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1 # The genetics of male pheromone preference difference between
2 # *Drosophila melanogaster* and *D. simulans*

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11 male mate choice, pheromones, courtship behavior, reproductive isolation, behavioral genetics
12

13 **Author contributions**
14 MPS and TLT conceived of the study and interpreted the data. MPS, AP, and JMR, designed the
15 assays and collected the data. MPS analyzed the data. All authors wrote the manuscript.

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26 **Abstract**

27 Species of flies in the genus *Drosophila* differ dramatically in their preferences for mates, but
28 little is known about the genetic or neurological underpinnings of this evolution. Recent
29 advances have been made to our understanding of one case: pheromone preference evolution
30 between the species *D. melanogaster* and *D. simulans*. Males of both species are very sensitive
31 to the pheromone 7,11-HD that is present only on the cuticle of female *D. melanogaster*. In one
32 species this cue activates courtship, and in the other it represses it. This change in valence was
33 recently shown to result from the modification of central processing neurons, rather than
34 changes in peripherally expressed receptors, but nothing is known about the genetic changes
35 that are responsible. In the current study, we show that a 1.35 Mb locus on the X chromosome
36 has a major effect on male 7,11-HD preference. Unfortunately, when this locus is divided, the
37 effect is largely lost. We instead attempt to filter the 159 genes within this region using our
38 newfound understanding of the neuronal underpinnings of this phenotype to identify and test
39 candidate genes. We present the results of these tests, and discuss the difficulty of identifying
40 the genetic architecture of behavioral traits and the potential of connecting these genetic
41 changes to the neuronal modifications that elicit different behaviors.

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42 **Introduction**

43 Understanding the proximate mechanisms of phenotypic divergence has long been a goal of
44 evolutionary biologists (Stern et al., 2009; Stern & Orgogozo, 2008). Advances in genome
45 sequencing have led to a recent boom in genotype-phenotype association studies, the large
46 majority being for morphological traits (Orgogozo & Martin, 2013). These findings have resulted
47 in a better understanding of the molecular underpinnings of morphological evolution, allowing
48 us to observe general patterns about the types of genetic changes commonly associated with
49 phenotypic change, the number of loci involved, and the general size of their effects
50 (Kittelmann et al., 2017; Martin & Orgogozo, 2013; Rebeiz & Williams, 2017). Comparatively,
51 we have fewer studies of the proximate mechanisms underlying behavioral divergence with
52 which to draw broad conclusions. This is unfortunate, because behaviors are important
53 phenotypes, particularly with respect to speciation and biodiversity. For example, differences in
54 host, habitat, and mating behaviors can form strong reproductive barriers between species
55 (Coyne & Orr, 1997, 2004).

56 Behaviors holistically involve the detection of stimuli via the peripheral nervous system,
57 sensory integration via central nervous system processing, and the coordinated production of a
58 behavioral output. It is therefore surprising that a large proportion of genes known to cause
59 behavioral divergence between species affect sensory perception at the periphery, rather than
60 the other molecular determinants of behavior (Auer et al., 2019; Cande et al., 2013; Leary et al.,
61 2012; McBride et al., 2014). This could indicate that changes at the periphery, mainly in
62 membrane-bound stimulus-detecting receptors, are favored targets of selection because they
63 can have drastic effects on specific phenotypes while minimizing pleiotropic effects (McBride,
64 2007). It is, however, difficult to generalize from so few examples. Moreover, it is quite possible
65 that this pattern is due mainly to ascertainment bias (Rockman, 2012). Indeed, many case
66 studies that have successfully mapped causal genes explaining behavioral divergence have used
67 a candidate-gene approach targeting sensory receptors, so it is plausible that other types of
68 changes are more common but have been overlooked. For example, recent studies that take
69 whole-genome approaches have identified important variants in genes affecting behavior that
70 act at the synapse (Kocher et al., 2018), or as hormonal neuromodulators (Bendesky et al.,

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71 2017). To make generalized predictions about the types of changes underlying behavioral
72 divergence, we need more studies of the genetic basis of behavior that take unbiased
73 approaches, preferably in systems with multiple comparable cases of evolution in similar
74 behaviors (i.e. "metamodel systems" sensu Kopp, 2009). With an active research community,
75 many genetic tools, and an easily manipulated life-history, the *Drosophila* species group
76 provides an excellent opportunity to make further progress, particularly because closely related
77 species differ dramatically in many behaviors.

