

# Behavior Genetics

## Pleiotropic effects of DDT resistance on male size and behaviour

--Manuscript Draft--

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<b>Abstract:</b>	<p>Understanding the evolution and spread of insecticide resistance requires knowing the relative fitness of resistant organisms. In the absence of insecticides, resistance is predicted to be costly. The <i>Drosophila melanogaster</i> DDT resistance allele (DDT-R) is associated with a male mating cost. This could be because resistant males are generally smaller, but DDT-R may also alter courtship behaviours. Here we tested for body size and courtship effects of DDT-R on mating success in competitive and non-competitive mating trials respectively. We also assessed relative aggression in resistant and susceptible males because aggression can also influence mating success. While the effect of DDT-R on male size partly contributed to reduced mating success, resistant males also had lower rates of courtship and were less aggressive than susceptible males. These differences contribute to the observed DDT-R mating costs. Additionally, these pleiotropic effects of DDT-R are consistent with the history and spread of resistance alleles in nature.</p>	
<b>Response to Reviewers:</b>	<p>Dear Dr Yong-Kyu Kim,</p> <p>Please find enclosed our revised MS that we hope will be sufficient for a final acceptance. There were only minor comments made by Reviewer #2 that needed to be addressed:</p> <p>1. A better rationale for why spa mutation tester females were used. Reply: There were a number of different mating assays conducted in the Smith et al (2011) study that this MS is based on, some of which involved sperm competition (and thus required scoring of offspring to determine paternity). Rather than use different tester females for the different tests, we opted for consistency within that previous</p>	

study and with this, our follow-up. We hope this clarifies our choice and thank you for your very helpful comments. We have added this information in lines 131-134.

2. Figure S1: There is a dotted line in A from "tap" to "lick" that lacks an arrow. Is this meant to be the beginning of the dotted line that goes from "lick" to "attempt"? Is there a way to draw this better so that the line doesn't go through "lick"?

Reply: We have revised S1 figure to clarify this transition ('tap' to 'attempt') as the line was partly obstructed by the box containing 'lick' in the previous version. We trust this amendment makes the figure more clear.

Sincerely,  
Nina Wedell (on behalf of the authors)

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# 1 **Pleiotropic effects of DDT resistance on male size and** 2 **behaviour**

3  
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23 **Running head: DDT-R affects male size and behaviour**

24

25 **Abstract**

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3 27 Understanding the evolution and spread of insecticide resistance requires knowing the  
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6 28 relative fitness of resistant organisms. In the absence of insecticides, resistance is predicted to  
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8 29 be costly. The *Drosophila melanogaster* DDT resistance allele (DDT-R) is associated with a  
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10 30 male mating cost. This could be because resistant males are generally smaller, but DDT-R  
11  
12 31 may also alter courtship behaviours. Here we tested for body size and courtship effects of  
13  
14 32 DDT-R on mating success in competitive and non-competitive mating trials respectively. We  
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16 33 also assessed relative aggression in resistant and susceptible males because aggression can  
17  
18 34 also influence mating success. While the effect of DDT-R on male size partly contributed to  
19  
20 35 reduced mating success, resistant males also had lower rates of courtship and were less  
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22 36 aggressive than susceptible males. These differences contribute to the observed DDT-R  
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24 37 mating costs. Additionally, these pleiotropic effects of DDT-R are consistent with the history  
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26 38 and spread of resistance alleles in nature.  
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35 40 **Keywords**

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37 41 mating success, insecticide resistance, aggression, courtship, body size, pleiotropy  
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## 44 Introduction

45  
46 A key question in the evolution and spread of insecticide resistance is the fitness of  
47 organisms carrying a resistance allele. Theory holds that, in the absence of insecticide,  
48 resistance should be costly (Crow 1957). However, evidence of pleiotropic fitness costs  
49 associated with insecticide resistance alleles is equivocal. Some studies have found that  
50 investment in resistance carries a fitness cost (Minkoff and Wilson 1992; Chevillon et al.  
51 1997; Boivin et al. 2001; Berticat et al. 2002; Rivero et al. 2011; Smith et al. 2011; Platt et al.  
52 2015), whereas others have failed to find any detrimental effects (Follett et al. 1993; Tang et  
53 al. 1999; Castañeda et al. 2011), and some have even demonstrated insecticide resistance  
54 alleles conferring pleiotropic fitness benefits (Omer et al. 1992; Arnaud and Haubruge 2002;  
55 McCart et al. 2005; Bielza et al. 2008). Furthermore, pleiotropic effects of resistance can be  
56 positive or negative, depending on the precise fitness components measured (Brewer and  
57 Trumble 1991), and these effects can also be sex-specific (Smith et al. 2011). Finally,  
58 resistance alleles can also show epistasis, where pleiotropic effects are mediated by the  
59 genotype (genetic background) of the insect (Hollingsworth et al. 1997; Oppert et al. 2000;  
60 Smith et al. 2011).

61 Both epistasis and sex-specific fitness effects have recently been reported for a DDT  
62 resistance allele in *Drosophila melanogaster* (McCart et al. 2005; Smith et al. 2011; Rostant  
63 et al. 2015; also see Hawkes et al. 2016). DDT resistance in *D. melanogaster* is conferred by  
64 the upregulation of a cytochrome P450 enzyme, CYP6G1 (Daborn et al. 2002). Resistant flies  
65 have tandemly duplicated *Cyp6g1* alleles that possess the LTR (Long Terminal Repeat) of an  
66 *Accord* retrotransposon inserted in the cis-regulatory region (Daborn et al. 2002). While there  
67 appears to be a benefit to females of carrying this resistant allele (DDT-R) (McCart et al.  
68 2005), a recent study (Smith et al. 2011) demonstrated a strong competitive mating  
69 disadvantage for DDT-R males in the *Canton-S* (CS) background (for additional evidence

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70 also see Rostant et al. 2015 and Hawkes et al. 2016). This may be because resistant males are  
71 smaller than susceptible males (Smith et al. 2011): body size is positively associated with  
72 male fitness in *D. melanogaster* (Partridge and Farquhar 1983; Partridge et al. 1987; Pitnick  
73 1991). However, this does not preclude the possibility that DDT-R could also affect other  
74 components of mating success, especially because resistance alleles affect behaviour  
75 (Rowland 1991; Foster et al. 2007; Foster et al. 2011).

