. Additionally, even though quantifying the fitness effects of a resistance allele in naive genetic backgrounds may yield insight into the original effects of the allele before potential compensatory evolution could ameliorate any costs, it does not inform us of the effects present in a

contemporary population that has coevolved with the resistance allele. By using recently caught flies from a population where the *BA* allele is naturally segregating, our fitness measures also include the contribution of any cost-ameliorating genetic architecture that has coevolved with the *BA* allele.

Overall, we found no significant difference between resistant and susceptible males in their pre-copulatory or post-copulatory competitive ability when assayed in paired competitions (Fig. 1). This is consistent with the results of Smith et al. (2011), who introgressed the *BA* allele into another recently caught genetic background and found no cost to males in the same assay. While there was no significant overall effect, there was an interaction between male mating success and experimental block that demonstrated variation in pre-copulatory competitive ability. The ability to secure matings is a key determinant of male fitness (Powell 1997; Hosken & House 2011). This interaction could be epistatic or genotype-by-environment (Hunt & Hosken 2014), or alternatively may simply be a statistical artefact of forcing competitions to have simple binomial outcomes as this potentially increases the standard error of the mean.

We found that resistant females exhibited a qualitatively similar increase in fecundity to that seen in *Canton-S* by McCart et al. (2005) (Fig. 2). The increase in offspring production can be apportioned directly to increased egg production in resistant females and not greater egg or larval viability. This contrasts with McCart et al.'s (2005) findings where resistant offspring were more viable. Larger females were also significantly more fecund than smaller females – a general trend in insects (Honek 1993). Male genotype did not have any significant effect on female offspring production, which is consistent with previous evidence that the female benefit may be a maternal effect (McCart & ffrench-Constant 2008).

The DDT resistance assay confirms that *BA* individuals of both sexes are indeed more resistant to DDT than their *M* counterparts. Additionally, there is significant sexual dimorphism in the relative level of resistance conferred by both alleles. Schmidt et al. (2010) introgressed the *BA* allele into the *Canton-S*

background and reported no sexual dimorphism in resistance conferred by the *M* allele and an approximately 1.3-fold increase in resistance conferred by the *BA* allele in females relative to males. Here, we show that *M* females are 5.5-fold more resistant than *M* males, and resistant *BA* females are 9.7-fold more resistant than *BA* males. This level of sexual dimorphism hints at the presence of genetic modifiers that have coevolved with the *Cyp6g1* locus allowing sex-specific gene expression in this population. Consistent with this, we found sexual dimorphism in relative *Cyp6g1* expression across both alleles. This sexual dimorphism may explain the lack of a detectable mating disadvantage for resistant males compared to the *Canton-S* strain, in which sexual dimorphism in resistance is less pronounced. IASC can be resolved by the evolution of the genetic architecture underlying the conflict whereby the genetic constraints preventing sexual dimorphism are removed (e.g. Harano et al. 2010; Hosken 2011). However, as most (if not all) phenotypic traits are genetically correlated with other traits, it is likely that networks of genetic correlation may to some extent always retain a certain level of constraint and IASC (Harano et al. 2010). When modifiers do evolve that ameliorate the costs of IASC they are expected to have a greater effect on the sex under stronger sexual selection (Pischedda & Chippindale 2005), but it is impossible to tell from our data whether the sex-specific expression of *Cyp6g1* we report here has any sex-linkage.

Our individual-level fitness assays suggest that the *BA* allele is not sexually antagonistic in this recently sampled population, where it appears to be selectively neutral in males but beneficial in females. Consistent with this, phenotypic and qRT-PCR assays revealed sex-specific resistance regulation. These data support the hypothesis that genomic modifiers offset at least some of the male resistance costs that exist in this population.

We sought to validate our individual-level fitness estimates in a more realistic context by measuring the frequency dynamics of the *BA* and *M* alleles in experimentally evolving populations that started at either an initially high or low frequency of the *BA* allele. We first parameterized the model of Rostant et al. (2015) with our fitness estimates to obtain quantitative predictions from our

fitness estimates. The parameterised model predicted that the *BA* allele should rapidly increase in frequency from a low initial frequency and increase marginally from a high initial frequency after 7 generations (dashed black lines, Fig. 3). While the trajectory of our initially low frequency populations qualitatively fits this pattern, we found a decrease in frequency across 7 generations in the initially high frequency populations that the model did not predict. These results indicate that there is balancing selection at the *Cyp6g1* locus. This is consistent with Rostant et al. (2015) who, when empirically testing the model parameterized with fitness estimates for *Canton-S* flies, also found that *BA* frequency converged to a stable intermediate value. As noted however, our population-level findings are inconsistent with the data from individual-level fitness assays.

How can we explain this apparent discrepancy? One explanation is that individual-level assays do not fully reflect fitness in more natural population settings. In a review of the literature investigating costs of resistance to *Bt* toxins in insects, Gassmann et al. (2009) found that trait-based approaches detected costs in 34% of experiments. In contrast, population-based approaches (those that track the frequency of resistance alleles in a population over time) detected fitness costs in 62% of experiments. Thus, costs to resistance could emerge in a population context that would not be detected in individual-based fitness assays. This may in part be due to the fact that male mating success can be dependent on social context (Billeter et al. 2012). It may also be the case that other mechanisms (e.g. overdominance) are responsible for the maintenance of the *M* allele in our experimental evolution populations. However, since our findings at the population level mirror those of Rostant et al. (2015) IASC seems the more likely explanation. The presence of polymorphism at the *Cyp6g1* locus in the original isolines themselves suggests this balancing selection may also be present in the source population. Our experimental evolution data are consistent with this idea, but further work is needed to clarify this possibility.

One potential proximate explanation for the apparent discrepancy between our individual-level and population-level data is that mating pairs remained *in*

copula for longer when males carried the susceptible *M* allele. As copulation duration can be used as a proxy for reproductive investment in males (Bretman et al. 2009; Lüpold et al. 2010), the effect of male genotype on copulation duration could represent lower reproductive investment by resistant *BA* males. Sperm is transferred relatively rapidly in *D. melanogaster*, and extended copulation duration probably achieves increased semen transfer and associated accessory gland protein effects (e.g. reduced female remating rate) (Gilchrist & Partridge 2000; Chapman & Davies 2004). Shorter copulation duration in resistant males could thus reduce the ability of these males to delay female remating, which would decrease resistant male fitness, but would not be detectable in the design of our fitness assays. It may, however, help explain the balancing selection observed in our experimental evolution populations where females had the opportunity to remate constantly across several days.

Conclusions

Using recently sampled *D. melanogaster* we show that an insecticide resistance allele at the *Cyp6g1* locus confers a pleiotropic fitness benefit to females in the form of increased reproductive output, and while having no direct detectable effect on male fitness, it does reduce male reproductive investment. Additionally, we find some sexual dimorphism in DDT resistance and relative *Cyp6g1* expression that could explain the lack of detectable sexual antagonism at the individual level. These individual-level data suggest the presence of genetic modifiers that at least partially ameliorate previously reported male mating costs. Nonetheless, we find evidence of balancing selection at the *Cyp6g1* locus in experimentally evolving populations that were not theoretically predicted. These population-level data suggest that, despite sexual dimorphism in resistance and gene expression, some sexual antagonism remains. Taken together, our results suggest that individual-level fitness assays may not capture sexually antagonistic fitness effects that emerge at the population level, and such effects can maintain resistance at the *Cyp6g1* locus in the absence of insecticide.

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Tables and figures

Table 1. qRT-PCR primer sequences

Transcript	**Direction**	**Sequence**
Cyp6g1	F	ACCCTTATGCAGGAGATTG
Cyp6g1	R	TAGGCTGTTAGCACGAATG
Rpl32	F	TAAGCTGTCGCACAAATGG
Rpl32	R	GGGCATCAGATACTGTCCC

Table 2. qRT-PCR thermal profile

Duration	**Temperature (°C)**	**Cycle number**
10:00	50	1
03:00	95	
00:20	95	40
00:20	60	
01:00	95	1
00:30	60	
00:30	95	

Table 3 MANOVA and univariate ANOVA of male precopulatory competitive ability. Significant *p* values in bold.

	MANOVA		
	Pillai's trace	$F_{3,108}$	*p*
Male genotype	0.059	2.208	0.091
Male size difference	0.035	1.277	0.286
Female size	0.119	4.801	**0.004**

	Male genotype (mean ± SE)		Univariate ANOVAs	
	Resistant	Susceptible	$F_{1,108}$	*p*
Log copulation latency (min)	3.897 ± 0.123	3.819 ± 0.152	0.162	0.688
Copulation duration (min)	17.646 ± 0.792	20.762 ± 0.967	6.349	**0.013**
No. of offspring	65.484 ± 1.946	64.813 ± 3.138	0.034	0.854

	Relative male size *β*	$F_{1,108}$	*p*
Log copulation latency (min)	-0.324	0.079	0.78
Copulation duration (min)	-14.439	3.226	0.075
No. of offspring	21.742	0.609	0.437

	Female size *β*	$F_{1,108}$	*p*
Log copulation latency (min)	2.239	0.608	0.437
Copulation duration (min)	68.392	14.059	**0.0003**
No. of offspring	-29.268	0.295	0.588

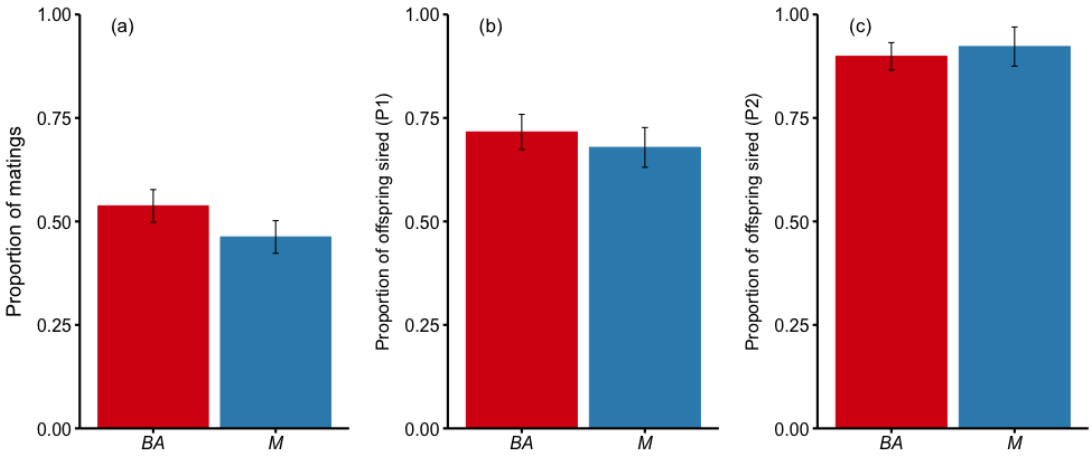

Figure 1 Competitive performance (±SE) of resistant *BA* and susceptible *M* males in the (a) precopulatory competitive assay, (b) sperm defence assay, and (c) sperm offence assay.

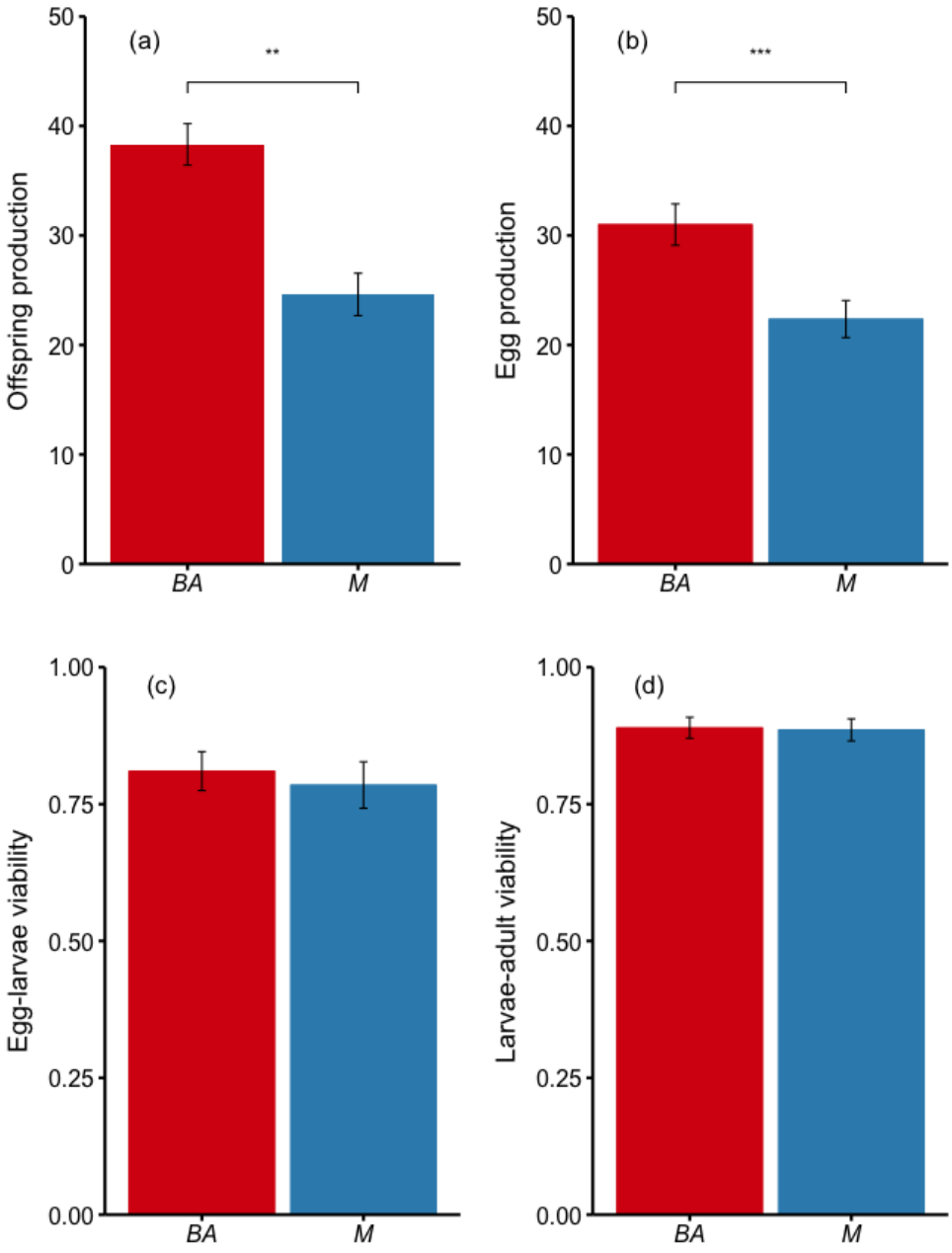

Figure 2 Means (±SE) of resistant *BA* and susceptible *M* females for (a) offspring production, (b) egg production, (c) egg to larvae viability, and (d) larvae to adult viability. (** - p=0.01, *** - p<0.01)

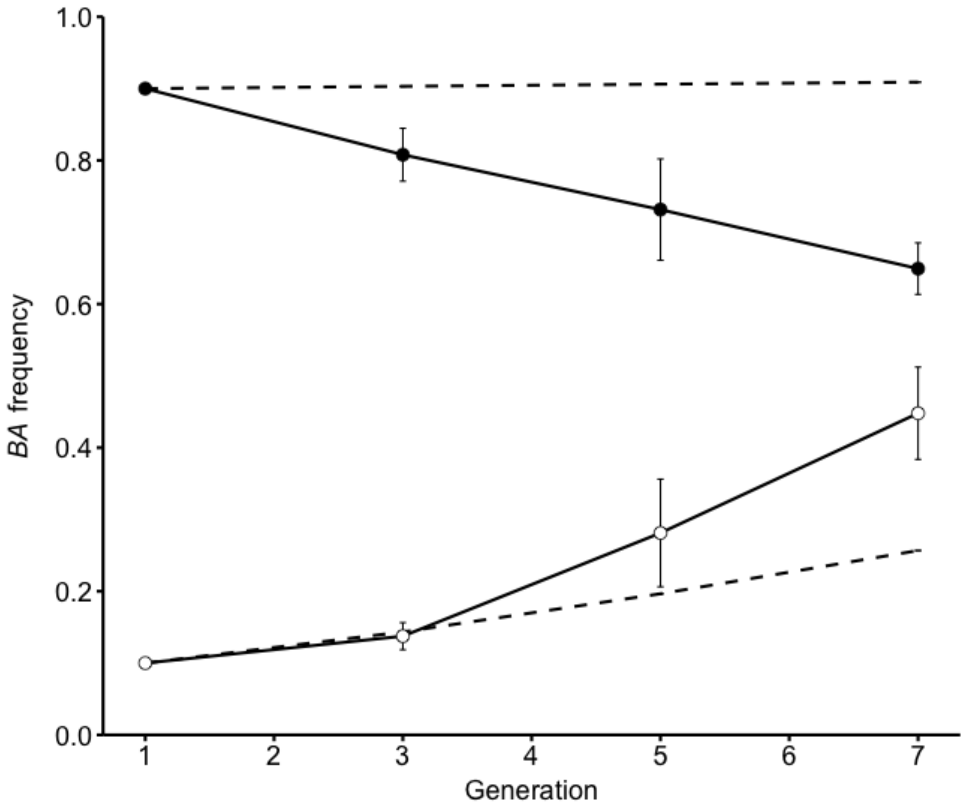

Figure 3. Mean frequency (±SE) of the resistant *BA* allele across 7 generations of experimental evolution from an initially high frequency (closed circles) and an initially low frequency (open circles). Black dashed lines indicate model predictions based on fitness assay data.

