SEX-SPECIFIC EFFECTS OF DDT RESISTANCE IN FLIES

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.

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ABSTRACT

In *D. melanogaster*, resistance to DDT is conferred by the upregulation of a cytochrome P450 enzyme, CYP6G1. Resistant flies have tandemly duplicated *Cyp6g1* alleles that possess the LTR (Long Terminal Repeat) of an *Accord* retrotransposon inserted in the cis-regulatory region, 291bp upstream of the transcription start site. This DDT resistance allele (DDT-R) has been shown to have pleiotropic fitness benefits for female flies in at least one genetic background and with evidence of sexually antagonistic selection at this locus. In this thesis, I first review the role of transposable elements in conferring insecticide resistance and the evidence to date regarding the pleiotropic effects of DDT-R in *D. melanogaster*. By conducting life history and behavioural tests on flies of two genetic backgrounds I examine the sex-specific effects of expressing DDT-R in the absence of DDT. Finally I develop a single locus population genetics model based on these sex-specific effects and test the model using replicate laboratory populations.

The first main finding is that DDT-R incurred a male mating cost that depended on the genetic background in which DDT-R was found and that this cost coincided with strong epistasis between genetic background and DDT-R that influenced male size (Chapter 3). Following on from this result, it was confirmed that the effect of DDT-R on male size does contribute to lowered mating success but does not fully explain this fitness cost (Chapter4). Additionally, resistant males were found to have a lowered rate of courtship behaviour driven by aborted chasing of females and lower male-male aggression than susceptible males (Chapter 4). Fitness assays in wild caught strain females revealed that DDT-R confers a fecundity increase but unlike previous work, no offspring viability increases were detected (Chapter 5). Thus as with male costs, specific pleiotropic female fitness benefits to resistance depend on genetic background. Modelling of DDT-R using a simple single-locus approach (Chapter 6) provides, for the first time, a unifying explanation for past and present DDT-R frequencies in nature and in old laboratory populations. The model is consistent with an old origin for the original DDT-R mutation held at low equilibrium frequency through balancing selection of a sexually antagonistic nature. It is also consistent with continued near fixation of DDT-R long after discontinued use and matches empirical observations in laboratory populations of the Canton-S background.

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AUTHOR'S DECLARATIONS

Wayne G. Rostant (WGR) was supported by a University of Exeter Studentship. Other than Chapter 3, which was co-authored by WGR, the thesis was written by WGR with comments and editing from Nina Wedell (NW) and David J. Hosken (DJH). The DDT-R flies used for Chapters 3 and 4 were prepared, via introgression and selection, by Damian T. Smith (DTS), while those of Chapters 5 and 6 were prepared using similar methods by WGR. Further contributors to each chapter are outlined below.

Chapter 2

This chapter was published as a review article in *Advances in Genetics* 78, 169-201, with NW and DJH as co-authors. WGR, NW and DJH thank Judith Mank for comments on an earlier draft of the manuscript.

Chapter 3

The data in this chapter was collected by DTS and WGR with assistance from Martin Yeo (MY), Rob Griffin (RG), Amanda Bretman (AB), Tom Price (TP), Jack Hollis, Conner-Benjamin Parker and Nicole Goodey. Mutant marker flies were supplied by Tracey Chapman. This chapter was published in the *Journal of Evolutionary Biology* 24, 1351-1362, with DTS as the primary author and DJH, WGR, MY, RG, AB, TP, Richard ffrench-Constant and NW as co-authors.

Chapter 4

The data in this chapter was collected by WGR, Jemima Bowyer, Jack Coupland and James Facey with assistance from MD Sharma, Daniel Brown and Ali Skeats.

Chapter 5

The data in this chapter was collected by WGR, Catherine Bradford and Sophie King.

Chapter 6

The model was developed by WGR. Data on present experimental population experiments was collected by WGR. Data from McCart (2006) was also used with the knowledge and consent of the author.

CHAPTER 1: General introduction

1.1 Sex-specific pleiotropic effects of insecticide resistance

A central question in the evolution of resistance is the fitness of the organism carrying a mutant allele of a resistance gene. Theory holds that, in the absence of insecticide, the majority of insecticide-resistant organisms should show some differential survival in comparison with "wild-type" organisms. That is, resistance should be costly (e.g. Crow 1957). However, empirical evidence on the pleiotropic fitness costs of insecticide resistance is equivocal.

There are a few empirical studies that confirm that investment in resistance entails a fitness cost (e.g. Minkoff and Wilson 1992; Carrière et al. 2001; Foster et al. 2003; Smith et al. 2011). On the other hand, some authors have failed to reveal any detrimental effects of insecticide resistance (e.g. Follett et al. 1993; Baker et al. 2008; Castañeda et al. 2011), and some have demonstrated pleiotropic fitness benefits (e.g. Omer et al. 1992; Mason 1998; Arnaud and Haubruge 2002; McCart et al. 2005; Bielza et al. 2008).

In other studies, some measures of fitness have been negatively affected, others positively (Brewer and Trumble 1991), and this may involve intralocus sexual antagonism (Bonduriansky and Chenoweth 2009), where resistance alleles have opposing fitness effects depending on the sex in which they reside. This has recently been documented for DDT resistance in *D. melanogaster*, where resistance confers a strong fecundity advantage to females, but a competitive mating disadvantage to males in at least one genetic background (McCart et al. 2005; Smith et al. 2011, reproduced herein as Chapter 3).

This reflects a conflict between shared and divergent aspects of the biology of the sexes. While shared traits (such as detoxification of xenobiotics) are assumed to be controlled by a common genetic machinery in both sexes (Lande 1980), the sexes are defined by strongly divergent reproductive strategies that generate sex-specific selection on many of these shared traits (Bonduriansky and Chenoweth 2009). In the case of DDT resistance, upregulation of *Cyp6g1* by DDT resistance alleles (DDT-R; see the following section) has positive fitness consequences for females in the absence of DDT, as reflected in increased reproductive output (McCart et al. 2005). At the same time, upregulation appears to have negative effects on male fitness in at least one

genetic background (Smith et al. 2011, reproduced herein as Chapter 3). Thus, in the absence of DDT, and the strong viability selection that it imposes, there will be conflict at the DDT-R locus with positive selection on females (coupled with negative selection on males) displacing males from their phenotypic optimum and reducing their fitness.

However this is only true if there is a negative inter-sexual genetic correlation for fitness at the locus. In a second genetic background DDT-R appears to have no effect on male fitness (Smith et al. 2011, reproduced herein as Chapter 3). This reflects epistasis, where the pleiotropic fitness effect is mediated by the genetic background of the insect in question. In the course of this thesis I will report on various aspects of DDT-R's sex-specific pleiotropic effects, including sexual antagonism and epistasis.

1.2 Study system

Fifty years ago, studies in *D. melanogaster* indicated that many genes contributed to DDT resistance (Crow 1957; Kikkawa 1961; Dapkus and Merrell 1977; Hallstrom 1985) including loci on all three major chromosomes. However, work on the Hikone-R strain indicated that resistance in this strain was largely conferred by a single dominant locus on chromosome II, and this was later found to be the cytochrome P450 gene *Cyp6g1* (Daborn et al. 2001).

Tissue-specific *Cyp6g1* expression patterns have been investigated using *in situ* hybridization (Chung et al. 2007; McCart and ffrench-Constant 2008; Chung et al. 2009), real time PCR (Chung et al. 2007; McCart and ffrench-Constant 2008) and microarrays (Table 1.1, Chintapalli et al. 2007), consistently revealing enrichment in the Malphigian tubules, midgut and fat bodies. This expression in excretory and digestive tissues is consistent with a role for the gene product, CYP6G1, in metabolising xenobiotics.

Homology modelling of the molecular structure of CYP6G1 has revealed an active site of high shape and chemical complementarity with the molecular characteristics of DDT (Jones et al. 2010). While the major products of DDT metabolism *in vivo* are still uncertain, the authors of that study suggest that CYP6G1 holds DDT in such a way as to allow reaction to any of a number of metabolites (e.g. DDD, DDA), which are less lipophilic than DDT and show less neurotoxicity when tested on sensory nerves.

Resistant *Cyp6g1* alleles (DDT-R) were found to have a defective copy of a transposable element (specifically, an *Accord*-LTR retrotransposon) inserted about 300 bp upstream of the transcription start site. Subsequently, the molecular mechanism of upregulation was identified with cis-regulatory sequences in the TE being primarily responsible for increased *Cyp6g1* transcription (Chung et al. 2007). One recent study has revealed a stronger genetic association between DDT-R (i.e. *Accord* LTR-inserted) *Cyp6g1* alleles with nicotine resistance than DDT resistance (Li et al. 2012) suggesting that the original function of the enzyme was to detoxify naturally-occurring plant allelochemicals.

Regardless of its original substrate, CYP6G1's proposed active site structure can accommodate differently shaped substrates ranging from imidacloprid to malathion (Jones et al. 2010). Empirically, increased expression of CYP6G1 is associated with cross-resistance to a number of pesticides other than DDT including imidacloprid, nitenpyram, and lufenuron (Daborn et al. 2002; Catania et al. 2004; Schlenke and Begun 2004; Daborn et al. 2007). Recently, heterologous expression of CYP6G1 in *Escherichia coli*, followed by detailed study of activity patterns and binding properties on a wide variety of insecticides has provided further evidence for its broad specificity (Cheesman et al. 2013).

This broad specificity may in part account for the prevalence of DDT-R alleles in nature. Catania et al. (2004) conducted a survey of the *Accord*-LTR insertion at *Cyp6g1* in 673 lines from 34 populations around the world. They found near fixation of the *Accord*-LTR-inserted alleles in non-African and North and Western African *D. melanogaster* populations (85 to100% of chromosomes sampled), with significantly lower frequencies in East African populations (32 to 55%). Also, a selective sweep at *Cyp6g1*, associated with strong recent selection, has been demonstrated (Catania et al. 2004; Schlenke and Begun 2004).

Variation in the *Accord*-LTR-inserted allele has also been found - diagnostic PCR revealed some variability in product size, and subsequent cloning and sequencing of the variants revealed an insertion of another TE, this time a partial *P* element, nested within the *Accord*-LTR in a New Delhi line (Catania et al. 2004). Furthermore, Emerson et al. (2008), using genome-wide tiling arrays, found copy number polymorphism in *D.*

melanogaster at *Cyp6g1*, with 13 of 15 lines tested showing a duplication encompassing both *Cyp6g1* and *Cyp6g2*.

Most recently, Schmidt et al. (2010) characterized copy number variation and further allelic variation at the *Cyp6g1* locus. Characterization of *Cyp6g1* copy number variation and TE insertion complexity in the *D. melanogaster* RK146 strain revealed two full-length copies of *Cyp6g1* (named *Cyp6g1-a* and *Cyp6g1-b*). A repeat unit was found between the two full-length copies that contained a fusion of partial copies of both *Cyp6g1* and *Cyp6g2*, the gene found downstream of *Cyp6g1-b*. Both copies of *Cyp6g1* were found to contain the LTR of the *Accord* element. Unexpectedly, a *HMS-Beagle* TE was found inserted into the *Accord*-LTR upstream of *Cyp6g1-a*. Testing of other *D. melanogaster* lines revealed that the partial *P* element found by Catania et al. (2004) was located upstream of *Cyp6g1-b*. Schmidt et al. (2010) also demonstrated an allelic progression of five different alleles, including the ancestral allele lacking any TE insertions and four alleles that involve duplication of *Cyp6g1*. They reason that these alleles represent multiple adaptive steps at *Cyp6g1*, with increased DDT resistance being demonstrated along the allelic progression. Their survey of *D. melanogaster* global populations showed that most flies in Europe, Asia, and the United States carry the *Cyp6g1* duplication and the double *Accord*-LTR (no *P* element) insertion or the *Cyp6g1* duplication and combined *Accord*-LTR insertion/*Accord*-LTR (with nested *HMS-Beagle*) insertion.

The question of the age of the original *Accord*-inserted allele remains open. In their survey, Schmidt et al. (2010) did not find any *Cyp6g1* alleles with the insertion that did not also represent a duplication, nor did they find any gene duplicates which did not also contain the *Accord* insertion. This strongly suggests that either the original insertion and duplication events occurred simultaneously, or, more likely, the original *Accord* insertion preceded the duplication. If the latter case is true, then we are yet to find the original *Accord*-inserted allele.

Catania et al. (2004) suggested that the lower than expected reduction in variability in microsatellites around the *Cyp6g1* locus could be explained most parsimoniously if the *Accord*-LTR insertion occurred at low frequency in African populations before the species' global expansion. This would imply that the insertion was already part of the genetic variation at this locus well before it permitted

adaptation to insecticide and may be an example of how TEs provide latent genetic variation facilitating adaptive responses to selection.

The absence of strong DDT selection since its ban in most countries globally over the past 30 years has not brought about the loss of DDT-R alleles in *D. melanogaster*, as might be expected from theory - overexpression of P450 genes must have a cost, and if the resistance-to-DDT benefit to balance this cost is not there, then selection should remove the resistance allele. The near fixation of *Accord*-inserted *Cyp6g1* alleles in worldwide populations (Catania et al. 2004) and the seemingly adaptive, on-going elaboration of these alleles through subsequent TE insertion increasing DDT resistance (Schmidt et al. 2010) thus poses a puzzle.

The most obvious explanation is that DDT-R alleles remain under strong xenobiotic selection, as they confer cross-resistance to other pesticides and naturally occurring allelochemicals. There are other explanations, however, and in the present thesis we examine in some detail the nature of DDT-R's sex-specific pleiotropic effects to determine whether sex-specific selection can account for the patterns in allele frequency seen in nature.

This work is motivated by previous studies which demonstrated a putative negative effect of *Cyp6g1* upregulation on male fitness (Drnevich et al. 2004) and, specifically the pleiotropic effects of DDT-R alleles on female fitness documented in McCart et al. (2005) , McCart (2006) and McCart and ffrench-Constant (2008). In these latter studies it was found that resistant females laid more eggs and that these eggs and their associated larvae enjoyed a higher fitness which then disappeared in the pupa. RNAi flies showed no obvious phenotypes (McCart and ffrench-Constant 2008) leaving no clues as to why excess *Cyp6g1* transcripts confer a fitness advantage when packaged into embryos. The authors of that study speculated that CYP6G1 may have antioxidant activity in its broadest sense, or that it may metabolise a specific insect growth hormone such as juvenile hormone (JH) and therefore alter embryonic and larval development. This latter possibility is intriguing, given that *Cyp6g1* has a broad catalytic profile (Daborn et al. 2007; Jones et al. 2010; Cheesman et al. 2013) and that its closest paralogue, *Cyp6g2* may be involved in JH synthesis (Chung et al. 2009).

1.3 A note on introgression

In the studies contained in this thesis, preparation of fly populations to investigate the pleiotropic effects of DDT resistance involves introgression of the allele of interest into different genetic backgrounds. In this procedure the DDT resistance allele (DDT-R) is introduced to a genetic background through repeated backcrossing and selection with the insecticide. The Canton-S flies used in Chapters 3 and 4 were prepared prior to my PhD, using a recently wild-caught isoline that possessed the resistance allele and seven generations of backcrossing (Smith et al. 2011).

Subsequently, these introgressed populations were lost through 'contamination' of population cages. That is, routine PCR diagnostic revealed variation at *Cyp6g1* in previously homozygous populations, indicating movement of flies of unknown background into the cages. This necessitated a repeat of the introgression procedure to prepare new populations for the subsequent studies found in Chapters 5 and 6. For these latter studies I chose to follow McCart et al. (2005) who used Hikone-R as the source of DDT-R and examined life-history traits after five generations of backcrossing and selection.

This potential hitchhiking of surrounding sequence with the *Cyp6g1* locus is an obvious drawback which can never fully be eliminated through backcrossing (see Naveira and Barbadilla 1990). However, as a test of the robustness of her findings McCart (2006) repeated a subset of her life-history assays on flies which had been backcrossed a further 15 generations, and found a consistent pleiotropic benefit to females. This added backcrossing would have had the effect of narrowing the genetic difference between resistant and susceptible flies and reinforces the claim that it is the variation at the *Cyp6g1* locus which results in the fitness effects observed.

1.4 Thesis outline

During the course of my PhD I conducted a number of life-history and behavioural assays to compare the sex-specific effects of DDT-R in the absence of DDT selection. The following chapters follow a logical sequence of enquiry beginning with a literature review (Chapter 2) followed by four 'data chapters' each of which involves a discrete but related study, and that have been so divided and written as to reflect the intention to publish each as independent manuscripts. Thus, there is some overlap between the

four chapters in introductory text and in the description of introgression alluded to in the previous section.

Chapter 2 reviews the phenomenon of Transposable elements (TEs) in insecticide resistance. This chapter was published earlier this year in *Advances in Genetics* and was co-authored by Nina Wedell and David Hosken, as stated in the Author's Declarations. In it I give brief overviews of the nature of TEs, their role as sources of endogenous, spontaneous mutation and their implication in various instances of insecticide resistance insecticide resistance. I also highlight areas of current research, some of which is presented in Chapters 3-6, and suggest avenues for future research on the DDT-R system in *Drosophila*, including the need for surveys of the prevalence and fitness consequences of DDT resistance alleles in other genetic backgrounds and in *D. simulans*.

Chapter 3 was published in the *Journal of Evolutionary Biology* (see Author's Declarations) and describes a series of fitness assays designed to determine the effect of DDT resistance alleles on male fitness determinants in two genetic backgrounds. Assays examined relative pre- and post-copulatory fitness of backcrossed resistant males when compared to males of the susceptible stock for each genetic background. We found that DDT-R conferred a male competitive mating cost in the Canton-S background but not when expressed in a second wild-caught isoline.

Chapter 4 builds on the results of Chapter 3 by examining the basis for the male competitive mating disadvantage found in DDT-R flies of the CS background. To this end I conducted three separate experiments to first explore whether size mediates competitive mating success in DDT-R flies and then demonstrate how DDT-R also affects two aspects of behavioural phenotype that relate to male competitive reproductive success, namely courtship and aggression.

Chapter 5 follows on the study of male fitness in the WC background in Chapters 3 and 4 by conducting simple life-history tests on WC females to investigate whether the female benefit documented in the Canton-S background by McCart et al. (2005) applies to this other genetic background. I find that, as in Canton-S females there is a fecundity benefit of DDT-R, but unlike Canton-S there is no evidence of an increase in early offspring viability.

In Chapter 6 I constructed a simple single-locus population genetic model in an attempt to generate predictions about the population level outcomes of the sex-specific effects documented in McCart et al. (2005) and in Chapters 3-5. The model dynamics predict the invasability (and rate of invasion) of susceptible populations by DDT-R, and analytical solutions for genotype (and allele) frequency equilibria. I applied parameter values from the previously mentioned empirical studies to predict the trajectories of DDT-R frequency in experimental populations of both Canton-S and WC background flies and conducted a simple test of the model using replicate small population cages (vials) initiated at different starting frequencies.

Chapter 7 is a discussion of the overall findings of the thesis with a description of on-going work on the DDT-R system here at the Centre for Ecology and Conservation, University of Exeter and suggestions for future avenues of research.

Table 1.1. *Cyp6g1* expression patterns from FlyAtlas microarray study (Chintapalli et al 2007). 'mRNA Signal' indicates how abundant the mRNA is. 'Present Call' indicates out of 4 Affymetrix arrays how many times it was detectably expressed. 'Enrichment' is an indicator of tissue specificity and measures how much greater the signal is compared to whole flies. Shading indicates enrichment > 4.

Tissue	mRNA Signal	Present Call	Enrichment
Brain	21 ± 2	4 of 4	0.00
Head	1469 ± 142	4 of 4	3.40
Eye	568 ± 38	4 of 4	1.30
Thoracicoabdominal ganglion	21 ± 8	2 of 4	0.00
Salivary gland	8 ± 4	0 of 4	0.02
Crop	21 ± 2	4 of 4	0.00
Midgut	1852 ± 33	4 of 4	4.20
Tubule	5542 ± 1174	4 of 4	12.70
Hindgut	145 ± 17	4 of 4	0.30
Heart	929 ± 103	4 of 4	2.13
Fat body	2456 ± 52	4 of 4	5.62
Ovary	1 ± 0	0 of 4	0.00
Testis	2 ± 1	1 of 4	0.00
Male accessory glands	27 ± 3	2 of 4	0.10
Virgin spermatheca	4664 ± 111	4 of 4	10.67
Mated spermatheca	2505 ± 286	4 of 4	5.73
Adult carcass	1297 ± 147	4 of 4	3.00
Larval CNS	3 ± 1	0 of 4	0.01
Larval Salivary gland	37 ± 3	4 of 4	0.09
Larval midgut	340 ± 108	4 of 4	0.78
Larval tubule	88 ± 19	4 of 4	0.20
Larval hindgut	30 ± 5	4 of 4	0.07
Larval fat body	4886 ± 667	4 of 4	11.20
Larval trachea	16 ± 7	3 of 4	0.04
Larval carcass	17 ± 7	3 of 4	0.04
S2 cells (growing)	2 ± 0	0 of 4	0.01
Whole fly	437 ± 53	4 of 4	

CHAPTER 2: Transposable elements and insecticide resistance

2.1 Abstract

Transposable elements (TEs) are mobile DNA sequences that are able to copy themselves within a host genome. They were initially characterized as selfish genes because of documented or presumed costs to host fitness, but it has become increasingly clear that not all TEs reduce host fitness. A good example of TEs benefiting hosts is seen with insecticide resistance, where in a number of cases, TE insertions near specific genes confer resistance to these man-made products. This is particularly true of *Accord* and associated TEs in *Drosophila melanogaster* and *Doc* insertions in *Drosophila simulans*. The first of these insertions also has sexually antagonistic fitness effects in the absence of insecticides, and although the magnitude of this effect depends on the genetic background in which *Accord* finds itself, this represents an excellent example of intralocus sexual conflict where the precise allele involved is well characterized. We discuss this finding and the role of TEs in insecticide resistance. We also highlight areas for further research, including the need for surveys of the prevalence and fitness consequences of the *Doc* insertion and how *Drosophila* can be used as models to investigate resistance in pest species.

2.2 Introduction

The concept of an essentially stable genome, with each specific genetic element confined to a single locus was developed during the first few decades of last century. This simple picture first came under serious challenge through the work of McClintock (1950, 1984) who, while analysing chromosome breakage in maize at Cornell University, first discovered what we now know as transposable elements (TEs). McClintock called these mobile elements “controlling elements,” a term which reveals her early assertion of their potential involvement in gene expression.

This view has turned out to be remarkably prescient. However, TEs have spent much of the time since their discovery under the monikers “junk DNA” and “selfish DNA,” revealing a general opinion that these mobile stretches of DNA played little if any part in evolution of their “hosts.” TEs were largely thought to have no influence on host genes and were interesting only insofar as their unique form of drive allowed them to invade host genomes and spread through populations. A recent review by

Biémont (2010) gives a good account of how prevailing views have come full circle to vindicate McClintock's proposal that TEs are crucial components of genomes and drivers of their evolution through their ability to affect gene expression. This journey from junk to critical agents of adaptive change has gathered pace as the sequencing of whole genomes has revealed the ubiquity and diversity of TEs.

There was an initial reluctance by many geneticists to accept that maize was not an anomalous case. That so-called jumping genes might exist in other genomes was difficult to reconcile with the fact that genetic maps had revealed remarkable homogeneity between individuals within species. The success of mapping of genes to precise positions on a chromosome was incompatible with genes moving around the genome. The isolation of bacterial TEs from *Escherichia coli* (Shapiro 1969) was the first step toward acceptance that TEs were a general feature of genomes.

In spite of these discoveries, it was not until the 1970s that scepticism over the fundamental importance of TEs finally began to erode (Biémont and Vieira 2006). The reason for this was the emergence of hybrid dysgenesis—a phenomenon observed when females of laboratory *Drosophila melanogaster* stocks were mated with males derived from natural populations. The progeny of these crosses displayed unusual germ line phenotypes including sterility, high mutation rate, and increased frequency of chromosomal aberration, while no such deficiencies exist in the reciprocal cross. The source of the dysgenesis turned out to be a TE called the *P* element which was present in wild strains but absent in laboratory strains.

Concurrent with a developing understanding of the ubiquity and importance of TEs within genomes has been the increased use of pesticides to control pest organisms, particularly from the 1950s onward (Wilson 2001). This strong pervasive selection over many generations has provided the theoretical conditions under which adaptation by major genes might be favoured, although early models suggested that strong selection, while necessary, is probably insufficient to favour major gene over polygenic adaptation (Lande 1983). Nevertheless, the overwhelming empirical evidence is that the evolution of pesticide resistance is most often associated with the spread of a major mutation (Wilson 2001), and it has been suggested that it is not the strength of selection per se, but the amount of phenotypic change required to achieve

adaptation which determines the genetic architecture of the adaptive response (Macnair 1991).

In light of these theoretical and empirical findings, it is perhaps unsurprising that TEs are increasingly being implicated in the adaptive response of organisms to man-made xenobiotics. In this review, we highlight the properties of TEs and insecticide resistance that make the former uniquely suited to the latter adaptive response. While drawing on several putative and several well documented examples of TE-mediated insecticide resistance from the literature, we focus primarily on the striking cases of DDT resistance in *D. melanogaster* and *Drosophila simulans* which have been particularly well studied. In both instances, the resistance phenotype has been conferred by parallel insertions of TEs near a cytochrome P450 gene.

2.3 Transposable elements

Definition and origin

TEs, simply put, are DNA sequences that have the capacity to transpose. That is, they change their chromosomal location from one position to another within the same genome, within a single cell (Kidwell and Lisch 2001; Hua-Van et al. 2011). They typically encode genes to promote this movement, in which case transpositional ability is intrinsic. These TEs are said to be autonomous and contrast with nonautonomous TEs which cannot transpose on their own, instead depending on the transposition machinery of other TEs (Kidwell and Lisch 2001; Wicker et al. 2007; Hua-Van et al. 2011).

While questions concerning the origin and early evolution of TEs may never be fully resolved, it does appear that their evolution has occurred primarily through the serial addition of domains, several of which seem likely to have evolved from bacteria (Kidwell and Lisch 2001). The question of a common origin for all TEs remains open (Wicker et al. 2007).

Classification of TEs

The first TE classification system was proposed by Finnegan (1989) and included two main TE classes which were distinguished by their transposition intermediate. Class I elements include those which transcribe via an RNA intermediate and, using a “copy-

and-paste" mechanism, establish new copies of themselves elsewhere in a genome. Class II elements, in contrast, excise from donor sites and move to new locations in a genome without use of an RNA intermediate, that is, they use a "cut-and-paste" method of transposition (Figure 2.1).

To cope with an expanded array of TEs with diverse characteristics, Wicker et al. (2007) proposed a classification scheme that built on Finnegan's original proposal (Table 2.1) by incorporating mechanistic and enzymatic criteria to the classification procedure. The original two classes were retained, and two subclasses within class II (DNA transposons) were formed to separate DNA transposons which leave the donor site (excision) to reintegrate elsewhere (subclass 1) from those which copy themselves for insertion (subclass 2). The next hierarchical ranking (i.e., order) marks differences in the insertion mechanism and thus organization and enzymology. Superfamilies within an order share a replication strategy but are distinguished by large-scale features such as the structure of protein or noncoding regions. Families within superfamilies are defined by DNA sequence conservation.

Transposition rates

The best data on TE transposition rates have come from laboratory experiments on *D. melanogaster* (Burt and Trivers 2006). Rates are variable, ranging from 2.9×10^{-6} per element per generation for P elements in inbred lines (Dominguez and Albornoz 1996) to 0.25 per *P* element in dysgenic crosses. However, they are typically low—estimates of 10^{-4} with order of magnitude variation have been found for LINEs and LTR retrotransposons in two separate experiments (Nuzhdin and Mackay 1995; Maside et al. 2000). That these estimates tend to be higher than excision rates (of the order 10^{-6}) implies that TEs should, in general, accumulate in genomes over evolutionarily trivial timescales (Burt and Trivers 2006). As a recent, well-cited example of this, *P* elements had been shown to have invaded all known wild populations of *D. melanogaster* in the matter of about 50 years (Anxolabéhère et al. 1988) after horizontal transfer from *Drosophila willistoni* (Daniels et al. 1990).

The abundance and distribution of TEs

TEs have been discovered and characterized in most species that have been adequately examined (Kidwell and Lisch 2001). They are more ubiquitous in eukaryotes where they are present in virtually all species investigated to date, with few exceptions (Wicker et al. 2007). In prokaryotes, on the other hand, more than 20% of sequenced genomes lack TEs or their remnants (Touchon and Rocha 2007). TEs also tend to be more abundant in eukaryote genomes, making up to 80% of the genome. For example, they comprise 60% of the maize genome (Messing and Dooner 2006), 45% of the human genome (Lander et al. 2001; Cordaux and Batzer 2009), and 15% of the *D. melanogaster* genome (Dowsett and Young 1982), while in prokaryotes, they form only a maximum of 10% of genomes (Hua-Van et al. 2011).

Thomas (1971) famously coined the term "C-value paradox" to define the then-curious lack of correlation between genome size (measured as DNA content or C-value) and the biological complexity of eukaryotes. Subsequently, it was found that, rather than correlating with gene content, genome size often correlates with quantities of TE and TE-derived DNA. In fact, because the abundance of TEs within a genome can vary widely (Biémont and Vieira 2006), they, in addition to repetitive DNA, are major determinants of genome size within taxa (Bennetzen 2005). For example, the genome size of barley is 10 times larger than that of rice (Argumuganathan and Earle 1991), a related grass with which it shows a great degree of synteny except that its genes are separated by large clusters of retrotransposons.

2.4 Effects of TEs on host fitness and evolution

TEs as selfish DNA

Selfish genetic elements (SGEs) may be defined as stretches of DNA that act narrowly to advance their own interests at the expense of the whole organism by ensuring that a disproportionate fraction of offspring carry the DNA in question (Burt and Trivers 2006). The concept of TEs as "selfish" or "parasitic" was codified in seminal papers by Doolittle and Sapienza (1980) and Orgel and Crick (1980), but while the view of TEs as SGEs is now widely accepted (Werren 2011), it perhaps obscures the continuum of interactions (from extreme parasitism to obligate mutualism) between host and TE (Kidwell and Lisch 2001) that often profoundly influence host genome evolution (Hurst

and Werren 2001; Kidwell and Lisch 2001; Biémont and Vieira 2006; Feschotte 2008; Biémont 2010; Hua-Van et al. 2011). Nevertheless, it is clear that the default view of TEs cannot be that they are simply functional parts of the genome. Brookfield (2005), in developing an analogy first made by Kidwell and Lisch (2001), describes the interaction of TEs and their hosts (and indeed between TEs within a host) in terms of the "ecology of the genome." He suggests that questions about TE numbers, diversity, and population dynamics within genomes have ecological parallels with species in communities, and ecology therefore provides insights into the biology of TEs.

While most SGEs compete for representation at a single locus, TEs accumulate by copying themselves to new genomic locations and it is this unique aspect of their drive that lies at the heart of their influence on host fitness and evolution. Because TEs can transpose at a frequency (typically 10^{-5} to 10^{-3} per element per generation) that is often much higher than classical nucleotide-base substitution rates (10^{-9} to 10^{-8}), they are powerful producers of the raw material for evolution (Biémont and Vieira 2006). The mutations caused by TE insertion and excision are also diverse, encompassing a broad spectrum from small-scale nucleotide changes to large chromosomal rearrangements (Kidwell and Lisch 2001; Hua-Van et al. 2011) including TE-mediated gene duplication (Jiang et al. 2004; Xiao et al. 2008; Yang et al. 2008). The combination of these two factors means that TEs may play an especially important role in evolution as the main source of spontaneous internal mutations (Kidwell and Lisch 2001; Li et al. 2007). For example, the high rate of new insertions of *Alu* and *LINE-1* elements (Xing et al. 2009) means that TE insertions are a significant source of mutations in humans (Cordaux and Batzer 2009). Additionally, 50–80% of mutations in *Drosophila* are the result of TE insertions (Green 1988; Finnegan 1992; Biémont and Vieira 2006).

Negative effects on hosts

As with other types of mutation, TE-induced changes will tend to be either harmful or neutral in their fitness effects on the host. TEs harm hosts in a number of ways. Insertions may disrupt coding sequences or cis-regulatory regions, while recombination between TE copies can result in deletions and rearrangements. On top of this are the costs to the host of transcription and translation of large numbers of TEs (Charlesworth et al. 1994; Kidwell and Lisch 2001; O'Donnell and Burns 2010). Fitness

reductions have been quantified for *P* element transposition in *D. melanogaster* (e.g. Fitzpatrick and Sved 1986; Mackay 1986, 1989; Mackay et al. 1992; Currie et al. 1998) where even nonlethal inserts tend to reduce host fitness by as much as 12.2% per insert when homozygous (Eanes et al. 1988; Mackay et al. 1992).

In most cases, highly deleterious insertions will be quickly removed by selection, but areas of the genome which experience low recombination might be expected to accumulate insertions that have even moderately harmful effects. Y chromosomes and neo-Y chromosomes, where TE fixation rates tend to be much higher than on X chromosomes or autosomes, present such a case—since recombination is suppressed, selection is expected to be less effective due to hitchhiking and other effects (Charlesworth and Charlesworth 2000). For example, TEs have accumulated at a very high abundance on the *Drosophila miranda* neo-Y chromosome and might have been involved in causing a loss of gene activity (Steinemann and Steinemann 1998).

TE population dynamics

While there is continuing debate as to which of the various sources of harm are more important (Burt and Trivers 2006), it is the interplay between selection for increased replication at the TE level, but against deleterious host fitness effects that is responsible for TE population dynamics. Most of the deleterious phenotypic effects of TEs will be removed from a population over time by purifying selection (Kidwell and Lisch 2001). Nonetheless, a population genetics model has shown that TEs can produce significant deleterious effects in the host and still spread in the population (Hickey 1982). Other models (e.g. Brookfield and Badge 1997) highlight the importance of host population demography on TE copy number. In these models, factors such as small host effective population size (N_e) attenuate the power of natural selection in regulating TE copy number. Empirical evidence suggests that these factors play an important part in TE copy number and distribution in natural populations (Charlesworth and Charlesworth 1995; Lockton et al. 2008; Lockton and Gaut 2010). Lynch and Conery (2003) suggest that many aspects of complex genomes such as TE abundance were indirect consequences of reduced N_e, producing less effective selection against mildly deleterious insertions.

While transposition rates tend to exceed excision rates, there is strong evidence that TE copy number is regulated. For example, the most abundant TE family still active in *D. melanogaster* is the retrotransposon *roo*, and there are only 60 full-length copies per haploid genome in the euchromatin (Kaminker et al. 2002). Charlesworth and Charlesworth (2010) list five kinds of processes which may be involved in regulating TE abundance: (1) self- and/or host regulation of transposition rates, (2) selection against mutations, (3) ectopic exchange, (4) direct negative fitness effects of transposition on host fitness, and (5) indirect effects of copy number on fitness. They conclude that while each of these processes is plausible, and they are not mutually exclusive, the ectopic exchange model seems to be most consistent with current evidence (Charlesworth et al. 1997; Charlesworth and Charlesworth 2010). Petrov et al. (2011) come to similar conclusions while examining the population frequencies of 755 TEs in six *D. melanogaster* populations.