76 Here, we test the size-mediated effect of DDT-R on competitive mating success and  
77 examine DDT-R effects on aspects of male behaviour. We initially conducted competitive  
78 mating trials, directly manipulating the size disparity between resistant and susceptible males,  
79 to investigate whether the size difference is sufficient to cause the DDT-R mating  
80 disadvantage. Secondly, we examined the courtship behaviour of DDT-R and susceptible  
81 males in a non-competitive context to quantify potential differences in the intensity, rate and  
82 sequence of behaviours that could generate differential mating success. Lastly, we  
83 investigated male-male aggression to see if DDT-R males differed from susceptible males  
84 (Dierick and Greenspan 2006).

86 **Material and methods**

87

88 **Introgression and population maintenance**

89

90 CS stock flies were initially homozygous for the ancestral (susceptible) *Cyp6g1* allele. The  
91 DDT-R allele *Cyp6g1-BA* (Schmidt et al. 2010) was introgressed using a separate wild-  
92 caught resistant strain for the initial cross (Smith et al. 2011). This was followed by repeated  
93 backcrossing for seven additional generations into stock CS flies. After each generation of  
94 backcrossed mating, developing progeny were subject to DDT selection by lacing rearing  
95 vials with 500  $\mu$ l of 4 $\mu$ g/mL DDT in acetone solution. Effectively, the dose is 2 $\mu$ g of DDT  
96 per vial, which has been shown to result in close to 90% 24-hr mortality in CS flies (Daborn  
97 et al 2001). After the backcrossing, mating pairs were established and the progeny of  
98 homozygous resistant crosses (RR $\times$ RR: PCR diagnostic according to Daborn et al. (2002))  
99 were subsequently used to found the corresponding DDT-R population (CS<sub>RR</sub>). Both  
100 populations (CS<sub>RR</sub> and susceptible, CS<sub>SS</sub>) were subsequently maintained at 25°C on complete  
101 Jazz-mix *Drosophila* food (Fisher, Pittsburgh, PA) in 30 $\times$ 30 $\times$ 30 cm population cages with  
102 12:12 h light:dark and humidity ~40%.

103 Experimental flies were collected as first instar larvae from Petri dishes containing  
104 1.5% agar in apple juice with yeast paste spread on a small area of the surface. With the  
105 exception of the size manipulation experiment, larvae were reared at a standard density of  
106 100 larvae per food vial (approximately 5 mL in 3  $\times$  7 cm vials). Virgin adult flies were held  
107 in narrow food vials (approximately 5 mL in 2  $\times$  9.5 cm circular vials) at a density of  
108 approximately 20 flies per vial.

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110 **Effect of size and resistance allele on mating success**

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2 112 To obtain males of various sizes for this experiment, larvae of both genotypes were reared at  
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5 113 two different densities of either 25 per vial or 150 flies per vial. Twenty four hours before the  
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7 114 experiment, we anaesthetised (using CO<sub>2</sub>) 2-4-day old virgin CS<sub>RR</sub> and CS<sub>SS</sub> males and sorted  
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10 115 them, under a dissecting microscope, into categories according to thorax length  
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12 116 measurements. Preliminary measurements had given modal thorax lengths of 1.07 mm for  
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14  
15 117 susceptible males and 0.98 for resistant males. We used these to define the three broad size  
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17 118 categories ('large' $\geq$ 1.07mm; 1.07mm>'medium' $>$ 0.98mm; 'small' $\leq$ 0.98mm). Individual  
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19 119 large males of each genotype were then randomly paired with small males of the other, as  
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22 120 were medium resistant with medium susceptible.

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24 121 Each pair was gently aspirated into a narrow polypropylene vial. Prior to this pairing  
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26  
27 122 off, we used blue and pink paint powder to identify individual males in a factorial way  
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29 123 (Champion de Crespigny and Wedell 2007; Smith et al. 2011) so that half the resistant and  
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32 124 susceptible males were blue and the other half were pink. Thus pink males always competed  
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34 125 against blue males, and resistant males always competed against susceptible males.  
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36 126 Experimental observers were blind to these treatments. On the day of the mating assay a  
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39 127 single virgin female was gently aspirated into each vial. Females were 3-5 days old and of a  
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41 128 wild-type background (Dahomey) into which the recessive *sparkling poliart* (*spa*) mutation  
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43  
44 129 had been recently backcrossed (Fricke et al. 2009). This tester strain was used for consistency  
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46 130 with previous studies on the effect of DDT-R on male competitive fitness (Smith et al. 2011).  
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48  
49 131 A number of different mating assays were conducted in Smith et al (2011), some of which  
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51 132 involved sperm competition (and thus required scoring of offspring to determine paternity).  
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54 133 Rather than use different tester females for the different tests, we opted for consistency within  
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56 134 the previous study and with this, our follow-up. For each replicate triad, at the onset of  
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58 135 copulation we immediately aspirated the unsuccessful male out of the vial and similarly  
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136 removed the successful male post-copulation. Wing size was measured as a surrogate of body  
137 size for all successful and unsuccessful males using SPOT BASIC 4.1 (Diagnostic  
138 instruments, Inc., Sterling Heights, MI, USA).