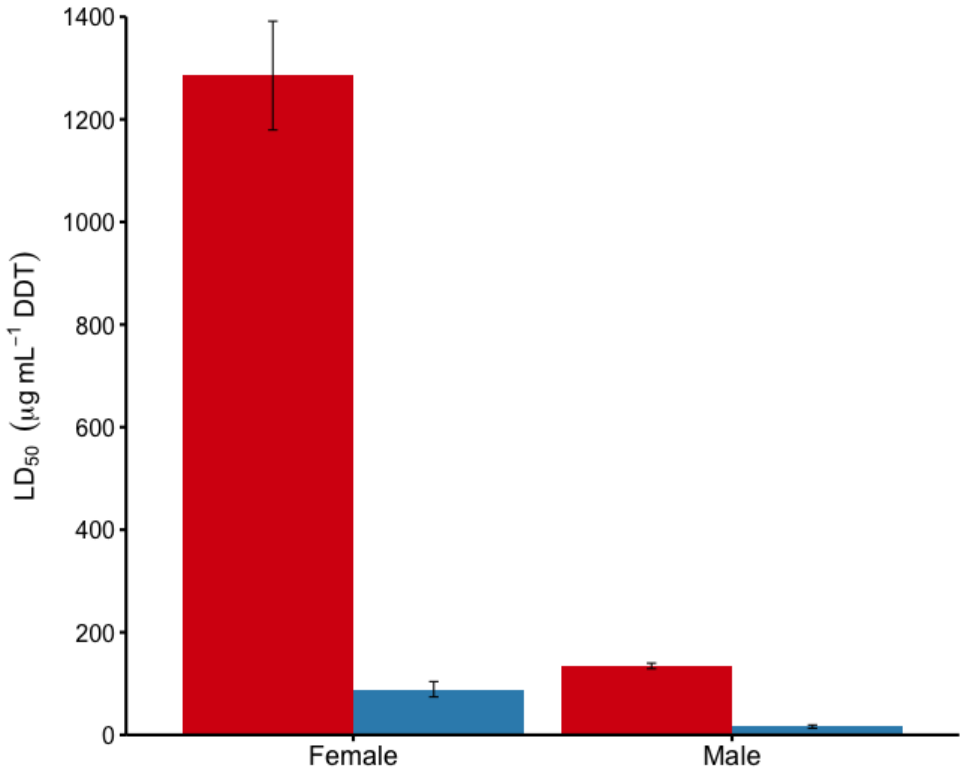

Figure 4. DDT resistance (LD_{50} ±95% CI) in males and females carrying the resistant *BA* allele (red bars) and the susceptible *M* allele (blue bars).

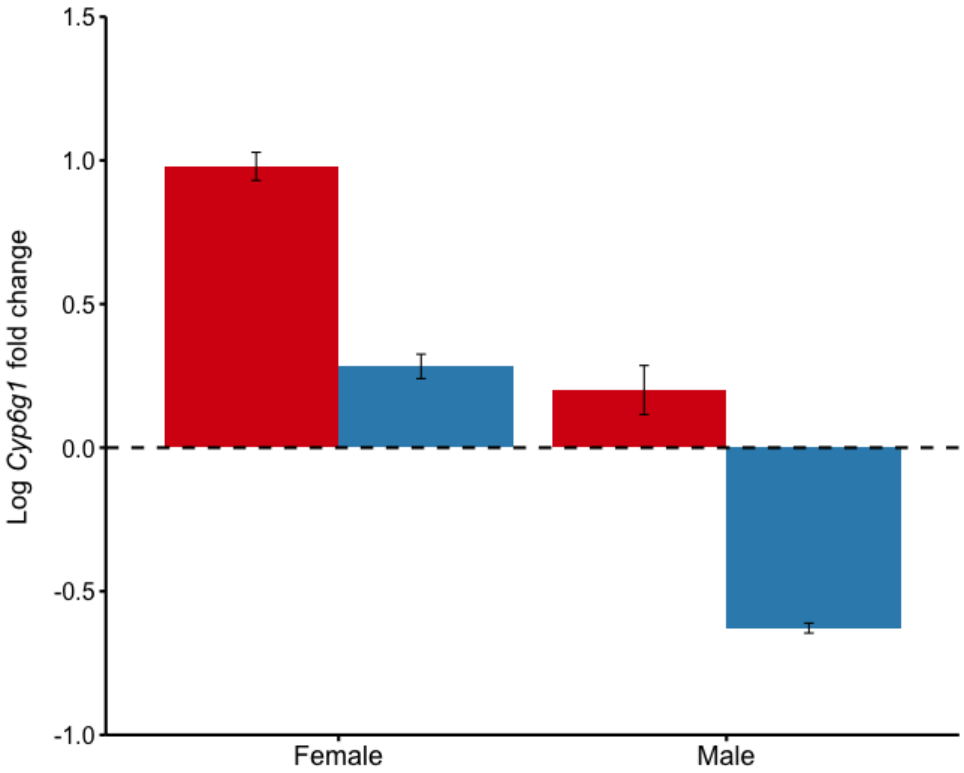

Figure 5. Relative *Cyp6g1* expression (±SE) in males and females carrying the resistant *BA* allele (red bars) and the susceptible *M* allele (blue bars).

Chapter 3

Overdominance does not explain balancing selection at the *Cyp6g1* locus

Abstract

Balancing selection is a pervasive force in maintaining genetic variation in wild populations and can occur as a result of several mechanisms including intralocus sexual conflict, sex-specific dominance patterns, and overdominance. The *Cyp6g1* locus in *Drosophila melanogaster* is the site of a series of alleles that confer resistance to a range of insecticides. One of these, the *BA* allele, can have sexually antagonistic effects on fitness in certain genetic backgrounds. In a recently collected Australian population the *BA* allele is subject to balancing selection when in competition with the susceptible *M* allele, and is associated with increased fecundity in resistant females but reduced reproductive investment in males. However, the sex-specific dominance patterns of the fitness effects of the *BA* allele are unknown and it is unclear whether overdominance may also play a role in generating balancing selection at the locus. Here, we use flies from the same Australian population as previous work to demonstrate that the *BA* allele confers a dominant fitness benefit in females, but find no evidence of additional costs to resistance or any discernable dominance pattern in males. Additionally, we find no evidence of heterozygote advantage in either sex regardless of the parental origin of the *BA* allele. Our results suggest that neither overdominance nor sex-specific dominance patterns help explain the observed balancing selection at the *Cyp6g1* locus in this population, and we suggest that quantitative genetic approaches may be required to uncover the force that maintains the polymorphism at this locus.

Introduction

Balancing selection can produce stable polymorphisms across the genome and may help explain the maintenance of a significant proportion of quantitative genetic variation in wild populations (Barton & Keightley 2002). Two mechanisms that can generate balancing selection are intralocus sexual conflict (IASC) and overdominance (Connallon & Clark 2014). IASC occurs when traits that share a common genetic basis across the sexes are subject to sexually antagonistic selection (Rice & Chippindale 2001; Chapman et al. 2003; Bonduriansky & Chenoweth 2009). IASC can constrain the sexes from reaching their respective phenotypic optima and result in a tug-of-war of allelic replacement until sex-linked trait expression can evolve (Rice 1984; Bonduriansky & Chenoweth 2009; Harano et al. 2010; Hosken 2011). Overdominance can result in balancing selection at a locus when the fitness of heterozygotes is higher than that of either homozygote, maintaining an intermediate frequency of both alleles. Cases of IASC or overdominance that result in balancing selection at known loci are rare, but recent work has shown that the *Cyp6g1* insecticide resistance locus in *Drosophila melanogaster* is subject to both IASC and balancing selection (McCart et al. 2005; Smith et al. 2011; Rostant et al. 2015; Hawkes et al. 2016; Chapter 2).

The cytochrome P450 gene *Cyp6g1* in *D. melanogaster* is the site of a series of at least four alleles that confer resistance to a broad range of insecticides (Bergé et al. 1998; Daborn et al. 2002; Le Goff et al. 2003; Chung et al. 2007; Daborn et al. 2007; Jones et al. 2010; Schmidt et al. 2010). These alleles confer increasing levels of resistance across the series as a result of *Cyp6g1* upregulation caused by a tandem duplication of the gene and cis-regulatory elements in several nested transposable element insertions (Daborn et al. 2002; Schmidt et al. 2010; Hawkes et al. 2016; Chapter 2). Notably, sexual dimorphism in resistance also increases across the series, consistent with a history of sexually antagonistic selection (Schmidt et al. 2010). The most well characterised allele with respect to its sex-specific effects on fitness is the resistant *BA* allele (McCart et al. 2005; Smith et al. 2011; Rostant et al. 2015; Hawkes et al. 2016; Chapter 2).

The *BA* allele is known to be sexually antagonistic in the old lab strain *Canton-S*, and is under balancing selection that is suspected to be the result of IASC in a population

of flies recently collected from Australia. In *Canton-S*, resistant females produce larger numbers of faster developing and more viable offspring (McCart et al. 2005), whereas resistant males secure a smaller proportion of matings (Smith et al. 2011). In the Australian population, we have shown that resistant females are more fecund, while resistant males are similar to susceptible males in standard single-bout mating and fertilisation success trials despite seeming to invest less in reproduction via shorter copulations (Bretman et al. 2009; Lüpold et al. 2010; Hawkes et al. 2016; Chapter 2). However, these single-bout trials do not fully replicate the natural conditions of the *D. melanogaster* mating system where both sexes mate multiply and female remating behaviour can be influenced by male seminal compounds transferred during copulation (Chapman & Davies 2004). Concordantly, we observed balancing selection that was able to maintain the *BA* allele at an intermediate frequency in the more natural mating conditions of experimental populations (when in competition with the insecticide susceptible *M* allele) (Hawkes et al. 2016; Chapter 2). If the reduced reproductive investment by resistant males is indeed indicative of a fitness cost of resistance related to competitive remating ability, we predict that it should translate to detectable fitness differences in assays where there is a more natural mating environment with the opportunity for competitive remating by both sexes.

While it appears that the *BA* allele is sexually antagonistic in the Australian population and that it is this antagonism that results in balancing selection, it may be the case that overdominance can also explain the maintenance of variation at the locus if heterozygote resistant individuals have higher fitness than either homozygote. The potential for heterozygote advantage is perhaps complicated by evidence that the sexual antagonism of the *BA* allele may be a maternal effect (Schmidt et al. 2010), in which case the source of the allele in heterozygotes (i.e. maternally (*BA/M)* or paternally (*M/BA*) inherited) may be an important consideration. Moreover, the dominance of an allele's fitness effect can alter its evolutionary trajectory (Paris et al. 2008), and sex-specific dominance alone can result in balancing selection (Barson et al. 2015). The insecticide resistance conferred by the *BA* allele itself is a dominant trait (Daborn et al. 2002), but this does not necessarily mean that the fitness effects are also dominant (Gassmann et al. 2009). The fitness effect of the *BA* allele in females appears to be dominant in *Canton-S* (McCart et al.

2005), but we do not know whether this result is generalisable to the contemporary Australian population. Additionally, there has been no investigation into the dominance patterns of male fitness effects of the *BA* allele. Knowing the dominance of the fitness effect of the *BA* allele in each sex would allow us to better understand the cause of balancing selection at the locus (Barson et al. 2015).

Here, using *D. melanogaster* from the same recently collected Australian population as previous work, we show that both male and female resistant heterozygotes (*BA/M* and *M/BA*) are equally as fit as resistant homozygotes (*BA/BA*). In females, the fitness benefit is dominant and all three resistant genotypes are more fecund than the susceptible genotype (*M/M*). In males, we do not find evidence for a cost of resistance that was predicted by previous work. In neither sex do we find evidence that the fitness effects of the *BA* allele depend on maternal or paternal origin of the allele. Our data suggest that overdominance does not explain the balancing selection at the *Cyp6g1* locus reported in this population, and we find no additional evidence that the counterselection against the *BA* allele is the result of a fitness cost in males.

Materials and methods

Experimental animals

Populations homozygous for either the susceptible *M* allele or the resistant *BA* allele were created from heterozygous isofemale lines (David et al. 2005) (n=15) such that genetic variation within populations was maximised but genetic variation between populations was minimised in order to control for the effect of genetic background (Gassmann et al. 2009) (detailed protocol outlined in Hawkes et al. (2016) and Chapter 2). Virgin adult males and females from these populations were crossed reciprocally to produce experimental heterozygotes, while experimental homozygotes were bred from virgin adults within each population. Standardised competitors and mates were collected from a population of *D. melanogaster* homozygous for the *sepia* mutation (obtained from the Bloomington Drosophila Stock Center). All experimental animals were 3-5 day old virgin adults at the start of experimentation, and were not anaesthetised within 24 hours of experimentation to avoid any effects of CO_2 on behaviour (Barron 2000).

Male fitness assays

To test for effects of the overdominance at the *Cyp6g1* locus in males we estimated reproductive success of homozygous and heterozygous males against a standardised competitor. One focal male (of genotype *BA*/*BA*, *BA*/*M* (maternally inherited *BA*), *M*/*BA* (paternally inherited *BA*), or *M*/*M*) and one *sepia* male were simultaneously introduced into a vial containing one *sepia* female and food/oviposition media. Experimental triplets were housed together for 7 days across 3 vials (transferred by gentle aspiration). All offspring from each vial were counted and the proportion of offspring sired by each male was scored by the assignment of paternity by eye colour. Housing the triplets together allowed for repeated bouts of both pre- and post-copulatory competition across the experimental period incorporating both male-male competition and female preference(Dougherty & Shuker 2014). Triplets in which any individuals died across the experimental period were discarded (12.5% of triplets, final sample sizes *BA/BA* n=33, *BA/M* n=33, *M/BA* n=37, *M/M* n=37).

Female fitness assays

To test for the effects of overdominance on female fitness we estimated the fecundity of homozygous and heterozygous females. Females (of genotype *BA*/*BA*, *BA*/*M* (maternally inherited *BA*), *M*/*BA* (paternally inherited *BA*), or *M*/*M*) were mated once to a standardised *sepia* male and allowed to oviposit for 7 days across 3 vials (transferred by gentle aspiration). All offspring that eclosed within 9 days of the first eclosion in a vial were collected and counted. Females that produced no offspring were excluded from analyses (n=6) since the cause of mating failure could not be determined (Greenway et al. 2015), as were females that died during the experimental period (n=13) (final sample sizes *BA/BA* n=35, *BA/M* n=33, *M/BA* n=36, *M/M* n=37).

Statistical analyses

All statistical analyses were performed in R version 3.1.2 (R Core Team 2012). Maximal Generalised Linear Models (GLMs) were fit with all explanatory variables and then simplified via stepwise term deletion (*Cyp6g1* genotype was always

retained as it is the primary variable under investigation). Models were fit with quasi-error structures when overdispersed. Pairwise comparisons of fitness measures across genotypes were implemented in *multcomp* (Hothorn et al. 2008).

The effects of *Cyp6g1* genotype on the proportion of offspring sired by males and female fecundity were estimated using GLMs fit with quasi-binomial and quasi-Poisson error structures, respectively. Full models were fit with *Cyp6g1* genotype, male size difference (focal male – competitor male), and female size as explanatory variables for males, and *Cyp6g1* genotype and female size for females. Pairwise Tukey HSD contrasts adjusted for multiple comparisons were then estimated to compare fitness measures between genotypes. To test whether the source of the *BA* allele had any effect on fitness measures the minimal GLMs were refitted with maternal and paternal alleles as separate variables (including the interaction between the two).

Results

Male fitness assays

Cyp6g1 genotype did not significantly influence the proportion of offspring sired by focal males (GLM, χ^2_{138}=0.04, p=0.94) (Figure 1). Additionally, neither the difference in size between the two males (GLM, χ^2_{136}=0.1, p=0.57) nor female size (GLM, χ^2_{135}=0.08, p=0.62) influenced the proportion of offspring sired. The *BA* allele had no significant effect on the proportion of offspring sired regardless of whether it was maternally inherited (GLM, χ^2_{138}=0.09, p=0.58) or paternally inherited (GLM, χ^2_{138}=0.00004, p=0.99) (Figure 1).

Female fitness assays

Cyp6g1 genotype significantly influenced offspring production (GLM, $F_{1,139}$=8.5, p<0.001) with susceptible females (*M*/*M*) producing significantly less offspring than resistant females of all genotypes (Tukey HSD, *BA*/*BA*, z=4.22, p<0.001; *BA*/*M*, z=4.31, p<0.001; *M*/*BA*, z=3.67, p<0.01) (Figure 2). Larger females produced significantly more offspring (GLM, $F_{1,137}$=4.56, p<0.05). The *BA* allele was associated with significantly higher fecundity both when it was maternally inherited (GLM, $F_{1,138}$=10.83, p<0.01) and paternally inherited (GLM, $F_{1,138}$=5.54, p=0.02), with an

interaction between parental origins reflecting the fact that the fitness benefit was dominant (GLM, $F_{1,137}$=7.87, p<0.01) (Figure 2).

Discussion

The insecticide-resistant *BA* allele of the cytochrome P450 gene *Cyp6g1* is subject to balancing selection in a population of *D. melanogaster* collected recently from Australia (Hawkes et al. 2016; Chapter 2). The allele is associated with increased female fecundity and reduced male reproductive investment, suggesting that the balancing selection may be the result of IASC (Hawkes et al. 2016; Chapter 2). Here, we show that the *BA* allele is not associated with any heterozygote advantage or any sex-specific dominance patterns for fitness that might help explain the observed balancing selection. Additionally, we do not find any evidence of a cost to resistance in males that was predicted from previous work.