Negating host fitness costs

1. Cost minimization at the level of TEs

The fate of a TE in its host population thus depends not only on transposition rate but also on host fitness effects, and TEs themselves should evolve to reduce host harm (Burt and Trivers 2006). Germ line specificity of transpositional activity, as demonstrated in a number of class I (e.g. *I* elements and *gypsy*) and class II (e.g. *P* elements and *hobo*) elements in *Drosophila*, is one such adaptation (Burt and Trivers 2006) since transposition within the soma does not benefit the TE but does damage the host (Charlesworth and Langley 1986).

Another damage-limiting strategy adopted by TEs is to insert preferentially into safe sites in the genome, as seen in *Ty1*, *Ty2*, *Ty3*, and *Ty4* retrotransposons in baker's yeast which target intergenic regions upstream of tRNA genes (Kim et al. 1998). Additionally, there are many TEs which integrate into gene-rich regions, but which use mechanisms that prevent the disruption of open-reading frames (ORFs) (Levin and Moran 2011). One example of this is seen in *D. melanogaster P* elements which tend to avoid disrupting ORFs by inserting within 500 bp upstream of host gene transcription start sites (Bellen et al. 2011). Other safe haven transpositions include insertion into other TEs and preferential insertion at or near telomeric chromosome ends. Examples

of the latter include the *HeTA*, *TART*, and *TAHRE* non-LTR retrotransposons which comprise the ends of *D. melanogaster* chromosomes (Biessmann et al. 1992; Levis et al. 1993; George et al. 2010).

It has also been proposed that some TEs may have evolved autoregulation of transposition rate to avoid the deleterious effects of uncontrolled transposition bursts (Burt and Trivers 2006; Hua-Van et al. 2011). Theory suggests that the circumstances under which such regulation would evolve are probably common, although unlikely to exist in unstructured random-mating hosts (Charlesworth and Langley 1986, 1989). Nevertheless, there are examples where self-regulation appears to be the case such as the *P* element-encoded repressor which represses transposition and excision (Robertson and Engels 1989).

2. Cost minimization at the level of the host: TE suppression

Hosts are not defenceless against harmful transposition. Many organisms have evolved complex mechanisms to deal with TEs. Small RNA-based mechanisms act to defend eukaryotic cells against TEs by posttranscriptional disruption of TE mRNA (Aravin et al. 2007; Malone and Hannon 2009; van Rij and Berezikov 2009). Another way in which some host taxa suppress their TEs is through epigenetic control, including methylation. In fact, it is widely thought that epigenetics, whose processes are commonly used by metazoans in cell lineage-specific gene regulation, first evolved to defend against foreign DNA including TEs (Hua-Van et al. 2011). This is one example of how the prolonged interaction of host and TE has ultimately benefited the host—it is far from the only one.

Beneficial effects of TEs

As with any other source of mutation, TEs can occasionally produce beneficial genetic alterations to host genomic DNA. A beneficial insertion would be expected to go to fixation within a population, and TE fixation has been observed, particularly in *D. melanogaster* (González and Petrov 2009). The *S* element(s) associated with the Hsp70 (heat-shock protein) genes in *D. melanogaster* is one possible example of a beneficial TE (Maside et al. 2002). While the functional significance of this insertion has not been elucidated, there is strong evidence of a selective sweep around it. Furthermore, the

insertion apparently occurs in a freely recombining region of the genome, which substantially lowers the probability of fixation via drift.

Co-option/domestication

In contrast to the benefits derived from genetic alteration of host genomic sequences *per se*, TE sequences themselves may be co-opted for host function, a process which has been called "domestication" or "exaptation" when TE-coding sequence function has been appropriated for host use. There are several examples of this in the literature, one of the most cited being the full domestication of the *Drosophila* telomeric retrotransposons *HeTA* and *TART* which function as telomerase to heal chromosome ends. Even noncoding TE sequences may be useful—one striking example of the fixation of a beneficial insertion which is of particular importance to this review is found in the evolution of DDT resistance in *D. melanogaster*. Here, an *Accord* retrotransposon-derived sequence inserted upstream of a cytochrome P450 gene has been shown to upregulate the detoxification enzyme and increase pesticide resistance (Daborn et al. 2002; Chung et al. 2007). The remainder of the review focuses on insecticide resistance and how TEs influence this.

2.5 Insecticide resistance

The rate of evolution

Given the evolutionary potential of TEs, perhaps it is not surprising that they play an important role in such key fitness traits as pesticide resistance. Over the past 100 years, there has been an increased use of toxic chemicals to control pest organisms, particularly from the 1950s onward (Wilson 2001). This strong, pervasive source of selection has demonstrated the tremendous capacity of populations to evolve resistance to toxins. Since the first insecticide resistance case was reported almost a century ago (Melander 1914), there have been thousands of cases of resistance in hundreds of species (Georghiou and Lagunes-Tejeda 1991; Whalon et al. 2008). Some of the most dramatic examples of microevolution in action have come from selection for chemical resistance (Hartl and Clark 1997), with resistance evolving in as few as 5–50 generations (May 1985) and toward rapid global fixation in many insect pest populations (Catania et al. 2004; Schlenke and Begun 2004; Whalon et al. 2008).

What is resistance?

From a functional point of view, insecticide resistance may be defined as the ability of an organism to survive a dose of insecticide that is lethal to a susceptible one (Georghiou and Saito 1983), and dynamically, it has also been described as the microevolutionary process whereby genetic adaptation through pesticide selection results in populations of susceptible insects being replaced by resistant ones over a period of time (Wilson 2001). The biochemical mechanisms and molecular genetics underlying resistance have been well studied and have been the subject of several books (Denholm et al. 1999; Ishaaya 2001; Clark and Yamaguchi 2002) and reviews (Feyereisen 1995; Oakeshott et al. 2003; ffrench-Constant et al. 2004).

The proximate biochemical mechanisms of resistance can be divided into four main categories (Wilson 2001). The first of these is behavioural resistance (i.e., avoidance of the insecticide), which may involve genetic changes, but is probably of minor importance even though it has been documented for a few species (Sparks et al. 1989). Reduction in the penetrative ability of the toxin is a second mechanism, but again this does not seem to be of major importance (Wilson 2001). Target-site inactivation (changes in the insecticides site of action) is a very important biochemical resistance mechanism (Hollingworth and Dong 2008; Wilson 2001). Every potent insecticide has one or more specific binding sites on critical macromolecules, and changes in the ability of the toxin to bind must affect its impact on the insect (Hollingworth and Dong 2008). Lastly, biotransformation, the metabolic breakdown of a toxin, is a common defence against natural xenobiotics (Li et al. 2007). It is therefore not surprising that, with the widespread use of synthetic organic agricultural chemicals, the enzymatic systems which originally evolved to detoxify phytotoxins should been enlisted to defend against insecticides (Wilson 2001). Three types of enzymes—esterases (through ester hydrolysis), cytochrome P450 monoxygenases (through oxidation), and glutathione transferases (through ester hydrolysis)—are commonly used to transform insecticides into less toxic products (Hollingworth and Dong 2008).

When an insecticide is first introduced, the target population largely consists of susceptible phenotypes (Roush and McKenzie 1987; Mallet 1989; Macnair 1991; McKenzie and Batterham 1994). Within the population, there will be a distribution of

susceptibility based on factors such as size, age, and physiological condition (McKenzie and Batterham 1994), which are generally polygenically inherited. Insecticide selection on this distribution will act via the phenotype and resistance will be polygenically inherited, combining pre-existing factors of primarily minor effect (such as size and developmental rate) (ffrench-Constant et al. 2004). This type of selection is seen in most laboratory studies (McKenzie and Batterham 1994; ffrench-Constant et al. 2004), which explains why early studies of DDT resistance (e.g. Crow 1957) determined that resistance evolution was a polygenic response.

This contrasts strongly with what has been found in natural populations, where resistance to particular insecticides often involves one or two major genes (Roush and McKenzie 1987; Field et al. 1988; Mallet 1989; Raymond et al. 1989; McKenzie and Batterham 1994; ffrench-Constant et al. 2004). This may represent detection bias, but another explanation could be that insecticides in the field tend to occur at concentrations which favour variation outside of the normal phenotypic distribution (i.e. rare resistant mutations of major effect). Natural populations are much larger than laboratory populations and so more likely to contain individuals with these rare mutations. A second reason for the preponderance of monogenic resistance in the wild may be evolutionary constraint resulting from opposing natural selection on multiple targets. The nature of the ultimate genetic changes which lead to monogenic resistance also varies with respect to the proximate biochemical mechanism involved (Wilson 2001).

Mechanisms

Target-site inactivation is usually effected by subtle changes in the target protein—it is therefore easy to understand the importance of point mutations for this resistance mechanism (Wilson 2001). An altered protein must retain at least some degree of normal function while decreasing its xenobiotic sensitivity, which explains the highly conserved nature of such changes (ffrench-Constant 1999; ffrench-Constant et al. 1998; Wilson 2001; Li et al. 2007). A striking illustration of this is the parallel evolution of cyclodiene resistance in a wide range of pest species and in *Drosophila*, which is a result of the same single amino acid substitution in the chloride ion channel pore of

the gamma-aminobutyric acid receptor protein (Thompson et al. 1993; ffrench-Constant et al. 1998).

Metabolic resistance, on the other hand, tends to involve the overexpression of existing metabolic enzymes either through gene amplification (i.e. gene duplication, which results in more gene product) or alterations in their regulatory systems, which increase transcription and/or stabilize mRNA (Wilson 2001; Li et al. 2007; Hollingworth and Dong 2008). Examples of resistance through gene copy increase are seen for esterase genes in mosquitoes and aphids, GSTs in the housefly and the aphid *Nilaparvata lugens* and cytochrome P450s in three dipterans including *D. melanogaster* and *D. simulans* and the potato aphid *Myzus persicae* (reviewed in Devonshire and Field 1991; Bass and Field 2011). A particularly striking example is provided by resistant *Culex pipiens quinquefasciatus* mosquitoes, where the esterase gene *B1* is amplified in a tandem array as much as 250-fold, conferring high organophosphate (OP) resistance (Mouchès et al. 1986, 1990; Karunaratne et al. 1993).

Gene upregulation is the most common process involved in P450-mediated insecticide resistance, but upregulation has also been documented for the other two major classes of detoxification enzymes already mentioned (Li et al. 2007). This is usually achieved through changes (point mutations or indels) in either cis- or trans-regulatory loci. An example of the former is provided by the P450 *Cyp6g1* gene in *D. melanogaster* where the insertion of a defective copy of the *Gypsy*-like LTR retrotransposon *Accord* in the 5' promoter region results in upregulation of the enzyme and cross-resistance to DDT, imidacloprid, nitenpyram, and lufenuron (Daborn et al. 2002; Catania et al. 2004; Schlenke and Begun 2004). As an example of the latter, overexpression of a GST allele in the resistant *Aedes egypti* GG strain is due largely to a loss-of-function mutation in an unidentified trans-acting repressor that represses mRNA transcription and/or decreases mRNA stability in the susceptible strains (Grant and Hammock 1992).

Costs of resistance?

A central question in the evolution of resistance is the fitness of the organism carrying a mutant allele of a resistance gene. Theory holds that, in the absence of insecticide,

the majority of insecticide-resistant organisms should show some differential survival in comparison with "wild-type" organisms. That is, resistance should be costly (e.g. Crow 1957). However, empirical evidence on the pleiotropic fitness effects of insecticide resistance appears to be equivocal. There are a few empirical studies that confirm that investment in resistance entails a fitness cost (Minkoff and Wilson 1992; Carrière et al. 1994, 1995, 2001; Yamamoto et al. 1995; Chevillon et al. 1997; Alyokhin and Ferro 1999; Boivin et al. 2001; Berticat et al. 2002; Foster et al. 2003; Rivero et al. 2011; Smith et al. 2011;). On the other hand, some authors have failed to reveal any detrimental effects of insecticide resistance (Follett et al. 1993; Tang et al. 1997, 1999; Baker et al. 1998, 2008; Castañeda et al. 2011), and some have demonstrated pleiotropic fitness benefits (Omer et al. 1992; Bloch and Wool 1994; White and Bell 1995; Mason 1998; Haubruge and Arnaud 2001; Arnaud and Haubruge 2002; McCart et al. 2005; Bielza et al. 2008).

In other studies, some measures of fitness have been negatively affected, others positively (Brewer and Trumble 1991), and this may even involve sexual antagonism, where resistance alleles have opposing fitness effects depending on which sex they reside. This has recently been documented for DDT resistance in *D. melanogaster*, where resistance confers a strong fecundity advantage to females, but a competitive mating disadvantage to males (McCart et al. 2005; Smith et al. 2011). In addition, how resistance alleles impact non-resistance-related fitness can depend on the strain being investigated (Chevillon et al. 1997; Hollingsworth et al. 1997; Oppert et al. 2000; Smith et al. 2011). This reflects epistasis, where the pleiotropic fitness effect is mediated by the genotype (or genetic background) of the insect in question.

2.6 TEs conferring insecticide resistance

Initial findings

Wilson (1993) was the first to speculate that TEs were implicated in insecticide resistance, although the evidence was indirect—he was able to generate Methoprene-resistant alleles in *D. melanogaster* using *P* element mutagenesis (Wilson and Turner 1992). Around the same time, Waters et al. (1992) found an association between *Drosophila* strains resistant to DDT and Malathion and a *17.6* TE insertion in the 3' region of a cytochrome P450 enzyme gene. In this case, it was found that the resistant

strains lacked the insertion, suggesting that resistance was a result of an excision of the TE. However, Delpuech et al. (1993) subsequently reported that the presence or absence of the *17.6* LTR was uncorrelated with resistance in 31 strains of *D. melanogaster* and *D. simulans*.

Wilson (2001) was less convinced about the possibility that TEs play a significant role in insecticide resistance in nature (notwithstanding the *P* element-induced resistance, he demonstrated in the laboratory a decade earlier) conceding that, at most, "TE mutagenesis may be important only for a few genes where resistance can result from severe underexpression or nonfunctional gene product." However, since his review, evidence has been steadily accumulating that TEs do, in fact, play an important part in the evolution of insecticide resistance.

The observation that TEs are frequently found within or in close proximity to resistance genes provides indirect evidence that TEs are involved in resistance-related adaptive genomic changes (Li et al. 2007). This inference was bolstered by the findings of Chen and Li (2007) who reported that TE insertions were enriched around and within xenobiotic-metabolizing P450 genes of both *Helicoverpa zea* moths and *D. melanogaster* flies. They also found that TE insertions were absent from essential housekeeping P450 genes in *D. melanogaster*, which might be expected since mutation of essential genes is more likely to be lethal and not simply reduce fitness. Taken together, these results indicate that TEs are also selectively retained within or in close proximity to xenobiotic-metabolizing P450 genes. Similarly, while a *Bari-1* element insertion occurs downstream of the P450 gene *Cyp12a4* in an Australian lufenuron-resistant *D. melanogaster* strain, its presence in lufenuron-susceptible strains suggests that while the insertion may be important, it is not the main cause of resistance (Bogwitz et al. 2005).

Recent studies provide more conclusive, direct evidence for a causative link between resistance and TEs. For example, insertion of a 2.3-kb LTR retrotransposon *Hel-1* in the putative Bt-toxin receptor gene *cadherin* leads to 3'-truncated nonfunctional cadherin protein and Bt resistance in a laboratory-selected *Heliothis virescens* strain (Gahan et al. 2001). Furthermore, parallel insertions of *Accord*-LTR or *Doc* non-LTR retrotransposon into the 5'-regulatory region of *Cyp6g1* in *D. melanogaster* or *D. simulans* are associated with *Cyp6g1* upregulation and DDT

resistance (Daborn et al. 2002; Schlenke and Begun 2004) (See section 2.6). Additionally, in *D. melanogaster*, insertion of a *Doc1420* retrotransposon into the second exon of the predicted gene *CG10618* (*CHKov1*, a putative choline kinase gene) generates two sets of altered transcripts and a novel polypeptide (Aminetzach et al. 2005). Whether through loss of original *CHKov1* function or through function of the new protein, the *Doc1420* insertion confers moderate OP resistance (Aminetzach et al. 2005).

Why are TEs so important?

Insecticide resistance results from very strong, persistent directional selection. TE-mediated changes in regulation can lead to massive and rapid changes in expression, responses that are potentially highly adaptive when an organism is faced with a major, pervasive, and novel mortality agent in the environment, like an insecticide. A useful contrast which illustrates this point is the essential absence of TEs involved in natural xenobiotic resistance—if we consider that mutational changes in plant allelochemicals are unlikely to bring about massive changes in mode of action or in toxicity, then mutational change associated with allelochemical resistance may be acquired more slowly as a result of the accumulation of small changes in structural genes (Li et al. 2007).

Application of insecticide tends to favour insecticide resistance, involving single genes of major effect rather than polygenic resistance (ffrench-Constant et al. 2004), and it has been found that most resistant field strains show monogenic resistance (Roush and McKenzie 1987). Where resistance genes are already involved in essential functions, as is often the case for metabolic enzymes, it is advantageous to maintain the quality of mRNA to allow wild-type function to be retained and instead regulate gene expression. TE insertion within regulatory regions of genes which confer resistance often results in upregulation, that is, increase in the quantity of mRNA. This may be because many TEs have built-in enhancer sequences related to their transposition (Zhang and Saier 2009) that have been co-opted by the host, but another possibility is that such spacing may move genes further from existing regulatory sequences (Schlenke and Begun 2004).

Mechanisms of resistance via TEs

ffrench-Constant et al. (2006) list four possible mechanisms whereby TE insertions might confer insecticide resistance. First, a TE insertion in the 5'-end of a gene may introduce a novel enhancer sequence. The *Accord*-LTR upstream of the cytochrome P450 gene *Cyp6g1* in *D. melanogaster* is one such case (Chung et al. 2007), and the *Cyp6g1* homolog in *D. simulans*—where the insertion is a complete *Doc* element—may also be one (Schlenke and Begun 2004). Another example of this mechanism is found in the mosquito *Culex quinquefasciatus* where the insertion of a miniature-inverted terminal repeat (*MITE*)-like element upstream of another cytochrome P450 gene is associated with increased pyrethroid resistance (Itokawa et al. 2010). The second mechanism involves increased mRNA stability via TE insertion in the 3'-end of a gene which increases the final pool of translatable RNA. Third, TEs might excise a gene and move it to a different genomic location away from local repressor elements normally responsible for shutting off expression or to a position proximal to an enhancer element. This position effect was demonstrated in principle by Berrada and Fournier (1997) who used *P* element-mediated transposition to initiate transcriptional overexpression of an artificially constructed acetylcholinesterase minigene in *D. melanogaster*. Finally, TE insertion might alter the pattern of resulting transcripts and potentially lead to a truncated gene product of novel function as appears to be the cases described by Gahan et al. (2001) and Aminetzach et al. (2005). One further mechanism not mentioned by ffrench-Constant et al. (2006) involves gene amplification. The transpositional mechanism of TEs may result in gene duplication through ectopic recombination (e.g. Yang et al. 2008) and consequent increase in gene product, or evolution of new gene function in the duplicated gene.

TE-mediated DDT resistance in D. melanogaster and D. simulans

Fifty years ago, studies in *D. melanogaster* indicated that many genes contributed to DDT resistance (Crow, 1957; Kikkawa 1961; Dapkus and Merrell 1977; Hallstrom 1985) including loci on all three major chromosomes. However, work on the Hikone-R strain indicated that resistance in this strain was largely conferred by a single dominant locus on chromosome II, and this was later found to be *Cyp6g1* (Daborn et al., 2001). Resistant alleles were found to have a defective copy of an *Accord*-LTR

retrotransposon inserted about 300 bp upstream of the transcription start site. Subsequently, the molecular mechanism of upregulation was identified (Chung et al., 2007) with cis-regulatory sequences in the *Accord*-LTR being responsible for increased *Cyp6g1* transcription.

Schlenke and Begun (2004), while investigating reduced heterozygosity around the *Cyp6g1* locus in *D. melanogaster* and *D. simulans*, found that another TE insertion, this time a full-length copy of the non-LTR retrotransposon *Doc*, occurred 200 bp upstream of the gene in Californian populations of the latter species. Once again, the insertion correlated with increased *Cyp6g1* expression compared with that found in African populations lacking the insertion. In contrast to the *Accord* insertion in *D. melanogaster* which is highly degenerate (comprising only the LTR), the *Doc* insertion in *D. simulans* is of an autonomous element, suggesting that it is a much more recent event. Selective sweeps at *Cyp6g1*, associated with strong recent selection, were demonstrated in both species (Catania et al. 2004; Schlenke and Begun 2004).

Catania et al. (2004) conducted a survey of the *Accord*-LTR insertion at *Cyp6g1* in 673 lines from 34 populations from around the world. They found near fixation of the *Accord*-LTR-inserted alleles in non-African and North and Western African *D. melanogaster* populations (85–100% of chromosomes sampled), with significantly lower frequencies in East African populations (32–55%). Variation in the Accord-LTR-inserted allele was also found— diagnostic PCR revealed some variability in product size, and subsequent cloning and sequencing of the variants revealed an insertion of a partial *P* element nested within the *Accord*-LTR in a New Delhi line (Catania et al. 2004). Furthermore, Emerson et al. (2008), using genome-wide tilling arrays, found copy number polymorphism in *D. melanogaster* at *Cyp6g1*, with 13 of 15 lines tested showing a duplication encompassing both *Cyp6g1* and *Cyp6g2*.

Most recently, Schmidt et al. (2010) characterized copy number variation and further allelic variation at the *Cyp6g1* locus. Characterization of *Cyp6g1* copy number variation and TE insertion complexity in the *D. melanogaster* RK146 strain revealed two full-length copies of *Cyp6g1*, named *Cyp6g1-a* and *Cyp6g1-b*. A repeat unit was found between the two full-length copies that contained a fusion of partial copies of both *Cyp6g1* and *Cyp6g2*, the gene found downstream of *Cyp6g1-b*. Both copies of *Cyp6g1* were found to contain the LTR of the *Accord* element. Unexpectedly, a *HMS*-

Beagle TE was found inserted into the *Accord*-LTR upstream of *Cyp6g1-a*. Testing of other *D. melanogaster* lines revealed that the partial *P* element found by Catania et al. (2004) was located upstream of *Cyp6g1-b*. Schmidt et al. (2010) also demonstrated an allelic progression of five different alleles, including the ancestral allele lacking any TE insertions and four alleles that involve duplication of *Cyp6g1* (Fig. 2.2). They reason that these alleles represent multiple adaptive steps at *Cyp6g1*, with increased DDT resistance being demonstrated along the allelic progression. Their survey of *D. melanogaster* global populations showed that most flies in Europe, Asia, and the United States carry the *Cyp6g1* duplication and the double *Accord*-LTR (no *P* element) insertion or the *Cyp6g1* duplication and combined *Accord*-LTR insertion/*Accord*-LTR (with nested *HMS-Beagle*) insertion.

The question of the age of the original *Accord*-inserted allele remains open. In their survey, Schmidt et al. (2010) did not find any *Cyp6g1* alleles with the insertion that did not also represent a duplication, nor did they find any gene duplicates which did not also contain the *Accord* insertion. This strongly suggests that either the original insertion and duplication events occurred simultaneously, or, more likely, the original *Accord* insertion preceded the duplication. If the latter, then we are yet to find the original *Accord*-inserted allele.

Catania et al. (2004) suggested that the lower than expected reduction in variability in microsatellites around the *Cyp6g1* locus could be explained most parsimoniously if the *Accord*-LTR insertion occurred at low frequency in African populations before the species' global expansion. This would imply that the insertion was already part of the genetic variation at this locus well before it permitted adaptation to insecticide and may be an example of how TEs provide latent genetic variation facilitating adaptive responses to selection.

The absence of strong DDT selection since its ban in most countries globally over the past 30 years has not brought about the loss of DDT-R alleles in *D. melanogaster*, as might be expected from theory—overexpression of P450 genes must have a cost, and if the resistance-to-DDT benefit to balance this cost is not there, then selection should remove the resistance allele. The near fixation of *Accord*-inserted *Cyp6g1* alleles in worldwide populations (Catania et al. 2004) and the seemingly adaptive, on-going elaboration of these alleles through subsequent TE insertion

increasing DDT resistance (Schmidt et al. 2010) thus poses a puzzle. The most obvious explanation is that these alleles confer cross-resistance to other pesticides and therefore remain under strong xenobiotic selection. There are other explanations, however.

2.7 Sex-specific effects of TEs independent of DDT resistance

The strong fitness benefit conferred by an *Accord*-inserted *Cyp6g1* allele to Canton-S strain females and pupae in the absence of pesticide (McCart et al. 2005) offers another explanation for the persistence of these resistance alleles at high frequencies in the wild. However, this nonresistance fitness benefit should not be viewed in isolation—Smith et al. (2011) very recently confirmed a competitive mating disadvantage to males carrying the same allele and in the same genetic background as used by McCart et al. (2005). This male fitness cost almost perfectly balances the benefit conferred to females. The presence of a DDT-R male fitness disadvantage was first suggested by Drnevich et al. (2004), who in breeding *D. melanogaster* males of high and low MCRS (male competitive reproductive success) discovered that high MCRS was associated with low expression of *Cyp6g1*.

This apparent sexually antagonistic selection at *Cyp6g1* is an excellent example of intralocus sexual conflict where the precise locus has been identified. Intralocus sexual conflict occurs when an allele has positive fitness effects in one sex and negative in the other sex (Bonduriansky and Chenoweth 2009; Hosken et al. 2009). Sexually antagonistic selection generally helps maintain genetic variation and has important implications for the history of the DDT-R allele. If the original *Accord*-inserted allele antedates insecticide use, as Catania et al. (2004) posit, sexually antagonistic selection may have accounted for its low frequency in populations prior to DDT's introduction—no *Accord*-inserted *Cyp6g1* alleles have been found from lines established in the 1930s (Schmidt et al. 2010).

It should be noted that the male fitness disadvantage of the *Accord*-inserted allele is not consistent across different genetic backgrounds. When the allele was introgressed into another *D. melanogaster* strain, males were still competitively disadvantaged, but not significantly so (Smith et al. 2011). As yet, the fitness effects of the *Accord* insertion in females of the latter genetic background have not been

examined. This latter strain had been established by Trudy MacKay in North Carolina, USA, from wild-caught flies in 2004 and contrasts with the Canton-S background which represents one of the earliest established laboratory populations (1930s). The lack of a significant male disadvantage in the more recently established strains may thus represent the evolution of male-specific modifiers in populations dominated by resistant *Cyp6g1* alleles (although this could also represent a statistical power issue). In this context, it is possible that DDT (and other insecticide) selection may have prompted not only rapid spread of the resistance allele but also male-specific counter-adaptation to reduce pleiotropic fitness costs of resistance. Interestingly, while homozygous resistant Canton-S males were significantly smaller than their susceptible counterparts, the converse was true for males of the wild-type genetic background (Smith et al. 2011), hinting that amelioration of the male fitness cost may involve loci influencing male body size.

Cohan et al. (1994) list two general ways in which pleiotropic fitness costs of an adaptive mutation may be ameliorated. The epistatic male DDT-R cost effect seen by Smith et al. (2011) may be an example of the "compensatory" mode, in which natural selection favours modifiers (at other loci) that compensate for the deleterious effects of the mutant allele (Fisher 1928). The other mode, known as "replacement," describes the case where there are multiple mutations which confer the same adaptation, but which vary in their pleiotropic fitness costs such that the original mutation is replaced by one which confers the same adaptive benefit at lower cost (Haldane 1932). Interestingly, this mode has been invoked to explain temporal allele replacement observed in the insecticide resistance gene *Ester* in the mosquito *C. pipiens* in southern France (Labbé et al. 2009). A similar scenario may exist in the *Cyp6g1* allelic progression described by Schmidt et al. (2010).

2.8 Ongoing and future research

In spite of observed female fitness benefits in the absence of DDT (McCart et al. 2005), recent simple models (our unpublished data; see Chapter 6) demonstrate that an *Accord*-inserted *Cyp6g1* (i.e., DDT-R) allele could have been kept at low frequency in populations before DDT was introduced, through male-associated fitness costs (Smith et al., 2011). The net effect of male and female pleiotropic fitness components, with

parameter values based on empirical work by Daborn et al. (2001), McCart et al. (2005), and Smith et al. (2011), was a very slow return to a stable polymorphism at the *Cyp6g1* locus (Fig. 2.3). These results are therefore consistent with continued high levels of DDT-R several years after the removal of DDT selection, a phenomenon which has been previously explained by invoking cross-resistance, a lack of fitness cost and low migratory rates (Catania et al. 2004; McCart et al. 2005).

Further work is required to determine the range of fitness effects of DDT-R in different genetic backgrounds and to further explore the population genetics of these alleles. We also need to investigate the underlying reasons for the competitive disadvantage observed in resistant males of Canton-S background— is it simply size mediated or are there other mechanisms (behavioural and/or physiological) which affect male fitness? Related to this, the demonstration of epistasis with regard to male fitness effects begs a more thorough investigation of the distribution of pleiotropic fitness effects in both sexes. How common is the DDT-R-associated male competitive disadvantage? How common is the female fitness benefit? In this respect, it would be useful to run population cage studies and observe temporal change in replicate laboratory populations with different initial *Cyp6g1* allele frequencies. This would also complement population genetic models. At the molecular level, there remains an opportunity to further investigate the genetic basis of DDT-R epistasis—what are the modifiers altering the male fitness costs? Although *D. melanogaster* is not a pest species, understanding the relative reproductive success of susceptible and resistant flies with differing genetic backgrounds could provide valuable baseline data to inform insecticide resistance management programs for pest species. Work to date suggests that using a single genetic background to test for effects may not be representative.

Given the great variation recently discovered at the *Cyp6g1* locus in *D. melanogaster* (Schmidt et al. 2010), the time is ripe to investigate the fitness effects of these newly discovered alleles. In the case of the more derived alleles, females have much higher DDT resistance than males (Schmidt et al. 2010), which points toward possible mitigation of the intralocus sexual conflict described in Smith et al. (2011). One hypothesis, easily testable through a fitness components approach and/or selection-based stability analysis *sensu* Raymond et al. (2011) (population cage experiments described above), is that the allelic succession may be partially driven by

the “replacement” mode of amelioration. However, intralocus conflict resolution like this presumably requires a change to genetic architecture, which makes resolution more difficult than it may appear (e.g. Harano et al. 2010).

It is unknown whether gene amplification or TE-induced cis-acting mutation has the greater effect on DDT resistance and associated pleiotropic fitness effects *in D. melanogaster*—dissecting the respective contributions to resistance/fitness would require single-copy TE-inserted *Cyp6g1* alleles, and these are yet to be found (Schmidt et al. 2010). The universal presence of TE insertions in both copies of all DDT-R alleles thus found suggests that the insertion occurred prior to, or concurrently with, the duplication event.

This parallels pyrethroid resistance in the mosquito *C. quinquefasciatus*. Here, resistance is associated with overexpression of another cytochrome P450 gene, *Cyp9m10* (Hardstone et al. 2010; Itokawa et al. 2010, 2011). As with *D. melanogaster* DDT-R, the constitutive upregulation occurs in haplotypes that have an upstream insertion of a TE (in this case a truncated copy of the MITE TE, *CuRE1*; Itokawa et al. 2010). Moreover, one of the resistant haplotypes also consists of a tandem repeat of the TE-inserted sequence (Itokawa et al. 2011). Unlike the *D. melanogaster* DDT-R system, the relative contributions of the TE insertion and gene amplification to resistance (and for that matter pleiotropic fitness) can easily be parsed out, since there are haplotypes that possess the former but not the latter. Itokawa et al. (2011) suggest that, based on the nonlinear resistance efficacy to *Cyp9m10* expression, resistance is disproportionately greater as a result of the cis-acting mutation (the TE insertion) occurring before the duplication event, than if the duplication had preceded the insertion. The parallels of these stories for two different enzymes, conferring resistance to two different insecticides in two distantly related species, underline the usefulness of intensive study of model insect systems. They also hint at a general pattern—tandem repeats, which are difficult to detect, could be commonly associated with TE insertion-induced insecticide resistance. This remains to be established.

Compared with the extensive work done on the *Accord*-inserted *Cyp6g1* in *D. melanogaster*, little is known about *Doc*-inserted *Cyp6g1* in *D. simulans*. It remains to be seen whether this mutation has a significant and consistent effect on resistance across different strains/genetic backgrounds. Furthermore, no work has been done to

examine potential pleiotropic fitness effects of this insertion, much less the presence of epistatic interactions or the possibility of intralocus sexual conflict, as has been demonstrated for *D. melanogaster*. A good first step may be to perform a worldwide survey akin to that of the *Accord*-LTR insertion by Catania et al. (2004). This would provide some indication of the geographic range of the *Doc*-inserted allele. Given the evidence for on-going and rapid adaptation at *Cyp6g1* in *D. melanogaster*, it may well be worth having a closer look at the variation which exists at this locus in *D. simulans*. Just how similar the responses of the two species are to similar selection also remains to be seen.

Another avenue of research involves gene by environment interaction as it relates to fitness costs of resistance in these model systems. The laboratory-based fitness component approach cannot fully encompass the full diversity of environments in which wild populations face selection, and this may be a reason why costs are not always detected—environmental factors such as natural enemies, resource limitation, overwintering, and different host plant have all been shown to increase resistance costs in various taxa (Carrière et al. 2001; Janmaat and Myers 2005; Raymond et al. 2005, 2007, 2011). Moving population cage experiments outdoors could increase the reality of the stability-selection approach, giving a better reflection of how well resistance genotypes perform under natural conditions. Just as the genetic background provided by the rest of the genome represents a genetic "environment" in which resistance alleles act, so does the presence of extragenomic DNA, including cytoplasmic endosymbionts. *Wolbachia* is a maternally transmitted intracellular bacterium found in a wide range of arthropods and nematodes (Werren 1997; Stouthamer et al. 1999). Its relationship with its host ranges from parasitic to symbiotic. At the parasitic end of the spectrum, it can have profound effects on host reproduction, displaying a range of phenotypes from male killing to feminization to cytoplasmic incompatibility (Werren 1997; Stouthamer et al. 1999). These strategies increase its transmission within a population, often at the expense of its host's fitness—the hallmark of an SGE. *Wolbachia* is found not only in *Drosophila* (where it has undergone a very recent expansion to near fixation in many populations), but also in many other insects including pest species—one recent estimate is that more than 66% of arthropod species harbour *Wolbachia* infections (Hilgenboecker et al. 2008).

Given its ubiquity and potentially profound effect on host fitness, *Wolbachia* cannot be ignored when examining pleiotropic effects of resistance. For example, *Wolbachia* has been implicated in directly modifying the cost of insecticide resistance in mosquitoes (Duron et al. 2006). Where insecticide resistance is conferred by a TE, we may find that intergenomic interactions (akin to epistasis) between the TE and intracellular endosymbionts are critical to the population genetics of insecticide resistance alleles.