139

#### 140 Male courtship behaviour

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142 Replicates of four homozygous crosses ( $CS_{RR} \text{♀} \times CS_{RR} \text{♂}$ ,  $CS_{RR} \text{♀} \times CS_{SS} \text{♂}$ ,  $CS_{SS} \text{♀} \times CS_{SS}$   
143  $\text{♂}$ ,  $CS_{SS} \text{♀} \times CS_{RR} \text{♂}$ ) were established. Each dyad consisted of one virgin male and one

144 virgin female in a shallow cylindrical arena, with courtship being video recorded from above.

145 Each arena consisted of a small plastic Petri dish 3.5 x 1cm (diameter x depth) with a secure  
146 lid and containing a small food cup (1.5mL Eppendorf cap) (Dierick and Greenspan 2006).

147 The food cup was filled with 2.0% agar in apple juice with yeast paste spread on a small area  
148 of the surface. Eight of these arenas could be arranged, in a 2 x 4 array, within the maximum  
149 field of view which allowed detailed recording of courtship behaviour under ambient light.

150 Arenas were separated from each other by white paper partitions. Twelve hours prior to each

151 assay virgin females were aspirated into each arena to adjust to their surroundings and

152 immediately prior to loading the males the array was placed under a high definition video

153 camera (Panasonic HD-SD90). Recording commenced and males were then aspirated into

154 each arena. Once a pair began copulating the arena was removed and replaced in the array by

155 a new arena containing another virgin female, repeating the assay. If there was no copulation

156 after 30 minutes the arena was removed and the male was classed as unsuccessful. Successful

157 males were retained for size measurement as above. All flies were 6 days old at the time of

158 assay.

159 Behavioural recordings were analysed for thirteen successful pairings of each cross.

160 Seven courtship behaviours were distinguished following the protocol of Ejima and Griffith

161 (2007) (Supplementary table S1). Continuous records were analysed, and the frequency and  
162 duration of each behaviour, as well as the times at which each behaviour stopped and started,  
163 was recorded.

164

#### 165 Male aggression

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167 Within-genotype aggression was video recorded between pairs of virgin CS<sub>SS</sub> and CS<sub>RR</sub>  
168 males within the arena setup described above, with the exception that a decapitated female  
169 was placed on the food surface of each arena immediately prior to the assay to aid in  
170 attracting males (Chen et al. 2002). The resistance status of the decapitated females in each  
171 arena was balanced across male genotypes. Flies reared in social environments have  
172 suppressed aggression (Hoffmann 1990), but this is reversible after just one day of isolation  
173 (Wang et al. 2008). Therefore experimental flies were individually isolated 24 hours before  
174 each assay. To further increase aggression levels, each individual male was then transferred,  
175 90 minutes before each assay, into foodless vials containing water-saturated cotton wool.  
176 This time-scale has been shown to increase aggression without revealing any underlying  
177 differences in starvation sensitivity (Edwards et al. 2006).

178 All flies were 5-8 days old during the experiment and were not exposed to anaesthesia  
179 for at least 24 hours prior to the assay. As in the courtship behaviour assay, an array of 8  
180 arenas (maximum) at a time was recorded. Two males of the same genotype (CS<sub>RR</sub> or CS<sub>SS</sub>)  
181 were gently aspirated into each arena. The flies were allowed to adjust for 15 minutes, and  
182 were then recorded for 10 minutes using the same camera as in the courtship behaviour assay.  
183 Flies were then anaesthetised and retained for size measurement as per the male size-effect  
184 assay. In this manner a total of 30 replicate pairs of each genotype were assayed for  
185 aggression. Four separate aggressive behaviours were defined following Chen et al. (2002)

186 (Supplementary table S1). From each 10 minute recording, the number of aggressive  
187 behavioural occurrences was noted.

188  
189 Statistical analyses

190  
191 Statistical analyses were performed in R 3.2.3 (R Core Team (2015) using the base stats  
192 package, except where otherwise stated. For univariate behavioural count and duration data  
193 we used generalized linear models (GLMs); or Generalized linear mixed-effects models  
194 (GLMMs) as implemented in package ‘lme4’ (Bates et al. 2015). Maximal models included  
195 male- and, where appropriate female-, resistance genotype as explanatory variables with male  
196 size as a covariate. Wherever appropriate, non-normal error structure was specified with  
197 default link functions. Overdispersion was accounted for by using quasi-likelihood to specify  
198 more appropriate variance functions. In all GLM or GLMM analyses stepwise model  
199 simplification of the maximal model with analysis of deviance was used to determine  
200 significant terms. Significance was adjusted for multiple univariate testing of courtship  
201 behaviours using the Benjamini-Hochberg method to control for false discovery rate  
202 (Benjamini and Hochberg 1995).

203 Overall courtship behavioural response was analysed within a compositional  
204 framework by permutational multivariate analysis of variance, using the `adonis2()`  
205 function in the ‘vegan’ package (Oksanen et al 2017). Prior to analysis, time spent in each  
206 courtship behaviour by each courting pair (sample) was transformed via the chi-square  
207 distance transformation in function `decostand()`, and a pairwise dissimilarity matrix  
208 constructed based on Euclidean distances. Use of chi-square distances has been shown to  
209 have favourable properties in the analysis of compositions (Jackson 1997), particularly when  
210 there are many essential zeros (Stewart 2016) as is the case with our behavioural data. After

1 211 checking for multivariate homogeneity of group variances using function `betadisper()`,  
2 212 the dissimilarity matrix was then subjected to permutational MANCOVA with all the same  
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4 213 explanatory terms as in the univariate GLMs. Significance of terms was determined by  
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7 214 stepwise model simplification of the maximal model using marginal permutation tests, with  
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10 215 pseudo-*F* ratios (McArdle & Anderson 2001).