78 Courtship preference is one behavior that varies dramatically among *Drosophila* species.
79 In *Drosophila,* males and females express a blend of cuticular hydrocarbons (CHCs), many of
80 which act as gustatory pheromones (Pardy et al., 2018). These CHCs are variable between
81 species (Jallon & David, 1987), and can act as sex pheromones (Ferveur & Sureau, 1996) and
82 species identification signals (Billeter et al., 2009). For example, *D. melanogaster* females
83 predominantly express 7,11-heptacosadiene (7,11-HD), while females in the closely-related
84 species, *D. simulans*, primarily express 7-tricosene (7T). The male responses to these
85 pheromones differ dramatically between species: *D. melanogaster* males willingly court
86 conspecific females, but the presence of 7,11-HD on the *D. melanogaster* female cuticle
87 suppresses courtship by *D. simulans* males (Billeter et al., 2009; Clowney et al., 2015; Seeholzer
88 et al., 2018). Male pheromone preference, therefore, constitutes an early barrier to
89 interspecific mating (Shahandeh et al., 2018).

90 Both *D. melanogaster* and *D. simulans* males detect 7,11-HD via gustatory receptor
91 neurons in the forelegs that express the ion channel *ppk23* (Lu et al., 2012, 2014; Thistle et al.,
92 2012; Toda et al., 2012). From there, the signal is propagated through two clusters of neurons
93 (vAB3 and maL neurons) that simultaneously excite and inhibit the P1 central courtship neurons
94 (Clowney et al., 2015). These P1 central courtship neurons act as command neurons, essentially
95 like an on/off switch for male courtship (Auer & Benton, 2016). A recent study found that the
96 evolution of the interactions among these neurons in the central nervous system causes the
97 difference in 7,11-HD preference, rather than the evolution of the peripheral nervous system.
98 (Seeholzer et al., 2018). In *D. melanogaster*, 7,11-HD promotes courtship because the excitation
99 of P1 neurons is greater than the inhibition; in *D. simulans*, the opposite seems to be the case

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100 (Seeholzer et al., 2018). However, the molecular changes underlying these differences in
101 neuronal interactions remain unknown. This detailed, if still incomplete, understanding of the
102 cellular basis of behavioral evolution presents an excellent opportunity to map the causal genes
103 and link evolution in behavior at the genetic, cellular, and organismal levels.
104 Here we present the results of a genotype-phenotype association study where we make
105 considerable progress toward identifying the loci underlying shifts in 7,11-HD preference
106 behavior between *D. simulans* and *D. melanogaster*. First, we confirm a previous result
107 demonstrating that a portion of the male preference phenotype maps to the X chromosome
108 (Kawanishi & Watanabe, 1981). We then show that the preference for 7,11-HD can be
109 recovered in hybrids with a single 1.35 Mb region of the *D. melanogaster* X chromosome. We
110 additionally present the results of two attempts to map this region to a causal gene; both a
111 fine-mapping and candidate gene approach were unsuccessful. Nonetheless, our findings have
112 identified a fraction of the *D. melanogaster* genome containing loci for further functional
113 investigation.
114
115 ## Methods
116 ### *Fly stocks and maintenance*
117 We maintained all fly strains in 25 mm diameter vials on standard cornmeal/molasses/yeast
118 medium at 25°C under a 12 h:12 h light/dark cycle. Under these conditions, we established non-
119 overlapping two-week lifecycles. Every 14 days, we transferred all of the emerged male and
120 female adult flies into vials containing fresh food, where they were allowed to oviposit for 1–3
121 days before being discarded. To test for species differences in male preference, we used two *D.*
122 *simulans* strains: simC167.4 (obtained from the UC San Diego *Drosophila* Stock Center; Stock #:
123 14021-0251.99) and *Lhr* (Brideau et al., 2006; Watanabe, 1979). We used four *D. melanogaster*
124 strains: Canton-S, DGRP-380 (MacKay et al., 2012), C(1)DX-LH_M and LH_M (Rice et al., 2005). To
125 screen portions of the X chromosome, we created duplication hybrids (see below) using a total
126 of 22 Dp(1;Y) strains listed in Table 1 (Cook et al., 2010).
127
128 ### *Hybrid crosses*