Although *D. melanogaster* is not a pest species, understanding the relative fitness of susceptible and resistant flies with differing genetic backgrounds and under different environments could provide valuable insights to inform insecticide resistance management programs for pest species. To this end, we urge the use of multiple avenues of investigation that include the laboratory-based, sex-specific fitness component approach, stability-selection experiments, and mathematical modelling to increase our understanding of insecticide resistance dynamics in natural populations.

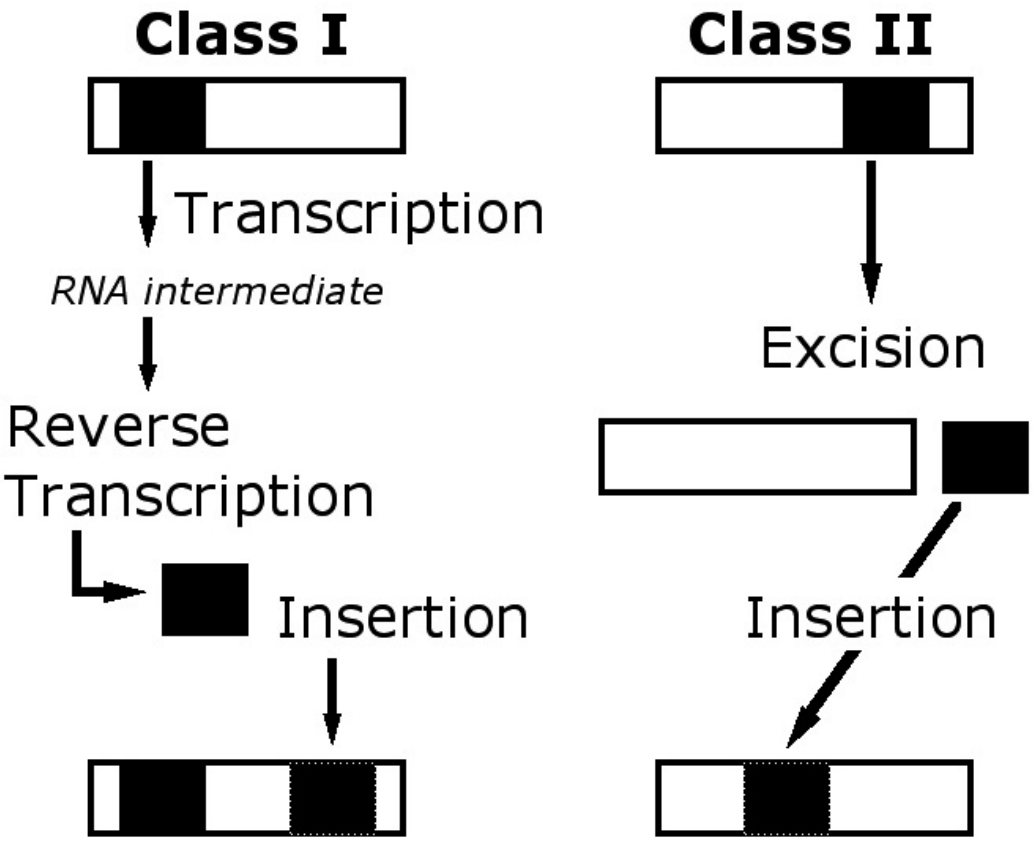

Figure 2.1 A diagram of the transpositional modes of the two major transposable element classes, class I and class II. Class I elements do not move once inserted into host DNA but use RNA intermediates to insert additional TE copies in new genomic locations. Class II elements can move by excision from host DNA, followed by reinsertion into a new location.

Table 2.1 The Transposable Element Classification System Proposed by Wicker et al. (2007).

Class	**Order**	**Superfamilies**
Class I (retrotransposons)	LTR (Long Terminal Repeat)	*Copia, Gypsy, Bel-Pao, Retrovirus, ERV*
	DIRS (*Dictyostelium* Intermediate Repeat Sequence)	*DIRS, Ngaro, VIPER*
	PLE (*Penelope*-like Elements)	*Penelope*
	LINE (Long Interspersed Nuclear Element)	*R2, RTE, Jockey, L1, I element*
	SINE (Short Interspersed Nuclear Element)	*tRNA, 7SL, 5S*
Class II (DNA transposons):		
subclass 1	TIR (Terminal Inverted Repeat)	*Tc1-Mariner, hAT, Mutator, Merlin, Transib, P element, PiggyBac, PIF-Harbinger, CACTA*
	Crypton	*Crypton*
subclass 2	Helitron	*Helitron*
	Maverick	*Maverick*

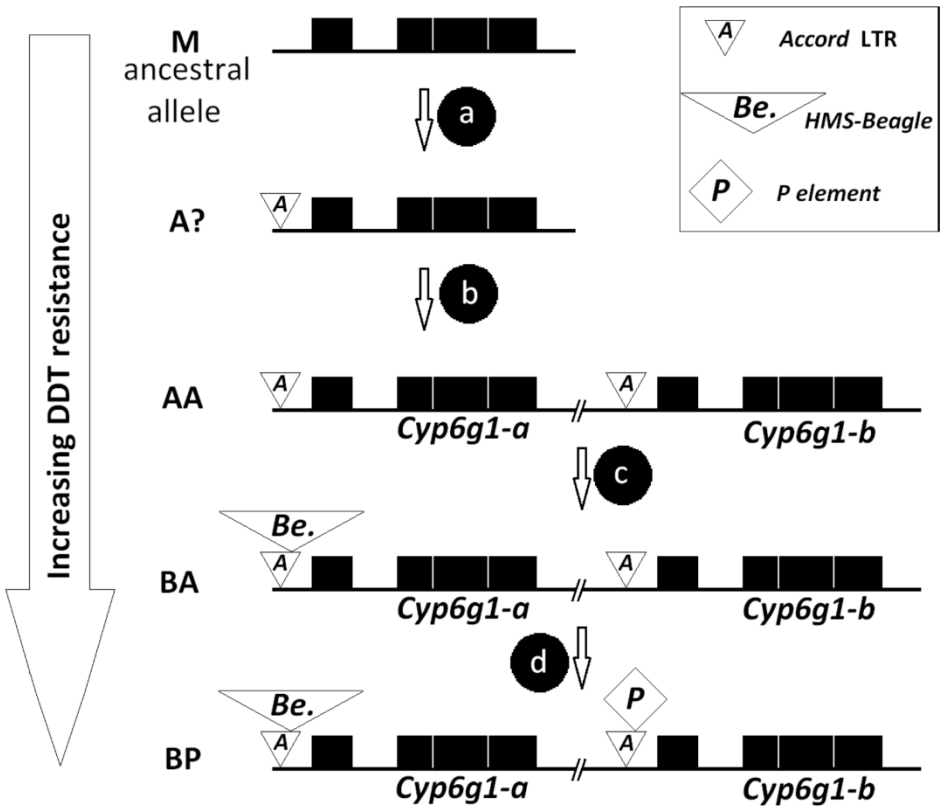

Figure 2.2 Allelic progression at the *Cyp6g1* locus in *D. melanogaster* as described by Schmidt et al. (2010). The ancestral allele (*Cyp6g1-[M]*) is found in DDT-susceptible strains such as Canton-S and is the only allele found in laboratory lines established in the 1930s. The most plausible sequence of changes to the ancestral allele are as follows: (a) Insertion of an *Accord* retrotransposon 5' of *Cyp6g1*, followed by excision (leaving only the LTR footprint), produces hypothetical allele *Cyp6g1-[A?]*. (b) Duplication of the *Accord* LTR-inserted allele produces a tandem repeat of two full-length *Cyp6g1* copies (*Cyp6g1- a* and *Cyp6g1-b*) separated by partial *Cyp6g1*/*Cyp6g2* repeat units and resulting in the DDT-resistant allele *Cyp6g1-[AA]*. (c) Insertion of an *HMS-Beagle* retrotransposon into the *Accord*-LTR found in *Cyp6g1-a* of *Cyp6g1-[AA]* produces the DDT-resistant allele *Cyp6g1-[BA].* (d) Insertion of a *P*-element DNA transposon into the *Accord*-LTR found in *Cyp6g1-b* of *Cyp6g1-[BA]* produces the DDT-resistant allele *Cyp6g1-[BP].*

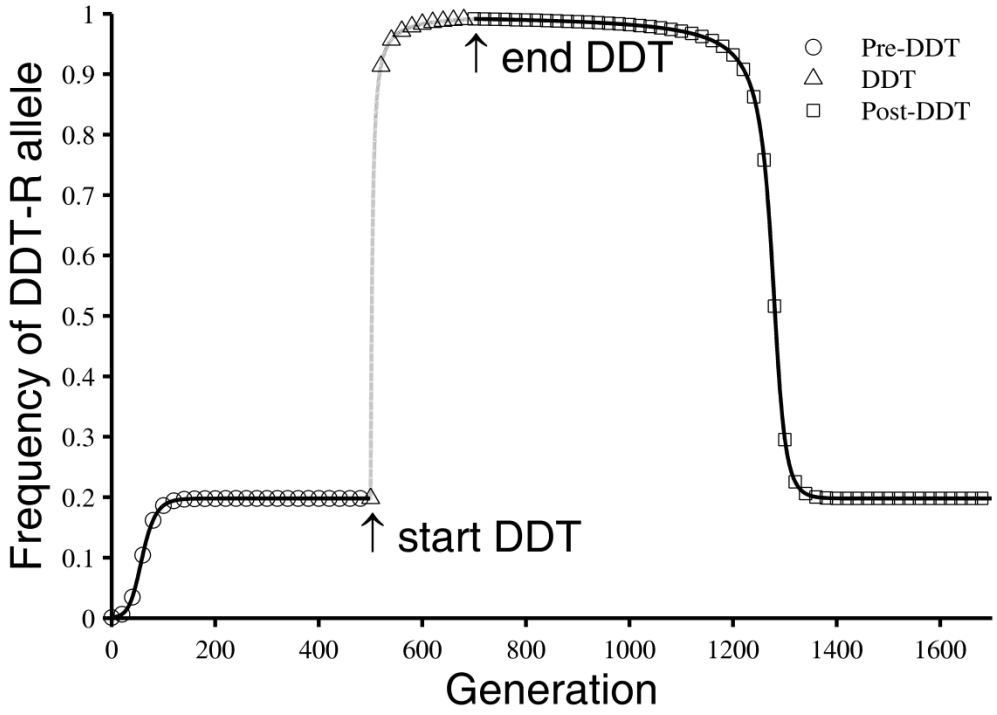

Figure 2.3 Results of a population genetic model showing the changes in DDT-R frequency prior to, during, and after DDT selection. Simulations incorporate the sexually antagonistic fitness effects of the allele in the absence of DDT. The initial allele frequency was set very low (initial DDT-R frequency is 0.001) to demonstrate that the balance of male and female fitness effects can move a rare allele to a stable polymorphism. Note that the fixation of the allele is very rapid when DDT use is introduced and the return to the polymorphic equilibrium after relaxing the strong directional selection for resistance (DDT) is initially slow. Symbols plotted every 20 generations.

CHAPTER 3: DDT resistance, epistasis and male fitness in flies

3.1 Abstract

In *Drosophila melanogaster*, the DDT resistance allele (DDT-R) is beneficial in the presence of DDT. Interestingly, DDT-R also elevates female fitness in the absence of DDT and existed in populations before DDT use. However, DDT-R did not spread regardless of DDT-independent selective advantages in females. We ask whether sexual antagonism could explain why DDT-R did not spread before pesticide use. We tested pre- and post-copulatory male fitness correlates in two genetic backgrounds into which we backcrossed the DDT-R allele. We found costs to DDT-R that depended on the genetic background in which DDT-R was found and documented strong epistasis between genetic background and DDT-R that influenced male size. Although it remains unclear whether DDT-R is generally sexually antagonistic, or whether the fitness costs noted would be sufficient to retard the spread of DDT-R in the absence of DDT, general fitness advantages to DDT-R in the absence of DDT may be unlikely.

3.2 Introduction

In addition to the direct impacts humans have had on the environment, we have also inadvertently imposed selection on many natural populations (Palumbi 2001). This selection has generated evolutionary responses in a wide range of organisms (Allendorf and Hard 2009). Notable cases include the evolutionary reduction of secondary sexual characters caused by big game hunting (Coltman et al. 2003), the evolution of antibiotic-resistant bacteria in hospitals (Hiramatsu 1995; Deurenberg et al. 2006), HIV evolution in response to common treatments (Little et al. 1999, 2002), and the evolution of life-history traits in many commercially exploited fishes (Ricker 1981). The spread of DDT (dichlorodiphenyltrichloroethane) resistance is another example of human-induced selection causing rapid evolution (Palumbi 2001). This occurred during the 1950s and 1960s when DDT was widely used as an agricultural pest-control agent and in an attempt to eradicate malaria.

Although the genetic mechanism for DDT resistance in most organisms is not known, in *Drosophila melanogaster*, resistance to DDT is caused by a single insertion of a transposable element (TE) close to a P450 gene – a family of genes that are known to be involved in detoxification of xenobiotics (Feyereisen 1999). The TE in question is the

Accord retrotransposon (a TE that uses RNA in stages of its transposition) inserted in the promoter region of the *Cyp6g1* gene, 291 bp upstream from the start of transcription (Daborn et al. 2002). The presence of the TE in this position is perfectly correlated with 10-100 times upregulation of *Cyp6g1* transcription and causes high levels of resistance to DDT and other insecticidal chemicals (Daborn et al. 2002). Furthermore, the 20-kb region surrounding the *Cyp6g1* allele that contains the TE has no DNA sequence variation in worldwide samples (Catania et al. 2004). Such a large region without variation surrounding an allele is highly suggestive of a strong and recent selective sweep (Catania et al. 2004), and it is likely that the extensive use of DDT in the 1950s and 1960s was the reason for this selection.

The *D. melanogaster* resistance allele (DDT-R) is especially interesting because in addition to benefits associated with resistance, females carrying DDT-R have a large fitness advantage over susceptible females in the absence of DDT (McCart et al. 2005). This was evident for a range of fitness determinants, with DDT-R females laying more eggs and a greater proportion of viable eggs than susceptible females. Additionally, resistant offspring also have higher larval and pupal viability as well as shorter development times. This represents a considerable fitness advantage for DDT-R females, with susceptible females having a relative fitness of 80% or less for these measures. However, in spite of this considerable fitness advantage to DDT-R females in the absence of DDT, and the fact that it was present in natural population before DDT use, the DDT-R allele did not spread until DDT use became common (Catania et al. 2004). With all else being equal, an allele with a selective advantage as large as that documented for female fitness should have spread, especially because the allele was present long before the use of DDT (i.e. it had sufficient time to spread). As this did not occur, something must have retarded the spread of the allele. There are a number of factors that could act as a brake, including covariance with other alleles under selection, unknown associated costs or the possibility that the allele has sexually antagonistic fitness effects. Sexual antagonism is well documented in *Drosophila* (Chippindale et al. 2001; Gibson et al. 2002) and if DDT-R behaved in this way, then the selective advantages seen in females could be balanced by costs in males, and the failure of the allele to spread in the absence of DDT would be understandable. To date, however, there has been no assessment of the effects of DDT-R on male fitness.

Here, we investigated possible fitness costs associated with DDT-R in male *D. melanogaster*. We tested DDT resistant and susceptible males for differences in pre- and post-copulatory components of male fitness, including male size. Male size is an important determinant of a male's ability to attain matings and hence male fitness in *D. melanogaster* (Bateman 1948; Partridge and Farquhar 1983; Partridge et al. 1987b; Markow 1988; Pitnick 1991). As pre-copulatory male fitness components, we assayed male mating success in competitive and noncompetitive environments. It is important to assay pre-copulatory mating success both with and without male-male competition, as both situations are likely to occur in the natural environment of *D. melanogaster*. In noncompetitive situations (with only a single male present), resistance to DDT could influence the number of males that mated compared with the number that did not. If fewer resistant males mated compared with susceptible males, this could represent a cost to DDT resistance. In competitive pre-copulatory environments (in this case, two males competing for mating), the first male to mate is successful. For post-copulatory male fitness components, we measured the siring success of males in sperm competition with a rival male. We measured both the sperm defence (P1: the paternity secured when the focal male is the first of two males to mate) and offence (P2: the siring success of the second of two males to mate) ability of resistant and susceptible males. Because sperm competition is the norm in *D. melanogaster* (Harshman and Clark 1998; Imhof et al. 1998; Snook and Hosken 2004), sperm competitiveness is also likely to be an important male fitness component. We conducted all assays in two genetic backgrounds.

3.3 Materials and methods

Backcrossing

To introgress the DDT resistance associated *Accord* element into two susceptible genetic backgrounds, we assigned a wild caught isoline known to have the *Accord* element and backcrossed it to the Canton-S (CS) and a wild caught (WC) susceptible genetic background [PCR diagnostic according to Daborn et al. (2002)]. The resistant and susceptible WC isolines were collected by Trudy MacKay in North Carolina, USA in 2004. We placed 50 males from the resistant line with 50 females from each of the

susceptible lines and allowed them to mate freely for 3 days. We did the same crosses with resistant females and susceptible males. We removed adults and laced the vials with DDT by rolling 500 µL of 4 µg mL^{-1} DDT (Sigma, St. Louis, MO, USA) in acetone on the inside of the vial until the acetone had completely evaporated. From the surviving larvae, we collected virgin adults and used them in the next generation of backcrossing with the susceptible line. We did this for seven generations of backcrossing. After the seventh generation of backcrossing, we mated surviving adults in individual pairs and allowed them to lay eggs. We diagnosed the parents for the presence of the *Accord* TE using PCR. Only the offspring of two homozygous parents possessing the *Accord* TE were used to create a homozygous-resistant population of the *Accord* TE backcrossed into the susceptible genetic backgrounds (n = 2). We used 15 adult pairs to start the resistant CS genetic background and 23 adult pairs to start the resistant WC genetic background, giving an N_e of 30 and 46 for the CS and WC genetic backgrounds, respectively. The starting *Ne* for resistant lines of each genetic background was relatively similar (well within the same order of magnitude), and so we do not expect differential inbreeding to influence our results. Furthermore, certain aspects of male fertility are extremely susceptible to inbreeding depression (e.g. Okada et al. 2011) and as we find no evidence of inbreeding depression in these characters (see below), we can be confident that there was no differential inbreeding influencing our results. Additional DDT-R alleles involving the *Accord* TE and another TEs inserted within the *Accord* have recently been identified (Schmidt et al. 2010). We used a DDT-R allele that contains the *Accord* TE but do not know whether this allele also contains other TEs.

Females used in all experiments had the recessive *sparkling poliert* (*spa*) mutation recently backcrossed into a wild-type Dahomey background (Fricke et al. 2009). Using spa females allowed us to assign paternity of offspring produced by either a wild-type or a spa male during the P1 and P2 assays. Females were polymorphic for the DDT-R allele, but we know of no reason to expect any bias in allele frequency amongst our treatments. We maintained the strains in 30 × 30 × 30 cm population cages (Bioquip, Knutsford, UK) and fed them on '*Drosophila* quick mix medium' (Blades Biological, Edenbridge, UK). For experimental flies, we collected first instar larvae from standard Petri dishes containing 1.5% agar in apple juice with yeast paste spread on a small area of the surface. Larval density can influence adult size (Miller and Thomas

1958), so we placed 100 larvae in each food vial (approximately 5 mL in 3 × 7 cm circular vials) to control larval density. Also, the number of potentially competing males present before mating influences male mating behaviour (Bretman et al. 2009). To standardize the competitive environment of males, we collected virgin adults and kept them in vials containing food at a density of approximately 20 flies per vial. We put females in experimental vials containing food 24 h before the start of experiments. All flies were 2-5 days old at the start of experiments. After the experiments, we estimated body size by measuring the wings of all flies using SPOT BASIC 4.1 (Diagnostic instruments, Inc., Sterling Heights, MI, USA) by measuring the distance between the intersection of the third longitudinal vein and the anterior cross vein, and the distal tip of the third longitudinal vein. We observed matings for approximately 6 h during the pre-copulatory competitive assay (PCC), pre-copulatory noncompetitive assay (PCN) and the first matings of the P1 and P2 assays. We reared flies and conducted experiments described below with both CS and WC genetic backgrounds at a constant temperature of 25°C.

Pre-copulatory competitive assay – PCC

We placed a single *spa* female in a vial with one resistant and one susceptible male. We used blue and pink paint powder to identify individual males following Champion de Crespigny and Wedell (2007) so that half the resistant and susceptible males were blue and the other half were pink. Pink males always competed against blue males, and resistant males always competed against susceptible males. After the start of copulation, we immediately aspirated the unsuccessful male out of the vial. We recorded the latency to copulation, copulation duration and whether the successful male was resistant or susceptible. Copulation duration has been used as a proxy for male ejaculate investment and can influence male fitness (Gilchrist and Partridge 2000; Bretman et al. 2009) and is therefore included in our analyses. It should be noted, however, that copulation duration does not necessarily correlate well with investment in different ejaculate components (Wigby et al. 2009; Lupold et al. 2011). After successful copulations, we allowed females to lay eggs for 5 days following copulation and counted the total number of offspring produced after 17 days from copulation.

This allowed all offspring to enclose without the risk of counting offspring from the next generation.

Pre-copulatory noncompetitive assay – PCN

We placed a resistant or a susceptible male individually in a vial with a single *spa* female and recorded latency to copulation and copulation duration. Copulation initiation is largely controlled by females (Markow 1996), but resisting copulation can be costly to females (Partridge and Fowler 1990). We use the framework of Jennions and Petrie (1997) to define female preference, where female 'choosiness' is defined as the time a female takes to examine a potential mate. No-choice designs (where only one male is presented to a female) are a standard way of testing female preference without the influence of male-male competition (Shackleton et al. 2005; Narraway et al. 2010; Sharma et al. 2010). In practice, males that mate sooner to females are considered more attractive. We allowed females to lay eggs for 5 days following copulation and counted all offspring produced after 17 days.

Sperm defence – P1

We mated a resistant or a susceptible male to a female as in the PCN assay. Twenty-four hours later, we gave the females the opportunity to remate to a *spa* male for 4 h every day until remating occurred. We measured the latency to copulation (as in the PCN assay) and copulation duration of both matings and the number of offspring produced before the female remated. Latency to copulation and copulation duration could be influenced by whether a male was resistant or susceptible ('male resistance status') in the same way as in the PCN assay. We used the *spa* phenotype to assign offspring produced over 5 days following the second mating to the first or second male to mate, where *spa* offspring belonged to the second male to mate. Male size was determined for both males and females as described earlier.

Sperm offence – P2

We conducted this assay in the same way as the P1 assay in all respects apart from the reversal of mating order of *spa* males with resistant and susceptible males. We used

the *spa* phenotype to assign offspring produced over 5 days following the second mating to the first or second male to mate, where *spa* offspring belonged to the first male to mate. Male size was determined for both males and females as described earlier.

Statistical analysis

We carried out all statistical analysis using R version 2.9.2 (R Development Core Team, 2009). We tested data for normal distribution and homogeneity of variance, where data did not conform to a normal distribution we transformed the data when possible or we used appropriate nonparametric tests or error distributions. We tested for differences in competitive mating success between resistant and susceptible males during the PCC assay using an exact binomial test. We built all multivariate analysis of covariance (MANCOVAs), generalized linear models (GLMs) and general linear mixed effects models (GLMMs) by including all relevant interactions. We removed all individuals that did not mate during the experiments for the MANOVA and GLM analyses that required mating to have occurred. When analysing the number of offspring or paternity of males, we excluded all individuals that did not produce any offspring. In the sperm competition assays, length of the female refractory period and the number of offspring produced before remating were significantly correlated, so we included the variable that told us most about male fitness; number of offspring produced. We reduced all models in a stepwise manner, removing the least significant term at each step, but we always retained male resistance status as a covariate in the models as this was the primary focus of the study. We used relative male size in cases when two males were competing against each other. In the PCC assay, relative male size was resistant male size minus susceptible male size, whereas in the sperm competition assays, relative male size was resistant or susceptible male size minus *spa* male size. The same process was used to calculate relative copulation duration in the P1 and P2 assays. Also, in the PCN, P1 and P2 assays, we used χ^2 contingency tests to determine whether the number of unsuccessful male matings was different from the expected distribution of no difference between resistant and susceptible males.

3.4 Results

We used both genetic backgrounds (CS and WC) for each of the assays described in the materials and methods section. We describe results in each assay first for the CS and then the WC genetic background. When resistant and susceptible males from either genetic background were competing directly against each other in the PCC assay, as a null hypothesis, we assumed resistant and susceptible males would mate in equal frequencies (i.e. there would be no difference between resistant and susceptible males).

Pre-copulatory competitive assay: CS background

Susceptible males mated significantly more often than resistant males (exact binomial test $Bin_{0.5}$, number of resistant matings 18 of 82 trials, $p < 0.001$, Figure 3.1a). To test for an effect of male resistance status on copulation latency, copulation duration or offspring production, we used a MANCOVA with copulation latency, copulation duration and number of offspring produced as response variables and male resistance status as an explanatory factor, with relative male size and female size as covariates. Female size was significant in the multivariate analysis because of its univariate effects on log- transformed copulation latency and copulation duration (Table 3.1). There was no effect of male resistance status on the multivariate combination of these characters (Table 3.1).

Pre-copulatory competitive assay: WC genetic background

In contrast to the CS genetic background, we saw no difference between the susceptible and resistant males in the frequency of mating in the WC genetic background (exact binomial test $Bin_{0.5}$, number of resistant matings 53 of 113 trials, $p = 0.573$, Figure 3.1b). We conducted the MANCOVA analysis for the WC genetic background as in the CS genetic background. None of the explanatory variables had a significant effect on the multivariate combination of the response variables, and this was true even after a stepwise model reduction (Table 3.1). The difference in the results between the CS and WC genetic backgrounds indicated that there was no consistency in cost to males across genetic backgrounds.

Pre-copulatory noncompetitive assay: CS genetic background

We found a significant multivariate effect of male resistance status when we conducted a MANCOVA with copulation latency, copulation duration and offspring production as response variables with male resistance status as an explanatory factor, and male size and female size as covariates in a noncompetitive assay (Table 3.2). The effect of male resistance status on the multivariate trait combination was driven by resistant males producing more offspring than susceptible males (Table 3.2). We found no relationship between male resistance status and the proportion of males that did not mate ($\chi^2_1 = 0.075$, $p = 0.784$).

Pre-copulatory noncompetitive assay: WC genetic background

In contrast to the previous assay using the CS genetic background, we found no effect of male resistance status when we conducted the same MANCOVA analysis in the WC genetic background. Male size had an effect on the multivariate combination of dependent variables in this model. The multivariate effect was because of a negative relationship between male size and log-transformed copulation latency (Table 3.2). Female size also had a significant effect in the multivariate model, and this was driven by larger females producing more offspring (Table 3.2). Additionally, unlike the CS genetic background, in this genetic background, resistant males were more likely to mate than susceptible males ($\chi^2_1 = 8.99$, $p = 0.003$).

Sperm defence (P1): CS genetic background

There was no significant effect of male resistance status on P1 when we conducted a quasibinomial GLM using P1 as the response variable with male resistance status as an explanatory factor and relative male size, relative copulation duration and female size as covariates ($F_{1,80} = 2.06$, $p = 0.154$, n = 82). Female size ($F_{1,78} = 0.033$, $p = 0.86$), relative copulation duration ($F_{1,79} = 0.335$, $p = 0.56$) and relative male size ($F_{1,80} = 0.543$, $p = 0.46$) did not influence P1. We also found no significant effect of male resistance status, relative male size or female size in a MANCOVA using copulation latency, copulation duration and number of offspring produced before remating as response

variables (Table 3.3). We found no relationship between male resistance status and the number of males that did not mate during the first mating of the P1 assay (χ^2_1 = 1.40, p = 0.237).

Sperm defence (P1): WC genetic background

Again, we found no significant effect of first male resistance status on P1 in the WC genetic background using a similar GLM model as above for the CS genetic background ($F_{1,155}$ = 0.008, p = 0.930, n = 158). Relative copulation duration had a significant positive relationship with P1 (b = 0.07, $F_{1,156}$ = 9.821, p = 0.002). Female size ($F_{1,153}$ = 0.1304, p = 0.72) and relative male size ($F_{1,154}$ = 0.275, p = 0.60) did not influence P1. In contrast to the CS genetic background, when using the same MANCOVA, we found a significant effect of male resistance status on the multivariate combination of dependent variables in the WC genetic background. Univariate analysis showed that this was because of resistant males having significantly shorter copulation durations than susceptible males (Table 3.3). Similarly to the CS genetic background, we found no relationship between male resistance status and the proportion of males that did not mate (χ^2_1 = 0.26, p = 0.609).

Overall, we found no effect of male resistance status on P1 in the CS or WC genetic background. We found that resistant males had significantly shorter copulation durations in the WC genetic background, which was not the case in the CS genetic background.

Sperm offence (P2): CS genetic background

Again, using a quasibinomial GLM with P2 as response variable and second male resistance status as an explanatory factor and relative male size, relative copulation duration and female size as covariates, we found no significant effect of male resistance status ($F_{1,79}$ = 0.14, p = 0.290, n = 81), relative male size ($F_{1,76}$ = 0.279, p = 0.60), relative copulation duration ($F_{1,78}$ = 0.869, p = 0.35) and female size ($F_{1,77}$ = 0.421, p = 0.52) on P2. In a MANCOVA using second male copulation duration and offspring produced before remating as response variables with male resistance status as a predictor and relative male size and female size as covariates, we also found no effect of second male resistance status on the multivariate combination of dependent

variables (Table 3.4). In the P2 assay, when resistant or susceptible males were attempting to mate with once-mated females, we found no difference in the proportion of resistant males that did not mate compared with susceptible males (χ^2_1 = 0.05, p = 0.799).

Sperm offence (P2): WC genetic background

Similarly, we found no effect of male resistance status on P2 ($F_{1,101}$ = 0.11, p = 0.741, n = 108). Relative male size ($F_{1,100}$ = 0.150, p = 0.70), relative copulation duration ($F_{1,99}$ = 0.012, p = 0.91) and female size ($F_{1,98}$ = 0.008, p = 0.93) also did not influence P2, using a quasibinomial GLM with P2 as response variable and male resistance status as an explanatory factor and relative male size and female size as covariates. We also found no effect of second male resistance status or either covariate on copulation duration or offspring produced before remating using the same MANCOVA analysis as with the CS genetic background (Table 3.4). Similarly to the CS genetic background, we found no relationship between male resistance status and the number of males that did not mate (χ^2_1 = 1.70, p = 0.192). During the P2 assay, we found no significant effect of male resistance status on P2 or any other component of male mating behaviour that we measured in either genetic background.

Male size: CS genetic background

In the above assays, we found size differences between resistant and susceptible males. In each genetic background, we pooled male size from all the above assays to determine whether there was an overall effect of resistance status on male size. In the CS genetic background, resistant males were smaller than susceptible males (GLMM with male size as response variable, male resistance status as explanatory variable and experiment as random factor χ^2_3 = 19.73, p < 0.001, mean resistant male wing measurement = 1.25 mm, mean susceptible male wing measurement = 1.28 mm).

Male size: WC genetic background

In contrast, in the WC genetic background, resistant males were larger than susceptible males (GLMM with male size as response variable, male resistance status

as explanatory variable and experiment as random factor $\chi^2_3 = 27.82$, $p < 0.001$. Mean resistant male wing measurement = 1.29 mm, mean susceptible male wing measurement = 1.27 mm).

3.5 Discussion

Following the extensive use of DDT, the *D. melanogaster* DDT-R allele spread rapidly and now occurs globally at very high frequencies (Daborn et al. 2002; Catania et al. 2004). Despite this allele being present prior to the use of DDT and conferring a significant fitness benefit to females in the absence of DDT (McCart et al. 2005), it did not occur at high frequencies prior to DDT use (Catania et al. 2004). One potential explanation for DDT-R only occurring at low frequencies despite female benefits is that it has sexually antagonistic effects. We investigated several important male fitness components to examine whether sexual antagonism could explain the relative rarity of DDT-R before the use of DDT. Although we found some evidence that DDT-R was costly in males, this depended on the genetic background in which the allele was expressed, and as a result, it is unclear whether the costs we detected could counter-act the female benefits to prevent the spread of DDT-R. However, the substantial epistasis between DDT-R and genetic background could have important consequences for the spread of this allele. We discuss our major findings and their main implications below.

In the CS genetic background, DDT-R males achieved only 22% of matings when competing directly against a susceptible male. Mating success is a major determinant of male fitness in *Drosophila* (Bateman 1948), and mating in competitive situations is likely to be important in natural environments (Powell 1997). In the CS genetic background where DDT-R males achieved fewer competitive matings, they were also smaller than susceptible males. Male size correlates with male mating success in *D. melanogaster* (Partridge and Farquhar 1983; Partridge et al. 1987b), so male size could be a mechanism driving the difference in competitive mating success in the CS genetic background. Regardless of mechanism, a difference in mating success of this magnitude would certainly retard the spread of DDT-R prior to the use of DDT. However, in the WC genetic background, we found no difference in competitive mating success between resistance and susceptible males. Furthermore, DDT-R males were larger than susceptible males in the WC background but had no mating

advantage, which suggests that male size is not the only factor responsible for the differences in male mating success in these binary competitive assays.

In noncompetitive mating trials, the CS DDT-R and susceptible males did not differ in their ability to mate with either virgin or once-mated females. In the WC genetic background on the other hand, DDT-R males were more successful at mating with virgin females, but males did not differ in their ability to mate with nonvirgin females. This benefit to DDT-R in the WC background means that males resistant to DDT may occasionally have higher fitness.

In the CS genetic background, we found that when controlling for male size, females mated to DDT-R males produced more offspring. However, if we did not control for male size, there was no difference in the number of offspring sired between the two male genotypes. We also found that in the WC genetic background, females mated to DDT-R and susceptible males did not differ in their productivity. In a WC background, male size influenced copulation latency and copulation duration; however, there was no effect of male resistance status on either of these variables. So overall, there was no (obvious) cost to DDT-R.

To summarize the pre-copulatory assays, susceptible males had greater mating success in the CS background, but there were no competitive mating differences between DDT-R and susceptible males in the WC background. Furthermore, WC DDT-R males were more successful at securing matings when not in competition with other males. The number of offspring produced also depended on the male genetic background and resistance status. In the CS genetic background, females mated to DDT-R males produced more offspring than susceptible males, but there were no differences in female productivity when DDT-R and susceptible males were of the WC genetic background. Overall, it is clear that the effects of DDT resistance on pre-copulatory male fitness differ between genetic backgrounds, but we could not find a consistent cost to DDT-R that would indicate sexually antagonistic selection is generally acting on the allele (Tables 3.2 and 3.5).

In the sperm competition assays, we found no significant differences in the sperm offence or defence (P1 or P2) ability of DDT-R and susceptible males in either genetic backgrounds. It is unlikely that our failure to find a difference was because of low statistical power, as the smallest sample size in our sperm competition assays was

82 triads. In the wild, *D. melanogaster* females mate multiply and regularly store sperm from more than one male concurrently (Harshman and Clark 1998; Imhof et al. 1998). Here, we have investigated doubly mated females, and although females may mate more than twice in their natural environment, sperm displacement /dumping effectively means only two ejaculates are ever really competing (Gromko et al. 1984; Snook and Hosken 2004; Manier et al. 2010). Non-sperm components of the ejaculate, such as accessory gland proteins, can also have dramatic effects on the outcome of sperm competition (Aigaki et al. 1991; Chapman et al. 2000, 2003). So although we see no net difference between DDT-R and susceptible males in their sperm competitive ability, it remains possible that specific mechanistic components of a male ejaculate may be affected by the resistance allele.