The positive effect of the *BA* allele on female fecundity reported here is consistent with other work in *Canton-S* (McCart et al. 2005) as well as our previous work using this same Australian population (Hawkes et al. 2016; Chapter 2). The exact mechanism of the benefit to female fitness is unknown, but there is some evidence that it may be a maternal effect of provisioning extra *Cyp6g1* mRNA to embryos (McCart et al. 2005; McCart & ffrench-Constant 2008). Consistent with this, we have previously shown that the *Cyp6g1* genotype of male mates does not affect female fecundity in assays where *BA* females produce more offspring (Hawkes et al. 2016; Chapter 2). These new data show that heterozygous resistant females produce more offspring than susceptible females regardless of whether they inherited the *BA* allele maternally or paternally i.e. it is a benefit of resistance *per se* and any indirect genetic benefit from having a resistant mother does not persist into adulthood (a pattern common to maternal effects, McCart et al. 2005).

We previously found indirect evidence of a male cost of resistance in the form of reduced reproductive investment, and hypothesised that the design of our fitness assays may have prevented such a cost from influencing our measures of male

competitive ability. Specifically, reduced reproductive investment by resistant males may reduce the magnitude of seminal fluid protein effects that delay female remating, which our single-bout assays of mating and fertilisation ability could not detect. In order to account for such potential effects, the male fitness assay reported here housed focal males with a female mate and a male competitor across several days before measuring the proportion of offspring sired by the focal male. This design allows the continual opportunity for pre- and post-copulatory sexual selection integrating both male-male competition and female preference, including female remating dynamics. Despite this, we did not find any direct evidence of an additional cost to resistance in males, consistent with our previous work (Hawkes et al. 2016; Chapter 2).

The dominance of an allele's fitness effect can influence its evolutionary trajectory and alter the parameter space in which a sexually antagonistic allele can be maintained at an intermediate frequency (Kidwell et al. 1977; Rice 1984; Fry 2010; Connallon & Clark 2014). This is particularly the case for sexually antagonistic loci when dominance patterns are sex-specific, for example when each allele is dominant in the sex for which it is beneficial (Fry 2010; Barson et al. 2015). We find no pattern of dominance in our male fitness measure while the fitness benefit of the *BA* allele in females is dominant. The dominance of the fitness effect in females should strengthen selection on the *BA* allele relative to a situation where heterozygotes have intermediate fitness or where the fitness effect is recessive. We also find no evidence of overdominance, instead male and female resistant heterozygotes have the same fitness as resistant homozygotes (and susceptible homozygotes in the case of males). This, too, should favour fixation of the *BA* allele and does not help explain the maintenance of variation at this locus in experimental populations.

The data presented here suggest that overdominance does not help explain the previously observed balancing selection between the *BA* and *M* alleles in this population. Conversely, a dominant fitness benefit in females should theoretically encourage the fixation of the *BA* allele, and the lack of any detectable fitness effect in males should not act as a counterselective force in opposition to the positive

selection on the *BA* allele in females. The precise nature of the selective force counteracting the observed dominant fecundity benefit in females remains elusive. One possibility is that reduced reproductive investment is indeed a cost to resistance in males, but epistatic interactions between the *BA* allele and the genetic background in males in this population are variable and large in magnitude relative to the fitness effect. This could make any fitness effect in males difficult to detect when using an approach where unrelated individuals are sampled from a population and genetic background is not explicitly controlled. To test this possibility, a quantitative genetic approach employing isogenic lines could be used to estimate intersexual genetic correlations for the fitness effect of the *BA* allele, where a negative correlation would be expected if IASC can truly explain the observed balancing selection. Additionally, a more functional test of the relationship between the *Cyp6g1* expression and male fitness across isogenic lines would also be instructive.

Conclusions

The *BA* allele of the *D. melanogaster* cytochrome P450 gene *Cyp6g1* is sexually antagonistic in an old lab strain and subject to balancing selection with sex-specific fitness effects in a recently collected Australian population. Here, we use male and female fitness assays in homozygotes and heterozygotes carrying the insecticide resistant *BA* and susceptible *M* alleles to show that the reported balancing selection cannot be explained by overdominance. We show that resistance confers a dominant fecundity benefit to females, and that the parental origin of the *BA* allele does not influence the fitness effects in either sex. Thus, the observed balancing selection is not explained by sex-specific patterns of dominance. Additionally, we extend previous work with a more realistic male fitness assay design that should be able to detect differences in competitive ability related to female remating behaviour, and do not find direct evidence of additional male costs to resistance. Despite indirect evidence of a cost of resistance in males, the precise nature of the counterselective force that maintains genetic variation at the *Cyp6g1* locus in this population remains elusive.

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Tables and Figures

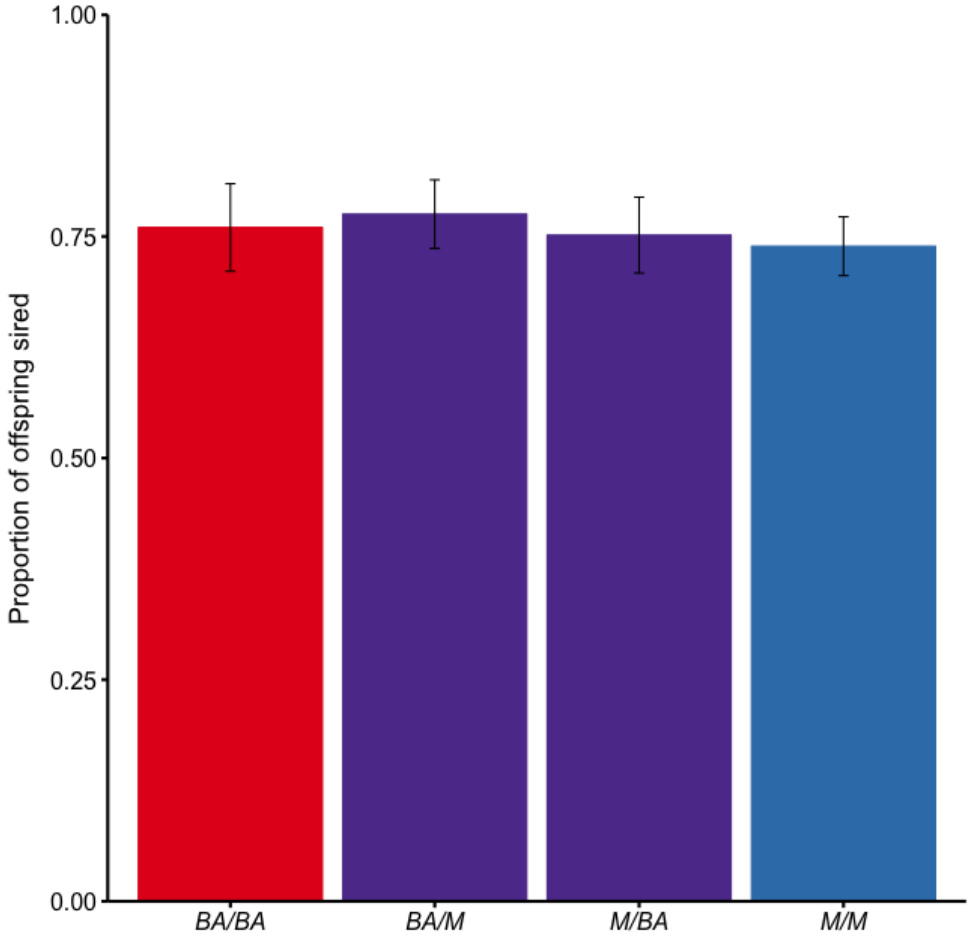

Figure 1 The proportion of offspring sired by focal homozygote and heterozygote males in competition with a standard *sepia* competitor (±SE).

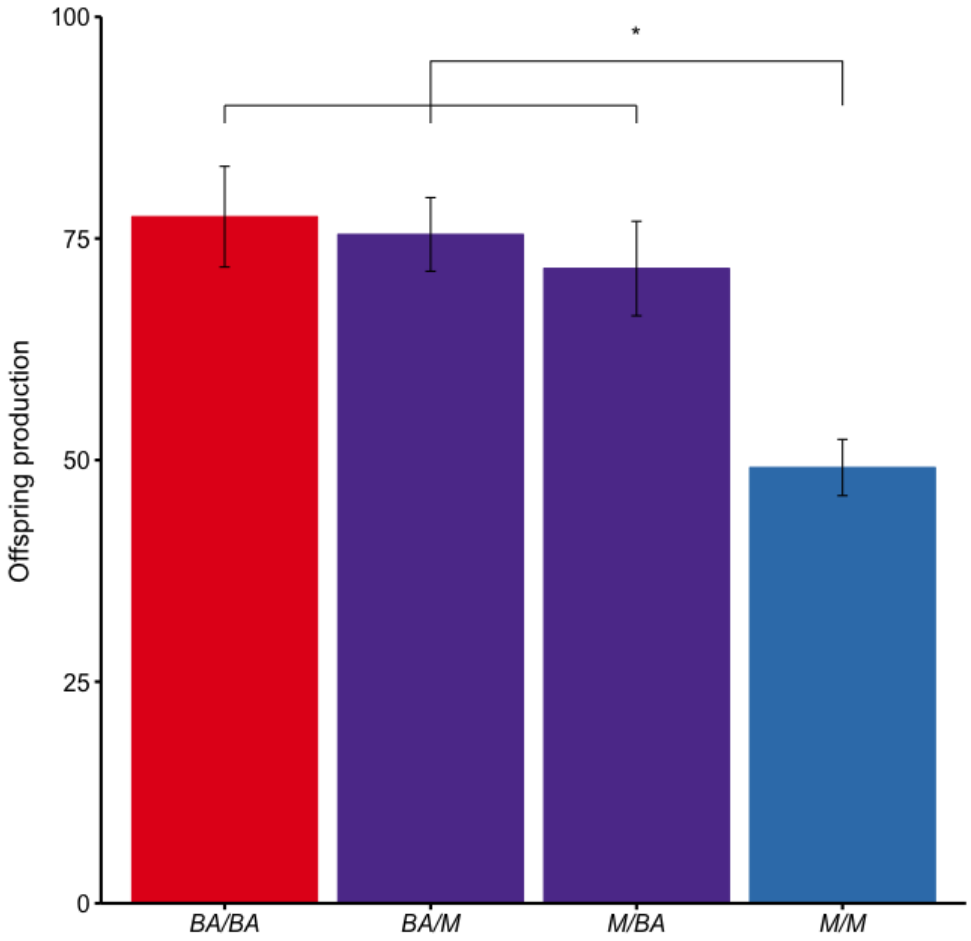

Figure 2 The number of offspring produced by homozygote and heterozygote females after a single mating (±SE). (***** indicates significant difference where $p<0.01$)

Chapter 4

Negative genetic correlations with female fitness and *Cyp6g1* expression reveal a male fitness cost of insecticide resistance

Abstract

Balancing selection at the *Cyp6g1* insecticide resistance locus is associated with sex-specific fitness effects of the resistant *BA* allele in a recently collected population of *Drosophila melanogaster*. While there is clear evidence of a fitness benefit to resistance in females, direct evidence for a fitness cost to resistance in males that would generate the observed balancing selection is lacking. Here we use isofemale lines and quantitative genetic analyses to directly test for sexually antagonistic fitness effects of the *BA* allele by estimating genetic fitness correlations between the sexes. Consistent with expectation we found a negative fitness association across the sexes, and a negative genetic correlation between *Cyp6g1* expression and male fitness revealing that male fitness is indeed related to *Cyp6g1* expression in this population. We argue that these genetic correlations and fitness estimates demonstrate the operation of intralocus sexual conflict over insecticide resistance that can explain the previously observed balancing selection at the *Cyp6g1* locus.

Introduction

Intralocus sexual conflict occurs when sexually antagonistic selection acts on sexually homologous traits and one or both sexes are constrained from reaching their phenotypic fitness optima (Rice & Chippindale 2001; Chapman et al. 2003; Bonduriansky & Chenoweth 2009). This conflict is characterized by a negative intersexual genetic correlation for fitness, and can generate balancing selection capable of maintaining genetic variation across the genome (Kidwell et al. 1977; Dean et al. 2012; Rostant et al. 2015). This could help explain how additive genetic variation is maintained in the face of selection (Barton & Keightley 2002). While IASC is an important evolutionary mechanism influencing male-female coevolution and the maintenance of genetic variation, there are few well-characterised examples where the precise loci subject to sexually antagonistic selection are known (e.g. Lonn et al. 2017) (though recent work employing transcriptomic approaches has yielded many candidate genes; Innocenti & Morrow 2010; Griffin et al. 2013). One documented allele is that of the insecticide resistance gene *Cyp6g1* in *Drosophila melanogaster* (McCart et al. 2005; Smith et al. 2011; Rostant et al. 2015; Hawkes et al. 2016).

The *D. melanogaster* cytochrome P450 gene *Cyp6g1* confers resistance to a broad range of insecticides (Bergé et al. 1998; Daborn et al. 2002; Le Goff et al. 2003; Jones et al. 2010). It is the site of an ancestral susceptible allele and a series of at least 4 resistance alleles that confer resistance via the upregulation of *Cyp6g1* expression as a result of gene duplication and cis-regulatory elements in nested transposable elements inserted upstream of the gene (Schmidt et al. 2010). Recent work has shown that one of these alleles, the resistant *BA* allele, is subject to IASC in certain genetic backgrounds (McCart et al. 2005; Smith et al. 2011; Rostant et al. 2015).

When introgressed into the old lab strain *Canton-S*, females carrying the resistant *BA* allele have higher fitness than those carrying the susceptible *M* allele, while in males the reverse is true (McCart et al. 2005; Smith et al. 2011; Rostant et al. 2015). In a recently collected Australian population, we have shown that there is balancing selection between the *BA* and *M* alleles, where

BA females have higher fecundity than *M* females but *BA* males exhibit reduced reproductive investment relative to *M* males (Hawkes et al. 2016; Chapter 2). However, the reduced reproductive investment does not seem to be enough to explain the balancing selection observed at the locus (Hawkes et al. 2016; Chapter 2, Chapter 3). Alternative hypotheses such as overdominance and sex-specific patterns of dominance are likewise unable to account for the observed balancing selection (Chapter 3). It may be the case that epistatic interactions between the *BA* allele and the genetic background in males are too large in magnitude relative to the fitness effect of the *BA* allele to detect using an approach where unrelated individuals are sampled from a genetically heterogenous population. If so, explicitly controlling for relatedness and estimating quantitative genetic parameters for the sex-specific fitness effects of the *BA* allele may stand a better chance of detecting IASC at the *Cyp6g1* locus in this population. Additionally, our previous work on the Australian population only categorically partitioned males into resistant (homozygous for the *BA* allele) or susceptible (homozygous the *M* allele) groups, and has not considered continuous variation in *Cyp6g1* expression that may better predict male fitness.

Here, we use isofemale lines to estimate the sex-specific fitness effects of the *BA* allele relative to the *M* allele while explicitly controlling for genetic background. We also use standard quantitative genetic analyses to estimate the intersexual genetic correlation for the fitness effects of the *BA* allele and the heritabilities of these effects. Finally, we use qRT-PCR to estimate the genetic correlation between *Cyp6g1* expression and fitness in resistant males relative to susceptible males.

Materials and methods

Experimental animals

Isofemale lines (isolines; David et al., 2005) (n=37) were founded by single gravid females collected from Margaret River, Australia. Isolines were maintained for approximately 30 generations of full-sib mating before genotyping (diagnostic PCR as per Schmidt et al. 2010) that revealed several isolines to be polymorphic at the *Cyp6g1* locus with both the susceptible *M*

and resistant *BA* allele segregating within them (n=15). Virgin adults were crossed within each polymorphic isoline and genotyped, with the offspring from homozygote crosses retained to produce a homozygous *M* (susceptible) and homozygous *BA* (resistant) sub-line of each isoline that otherwise shared the same genetic background. These paired homozygous isolines (*BA* n=15; *M* n=15) were maintained for at least 5 generations of full-sib mating before experiments began. Hereafter, "isoline" will refer to both genotype versions of each original isoline i.e. there are n=15 isolines each with a *BA* sub-line and a *M* sub-line.

All flies were maintained on a cornmeal based medium (standard Jazz mix, Applied Scientific) and housed in incubators at 25°C on a 12:12h light:dark cycle. Experimental animals were collected as first instar larvae, housed at a standard density, were all 3-5 day old virgin adults at the start of experimentation, and were not anaesthetised within 24 hours of experimentation to avoid any effects of CO_2 on behaviour (Barron 2000). Where specified, *sepia* competitors and mates were collected from a population of *D. melanogaster* homozygous for the recessive *sepia* mutation (obtained from the Bloomington Drosophila Stock Center).

Male fitness assays

To test for the effect of the *BA* allele on male fitness we estimated male fitness for individual *BA* and *M* males from each isoline as the proportion of offspring sired when in competition with a standard competitor (a *sepia* male). There is evidence that the *BA* allele can lower male mating success (Smith et al. 2011) and reduce investment in post-copulatory components of reproduction (Hawkes et al., 2016; Chapter 2), and so our measure integrates both pre- and post-copulatory selection. Focal males and *sepia* competitors were simultaneously aspirated into vials containing a *sepia* female and housed together as triplets for 7 days of interaction and oviposition. Eggs laid during the interaction period were allowed to develop and offspring that eclosed successfully within 9 days of the first eclosion were counted and assigned to sires based on eye colour.

Female fitness assays

To test for the effect of the *BA* allele on female fitness we estimated female fitness for individual *BA* and *M* females from each isoline as the number of offspring produced after a single mating to a standard mate (a *sepia* male). Individual *sepia* males were introduced to vials containing focal females and observed until mating occurred. Once mating had ended the *sepia* male was removed and females were allowed to oviposit for 7 days. All offspring that eclosed successfully within 9 days of the first eclosion were counted.