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12 216 Courtship behavioural sequences were analysed as discrete event single-order Markov  
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14 217 Chains, testing for the existence of non-random temporal associations among the seven  
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17 218 different behaviours. Transition matrices were constructed by tabulating all instances in  
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19 219 which one behaviour led to another. These were pooled for all males of each genotype to give  
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22 220 two overall transition matrices, one for resistant males and one for susceptible males.  
23  
24 221 Transition categories that never occurred (e.g. decamp→lick) were considered structural  
25  
26 222 zeros (West and Hankin 2008) and not included in subsequent analysis. A generalisation of  
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29 223 Fisher's Exact test which can cope with structural zeros is implemented in R package  
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31 224 'aylmer' (West and Hankin 2008) and was used to test for non-randomness (stereotypical  
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33 225 structure) in the sequence of behaviours both at the level of the whole matrix and for each  
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36 226 possible transition. Markov Chain Monte Carlo (MCMC) was used to explore the space of  
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39 227 permissible matrices and approximate the *p*-value (West and Hankin 2008).

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229 **Results**

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231 Effects of size and resistance allele on mating success

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233 Of the 187 successful competitive trials, susceptible males won the majority (120) of

234 matings. A maximal GLM model of the binary response (susceptible or resistant male wins)

235 was fitted as a function of size ratio (i.e. susceptible male wing size/resistant male wing size),

236 along with susceptible male wing size as a covariate and susceptible male colour with

237 interactions, using binomial error structure. Stepwise model simplification revealed a sole

238 significant main effect of the size ratio on whether a resistant or susceptible male won a

239 competitive trial (Fig. 1a;  $\chi^2_1 = 5.204$ ,  $p = 0.023$ , binomial errors). Susceptible males have a

240 greater than 50% chance of winning a competitive trial when the susceptible/resistant size

241 ratio is at least 0.9. Further examination was carried out by dividing the trials by post-hoc

242 wing size measurements into three categories: “Matched”, which consisted of closely sized

243 males (within  $\pm 2.5\%$  of each other); “Smaller SS”, where the susceptible male was more than

244 2.5% smaller than the resistant; and “Larger SS”, where the susceptible was more than 2.5%

245 larger than the resistant. In the latter category susceptible males won the significant majority

246 of trials (Exact Binomial Test, 52 successes from 73 trials,  $p < 0.001$ ) but there was no

247 significant departure from a null of 50% for either the “Matched” (Exact Binomial Test, 32

248 successes from 55 trials,  $p = 0.28$ ) or “Smaller SS” (Exact Binomial Test, 31 successes from

249 50 trials,  $p = 0.12$ ) categories (Fig. 1b). Thus there is nullification, but no reversal of the

250 susceptible mating advantage when resistant males are larger than susceptible males.

251 Model simplification of log-transformed copulation latency as a function of wing size

252 ratio and susceptible male colour yielded a null minimum adequate model. Thus the size

253 difference of the competing males did not have any effect on copulation latency (log-  
254 transformed latency,  $F_{1,185} = 1.751$ ,  $p = 0.19$ , normal errors).

255

256 Male courtship behaviour

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258 Both resistant and susceptible males displayed the full repertoire of courtship behaviours  
259 (Ejima and Griffith 2007). However, two behaviours were very rare (fencing: 81% zero  
260 cases; tapping: 73% zero cases) and so were removed from subsequent multivariate and  
261 univariate analyses. Prior to permutational MANCOVA on transformed behavioural data,  
262 multivariate outliers were detected and the worst six removed to minimize their influence on  
263 subsequent tests. These samples coincided with courtship durations < 45 seconds long and  
264 were equally distributed between RR and SS male treatments. Their removal ensured  
265 multivariate homogeneity of variances, which was confirmed for groups defined both by  
266 male resistance status (Permutation dispersion test, pseudo- $F_{1,44} = 1.414$ , N.perm = 999,  $p =$   
267 0.243) and female resistance status (Permutation dispersion test, pseudo- $F_{1,44} = 0.091$ ,  
268 N.perm = 999,  $p = 0.788$ ). After stepwise removal of all other explanatory terms due to non-  
269 significance, there was a significant multivariate effect of male resistance status  
270 (Permutational MANOVA marginal test, pseudo- $F_{1,43} = 4.550$ , N.perm =  $2 \times 10^5$ ,  $p = 0.012$ )  
271 and a marginally significant effect of female resistance (Permutation MANOVA marginal  
272 test, pseudo-  $F_{1,43} = 3.006$  , N.perm =  $2 \times 10^5$ ,  $p = 0.048$ ) on courtship behaviour.

273 None of the GLM models revealed any significant effects of female resistance status  
274 and male size, nor were any interactions that included these terms. However, male resistance  
275 status altered copulation latency and this effect was driven by time from first courtship to  
276 copulation i.e. ‘courtship duration’ (Table 1). Thus resistant males are slower to copulate

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3 277 once courtship has commenced (Fig. 2a). Resistant males also decamped more (Fig. 2b), had  
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5 278 lower rates of wing vibration (Fig. 3a), chasing (Fig. 3b) and copulation attempts (Fig. 3c).

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7 279 Twenty nine different behavioural transitions were observed, the most frequent being  
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9 280 chase→ wing vibration (resistant count = 246; susceptible count = 192) and wing  
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11 281 vibration→attempt copulation (resistant count = 79; susceptible count = 81). Results of the  
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13 282 generalised Fisher's Exact Test show departure from independence for both the resistant ( $p <$   
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15 283 0.001) and susceptible ( $p < 0.001$ ) matrices, indicating the presence of stereotypical  
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17 284 behavioural sequences. All significant transitions are shown in kinematic diagrams of  
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19 285 resistant and susceptible male courtship behaviour (Supplementary Fig. S1). Overall patterns  
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21 286 of behaviour were similar for both genotypes with males tending to move from chasing to  
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23 287 wing vibration followed by genital licking and/or attempted copulation. When an attempt  
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25 288 failed, the male would chase the female if she moved away, or transition back to wing  
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27 289 vibration. Key differences in the patterns of the two male genotypes include transitions away  
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29 290 from and returning to the female (i.e. decamping). Resistant males were more likely to  
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31 291 decamp following a chase with a significant 19% of resistant chases ending with the male  
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33 292 decamped (Supplementary Table S2) as opposed to a non-significant 7% of susceptible  
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35 293 chases (Supplementary Table S3).