During the sperm defence (P1) assay, DDT-R and susceptible males did not differ in the number of offspring their mates produced before remating. Specific components of the *D. melanogaster* ejaculate are responsible for the female refractory period (a period of reduced female receptivity), and during this time, females lay eggs. While there is variation in expression of the main gene responsible for the refractory period (*Acp70A*, or "sexpeptide") (Smith et al. 2009), the number of offspring production during the refractory period is not influenced by DDT-R. In sum, we find no evidence that DDT-R influences male sperm competitive ability, or their ability to manipulate females' productivity or likelihood of remating.

DDT-R and susceptible males differed in size in both genetic backgrounds, but the direction of the difference depended on genetic background. In the CS genetic background, DDT-R males were smaller, whereas in the WC genetic background, DDT-R males were larger than susceptible males. Size is a very plastic trait in *Drosophila* and although environmental heterogeneity is impossible to totally avoid, we reared all males under constant larval density to minimize differences in developmental environments. Furthermore, when data from all experiments were pooled (by genetic background), the effect of resistance on size was highly significant. As noted above, male size is a major determinant of male fitness in *D. melanogaster* (Partridge & Farquhar 1983; Partridge et al. 1987a,b; Pitnick 1991; Stearns 1992; Roff 2002). Therefore, in genetic backgrounds where DDT-R males are smaller, this could represent a cost to resistance, as we noted in the PCC assays. However, it is currently

unclear whether DDT-R males are usually smaller or not as we only sampled two genetic backgrounds, but if it were so, this could represent the sexually antagonistic effect we postulated as one potential brake on the spread of DDT-R. We do not know why there were such dramatic size differences between DDT-R and susceptible males, or why the strong epistasis we recorded exists. An obvious explanation is that the *Cyp6g1* allele responsible for DDT resistance is also involved in developmental pathways that affect body size, perhaps via resource acquisition. This would not be surprising as size is affected by many loci, which generates numerous potential interactions (Wade 2000). Furthermore, size-affecting loci are likely to include those associated with metabolism, which are the pathways where P450 enzymes such as *Cyp6g1* act (Feyereisen 1999). However, to the best of our knowledge, *Cyp6g1* is not known to map to regions that influence male body size.

Overall, we found some evidence that DDT-R is costly to males (Table 3.5). Our results suggest reduced mating success in the CS genetic background, and this is the same genetic background was that used by McCart et al. (2005) who found strong positive effects of DDT-R on female fitness. It is unclear whether the female benefits they report are universal (across genetic backgrounds), which may also explain why DDT-R did not spread prior to the use of DDT. Perhaps in other backgrounds, there is no female fitness advantage. Our results suggest that it is important not to overemphasize findings using only a single genetic background, as not accounting for genetic complexities such as epistasis may lead to misleading conclusions (e.g. Arnqvist et al. 2010). Nevertheless, in the CS background, we provide some evidence for the sexual antagonism that could (also) retard the spread of DDT-R in the absence of DDT. Similarly, in a recent study, it was also found that DDT-resistant *D. melanogaster* males with higher expression levels of *Cyp6g1* (deriving from several isofemale lines) suffered reduced reproductive success when in competition with *ebony* males, but it could not be determined whether this was because of resistant males being poor sperm competitors and/or less able to obtain copulations in premating competition (Drnevich et al. 2004). Hence, the mating costs we report for DDT-R males could account for the relatively low frequency of this allele before DDT use. This inference is (weakly) supported by comparing the relative fitness of DDT-R and susceptible males and females from the two studies. Using egg-production figures from McCart et al.

(2005) [to avoid assigning offspring fitness to parents (Wolf and Wade 2001)], we find the relative (to homozygous DDT-R females) fitness of homozygous susceptible females was 0.25. In our study, using male competitive mating success as a measure of fitness, the relative fitness of homozygous DDT-R CS males was 0.28 when compared with homozygous susceptible males. Although we acknowledge these are very imprecise calculations based on assumptions that are unlikely to be true, the relative fitness advantage and disadvantage are remarkably similar. More accurate assessment of net fitness of DDT-R males and females in a range of backgrounds is needed, but in any case, our data indicate that epistasis between the *Accord* TE and fly genetic background influences a range of fitness surrogates and makes it unlikely that DDT-R is always (or perhaps even often) beneficial in the absence of DDT. Whether or not different DDT-R alleles affect the costs and benefits of DDT-R remains to be established.

To conclude, we have found costs to DDT resistance in male competitive mating ability in one genetic background and these may explain why the DDT-R allele was relatively rare before the use of DDT as a pesticide. However, strong epistasis between the DDT-R allele and the genetic background in which it finds itself complicates matters. Additionally, the epistatic interactions documented here suggest that the female fitness advantages to DDT-R previously identified in the absence of DDT may not be universal. Study of DDT-R in more genetic backgrounds will provide clearer insight into its sexually antagonistic and epistatic fitness effects, and into the low frequency of DDT-R in *D. melanogaster* populations before the widespread use of DDT.

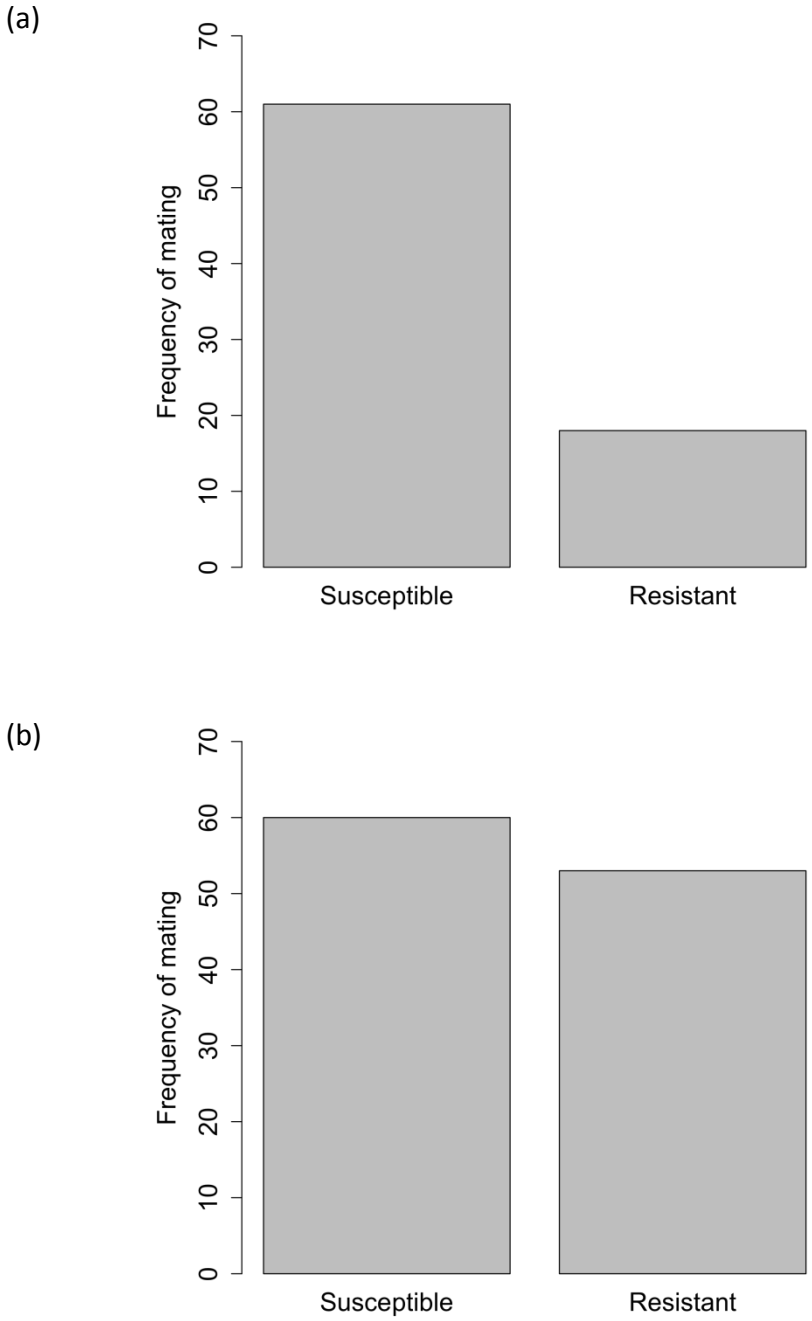

Figure 3.1 (a) The number of matings achieved by susceptible or resistant males in the Canton-S (CS) genetic background under pre-copulatory competitive (PCC) conditions. The resistant males mate significantly less often than the susceptible males in the CS genetic background. This represents a cost to males when they are competing with another male for matings. This situation is very likely to occur in the wild. (b) The number of matings achieved by susceptible or resistant males in the wild caught (WC) genetic background under PCC conditions. There was no difference in the number of matings achieved by resistant and susceptible males. In this WC genetic background,

the resistant males were significantly larger than susceptible males. This is in contrast to the CS genetic background, suggesting that there may be epistatic interactions with the DDT resistance associated allele affecting male size.

Table 3.1 Summary of MANOVA analysis and univariate ANOVA analysis of the pre-copulatory competitive (PCC) assay. We used offspring produced, log copulation latency and copulation duration as response variables with male resistance status as an explanatory factor and relative male size and female size as covariates. Female size significantly influenced the multivariate combination of the response variables in the Canton-S (CS) genetic background because of its univariate effect on copulation duration. None of the other explanatory variables or covariates significantly influenced the multivariate combination of response variables. Bold values represent $P < 0.05$.

Pre-copulatory competitive assay

Genetic background	Canton-S				Wild caught			
	MANOVA				MANOVA			
	Pillai's trace	$F_{(3,73)}$	P		Pillai's trace	$F_{(3,80)}$	P	
Male resistance status	0.023	0.563	0.641		0.068	1.945	0.129	
Relative male size	0.008	0.198	0.897		0.023	0.639	0.592	
Female size	0.144	4.088	**0.010**		0.082	2.395	0.074	
	Male resistance status (Mean ± SE)		Univariate ANOVAs		Male resistance status (Mean ± SE)		Univariate ANOVAs	
	Resistant	Susceptible	$F_{(1,75)}$	P	Resistant	Susceptible	$F_{(1,82)}$	P
Offspring produced	42.222 ± 3.932	37.525 ± 1.863	1.321	0.254	36.114 ± 1.553	37.739 ± 1.603	0.558	0.457
Copulation latency (min)*	3.466 ± 0.227	3.491 ± 0.136	0.008	0.928	3.532 ± 0.156	3.325 ± 0.150	1.460	0.230
Copulation duration (min)	16.444 ± 0.933	16.311 ± 0.605	0.013	0.911	13.659 ± 0.654	15.652 ± 0.818	4.694	**0.033**
	Relative male size β				Relative male size β			
Offspring produced	3.082		0.040	0.843	20.934		1.713	0.194
Copulation latency (min)	0.788		0.333	0.565	0.486		0.157	0.693
Copulation duration (min)	-2.865		0.235	0.629	3.549		0.283	0.596
	Female size β				Female size β			
Offspring produced	-4.146		0.045	0.832	-10.440		0.255	0.615
Copulation latency (min)*	4.956		4.101	**0.046**	5.268		6.479	**0.013**
Copulation duration (min)	27.200		6.622	**0.012**	2.172		0.064	0.801

*log copulation latency

Table 3.2 Summary of MANOVA analysis and univariate ANOVA analysis of the pre-copulatory noncompetitive (PCN) assay. We used offspring produced, log copulation latency and copulation duration as response variables with male resistance status as an explanatory factor and relative male size and female size as covariates. In the Canton-S (CS) genetic background, male resistance status significantly influenced the multivariate combination of the response variables because of its univariate influence on the number of offspring produced. In the wild caught (WC) genetic background, male size and female size significantly influenced the multivariate combination of the response variables because of their univariate influence on copulation duration and number of offspring produced respectively. Bold values represent $P < 0.05$.

Pre-copulatory non-competitive assay

Genetic background	Canton-S				Wild caught			
	MANOVA				MANOVA			
	Pillai's trace	$F_{(3,122)}$	P		Pillai's trace	$F_{(1,164)}$	P	
Male resistance status	0.076	3.366	**0.021**		0.035	2.016	0.114	
Male size	0.048	2.059	0.109		0.125	8.025	**<0.001**	
Female size	0.046	1.937	0.127		0.050	2.925	**0.035**	
	Male resistance status (Mean ± SE)		Univariate ANOVAs		Male resistance status (Mean ± SE)		Univariate ANOVAs	
	Resistant	Susceptible	$F_{(1,124)}$	P	Resistant	Susceptible	$F_{(1,170)}$	P
Offspring produced	53.117 ± 2.103	47.514 ± 1.822	4.061	**0.046**	43.915 ± 1.473	42.544 ± 1.329	0.434	0.511
Copulation latency (min)*	3.176 ± 0.126	3.479 ± 0.145	2.560	0.112	4.078 ± 0.096	4.354 ± 0.124	3.202	0.075
Copulation duration (min)	17.717 ± 0.407	18.471 ± 0.497	1.407	0.238	15.779 ± 0.457	13.474 ± 0.591	10.629	**0.001**
	Male size β				Male size β			
Offspring produced	-1.483		0.150	0.699	-29.530		2.241	0.136
Copulation latency (min)*	-3.301		3.921	**0.050**	-5.004		7.166	**0.008**
Copulation duration (min)	-9.071		2.547	0.113	-37.065		17.629	**<0.001**
	Female size β				Female size β			
Offspring produced	81.770		5.419	**0.022**	41.230		7.188	**0.008**
Copulation latency (min)*	1.685		0.102	0.751	-0.234		0.224	0.637
Copulation duration (min)	1.689		0.005	0.945	7.575		1.238	0.267

*log copulation latency

Table 3.3 Summary of MANOVA and univariate ANOVA analysis of the P1 assay. We used offspring produced before remating, log copulation latency and copulation duration as response variables with male resistance status as an explanatory factor and first male size and female size as covariates. In the Canton-S (CS) genetic background, male resistance status did not significantly influence the multivariate combination of response variables, nor did either of the covariates. In the wild caught (WC) genetic background, male resistance status significantly influenced the multivariate combination of response variables. Univariate analysis showed that the multivariate effect of male resistance status was because of its univariate effect on copulation duration. Bold values represent $P < 0.05$.

Sperm defence (P1)

Genetic background	Canton-S				Wild caught			
	MANOVA				MANOVA			
	Pillai's trace	$F_{(3,80)}$	P		Pillai's trace	$F_{(3,154)}$	P	
Male resistance status	0.017	0.469	0.705		0.168	10.338	**<0.001**	
1st male size	0.021	0.585	0.627		0.005	0.233	0.874	
Female size	0.038	1.066	0.368		0.025	1.334	0.265	
	Male resistance status (Mean ± SE)		Univariate ANOVAs		Male resistance status (Mean ± SE)		Univariate ANOVAs	
	Resistant	Susceptible	$F_{(1,82)}$	P	Resistant	Susceptible	$F_{(1,82)}$	P
Offspring produced before remating	39.267 ± 3.451	44.195 ± 3.490	0.989	0.323	86.047 ± 3.865	91.351 ± 3.149	1.076	0.301
Copulation latency (min)	30.956 ± 6.282	34.537 ± 6.305	0.159	0.691	75.756 ± 10.229	70.581 ± 11.914	0.112	0.739
Copulation duration (min)	16.289 ± 0.531	16.488 ± 0.596	0.064	0.801	12.023 ± 0.401	15.081 ± 0.363	30.718	**<0.001**
	1st male size β				1st male size β			
Offspring produced before remating	23.210		0.063	0.802	-26.460		0.500	0.481
Copulation latency (min)	-79.450		0.685	0.410	-53.980		0.428	0.514
Copulation duration (min)	7.850		0.679	0.412	-3.754		0.044	0.835
	Female size β				Female size β			
Offspring produced before remating	29.770		0.597	0.442	9.351		0.006	0.940
Copulation latency (min)	31.760		0.056	0.813	340.000		3.967	**0.048**
Copulation duration (min)	11.420		2.922	0.091	-0.236		0.018	0.893

Table 3.4 Summary of MANOVA and univariate ANOVA analysis of the P2 assay. We used offspring produced before remating and copulation duration of the second mating as response variables and male resistance status as an explanatory factor with relative male size and female size as covariates. Neither the male resistance status, relative male size nor female size significantly influenced the multivariate combination of the response variables in either the Canton-S (CS) or wild caught (WC) genetic background. Bold values represent $P < 0.05$.

Sperm offence (P2)

Genetic background	Canton-S				Wild caught			
	MANOVA				MANOVA			
	Pillai's trace	$F_{(2,78)}$	P		Pillai's trace	$F_{(2,103)}$	P	
Male resistance status	0.009	0.358	0.700		0.021	1.116	0.332	
Relative male size	0.064	2.674	0.075		0.040	2.130	0.124	
Female size	0.014	0.557	0.575		0.003	0.163	0.850	
	Male resistance status (Mean ± SE)		Univariate ANOVAs		Male resistance status (Mean ± SE)		Univariate ANOVAs	
	Resistant	Susceptible	$F_{(1,79)}$	P	Resistant	Susceptible	$F_{(1,104)}$	P
Offspring produced before remating	56.923 ± 6.315	61.545 ±5.378	0.314	0.577	43.196 ± 1.766	42.702 ± 1.764	0.040	0.842
Copulation duration (min)	20.667 ± 0.750	20.455 ± 0.770	0.040	0.842	17.882 ± 0.652	19.316 ± 0.678	2.256	0.136
	Relative male size β				Relative male size β			
Offspring produced before remating	-73.502		1.737	0.191	41.878		4.210	**0.043**
Copulation duration (min)	-15.582		3.703	0.058	-2.103		0.009	0.926
	Female size β				Female size β			
Offspring produced before remating	-38.070		0.115	0.736	9.366		0.188	0.665
Copulation duration (min)	12.466		1.001	0.320	-0.231		0.058	0.810

Table 3.5 Summary of the difference in relative fitness between resistant and susceptible males. In the CS genetic background, there was a cost to resistant males in the PCC assay but a benefit to resistance in the PCN assay. In the WC genetic background, there were no differences between resistant and susceptible males.

	Fitness change due to DDT resistance			
Genetic background	PCC	PCN	P1	P2
CS	-	+	0	0
WC	0	0	0	0

CS, Canton-S; WC, wild caught; PCC, precopulatory competitive assay; PCN, precopulatory noncompetitive assay; P1, sperm defence; P2, sperm offence; '**-**', cost to resistance; '**+**', benefit to resistance; '0', no fitness change because of resistance.

CHAPTER 4: Pleiotropic effects of DDT resistance on male size and behaviour

4.1 Abstract

The DDT resistance allele (DDT-R) in *Drosophila melanogaster* is associated with a male mating cost in the absence of DDT, when expressed in the Canton-S genetic background. Resistant males are also significantly smaller than susceptible males. However, it is uncertain whether the mating cost is mediated by the size effect or if DDT-R also affects aspects of male behavioural phenotype which decrease mating success. We tested the former by directly manipulating size disparity between resistant and susceptible males in competitive mating trials. We also made detailed observations of courtship behaviour by resistant and susceptible males in non-competitive mating trials. Finally, we counted aggressive acts in pairs of resistant and pairs of susceptible males to determine levels of male-male aggression within each genotype. We found that the effect of DDT-R on male size does contribute to lowered mating success but does not fully explain this fitness cost. Resistant males were also found to have a lowered rate of courtship behaviour driven by aborted chasing of the female. Susceptible males demonstrated higher levels of aggression than resistant males overall, with greatly enhanced aggression in evening trials. These behavioural differences may constitute the other phenotypic components that, along with male size, result in the DDT-R mating costs previously observed.

4.2 Introduction

A central question in the evolution and spread of insecticide resistance is the fitness of the organism carrying a mutant allele of a resistance gene. Theory holds that, in the absence of insecticide, the majority of insecticide-resistant organisms should show some differential survival in comparison with “wild-type” organisms. That is, resistance should be costly (e.g., Crow 1957). However, empirical evidence of pleiotropic fitness costs associated with insecticide resistance is equivocal.

There are a few studies that confirm that investment in resistance carries a fitness cost (Alyokhin and Ferro 1999; Berticat et al. 2002; Boivin et al. 2001; Carrière et al. 1994, 1995, 2001; Chevillon et al. 1997; Foster et al. 2003; Minkoff and Wilson 1992; Rivero et al. 2011; Smith et al. 2011; Yamamoto et al. 1995). On the other hand,

some authors have failed to reveal any detrimental effects of carrying insecticide resistance alleles (Baker et al. 1998, 2008; Castañeda et al. 2011; Follett et al. 1993; Tang et al. 1997, 1999), and some have even demonstrated pleiotropic fitness benefits (Arnaud and Haubruge 2002; Bielza et al. 2008; Bloch and Wool 1994; Haubruge and Arnaud 2001; Mason 1998; McCart et al. 2005; Omer et al. 1992; White and Bell 1995). In other studies, some measures of fitness have been negatively affected, others positively (Brewer and Trumble 1991), and this may even involve sex-specific effects, where resistance alleles have different impacts on fitness depending on the sex in which they reside. In addition, how resistance alleles impact on nonresistance-related fitness can depend on the strain being investigated (Chevillon et al. 1997; Hollingsworth et al. 1997; Oppert et al. 2000; Smith et al. 2011). This reflects epistasis, where the pleiotropic effect is mediated by the genotype (or genetic background) of the insect in question.

Both epistasis and sex-specific fitness effects have recently been reported for a DDT (dichlorodiphenyltrichloroethane) resistance allele in *Drosophila melanogaster* (McCart et al. 2005; Smith et al. 2011). In *D. melanogaster* DDT resistance is conferred by the upregulation of a cytochrome P450 enzyme, CYP6G1 (Daborn et al. 2002). Resistant flies have tandemly duplicated C*yp6g1* alleles that possess the LTR (Long Terminal Repeat) of an *Accord* retrotransposon inserted in the cis-regulatory region (Daborn et al. 2002). These DDT resistance alleles (DDT-R) have been subject to further, purportedly adaptive, sequence evolution (Schmidt et al. 2010) and have gone to near fixation in many natural populations (Catania et al. 2004). Susceptible flies have the ancestral allele, which has neither duplication nor TE (transposable element)-derived enhancer sequences.

While there appears to be a consistent benefit of DDT-R to females (McCart et al. 2005; Chapter 5 of this thesis), a competitive mating cost was recently documented for DDT resistance (Smith et al. 2011: Chapter 3 of this thesis). In the two different genetic strains for which DDT-R life-history analysis has been undertaken (Canton-S and WC) DDT-R females have demonstrably increased fecundity. DDT-R females of the Canton-S background also showed an early offspring viability increase which bears the hallmark of maternal provisioning to the embryo (McCart and ffrench-Constant 2008). Interestingly, an earlier study of another laboratory-adapted population (Ives) found

that male genotypes with low mating success in a competitive setting (MCRS) had significantly higher (about two-fold) expression of *Cyp6g1* (Drnevich et al. 2004). Although the differences in expression may not have been related to the presence of the DDT-R (which was not tested), this finding suggested that there could be a trade-off between insecticide resistance and male fitness

A more recent male fitness study (Smith et al. 2011) demonstrated a strong pre-copulatory competitive mating disadvantage for resistant males (made resistant through introgression of the DDT-R allele) of the old laboratory strain Canton-S (CS), but not when the same allele was examined in the more recently wild-caught strain (WC). Resistant and susceptible males differed in size in both genetic backgrounds, but the direction of the difference depended on genetic background. In the CS genetic background, resistant males were smaller, whereas in the WC genetic background, resistant males were larger than susceptible males. Male size is a major determinant of male fitness in *D. melanogaster* (Partridge and Farquhar 1983; Partridge et al. 1987a,b; Pitnick 1991; Stearns 1992; Roff 2002). Therefore, in genetic backgrounds where resistant males are smaller, size could represent the cost to resistance resulting in reduced mating success in a competitive situation. However, this does not preclude the possibility that DDT-R could be affecting some other phenotypic component which affects pre-copulatory mating success. Resistance alleles have been shown to affect insect fitness through behavioural changes in the past (Rowland 1991; Foster et al. 2007; Foster et al. 2011). Furthermore, another cytochrome P450, *Cyp6a20*, is found on the same chromosome arm as *Cyp6g1* and has been implicated in mediating aggressive behaviour in *D. melanogaster* (Diereck and Greenspan 2006; Wang et al. 2008) in the absence of DDT resistance alleles.

Here, we examine the behavioural basis for the male competitive mating disadvantage found in DDT-R flies of the CS background. To this end we performed three separate experiments to first explore whether size mediates competitive mating success in DDT-R flies and then investigated how DDT-R also affects other aspects of mating behaviour. In the first experiment we conducted the same competitive mating assay as Smith et al. (2011), but directly manipulated the size disparity between resistant and susceptible males to investigate whether the size difference is sufficient to cause the DDT-R mating disadvantage. Secondly, we examined, in detail, the

courtship behaviour of DDT-R and susceptible males in a non-competitive context to quantify potential differences in the intensity, rate and sequence of behaviours that may lead to differential mating success. Lastly, we investigate within-genotype (resistant or susceptible) male-male aggression by quantifying aggressive behavioural interactions in a simple two-male arena assay similar to that of Diereck and Greenspan (2006).

4.3 Materials and methods

Introgression and population maintenance

CS stock flies were initially homozygous for the ancestral (susceptible) *Cyp6g1* allele. The DDT-R allele *Cyp6g1-BA* (Schmidt et al. 2010) was introgressed into this stock using a separate wild-caught resistant strain for the initial cross (Smith et al. 2011). This was followed by repeated backcrossing for seven more generations into stock CS flies. After each generation of backcrossed mating, developing progeny were subject to DDT selection by lacing rearing jars with 4µg/mL DDT in acetone solution. After the backcrossing, mating pairs were established and the progeny of homozygous resistant crosses (RR×RR) were subsequently used to found the corresponding DDT-R population (CS_{RR}). Both populations (CS_{RR} and CS_{SS}) were subsequently maintained at 25°C on complete Jazz-mix *Drosophila* food (Fisher, Pittsburgh, PA) in 30×30×30 cm population cages with 12:12 h light:dark and humidity ~40%. Experimental flies were collected as first instar larvae from Petri dishes containing 1.5% agar in apple juice with yeast paste spread on a small area of the surface. Larval rearing was kept at a standard density of 100 larvae per food vial (approximately 5 mL in 3 × 7 cm circular vials). Virgin adult flies were held in narrow food vials (approximately 5 mL in 2 × 9.5 cm circular vials) at a density of approximately 20 flies per vial.

Effect of size and resistance allele on mating success

Twenty four hours before the first experiment, we anaesthetised (using CO_2) 2-4-day old virgin CS_{RR} (resistant) and CS_{SS} (susceptible) males and sorted them, under a dissecting microscope, into categories according to thorax length measurements. Previous measurements had given modal thorax lengths of 1.07 mm for susceptible

males and 0.98 for resistant males which we used to define the three broad size categories ('large'≥1.07mm; 1.07mm>'medium'>0.98mm; 'small'≤0.98mm). Individual large males of each genotype were then randomly paired with small males of the other, as were medium resistant with medium susceptible. Each pair was gently aspirated into a narrow polypropylene vial. Prior to this pairing off, we used blue and pink paint powder to identify individual males in a factorial way following Champion de Crespigny and Wedell (2007) and Smith et al. (2011), so that half the resistant and susceptible males were blue and the other half were pink. Thus pink males always competed against blue males, and resistant males always competed against susceptible males. Experimental observers were blind to these treatments. On the day of the mating assay a single virgin female was gently aspirated into each vial. Consistent with the precopulatory competitive assay (PCC) of Smith et al. (2011), the females used were 3-5 days old and of a wild-type background (Dahomey) into which the recessive *sparkling poliert* (*spa*) mutation had been recently backcrossed (Fricke et al. 2009). For each replicate triad, at the onset of copulation we immediately aspirated the unsuccessful male out of the vial and similarly removed the successful male post-copulation. SPOT BASIC 4.1 (Diagnostic instruments, Inc., Sterling Heights, MI, USA) was then used to measure wing size as a surrogate of body size for all successful and unsuccessful males.

Male courtship behaviour

Replicates of four homozygous crosses (CS_{RR} ♀ × CS_{RR} ♂, CS_{RR} ♀ × CS_{SS} ♂, CS_{SS} ♀ × CS_{SS} ♂, CS_{SS} ♀ × CS_{RR} ♂) were established. Each dyad consisted of one virgin male and one virgin female in a shallow cylindrical arena, with courtship being video recorded from above. Each arena, similar to that used in Diereck and Greenspan (2006), consisted of a small plastic Petri dish 3.5 x 1cm (diameter x depth) with a secure lid and containing a small food cup (1.5mL Eppendorf cap). The food cup was filled with 2.0% agar in apple juice with yeast paste spread on a small area of the surface. Eight of these arenas could be arranged, in a 2 × 4 array, within the maximum field of view which allowed detailed recording of courtship behaviour under ambient light. Arenas were separated from each other by white paper partitions. Twelve hours prior to each assay virgin females were aspirated, via loading holes in the lids, into each arena to adjust to their

surroundings. After loading, each hole was covered by transparent adhesive tape. Following the 12 hour adjustment period and immediately prior to loading the males the array was placed under a high definition video camera (Panasonic HD-SD90). Recording commenced and males were then aspirated into each arena. Once a pair began copulating the arena was removed and replaced in the array by a new arena containing another virgin female, repeating the assay. If there was no copulating after 30 minutes the arena was removed and the male was classed as unsuccessful. Successful males were retained for size measurement as above. All flies were 6 days old at the time of assay.

Behavioural recordings were analysed for thirteen successful pairings of each cross. Courtship behaviours were distinguished following the protocol of Ejima and Griffith (2007) and are described in Table 4.1. Continuous records were analysed, and the frequency and duration of each behaviour as well as the times at which each behaviour stopped and started was recorded.

Male aggression

Within-genotype aggression was video recorded between pairs of virgin CS_{SS} and CS_{RR} males within the arena setup described above, with the exception that a decapitated female was placed on the food surface of each arena immediately prior to the assay to aid in attracting males (Chen et al. 2002). The resistance status of the decapitated females in each arena was balanced across genotypes. Flies reared in social environments have been shown to have suppressed aggression (Hoffman 1990). However, this is reversible after just one day of isolation (Wang et al. 2007). Therefore, for the purpose of assessing within-genotype aggression, experimental flies were individually isolated 24 hours before each assay. To further increase aggression levels, each individual male was then transferred, 90 minutes before each assay, into foodless vials containing water-saturated cotton wool. This time-scale has been shown to increase aggression without revealing any underlying differences in starvation sensitivity (Edwards et al. 2006).

All flies were 5-8 days old during the experiment and were not exposed to anaesthesia for at least 24 hours prior to the assay. As in the courtship behaviour assay, an array of 8 arenas (maximum) at a time was recorded. Two males of the same

genotype (CS_{RR} or CS_{SS}) were gently aspirated into each arena. The flies were allowed to adjust for 15 minutes, and were then recorded for 10 minutes using the same camera as the courtship behaviour assay. Flies were then anesthetised and retained for size measurement as per the male size-effect assay. In this manner a total of 30 replicate pairs of each genotype were assayed for aggression in three arrays per day (one each in the morning, afternoon and evening), over the course of three days. Four separate aggressive behaviours were defined following Chen et al. (2002). From each 10 minute recording, the frequency of aggressive behavioural occurrences was noted.

Statistical analysis

All statistical analyses were conducted using R (version 2.13.0). For behavioural frequency and duration data we used generalized linear models (GLMs). Maximal models included male- and, where appropriate female-, resistance genotype as explanatory variables with male size as a covariate. Wherever appropriate, non-normal error structure was specified with default link functions (e.g. Poisson error structure fitted with a log-link for count data, and Binomial error using logit-link for proportion data). Overdispersion was accounted for by using quasi-likelihood to specify more appropriate variance functions. In all GLM analyses stepwise model simplification of the maximal model with analysis of deviance was used to determine significant terms.

Courtship behavioural sequences were analysed as discrete event single-order Markov Chains, testing for the existence of non-random temporal associations among the seven different behaviours. Toward this goal, transition matrices were constructed by tabulating all instances in which one behaviour led to another. These were pooled for all males of each genotype to give two overall transition matrices, one for resistant males and one for susceptible males. Transition categories which never occurred (e.g. decamp→lick) were considered structural zeros (West and Hankin 2008) and not included in subsequent analysis. A generalisation of Fisher's Exact test which can cope with structural zeros is implemented in R package 'aylmer.test' (West and Hankin 2008) and was used here to test for non-randomness (stereotypical structure) in the sequence of behaviours both at the level of the whole matrix and for each possible transition. The p-value in Fisher's test is normally calculated by summing the probabilities of all permissible matrices (i.e. matrices with the same marginal totals)

with equal or more extreme arrangement than that observed (Agresti 2002; West and Hankin 2008). In the case of the present analysis, the total number of transitions is extremely large, making it impractical to enumerate all possible matrices. Thus Markov Chain Monte Carlo (MCMC) was used to explore the space of permissible matrices and approximate the p-value (West and Hankin 2008).

4.4 Results

Effects of size and resistance allele on mating success

Of the 187 successful competitive trials, susceptible males won the majority (120). A maximal GLM model of the binary response (susceptible or resistant male wins) was fitted as a function of size ratio (i.e. susceptible male wing size/resistant male wing size), along with susceptible male wing size as a covariate and susceptible male colour with interactions, using binomial error structure. Stepwise model simplification revealed a sole significant main effect of the size ratio on whether a resistant or susceptible male won a competitive trial (Figure 4.1a; $\chi^2_1 = 5.204$, $p = 0.023$, binomial errors). According to this minimal adequate model, susceptible males have a greater than 50% chance of winning a competitive trial when the susceptible/resistant size ratio is at least 0.9. Further examination of the data was carried out by dividing the trials by post-hoc size measurements into three categories. These were “Matched”, which consisted of closely sized males (within ±2.5% of each other); “Smaller SS”, where the susceptible male was more than 2.5% smaller than the resistant; and “Larger SS”, where the susceptible was more than 2.5% larger than the resistant. In the latter category susceptible males won the significant majority of trials (Figure 4.1b; Exact Binomial Test, 52 successes from 73 trials, $p < 0.001$) but there was no significant departure from a null of 50% for either the “Matched” (Figure 4.1b; Exact Binomial Test, 32 successes from 55 trials, $p = 0.28$) or “Smaller SS” (Figure 4.1b; Exact Binomial Test, 31 successes from 50 trials, $p = 0.12$) categories. Thus there is nullification, but no reversal of the susceptible mating advantage when resistant males are larger than susceptible males.

Model simplification of log-transformed copulation latency as a function of wing size ratio and susceptible male colour yielded a null minimum adequate model.

