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DDT resistance, epistasis and male fitness in flies

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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.

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 & 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 up regulation 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 egg and a greater proportion of viable eggs than susceptible females. Additionally, resistant offspring also have

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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 0.8 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 affects 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 & Farquhar, 1983; Partridge *et al.*, 1987b; Markow, 1988; Pitnick, 1991). As precopulatory male fitness components, we assayed male mating success in competitive and non-competitive environments. It is important to assay precopulatory 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 precopulatory 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 & Clark, 1998; Imhof *et al.*, 1998; Snook & Hosken, 2004), sperm competitiveness is also likely to be an important male fitness component. We conducted all assays in two genetic backgrounds.

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 N_e 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 amount 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 & 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 precopulatory competitive assay (PCC), precopulatory 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.

Precopulatory 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 & 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 & 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.

Precopulatory 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 (Patridge & Fowler, 1990). We use the framework of Jennions & 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) models 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 occured. 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.

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).

Precopulatory 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, $P < 0.001$, Fig. 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 1). There was no effect of male resistance status on the multivariate combination of these characters (Table 1).

Precopulatory 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, $P = 0.573$, Fig. 1b).

We conduced the MANCOVA analysis for the WC genetic background as in the CS genetic background.

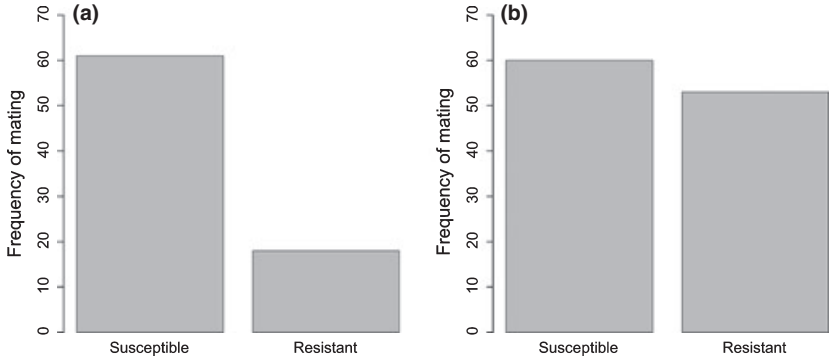

Fig. 1 (a) The number of matings achieved by susceptible or resistant males in the Canton-S (CS) genetic background under precopulatory competitive (PCC) conditions. The resistant males mate significantly less often than the susceptible males in the CS genetic background. This represents a cost to males 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 1 Summary of MANOVA analysis and univariate ANOVA analysis of the precopulatory 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 $P < 0.05$.

Genetic background	Precopulatory competitive assay					
	CS			Wild caught (WC)		
	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	$F_{1,75}$	P	Relative male size β	$F_{1,82}$	P
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	$F_{1,75}$	P	Female size β	$F_{1,82}$	P
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.

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 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.

Precopulatory 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 2). The effect of male resistance status on the multivariate trait combination was driven by resistant males producing more offspring than susceptible males (Table 2). We found no relationship between male resistance status and the proportion of males that did not mate ($\chi^2_1 = 0.075$, $P = 0.784$).

Precopulatory 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 CS 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 2). Female size also had a significant effect in the multivariate model, and this was driven by larger females producing more offspring (Table 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

Table 2 Summary of MANOVA analysis and univariate ANOVA analysis of the precopulatory 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 $P < 0.05$.

Genetic background	Precopulatory noncompetitive assay					
	CS			WC		
	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	$F_{1,124}$	P		Male size β	$F_{1,170}$	P	
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	$F_{1,124}$	P		Female size β	$F_{1,170}$	P	
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.

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). 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 (β = 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). 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

Table 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 $P < 0.05$.

Genetic background	Sperm defence (P1)					
	CS			WC		
	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**
First 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	$F_{1,156}$	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**

	First male size β	$F_{1,82}$	P	First male size β	$F_{1,156}$	P
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	$F_{1,82}$	P	Female size β	$F_{1,156}$	P
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

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 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 ($\chi^2_1 = 0.05$, $P = 0.799$).

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 4). Similarly to the CS genetic background, we found no relationship between male resistance status and the number of males that did not mate ($\chi^2_1 = 1.70$, $P = 0.192$). 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

Table 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 $P < 0.05$.

Genetic background	Sperm offence (P2)					
	CS			WC		
	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	$F_{1,79}$	P	Relative male size β	$F_{1,104}$	P
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	$F_{1,79}$	P	Female size β	$F_{1,104}$	P
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

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 $\chi^2_3 = 19.73$, $P < 0.001$, 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).

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 & 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 precopulatory 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 precopulatory 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 (Table 2).

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 & 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 & Hosken, 2004; Manier *et al.*, 2010). Nonsperm 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 'sex peptide') (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, nor 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 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 & 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 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.

Table 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	PI	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.

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.

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