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129 To confirm the role of the X chromosome in male mate discrimination, we needed to make F1
130 hybrid males by crossing *D. melanogaster* females to *D. simulans* males (hybrid offspring would
131 then have a *D. melanogaster* X) and by the reciprocal cross (hybrids would have the same
132 autosomal genotype, but the *D. simulans* X). *D. melanogaster*/*D. simulans* hybrid males
133 normally die during development, while hybrid females are infertile (Watanabe, 1979). The *Lhr*
134 strain of *D. simulans* rescues male viability, allowing us to collect living male and female hybrid
135 offspring (Figure 1A). However, crossing females from this *D. simulans* hybrid male rescue strain
136 to *D. melanogaster* males never yielded offspring, probably because of very strong pre-mating
137 isolation. Instead, we used genetic tools (see below) to make these same genotypes while only
138 crossing *D. melanogaster* females to *D. simulans* males. In this crossing direction, we found
139 hybrids could be made using the following steps. First, we collected 20 *D. simulans* males as
140 virgins and aged them for 7-12 days. We collected 10 *D. melanogaster* females as very young
141 virgins, just 2-4 hours after eclosion. We immediately combined 10 very young *D. melanogaster*
142 virgins with 20 aged *D. simulans* males in a vial with food media. We pushed a foam plug into
143 the vial, leaving only 1-2 cm of space above the food surface. We held these hybrid cross vials in
144 this manner for 2-3 days before transferring the flies to a new vial with fresh food to oviposit.
145 To create male hybrids with the *D. melanogaster* X chromosome (melX), we set up the
146 above crosses using the LH_M strain of *D. melanogaster* and the *Lhr* strain of *D. simulans*. To
147 create male hybrids with the X chromosome of *D. simulans* (simX), we set up the same cross
148 using the C(1)DX-LH_M strain of *D. melanogaster* and the *Lhr* strain of *D. simulans*. Females of the
149 C(1)DX-LH_M strain have a compound X chromosome and a Y chromosome in an LH_M autosomal
150 background. The male offspring of this cross inherit their X chromosome from the father, while
151 their Y chromosome and cytoplasm are inherited from the mother (Figure 1B). Thus, simX and
152 melX hybrids only differ in their sex chromosomes. By directly comparing them we can isolate
153 the effect of the sex chromosomes (primarily the X chromosome) on male courtship preference
154 behavior.
155
156 ***Male courtship assays***
157 For all courtship assays, we aged virgin males and females in single-sex vials for 4 days at 25°C

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158 in densities of 10 and 20, respectively. We gently aspirated a single experimental male into a 25
159 mm diameter vial with standard cornmeal/molasses/yeast medium 24 hours before
160 observation. On the morning of observation, we aspirated a single female into the vial and
161 pushed a foam plug down into the vial, leaving a space of 1-3 cm above the food surface. This
162 limited space ensures that flies interact within the observation time period. We observed flies
163 for 30 minutes, collecting minute-by-minute courtship data by manually scoring each pair for
164 three easily observed stages of courtship: singing (single wing extensions and vibration),
165 attempted copulation, and successful copulation. We scored pairs that exhibited multiple
166 stages within a single minute once within that minute. We conducted 2 observations per day at
167 room temperature between the hours of 9 and 11 AM (0-2 hours after fly incubator lights turn
168 on). All observers were blind to both male and female genotype (see below).
169 We tested the male courtship behavior of the following strains: *D. melanogaster* (LH_M,
170 Canton-S, RNAi strains), *D. simulans* (*Lhr* and simC167.4), and various melX and simX F1 hybrids.
171 We measured male courtship towards three types of females, *D. melanogaster*, *D. simulans*,
172 and F1 hybrid females, using no-choice (single female) assays. All *D. simulans* female courtship
173 objects were of the simC167.4 strain. Female courtship objects for *D. melanogaster* were of the
174 DGRP-380 strain when testing males of the LH_M, *Lhr*, melX, simX, and duplication hybrid
175 genotypes (i.e. when testing for an effect of the X chromosome), but we used females from the
176 Canton-S strain with Canton-S males, RNAi males and controls, and gene aberration melX
177 hybrids (see below), because these additional data were collected during a separate follow-up
178 experiment. We made F1 hybrid females using the methods described above (i.e. from a cross
179 between LH_M and *Lhr*; Figure 1A).
180

181 ***Duplication hybrid crosses***

182 Mapping the genes responsible for this evolved behavior is very challenging for several reasons.
183 QTL analysis is not possible using these species, as hybrid males are inviable and hybrid females
184 are infertile. One way around this problem is to use large engineered deletions to make loci
185 hemizygous rather than heterozygous in F1 hybrids, exposing recessive or additive alleles from
186 the *D. simulans* parent (Laturney & Moehring, 2012; Moehring & Mackay, 2004; Pardy et al.,