Thus the size difference of the competing males did not have any effect on copulation latency (log-transformed latency, $F_{1,185} = 1.751$, $p = 0.19$, normal errors). Using the subset of trials for which there was copulation duration data (n = 183), model simplification of copulation duration as a function of size ratio and male genotype yielded a sole significant effect of male genotype on duration, after removal of two outliers (Figure 4.2; null ($F_{1,180} = 6.0747$, $p = 0.015$, normal errors). Resistant males had shorter copulations (974±38 sec, mean ± s.e.) than susceptible males (1090±29 sec, mean ± s.e.).

Male courtship behaviour

Both resistant and susceptible males displayed the full repertoire of courtship behaviours described in the methods. None of the GLM models revealed any significant effects of female genotype or male size or their interactions with each other and male genotype. Thus only the main effects of male genotype on courtship behaviours are reported here. There was a strong significant effect of male resistance genotype on copulation latency (in seconds) (Figure 4.3a; $F_{1,50} = 14.236$, $p < 0.001$, Gamma errors) with resistant males (mean latency = 823sec, standard error interval = (743,923)) taking almost twice as long to achieve copulation as susceptible males (mean latency = 449 sec, standard error interval = (407,502)). When decomposed into courtship latency and adjusted copulation latency (time from first courtship behaviour to copulation) we find no effect of resistance status on the former (Figure 4.3b; $F_{1,50} = 0.8472$, $p = 0.36$, quasipoisson errors), but a highly significant effect on the latter (Figure 4.3c; $F_{1,50} = 11.471$, $p = 0.001$, quasipoisson errors) . In summary, resistant males take longer than susceptible males to achieve copulation once courtship has been initiated.

Once courtship began there was no difference between the male genotypes in the proportion of time spent actively courting time versus away (decamped) from the female ($F_{1,50}= 2.3412$; $p = 0.14$, quasibinomial errors). There was a significant effect of male genotype on the frequency of decamping events relative to the total number of behavioural events (Figure 4.4; $F_{1,50}= 7.959$, $p = 0.007$, quasibinomial errors) with resistant males having a higher percentage of decamping events (11%) than susceptible males (6%).

The most common courtship behaviour, in terms of relative frequency, was wing vibration but there was no effect of male genotype on either proportion of time spent on wing vibrating (logit-transformed proportions, $F_{1,50} = 3.1183$, $p = 0.08$, normal errors) or in the relative frequency of wing vibrating events ($\chi^2_1 = 0.47196$, $p = 0.49$, binomial errors). In contrast, there was a significant effect of male genotype on the wing vibration rate (events per minute) after removal of a single outlier (Figure 4.5a; $F_{1,49} = 6.831$, $p = 0.010$, Gamma errors) with resistant males having a lower rate (2.44 min^{-1},standard error interval = (2.16, 2.80)) than susceptible males (4.07 min^{-1}, standard error interval = (3.62, 4.66)).

The second most frequent courtship behaviour, and the one which accounted for the greatest proportion of time, was chasing. There was no significant difference between resistant and susceptible males in the proportion of time spent chasing females ($F_{1,50} = 0.0671$, $p = 0.80$, quasibinomial errors) or in the relative frequency of chases (logit-transformed relative frequency, $F_{1,50} = 1.012$, $p = 0.32$, normal errors). As with wing vibrations, there was a significant effect of male genotype on the chase rate after removal of a single outlier (Figure 4.5b; $F_{1,49} = 17.934$, $p < 0.001$, normal errors) with resistant males having a lower rate (1.55 min^{-1},standard error interval = (1.31, 1.79)) than susceptible males (2.99 min^{-1},standard error interval = (2.74, 3.22)).

Both resistant and susceptible males required the same number of attempted copulations to achieve success ($F_{1,50} = 0.003$, $p = 0.96$, quasipoisson errors). Similarly, there was no difference in the relative frequency of attempts (logit-transformed relative frequency, $F_{1,50} = 1.470$, $p = 0.23$, normal errors). There was, however, a significant effect of male genotype on the attempted copulation rate (Figure 4.5c; $F_{1,48} = 9.049$, $p = 0.004$, Gamma errors) after removal of two outliers, with resistant males having a lower rate (0.82 min^{-1},standard error interval = (0.69, 0.99)) than susceptible males (1.76 min^{-1}, standard error interval = (1.50, 2.14)).

Genital licking was a frequent behaviour with very short bouts. There was a significant difference in the proportion of time spent genital licking, ($F_{1,50} = 4.369$, $p = 0.042$, quasibinomial errors) with resistant males spending a smaller proportion of their time on this behaviour than susceptible males, but in both cases licking did not occur often (resistant estimate = 0.6%; susceptible estimate = 1.0%). No difference was found between resistant and susceptible males in the relative frequency of this

behaviour (χ^2_1= 0.0002, p = 0.99, binomial errors). Genital licking rates were highly non-normal and heteroscedastic and no transformation or use of alternative error structure allowed use of the GLM framework. However, a simple non-parametric test failed to find any significant difference in rates between resistant and susceptible males (Wilcoxon rank-sum test, W = 252, Z = -1.580, p = 0.12).

A total of 1651 individual behavioural transition events were observed, including 963 during the 26 courtship pairs involving resistant males (Table 4.2) and 688 during the 26 susceptible male courtships (Table 4.3). Twenty nine (29) different transitions were observed, the most frequent being chase→ wing vibration (resistant freq. = 246; susceptible freq. = 192) and wing vibration→attempt copulation (resistant freq. = 79; susceptible freq. = 81). Results of the generalised Fisher's Exact Test show departure from independence for both the resistant (p < 0.001) and susceptible (p < 0.001) matrices, indicating the presence of stereotypical behavioural sequences. When similar tests were conducted to examine if individual transitions happened more frequently than expected by chance, (following Silvapulle and Sen 2005; West and Hankin 2008), resistant males showed eight significant transitions (Table 4.2) and susceptible males nine (Table 4.3). The significant transitions (p < 0.05) which they have in common include attempt copulation→chase, chase→ wing vibration, lick→attempt copulation, wing vibration→attempt copulation and wing vibration→lick. Resistant males had a further three significant transitions which are not found in susceptible males. These were chase→decamp, chase→wing vibration and fence→wing vibration while susceptible males have four significant transitions which were not significant in resistant males viz. decamp→fence, decamp→tap, fence→decamp and lick→chase.

All significant transitions are shown in kinematic diagrams of resistant and susceptible male courtship behaviour (Figure 4.6). For simplicity, and to highlight the most common transitions, only those which occurred at least 10% of the time are represented. Overall patterns of behaviour were similar for both male genotypes, with males tending to move from chasing to wing vibration followed by genital licking and/ or attempted copulation. If the attempt failed, the male would chase the female if she moved away, or transition back to wing vibration. Key differences in the patterns of the two male genotypes include transitions away from and returning to the female (i.e.

decamping). Resistant males were more likely to decamp following a chase. A significant 19% of resistant chases ended with the male decamped as opposed to a non-significant 7% of susceptible chases. Additionally, there appeared to be no stereotypical route back to the female for resistant males as there was no significant transitions from a decamped state even though the probability of transitioning to wing vibration was large (0.59) and similar to that of susceptible males (0.57). Another key difference was the probability of attempting copulation following wing vibration which was a significant transition in both cases but higher in susceptible males (0.30) than in resistant males (0.22).

Aggression

Thirty four (34) pairs of each male genotype were assayed for aggression. Aggressive behaviours were observed in 33 of the susceptible pairs and 25 of the resistant pairs, revealing a significant association between male genotype and the presence of aggression (Fisher's Exact test, $p = 0.013$). A maximal GLM model of the total number of aggressive behaviours was fitted as a function of male genotype, decapitated female genotype and time of day (three levels: Morning, Afternoon and Evening) with all interactions, using a quasipoisson error structure. During model simplification it was found that morning and afternoon results were not significantly different so a new factor was created with two levels ('Early' = Morning and Afternoon; and 'Late' = Evening). The minimal adequate model included a significant interaction between male genotype and this new factor (Figure 4.7; $F_{1,64} = 4.602$, $p = 0.036$, quasipoisson errors). After removal of this interaction there were still significant main effects of male genotype ($F_{1,65} = 17.117$, $p < 0.001$) and time of day ($F_{1,65} = 13.607$, $p < 0.001$). In summary, resistant males displayed lower aggression than susceptible males and while time of day does not affect aggression levels in resistant males, susceptible males show much-elevated aggression in the evening.

Complete wing size data was obtained for 60 of the 68 pairs permitting the size disparity between males to be calculated. When size disparity (and its interactions terms) was added to the minimum adequate model using this subset of the data there was no significant change in deviance. The disparity in size between competing males had no effect on total aggression levels. Similarly there was no effect of size disparity,

male genotype or their interactions on the proportion of aggressive acts that were high intensity (boxing and head butting) as opposed to low (wing threat and chase).

4.5 Discussion

We have demonstrated that the effect of the DDT resistance allele on male size previously documented (Smith et al. 2011) is an important phenotypic component mediating the DDT-R male mating cost in the CS background, but is insufficient to explain the magnitude of this cost. We have also identified striking differences in courtship and aggressive behaviour between resistant and susceptible males that also potentially contributes to differential male mating success in a competitive setting.

Our previous results (Smith et al. 2011) provided some evidence, without making a causative link, that the DDT-R mating disadvantage was an outcome of the resistance allele's effect on male size. There, DDT-R males were smaller than their susceptible counterparts in the CS background and this was a robust and highly significant finding across experiments. We suggested that this size difference was the pleiotropic phenotypic effect which mediated the competitive mating cost of resistance in this genetic background. This was further supported by the lack of a mating disadvantage seen in another background (WC), where the size effect was in the opposite direction. In the current study, by directly selecting the relative sizes of competing males, we confirm an effect of a male size disparity on the probability of susceptible (or resistant) males winning competitive mating trials between CS males. Moreover, we show that reversal of this usual size disparity eliminates the mating disadvantage in DDT-R males. This lends support to the hypothesis that the pleiotropic effect of DDT-R on size is at least partly responsible for the fitness costs observed for DDT-R.

However, if the competitive mating disadvantage conferred to DDT-R males was solely a result of pleiotropic effects on size, the *a priori* expectation was that large resistant males have a competitive advantage when competing against smaller susceptible males. This was not seen. In fact, large resistant males still lost most of their trials against small susceptible males (31 out of 50), even though this was not a significant departure from a binomial 0.5 expectation. According to the logistic model the probability of resistant males winning a trial does not exceed 50% until the

susceptible/resistant size ratio drops below 0.9. This suggests an effect of DDT resistance status on male competitive mating success over and above the effect of the resistance allele on size and motivated an assessment of potential behavioural effects of carrying the resistance allele.

In the non-competitive courtship behaviour assay there was no difference in the courtship latency of resistant and susceptible males. This suggests that DDT-R did not affect males' detection of or attraction to females of the same genetic background (CS). Once courtship had been initiated, however, resistant males took a much longer time to copulate than their susceptible counterparts. This resulted in an overall two-fold increase in copulation latency in resistant compared to susceptible males - a difference which, if extrapolated to a more natural scenario of competition for females, would constitute a very significant fitness cost. When courtship behaviour was examined in more detail, there were large and significant differences in many behaviours (Table 4.4). Interestingly, both resistant and susceptible males required the same number of attempted copulations to achieve intromission and attempted copulation at the same frequency relative to all courtship behaviours. However, attempted copulations occurred at a slower rate (per unit time) in resistant males and this points towards differences in other key behaviours in the lead up to the terminal event (i.e. successful intromission).

Genital licking typically preceded an attempted copulation and resistant males spent a smaller proportion of their time engaged in this behaviour. However, bouts of licking were short and thus constituted a tiny fraction of the courtship time budget for both genotypes. More importantly, genotypes did not differ in the relative frequency or rate of this behaviour. Courtship song (wing vibration) tended to precede either genital licking or a direct attempt at copulation. Once again, genotypes did not differ in the relative frequency, or in the proportion of time spent wing vibrating. Critically though, resistant males performed this behaviour at a lower rate. This follows the same pattern as chasing, where a depressed chase rate was observed in resistant males.

Decamping was the major behaviour showing a significant difference in terms of relative frequency – in resistant males more than 11% of behavioural events were decamps, which is almost twice that found for susceptible males. This implies a

significant difference in the structure of courtship of resistant and susceptible males, one borne out in the behavioural sequence analysis. As expected, overall transition matrices were found to be significantly non-random, indicating stereotypical sequences of behaviour for both resistant and susceptible male courtship – something that is well documented in *Drosophila* (Spieth 1974). While the overall sequences of behaviour were similar for both male genotypes, there was a much higher probability of a resistant male's chase ending in decamping i.e. movement away from the female – a significant 19% of resistant male chases ended this way. In contrast only 7% of susceptible male chases ended in decamping representing a non-significant transition. Taken together, resistant males decamp following a chase more often than by chance and much more often than susceptible males for which this is a random sequence. This corroborates the observed difference in relative frequency of decamping events.

The tendency for resistant males to break off from the normal courtship sequence is interesting and could be the cause of the depressed rates of courtship behaviours such as chasing and wing vibration. This is reinforced when we consider that after decamping, there was no non-random route back to the active courtship sequence for either resistant or susceptible males. Once a male decamped he was unlikely to re-enter the sequence where he left off. Susceptible males were more likely to follow courtship song with a copulation attempt (30% of transitions from wing vibration were of this type) compared to resistant males (22% of transitions from wing vibration). This combination of disrupted courtship sequence through surrendered chases and lowered wing vibration to attempt copulation probability ultimately accounts for the increased copulation latency observed in resistant males.

Overall aggression levels in susceptible males were found to be much higher than in resistant males. In early trials (morning and afternoon), counts of aggressive acts were more than 50% higher in susceptible males than resistant males. The difference was greatly magnified during evening trials, with more than 4 times as many aggressive acts in susceptible males as in resistant males. The evening effect was driven solely by increased aggression in susceptible males, with resistant males showing no difference in aggression levels. This is an intriguing result, suggesting some disruption by DDT-R of the normal temporal variation in aggressive behaviour. Is it

possible that *Cyp6g1* overexpression may alter normal circadian patterns of aggressive (and other) behaviours?

It is important to note that while these results were striking, the current setup aimed to maximise aggression levels by priming males before the trial. This priming involves isolation and starvation, and so it is conceivable that realized aggression levels in a different social and environmental context may be low enough as to obscure any differences between resistant and susceptible males. This has implications for how increased aggression levels may impact male success in competition for females. Observations under field conditions suggest that, in nature, fighting in *D. melanogaster* might be unimportant in acquiring a mating advantage (Partridge et al. 1987b; Taylor and Kekic 1988). Nevertheless, under some conditions, aggression has been shown to confer a mating advantage for territorial males (Hoffmann and Cacoyianni 1990). Such conditions include defendable food sources and crucially, the occurrence of only a small number of other males. Thus, in terms of explaining the observed DDT-R male mating cost described by Smith et al. (2011), invoking DDT-R effects on aggression is justified.

Outstanding questions include how these DDT-R-influenced differences in size, aggression and courtship behaviour relate to each other and how they are integrated to affect male fitness. Studies on the role of the gene *fruitless* suggest that aggression and mating behaviour are genetically closely linked in *D. melanogaster* (Vrontou et al. 2006). Zwarts et al. (2011) found that the genetic architecture of *D. melanogaster* aggression is dominated by pervasive pleiotropy, extensive epistasis, and a large mutational target size. This situation is most likely replicated for other complex behaviours such as courtship. Studies of aggression (Edwards et al. 2006) and mating behaviour (Mackay et al. 2005) in *D. melanogaster* have revealed large numbers of differentially expressed genes (as much as 10% of the genome) in response to artificial selection. If such a large proportion of the genome affects any one trait, the same genes must affect multiple traits. Thus, genes affecting behaviour are also likely to be involved in neurogenesis, metabolism, development, and general cellular processes, and many of the same genes may affect multiple behaviours (Edwards et al. 2006). Moreover there was a large overlap in the genes (878 probe sets) which responded to selection in these two studies, more so than expected by chance (Edwards et al. 2006).

Two of the genes described by Mackay et al. (2005) and Edwards et al. (2006), *Pigment dispersing factor* and *chryptochome*, were not only differentially expressed between lines selected for increased and decreased mating speed, and between lines selected for different levels of aggressive behaviour, but had been initially defined based on their involvement in circadian rhythm.

Some P450s may be able to metabolize both xenobiotics and endogenous compounds (Scott 2008) and this might be the case for *Cyp6g1*, which has broad specificity (Daborn et al. 2002). *Cyp6g1* is expressed mainly in the midgut, malphigian tubules and fat body of larvae and adult *D. melanogaster* (Chintapalli et al. 2007; Chung et al. 2007; Chung et al. 2009). These tissues are associated with digestion, excretion and osmoregulation. They are also, critically, sites of steroid hormone metabolism (Canavaso et al. 2001). The combination of *Cyp6g1*'s broad specificity and expression in these tissues open the possibility that its overexpression increases metabolism of one or more endogenous substrates affecting development and behaviour. While Chung et al. (2009) did not detect any endogenous *Cyp6g1* expression in embryos, a previous study (McCart and ffrench-Constant 2008) had shown that eggs laid by DDT resistant females contained 20-fold more *Cyp6g1* RNA prior to the start of endogenous transcription. This suggests that the early viability advantage conferred to offspring of resistant females (McCart et al. 2005) may be a result of maternal provisioning of *Cyp6g1* transcripts, implicating *Cyp6g1* in early developmental processes.

Intriguingly, a recent study also found a more than 4-fold higher *Cyp6g1* expression level in brains of European over African populations of *D. melanogaster* (Catalán et al. 2012). As all the European, but than half of the African populations used in that study possessed the *Accord* insertion (i.e. the DDT-R allele) (Müller et al. 2011), it is likely that DDT-R alleles are responsible for the overexpression and may directly affect behaviour through increased CYP6G1 activity on some endogenous substrate in the brain.

Another possibility, which cannot be discounted, is that there is an energetic cost to *Cyp6g1* overexpression. A trade-off between resistance and energetic reserves has been found in recent studies of resistant *Culex pipiens* mosquitos (Hardstone et al. 2010; Rivero et al. 2011). While such a trade-off might explain a number of

phenomena in DDT-R CS males, such as their smaller size, increased decamping after chases and lower aggression (aggressive acts can be energetically taxing), it would be harder to explain the increased reproductive output of DDT-R females (McCart et al. 2005; Chapter 5) or the lack of DDT-R in males when expressed in a different genetic background (Smith et al. 2011).

The present study suggests that several elements of inter- and intrasexual selection may play a role in determining the fitness of DDT-R males. As yet it is not certain how the different aspects of DDT-R male phenotype are integrated to result in the observed pre-copulatory mating cost. The first step to understanding this will involve more detailed analysis of courtship in a competitive context. The presence of a competing male might reinforce the decamping effect through so-called 'loser effects' i.e. where losers fail to win subsequent fights (Yurkovic et al. 2006). It could also potentially diminish it through social facilitation of active courtship behaviours. Additionally, it would be interesting to see if the male mating costs scale up to population level phenomena, especially given the reduced inter-male aggression levels observed at higher densities (Hoffmann 1990; Wang et al. 2008) and recent evidence of social niche construction through aggression in *D. melanogaster* (Saltz and Foley 2011).

In summary, this study provided evidence of multiple effects of DDT-R on male non-resistance phenotype in *Drosophila melanogaster* for a range of behaviours closely linked to male fitness. We have confirmed that the competitive mating cost previously reported for DDT-R males is at least partly mediated by pleiotropic size effects. We have also discovered large behavioural impacts of DDT-R, including reduced male aggression and increased probability of aborted courtship sequences that are potentially important in generating the DDT-R mating disadvantage of CS males.

Table 4.1 Behaviours displayed by male *D. melanogaster* in courtship and aggression assays.

Designation	**Behaviour** (as defined in *Ejima and Griffith 2007 and †Chen et al. 2002)
Courtship:	
Tapping *	Male touches female's body with his foreleg
Fencing	Male and female hit each other's forelegs
Chasing	Male follows female
Wing vibration *	Male extends and vibrates wing, producing courtship song
Genitalia licking *	Male extends proboscis and licks the female's genitalia
Attempted copulation *	Male grasps female with forelegs and curls tip of his abdomen
Decamping	Male walks away from female, so no courtship is taking place
Aggression†:	
Wing threat	Male quickly raises both wings to a 45° angle towards opponent
Lunging	One male rears up on hind legs and snaps down on the other
Holding	One male grasps the opponent with forelegs and tries to immobilize
Tussling	Both males tumble over each other, sometimes leaving food surface

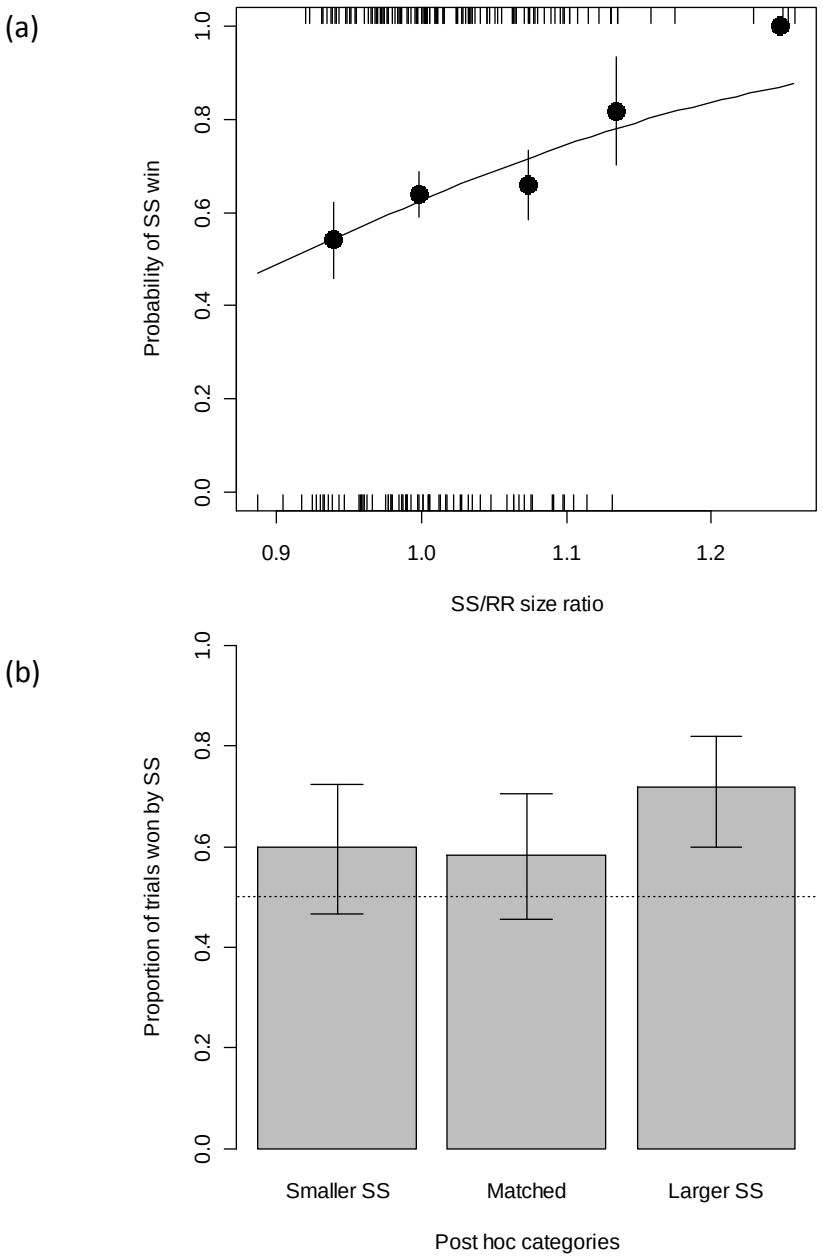

Figure 4.1 The effect relative size on whether a susceptible or resistant male wins in competitive trials. (a) Logistic plot: the curve represents the fit of the logistic model of susceptible male win probability as a function of the susceptible/resistant wing size ratio (SS/RR). Points show empirical probabilities (+/- s.e.) of a susceptible male win. Rugs (vertical lines) at the top and bottom of the graph show the empirical distribution of binary win data (susceptible win = 1; resistant win = 0). (b) Probability of susceptible male win, with 95% binomial confidence intervals, when competitive trial data is divided into three post-hoc categories, namely 'Smaller SS' where the susceptible/resistant wing size ratio < 0.975; 'Matched' where 0.975 ≤

susceptible/resistant wing size ratio ≤ 1.025; and ‘Larger SS’ where susceptible/resistant wing size ratio > 1.025.

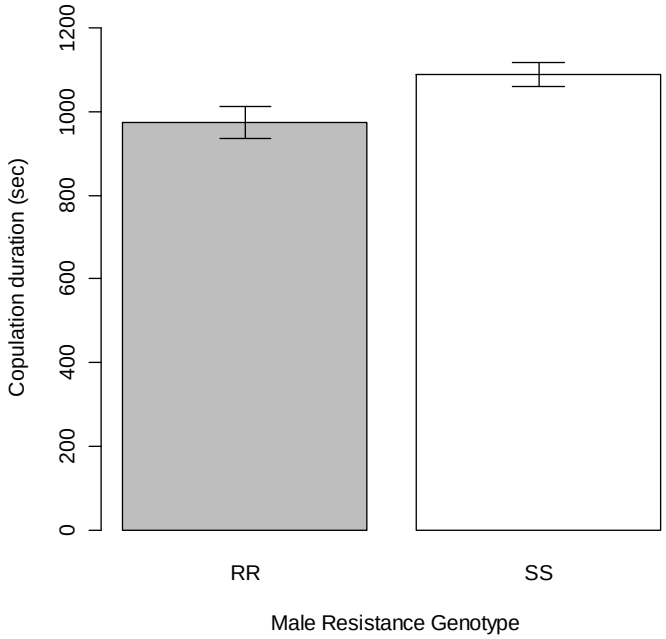

Figure 4.2 Effect of male resistance genotype on copulation duration. Mean time in seconds with standard errors shown. Resistant = 'RR'; susceptible = 'SS'.

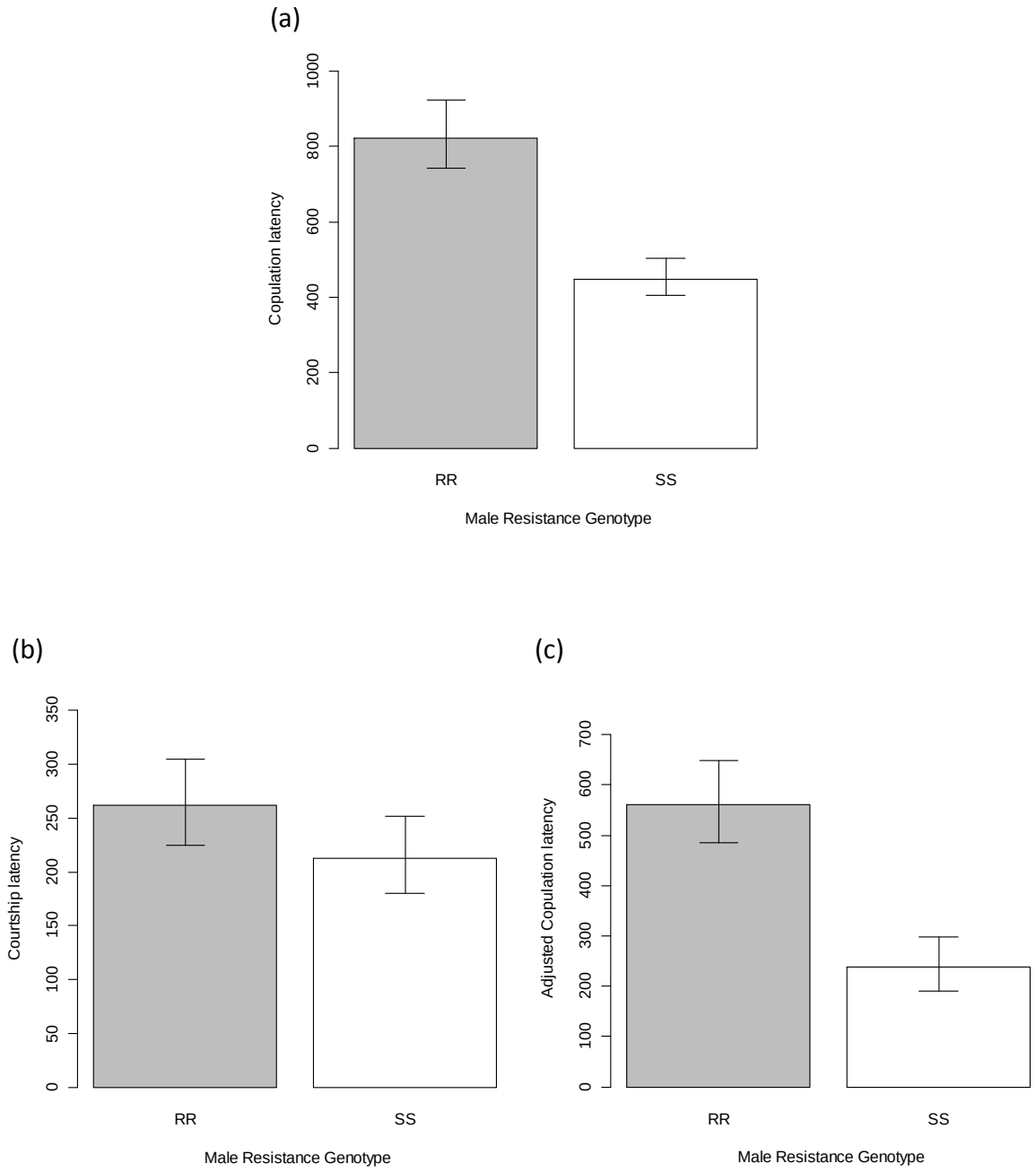

Figure 4.3 Effect of male resistance genotype on (a) total copulation latency, (b) courtship latency and (c) adjusted copulation latency (time taken from first courtship behaviour to successful copulation). Mean time in seconds with standard errors shown. Resistant = 'RR'; susceptible = 'SS'.

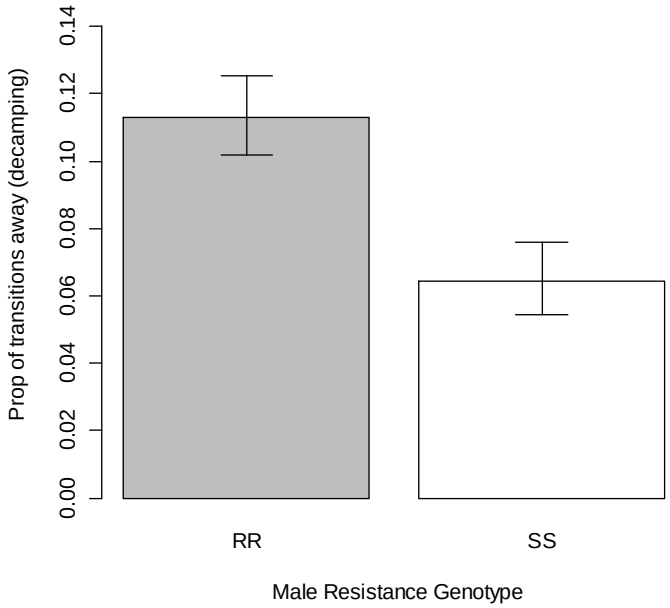

Figure 4.4 Effect of male resistance genotype on the proportion of behavioural events that are decamping events. Mean proportions with standard errors shown. Resistant = 'RR'; susceptible = 'SS'.

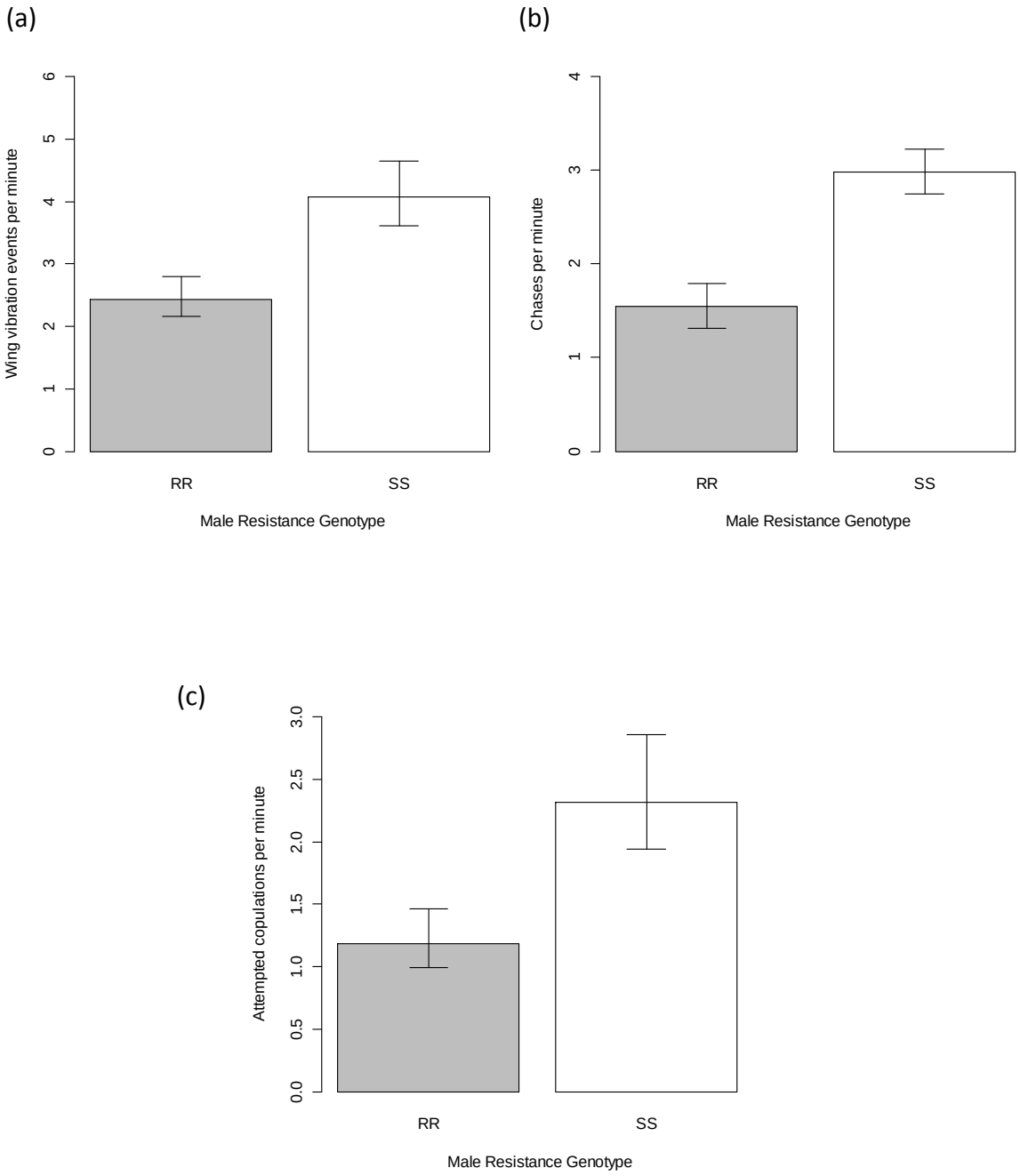

Figure 4.5 Effect of male resistance genotype on (a) wing vibration rate, (b) chase rate and (c) attempted copulation rate. Mean rate (events per minute) with standard errors shown. Resistant = 'RR'; susceptible = 'SS'.