Cyp6g1 expression

In order to examine the relationship between *Cyp6g1* expression and male fitness we estimated relative *Cyp6g1* transcript abundance of *BA* compared to *M* males within isolines using qRT-PCR. RNA was extracted from pooled samples of 10 whole males of both genotypes from each isoline homogenised in RNAlater (Fisher) using the Purelink RNA Mini Kit (Ambion) standard protocol (3 biological replicates per genotype per isoline). qRT-PCR was performed using the Brilliant III Ultra-Fast SYBR kit (Agilent) on a Stratagene Mx3000P in technical triplicates. *Cyp6g1* transcript abundance of *BA* males relative to *M* males was estimated within isolines using the efficiency-calibrated method of Pfaffl (2001), normalised by the reference gene *Rpl32*. Baseline fluorescence-corrected efficiencies and Cq values were estimated using LinRegPCR version 2016.0 (Ruijter et al. 2009) (mean±SD efficiencies; *Cyp6g1* 1.89±0.01, *Rpl32* 1.91±0.02).

Genetic correlations and heritabilities

While all fitness data were estimated for both genotypes within each isoline, the intersexual genetic correlation of fitness (r_{MF}) and the genetic correlation between relative *Cyp6g1* expression and male fitness (r_G) were estimated using a relative measure of the fitness effect of the *BA* allele. The mean within-isoline *M* fitness was subtracted from each individual *BA* fitness measure (proportion of offspring sired for males, fecundity for females) (Eq 1), yielding a relative measure of the fitness effect of the *BA* within each isoline.

$$\omega_{i,j}^{rel} = \omega_{i,j}^{BA} - \bar{\omega}_j^M \quad (1)$$

Where $\omega_{i,j}^{rel}$ is the new relative fitness measure of individual *i* in isoline *j*, ω_i^{BA} is the original fitness measure for *BA* individual *i* in isoline *j*, and $\bar{\omega}_j^M$ is the mean of the fitness measures of all *M* individuals in isoline *j*. Our qRT-PCR protocol yielded similarly relative measures for the effect of the *BA* allele on *Cyp6g1* expression. These relative measures allow us to explicitly test how the *BA* allele influences fitness and *Cyp6g1* expression within and across isolines.

Genetic correlations were estimated using the delete-one jackknife method (Roff & Preziosi 1994) as estimates of quantitative genetic parameters can be sensitive to outliers when calculated from a low number of isolines. The jackknife procedure limits this risk by re-calculating the correlation estimate sequentially after removing each isoline in turn to account for isolines that contribute a disproportionate amount of variance.

Broad-sense heritabilities (H^2) of the effects of the *BA* allele relative to the *M* allele were estimated as the coefficient of intraclass correlation (Hoffmann & Parsons 1988, David et al. 2005). Standard errors were calculated according to Becker (1984).

Statistical analysis

All statistical analyses were performed in R version 3.1.2 (R Core Team 2012). General Linear Mixed Models (GLMMs) were implemented in *lme4* (Bates et al. 2015). All fitness data were standardised by *Z*-transformation to allow formal comparison between variables on different scales.

Male and female fitness *Z*-scores were analysed using GLMMs fit with isoline, genotype, and the interaction between the two as fixed effects, and a random effects structure that allows genotype-specific variance among isolines with a random intercept and slope. Main effects and their interactions were tested for

significance using the Anova function in the *car* package (Fox & Weisberg 2011).

Genetic correlations and heritabilities were considered statistically significant if the estimate divided by the standard error exceeded 1.96 (Gershman et al. 2010) .

Results

Male fitness - proportion of offspring sired

The resistant *BA* allele significantly reduced the proportion of offspring sired by males relative to the susceptible *M* allele (total n=225; mean±SE n per isoline=15±1.4; GLMM, χ^2_1=4.75, p=0.029; Figure 1A), and the proportion of offspring sired differed significantly among isolines (GLMM, χ^2_{14}=27.94, p=0.014; Figure 1A). The interaction between genotype and isoline did not significantly influence the proportion of offspring sired (GLMM, χ^2_{14}=13.96, p=0.45; Figure 1A).

Female fitness – fecundity

The resistant *BA* allele significantly increased female fecundity relative to the susceptible *M* allele (total n=459; mean±SE n per isoline=30.6±0.96; GLMM, χ^2_1=20.90, p<0.001; Figure 1B), and fecundity differed significantly among isolines (GLMM, χ^2_{14}=139.38, p<0.001; Figure 1B). The interaction between genotype and isoline did not significantly influence fecundity (GLMM, χ^2_{14}=16.36, p=0.29; Figure 1B).

Intersexual genetic correlation of fitness

The genetic correlation of the effect of the *BA* allele on fitness between the sexes was significantly negative (r_{MF}=-0.29±0.05; Figure 2). The relative changes in fitness between resistant and susceptible individuals within-isolines were significantly and highly heritable in both males (H^2=0.44±0.12) and females (H^2=0.47±0.10).

Cyp6g1 expression and male fitness

The genetic correlation between *Cyp6g1* expression and the proportion of offspring sired by resistant *BA* males relative to susceptible *M* males was also weakly but significantly negative (r_G=-0.281±0.101; Figure 3). Relative *Cyp6g1* expression was also highly heritable (h^2=0.986±0.006).

Discussion

Here we present evidence for the operation of intralocus sexual conflict (IASC) at the *Cyp6g1* locus in a recently collected Australian population of *D. melanogaster*. We find that when explicitly controlling for genetic background using isofemale lines (isolines), the resistant *BA* allele confers a fitness benefit to females but a fitness cost to males as evidenced by the negative intersexual fitness correlation. This direct evidence of a cost to fitness in males stands in contrast to previous work on this population that did not explicitly control for genetic background and found only indirect evidence of a male cost. The negative intersexual genetic correlation (r_{MF}) for these sex-specific fitness effects is a hallmark of IASC and importantly, the negative genetic correlation (r_G) between relative *Cyp6g1* expression and male fitness is consistent with the idea that it is insecticide resistance that is causative in generating the fitness effects.

In this population we have previously reported balancing selection at the *Cyp6g1* locus in experimental populations where flies were free to interact. This was associated with sex-specific fitness effects, with females carrying the resistant *BA* allele being more fecund than their susceptible *M* counterparts, but resistant *BA* males exhibit lower reproductive investment than susceptible *M* males (Hawkes et al. 2016; Chapter 2). While this reduction in reproductive investment is indirect evidence of a male fitness cost, it cannot fully explain the observed balancing selection in our population cages, nor can overdominance or sex-specific patterns of dominance (Hawkes et al. 2016; Chapter 2; Chapter 3). This previous work sampled flies from a genetically heterogenous population founded by the isolines used in the present study. As such, previously the genetic background was not explicitly controlled across both *Cyp6g1* genotypes, increasing the potential for any fitness effects of the *BA* allele to be masked by epistasis. Here we explicitly control for

genetic background by comparing the fitness of *BA* individuals to *M* individuals within multiple isolines affording greater precision in estimating the fitness effects of the *BA* allele (Gassmann et al. 2009).

Resistant *BA* females had relatively higher fitness than susceptible *M* females as measured by fecundity, consistent with previous work in *Canton-S* (McCart et al. 2005) and this same Australian population (Hawkes et al. 2016; Chapter 2; Chapter 3). Female fecundity varied significantly among isolines as one might expect; isolines capture and maintain subsets of the genetic variation and covariation present in the source population within highly inbred lineages that generally exhibit much higher between-line than within-line genetic variance (David et al. 2005). Unlike females however, we found that resistant *BA* males were less fit, siring a lower proportion of offspring than susceptible *M* males against a standard competitor during sustained male-male competition. There is evidence that the *BA* allele reduces male mating success in *Canton-S* (Smith et al. 2011), and that it lowers post-copulatory reproductive investment in the Australian population we are investigating (Hawkes et al. 2016; Chapter 2). Here we use a measure of male fitness (proportion of offspring sired during sustained male-male competition) that includes both pre- and post-copulatory components of mating and fertilization success that are relevant to male fitness. We have previously used this measure to investigate the fitness effects of the *BA* allele in males without explicitly controlling for genetic background as we have here, and did not find an effect (Chapter 3). We suspect that this may have been due to significant epistasis making the modest fitness cost to males difficult to detect when sampling unrelated individuals from a genetically heterogenous population. As with female fecundity, the proportion of offspring sired varied significantly among isolines. When using these population average fitness component estimates to parameterise the mathematical model outlined in Chapter 2 (relative resistant male fitness = 0.83, relative resistant female fitness = 1.22), the *BA* allele is predicted to be maintained at an equilibrium frequency of 0.47.

Our quantitative genetic parameter estimates show that the fitness effect of the *BA* allele is negatively correlated between the sexes, and that the fitness

effect in both males and females is highly heritable. Using isolines to estimate genetic correlations tends to be biased towards positive estimates (Rose 1984), and so our negative estimates are likely conservative. Conversely, using isolines can overestimate heritability (particularly more than a few generations after line establishment) and so our heritability estimates should be interpreted cautiously (Hoffmann & Parsons 1988). Taken together, these estimates suggest the presence of epistatic interactions between the *BA* allele and genetic background that allow for the operation of IASC at the *Cyp6g1* locus in this population. This contrasts with our non-relative fitness data where we did not directly detect a significant interaction between *Cyp6g1* genotype and isoline influencing fitness component measures for either sex. A negative intersexual genetic correlation for fitness is characteristic of IASC, and the magnitude of our estimate suggests that the IASC we have detected is a case of attenuated conflict that is possibly in the process of resolution (Bonduriansky & Chenoweth 2009). This is consistent with our previous work showing relatively high levels of sexual dimorphism in *Cyp6g1* expression in this population relative to *Canton-S*, where IASC is more pronounced (Hawkes et al. 2016; Chapter 2). Additionally, the presence of an allelic series itself featuring increasing sexual dimorphism in insecticide resistance across the series implies a history of sexually antagonistic selection (Schmidt et al. 2010). These, combined with the fact that the structural mutations across the series involve gene duplication, are reasons to suggest that this resolution may at least partially be occurring via sex-limited gene expression (Bonduriansky & Chenoweth 2009; Schmidt et al. 2010; but see Hosken 2011).

Our quantitative genetic parameter estimates also show that the relative male fitness of *BA* males compared *M* males is negatively genetically correlated with the relative expression of *Cyp6g1* in *BA* males compared to *M* males i.e. higher upregulation of *Cyp6g1* tends to result in lower male fitness. *Cyp6g1* upregulation has been negatively associated with competitive male reproductive success in *D. melanogaster* previously (Drnevich et al. 2004), and our genetic correlation shows that *Cyp6g1* expression is indeed related to the observed male fitness cost in this population. In order to definitively

demonstrate IASC at the *Cyp6g1* locus in this population we must now estimate the intersexual genetic correlation for *Cyp6g1* expression and the genetic correlation between *Cyp6g1* expression and female fitness directly.

Conclusions

Here we have used isofemale lines to demonstrate highly heritable and sexually antagonistic fitness effects of the *Cyp6g1 BA* allele that are negatively genetically correlated between the sexes in a recently collected population of *Drosophila melanogaster*. These data, along with a negative genetic correlation between *Cyp6g1* expression and male fitness reveal a previously elusive male fitness cost of resistance, and suggest that intralocus sexual conflict over resistance may explain balancing selection at the *Cyp6g1* locus in this population.

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Tables and Figures

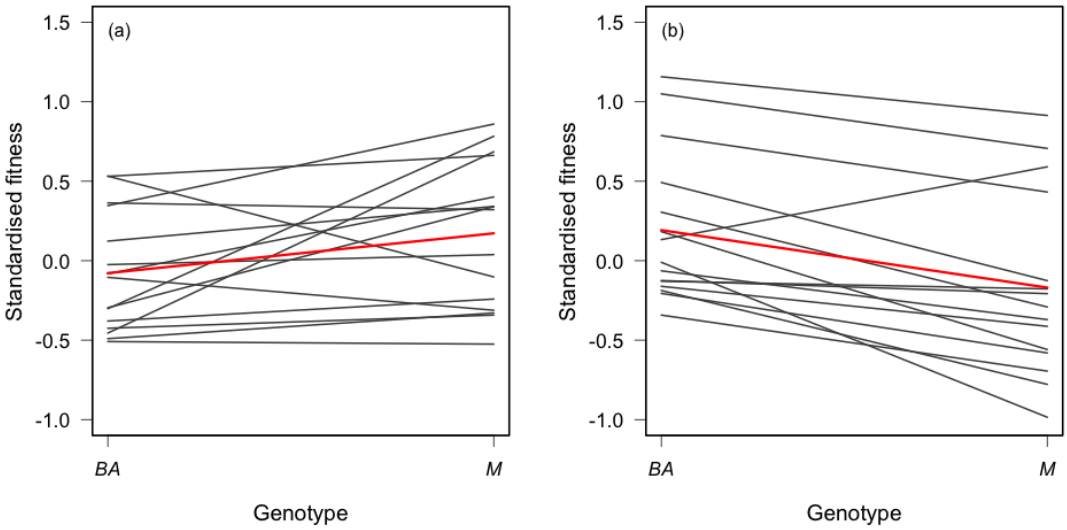

Figure 1 Interaction plot for the effects of isoline and genotype on standardised fitness means for individual isolines in (a) males (proportion of offspring sired), and (b) females (fecundity). Grey lines represent individual isoline means, and the red line represents the mean of all isolines.

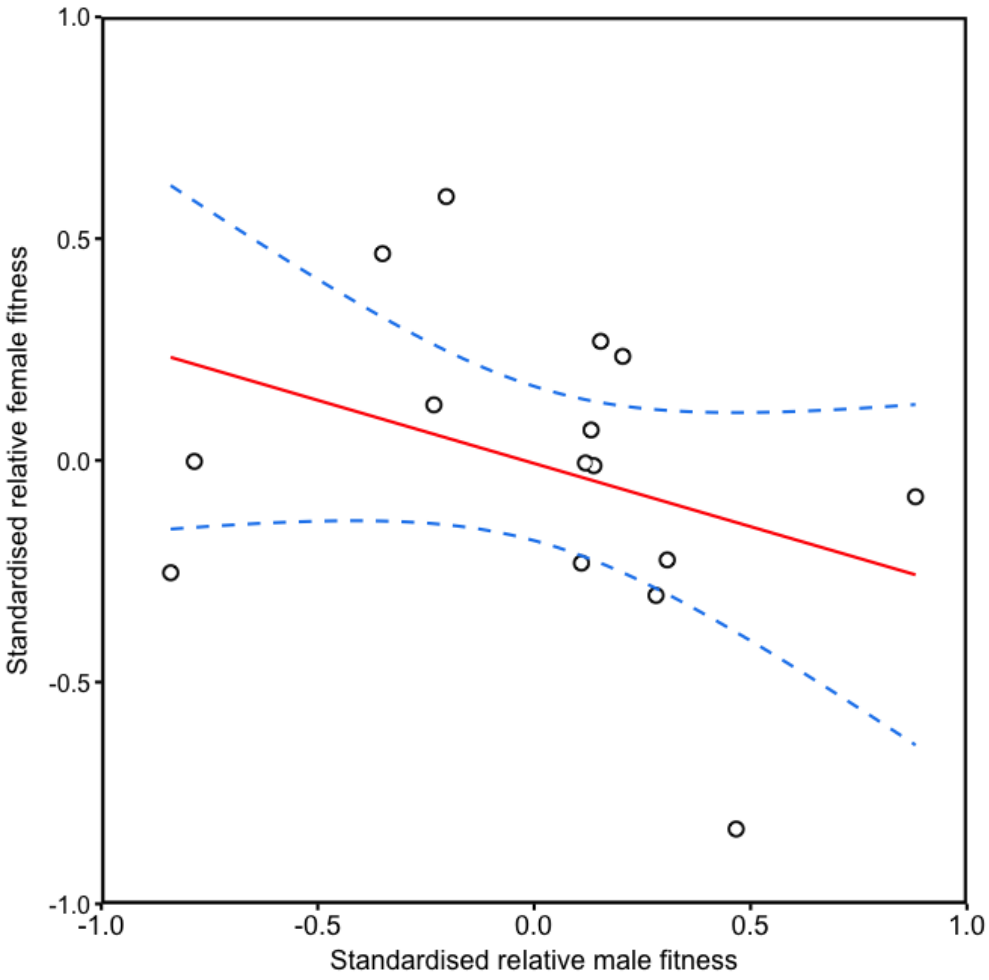

Figure 2 Intersexual genetic correlation between standardised relative male fitness (proportion of offspring sired) and standardised female fitness (fecundity). Solid red line represents the intersexual genetic correlation (r_{MF}) estimate, dashed blue lines represent 95% CI.