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187 2018; Ryder et al., 2004). However, a deletion screen is not possible for an X-linked behavior
188 present only in males because the X is already hemizygous, so large deletions are lethal.
189 Instead, we used a set of transgenic strains (Cook et al., 2010) to create duplication hybrid
190 males – hybrids with a complete *D. simulans* X chromosome and an additional segment of the
191 *D. melanogaster* X chromosome translocated to *D. melanogaster* Y chromosome. (Figure 1C,
192 Table 1A). This method has been used to map hybrid incompatibility loci (Cattani & Presgraves,
193 2012), but to our knowledge has not been used to map differences in morphology or behavior.
194 The primary caveat to this method is the inability to detect *D. melanogaster* loci that are
195 recessive to their *D. simulans* counterparts. Because F1 hybrid males are hemizygous, it is
196 impossible to assess dominance a priori. Despite this limitation, the primary advantage to this
197 method is our ability to assay a large portion of the X chromosome (80%) using just 16 DP(1;Y)
198 hybrid strains. To create these hybrids, we crossed *D. melanogaster* males from a DP(1;Y) strain
199 to *D. melanogaster* females from the C(1)DX-LH_M strain (Figure 1C). We then took the resulting
200 female offspring, which carry a *D. melanogaster* compound X chromosome and a *D.*
201 *melanogaster* Y chromosome that has a translocated segment of the X chromosome, and
202 crossed them to *D. simulans Lhr* males using the hybrid cross methods described above. The
203 DP(1;Y) Y chromosome is marked with the dominant visible *Bar* mutation, so inheritance of this
204 chromosome is easy to track. After assaying our original 16 duplication hybrid strains, we tested
205 additional strains with duplications that partially overlap a region we identified in the initial
206 screen in an attempt to fine-map loci within DP(1;Y) segments of interest (Table 1B).
207

208 ***Perfuming D. simulans females with 7,11-HD***

209 Adapting the methods of Thistle et al. (2012), we perfumed *D. simulans* females with synthetic
210 7,11-HD to test for a role in courtship behavior for a duplication of interest (BSC100). To
211 perfume females, we placed 20 simC167.4 females into an empty 25 mm diameter vial. We had
212 previously added either 40 μL of ethanol (sham treatment), or 200 or 400 μg of 7,11-HD
213 dissolved in 40 μL ethanol (perfume treatment) to the vial, and allowed the liquid to evaporate.
214 We then vortexed the vials on the highest setting for three 20-second intervals separated by 20
215 seconds of recovery. We allowed females to recover from vortex mixing for 30 minutes before

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216 loading them into courtship vials. We conducted courtship assays as described above. We
217 detected no difference in courtship between our 200 and 400 μg perfuming treatments (χ^2=
218 0.083, df = 1, p = 0.773), so we combined them into a single perfume treatment in our final
219 analysis.
220

221 **Selecting and testing candidate genes for validation**

222 The BSC100 duplication region that has a significant effect on male courtship behavior in a
223 hybrid background (see results) contains 159 genes (Table S1). In order to select appropriate
224 candidate genes to test for a role in divergent male courtship behaviors, we used modENCODE
225 expression data (Graveley et al., 2011) to identify genes expressed in the *D. melanogaster*
226 central nervous system, a justification set by the findings of Seehozer et al. (2018). We further
227 filtered this list of genes, obtained from FlyBase (Dos Santos et al., 2015), for biological function
228 in nervous system development/function, or transcription factor activity to exclude any genes
229 not specific to the nervous system (i.e. cell maintenance loci). Finally, we selected any of the 25
230 remaining genes that have known *Fruitless* binding sites or interactions, as *Fruitless* is
231 responsible for the male specific wiring of the central nervous system (Goto et al., 2011; Manoli
232 et al., 2005; Vernes, 2014). This left us with a list of 6 candidate genes. For 4 of the 6 genes, we
233 were able to procure a non-lethal aberration (Table 2A). We crossed females of these strains to
234 *D. simulans Lhr* males to create melX hybrids with individual gene knockouts. Two of these
235 knockouts (*Smr* and *pot*) are held over a balancer chromosome in females, and thus produce
236 two types of melX hybrid males: those carrying a balancer (intact) X chromosome, and those
237 carrying a defective X-linked allele. Unfortunately, *pot* defective hybrid males were not viable,
238 and thus could not be observed (Table S3). For *Smr*, we compared both balancer hybrids and
239 *Smr*$^-$ hybrids paired with *D. melanogaster* and *D. simulans* females in the courtship assays
240 described above. For the remaining, unbalanced strains (*Ten-a* and *Pde9*), we compared
241 courtship of knockout hybrid males toward *D. simulans* and *D. melanogaster* females. *Smr* and
242 *Pde9* hybrids presented an extra challenge, as males have white eyes (Table 2A), and thus,
243 difficulty tracking a female courtship target. To remedy this challenge, we observed these males
244 as above, but in a smaller reduced arena to increase interaction between visually impaired