Table 4.2 Overall behavioural transition matrix for resistant male courtship showing the frequency of each transition summed over 26 replicate trials. Transitions which occurred more frequently than by chance, as tested using a modified version of Fisher's Exact test (see text) are indicated in bold. Structural zeros are indicated by dashes.

	Following behaviour							
Preceding behaviour	attempt copulation	chase	decamp	fence	lick	tap	wing vibration	**Row Totals**
attempt copulation	-	**60**	7	-	-	-	11	78
chase	5	-	**62**	**1**	2	2	**246**	318
decamp	0	45	-	0	-	1	66	112
fence	-	1	0	-	-	-	**5**	6
lick	**20**	42	5	-	-	-	15	82
tap	0	3	-	-	-	-	3	6
wing vibration	**79**	162	38	-	**80**	2	-	361
Column Totals	104	313	112	1	82	5	346	963

Table 4.3 Overall behavioural transition matrix for susceptible male courtship showing the frequency of each transition summed over 26 replicate trials. Transitions which occurred more frequently than by chance, as tested using a modified version of Fisher's Exact test (see text) are indicated in bold. Structural zeros are indicated by dashes.

	Following behaviour							
Preceding behaviour	attempt copulation	chase	decamp	fence	lick	tap	wing vibration	**Row Totals**
attempt copulation	-	**57**	4	-	-	-	16	77
chase	8	-	16	0	3	0	**192**	219
decamp	2	13	-	**3**	-	**2**	26	46
fence	-	2	**2**	-	-	-	3	7
lick	**11**	**29**	2	-	-	-	17	59
tap	1	2	-	-	-	-	5	8
wing vibration	**81**	110	22	-	**56**	3	-	272
Column Totals	103	213	46	3	59	5	259	688

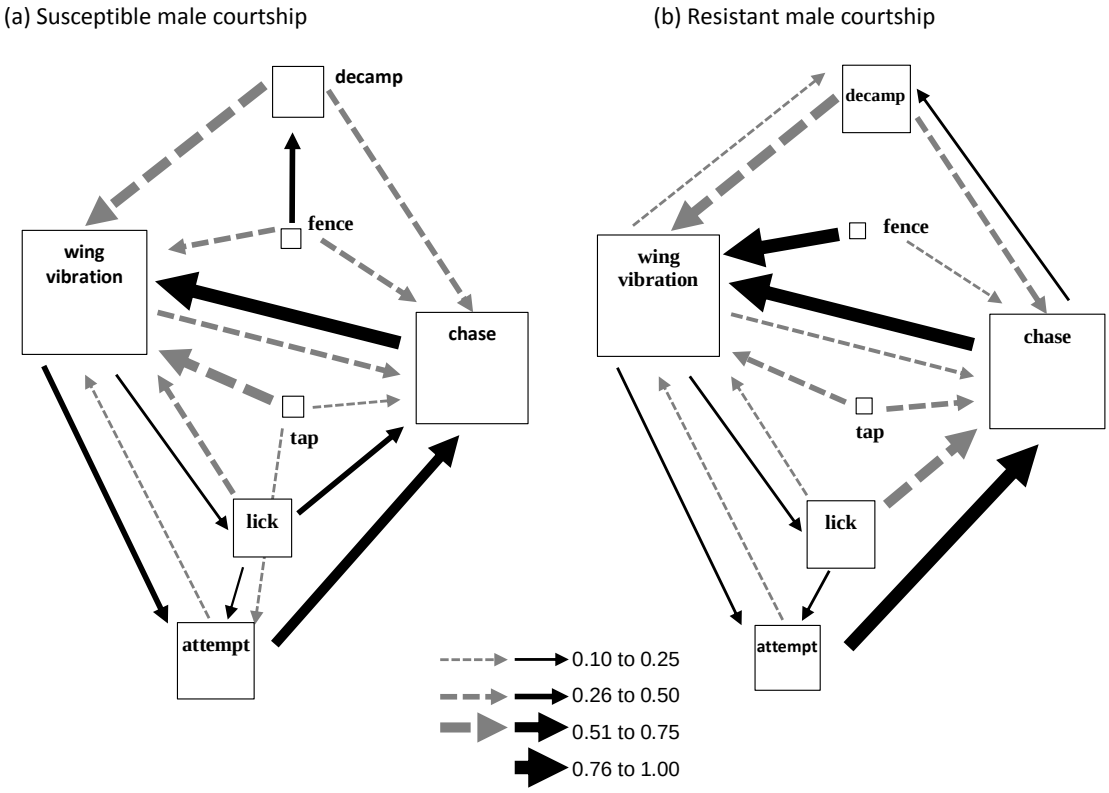

Figure 4.6 Kinematic diagram of behavioural transitions that occurred more than 10% of the time for (a) susceptible males and (b) resistant males during courtship. Arrow thickness indicates probability of occurrence. Solid, black arrows represent those transitions which occurred more frequently than expected by chance ($p < 0.05$) and grey dashed arrows show non-significant transitions ($p > 0.05$). Box size indicates frequency of behaviour.

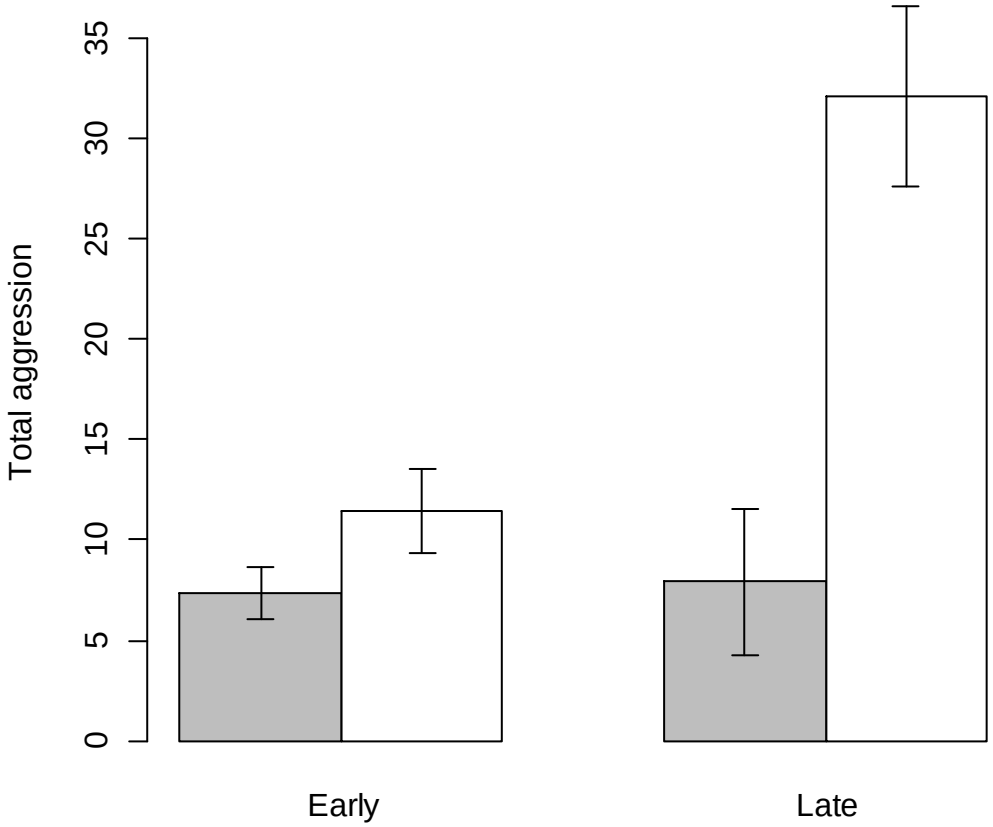

Figure 4.7 Total aggression (counts of all aggressive behaviours) observed in pairs of resistant (grey bars) and susceptible (white bars) males during two periods of the day.

Table 4.4 Qualitative summary of behavioural responses to possession of DDT-R allele. ↑ represents increase in resistant males relative to susceptible males. ↓ represents decrease in resistant males relative to susceptible males. Dash indicates no difference between resistant and susceptible males. Blank cells = not applicable.

Behavioural response	**Proportion of time**	**Relative frequency**	**Rate**	**Absolute measure**
Decamping	-	↑	-	
Chasing	-	-	↓	
Wing vibration	-	-	↓	
Genital licking	↓	-	-	
Attempted copulation		-	↓	
Copulation latency (total)				↑
Courtship latency				-
Adjusted Copulation latency				↑
Aggression				↓

Chapter 5: Consistent benefit of DDT resistance to female fitness

5.1 Abstract

In *Drosophila melanogaster* resistance to the insecticide DDT is conferred by the upregulation of a cytochrome P450 enzyme, CYP6G1. Previous studies of one resistance allele (DDT-R) in the Canton-S strain have demonstrated pleiotropic fitness benefits of DDT-R in females, but costs in some male backgrounds. It is not known if the female fitness benefits extend to other genetic backgrounds. Here we conducted fitness assays in wild caught strain females in order to examine the possibility of general fitness benefits. We show that DDT-R confers a fecundity increase but unlike previous work, no offspring viability increases were detected. Thus as with male costs, specific pleiotropic female fitness benefits to resistance depend on genetic background.

5.2 Introduction

The microevolutionary dynamics of insecticide resistance depends on the fitness of the organism carrying a resistant allele relative to the susceptible allele. The effect of resistance mutations on non-resistance-related traits has important implications for the frequency trajectories of resistance alleles, particularly following discontinuation of insecticide application. Theory holds that resistance should be costly (Crow 1957), with resistance alleles conferring some fitness disadvantage in the absence of the selecting agent (i.e. insecticide). However, empirical evidence of the pleiotropic fitness effects of insecticide resistance appears to be equivocal with examples of resistance-related fitness costs (Minkoff and Wilson 1992; Carriére et al. 1994; Chevillon et al. 1997; Alyokhin and Ferro 1999; Carriére et al. 2001; Foster et al. 2003; Rivero et al. 2011; Smith et al. 2010) and benefits (Omer et al. 1992; Bloch and Wool 1994; White and Bell 1995; Mason 1998; Haubruge and Arnaud 2001; McCart et al. 2005).

In *Drosophila melanogaster* DDT (dichlorodiphenyltrichloroethane) resistance is conferred by the upregulation of a cytochrome P450 enzyme, CYP6G1. Resistant flies have tandemly duplicated *Cyp6g1*alleles that possess the LTR (Long Terminal Repeat) of an *Accord* retrotransposon inserted in the cis-regulatory region (Daborn et al. 2002), whereas susceptible flies have the ancestral allele that has neither duplication nor TE (transposable element)-derived enhancer sequences.

An epistatic male fitness cost was recently documented for DDT resistance (Smith et al. 2011). Here a DDT-resistant allele (DDT-R) conferred a strong competitive mating disadvantage in an old laboratory strain of *D. melanogaster* (Canton-S) but not when the same allele was examined in a more recently wild-caught strain (WC). This follows on from earlier work demonstrating strong pleiotropic fitness benefits of DDT-R in Canton-S females, including enhanced fecundity and maternally induced increases in egg and larval viability (McCart et al. 2005). This represents a striking example of intralocus sexual conflict, where the allele has positive fitness effects in one sex but negative in the other sex (Bonduriansky and Chenoweth 2009; Hosken et al. 2009), and where the precise locus under sexually antagonistic selection (*Cyp6g1*) has been identified.

Given the strong sex-specific fitness effect of DDT-R and the varying fitness consequences for males of different strains, it remains to be seen whether the reported fitness benefits to females are consistent across different genetic backgrounds. Here we report on a number of DDT-R fitness assays in WC females (Smith et al. 2011) in order to assess this.

5.3 Materials and methods

Genetic background and introgression

WC stock flies were initially homozygous for the ancestral *Cyp6g1* allele (SS) and DDT-R was introgressed into the WC background using Hikone-R flies (McCart et al. 2005; Smith et al. 2011) for the initial cross. This was followed by repeated backcrossing for five generations into stock WC flies (WC_{SS}). After each generation of backcrossed mating, developing progeny were subject to DDT selection by lacing rearing jars with 60µg/ml DDT in acetone solution. After backcrossing, mating pairs were set up and progeny of RR×RR crosses were then used to found the corresponding DDT-R population (WC_{RR}). Both populations (WC_{RR} and WC_{SS}) were subsequently maintained at 25°C on complete Jazz-mix *Drosophila* food (Fisher, Pittsburgh, PA) in 30 × 30 × 30 cm population cages with 12:12 h light:dark. Experimental flies were collected as first instar larvae from Petri dishes containing 1.5% agar in apple juice with yeast paste spread on a small area of the surface. Larval rearing was kept at a standard density of 100 larvae per food vial. Virgin adult flies were held in vials containing food at a density

of approximately 20 flies per vial. All flies were two to five days old at the start of each experiment.

Female fitness assays

Blind mating trials of all four homozygous crosses (WC_{RR}♀×WC_{RR}♂, WC_{RR}♀×WC_{SS}♂, WC_{SS}♀×WC_{SS}♂, WC_{SS}♀×WC_{RR}♂) were set up, with dyads consisting of one virgin male and one virgin female in a narrow polypropylene vial. In the first experiment 20 replicates of each cross were initiated and 73 of 80 pairs mated. Males were removed after copulation and females allowed to oviposit in the vial for 24 hours. Each female was moved (at 24 hour intervals) twice into new oviposition vials and then removed for size measurement. Adult offspring were collected and counted at 5 days from the first eclosion. In the second experiment 40 replicates of each cross were initiated and 127 of 160 pairs mated. Males were removed as before and, at 24 hours post-mating, females were placed to oviposit for 24 hours each on a series of three egg laying cups. Total eggs in each cup was assessed immediately upon removal of the female as a measure of fecundity, while in the first two cups the number of unhatched eggs was assessed upon removal of the female and 24 hours later to give a measure of egg viability. All larvae were removed from each cup immediately after counting and placed on new food in a separate vial to develop, with the number of eclosed offspring assessed as above. After the experiments, we used SPOT BASIC 4.1 (Diagnostic instruments, Inc., Sterling Heights, MI, USA) to measure wing size as a surrogate of body size for all mated flies.

Statistical analysis

We conducted analyses using generalized linear models (GLM) in R (version 2.13.0) with male and female wing size as covariates. Fecundity data was analysed using Poisson error structure fitted with a log-link. Proportion data, including egg and larval/pupal viability were analysed using binomial error structure with a logit link. Overdispersion in the fecundity and viability data was accounted for by using quasi-likelihood to specify more appropriate variance functions. For the purpose of eclosed offspring counts, negative binomial errors were used as a better fit for the highly overdispersed data, giving smaller model residual deviance. In all analyses stepwise model simplification with analysis of deviance was used to determine significant terms.

5.4 Results

Integrated fitness effect – adult offspring production

There were 64 crosses in the first experiment that yielded at least one eclosed offspring. For these data, model simplification of a GLM of total eclosed offspring against male genotype, female genotype and female size (and all interactions) revealed a significant effect of female genotype on the number of offspring eclosed, with resistant females producing more offspring (Figure 5.1a; $\chi^2_1 = 4.39$, $p = 0.036$). This represents a resistance-related fitness advantage similar to that found in Canton-S flies (McCart et al. 2005) (Figure 5.1b; $\chi^2_1 = 20.99$, $p < 0.001$).

Female fitness components

Full fecundity data was obtained for 115 females. Model simplification of a GLM of fecundity against male genotype, female genotype and female size (and all interactions) revealed a significant effect of female resistance genotype on number of eggs laid (Figure 5.2a; $F_{1,113} = 15.45$, $p < 0.001$) with resistant females (mean eggs laid = 23.92, standard error interval = (22.06, 25.93)) laying more eggs than susceptible females (mean eggs laid = 14.06, standard error interval = (12.56,15.72)). Egg viability data was obtained for 81 females, and simplification of the GLM of egg viability against the same explanatory variables yielded a null minimum adequate model, with no significant effect of female genotype (Figure 5.2b; $F_{1,79} = 0.5234$, $p = 0.47$). Larvae were collected from 82 females and there was no significant effect of female genotype on combined larval-pupal viability (Figure 5.2c; $F_{1,80} = 2.34$, $p = 0.13$).

4. DISCUSSION

As with Canton-S flies (McCart et al. 2005) we found that DDT-R confers a strong fecundity advantage to resistant females in the WC background. However, in contrast to Canton-S findings, we failed to find any significant viability benefit either at the egg or larval to pupal stages. Instead, the increased fecundity appears to be solely responsible for an increased number of offspring surviving to adulthood for resistant females relative to susceptible females. This is borne out by the relative fitness measure for fecundity (SS/RR= 0.59), which closely matches that for eclosed offspring (SS/RR= 0.61).

A previous study on Canton-S flies suggested a link between the maternal contribution of resistant mothers to increased offspring viability and the provisioning of embryos with increased *Cyp6g1* mRNA transcripts (McCart and ffrench-Constant 2008). It was shown that eggs laid by resistant females contain 20-fold more *Cyp6g1* RNA prior to the start of endogenous transcription in the embryo. If this link is causative, our failure to find any offspring viability effect suggests that resistant WC females may not be packaging higher levels of *Cyp6g1* RNA into their eggs.

Strong epistatic effects of DDT-R on male fitness costs have been demonstrated in the two genetic backgrounds investigated (Smith et al. 2011). In contrast, we demonstrate here that the effect of genetic background is more subtle in females, with DDT-R conferring a similarly large advantage to WC females as found in Canton-S females. A consistent female fitness benefit would help to explain the near fixation of DDT-R alleles worldwide, even in the absence of DDT, provided that male costs are smaller than female benefits and/or are rare. If the original *Cyp6g1* mutation is truly old, arising prior to the out-of-Africa *D. melanogaster* habitat expansion event (Catania et al. 2004), it may have been held at very low frequencies by male costs.

Surveys have found fixation of ancestral copies in old laboratory strains (Catania et al. 2004; Schmidt et al. 2010) suggesting that they (including Canton-S) were derived from wild populations where DDT-R was absent or rare. Hence there would have been little selection for cost amelioration in males of the Canton-S background. The onset of strong directional selection with the use of insecticides would have rapidly increased the frequency of DDT-R, favouring any mutation that mitigated male costs. In this context, it is unsurprising that DDT-R-associated male costs exist for the old laboratory strain and are absent from the contemporary wild-caught strain.

The present study reinforces the need to obtain resistance-induced pleiotropic fitness measures across multiple genetic backgrounds and in both sexes if we are to fully understand the population genetic dynamics of insecticide resistance. While this study builds on a well-researched non-pest study system, these lessons are also applicable to pest species where resistance genes have already been identified.

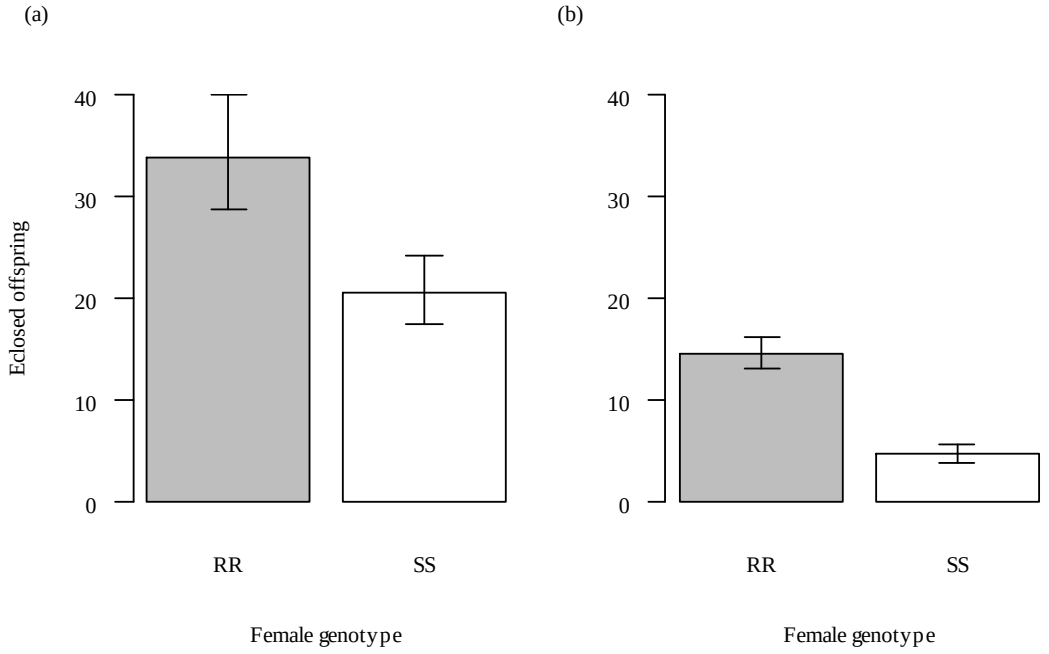

Figure 5.1 Total adult offspring production (means ± s.e.) over three days of laying post mating by resistant (RR) and susceptible (SS) females of (a) WC background; and (b) Canton-S background (from McCart et al. (2005)).

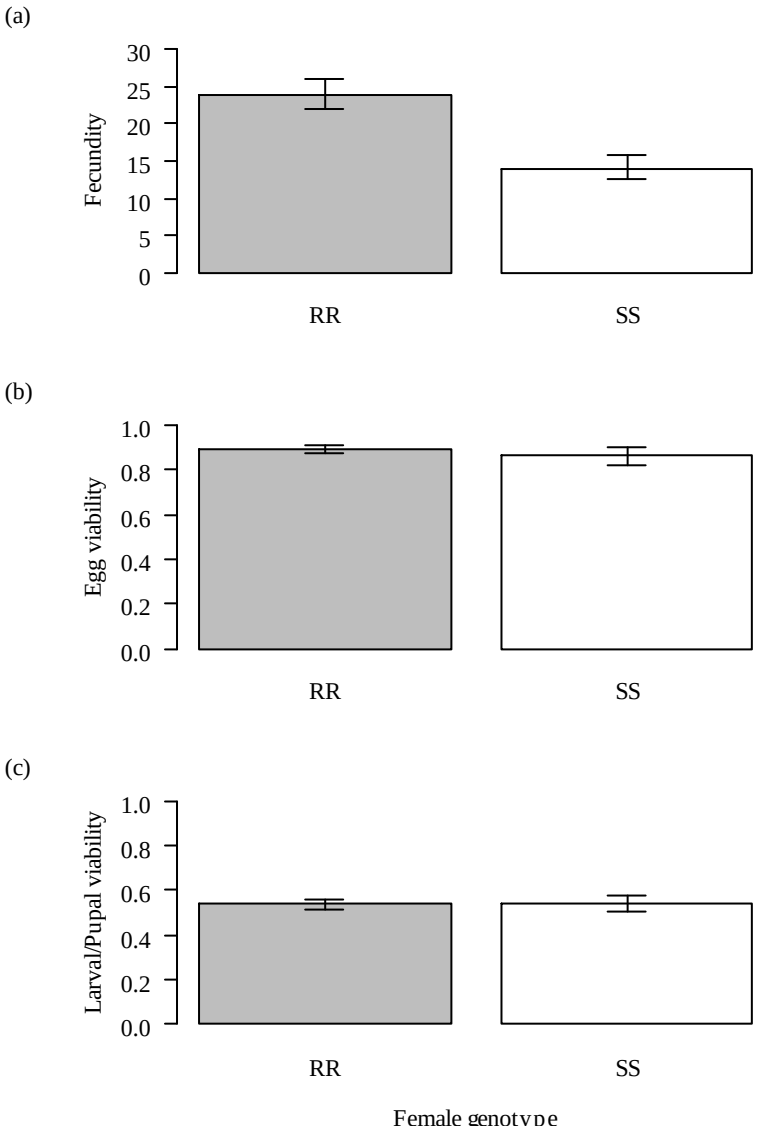

Figure 5.2 Absolute fitness component measures for resistant (RR) and susceptible (SS) females of the WC background (means ± s.e.). (a) Total fecundity. (b) Egg viability. (c) Combined larval and pupal viability. Resistant females are more fecund (a) but there is no difference in offspring viability ((b) and (c)).

CHAPTER 6: The spread of an insecticide resistance allele with sexually antagonistic effects: a model and a test

6.1 Abstract

The DDT resistance allele (DDT-R) in *Drosophila melanogaster* exerts sex-specific pleiotropic effects on individual fitness, including benefits to females and costs to males that are genetic background dependent. We present a population genetic model of this system to reflect these sex-specific pleiotropic effects, and examine the model's analytical behaviour and its outcomes for parameter values that represent the range of empirical observations found for males and females in previous studies. We also test the predictions of the model using replicate laboratory populations of two previously studied genetic backgrounds (Canton-S and WC). The model dynamics predict the rate of invasion of susceptible populations by DDT-R, solutions for allele frequency equilibria and conditions for stable polymorphisms. In so doing, a single unifying explanation is given for past and present DDT-R frequencies observed in natural and laboratory populations. Model predictions are qualitatively met in populations of Canton-S flies, demonstrating for the first time maintenance of variation at a known autosomal locus (with naturally occurring alleles) through sexually antagonistic selection. Predictions that DDT-R should move toward fixation in WC flies were not met in the laboratory populations, suggesting an as yet unspecified cost to DDT-R in this genetic background.

6.2 Introduction

The widespread use of toxic chemicals to control insect pests has resulted in strong, pervasive directional selection for insecticide resistance in both pest and non-pest species. While insecticide resistance poses a major economic problem and deservedly receives much applied research focus, it is also a biologically interesting phenomenon in its own right (Wilson 2001), with the potential to shed light on fundamental questions of evolutionary dynamics. This human-induced selection has led to some of the most dramatic examples of evolution in action, with illustrative powers resulting from several key factors. Firstly, the magnitude and ubiquity of insecticide-selection means that the evolution of resistance is both a temporally observable and spatially

expansive phenomenon, with resistance evolving in as few as 5-50 generations (May 1985; Palumbi 2001), and moving toward global fixation in many insects (Whalon et al. 2008; Catania et al. 2004; Schlenke and Begun 2004). Additionally, the overwhelming empirical evidence indicates that the evolution of pesticide resistance is typically associated with the spread of a major mutation (Wilson 2001; Rostant et al. 2012), which permits the modelling of resistance dynamics within a reasonably tractable population genetics (one to a few genes) framework.

A central question in the evolution of insecticide resistance is the fitness of the organism carrying a resistance gene in the absence of the pesticide. Theory holds that in that situation, insecticide-resistant organisms should show reduced fitness in comparison to the 'wildtype', because otherwise the resistance gene would already have been at appreciable frequencies prior to the use of insecticides. That is, resistance is assumed to be costly (e.g. Crow 1957). In reality, the evidence for pleiotropic fitness effects of insecticide resistance is equivocal. While some studies have confirmed fitness costs of resistance (Minkoff and Wilson 1992; Carrière et al. 1994, 1995; Yamamoto et al. 1995; Chevillon 1997; Alyokhin and Ferro 1999; Boivin et al. 2001; Carrière et al. 2001; Foster 2003; Rivero et al. 2011; Smith et al. 2011), others have failed to reveal any deleterious effects of insecticide resistance (Follett et al. 1993; Tang et al. 1997, 1999; Baker et al. 1998), and some have demonstrated pleiotropic fitness benefits (Omer et al. 1992; Bloch and Wool 1994; White and Bell 1995; Mason 1998; Haubruge and Arnaud 2001, 2002; McCart et al. 2005). The picture is further complicated by studies where resistance has opposing pleiotropic effects on different fitness components (Brewer and Trumble 1991). How resistance alleles impact non-resistance-related fitness can also depend on the genetic background being investigated (Smith et al. 2011). This reflects some background epistasis, where the pleiotropic fitness effect is mediated by the genotype (or genetic background) of the insect in question. The interaction of resistance alleles with the rest of the genome may even involve sexual antagonism, where resistance alleles have opposing fitness effects depending on the sex they find themselves in. Both epistasis and sexually antagonistic fitness effects have recently been documented for DDT (dichlorodiphenyltrichloroethane) resistance in *Drosophila melanogaster*, where resistance confers a strong fecundity advantage to females (McCart et al. 2005) and a

background-dependent male fitness effect – there is a competitive mating disadvantage to resistant males in at least one genetic background (Canton-S), but no such male disadvantage in another (WC) (Smith et al. 2011).

In *D. melanogaster*, resistance to DDT is conferred by the upregulation of a cytochrome P450 enzyme, CYP6G1. Resistant flies have tandemly duplicated *Cyp6g1*alleles that possess the LTR (Long Terminal Repeat) of an *Accord* retrotransposon inserted in the cis-regulatory region, 291bp upstream of the transcription start site (Daborn et al. 2002). Several of these mutant alleles have been characterised, and all differ from the ancestral susceptible *Cyp6g1* in having both the tandem repeat and *Accord* LTR insertion, while more derived alleles possess additional TE (transposable element) insertions (Schmidt et al. 2010). Surveys of contemporary natural and old (collected prior to the 1930's) laboratory populations have revealed near global replacement of the ancestral susceptible *Cyp6g1* by resistance alleles subsequent to the heaviest DDT use in the 1950's and 1960's (Catania et al. 2004), with the more derived alleles in the evolutionary sequence conferring ever higher resistance (Schmidt et al. 2010).

Catania et al. (2004) point to evidence that the *Accord* LTR insertion is an old mutation. This appears to be at odds with the absence of the resistance alleles from old laboratory populations, in spite of its demonstrably large non-resistance fitness advantage in Canton-S females (McCart et al. 2005). Smith et al. (2011) suggested that sexually antagonistic selection may have prevented the fixation of the allele prior to the introduction of DDT. This inference was supported by the relative mating disadvantage for the most common resistance allele in the Canton-S strain, which could retard the spread and fixation of the allele in the absence of DDT selection. Consistent with this, previous one-locus models incorporating sexually antagonistic selection have demonstrated that such selection can maintain genetic variation at the selected locus (e.g. Kidwell et al. 1977; Gavrilets and Rice 2006).

Here we have presented a population genetic model of the *D. melanogaster* DDT resistance system, incorporating sex-specific pleiotropic effects, and examine the model's behaviour over parameter values that represent the range of empirical observations found for males and females in previous studies. We were specifically interested in the invasion dynamics of the resistance alleles both in the presence and

absence of insecticide selection. Our main aims were to 1) examine how sexually antagonistic selection affects the rate of allele replacement from an initially susceptible population, 2) determine whether, and under what conditions, sexual antagonistic selection could produce a stable polymorphism at *Cyp6g1* in the absence of insecticide selection, and 3) examine the influence of sex-specific selection on the rate of return to such an equilibrium from near fixation of the resistance allele. We also empirically tested the model predictions by examining *Cyp6g1* genotype frequency trajectories in replicate laboratory populations in the absence of DDT selection, using the two genetic backgrounds investigated by Smith et al. (2011).

6.3 Material and Methods

The model

Given the different magnitudes and directions of selection acting at the *Cyp6g1* locus in males and females, it is difficult to predict the invasibility of susceptible populations or how DDT-R frequencies will change in the absence of insecticide selection. We modelled the frequency of DDT-R over time in *Drosophila melanogaster* using selection estimates from published fitness determinants. We derived fitness components for DDT-R females from McCart et al. (2005) who investigated female fitness (fecundity as well as egg, larval and pupal viability) in one genetic background (Canton-S). Male fitness was derived from our previous work that employed two genetic backgrounds (Smith et al. 2011). By combining this information in a simply parameterized, non-linear recursion model we generated predictions of allele frequencies over time. Additionally, we considered the effect of including a period of selection with pesticide on allele trajectories, and then by removing DDT selection (as this mimics the current situation), asked if DDT-R could be retained in the absence of this strong source of selection. All aspects of the model were executed using MATLAB.

1. Building the model

The model terms with default parameter values are outlined in Table 6.1. Given that there is a competitive mating disadvantage of DDT-R on Canton-S males (Smith et al 2011), we need to calculate the probability that a mating male has a specific genotype. We do this using the parameter *m* which represents the size of the mating

disadvantage. The proportion of fathers who carry each genotype is given by the following equations,

$$y_{RR} = \frac{mx_{RR}}{m(x_{RR} + x_{RS}) + x_{SS}}$$

$$y_{RS} = \frac{mx_{RS}}{m(x_{RR} + x_{RS}) + x_{SS}}$$

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

where R represents the DDT-R allele and S the susceptible allele. Here we assumed that heterozygote males (RS) experience the same mating disadvantage (m) as homozygous DDT-R males (RR). This assumption is based on the dominant nature of the DDT-R allele with respect to both the resistance (Daborn et al. 2002) and female fitness (McCart et al. 2005) phenotypes. Male mating probabilities (y_{ij}) vary with population genotype frequency (x_{ij}) for different values of m (Figure 6.1). For m = 1 (i.e. no mating disadvantage, as has been found for WC background males), male mating genotype probabilities are equivalent to the genotype frequencies i.e. $y_{ij} = x_{ij}$. Provided there are both resistant and susceptible males in a population, as m decreases, the proportion of DDT-R fathers (y_{RR} and y_{RS}) will be biased downwards ($y_{RR} < x_{RR}$ and $y_{RS} < x_{RS}$) and the proportion of DDT susceptible fathers (y_{SS}) biased upward ($y_{SS} > x_{SS}$).

Now we can calculate the relative mating frequencies (denoted by λ) in our population using the DDT-R genotype frequency and male mating probabilities as follows,

$$\lambda_{RRRR} = x_{RR} y_{RR}$$

$$\lambda_{RRRS} = x_{RR} y_{RS}$$

$$\lambda_{RRSS} = x_{RR} y_{SS}$$

$$\lambda_{RSRR} = x_{RS} y_{RR}$$

$$\lambda_{RSRS} = x_{RS} y_{RS}$$

$$\lambda_{RSSS} = x_{RS} y_{SS}$$

$$\lambda_{SSRR} = x_{SS} y_{RR}$$

$$\lambda_{SSRS} = x_{SS} y_{RS}$$
$$\lambda_{SSSS} = x_{SS} y_{SS} \quad (2)$$

where the mating frequency subscripts are listed in the order female genotype, male genotype.

Next, DDT-R fitness effects (summarised in Table 6.2) need to be incorporated into the model in order to predict the genotypic frequencies from one generation to the next. The relative numbers of each genotype eclosing in the next generation can then be calculated, taking into account the mating probabilities and fitness consequences as follows,

$$n_{RR} = FP\left(\lambda_{RRRR} + \tfrac{1}{2}\lambda_{RRRS} + \tfrac{1}{2}\lambda_{RSRR} + \tfrac{1}{4}\lambda_{RSRS}\right)$$
$$n_{RS} = FP\left(\lambda_{RRSS} + \tfrac{1}{2}\lambda_{RRRS} + \tfrac{1}{2}\lambda_{RSRR} + \tfrac{1}{2}\lambda_{RSRS} + \tfrac{1}{2}\lambda_{RSSS}\right) + P\left(\lambda_{SSRR} + \tfrac{1}{2}\lambda_{SSRS}\right)$$
$$n_{SS} = F\left(\tfrac{1}{4}\lambda_{RSRS} + \tfrac{1}{2}\lambda_{RSSS}\right) + \tfrac{1}{2}\lambda_{SSRS} + \lambda_{SSSS} \quad (3)$$

Where $F = f \times e \times l$. f is the relative fecundity of DDT-R females compared to susceptible females; e is the relative viability of eggs laid by DDT-R females; l is the relative viability of larvae of DDT-R females compared to susceptible females; and P is the relative pupal viability of DDT-R flies compared to susceptible flies.