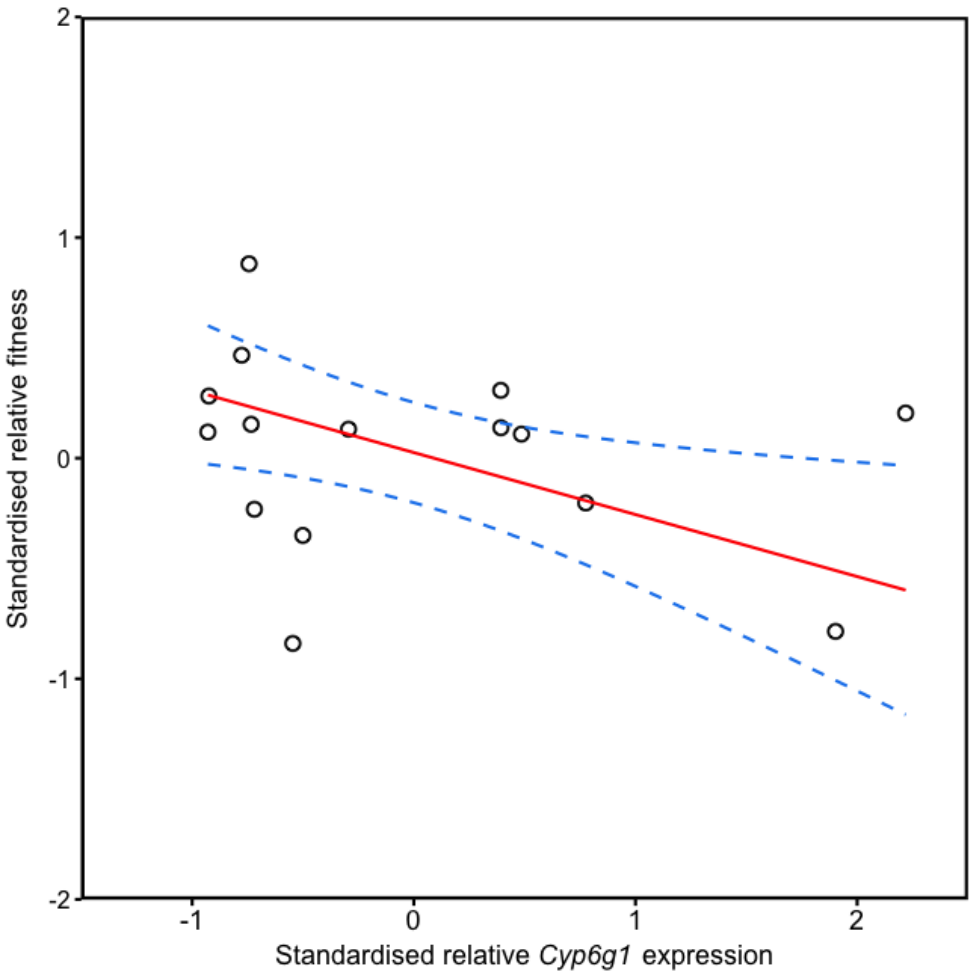

Figure 3 Genetic correlation between standardised relative male *Cyp6g1* expression and standardised relative male fitness (proportion of offspring sired). Solid red line represents the genetic correlation (r_G) estimate, dashed blue lines represent 95% CI.

Chapter 5

Sex-specific fitness effects of resistance across an adaptive walk

Abstract

The cytochrome P450 gene *Cyp6g1* is the locus of a series of insecticide resistance-conferring alleles in the fruit fly *Drosophila melanogaster*, one of which has been associated with sexually antagonistic fitness effects in some genetic backgrounds. This allelic series features a tandem duplication of the *Cyp6g1* gene and several nested transposable element insertions, and results in both increased resistance and increased sexual dimorphism in resistance across the series. It is therefore possible that the allelic replacement at the *Cyp6g1* locus has been in part driven by selection on both (i) insecticide resistance, and (ii) the resolution of intralocus sexual conflict over resistance. However, the dynamics of the sex-specific fitness effects across the series are unknown. Here, we ask whether more derived *Cyp6g1* alleles are less sexually antagonistic by back-crossing three *Cyp6g1* alleles (*AA*, *BA*, and *BP*) to a single genetic background and assaying male and female fitness across the series. We find that resistance is costlier to females than males of all three genotypes, and that more derived alleles are costlier to both sexes. However, we do not find any evidence of sexual antagonism in this genetic background. These results conflict with most previous work in other genetic backgrounds that have broadly found that *Cyp6g1* upregulation is deleterious or neutral for males but beneficial for females. While the explanation for these reversed sex-specific fitness effects is unclear, we suggest that the decreased fitness of resistant animals of both sexes across the series is the result of an increased metabolic cost of *Cyp6g1* upregulation.

Introduction

Insecticide resistance should theoretically be costly such that resistant individuals have lower fitness than susceptible individuals in the absence of insecticide (Crow 1957; Coustau et al. 2000; Hall et al. 2004). While this expected cost varies with the mechanism of resistance (Coustau et al. 2000) and whether resistance is conferred by *de novo* mutation or selection on standing genetic variation (ffrench-Constant & Bass 2017), resistance conferred by the constitutive overexpression of metabolic enzymes is expected to incur energetic costs related to the production and maintenance of high levels of protein product (Carriere et al. 1994). However, costs are not immutable and can be ameliorated by the evolution of genomic modifiers elsewhere in the genome (e.g. McKenzie & Purvis 1984) or by the replacement of costly resistance alleles with less costly alleles (e.g. Chevillon et al. 1997) (Coustau et al. 2000). Recent work has highlighted that sex is a major factor that can complicate our expectation of resistance costs (McCart et al. 2005; Smith et al. 2011; Hawkes et al. 2016). Where insecticide resistance genes are associated with sexually antagonistic fitness effects (intralocus sexual conflict; IASC) both sexes can be constrained from reaching their respective phenotypic optima for resistance expression (Rice & Chippindale 2001; Bonduriansky & Chenoweth 2009), and balancing selection can maintain resistance in populations even in the absence of direct selection from insecticides (Kidwell et al. 1977; Dean et al. 2012; Rostant et al. 2015; Hawkes et al. 2016). Any amelioration of the costs of resistance in one sex can thus be constrained by the fitness effects in the other until sex-limited expression evolves, leading to a ‘tug-of-war’ of allelic replacement (Rice & Chippindale 2001; Harano et al. 2010). The *Cyp6g1* gene in the fruit fly *Drosophila melanogaster* is the locus of a series of insecticide resistance alleles that have been associated with sex-specific (and in some cases, sexually antagonistic) fitness effects in several genetic backgrounds (McCart et al. 2005; Smith et al. 2011; Hawkes et al. 2016). However, it is unclear whether the adaptive walk at this locus has occurred solely due to direct selection imposed by insecticides, or whether the allelic series represents a process of cost amelioration and IASC resolution via allelic replacement.

The cytochrome P450 gene *Cyp6g1* confers resistance to a range of insecticides when upregulated including neonicotinoids and the organochloride DDT (Daborn et al. 2002; Daborn et al. 2007). The locus is the site of an allelic series consisting of an

ancestral insecticide susceptible allele (*M*), as well as at least 3 resistance-conferring alleles (*AA*, *BA*, *BP*) (Schmidt et al. 2010). The *M* allele is a single copy of the ancestral *Cyp6g1* gene, and across the series an *Accord* transposable element (TE) has inserted in the *Cyp6g1* promotor region followed by a tandem duplication of the entire gene (*M->AA*), a *HMS Beagle* TE has inserted within the first copy of the *Accord* insertion (*AA->BA*), and a P-element has inserted within the second copy of the *Accord* insertion (*BA->BP*) (Schmidt et al. 2010). There is also evidence for multiple *BP*-derived alleles with variably degenerate P-elements (collectively named *BP*∆) (Schmidt et al. 2010). Upregulation of *Cyp6g1* expression in resistant individuals is achieved by cis-regulatory elements within these TE insertions (Daborn et al. 2002; Chung et al. 2007). The level of resistance conferred by each allele increases across the series, as does the degree of sexual dimorphism in resistance (Schmidt et al. 2010). This sexual dimorphism suggests that the elaboration of *Cyp6g1* across the series may be related the resolution of IASC via allelic replacement including gene duplication and sex-limited gene expression.

The *Cyp6g1-BA* allele is strongly sexually antagonistic when introgressed into the old lab strain *Canton-S* (McCart et al. 2005; Smith et al. 2011; Rostant et al. 2015) and weakly sexually antagonistic in a population of flies caught recently in Australia (Hawkes et al. 2016; Chapter 4). In both cases, resistant females have higher fecundity than susceptible females, while resistant males have lower mating and/or fertilisation success than susceptible males. The female fitness benefit is dominant (Chapter 3) and appears to be a maternal effect of the result of increased *Cyp6g1* mRNA provisioning to the eggs of resistant females (McCart & ffrench-Constant 2008). Epistasis can influence the fitness effects of the *BA* allele in both sexes (Weinreich et al. 2005; Smith et al. 2011; Chapter 4), but it is unknown how these fitness effects change across the allelic series when controlling for the genetic background. Generally, it is expected that the degree of resistance-associated fitness costs should be proportional to the ratio of resistance between resistant and susceptible individuals (Gassmann et al. 2009), but this should equally hold true for fitness benefits if they are positively genetically correlated with resistance expression. Thus, where fitness effects are sexually antagonistic (or sex-specific) and more derived alleles confer higher levels of resistance via constitutive overexpression (as is the case with *Cyp6g1*), we might expect the higher-fitness

resistant sex to exhibit increased benefits across the series while the lower-fitness resistant sex incurs increased costs. However, the increased sexual dimorphism in resistance across the allelic series at the *Cyp6g1* locus may instead indicate that IASC is in the process of resolution, and any increase in resistant female fitness will occur at a faster rate than any decrease in resistant male fitness. The allelic series at the *Cyp6g1* locus offers an opportunity to study the dynamics of both the sex-specific fitness effects of resistance, as well as the potential for sex-specific resistance cost amelioration across the series. Specifically, genetic backgrounds where *Cyp6g1* resistance is sexually antagonistic, such as *Canton-S*, offer an excellent opportunity to investigate the dynamics of IASC across the so-called 'tug-of-war' of allelic replacement.

Here, we ask whether the more derived alleles of the *Cyp6g1* allelic series ameliorate any potential fitness costs or resolve any sexual antagonism at the locus by assaying the sex-specific fitness effects of *Cyp6g1*-based resistance across the series. We had intended to perform these comparisons by back-crossing the *AA*, *BA*, and *BP* alleles to the *Canton-S* genetic background, where *Cyp6g1* expression has sexually antagonistic effects on fitness. However, after experimentation concluded we were notified by the Bloomington Drosophila Stock Center that the *Canton-S* stock with which they had supplied us had been inadvertently crossed with a second, unknown strain of *D. melanogaster* also homozygous for the *M* allele. As a result of this crossing, the data presented here cannot be directly compared with other work using the *Canton-S* genetic background, and we do not have any prior evidence that the *Cyp6g1*-conferred resistance is sexually antagonistic in this new hybrid genetic background (which hereafter will be referred to as *Canton-X*). In the *Canton-X* genetic background we find that the relative fitness of resistant individuals declines across the series in both sexes, and that resistant females have lower relative fitness than resistant males across all alleles. While we find evidence of sex-specific fitness effects, none of the alleles appear to be sexually antagonistic and all were predicted to become either lost or fixed by selection in a single-locus recursive population genetic model allowing for sex-specific fitness effects.

Materials and methods

Back-cross populations

Three experimental populations were produced by back-crossing donor resistant strains homozygous for either the *AA* (isofemale line collected from Margaret River, Australia), *BA* (Hikone-R), or *BP* allele (isofemale line collected from Melbourne, Australia) to the naturally susceptible *Canton-X* (homozygous for the *M* allele; obtained from the Bloomington Drosophila Stock Center) for five generations after an initial reciprocal cross. In the initial cross and each subsequent generation of back-crossing 50 virgin males or females from the back-cross population (or donor population in the initial cross) were housed reciprocally with 50 *Canton-X* virgin males or females in glass vials for 72 hours. After the interaction and oviposition period adults were removed and the vials were laced with 500uL of 80ug/mL^{-1} DDT/acetone solution to ensure that only DDT-resistant offspring eclosed (DDT LD_{50}±SE: *Canton-X*♂ 10.89±4.99ug/mL^{-1}; *Canton-X*♀ 18.71±1.72ug/mL^{-1}). Eclosing offspring were collected as virgin adults under light CO_2 anaesthesia to be used for the next generation of back-crossing. After five generations of back-crossing virgin adults were crossed within each population and the offspring of homozygous crosses (diagonostic PCR as per Schmidt et al. 2010) were retained to produce a homozygous susceptible and resistant population for each back-cross population. The result was three populations of *Canton-X* homozygous for either the *AA*, *BA*, or *BP* allele, each with a paired population of *Canton-X* homozygous for the susceptible *M* allele. In this way we minimise the effect of differences in genetic background between resistant and susceptible populations by sourcing each susceptible population from the same back-cross lineage as their paired resistant population.

Experimental animals

Focal experimental insects were collected from the three resistant and three susceptible populations described above as first instar larvae, housed at a standard density, were all 3-5 day old virgin adults at the start of experimentation, and were not anaesthetised within 24 hours of experimentation to avoid CO_2-related behavioural effects (Barron 2000). Where specified, *sepia* competitors and mates were collected from a population of *D. melanogaster* homozygous for the recessive *sepia* mutation (obtained from the Bloomington Drosophila Stock Center). All flies

were maintained on a cornmeal based medium (standard Jazz mix, Applied Scientific) and housed in incubators at 25°C on a 12:12h light:dark cycle.

Male fitness assays

To test the effect of the three resistant alleles on male fitness we measured the proportion of offspring sired by resistant and susceptible males against a standard competitor (*sepia* males) in paired competitions. Focal and competitor males were simultaneously aspirated into vials containing a single *sepia* female and housed together as triplets for 7 days of interaction and oviposition. All offspring that successfully eclosed within 9 days of the first eclosion in a vial were counted and assigned to their sire based on eye colour.

Female fitness assays

To test for the effect of the three resistant alleles on female fitness we measured offspring production for resistant ant susceptible females after a single mating to a standard mate (*sepia* males). Males were introduced to vials containing individual focal females and observed. Once mating had concluded the males were removed and the focal females allowed to oviposit across seven days. All offspring that successfully eclosed within 9 days of the first eclosion in a vial were counted.

Statistical analyses

All statistical analyses were performed in R version 3.1.2 (R Core Team 2012). Terms in General/Generalised Lienear Models (GLMs) were tested for significance using the Anova function in the *car* package (Fox & Weisberg 2011). Male and female fitness were compared between genotypes of each allele using Generalised Linear Models (GLMs) fit with quasi-binomial and quasi-Poisson error structures, respectively, to account for overdispersion. GLMs were fit with genotype as a main effect. To compare the effect of the resistant alleles on male and female fitness across the allelic series we converted the fitness values of resistant males and females into relative values by dividing them by the average fitness of their paired susceptible population. These relative values were then compared across sexes and alleles using GLMs fit with sex, allele, and the interaction between the two as main effects. Subsequent comparisons of the relative fitness between specific alleles

within each sex were tested with Tukey contrasts adjusted for multiple comparisons using the *lsmeans* package.

Results

Male fitness

Resistant males sired a significantly higher proportion of offspring than susceptible males when carrying the *AA* allele (n=97, GLM: χ^2_1=4.623, *p*=0.031; Figure 1) but were not significantly different from susceptible males when carrying the *BA* allele (n=91, GLM: χ^2_1=0.046, *p*=0.829; Figure 1) or the *BP* allele (n=92, GLM: χ^2_1=0.041, *p*=0.839; Figure 1).

Female fitness

Resistant females produced significantly fewer offspring than susceptible females when carrying the *BA* (n=85, GLM: χ^2_1=4.908, *p*=0.0267; Figure 2) and *BP* (n=87, GLM: χ^2_1=4.266, *p*=0.039; Figure 2) alleles, but were not significantly different from susceptible females when carrying the the *AA* allele (n=86, GLM: χ^2_1=0.031, *p*=0.86; Figure 2).

Relative fitness across the allelic series

The relative fitness of resistant males was significantly higher than that of resistant females across the 3 alleles (n=538, GLM: χ^2_1=8.952, *p*=0.003; Figure 3). The relative fitness of resistant individuals of both sexes declined significantly across the series (GLM: χ^2_1=7.967, *p*=0.02; Figure 3). These effects of sex and allele did not interact (GLM: χ^2_1=0.094, *p*=0.954; Figure 3). The relative fitness of *AA* males and females was not significantly different to that of *BA* males and females (LSMeans: *z*-ratio=2.059, *p*=0.099) but was significantly higher than that of *BP* males and females (LSMeans: *z*-ratio=2.669-, *p*=0.02), while the latter two alleles were not significantly different across both sexes (LSMeans: *z*-ratio=0.599, *p*=0.82).

Discussion

The *Cyp6g1* locus of *Drosophila melanogaster* is the site of a series of several insecticide resistance-conferring alleles (Schmidt et al. 2010), one of which has been associated with sexually antagonistic fitness effects in some genetic backgrounds

(McCart et al. 2005; Smith et al. 2011; Rostant et al. 2015; Hawkes et al. 2016). It is unknown how this intralocus sexual conflict (IASC) progresses across the allelic series, and whether the fitness costs observed in males may be ameliorated with more derived alleles. Here, we backcrossed the *AA*, *BA*, and *BP* alleles to a single genetic background naturally homozygous for the *M* allele, and assayed the sex-specific fitness effects of resistance across the allelic series. We show that more derived alleles across the allelic series are costlier to carry for both males and females, and that all alleles are costlier for females than males. These effects are found in a hybrid genetic background resulting from a cross of the naturally susceptible *Canton-S* with an unknown but also naturally susceptible strain of *D. melanogaster*.

Our male fitness measures showed that across the allelic series the relative fitness of resistant males declined in the more derived alleles. Males carrying the *AA* allele sire a significantly higher proportion of offspring than susceptible males, but there is no significant difference between resistant and susceptible males when carrying the *BA* and *BP* alleles. Previous work has found that resistant males carrying the *BA* allele have either lower or equal fitness relative to susceptible males depending on the genetic background (Smith et al. 2011; Hawkes et al. 2016), and our current results are consistent with this previous finding. However, our results reveal that in the *Canton-X* genetic background where homozygous *BA* and *M* males are equally fit, resistant males carrying the less derived *AA* allele have higher fitness than susceptible males. This suggests that the increased *Cyp6g1* expression in *BA* males relative to the *AA* males (Schmidt et al. 2010) is accompanied by a reduction in the fitness of resistant males (despite not incurring an overall fitness cost). This is highlighted when comparing the relative fitness of resistant males to susceptible males across the series, where *AA* males have the highest relative fitness, *BP* males have the lowest, while *BA* males are intermediate. The functional mechanism that explains the fitness cost to males in terms of reduced mating success in the *Canton-S* background is altered social behaviour typified by lower aggression and less intense courtship (Rostant et al. 2017). Considering the evidence that overexpression of *Cyp6g1 per se* lowers male mating success in genetic backgrounds where there is a fitness effect (Drnevich et al. 2004) (Chapter 3), it is

unclear what mechanism might explain a male fitness benefit to resistance in the absence of insecticide.