#### 36 37 38 39 294 Aggression

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48 297 Thirty four pairs of each male genotype were assayed for aggression. Aggressive behaviours  
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50 298 were observed in 33 of the susceptible pairs and 25 of the resistant pairs, revealing a  
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52 299 significant association between male genotype and the presence of aggression (Fisher's Exact  
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54 300 test,  $p = 0.013$ ). Complete wing size data was obtained for 60 of the 68 pairs, permitting the  
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56 301 size disparity between males to be calculated. A maximal GLMM model of the total number  
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302 of aggressive behaviours was fitted as a function of male genotype, decapitated female  
303 genotype and size disparity with all interactions, using a negative binomial error structure and  
304 time of day as a random factor with three levels (morning, afternoon, evening). The minimal  
305 adequate model included only male genotype as a significant factor (Fig. 4;  $\chi^2_1 = 15.512$ ,  $p <$   
306 0.001, negative binomial errors). While resistant males displayed lower aggression than  
307 susceptible males, disparity in size between competing males had no effect on total  
308 aggression levels. Similarly there was no effect of size disparity, male genotype or their  
309 interactions on the proportion of aggressive acts that were high intensity (boxing and head  
310 butting) as opposed to low intensity (wing threat and chase).

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## 312 Discussion

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314 DDT-R can have sexually antagonistic fitness effects in the absence of DDT (Smith et al.  
315 2011; Rostant et al. 2015; Hawkes et al. 2016), but the phenotypic cause of lower fitness in  
316 DDT-R males is not clear. Here we show that the effect of DDT-R on male size previously  
317 documented (Smith et al. 2011) is an important mediator of the mating cost for DDT-R  
318 males, but is insufficient to explain the magnitude of this cost found in the *Canton-S* genetic  
319 background. We also identified differences in courtship and aggression between resistant and  
320 susceptible males that are likely to also contribute to differential male mating success. Our  
321 previous results (Smith et al. 2007; Rostant et al. 2015) suggested that the DDT-R mating  
322 disadvantage was a possible outcome of the DDT-R size effect. Here, by directly  
323 manipulating the relative sizes of competing males, we confirmed that male size influences  
324 the probability of winning competitive mating trials. Moreover, we show that reversal of the  
325 DDT-R size disparity eliminates the mating disadvantage of these males. However, if the  
326 competitive mating disadvantage conferred to DDT-R males was solely a result of pleiotropic  
327 size effects of carrying the resistance allele, then larger resistant males should have a  
328 competitive advantage against smaller susceptible males. This was not seen. In fact, large  
329 resistant males still lost 62% of their trials against small susceptible males, although the  
330 probability of resistant males winning a trial does not exceed 50% until the  
331 susceptible/resistant size ratio drops below 0.9. This suggests an effect of DDT resistance  
332 status on male competitive mating success over and above the effect of DDT-R on size.

333 Our analysis of courtship suggests why this might be, because resistant males showed  
334 a two-fold increase in copulation latency compared to susceptible males. Copulation latency  
335 is one measure of male-attractiveness (Taylor et al. 2008; Okada et al. 2011) indicating that  
336 DDT-R males are less attractive. This points towards differences in other key behaviours in

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337 the lead up to successful intromission (Table 1) with resistant males performing courtship  
338 song (wing vibration) at a lower rate and chasing females at a lower rate. In fact, male  
339 resistance status had an overall significant multivariate effect on courtship behaviour. There  
340 is also the possibility that DDT-R also alters fly cuticular hydrocarbons, another trait that  
341 affects male attractiveness (Ingleby et al. 2014). Interestingly, while we also detected a  
342 marginally significant multivariate effect of female resistance on courtship behaviour,  
343 subsequent univariate tests failed to indicate any effect on specific behaviours, suggesting  
344 more subtle differences that may require a fine-grained examination of interactions from the  
345 female perspective and/or greater replication.

346         Decamping (effectively aborting mating attempts already initiated) was the major  
347 behavioural difference between resistant and susceptible males. This suggests differences in  
348 the structure of courtship caused by DDT-R and this is borne out in the behavioural sequence  
349 analysis. Overall transition matrices were found to be significantly non-random, consistent  
350 with well documented stereotypical sequences of courtship behaviour (Spieth 1974).  
351 However, while the overall sequences of behaviour were similar for both male genotypes,  
352 there was a much higher probability of a DDT-R male's chase ending in decamping and these  
353 males decamp more often than by chance and much more often than susceptible males.  
354 Furthermore, susceptible males were more likely to follow courtship song (as indicated by  
355 wing movement) with a copulation attempt than the DDT-R males. This disrupted courtship  
356 sequence and higher incidence of decamping probably accounts for the increased copulation  
357 latency and lower mating success of DDT-R males.

358         Aggression levels were also much lower in DDT-R males. While these results were  
359 stark, it is worth noting that the experimental protocol maximised aggression levels by  
360 priming males before the trial (through isolation and starvation). It is possible therefore that  
361 differences in realised aggression may not be as apparent in other social or environmental

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362 contexts. Nonetheless this finding could also explain fitness decreases in DDT-R males as  
363 previous observations suggest that aggression can confer a mating advantage for territorial  
364 males (Hoffmann and Cacoyianni 1990; Baxter et al. 2015).