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245 males and females. To modify the courtship arena for these males, we inserted a single piece of
246 plastic vertically into the media, dividing the vial in half, before pushing a foam plug down into
247 the vial, resting 1-2 cm from the food surface.
248 Because the two remaining genes were either lethal in males (*cacophony*), or had no
249 aberration available at all (*Ir11a*), we knocked down expression in *D. melanogaster* flies using
250 RNAi under the control of the Gal4/UAS system (Perkins et al., 2015). For RNAi knock-down
251 strains, we compared progeny of RNAi lines crossed with the pan-neuronal elav-Gal4 stock
252 (Table 2B). The elav-Gal4 insertion is maintained in heterozygotes over a balancer
253 chromosome. Thus, this cross yields RNAi males, which express hairpin RNAi for the target gene
254 in all of their neurons, and control males, which encode for, but do not express the hairpin RNAi
255 because they lack Gal4 expression in neurons. Control males express a dominant visible marker
256 contained on the balancer chromosome they inherit instead of the Gal4 insertion. In this way,
257 for males paired with both *D. melanogaster* and *D. simulans* females, we compare the courtship
258 of males where expression of the gene of interest is reduced in neurons, to males of the same
259 background without reduced expression in the nervous system. While knocking out (or down)
260 expression of these 6 candidate genes allows us to compare the specific effects of loss of
261 function of *D. melanogaster* alleles to functioning alleles in their respective backgrounds
262 (hybrid for knock-outs, *D. melanogaster* for RNAi knockdown), it is not immediately clear what
263 phenotype to expect during these tests. This presents another challenge in interpreting these
264 results (see Discussion).
265
266 ***Data analysis***
267 From the minute-by-minute courtship data, for each male-female combination (see below), we
268 collected binomial data (court/did not court) to determine the courtship frequency (CF) of male
269 genotypes. We only considered males that spent 10% or more of the total assay time (i.e., >3
270 min) in one of the three courtship stages as successfully displaying courtship. For male
271 genotypes, unless otherwise stated, we used Fisher's exact test to compare the proportion of
272 males that courted a given female type followed by posthoc analysis with sequential Bonferroni
273 tests (Holm, 1979).

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274 For each male that displayed courtship towards a female target, we also calculated the
275 total percent of assay time (30 minutes) that a male spent courting as a proxy for male
276 courtship effort (CE). For males that mated with females, we calculated CE as the percent of
277 time a male spent courting from the start of the assay until the time of copulation. Unlike with
278 CF, we used no minimum threshold for CE. CE is representative of male investment in any given
279 female, another indication of male choice (Edward & Chapman, 2011). Because the courtship
280 effort distributions are highly skewed, we report the median values for comparison across
281 strains. For each male genotype, we compared courtship effort between female genotypes
282 using the Mann-Whitney U test followed by posthoc analysis with sequential Bonferroni tests
283 (Holm, 1979).
284