The relative resistance of female fitness followed the same pattern across the allelic series as that of male fitness. While the pattern was the same, the relative fitness values themselves were not. Resistant females carrying the *AA* allele did not produce a significantly different number of offspring to susceptible females, but both *BA* and *BP* females produced significantly fewer offspring than susceptible females. This contrasts with all previous work that has consistently shown a fitness benefit to resistance in females carrying the *BA* alleles in all investigated genetic backgrounds (McCart et al. 2005; Hawkes et al. 2016) (Chapters 1, 2, and 3). Evidence suggests that the fecundity benefit conferred by the *BA* allele in other genetic backgrounds is a maternal effect of provisioning extra *Cyp6g1* mRNA into eggs (McCart & ffrench-Constant 2008). If this same maternal effect is present in this genetic background, it appears that other pleiotropic costs to resistance are greater in magnitude. As with males, the decreasing relative fitness of resistant females across the allelic series is consistent with the increased energetic cost of higher *Cyp6g1* expression.

Our data reveal sex-specific fitness effects of all three alleles where the *AA* allele is beneficial in males but neutral in females, the *BA* allele is neutral in males but deleterious in females, and the *BP* allele is neutral in males but deleterious in females. When the sex-specific mean relative fitness values of each allele are used to parameterise the mathematical model outlined in Chapter 1, the *AA* allele is predicted to go to fixation while both the *BA* and *BP* alleles are predicted to be lost from the population. While these fitness effects could explain the near global fixation of the *Accord* insertion, they are less able to explain the prevalence of the *BA* allele in wild populations. However, our lack of knowledge regarding the provenance of the *Canton-X* genetic background limits the conclusions we can draw from comparisons with wild populations.

The lack of sexual antagonism in our fitness measures combined with the lack of any sex differences in the reduction in relative fitness across the allelic series suggests that the genetic elaboration at the *Cyp6g1* locus would be primarily favoured by the selection imposed by insecticide use rather than as part of a process of IASC

resolution in the *Canton-X* background. While the decreasing fitness effect across the series can then be explained simply by an increasing energetic cost of resistance, the explanation for the sex differences in relative fitness for each allele is less clear. If any cost amelioration or IASC resolution occurs across the series in the genetic backgrounds where such costs and conflict are present, it is likely to be the result of epistatic interactions with genetic modifiers elsewhere in the genome that evolved to offset potential costs and promote sex-linked resistance. This suggestion is supported by previous work showing that epistatic effects play an important role in determining the magnitude of the *BA* allele's fitness effects (Smith et al. 2011) (Chapter 3).

Conclusions

The cytochrome P450 gene *Cyp6g1* is the locus of an allelic series where one allele has been associated with sexually antagonistic fitness effects in certain genetic backgrounds. Here, we back-cross three of the alleles from the series into a single genetic background to determine the dynamics of the sex-specific fitness effects across the series, and whether selection to resolve the previously reported intralocus sexual conflict can in part explain the replacement of these alleles in the wild. While we find no evidence of sexual conflict in this genetic background, we do find sex-specific fitness effects where resistant females have consistently lower relative fitness than resistant males across all three alleles, and both sexes experience lower relative fitness when carrying more derived alleles. These fitness effects are consistent with an increased energetic cost of resistance as a result of overexpression of *Cyp6g1*. The decreased fitness of the resistant insects of both sexes across the allelic series suggests that the resolution of any sexual conflict observed in other genetic backgrounds may be dependent on epistasis.

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Tables and Figures

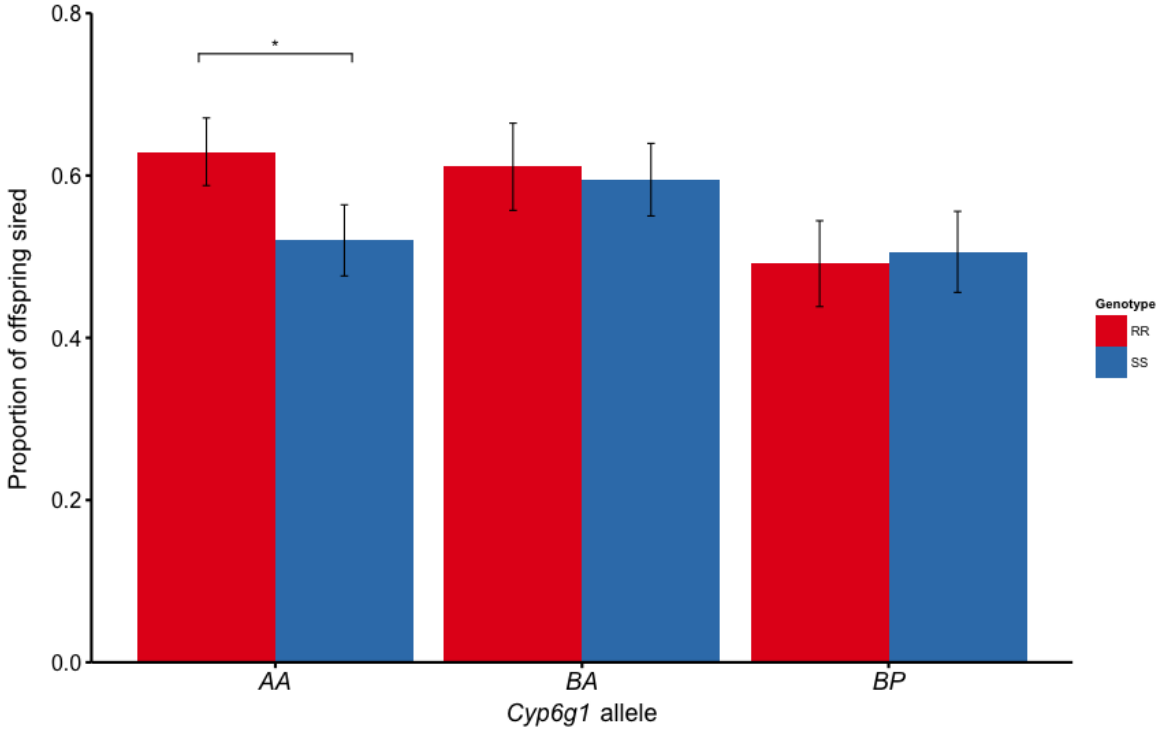

Figure 1 Proportion of offspring sired by resistant and susceptible males across the three allele back-cross populations (±SE) (* denotes significance at $p<0.05$).

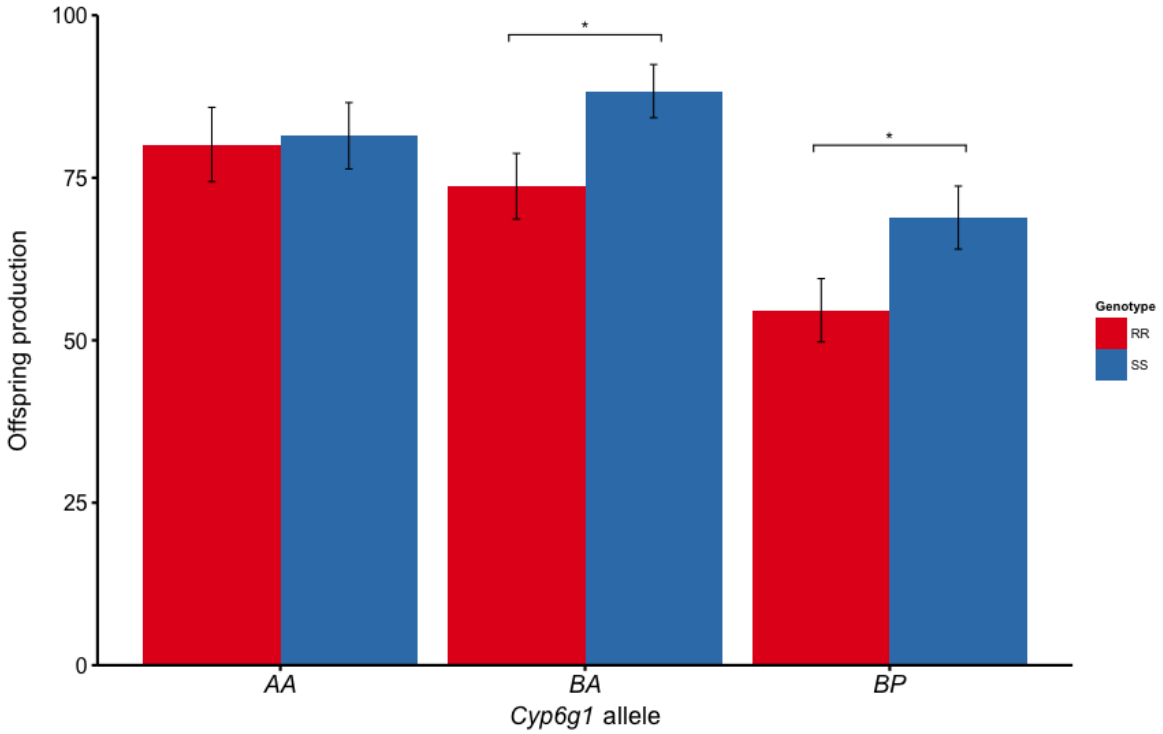

Figure 2 Offspring production by resistant and susceptible females across the three allele back-cross populations (±SE) (* denotes significance at $p<0.05$).

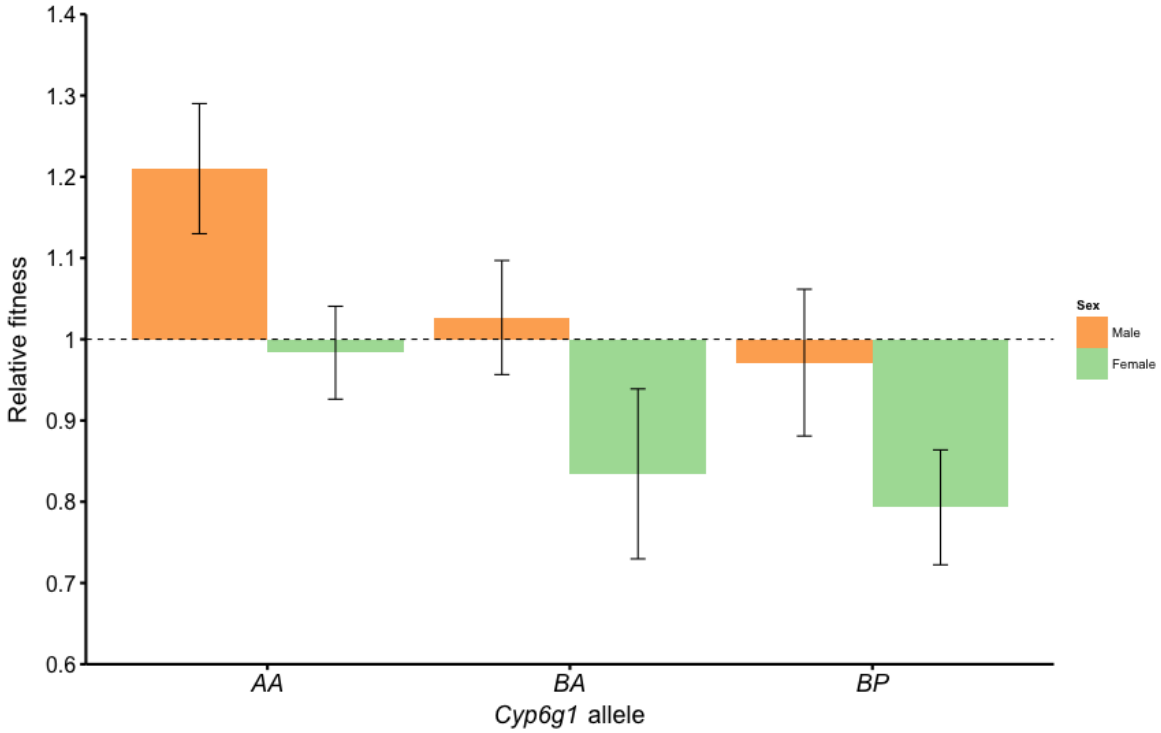

Figure 3 Relative fitness of resistant males and females across the three allele back-cross populations (±SE).

Chapter 6
Sexual selection drives the independent evolution of wing interference patterns between the sexes

Abstract

The seemingly transparent wings of many small wasps and flies have recently been found to display dramatic structural coloration. These structural colours (wing interference patterns: WIPs) may be involved in species recognition and mate choice, but as yet little is known about the evolutionary processes that shape them. As WIP colour is determined by wing structure they may also be subject to contrasting natural and sexual selection between the sexes (e.g. flight capability vs. attractive colouration), making them a potential candidate for intralocus sexual conflict. Additionally, existing research has been restricted by analysing WIPs without due consideration of how they are actually perceived by the species under study and how to best measure colour. Here, we use calibrated digital imaging and a model of *Drosophila* vision to compare WIPs of male and female *Drosophila simulans* from replicate populations forced to evolve with elevated or relaxed sexual selection for 68 generations. We show for the first time that WIPs modeled in *Drosophila* vision evolve in response to sexual selection differently between the sexes, suggesting that they are not subject to acute intralocus sexual conflict.

Introduction

Animal colour patterns are important sources of information used in a range of signalling contexts including species recognition (Barraclough et al. 1995), intrasexual competition (Siefferman & Hill 2005), and mate choice (Houde 1997). When colour patterns are subject to mate choice, colouration covaries with sexual fitness components, and can be part of multi-modal courtship behaviours that expose the patterns to the target of the display (Candolin 2003). Wing interference patterns (WIPs) are a newly discovered visual component of many insect wings that are thought to act as visual displays. They have been recorded in several *Drosophila* species (Shevtsova et al. 2011) and possibly represent previously unrecognised sexual signals in otherwise well-described *Drosophila* courtship displays, which also involve species-specific movement, song, olfaction, and taste (Greenspan & Ferveur 2000; Shevtsova et al. 2011; Katayama et al. 2014). WIPs are a form of structural colouration produced by thin-film interference where light striking the wing is refracted and reflected in such a way that the wavelength of the reflected light is dependent on the thickness of the chitinous membrane of the wing (Shevtsova et al. 2011). As a result, variation in wing thickness, along with other structural variation including hair placement and venation, determines variation in reflected colour (Shevtsova et al. 2011).

Recent work shows that WIPs within the human-visible spectrum are heritable and subject to sexual selection via female mate choice in *D. melanogaster* (Katayama et al. 2014), but generally little is known about the selective forces that shape WIPs and how they might respond to any such selection. Despite evidence that WIPs can be sexual signals, all work to date has used uncalibrated digital images where pixel colour values do not correspond linearly with radiance, making objective colour measurement extremely problematic (Stevens et al. 2007). Additionally, no work has yet investigated WIPs explicitly within the spectral sensitivities of the photoreceptors in the *Drosophila* visual system (including UV light), and so drawing clear biological conclusions about which WIP elements are under selection and how they might evolve is currently not possible.

The *Drosophila* eye contains five main types of photoreceptor, each expressing a single opsin gene; rhodopsins 1 and 3 through 6 (Rh1 and Rh3 through Rh6) (Schnaitmann et al. 2013). One is thought to be achromatic with broadband spectral sensitivity to both human-visible and UV light (Rh1), although this may also be used in colour processing (Schnaitmann et al. 2013), two have narrow peak sensitivities in the human-visible spectrum roughly corresponding to green (Rh6) and blue (Rh5) light, and two have narrow peak sensitivities in the UV spectrum at shorter (Rh3) and longer (Rh4) wavelengths (Rister et al. 2013; Schnaitmann et al. 2013). These photoreceptors are arranged into bundles of cells called ommatidia, consisting of a central column of two narrow peak photoreceptors (Rh6 and Rh4, or Rh5 and Rh3) encircled by six Rh1 photoreceptors. Almost all ommatidia are defined as being either 'pale' (expressing Rh3 and Rh6) or 'yellow' (expressing Rh4 and Rh6) (Hardie 1979; Salcedo et al. 1999; Wernet et al. 2007; Rister et al. 2013). Any investigation of *Drosophila* WIPs needs to take into account these attributes of the visual system if we are to understand any potential signalling roles they might have.

Mating signals and sexual selection have been extensively investigated in *D. simulans* (Spieth 1974; Markow et al. 2009; Taylor et al. 2007; Taylor et al. 2008; Sharma et al. 2010; Taylor et al. 2010; Ingleby et al. 2012; Sharma et al. 2012; Fiona C. Ingleby et al. 2013; F. C. Ingleby et al. 2013), but WIPs have not yet been incorporated into this framework. Female *D. simulans* are polyandrous, have a strong preference for certain male genotypes, but do not show clear mate-preference based on male size, and largely determine whether copulation occurs (Spieth 1974; Taylor et al. 2007; Taylor et al. 2008; Sharma et al. 2010; Taylor et al. 2010; Ingleby et al. 2012; Sharma et al. 2012; F. C. Ingleby et al. 2013). Here we investigated the impacts of sexual selection on WIPs in *D. simulans*, and whether any response to selection was correlated between the sexes. Calibrated digital imaging with Drosophila colour-vision modelling was used to capture WIP colour data as per-pixel 'cone-catch quanta' that describe the degree to which the photoreceptors of the *Drosophila* eye are expected to respond to the light reflected from each wing (Figure 1). WIP colours can vary dramatically within and between wings,

however the exact nature of *Drosophila* colour processing is poorly understood. We therefore measured WIP brightness and colour using a range of methods that make few assumptions concerning the nature of visual processing (i.e. about the luminance (perceived brightness) of the wings, their contrast and so on: see methods). Using male and female wings from experimental populations that had evolved with and without sexual selection (polyandrous and monogamous populations respectively), we provide the first direct evidence that sexual selection can drive the evolution of WIPs within wavelengths of light visible to the *Drosophila* visual system.