To obtain the frequency of the genotypes in the next generation we use the following recursions,

$$x'_{RR} = \frac{n_{RR}}{n_{RR} + n_{RS} + n_{SS}}$$

$$x'_{RS} = \frac{n_{RS}}{n_{RR} + n_{RS} + n_{SS}}$$

$$x'_{SS} = \frac{n_{SS}}{n_{RR} + n_{RS} + n_{SS}} \quad (4)$$

Now we would like to examine the dynamics of the model, beginning with solving for frequency equilibria ($\hat{x}_{RR}$, $\hat{x}_{RS}$, $\hat{x}_{SS}$) by letting $x' = x$ for each genotype. Because the three genotype frequencies must necessarily sum to unity, this non-linear

system is effectively a two-variable (x_{RR} , x_{RS}) model and is fully described by the first two recursions in (4). If we represent the functions x'_{RR} and x'_{RS} by g_1 and g_2, respectively, then there are two conditions, namely g_1 ($\hat{x}_{RR}$, $\hat{x}_{RS}$) = $\hat{x}_{RR}$ and g_2 ($\hat{x}_{RR}$, $\hat{x}_{RS}$) = $\hat{x}_{RS}$ which must be satisfied simultaneously at any equilibrium. This was done to obtain an analytical equilibrium solution for x_R .

2. Parameterising the model

All initial fitness parameter estimates were derived from assays conducted by McCart et al. (2005) and McCart (2006) (Canton-S: f, e, l, P), Smith et al. (2011) (Canton-S and WC: m) and in Chapter 5 (WC: f, e, l, P) .The relative competitive male mating success, m, was derived as the number of mating trials won by resistant males divided by the number won by susceptible males. Relative fecundity, f, was derived by dividing the egg count of resistant females by that of susceptible females. The relative viability measures (e, l, P) were derived by dividing the resistant viability by the susceptible viability.

3. Adding DDT selection

To simulate a prolonged period of pesticide selection, the model was initially run for 200 generations, starting at low DDT-R frequency (x_{RR} = 0, x_{RS} = 0.001) with all parameters set to default (Canton-S) values in the first case. This represents an initially susceptible population into which the DDT-R allele has been introduced at very low frequency and is allowed to go to the internal equilibrium representing the situation prior to the use of DDT in the 1940s. After this initial phase a period of 'DDT selection' was added by introducing a viability advantage, D = 5, representing the mortality ratio of susceptible to resistant flies in the presence of DDT. This ratio is conservative compared to the DDT resistance ratios of Daborn et al. (2001). As the DDT resistance phenotype is dominant, this added viability advantage was assigned to both RR and RS flies. DDT selection was applied for 300 generations after which time D was set to zero and the model run until previous internal equilibrium was achieved. To demonstrate these dynamics in a hypothetical genetic background where sexually antagonistic selection would maintain DDT-R at less than 10%, a second simulation was run with

fitness parameters set at m = 0.5, F = 1.5 and P = 1.05. The same intial genotype frequencies and number of 'pre-DDT' and 'DDT selection' generations were applied. Once again the model was run post-DDT until thee non-DDT equilibrium was achieved.

Empirical tests of model in replicate laboratory populations

Our model gives specific predictions about the speed with which DDT-R alleles can invade a susceptible population and DDT-R frequency equilibria with the parameter settings employed. How well this describes changes in allele frequencies in real populations is uncertain. As a qualitative test of the model we set up replicate fly populations of two genetic backgrounds at known initial DDT-R frequencies and propagated them for five non-overlapping generations to examine DDT-R frequency trajectories over time. These two genetic backgrounds have been used in the past (McCart et al. 2005; McCart 2006; Smith et al. 2011; Chapter 5) to examine the pleiotropic fitness effects of DDT-R alleles in *D. melanogaster*, yielding information on the individual male and female fitness parameters which the model required.

1. Preparing the stock populations- introgression of the resistance allele

CS flies were supplied by Bloomington Stock Center in 2011, while the WC background is derived from one of several isofemale lines that were collected in 2004 by Trudy Mackay in North Carolina, inbred by full sib mating for 20 generations and donated to us by Frank Jiggins. Both stocks were initially homozygous for the ancestral *Cyp6g1* allele (designated *Cyp6g1-M* by Schmidt et al. (2010) and referred to as DDT-S herein) as confirmed using PCR diagnostics (Daborn et al. 2002). For the purpose of introgression, we followed McCart et al. (2005) in using Hikone-R flies (supplied by Bloomington in 2011) which are homozygous for the most common resistance-associated *Cyp6g1* allele (designated *Cyp6g1-BA* in Schmidt et al. 2010 and referred to herein as DDT-R) as confirmed using another PCR diagnostic (Schmidt et al. 2010).

DDT-R was introduced to each susceptible background by two replicate crosses each of 25 susceptible stock females × 25 Hikone-R males and the reciprocal 25 Hikone-R females × 25 susceptible stock males. The 50 flies for each replicate cross were placed in a 10 cm x 6cm glass jar containing *Drosophila* quick mix medium (Blades Biological), allowed to mate and oviposit for 72 hours and then moved on to a similarly prepared jar – each

replicate was moved on twice to maximize offspring production. Immediately following removal of parental flies the inner surface of each jar was laced with DDT by pipetting 500 µl of 60µg/ml DDT in acetone solution and rolling until the acetone had fully evaporated. F1 larvae that survived and developed into adults were then backcrossed with the relevant susceptible stock as above. This backcrossing, combined with DDT selection, was carried out for 5 generations after which offspring were mated in individual pairs and allowed to lay eggs. The parents were then diagnosed for the presence of DDT-R alleles using the Daborn et al. (2002) PCR diagnostic. The offspring of homozygous DDT-R crosses were then used to found the corresponding DDT-R populations. All four populations (DDT-R and DDT-S of CS and WC backgrounds) were subsequently maintained in 30 × 30 × 30 cm population cages.

2. Present experimental populations

For each genetic background, 8 low frequency (LF) populations (initial DDT-R allele frequency 10%), 2 mid frequency (MF) populations (initial DDT-R allele frequency 50%) and 2 high frequency (HF) populations (initial DDT-R allele frequencies CS: 90%; WC: 67% and 80%) were prepared in the following manner. Each replicate population was started with two hundred 3-5 day old virgin flies at an even sex ratio with *Cyp6g1* genotypes at Hardy-Weinberg equilibrium frequencies (RR:RS:SS was 2:36:162 and 50:100:50 for LF and MF replicates respectively) with the exception of the WC HF replicates which were not started at Hardy-Weinberg due to insufficient adult virgins at the start of the experiment. Populations were reared in vials (diameter 4.5cm and height 12cm) with adult flies left to mate and lay eggs for 72h, at which time the adults were removed, to limit larval density, and stored at -20°C. Larvae were allowed to develop, pupate and eclose, and were collected as virgins for four days after initial eclosions. Eighty flies of each sex (n = 160) from the second and third day of eclosion were then randomly selected to act as parental flies for the next generation. Non-parental flies (i.e. offspring that were not members of the selected 160) were frozen. The process was repeated for 4 more generations, after which the populations were terminated. To determine the frequency of *Cyp6g1* genotypes at the end of this period about 50 individual 5th generation flies were analysed by PCR (Daborn et al. 2002) for the presence/absence of the *Accord* LTR-inserted allele.

3. McCart (2006) experimental populations

The original aim of this population cage experiment was to determine if DDT-R conferred an overall pleiotropic fitness advantage at the population level. Two sets of population cages were established using either 50 RR 5-generation-backcrossed virgin females or males crossed to 50 SS males or 50 SS virgin females (RR × SS and SS × RR). For each set, three replicate cages were run for a total of 6 replicate populations. Flies were left to mate and lay eggs for 72h at which time the adults were removed to limit larval density. Following the emergence of the next generation, adult flies were collected for seven days and then used to found a new cage for the next generation. The populations were maintained in this manner for 10 generations. At each generation 80-120 adult offspring were taken from the transfer population to be analysed by the Daborn et al. (2002) PCR diagnostic.

6.4 Results

Model dynamics and equilibria

The model yields at least two solutions (the boundary equilibria, where DDT-R is absent or fixed) and, under certain fitness parameter values a third, internal equilibrium (intermediate DDT-R frequency). It can be shown that for a stable internal equilibrium to exist the following inequalities must be true:

$$P > \frac{2}{m+F} \tag{5}$$

$$P < \frac{m+F}{2mF} \tag{6}$$

The stability of each boundary equilibrium also depends on these inequalities. If inequality (5) is reversed, then the lower boundary equilibrium is stable i.e. DDT-R cannot invade a susceptible population and $\hat{x}_{SS} = 1$. Correspondingly, if inequality (6) is reversed, then the upper boundary equilibrium is stable. That is, DDT-R at any initial frequency will go to fixation ($\hat{x}_{RR} = 1$). We can graphically represent the three regions of parameter space (Figure 6.2).

If inequalities (5) and (6) are true, explicit solutions for all internal equilibria are as follows,

$$\hat{x}_{RR} = \frac{-(Fm^2\alpha - F^2 - m^2 - 2Fm\alpha + F^2m\alpha + Fm^2P + F^2mP - 2F^2m^2P\alpha + Fm^2P\alpha + F^2mP\alpha)}{Fm\beta}$$

$$\hat{x}_{RS} = \frac{(Fm^2\alpha - F^2 - m^2 - 2Fm\alpha - 2Fm + F^2m\alpha + 2Fm^2P + 2F^2mP - 2F^2m^2P\alpha + Fm^2P\alpha + F^2mP\alpha)}{Fm\beta}$$

$$\hat{x}_{SS} = \frac{F + m - 2FmP}{\beta}$$

Where, $$\alpha = \frac{\sqrt{(F+m)(F-m)^2(F+m-2FmP)}}{Fm\beta}$$

And, $$\beta = F + m + FP + mP - 2FmP - 2 \quad (7)$$

We can calculate the expected equilibria for the default parameter values (see Table 6.1). These values (m = 0.28, f = 2.13; e = 1.57; l = 1.13; P = 1.12) satisfy inequalities (5) and (6) and the stable internal equilibrium occurs at $\hat{x}_{RR}$ = 0.09, $\hat{x}_{RS}$ = 0.51, $\hat{x}_{SS}$ = 0.40. For our model, this is globally stable, which means that regardless of the starting frequency (as long as it is neither 0 nor 1), the DDT-R allele frequency will go to a stable equilibrium of 0.34 in a population of *Canton-S* background flies in the absence of DDT selection (Figure 6.3). The initially high frequency mirrors the situation in the wild where DDT-R has reached near fixation in many global populations. It should be noted that it takes considerably longer to approach the internal equilibrium when starting from an initially high DDT-R frequency when compared to an initially low DDT-R frequency (Figure 6.3) – this demonstrates that, for this model, it is far easier for the resistant allele to invade a susceptible population than for the susceptible allele to invade a resistant population. This has implications for how DDT-R frequency will respond after a bout of strong selection with pesticides.

When simulating DDT selection in a Canton-S background the added DDT-R viability advantage, D=5, tends to push DDT-R allele away from the internal equilibrium towards fixation (Figure 6.4). This makes intuitive sense, but it should be noted that for this fixation to occur, the product of this parameter and the pupal viability parameter (i.e. $P \times D$) must be exceed the upper surface in Figure 6.2 (i.e. $PD > \frac{m+F}{2mF}$, c.f. with inequality (6)). As long as complete fixation is not achieved, then removal of pesticide selection does allow a return to the internal equilibrium, but at a very slow rate – it takes more than 300 generations for this to occur.

In the hypothetical genetic background, sexually antagonistic selection maintains DDT-R at an allelic frequency of about 0.099 in the absence of DDT, until DDT drives it to near fixation (Figure 6.5). In this case, subsequent return to the low internal equilibrium is even slower than found for the Canton-S parameter values – the recovery period is about 1000 generations which is more than three times the selection period.

Allele frequency trajectories in laboratory population

1. Canton-S background results

Based on an expected 34% DDT-R equilibrium frequency in the absence of DDT selection, predictions of the CS experimental laboratory populations included an increase in DDT-R frequency in CS LF populations with decreases in DDT-R frequency in the CS MF, CS HF and McCart (2006) populations.

Of the present Canton-S experimental populations 7 of the 8 LF populations (start at 10%) increased in DDT-R frequency while both MF populations (start at 50%) and both HF populations (start at 90%) decreased in DDT-R frequency by the 5^{th} generation (Figure 6.6). Additionally, 5 of the 6 McCart (2006) populations (start at 50%, non-Hardy-Weinberg) experienced a drop in DDT-R frequency by the 10^{th} generation (Figures 6.6 and 6.7). Given an expected equilibrium of 34% in the absence of DDT selection, this means that, overall, 16 of the 18 Canton-S populations experienced a shift in allele frequency in the expected direction, representing a qualitative match to model predictions (one-sided exact binomial test, $p < 0.001$).

Using *t* tests of logit-transformed frequency data, there are no significant differences between data and model predictions for the MF ($p = 0.11$), HF ($p = 0.15$) and McCart (2006) ($p = 0.25$) populations (Figures 6.6 and 6.7) at the termination of each experiment. However, the final frequencies in the LF populations were significantly lower than predicted by the model (one-sided *t*-test, $p < 0.001$). Examination of the McCart (2006) allele frequency trajectories confirms that there is an initial drop in allele frequencies from generations 1 to 5 that is steeper than predicted by the model but thereafter allele frequencies stabilise to match the model (Figure 6.7). Again, using *t* tests of logit-transformed frequency data, the departure of

the empirical frequencies from model predictions at generations 2 to 5 are significant ($p < 0.05$) while those from generations 6 to 10 are not ($p > 0.05$).

2. WC background results

Recent fitness assays on WC females have revealed a DDT-R fecundity advantage, f= 1.70, but no viability advantage ($e = l = P = 1$) (Chapter 5). This, coupled with a lack of male competitive disadvantage ($m = 1$) (Smith et al. 2011), means that DDT-R should ultimately go to fixation when introduced to a susceptible population. Therefore, the predictions of the WC experimental laboratory populations were for increases in DDT-R frequency in all (LF, MF, HF) populations after five generations.

Empirically, 6 of the 8 WC LF (start at 10%) populations showed an increase, with all MF (start at 50%) and HF (start at 67% and 80%) populations showing decreases (Figure 6.8). This means that, overall, only half of the 12 WC populations experienced a shift in allele frequency in the expected direction, representing a qualitative departure from model predictions (one-sided exact binomial test, $p = 0.61$).

Using one-sample t tests of logit-transformed frequency data, the final frequencies in the LF populations (one-sided t-test, $p = 0.007$) and MF populations (one-sided t-test, $p = 0.025$) were significantly lower than predicted by the model. The two HF populations were not replicates but exact binomial tests on each reveal that both the 67% population ($p < 0.001$) and the 80% population ($p < 0.001$) had significantly lower final frequencies than predicted (one-sided exact binomial test).

6.5 Discussion

We present here a simple one-locus, two-allele population genetics model of the *Drsosophila melanogaster* DDT resistance gene *Cyp6g1* which is found on autosome 2R. Previous empirical work on this system has examined various pleiotropic fitness effects for males and females, revealing both genetic background dependent (epistatic) and sexually antagonistic effects of resistance. These have included a consistent female benefit through increased fecundity and background-dependent enhanced offspring viability (McCart et al. 2005; Chapter 5), and a background-dependent male mating cost (Smith et al. 2011). These studies yielded individual-based estimates of various fitness determinants (e.g. mating probabilities, fecundity and

viability), which we used to parameterise the model. By combining the empirical evidence of sex-specific pleiotropic fitness within a theoretical framework we have, for the first time, made explicit predictions of the evolutionary dynamics of a naturally occurring sexually antagonistic allele.

An examination of general model dynamics reveals that, in addition to the boundary equilibria (fixation of either resistant or susceptible allele), there is at most one possible internal equilibrium (polymorphism) for any parameter value combination. This conforms to previous, generalized single-autosomal-locus models that incorporate different fitness effects for males and females (Karlin 1972; Kidwell et al. 1977; Kokko and Brooks 2003; Gavrilets and Rice 2006). We find that the conditions (parameter value combinations) required for the successful invasion of a monomorphic (all homozygous resistant or susceptible) population by the rare allele and for establishment of a stable polymorphism are clearly defined by simple inequalities.

These inequalities define a large area of parameter space that results in stable polymorphisms, suggesting that intralocus conflict can maintain variation at this locus under a wide range of conditions (Figure 6.2). Moreover, the parameter space for which sexually antagonistic polymorphisms are expected encompasses a wide range of parameter values that are empirically realistic for this system. This contrasts with the findings of previous simple sexually antagonistic models (Kidwell et al. 1977; Prout 2000; Patten and Haig 2009) where the conditions for a polymorphic equilibrium, given reasonable assumptions on strength of selection and dominance, were restrictive. This fundamental difference results from the fact that, in previous models, 'reasonable' selection coefficients on both sexes tend to be very small, severely constricting the parameter space that allows for polymorphism (Fry 2010). In the present specific model, in-depth knowledge of the DDT-R system dictates selection on both sexes that is orders of magnitude stronger than might otherwise be assumed.

It appears that the *Accord* LTR insertion into *Cyp6g1* is an old mutation and has only been recently co-opted for insecticide resistance (Catania et al. 2004). If this is true, then DDT-R alleles must have been kept at very low frequencies before this co-option as they are absent from all laboratory populations founded in the first decades of *D. melanogaster*'s use as a model organism. Our model provides a possible

explanation for how variation at *Cyp6g1* could have been maintained - via intralocus sexual conflict - prior to the 1940's when the widespread use of insecticides including DDT began. Applying Canton-S specific parameter estimates to the model without DDT selection results in a stable polymorphism of 34% DDT-R frequency with relatively quick invasion of susceptible populations and slow loss from resistant populations.

Given that the net fitness effect of DDT-R is mediated by the genetic background in which it is expressed (Smith et al. 2011), there needs to be a more thorough examination of DDT-R fitness effects in other genetic backgrounds. However, this epistatic effect does prompt speculation that the mutation may have arisen in a genetic background which allowed persistence at a low stable equilibrium. While a sexually antagonistic polymorphism at *Cyp6g1* is predicted for the Canton-S background, the predicted pre-pesticide allele frequency of 34% seems too high given that resistance alleles were not sampled from the wild prior to the 1940's. Conditions near the lower boundary equation (5) would yield low equilibrium frequencies accounting for the lack of detection before DDT use, while still permitting similar recovery dynamics (i.e. slow return to the equilibrium) after the removal of strong pesticide selection. This scenario is demonstrated in Figure 5 for a hypothetical genetic background where sexually antagonistic selection maintains DDT-R at an allelic frequency of about 0.099 until DDT selection drives it to near fixation. In this case, subsequent return to the low internal equilibrium is even slower than found for the Canton-S parameter values – the recovery period is more than three times the selection period.

Our model makes explicit predictions about the expected change in DDT-R allele frequency with time (generation), given fitness parameter values and initial allele frequencies. We confronted the model with data from experimental laboratory populations to see how well these predictions held, using two different genetic backgrounds and various starting frequencies in the absence of DDT selection. For the Canton-S background, given a predicted equilibrium of 34%, we expected that there would be increases in DDT-R frequencies when started at 10% with decreases when started at 50% and 90%. Canton-S population data from two different experiments (present and from McCart 2006) across two different laboratories (University of Exeter and University of Bath, respectively) qualitatively matches these expectations. This

demonstrates that, as predicted by our model, variation at *Cyp6g1* is maintained by sexually antagonistic selection at the locus in Canton-S populations. This is a clear demonstration of intralocus sexual conflict acting as a mechanism to maintain genetic variation. Furthermore, in three of the four treatments (present MF (50%), present HF (90%) and McCart 2006) there was no statistical difference between predicted and actual final frequencies. Low replication (n = 2) means that this agreement may be an artefact of low statistical power for the MF and HF populations, but this is unlikely to be a factor in the McCart (2006) populations (n = 6). Overall, there were lower than predicted final DDT-R frequencies in the Canton-S LF populations, indicating that actual invasion of susceptible populations by DDT-R was not as rapid as predicted by the model.

In contrast to the Canton-S populations, there was no broad agreement in direction of allele frequencies over time between model predictions and data for the WC populations. For the WC background we expected the stable equilibrium to be 100% i.e. there should be an increase in DDT-R frequencies when started at any population frequency. In fact, WC populations showed a similar pattern to Canton-S populations with increasing DDT-R frequency for most LF populations (as expected), but decreasing DDT-R frequency in all of the MF and HF populations. This indicates that variation at *Cyp6g1* is maintained in WC populations and suggests that the equilibrium frequency lies somewhere between 10% and 50%. It should be reiterated that the model's dynamics were originally formulated to reflect the sex-specific effects of DDT-R in the Canton-S background and there may be pleiotropic effects in the WC background which have not been accounted for. The absence in WC of a clear maternal effect of DDT-R on offspring viability (Chapter 5) and the opposing effects of DDT-R on male size in the different backgrounds reflect just two key differences. In addition, it is conceivable that, as in Canton-S males (Chapter 4), there are important effects of DDT-R on male behaviour which confer a mating disadvantage when the size effect is removed. Overall, given that DDT-R has not received as much study in the WC background as in Canton-S it is perhaps not surprising that the modelled predictions for Canton-S appear much more reasonable.

One key overarching assumption of our modelling approach is that parameter values (and even model functions) generated by largely individual-based fitness assays

can be used to predict average relative fitness at the population level and thus evolutionary dynamics. Although the qualitative predictions for Canton-S were met, the deviations from expected frequencies in the Canton-S LF and WC populations may be a result of properties that only emerge at the population scale. In this regard, there are two different but related factors which could, and almost certainly do, result in violation of this assumption of the model and may account for the discrepancies between model and population data. The first of these is the effect of density. The experimental populations were held at high density and certainly at much higher density than the assays originally used to derive the different fitness parameters. McCart (2006) did find that the DDT-R fecundity advantage (f) was insensitive to density effects. However, none of the other parameters in the model (m, e, l, P) have been investigated at densities approaching the experimental population setup.

The second factor is the effect of social context. As an example, we have modelled the relative competitive mating success of DDT-R males, m, as a strict parameter which applies regardless of the social context in which males find themselves competing for matings. This was calculated as the ratio of mating trials won by resistant males divided by those won by susceptible males where a trial consisted of simple triad of one standard background female and one male of each genotype (i.e. ♀, $♂_{RR}$, $♂_{SS}$) (Smith et al. 2011). This not only represents an unrealistically low density (compared to both natural and experimental populations) as discussed above, it gives information on relative mating probability at only one male genotype ratio (1:1) . It is far from certain that relative mating probability will be the same at different genotype ratios (e.g. see Billeter et al. 2012).

In spite of the potential limitations of the model, broad agreement of the predictions with laboratory population data for the well-studied Canton-S background lends direct empirical support for the assertion that a sexually antagonistic allele can invade a population and for the theory that sexually antagonistic selection can maintain variation at a specific locus (Rice 1984; Parker and Partridge 1998; Bonduriansky and Chenoweth 2009). Previous support for this theory has included negative genetic correlations for fitness of males and females (Chippindale et al. 2001; Fedorka and Mousseau 2004; Pischedda and Chippindale 2006; Brommer et al. 2007; Foerster et al. 2007; Delcourt et al. 2009, Mokkonen et al. 2011) and experimental

evolution using sex-limited selection (Prasad et al. 2007; Morrow et al. 2008).To date, however, only one other study has characterized the evolutionary dynamics of a specific sexually antagonistic allele (Dean et al. 2012). Moreover, in that study balancing selection was demonstrated in an experimentally derived allele whereas the present study applies to resistance alleles which occur in nature.

Our model does not accommodate adaptive amelioration of DDT resistance costs but the maintenance of high resistance frequencies over a long time span would increase potential for resolution of the sexual conflict. Cohan et al. (1994) list two general ways in which pleiotropic fitness costs of an adaptive mutation may be ameliorated. The epistatic nature of the male DDT-R cost effect seen by Smith et al. (2011) may be an example of the 'compensatory' mode, in which natural selection favours modifiers (at other loci) that compensate for the deleterious effects of the mutant allele (Fisher 1928). The other mode, known as 'replacement' describes the case where there are multiple mutations which confer the same adaptation, but which vary in their pleiotropic fitness costs such that the original mutation is replaced by one which confers the same adaptive benefit at lower cost (Haldane 1932). Interestingly, this mode has been invoked to explain allele replacement observed in the insecticide resistance gene *Ester* in the mosquito *Culex pipiens* in southern France (Labbé et al. 2009). A similar scenario may exist in the *Cyp6g1* allelic progression described by Schmidt et al. (2010), although increasing insecticide resistance has been cited as the cause.

Taken together, our model provides, for the first time, a single unifying explanation for a range of somewhat discordant evidence surrounding DDT-R. Our theoretical treatment is consistent with an old origin for the original DDT-R mutation (Catania et al. 2004) held at low equilibrium frequency through balancing selection of a sexually antagonistic nature. This would explain the failure to capture variation at *Cyp6g1* when wild populations were sampled in the early part of the 20th century. The model also allows for rapid increase in frequency to near-fixation in the face of intense directional selection through DDT use which overwhelms the intralocus conflict at *Cyp6g1*. Finally, it predicts the residual maintenance of high DDT-R frequency long after the removal of the directional selection. It is important to note that the retention of high DDT-R frequencies in the wild, long after discontinued DDT use, is also

consistent with other previously mooted explanations such as cross-resistance to other insecticides (Daborn et al. 2001; McCart et al. 2005) which are likely to have helped maintain high DDT-R frequencies.

The present study highlights the need to understand how the pleiotropic fitness effects of insecticide resistance genes translate to population genetic outcomes. We have also demonstrated a specific instance of variation maintenance through sexual antagonism. This has important implications for applied aspects of resistance including insect pest management and shows the potential of insect resistance systems to shed light on fundamental questions of evolutionary dynamics.

Table 6.1 Model terms.

term and default **Canton-S values**	**definition**
x_R	DDT-R (i.e. *Accord* LTR-inserted) allele frequency
$m = 0.28$	relative competitive mating success of DDT-R males compared to susceptible males
$f = 2.13$	relative fecundity of DDT-R females compared to susceptible females
$e = 1.57$	viability advantage of eggs laid by DDT-R females (*RR* and *RS*) compared to susceptible (*SS*) females
$l = 1.13$	viability advantage of larvae of DDT-R females (*RR* and *RS*) compared to susceptible (*SS*) females
$P = 1.12$	pupal viability advantage of DDT-R flies (*RR* and *RS*) compared to susceptible (*SS*) flies
y_{RR} , y_{RS} , y_{SS}	probability that a mating male has a particular DDT-R genotype: see equations (1)
x_{RR} , x_{RS} , x_{SS}	DDT-R genotype frequencies: see equations (4)
$D = 5$	DDT Resistance ratio of DDT-R (*RR* and *RS*) to susceptible (*SS*) flies (Mortality of susceptible flies/Mortality of DDT-R allele carrying flies).

Table 6.2 Calculating the contribution of each kind of mated pair to each genotype of adult offspring. Red font indicates DDT-R fitness effects. Note that pupal viability, *P*, is a function of offspring genotype whereas other fitness effects are derived solely from the mother. The numbers to the right are the proportions of offspring from each cross that have a particular genotype (in columns *RR, RS, SS*).

			RR	*RS*	*SS*
Cross ♀ × ♂	Frequency	DDT-R fitness effects ↓	*P*		none
RR × *RR*	λ_{RRRR}	$F = f \times e \times l$	1		
RR × *RS*	λ_{RRRS}	$F = f \times e \times l$	½	½	
RR × *SS*	λ_{RRSS}	$F = f \times e \times l$		1	
RS × *RR*	λ_{RSRR}	$F = f \times e \times l$	½	½	
RS × *RS*	λ_{RSRS}	$F = f \times e \times l$	¼	½	¼
RS × *SS*	λ_{RSSS}	$F = f \times e \times l$		½	½
SS × *RR*	λ_{SSRR}	none		1	
SS × *RS*	λ_{SSRS}	none		½	½
SS × *SS*	λ_{SSSS}	none			1

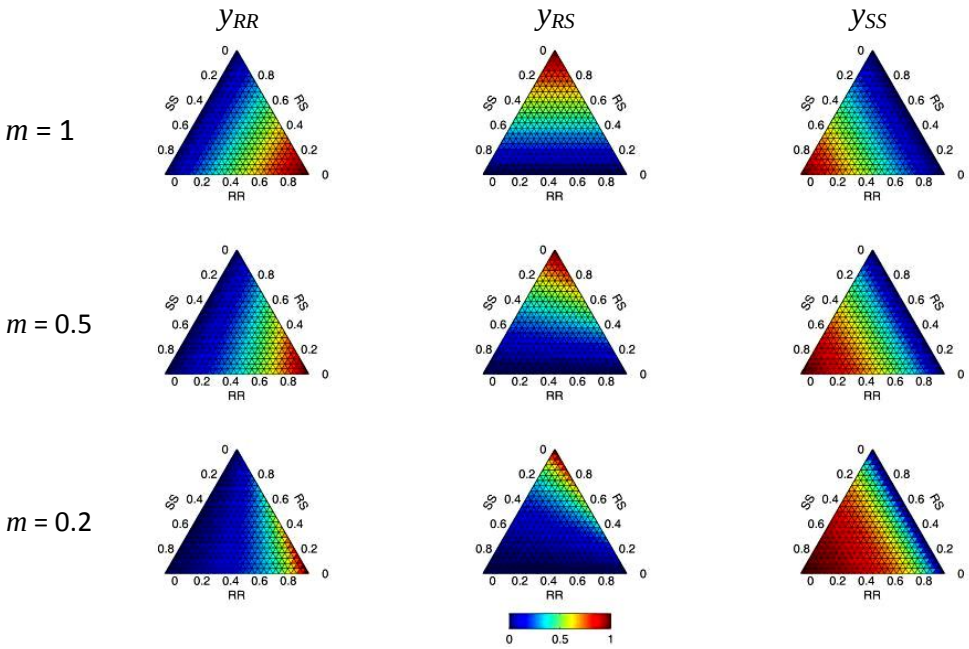

Figure 6.1 The relationship between the population frequency of DDT-R genotypes, x_{ij} (axes on individual ternary plots), and the probability, y_{ij} (denoted by colour, according to the legend), that a mating male has a particular genotype, for three different values of m. Left, middle and right column of plots represent y_{RR}, y_{RS} and y_{SS} respectively. Top row: m=1; middle row: m=0.5; bottom row: m=0.2. When m=1 male mating probabilities are equal to population genotype frequencies. As m decreases y_{SS} is biased upward while y_{RR} and y_{RS} are biased downward.

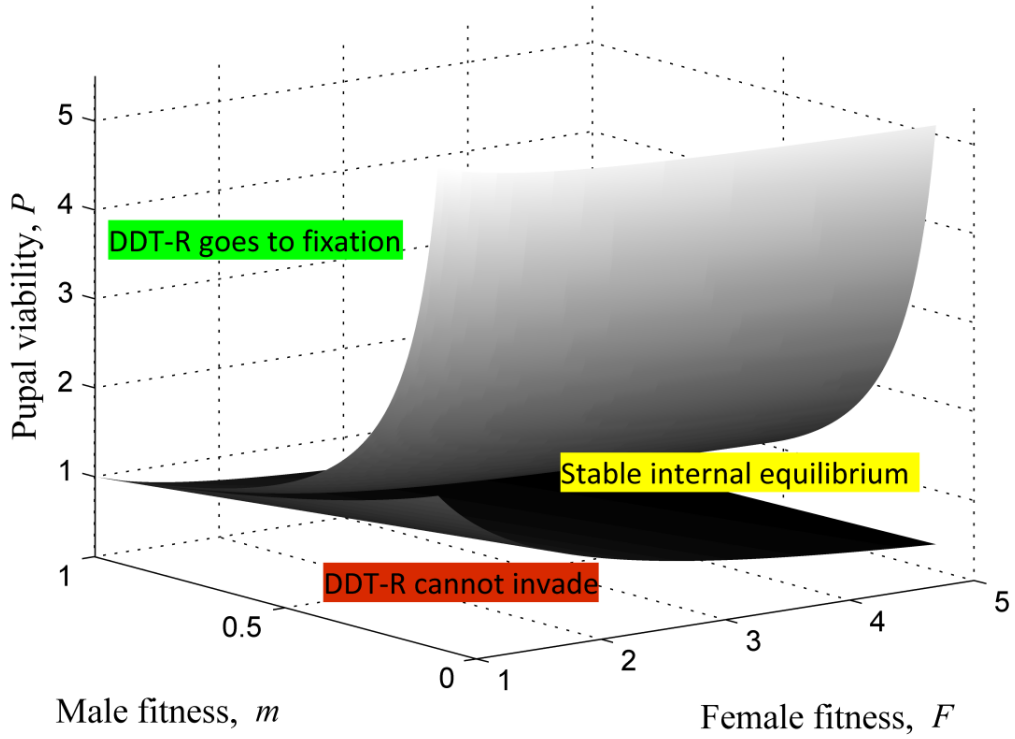

Figure 6.2. The model parameter space showing three different equilibrium regions. The upper surface is $P = \frac{m+F}{2mF}$, which if exceeded results in DDT-R fixation. The lower surface is $P = \frac{2}{m+F}$, below which DDT-R cannot invade. A stable internal equilibrium, where both resistant and susceptible genotypes co-occur, exists in the envelope between the two surfaces i.e. where $\frac{2}{m+F} < P < \frac{m+F}{2mF}$.