285 Results

286 ***A significant conspecific courtship preference between D. melanogaster and D. simulans***
287 For both *D. melanogaster* strains that we assayed, we detected significantly higher courtship
288 frequencies when males were paired with females of their own species (Figure 2A, Table S2).
289 Both LH_M and Canton-S courted *D. melanogaster* females significantly more frequently than *D.*
290 *simulans* females (LH_M: p = 0.0003; Canton-S: p = 0.0032). Likewise, both *D. simulans* strains
291 displayed higher courtship frequencies with *D. simulans* females than *D. melanogaster* females
292 (*Lhr:* p = 5.34E-09; simC167.4: p = 5.47E-12). There were no differences in the courtship
293 frequencies among strains of the same species (p = 1 for both *D. melanogaster* and *D.*
294 *simulans*). Unsurprisingly, when we calculated a consensus p-value (Rice, 1990), which tests the
295 combined effect of independent tests of the same hypothesis, for the two *D. melanogaster*
296 strains and the two *D. simulans* strains, conspecific courtship preferences remained highly
297 significant (p = 1.61E-5 and p < 1.00E-25, respectively), suggesting this is indeed a species-level,
298 rather than strain-specific difference.
299 We also observed each *D. melanogaster* and *D. simulans* strain with F1 hybrid females
300 (Figure 2A, Table S2). These females still produce 7,11-HD, due to a single functioning copy of
301 *desatF* (Shirangi et al., 2009), and concordantly, *D. melanogaster* and *D. simulans* males court
302 them similarly to *D. melanogaster* females. We found that our *D. melanogaster* strains were

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303 just as likely to court F1 hybrid females as they were to court *D. melanogaster* (p = 0.6486 for
304 LH_M, and p = 6724 for Canton-S), and these comparisons remained non-significant when we
305 calculated a consensus p-value (p = 0.7980). Conversely, for one of our *D. simulans* strains,
306 simC167.4, we found that males court F1 hybrid females significantly less often than *D.*
307 *simulans* females (p = 5.59E-07). *Lhr* males had a much lower courtship frequency with F1
308 hybrid females compared to *D. simulans* females (20% vs. 65%, respectively), but this difference
309 was not significant, likely due to small sample size (p = 0.1003 and N = 10). Supporting this, a
310 consensus p-value for both *D. simulans* strains found that *D. simulans* overall had a significantly
311 higher courtship frequency with *D. simulans* females than with F1 females (p = 9.93E-07). For
312 both *D. simulans* strains, we detected no difference in courtship frequency toward F1 and *D.*
313 *melanogaster* females, as all had relatively low courtship frequencies (for *Lhr* p = 0.1230, and
314 for simC167.4 p = 0.2089; and consensus p = 0.1197).
315 We find less striking differences when we calculate the courtship effort of those males
316 that did court (Figure 2B, Table S2). For example, once they began courting, Canton-S males
317 courted all three female types with equal vigor (p = 1 for all comparisons). LH_M males, however,
318 courted *D. melanogaster* with much higher vigor than *D. simulans* females (p = 0.0011). Thus,
319 unlike courtship frequency, courtship effort appears to have strain-specific effects within the *D.*
320 *melanogaster* strains we surveyed. For *D. simulans*, we detected a nearly significant increase in
321 courtship effort for simC167.4 males that courted *D. simulans* females compared to males that
322 courted F1 females (p = 0.0506). We are unable to detect significant differences in courtship
323 effort for *Lhr* males among any comparisons, or for simC4 male comparisons involving *D.*
324 *melanogaster* females, because so few males courted *D. melanogaster* females (and F1 females
325 for *Lhr*).
326
327 ***The X-chromosome partially explains differences in courtship behavior***
328 Qualitatively, the behavior of hybrid males largely replicates the behavior of the X chromosome
329 donating parent (Figure 2, Table S2). Like males of the *D. melanogaster* parent strain (LH_M),
330 melX hybrid males court both *D. melanogaster* and F1 hybrid females significantly more often
331 than *D. simulans* females (*D. melanogaster*: p = 0.0052, F1: p = 0.0005 Figure 2A). Also like the

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332 LH_M parent strain, melX males court F1 hybrid females and *D. melanogaster* females at similar
333 frequencies (p = 0.6724). Quantitatively, however, the behavior of melX males does not entirely
334 replicate that of the *D. melanogaster* parent strain. For instance, although still significantly
335 higher than when paired with *D. simulans* females, melX males court both *D. melanogaster*
336 females and F1 females at significantly lower frequencies than LH_M (p = 9.92E-07 and p =
337 0.0022, respectively). With respect to courtship effort, melX males court each female
338 indistinguishably (all p > 0.8541, Figure 2B).
339 The behavior of simX males more closely reproduces that of the *D. simulans* parent
340 strain (*Lhr*). Like *Lhr*, simX males court *D. simulans* females at much higher frequencies than *D.*
341 *melanogaster* females (p = 1.32E-15, Figure 2A) and F1 females (p = 4.03E-14). Unlike the *Lhr*
342 parent strain, simX males court F1 females significantly more frequently than *D. melanogaster*
343 females (p = 1.57E-05). Courtship towards *D. melanogaster* was still too rare to detect
344 differences in courtship effort compared to *D. simulans* or F1 females (both p = 1, Figure 2B),
345 but simX males courted *D. simulans* with significantly higher effort than F1 females (p = 0.0005,
346 Figure 2B). Quantitatively, simX males behave very similarly to their *D. simulans* parents. simX
347 males court *D. melanogaster* and F1 females with frequencies equivalent to that of *Lhr* males (p
348 = 0.5876 and p = 0.7190, respectively), but they court *D. simulans* females at a higher frequency
349 (p = 0.0373). We suspect this latter result is a byproduct of increased heterozygosity relative to
350 the inbred *Lhr* parent strain.
351