Results

The effect of sexual selection on the brightness of wings (luminance measured by the mean cone-catch values of the broadband photoreceptor Rh1) was dependent on sex (GLMM, χ^2_1=9.63, p=0.002). The WIPs of males evolving with sexual selection had significantly higher mean luminance values than the WIPs of males evolving without sexual selection (LSMeans, t ratio=3.93, p<0.001; Figure 2). In contrast, there was no difference in mean luminance between the WIPs of females evolving with or without sexual selection (LSMeans, t ratio=0.44, p=0.97).

The effect of sexual selection on the brightness contrast (luminance contrast measured by the standard deviation of the cone-catch values of the broadband receptor Rh1) was also dependent on sex (GLMM, χ^2_1=6.84, p=0.009). The WIPs of males evolving with sexual selection had significantly higher luminance contrast than those of males evolving without sexual selection (LSMeans, t ratio=4.69, p<0.001; Figure 3), but there was no difference between the WIPs of females evolving with or without sexual selection (LSMeans, t ratio=1.02, p=0.74; Figure 2).

While there were clear differences in luminosity between the WIPs of males from populations that had evolved with and without sexual selection, comparing individual photoreceptor stimulation does not accurately reflect the neurological processes involved in colour vision. Colour discrimination in *Drosophila* vision is best explained by a system of opponent colour

processing, where neurons receive antagonistic input from two or more photoreceptors and the contrast between these inputs is used to process colour information (Schnaitmann et al. 2013). To better represent this process we calculated four 'opponent channels' that have been empirically validated to accurately describe *Drosophila* colour discrimination (Rh5-Rh3, Rh6-Rh4, Rh6-Rh1, and Rh1-Rh4; Schnaitmann et al. 2013) by dividing the cone-catch quanta values of a focal photoreceptor by the sum of the cone-catch quanta values of that photoreceptor and a second comparator photoreceptor (e.g. Rh5/(Rh3+Rh5)) (Kelber et al. 2003). We generated images of these opponent channels from cone-catch data and measured the mean hue (average opponent channel pixel values across the wing) and colour contrast (standard deviation in opponent channel pixel values across the entire wing). Due to the high correlation between these four opponent channels (see methods), we used principal component analyses to extract one significant principal component that explained 90.82% of the variation in the average hue opponent channel values (Table 1), and one significant principle component that explained 79.94% of the variation in colour contrast values (Table 2).

The principal component for average hue described variation in the opponency of long versus short wavelength photoreceptors (Rh5 versus Rh3, and Rh6 versus Rh4), and opponency of narrowband photoreceptors in yellow ommatidia versus broadband photoreceptors (Rh6 against Rh1, and Rh1 against Rh4). The Rh1-Rh4 channel was significantly negatively loaded to this principal component while the remaining 3 channels were significantly positively loaded (Table 1). Thus, higher principal component scores indicate higher reflectance of longer wavelength light (measured by Rh5 and Rh6) relative to shorter wavelength (measured by Rh3 and Rh4), and this relationship becomes stronger as wing luminosity (measured by Rh1) increases. Again, we found that the effect of sexual selection on WIPs was different for males and females (GLMM, χ^2_1=7.51, p=0.006). The WIPs of males evolving with sexual selection differ significantly from those of males evolving without (LSMeans, t ratio=3.79, p=0.001), showing stronger biases towards longer wavelength light (i.e. human-visible spectrum), and towards Rh6 and Rh1 in opponency to Rh1 and Rh4, respectively (Figure 4). In

contrast, the average hues of female WIPs from either evolution treatment were indistinguishable (LSMeans, *t* ratio=0.06, p=0.99) (Figure 4).

The principal component for colour contrast describes variation in the same opponency channels as the component for average hue. All four opponent channels were significantly and positively loaded to this principal component (Table 2), and higher principal component scores therefore indicate higher colour contrast levels in all opponent channels. Once again, we found that the effect of sexual selection on WIPs was different for males and females (GLMM, χ^2_1=19.257, p<0.0001). The WIPs of males evolving with sexual selection have significantly higher levels of colour contrast than those of males without (LSMeans, *t* ratio=5.419, p<0.0001). In contrast, the colour contrast of female WIPs from both selection regimes were indistinguishable (LSMeans, *t* ratio=0.307, p=0.99) (Figure 4).

Discussion

Here we have used calibrated digital images of *D. simulans* wings in both visible and UV spectra and a model of *Drosophila* vision to reveal that male WIPs evolve in response to sexual selection within wavelengths of light visible to the *Drosophila* visual system. These results also indicate significant additive genetic variation for WIPs and confirm findings of heritable male attractiveness (Taylor et al. 2007). While these findings are consistent with those for *D. melanogaster* (Katayama et al. 2014) where evidence was found for sexual selection on WIP hue and saturation, that study did not use the explicit model of fly vision or the precise colour measurement we employed here. Studies of sexual colouration in other systems show that failure to consider the appropriate visual system and colour measurement can lead to erroneous conclusions about sexual selection (Bennett et al. 1997).

By employing experimental evolution we have explicitly shown that WIPs evolve via sexual selection as males evolving with mate choice and competition have significantly different aspects of wing colouration than males evolving without sexual selection. Sexual selection resulted in male wings eliciting a stronger mean response in green and blue-sensitive fly

photoreceptors relative to the UV-sensitive photoreceptors than the wings of males evolving without sexual selection. The wings of males evolving under sexual selection were also more luminous (were brighter) in general, yielding higher mean cone-catch values for the photoreceptor rhodopsin (Rh) 1 which has broadband spectral sensitivity to both visible and UV light, and also had higher luminance contrast. Sexual selection therefore seems to favour male wings that reflect more light in the human-visible green and blue wavelength regions, perceived as longer wavelength by the *Drosophila* visual system. However, interpretation of the bias of the evolutionary response away from the UV spectrum must be tempered by the low levels of UV light emitted in the controlled environment chambers that housed our populations - this may have constrained evolutionary responses towards the visible spectrum. Despite this, using a standard measure of male attractiveness, males evolving with sexual selection were more attractive to females after 55 generations (Duffy et al. 2016) – they mated faster, and because females determine whether copulation occurs or not (Spieth 1974), mating should be faster with more attractive males (Taylor et al. 2007; Taylor et al. 2010; Sharma et al. 2012).

Higher luminance and colour contrast (i.e. variation of WIP luminance and hues) in males evolving with sexual selection can potentially be explained by trade-offs with other sexually and naturally selected phenotypic optima for wing morphology (e.g. flight performance, or acoustic attractiveness in courtship displays) (Radwan 2008; Radwan et al. 2016). If selection on wing thickness (which affects WIPs; Shevtsova et al. 2011; Katayama et al. 2014) in these other contexts is to some degree orthogonal to selection on WIP colouration from sexual selection, then relaxing sexual selection on WIP colouration could allow these other sources of selection to erode variation in WIP hues that is only relevant in a sexual context. That males from non-sexual selection populations evolve to be more like females - see the principle component analyses - implies that WIPs are costly, which is typical for many sexual traits (Kotiaho 2001). Furthermore, because the mating environment from which our experimental populations were derived includes sexual selection (Taylor et al. 2008), the evolution we detect is probably best

explained by the relaxing of sexual selection on males in the monogamous populations.

In contrast to males, female wings have the same mean colouration and colour contrast regardless of the selective regime under which they evolved. This is perhaps unsurprising as sexual selection is typically stronger on males (Shuster & Wade 2003; Hosken & House 2011) and our selection protocol only manipulated the opportunity for sexual selection on them. Furthermore, similar sex-specific responses to sexual selection have been found in other *D. simulans* studies (Sharma et al. 2012).

Taken together, our data suggest that sexual selection drives the evolution of a suite of WIP elements in male *D. simulans*. Specifically, sexual selection favours bright, high contrast, longwave-shifted male WIPs. This finding is further supported by converting raw colour data into empirically validated opponency channels that reflect the neurological processing of colour discrimination in *Drosophila* (Schnaitmann et al. 2013). These data suggest that differences between treatments and sexes are indeed an evolutionary response to sexual selection (and its relaxation) on males, and that any intersexual genetic correlation underlying WIPs does not appear to be strong enough to prevent detectably independent sexual evolution. Intralocus sexual conflict is a frequent constraint preventing the sexes from reaching sex-specific fitness optima (Rice & Chippindale 2001), but in the *D simulans* we study, its effects generally seem to be limited (Taylor et al. 2010; Sharma et al. 2012) consistent with the sex-specific WIP findings.

Despite the caveats discussed above, we provide strong evidence for the evolution of WIPs through sexual selection, and we are confident that effects are from female mate choice because males from sexual selection lines were more attractive to females. In any case WIPs are a novel sexual signal that has until very recently been overlooked in sexual selection research, even in well-studied taxa like *Drosophila*. Our results therefore provide direct evidence that WIPs can evolve in response to sexual selection, and additionally

underline the importance of considering the visual sensitivities of intended targets when investigating sexual signals.

Materials and methods

Experimental populations

To investigate the ability of sexual selection to drive the evolution of WIPs, we established replicate experimental populations of *D. simulans* that evolved under either enforced monogamy (1♂:1♀, relaxed sexual selection on males) (n=4), or under enforced polyandry (4♂:1♀, elevated sexual selection on males) (n=4) for 68 non-overlapping generations. This is a standard technique for manipulating the opportunity for sexual selection and allows the action of both pre- and post-copulatory selection (Holland & Rice 1999; Hosken et al. 2001; Crudgington et al. 2005; Ilszer et al. 2006; Sharma et al. 2012).

In each generation, males and females were housed in mating vials at their treatment-specific sex ratio for six days (SS+ n=60 per replicate; SS- n=64 per replicate). More mating vials were included in the SS- treatment to equalise the effective population size (N_e) between the treatments (Sharma et al. 2012). Females were then haphazardly selected to be transferred to treatment- and replicate-specific oviposition vials and housed at a standardised density for 48 hours. Virgin adults were collected from oviposition vials after eclosion under light CO_2 anaesthesia and separated by sex before being haphazardly assigned into new mating vials for the next generation. Before wings were dissected and photographed all experimental populations were reared for a single generation in mating vials at a standard density (2♂:2♀) to reduce the likelihood of environmental or maternal effects confounding the results (Magalhães et al. 2011).

Experimental populations were derived from a stock population of *D. simulans* established from flies originally collected in Australia in 2004 (supplied by the Centre of Environmental Stress and Adaptation Research, La Trobe University, Australia) after screening with tetracycline to eliminate *Wolbachia* infection. *Wolbachia* infection has been associated with several deleterious effects on fitness in *D. simulans* (Snook et al. 2000; Champion de Crespigny

& Wedell 2006), and can induce cytoplasmic incompatibility in crosses with differences in infection status or strain (Werren 1997; Werren et al. 2008). All flies were housed at a temperature of 25°C under a 12:12hr light:dark cycle on an oatmeal based food media.

We dissected and photographed a total of 480 pairs of wings from 240 individuals. 36 wings were excluded from analyses due to objects obscuring the wing (e.g. fibres) or wing damage. Final sample sizes were: males evolving with sexual selection n=55; males without sexual selection n=58; females with sexual selection n=57; and females without sexual selection n=56 (all groups consisted of individuals sampled from 4 replicate populations).

Wing interference pattern imaging

Wings were photographed in a custom-built assembly using a calibrated Canon 7D camera that had been converted to full-spectrum sensitivity by replacing the sensor's visible-band pass filter with a quartz sheet (conversion by Advanced Camera Systems, Norfolk, UK). The camera was fitted with a Novoflex Noflexar 35mm lens that transmits in the visible and ultraviolet (UV) range, reverse-mounted on a helicoid to achieve a suitable magnification. Photographs were taken through a Baader UV/IR cut filter that transmits in the human visible range (400-700 nm), and then through a Baader Venus-U filter that only transmits in the UV (UV, 310-390 nm) range.

Wing interference patterns change dramatically as the angle of the wing, light source and viewing angle change under direct (e.g. point source) illumination. We therefore used a custom-built lighting system that provided uniform, diffuse lighting to create standardised illumination and viewing conditions. The lighting assembly used an Iwasaki eyeColor metal halide arc lamp modified to emit UV light by removal of its UV/IR filter. This bulb is designed to match the Commission on Illumination (CIE) standard D65 illuminant, so recreates natural illumination. The bulb was positioned inside a stainless-steel spherical reflector directly below the sample that focussed light onto a ring of raw white polytetrafluoroethylene plastic sheet around the lens, simulating a ring-flash.

Critically, this light source created standardised and uniformly diffuse illumination that matches natural conditions. The dorsal surfaces of wings were photographed in pairs on a dark, spectrally flat polymethyl methacrylate background that contained a scale-bar.

Image processing

Most imaging systems create photographs for viewing on non-linear, low dynamic range displays using 8-bits per channel colour spaces. However, such images are also non-linear, meaning the pixel values do not correspond linearly with radiance, which in turn makes them unsuitable for objective colour measurement (Stevens et al. 2007). Standard Red-Green-Blue (RGB) systems are also unsuitable for modelling *Drosophila* vision because they do not capture the UV portion of the spectrum to which *Drosophila* are sensitive, and previous analyses have included the red portion of the spectrum, which they are unable to detect (Briscoe & Chittka 2001). We therefore processed our whole-wing images using our Multispectral Image Analysis and Calibration Toolbox for ImageJ (Schneider et al. 2012), which enables image calibration, first controlling for lighting conditions and then converting images to animal cone-catch quanta (Troscianko & Stevens 2015).

We used the toolbox to combine the visible and ultraviolet whole-wing images into aligned, normalised multispectral stacks, and then used a cone-mapping approach to convert these images to "*Drosophila* vision" (i.e. *Drosophila* cone-catch quanta). Images were normalised (i.e. converted to relative reflectance images that control for lighting conditions) by measuring the background grey in each image, which was in turn calibrated against a Spectralon 99% reflectance standard (Labsphere). Briefly, the cone-mapping process uses the known spectral sensitivities of the camera to estimate the camera's response to a database of thousands of natural reflectance spectra illuminated using the CIE standard D65 illuminant following the von Kries correction. In addition, the *Drosophila* cone-catch quanta were calculated for the same illuminant using *Drosophila* spectral sensitivities (Salcedo et al. 1999; Schnaitmann et al. 2013). A polynomial model was then fitted between camera and *Drosophila*

vision. The model reported R^2 values >0.993 for all five receptor classes. For more information on the methodology see Troscianko & Stevens (2015).

Mean hue values were calculated for each wing that described the overall wing hue, and in addition the standard deviation in hue values for each wing was calculated in each hue channel to measure the colour complexity present in each wing display (e.g. wings of a single shade of grey would have a low variation in hue, while wings containing multiple shades of grey would have a high variation in hue). Cone-catch quanta were converted to opponent hue channels by dividing the values of a primary hue channel by the sum of that primary channel and a second opponent hue channel (e.g. the green-blue opponent channel was calculated as: Rh6/(Rh6+Rh5)) (Kelber et al. 2003).

Statistical analyses

All statistical analyses were performed in R version 3.1.2 (R Core Team 2012). General Linear Mixed Models (GLMM) were implemented in *lme4* (Bates et al. 2015).

Cone-catch quanta means and standard deviations were compared between sexes and treatments with GLMMs fit with sex, treatment, and their interaction as fixed effects, and population replicate and individual fly ID as random effects. Fixed effects were tested for significance using the Anova function in the *car* package (Fox & Weisberg 2011). Where a significant sex by treatment interaction was present, Tukey contrasts adjusted for multiple comparisons were obtained from the GLMMs using the *lsmeans* package (Lenth 2016). Where no significant interaction between sex and treatment was found, significant GLMM terms explaining differences in WIP traits are reported. We present cone-catch quanta as proportional luminance values that describe the proportion of light reflected by the wing (i.e. the cone catch).

Principal component analyses (PCA) were conducted on opponent channel data to reduce the dimensionality of the dataset and account for high levels of correlation between the cone-catch values across opponent channels. Principal components (PCs) derived from the PCAs were considered

biologically significant if their associated eigenvalue was greater than 1.0 (Norman & Streiner 2008), and the loading of PCs to each CHC peak was considered significant if greater than 0.35 (Tabachnick & Fidell 2013). Statistical testing of the principal component data was conducted in the same manner as for the cone-catch quanta data.

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Tables and Figures

Table 1 Principal component loadings and eigenvalues for mean hue. Significant values in bold (see methods).

	PC1	PC2	PC3	PC4
Eigenvalue	**1.906**	0.55036	0.25385	0.003604
% variance explained	90.82	7.572	1.611	0
Opponent channel loadings				
Rh5-Rh3	**0.4612392**	**-0.8602175**	-0.2174468	0.001113033
Rh6-Rh4	**0.5198955**	0.2029763	0.2958405	**-0.775233978**
Rh6-Rh1	**0.4992545**	**0.4496475**	**-0.7188915**	0.178205136
Rh1-Rh4	**-0.517409**	-0.1290101	**-0.590246**	**-0.606014826**

Table 2 Principal component loadings and eigenvalues for colour contrast. Significant values in bold (see methods).