365 To date the underlying developmental and genetic pathways by which DDT-R affects  
366 male size, aggression and courtship behaviour are not clear. However it seems apparent that  
367 upregulation of *Cyp6g1* influences both male size and behaviour in the CS background. This  
368 inference is corroborated by findings in another genetic background (Ives) where male  
369 genotypes with low competitive mating success had significantly higher expression of  
370 *Cyp6g1* irrespective of DDT-R (which was not examined) (Drnevich et al. 2004). Future  
371 transcriptome studies that include quantifying the expression levels of CYP6G1 and other  
372 genes implicated in regulating behaviours in resistant and susceptible CS flies are needed to  
373 evaluate their association with male reproductive behaviours and size variation (and see  
374 Hawkes et al. 2016).

375 The present study suggests that both male-male competition and female choice  
376 influence the mating success of DDT-R males. As yet it is not clear how the different aspects  
377 of DDT-R-male phenotype are integrated to cause the observed pre-copulatory mating cost.  
378 However, we have provided evidence of multiple effects of DDT-R on male behaviours  
379 closely linked to fitness and confirm the mating cost previously reported for DDT-R males is  
380 at least partly mediated by pleiotropic size and behavioural effects. These differences are  
381 likely to explain why DDT-R did not fix prior to the use of DDT despite increasing female  
382 fitness (Rostant et al. 2015).

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390

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395 **Ethical approval:** This article does not contain any studies with human participants or  
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536 **Tables**

537 **Table 1.**

538 Summary of courtship behavioural responses to possession of DDT-R allele. ↑ represents  
 539 increase in resistant males relative to susceptible males. ↓ represents decrease in resistant  
 540 males relative to susceptible males. Dash indicates no difference between resistant and  
 541 susceptible males. GLM error family (with any transformations of response variable), test  
 542 statistic and *p* values given, except in the case of genital licking rate for which a  
 543 nonparametric test was required. Adjusted *p* values (*p<sub>adj</sub>*) are Benjamini-Hochberg corrected  
 544 for multiple testing.

<b>Behavioural response</b>	<b>Measure</b>	<b>Effect</b> (RR male relative to SS)	<b>Test summary</b> Test, Error family, test statistic <i>p</i> value, (adjusted <i>p</i> value)
Copulation latency	Absolute (seconds)	↑	GLM, gamma, $F_{1,50} = 14.236$ $p < 0.001$ , ( $p_{adj} = 0.004$ )
Courtship latency	Absolute (seconds)	-	GLM, quasipoisson, $F_{1,50} = 0.8472$ $p = 0.36$ , ( $p_{adj} = 0.473$ )
Courtship duration	Absolute (seconds)	↑	GLM, quasipoisson, $F_{1,50} = 11.471$ $p = 0.001$ , ( $p_{adj} = 0.008$ )
Decamping	Proportion of time	-	GLM, quasibinomial, $F_{1,50} = 2.3412$ $p = 0.132$ , ( $p_{adj} = 0.225$ )
	Relative frequency	↑	GLM, quasibinomial, $F_{1,50} = 7.959$ $p = 0.007$ , ( $p_{adj} = 0.023$ )
Wing vibration	Proportion of time (logit-transformed)	-	GLM, Gaussian, $F_{1,50} = 3.1183$ $p = 0.082$ , ( $p_{adj} = 0.175$ )
	Relative frequency	-	GLM, binomial, $\chi^2_1 = 0.47196$ $p = 0.49$ , ( $p_{adj} = 0.598$ )
	Rate (min <sup>-1</sup> )	↓	GLM, gamma, $F_{1,49} = 6.831$ , $p = 0.012$ , ( $p_{adj} = 0.034$ )
Chasing	Proportion of time	-	GLM, quasibinomial, $F_{1,50} = 0.0671$ $p = 0.797$ , ( $p_{adj} = 0.903$ )
	Relative frequency (logit-transformed)	-	GLM, Gaussian, $F_{1,50} = 1.012$ $p = 0.319$ , ( $p_{adj} = 0.452$ )

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	Rate (min <sup>-1</sup> )	↓	GLM, Gaussian, $F_{1,49} = 17.934$ , $p < 0.001$ , ( $p_{adj} = 0.004$ )
Attempted copulation	Absolute (count)	-	GLM, quasipoisson, $F_{1,50} = 0.003$ $p = 0.96$ , ( $p_{adj} = 0.990$ )
	Relative frequency (logit-transformed)	-	GLM, Gaussian, $F_{1,50} = 1.470$ $p = 0.230$ , ( $p_{adj} = 0.355$ )
	Rate (min <sup>-1</sup> )	↓	GLM, gamma, $F_{1,48} = 9.049$ $p = 0.004$ , ( $p_{adj} = 0.019$ )
Genital licking	Proportion of time	-	GLM, quasibinomial, $F_{1,50} = 4.369$ $p = 0.042$ , ( $p_{adj} = 0.102$ )
	Relative frequency	-	GLM, binomial, $\chi^2_1 = 0.0002$ $p = 0.986$ , ( $p_{adj} = 0.990$ )
	Rate (min <sup>-1</sup> )	-	Wilcoxon rank-sum test, $W = 252$ , $Z = -1.580$ $p = 0.12$ , ( $p_{adj} = 0.225$ )

546  
547

548 **Figure captions**

549

550 **Fig. 1.**

551 The effect of relative size on whether a susceptible or resistant male wins in competitive  
552 trials. (a) Logistic plot: the curve represents the fit of the logistic model of susceptible male  
553 win probability as a function of the susceptible/resistant wing size ratio (SS/RR). Points show  
554 empirical probabilities (+/- s.e.) of a susceptible male win. Rugs at the top and bottom of the  
555 graph show the empirical distribution of binary win data. (b) Probability of susceptible male  
556 win, with 95% binomial confidence intervals, when competitive trial data is divided into  
557 three post-hoc categories. Asterisks represent significant departure from expectation of 50%  
558 (Exact binomial test) indicated by dotted line: '\*\*\*'  $p < 0.001$ .