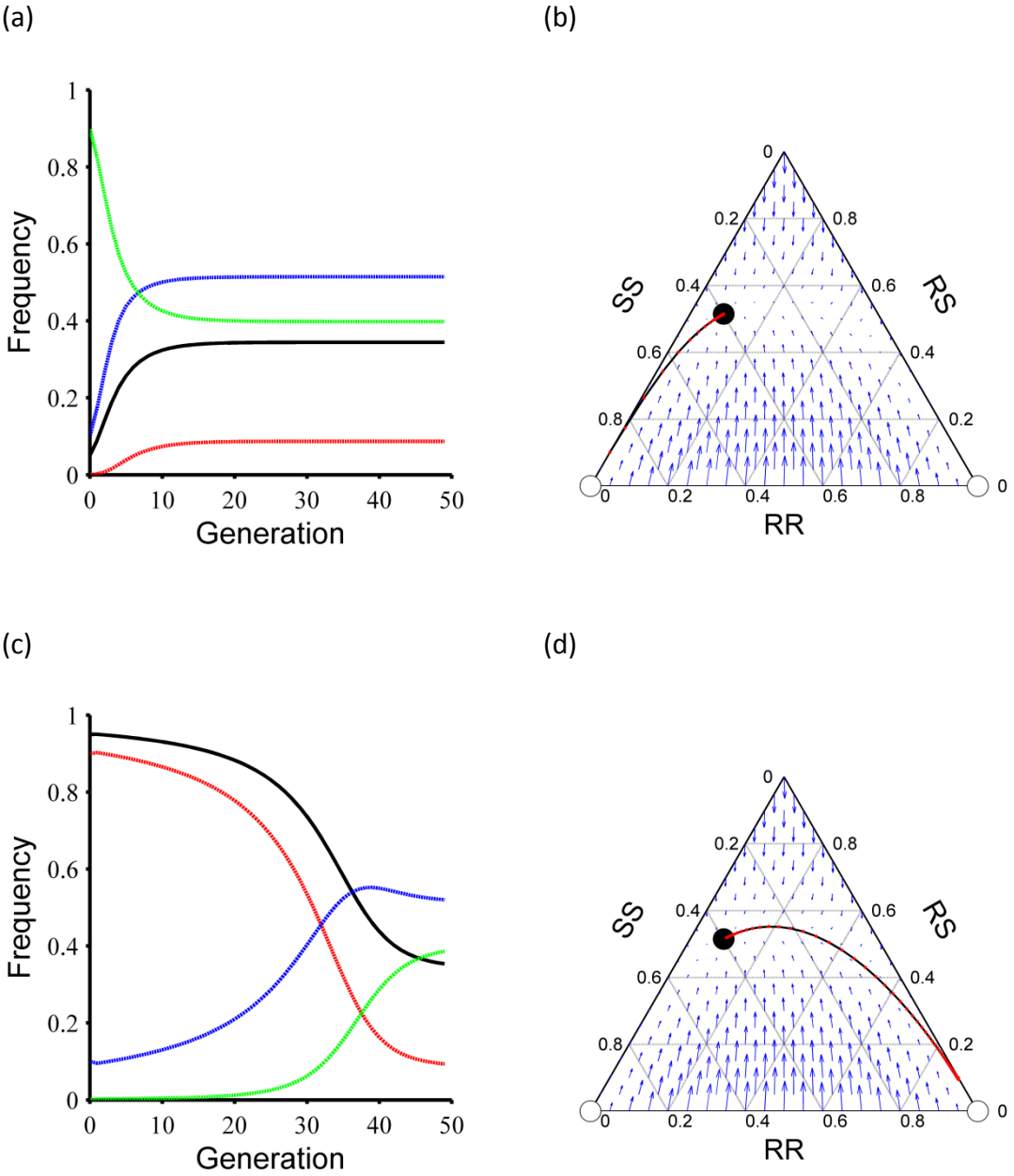

Figure 6.3 DDT-R Genotype and allele trajectories over 50 generations of the model with fitness parameters at default Canton-S values (Table 6.1) approach a stable internal equilibrium. (a) and (b): initial genotype frequencies x_{RR} = 0, x_{RS} = 0.1, x_{SS} = 0.9; (c) and (d): initial genotype frequencies x_{RR} = 0.9, x_{RS} = 0.1, x_{SS} = 0. In plots (a) and (c) the red line represents the frequency of x_{RR}, the blue line x_{RS}, the green lines x_{SS}, and the black line is DDT-R. Ternary plots (b) and (d) show genotype trajectory (red dots connected by black lines), equilibria (open circles are unstable equilibria, black circle is stable equilibrium) and genotype vector field (blue arrows).

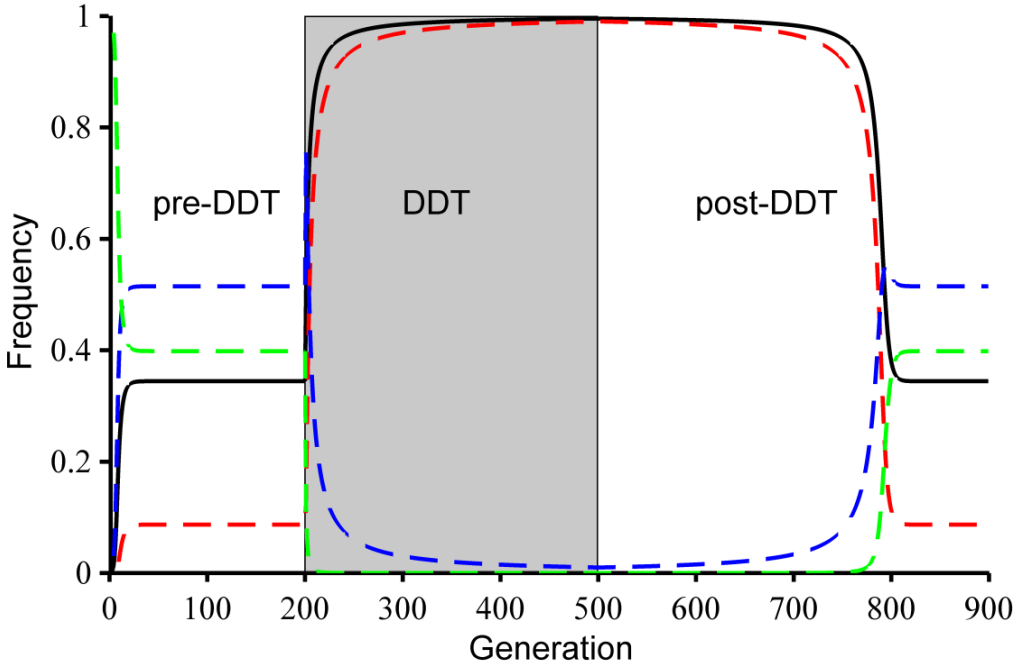

Figure 6.4 The effect of added DDT viability selection on DDT-R genotype and allele trajectories with fitness parameters at default Canton-S values (Table 6.1) starting from initial genotype frequencies $x_{RR} = 0$, $x_{RS} = 0.001$, $x_{SS} = 0.999$. The red line is the frequency of x_{RR}, the blue line is x_{RS}, the green line is x_{SS}, and the black line is DDT-R. The internal equilibrium of 34% in the absence of DDT selection is achieved within the first 20 generations (in the 'pre-DDT' period). DDT selection (shaded area) starts at generation 201 and ends at generation 500, by which time DDT-R has acquired a frequency greater than 99%. More than 300 subsequent generations are required 'post- DDT' for the stable internal equilibrium to be regained.

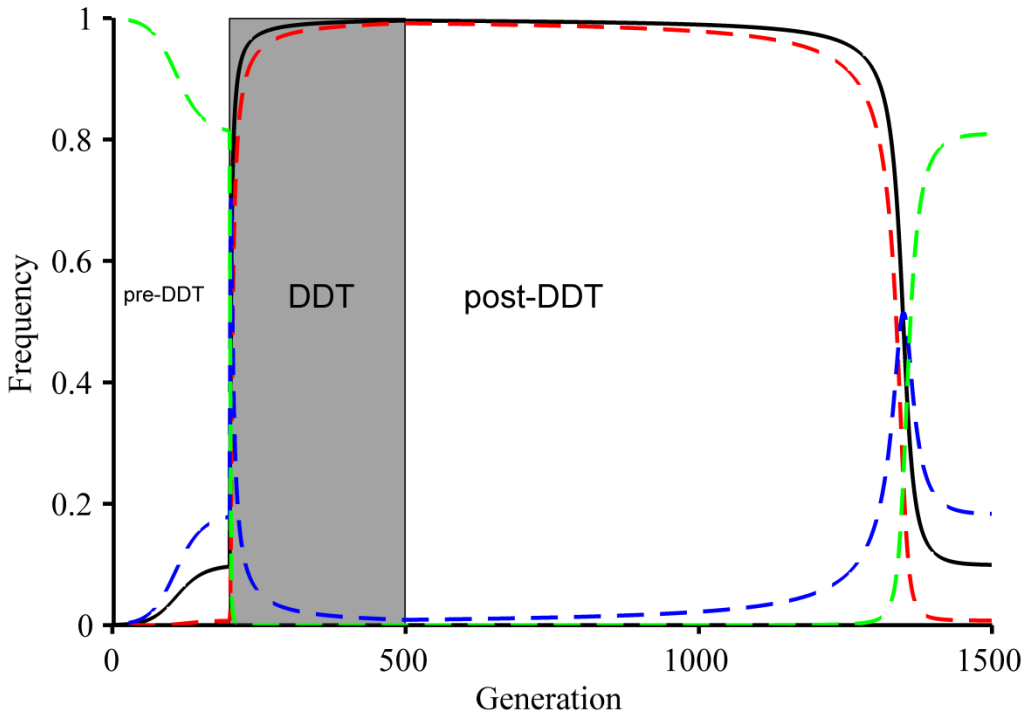

Figure 6.5 The effect of added DDT viability selection on DDT-R genotype and allele trajectories for hypothetical low equilibrium fitness parameters (m = 0.5, F = 1.5, P = 1.05, D = 5) starting from initial genotype frequencies x_{RR} = 0, x_{RS} = 0.001, x_{SS} = 0.999. The red line is the frequency of x_{RR} , the blue line is x_{RS} , the green line is x_{SS} , and the black line is DDT-R. The internal equilibrium of 9.9% in the absence of DDT selection is achieved within the first 200 generations (in the 'pre-DDT' period). As with Figure 6.4, DDT selection (shaded area) starts at generation 201 and ends at generation 500 at which time DDT-R has acquired a frequency of greater than 99%. More than 1000 generations are required 'post-DDT' selection for the stable internal equilibrium to be regained c.f. Figure 6.4).

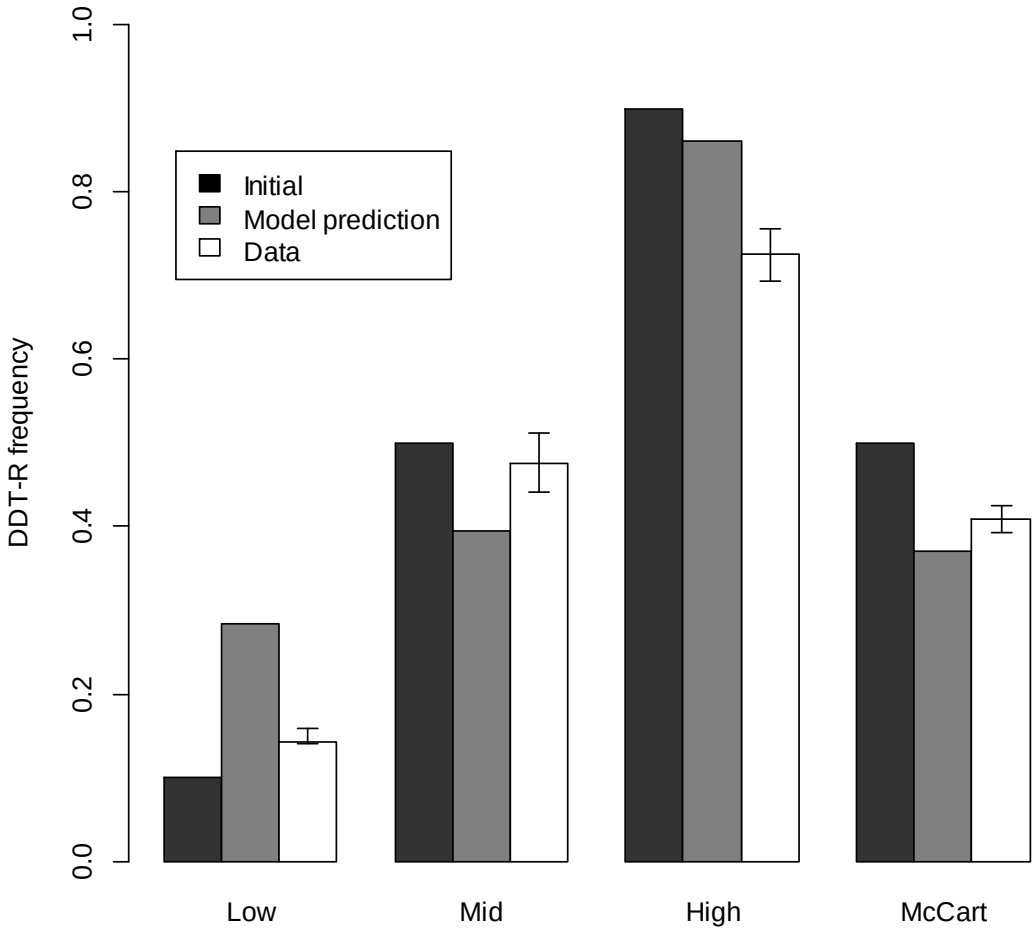

Figure 6.6 Comparison of final DDT-R frequencies from present Canton-S experimental populations (Low, Mid, High) and McCart (2006) populations with initial and model prediction frequencies. Empirical data (open bars) is presented as mean frequency with standard error bars. Present population data is for generation 5 while McCart population data is for generation 10.

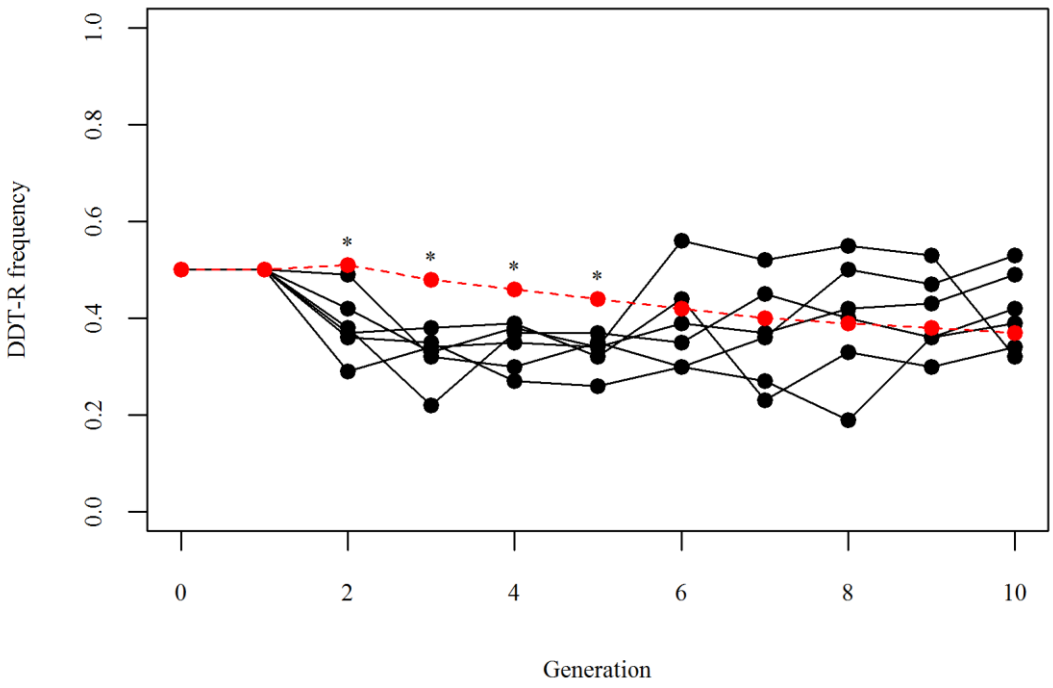

Figure 6.7 Comparison of McCart (2006) Canon-S population cage allele trajectories with model predictions. Black lines represent DDT-R allele frequencies over 10 generations. All 6 population cages started at x_R = 0.5 (generation 0) with either 50 RR males and 50 SS females or the reciprocal cross. Hence all genotypes were RS in generation 1. Red dashed line and square symbols represent the allele frequencies predicted. Population cage frequencies are significantly different from model predictions at generations 2 -5 (asterisks, t-tests of logit transformed frequency, p > 0.05) but match model predictions thereafter.

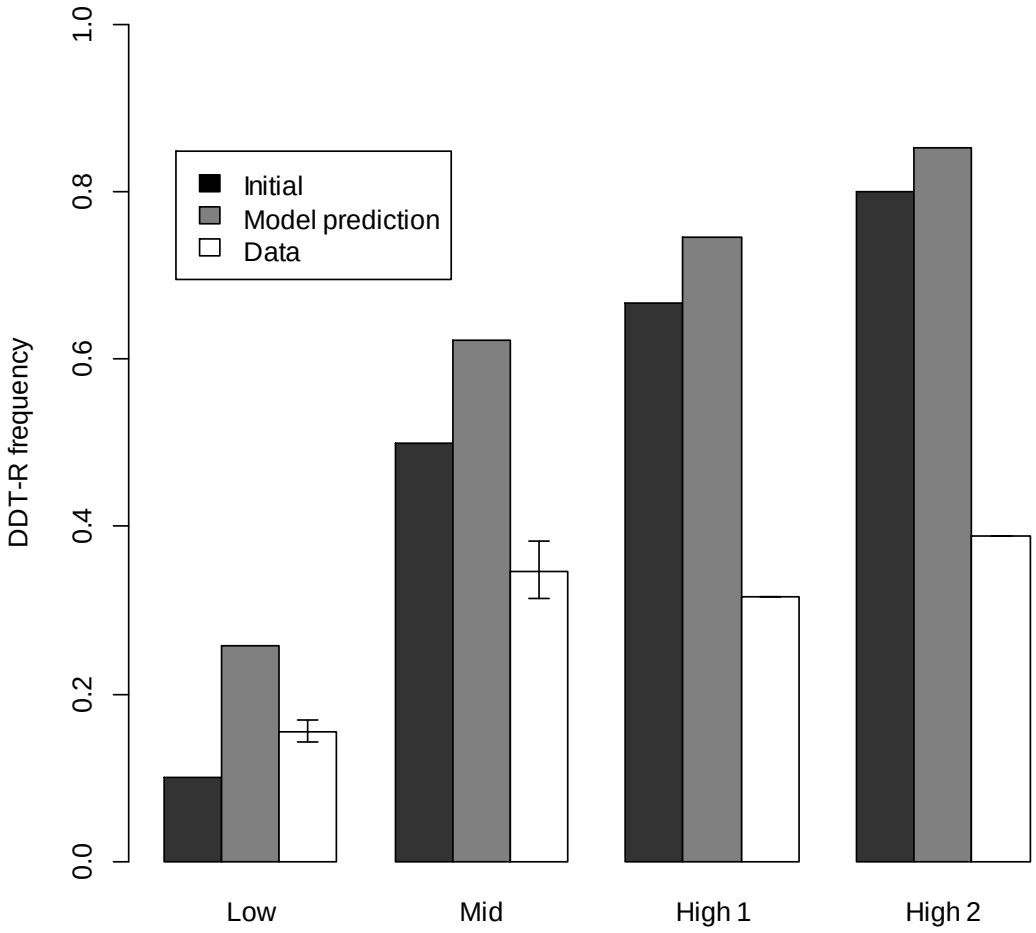

Figure 6.8 Comparison of final DDT-R frequencies from present WC experimental populations with initial and model prediction frequencies. Empirical data (open bars) is presented as mean frequency (at generation 5) with standard error bars. 'High 1' population started at 67% and 'High 2' population started at 80%.

CHAPTER 7: General discussion

Although theory holds that insecticide resistance should be costly in the absence of insecticide (e.g. Crow 1957), empirical evidence on the pleiotropic fitness costs of insecticide resistance appears to be equivocal. DDT resistance in *Drosophila melanogaster* was originally cited as a counterintuitive example, with DDT-R providing strong female and viability benefits (McCart et al. 2005). However, the work contained in this thesis shows that the story is not so simple and highlights the need for careful examination of the effect of a resistance allele in the context of its genetic environment. It is critical that the interaction of a resistance allele with the rest of the genome be considered if an understanding of resistance dynamics at the population scale is to be reached. Both the overall genetic background (McCart et al. 2005; Chapters 3,5-6) and the sex (Chapters 3,5-6) in which DDT-R was expressed played an important part in individual phenotypes and consequently the fitness (Chapters 3-5) and population genetics (Chapter 6) observed.

Evidence of a dramatic male fitness cost to DDT-R that depended on the genetic background was correlated with strong epistasis between genetic background and DDT-R that influenced male size (Chapter 3). The coincidence of a negative size effect with decreased mating success in Canton-S flies, and the absence of a mating disadvantage in the WC background where the DDT-R size effect was reversed was a key observation. It prompted speculation that the pre-copulatory male cost was driven purely through the pleiotropic effect of DDT-R on size, and motivated the closer investigation of the male DDT-R phenotype documented in Chapter 4.

Chapter 4 provides evidence of multiple effects of DDT-R on Canton-S male phenotype. Manipulation of the size disparity between resistant and susceptible males confirmed that the competitive mating cost previously seen was at least partly mediated by pleiotropic size effects. This in itself was not surprising, given the importance of male size on fitness found in previous studies (Partridge and Farquhar 1983; Partridge et al. 1987a,b; Pitnick 1991; Stearns 1992; Roff 2002). However, reversal of the normal size disparity (by competing larger resistant males with smaller susceptible males) did not result in a reversal of male fortunes, suggesting an effect of DDT resistance status on male competitive mating success over and above the effect of the resistance allele on size. This was borne out by the discovery of large behavioural

impacts, including reduced male aggression and increased probability of aborted courtship sequences. These are potentially important factors in generating the DDT-R mating disadvantage in the Canton-S background and beg the question of how these DDT-R-influenced differences in size, aggression and courtship behaviour relate to each other. It is possible that both reduced aggression and disrupted courtship sequence in resistant males may be part of a general syndrome of reduced activity related to an energetic cost of *Cyp6g1* overexpression. However, there was no difference between resistant and susceptible males in courtship latency, nor would this explain the increased reproductive output of DDT-R females (McCart et al. 2005; Chapter 5) or the lack of DDT-R costs in males when expressed in a different genetic background (Smith et al. 2011). It is also possible that *Cyp6g1* acts on endogenous substrates involved in biochemical pathways common to both aggression and courtship behaviour. It would be interesting to repeat the size/ behavioural assays in the WC genetic background to determine, firstly, if the positive effect of size is sufficient to prevent any mating costs in DDT-R males of this genetic background, and secondly whether DDT-R has a consistent effect on male behaviour.

The reductionist approach used herein to investigate different effects of DDT-R on male phenotype does not permit an understanding of how they are integrated to affect male fitness in a population. The first step in understanding how these non-independent phenotypic components interact to affect mating success will require more detailed analysis of courtship in a competitive context. Larval rearing density may also potentially affect the strength of the size disparity between resistant and susceptible males. Additionally, it would be interesting to see if the male mating costs scale up to population level phenomena, especially given the reduced inter-male aggression levels observed in *D. melanogaster* at higher densities (Hoffmann and Cacoyianni 1990; Wang et al. 2008). This could be investigated using mass mating assays of marked males and females, with treatments consisting of different densities and DDT-R frequencies.

The coordinated effect of DDT-R on male size and behaviour underlines the importance of investigating multiple aspects of phenotype when teasing out the pleiotropic effects of resistance. The behavioural effects of these resistance alleles highlight an underappreciated avenue for research, with only a handful of studies to

date examining the behavioural effects of resistance (e.g. Rowland 1991; Foster et al. 2007; Foster et al. 2011). Systems such as DDT-R in *D. melanogaster* have great potential for understanding how single mutations (a situation which commonly prevails in the evolution of resistance in the wild (Wilson 2001)) affect behaviour through pleiotropy and epistasis. In this respect, chapter 4 documents a number of interesting results that warrant further investigation.

The interaction of time-of-day with resistance genotype on male aggression (Figure 4.7) is one example of an unexpected result that demands further study. While most of the work on DDT-R effects herein have assumed differences in constitutive expression levels of *Cyp6g1*, cytochrome P450s in general, and *Cyp6g1* in particular have been shown to be inducible (e.g. Festucci-Buselli et al. 2005, Le Goff et al. 2006) and appear to be regulated by the circadian clock (Ueda et al. 2002; Wijnen and Young 2006; Hooven et al. 2009; Beaver et al. 2010). The plasticity this provides may be an adaptation to the increased oxidative stress (Lewis 2002) that upregulation of P450s can induce. Beaver et al. (2010) suggest that coordinating the upregulation of P450s with the temporal window when the individual is active and ingesting food, and thus most likely to be exposed to toxins, would minimize this oxidative stress. Constitutive upregulation of *Cyp6g1* in resistant males may be swamping this circadian rhythm in expression. Given the dramatic differential effects on aggression levels observed, it would be interesting to examine if there is also an effect of DDT-R on the circadian rhythm of courtship behaviour.

The expression of *Cyp6a20*, located on the same chromosomal arm as *Cyp6g1*, may be an important consideration in future work on the latter's involvement in modulating aggressive behaviour. Diereck and Greenspan (2006) found that a *D. melanogaster* line mutant for *Cyp6a20* (lowered expression levels as measured in heads) showed increased aggression. In another study, Wang et al. (2008) found that social experience increased *Cyp6a20* expression and decreased aggressiveness in a reversible manner. They suggest that *Cyp6a20* might be associated with pheromone sensing, and that sensitivity to these pheromones provides a mechanism by which social experience modulates aggressive behaviour. Moreover, *Cyp6a20* has also been shown to undergo circadian fluctuation (McDonald and Rosbash 2001; Ueda et al. 2002). Given their physical proximity, it is possible that *Cyp6g1* upregulation in DDT-R

flies interferes with the normal expression pattern of *Cyp6a20*, for example through co-regulation. Alternatively, enhanced *Cyp6g1* expression may act on an endogenous substrate common to the putative *Cyp6a20* pheromonal sensing pathway.

Epistatic effects of DDT-R on female fitness were also observed which point to another interesting possibility. If the results of McCart et al. (2005) and Chapter 5 are compared, there is a maternal DDT-R effect on offspring viability seen in the Canton-S background that is not apparent in the WC background. That this correlates with presence of a male fitness cost in the former, but absence in the latter presents an intriguing hypothesis that there is a maternal component to adult male mating costs. Enhanced provisioning of *Cyp6g1* transcripts by DDT-R females to their embryos may not only affect early offspring viability, as suggested by McCart and ffrench-Constant (2008), but it may trade-off with normal male development, resulting, for example in smaller size. At present we are conducting experiments to investigate this possibility.

The model presented in Chapter 6 provides, for the first time, a single unifying explanation for a range of somewhat discordant evidence surrounding DDT-R. The theoretical treatment is consistent with an old origin for the original DDT-R mutation (Catania et al. 2004) held at low equilibrium frequency through balancing selection of a sexually antagonistic nature. This would explain the failure to capture variation at *Cyp6g1* when wild populations were sampled in the early part of the 20th century. The model also allows for rapid increase in frequency to near-fixation in the face of intense directional selection through DDT use which overwhelms the intralocus conflict at *Cyp6g1*. Finally, it predicts the residual maintenance of high DDT-R frequency long after the removal of the directional selection. It is important to note that the retention of high DDT-R frequencies in the wild, long after discontinued DDT use, is also consistent with other previously mooted explanations such as cross-resistance to other insecticides (Daborn et al. 2001; McCart et al. 2005) which are likely to have helped maintain high DDT-R frequencies. Integration of the individual-based sex-specific fitness determinants of DDT-R into a single theoretical framework, and demonstrating its aptness for the Canton-S background in a simple empirical test, presents a case study that can be instructive in two broad areas. It not only has important lessons for applied aspects of resistance, including insect pest management, but shows the potential of insect resistance systems to shed light on fundamental questions of

evolutionary dynamics. In this latter respect, it is only the second instance (and the first involving alleles that occur in nature, c.f. Dean et al. 2012) demonstrating variation maintenance at a known locus through sexual antagonism.

Further work is required to determine the range of fitness effects of DDT-R in different genetic backgrounds and to further explore the population genetics of these alleles. How common is the DDT-R-associated male competitive disadvantage? How common is the female fitness benefit? At the molecular level, there remains an opportunity to further investigate the genetic basis of DDT-R epistasis—what are the modifiers altering the male fitness costs? Although *D. melanogaster* is not a pest species, understanding the relative reproductive success of susceptible and resistant flies with differing genetic backgrounds could provide valuable baseline data to inform insecticide resistance management programs for pest species. Work to date suggests that using a single genetic background to test for effects may not be representative.

Given the great variation recently discovered at the *Cyp6g1* locus in *D. melanogaster* (Schmidt et al. 2010), the time is ripe to investigate the fitness effects of these newly discovered alleles and the potential role for sexually antagonistic selection in maintaining this variation. In that study, it was observed that females had much higher DDT resistance than males and that this difference increased for the most derived resistance alleles that conferred the highest resistance. This may be due to increasingly sex-biased *Cyp6g1* expression as one proceeds along the allelic progression. If so, this could reflect males trading off insecticide resistance for increased mating success, resulting in divergent selection on *Cyp6g1* expression levels in the two sexes. In this scenario, it is plausible that sex-biased gene expression attenuates the intralocus sexual conflict (Bonduriansky and Chenoweth 2009) at *Cyp6g1* for the more derived alleles. This would presumably involve the evolution of sex-linked modifiers or alternative splicing mechanisms (McIntyre et al. 2006). Another hypothesis is that the *Cyp6g1* allelic succession itself may be partially driven by the "replacement" mode of amelioration. However, this does not fit as well with the observation of sex-biased resistance. Nevertheless both hypotheses highlight the possibility that ongoing evolution at *Cyp6g1* (Schmidt et al. 2010) need not only involve insecticide resistance but may include elements of intralocus sexual conflict including mechanisms of conflict resolution.

It is unknown whether gene amplification or TE-induced cis-acting mutation has the greater effect on DDT resistance and associated pleiotropic fitness effects in *D. melanogaster*—dissecting the respective contributions to resistance/fitness would require single-copy TE-inserted *Cyp6g1* alleles, and these are yet to be found (Schmidt et al. 2010). The universal presence of TE insertions in both copies of all DDT-R alleles thus found suggests that the insertion occurred prior to, or concurrently with, the duplication event.

This parallels pyrethroid resistance in the mosquito *Culex quinquefasciatus*. Here, resistance is associated with overexpression of another cytochrome P450 gene, *Cyp9m10* (Hardstone et al. 2010; Itokawa et al. 2010, 2011). As with *D. melanogaster* DDT-R, the constitutive upregulation occurs in haplotypes that have an upstream insertion of a TE (in this case a truncated copy of the MITE TE, *CuRE1*; Itokawa et al. 2010). Moreover, one of the resistant haplotypes also consists of a tandem repeat of the TE-inserted sequence (Itokawa et al. 2011). Unlike the *D. melanogaster* DDT-R system, the relative contributions of the TE insertion and gene amplification to resistance (and for that matter pleiotropic fitness) can easily be parsed out, since there are haplotypes that possess the former but not the latter. Itokawa et al. (2011) suggest that, based on the nonlinear resistance efficacy to *Cyp9m10* expression, the resistance phenotype is disproportionately stronger as a result of the cis-acting mutation (the TE insertion) occurring before the duplication event, than if the duplication had preceded the insertion. It would be interesting to examine if there are also sex-specific fitness effects at *Cyp9m10* in *C. quinquefasciatus*. Such eerily similar stories for two different enzymes, conferring resistance to two different insecticides in two distantly related species, underline the usefulness of intensive study of model insect systems. They also hint at a general pattern—tandem repeats, which are difficult to detect, could be commonly associated with TE insertion-induced insecticide resistance.

Schlenke and Begun (2004), while investigating reduced heterozygosity around the *Cyp6g1* locus in *D. melanogaster* and *D. simulans*, found that another TE insertion, this time a full-length copy of the non-LTR retrotransposon *Doc*, occurred 200 bp upstream of the gene in Californian populations of the latter species. Once again, the insertion correlated with increased *Cyp6g1* expression compared with that found in African populations lacking the insertion. This provides a striking example of parallel

evolution. In contrast to the *Accord* insertion in *D. melanogaster* which is highly degenerate (comprising only the LTR), the *Doc* insertion in *D. simulans* is of an autonomous element, suggesting that it is a much more recent event. Selective sweeps at *Cyp6g1*, associated with strong recent selection, were demonstrated in both species (Catania et al. 2004; Schlenke and Begun 2004).

Compared with the extensive work done on the *Accord*-inserted *Cyp6g1* in *D. melanogaster*, little is known about *Doc*-inserted *Cyp6g1* in *D. simulans*. It remains to be seen whether this mutation has a significant and consistent effect on resistance across different strains/genetic backgrounds. Furthermore, no work has been done to examine potential pleiotropic fitness effects of this insertion, much less the presence of epistatic interactions or the possibility of intralocus sexual conflict, as has been demonstrated for *D. melanogaster*. A good first step may be to perform a worldwide survey akin to that of the *Accord*-LTR insertion by Catania et al. (2004). This would provide some indication of the geographic range of the *Doc*-inserted allele. Given the evidence for on-going and rapid adaptation at *Cyp6g1* in *D. melanogaster*, it may well be worth having a closer look at the variation which exists at this locus in *D. simulans*. Just how similar the responses of the two species are to similar selection also remains to be seen.

Another avenue of research involves gene by environment (G × E) interaction as it relates to fitness costs of resistance in these model systems. The laboratory-based fitness component approach cannot fully encompass the full diversity of environments in which wild populations face selection, and this may be a reason why costs are not always detected—environmental factors such as natural enemies, resource limitation, overwintering, and different host plant have all been shown to increase resistance costs in various taxa (Carrière et al. 2001; Janmaat and Myers 2005; Raymond et al. 2005, 2007, 2011). Moving population cage experiments outdoors could increase the reality of the stability-selection approach, giving a better reflection of how well resistance genotypes perform under natural conditions. Just as the genetic background provided by the rest of the genome represents a genetic “environment” in which resistance alleles act, so does the presence of extragenomic DNA, including cytoplasmic endosymbionts. *Wolbachia* is a maternally transmitted intracellular bacterium found in a wide range of arthropods and nematodes (Werren 1997;

Stouthamer et al. 1999). Its relationship with its host ranges from parasitic to symbiotic. At the parasitic end of the spectrum, it can have profound effects on host reproduction, displaying a range of phenotypes from male killing to feminization to cytoplasmic incompatibility (Werren, 1997; Stouthamer et al. 1999). These strategies increase its transmission within a population, often at the expense of its host's fitness—the hallmark of an SGE. *Wolbachia* is found not only in *Drosophila* (where it has undergone a very recent expansion to near fixation in many populations), but also in many other insects including pest species—one recent estimate is that more than 66% of arthropod species harbour *Wolbachia* infections (Hilgenboecker et al. 2008). Given its ubiquity and potentially profound effect on host fitness, *Wolbachia* cannot be ignored when examining pleiotropic effects of resistance. For example, *Wolbachia* has been implicated in directly modifying the cost of insecticide resistance in mosquitoes (Duron et al. 2006). Where insecticide resistance is conferred by a TE, we may find that intergenomic interactions (akin to epistasis) between the TE and intracellular endosymbionts are critical to the population genetics of insecticide resistance alleles.

Although *D. melanogaster* is not a pest species, understanding the relative fitness of susceptible and resistant flies with differing genetic backgrounds and under different environments could provide valuable insights to inform insecticide resistance management programs for pest species. To this end, we urge the use of multiple avenues of investigation that include the laboratory-based, sex-specific fitness component approach, stability-selection experiments, and mathematical modelling to increase our understanding of insecticide resistance dynamics in natural populations.

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