	PC1	PC2	PC3	PC4
Eigenvalue	**3.19747867**	0.57029664	0.21800001	0.01422468
% variance explained	79.94	14.26	5.45	0.356
Opponent channel loadings				
Rh5-Rh3	**0.4337565**	**0.79945583**	**-0.392925**	-0.135409
Rh6-Rh4	**0.5393147**	-0.19742944	**0.4330702**	**-0.6947024**
Rh6-Rh1	**0.4749943**	**-0.56499812**	**-0.6647992**	0.1148894
Rh1-Rh4	**0.5434845**	0.05166349	**-0.4648679**	**0.6970318**

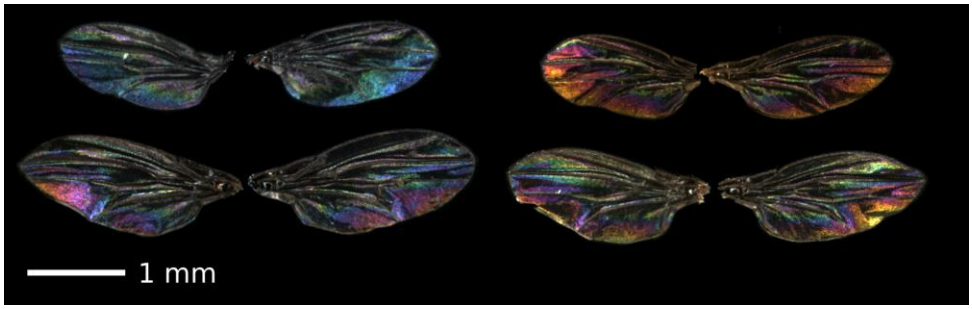

Figure 1 Examples of *Drosophila* wing interference patterns (WIPs) photographed in this study. The figure shows false-colour where *Drosophila* Rh6 is shown in red, Rh5 is shown in green, and Rh4 is shown in blue. Therefore any blue colours show ultraviolet peaks, while red and green show green and blue peaks respectively.

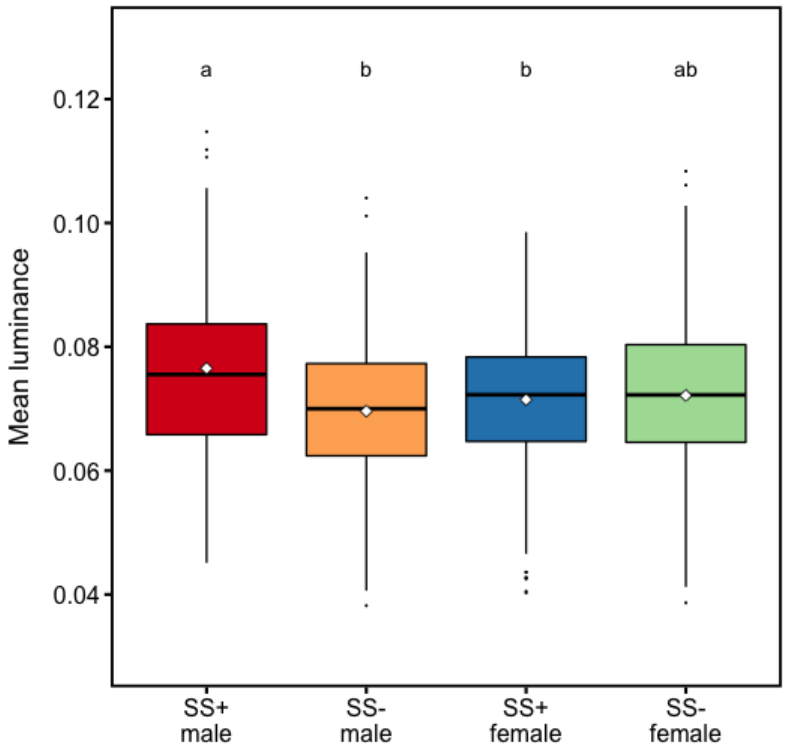

Figure 2 Mean luminance of WIPs as measured by average stimulation of the broadband Rh1 photoreceptor in the *Drosophila* visual system. Boxes represent the interquartile range, black bars are medians, white diamonds are means. Differences in letter annotation denote significance at $p<0.05$.

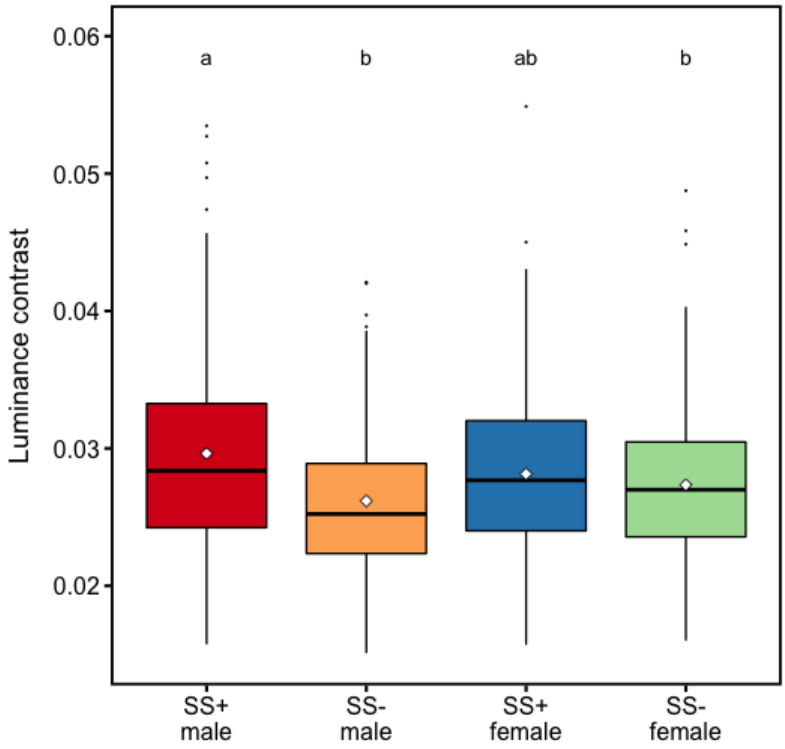

Figure 3 Luminance contrast of WIPs as measured by the standard deviation of the average stimulation the broadband Rh1 photoreceptor in the *Drosophila* visual system. Boxes represent the interquartile range, black bars are medians, white diamonds are means. Differences in letter annotation denote significance at $p<0.05$.

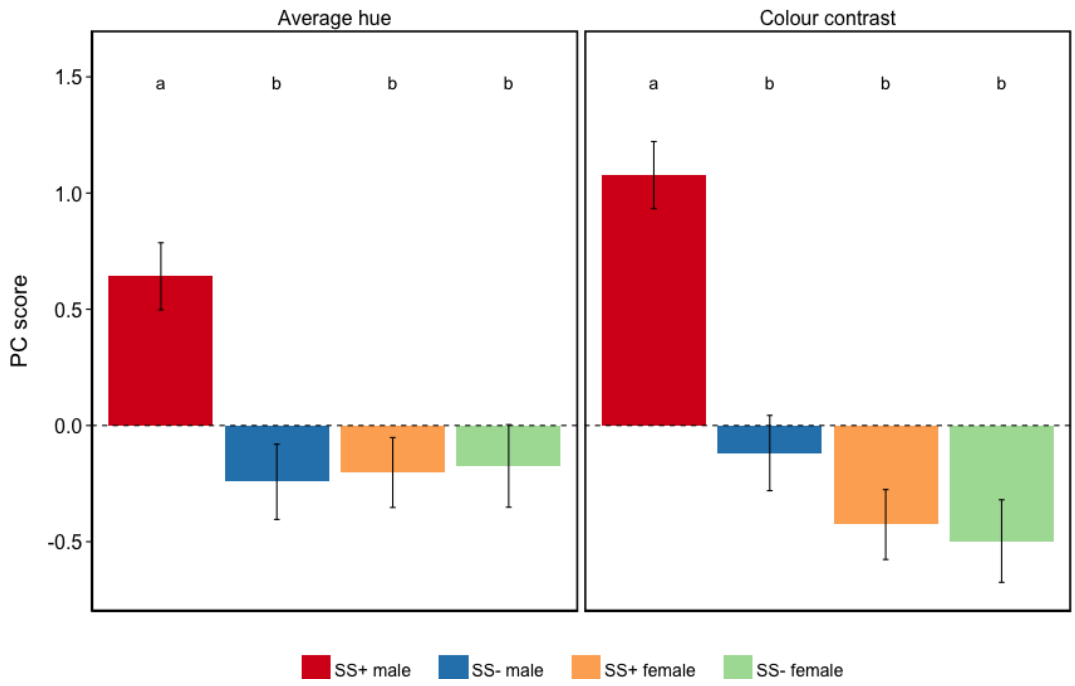

Figure 4 Principal component (PC1) means and standard errors explaining variation in the opponent channels Rh5-Rh3, Rh6-Rh4, Rh6-Rh4, and Rh1-Rh4 for both mean hue (left) and colour contrast (right). Differences in letter annotation denote significance at $p<0.05$.

Chapter 7

General discussion

Intralocus sexual conflict (IASC) occurs as a consequence of males and females being shaped by natural selection to pursue divergent life histories and reproductive strategies while still sharing a genome (Parker 1979). IASC has implications for a range of fundamental evolutionary processes, ultimately playing a large role in both the evolution of sexual dimorphism and in maintaining additive genetic variation in wild populations (Rice & Chippindale 2001; Chapman et al. 2003; Arnqvist & Rowe 2005; Bonduriansky & Chenoweth 2009). Despite this, there is little empirical evidence of the operation and resolution of IASC that simultaneously captures information about (i) the traits subject to sexually antagonistic selection, (ii) the genetic architecture underlying these traits, and (iii) the dynamics of the conflict across mutational steps. In this thesis I have presented research in which we investigated a trait that is subject to sexually antagonistic selection in certain genetic backgrounds where the genetic architecture underlying the conflict is known, and there are multiple mutational steps across which to track the progress of any sexual conflict. Additionally, we have explored the potential for IASC in wing interference patterns, a newly discovered trait subject to mate choice in *Drosophila* (Shevtsova & Hansson 2011). Sexual signals are classic examples of traits with a history of sexually antagonistic selection and IASC (Chippindale et al. 2001; Chapman et al. 2003; Arnqvist & Rowe 2005; Bonduriansky & Chenoweth 2009), but we know very little about wing interference patterns, their underlying genetic architecture, or the evolutionary forces that shape them.

Chapter 2 explores the presence of intralocus sexual conflict over the *Cyp6g1-BA* allele in a genetically heterogeneous and recently collected genetic background using a combination of trait- and population-based approaches. The *BA* allele is sexually antagonistic when back-crossed to the old isogenic lab strain Canton-S (McCart et al. 2005; Smith et al. 2011; Rostant et al. 2015) whose collection

predates the widespread use of insecticides, and so is naïve to both the selection imposed by insecticides and any genetic modifiers that might have since evolved to offset costs to resistance. It was not known if this sexual antagonism is present in modern genomes that have been exposed to the selection imposed by insecticides. Smith et al. (2011) back-crossed the *BA* allele to a recently collected isogenic background and did not detect a male cost to resistance, but it is unclear if this would be true in genetically heterogenous populations where epistasis may alter the degree or sign of any fitness effects. We found that the *BA* allele confers a fecundity benefit to females as a result of increased egg production, and found indirect evidence of a cost of resistance to males in the form of reduced reproductive investment. However, we found no differences in pre- or post-copulatory competitive ability in resistant and susceptible males in paired competitive bouts. We suspect that the design of our male fitness assays may have prevented the reduction in reproductive investment from having any impact on fertilisation success as males were only exposed to single bouts of postcopulatory competition. Where decreased reproductive investment alters seminal protein effects on female remating behaviour, repeated bouts or constant exposure to competition would be required to detect these effects. We detected significant sexual dimorphism in both resistance to DDT and *Cyp6g1* expression for both the *BA* and *M* allele. A mathematical model parameterised with our fitness estimates predicted that the *BA* allele should go to fixation, but experimental evolution revealed that the *BA* allele was subject to balancing selection. This balancing selection, combined with the indirect evidence of a male cost and significant sexual dimorphism, suggests that attenuated IASC may be operating over the *BA* allele in this population. However, there are several alternative explanations that could also produce this balancing selection, and these were the focus of Chapter 3.

In Chapter 3 we test the possibility that the balancing selection detected in Chapter 2 could be explained by overdominance or patterns of sex-specific dominance for the fitness effects of the *BA* allele. To do this we assayed sex-specific fitness measures for both homozygotes (*BA/BA, M/M*) and heterozygotes of both parental origin (*BA/M*, and *M/BA*). Due to possible limitations with the male fitness measures used in Chapter 2, we used a modified male fitness assay that integrates constant

exposure to both pre- and post-copulatory sexual selection to test for fitness effects that the assay design in Chapter 2 may have overlooked. We found no additional evidence for a male cost of resistance, and revealed that neither overdominance nor sex-specific patterns of dominance could explain the balancing selection we previously detected at the *Cyp6g1* locus. This data rules out all the major alternative explanations for the observed balancing selection at the *Cyp6g1* locus but also fails to provide additional evidence for the operation of IASC despite an experimental design that should be more sensitive to detecting male costs. As the data from Chapter 2 suggests that any IASC in this genetic background is likely to be attenuated, where the male costs are relatively small in magnitude, it may have been necessary to more stringently control for the genetic background between resistant and susceptible individuals in order to detect any effect. Alternatively, it may be that the average level of *Cyp6g1* expression in this population does not result in an average cost at the population level, but there still exists a negative intersexual genetic correlation for the fitness effect of the *BA* allele such that the most resistant males are the least fit. To test both of these possibilities we opted to employ isofemale lines to estimate these quantitative genetic parameters in Chapter 4.

Chapter 4 explores whether explicitly controlling for the genetic background using isofemale lines from the same population as Chapters 2 and 3 would allow us to detect IASC over the *Cyp6g1-BA* allele and explain the balancing selection we observed in Chapter 2. We estimate a negative genetic correlation between the fitness effect of the *BA* allele between the sexes, as well as a negative genetic correlation between relative *Cyp6g1* expression and relative male fitness between resistant and susceptible males. We again detect an overall fecundity benefit to resistant females, but in this instance we also find direct evidence for an overall cost to resistance in males. When used to parameterise the mathematical model from Chapter 2, the magnitudes of these sexually antagonistic fitness effects are predicted to maintain the *BA* allele at an intermediate frequency.

Taken together, the data from Chapters 2-4 reveal that even after decades of strong directional selection imposed by insecticides (that likely mask any sexual antagonism

in the wild) an attenuated level of IASC still operates over the *BA* allele and the expression of *Cyp6g1*. One factor that we have not investigated but which can influence the costs of resistance is the presence of any gene by environment interactions (Janmaat & Myers 2005). As all our insecticide resistance experiments have been conducted in the relative luxury of the lab, experimental animals are likely to be in good condition and not facing any significant challenges in terms of viability or survival. It may be the case that the cost of the *BA* allele in males is more apparent when males are in poorer condition or competing in a more wild-like environment, and indeed this may help explain the discrepancy between our trait- and population-level experiments in Chapter 2. In the future it would also be interesting to investigate the sex-specific fitness effects of the other *Cyp6g1* alleles in this and other recently collected genetic backgrounds to examine whether more derived alleles (i.e. the recently evolved *de novo* mutations) exhibit less attenuated IASC. To a similar end, we then chose to examine this question in a genetic background in which strong IASC over the *BA* allele had already been documented.

Chapter 5 attempts to characterise the dynamics of IASC as it progresses across the *Cyp6g1* series using the *AA*, *BA*, and *BP* alleles back-crossed for five generations to a single genetic background. We had intended this genetic background to be *Canton-S*, in which the *BA* allele is strongly sexually antagonistic (McCart et al. 2005; Smith et al. 2011; Rostant et al. 2015), but after experimentation had concluded we were informed by the Bloomington Drosophila Stock Center that the *Canton-S* stock with which they had supplied us had been inadvertently crossed with a second, unknown susceptible strain. In this new hybrid genetic background (*Canton-X*), we found no evidence of sexual antagonism across the series, but observe that resistance becomes costlier across the series for both sexes, and that the resistance conferred by all three alleles is more costly for females than males. While the reduction in fitness across the series in both sexes can be simply explained as an energetic cost of expressing higher levels of *Cyp6g1*, females experiencing higher costs across all the alleles is more difficult to explain in the context of previous work. In all previous work where sex-specific fitness effects have been assayed, *Cyp6g1* alleles tend to be beneficial for females and deleterious or neutral for males. The reversal of this trend in this hybrid genetic background is

puzzling, and our lack of knowledge about the provenance of the genetic background makes it even more difficult to interpret. However, if females are resource limited for fitness in the *Canton-X* genetic background while males are female limited, the energetic costs of resistance would be more acutely costly to females. The existence of these sex-specific fitness effects, while not sexually antagonistic, highlights the importance of considering sex when investigating the fitness effects of insecticide resistance.

Chapter 6 investigates the potential for IASC in wing interference patterns (WIPs) by testing whether WIPs can evolve independently between the sexes in *Drosophila simulans*. Using calibrated digital images and a model of *Drosophila* vision we compared the the WIPs of male and female *Drosophila simulans* from replicate populations forced to evolve with elevated or relaxed sexual selection on males for 68 generations. We found that male WIPs evolved to be brighter, higher contrast, and longwave-shifted in response to sexual selection, whereas female WIPs showed no associated response. This is the first direct evidence that WIPs can evolve in response to sexual selection in *Drosophila*, that WIPs are sexually selected in *D. simulans*, and that WIPs do not appear to be constrained from detectable independent evolution by acute IASC. WIPs are structural colouration based on wing thickness and as such they are subject to both sexual selection (mate choice on males) and natural selection (for flight performance in both sexes). As WIPs and flight capability are both mediated by wing morphology they are likely governed by the same underlying genetic architecture. This positive genetic correlation, combined with the need to maintain flight capability, likely places an upper limit on the resolution of any sexual conflict that makes WIPs an exciting new avenue to investigate IASC.

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