559

560

561 **Fig. 2.**

562 Effect of male resistance genotype on (a) total copulation latency, and (b) the proportion of  
563 behavioural events that are decamping events. Asterisks represent significance of main effect  
564 of male genotype in GLM: '\*\*'  $p < 0.01$  '\*\*\*'  $p < 0.001$ .

565

566

567 **Fig. 3.**

568 Effect of male resistance genotype on rates ( $\text{min}^{-1}$ ) of three common courtship behaviours (a)  
569 wing vibration, (b) chase, and (c) attempted copulation. Asterisks represent significance of  
570 main effect of male genotype in GLM: '\*'  $p < 0.05$  '\*\*'  $p < 0.01$  '\*\*\*'  $p < 0.001$ .

571

572

573 **Fig. 4.**

574 Counts of all aggressive behaviours observed in pairs of resistant and susceptible males.

575 Asterisk represents significance of main effect of male genotype in GLMM: ‘\*\*\*’  $p < 0.001$ .

576

577 **Fig. S1.**

578 Kinematic diagram of behavioural transitions that occurred more than 10% of the time for (a)

579 susceptible males and (b) resistant males during courtship. Arrow thickness indicates probability of

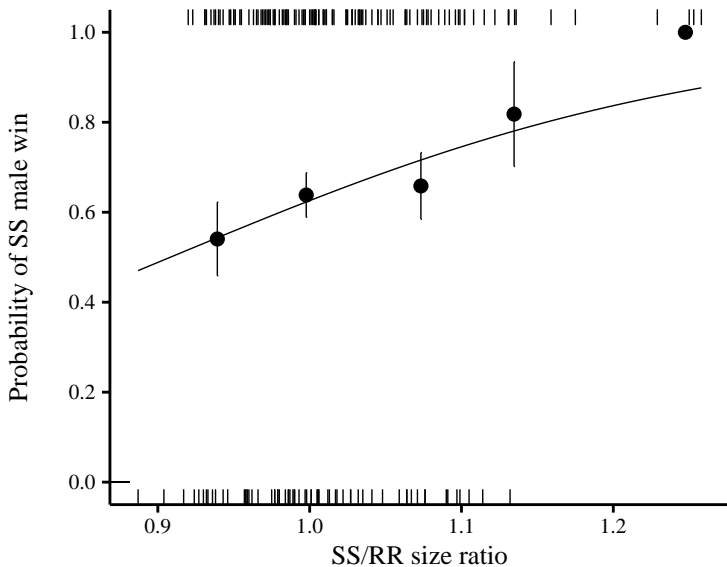
580 occurrence. Solid, black arrows represent those transitions which occurred more frequently than

581 expected by chance ( $p < 0.05$ ) and grey dashed arrows show non-significant transitions ( $p > 0.05$ ).

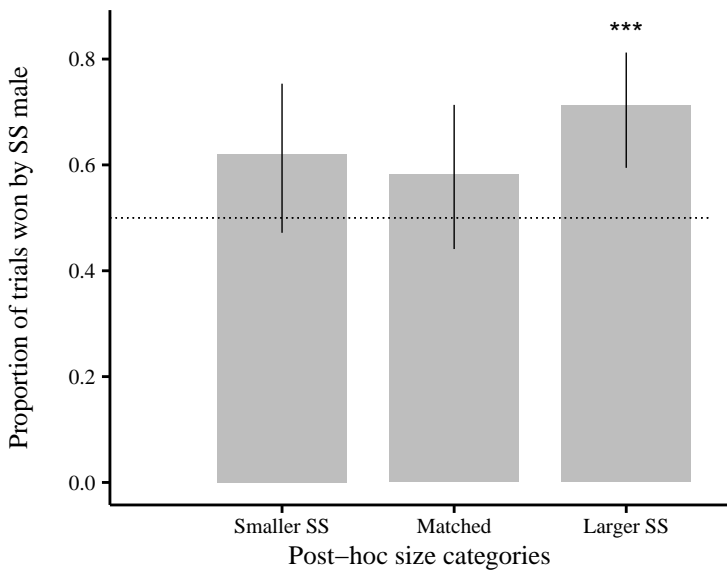
582 Box size indicates frequency of behaviour.

Figure 1

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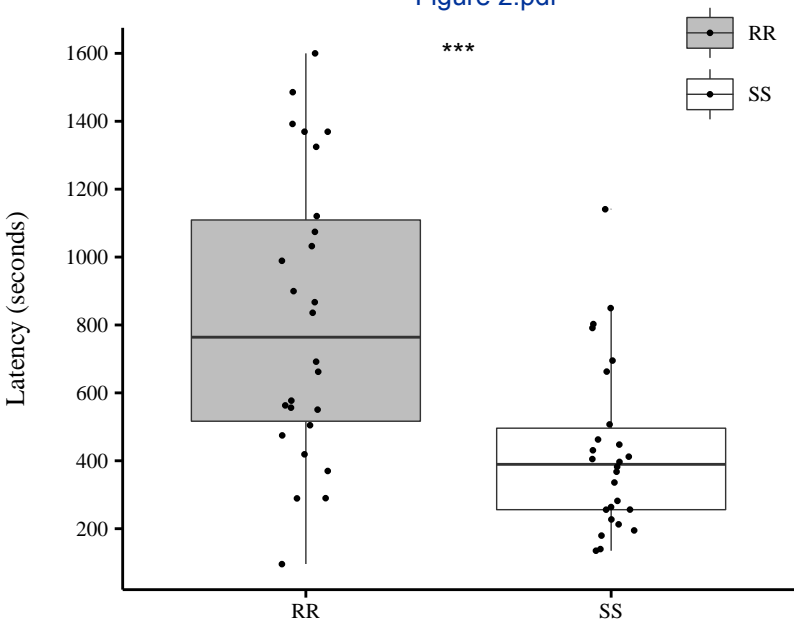