352 ***A single region of the D. melanogaster X chromosome changes simX hybrid courtship***
353 ***behavior***

354 To test specific regions of the X chromosome for their role in courtship preference differences,
355 we measured the courtship behavior of 16 duplication hybrids (Table 1A, Figure 1C). These
356 duplication hybrids are simX hybrids made heterozygous for one stretch of the *D. melanogaster*
357 X chromosome. We observed 15 of these strains with *D. melanogaster* females, and
358 interestingly, none displayed courtship (N = 6-20 for each, N = 179 total, Table S2). All 16 of the
359 duplication hybrid strains did court both *D. simulans* and F1 females, however. We detected
360 significant variation in the amount of courtship these lines displayed to both female courtship

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361 targets (χ^2= 55.36, df = 15, p-value = 1.55e-06 for *D. simulans* females, N = 709 total; and χ^2=
362 66.58, df = 15, p-value = 1.80e-08 for F1 females, N = 759 total).
363 On average, the duplication hybrid lines had significantly higher courtship frequencies
364 toward *D. simulans* females than toward F1 females (average CF = 35.86% for *D. simulans*
365 females, average CF = 19.30% for F1 females, p = 5.43E-08, N = 16). This pattern is consistent
366 with what we see for simX hybrids without X-linked duplications, which similarly courted *D.*
367 *simulans* females more often than F1 females. However, courtship frequencies with both
368 females are significantly higher for simX males compared to duplication hybrids (average simX
369 CF with F1 females = 30%, Student's t = -3.3664, df = 15, p = 0.0042; average simX CF with *D.*
370 *simulans* females = 89%, Student's t = -12.891, df = 15, p = 1.614e-09). In total, 15 of the 16
371 duplication hybrid genotypes courted *D. simulans* with higher frequency than F1 females
372 (Figure 3A, Table S2); three of these lines showed a significant preference for *D. simulans*
373 females after correction for multiple tests (p = 0.0415 for BSC296, p = 0.0061 for BSC277, and p
374 = 0.0437, for BSC200). Only one duplication hybrid strain, BSC100, courted F1 hybrids with
375 higher frequency than *D. simulans* hybrids (p = 0.0136). In general, the duplication hybrid
376 strains courted *D. simulans* females with greater effort than F1 hybrid females (grand median
377 CE = 17% for *D. simulans* females, and the grand median CE = 10% for F1 females, p = 8.78E-05).
378 Again, BSC100 duplication hybrids were the only hybrids to display higher courtship effort
379 toward F1 females (CE = 30%) than toward *D. simulans* females (CE = 13.33%), although this
380 difference was not significant after correcting for multiple comparisons (p = 0.0976, Figure 3B,
381 Table S4).
382
383 ***BSC100 duplication hybrid males prefer females with the cuticular hydrocarbon 7,11-HD***
384 To verify that CHCs are involved in the courtship behaviors that BSC100 duplication hybrid
385 males exhibited, we perfumed *D. simulans* females with 7,11-HD. When we observed BSC100
386 duplication hybrid males with perfumed *D. simulans* females, we saw a significant increase in
387 courtship frequency relative to sham-perfumed *D. simulans* females (p = 0.0400, Figure 3C,
388 Table S2). BSC100 hybrid courtship frequency increased from 16% with *D. simulans* sham-
389 perfumed females, to 40% with *D. simulans* females perfumed with 7,11-HD. Note, these