(b)



(a) Figure 2

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(b)

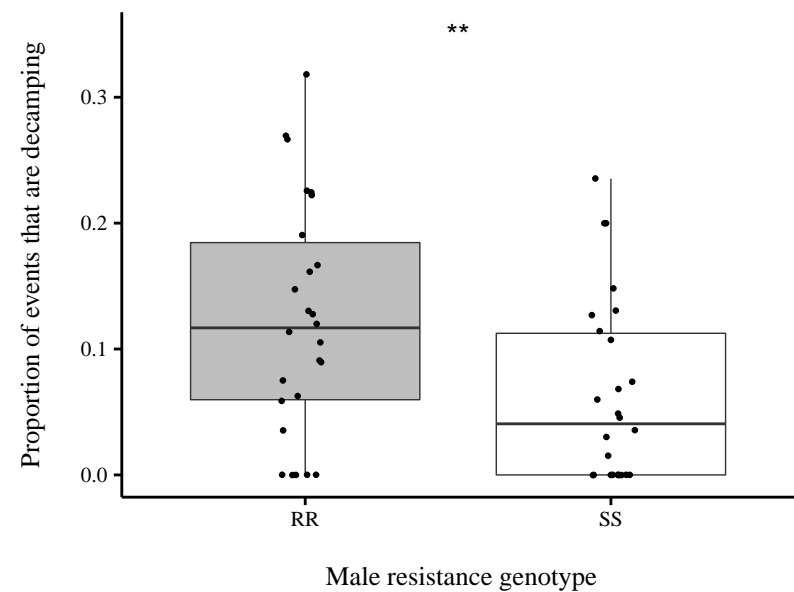
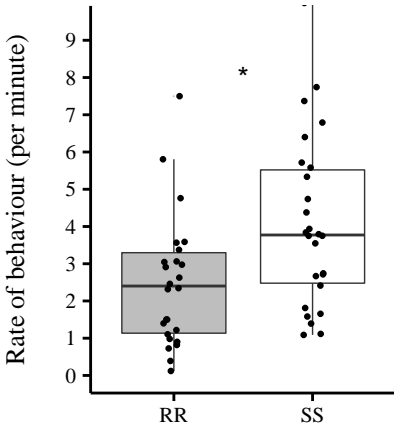
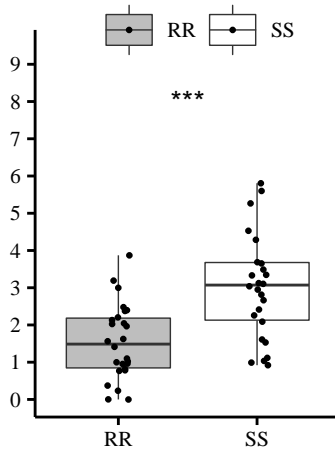




Figure 3 (a) Wing vibration

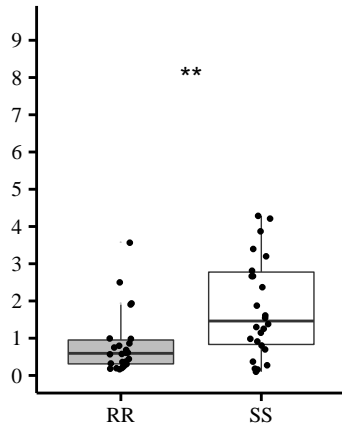


(b) Chasing



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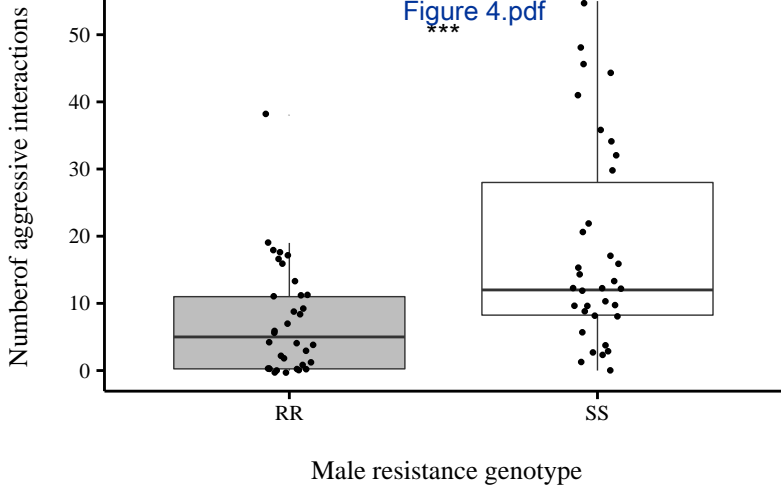
(c) Attempted copulation



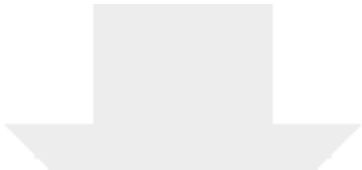
Male resistance genotype

Figure 4

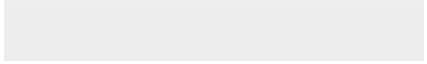
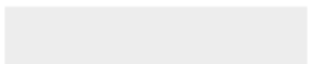
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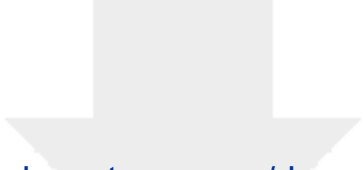
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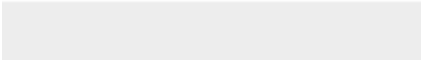

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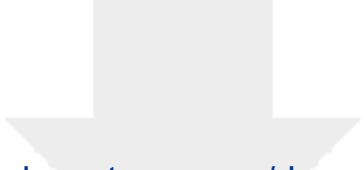
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