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390 courtship frequencies are similar to those seen when BSC100 hybrid males were paired with *D.*
391 *simulans* females (CF = 17.91%, p = 1) and F1 females (CF = 45.07%, p = 0.8603), respectively.
392 Again, the courtship effort data supports this conclusion (Figure 3D). We observed significantly
393 higher courtship effort toward *D. simulans* females perfumed with 7,11-HD (CE = 30%) when
394 compared to sham-perfumed *D. simulans* females (CE = 3%, p = 0.0014). Encouragingly, the
395 courtship effort we observed toward 7,11-HD-perfumed *D. simulans* females is even higher
396 than that of BSC100 hybrid male courtship effort toward F1 females (p = 0.0062). Additionally,
397 BSC100 hybrid males court sham-perfumed *D. simulans* and non-manipulated *D. simulans* with
398 equal effort (p = 0.4451).
399

400 ***Fine-mapping the BSC100 region of the X chromosome***

401 The BSC100 X chromosome segment spans ~1.35 Mb, from position 11,557,017 to 12,903,175
402 of the *D. melanogaster* X chromosome. This locus contains 159 genes (Table S1). To further
403 refine this region, we measured the courtship behavior of 6 additional duplication hybrids
404 heterozygous for various sections of the X chromosome within our region of interest (Figure 4A,
405 Table 1B). The 6 overlapping duplication hybrids had an average courtship frequency of 42%
406 with *D. simulans* and 20.22% with F1 females, comparable to the original 16 duplication
407 hybrids. Five of the strains (BSC47, BSC49, BSC54, BSC315, and BSC317) had higher courtship
408 frequencies with *D. simulans* females than with F1 females (Figure 4B, Table S2), with three
409 (BSC49, BSC315, and BSC317) being significant (p = 0.0002, p = 0.0462, and p = 0.0057
410 respectively). Overall, the overlapping duplication hybrid strains also displayed significantly
411 higher courtship effort toward *D. simulans* females (CE = 20%) than toward F1 females (CE =
412 12.5%, p = 0.0029). The same three strains (BSC49, BSC315, and BSC317) that courted *D.*
413 *simulans* females with higher frequencies than F1 females also displayed higher courtship effort
414 towards *D. simulans* females (p = 0.0460, p = 0.0408, and p = 0.0408, respectively).
415 A single strain, BSC101, displayed a different pattern than the others. Interestingly, this
416 duplication segment spans 98% of the region covered by BSC100, while the other duplications
417 cover various smaller portions of the region, ranging from 22% to 76% coverage (Figure 4A).
418 BSC101 had a higher courtship frequency when paired with F1 females (CF = 37.04%) than

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419 when paired with *D. simulans* females (CF = 29.63%), though not significantly so (p = 1 after
420 correcting for multiple comparisons). However, BSC101 duplication hybrids also did not differ
421 from BSC100 duplication hybrids with respect to courtship frequency towards either *D.*
422 *simulans* or F1 females (p = 0.2663 and p = 0.5028, respectively). Likewise, BSC101 duplication
423 hybrids were the only strain to display higher effort when paired with F1 females (CE = 26.67%)
424 than when paired with *D. simulans* females (CE = 10%), though not significantly so (p = 0.2067).
425 These numbers are also comparable to the courtship effort of BSC100 males towards each
426 female type.
427
428 ***Testing candidate genes***
429 The BSC100 region is known to contain 159 genes (Table S1). To reduce this list to a testable
430 number, we focused on genes with neurological functions and *fruitless* binding sites (see
431 Methods). This produced 6 candidate genes: *papillote* (pot), *cacophony* (cac), *Tenascin-a* (*Ten-*
432 *a*), *Smrtr* (*Smr*), *Ionotropic receptor 11a* (*Ir11a*), and *Phosphodiesterase 9* (*Pde9*, Figure 5). We
433 used loss-of-function mutations or RNAi to investigate 5 of these 6 genes further
434 (unfortunately, *pot* loss-of-function hybrid males were inviable).
435 For *Smr*, the hybrid cross between the *D. melanogaster* knockout strain produced
436 balancer hybrid (melX males with the X chromosome intact) and *Smr*$^-$ hybrid males (melX males
437 with the X chromosome lacking a functional *Smr* gene). *Smr* balancer hybrids courted *D.*
438 *simulans* and *D. melanogaster* at similar frequencies (p = 1, Figure 6A, Table S2), and with
439 similar efforts (p = 0.1136, Figure 6B). The same is true for *Smr*$^-$ hybrid males (p = 1 for both CF
440 and CE). However, these males courted both females at significantly higher frequencies than
441 balancer hybrids (p = 0.0073 for *D. simulans*, and p = 0.0135 for *D. melanogaster*). Despite
442 quantitative differences, both *Smr*$^-$ and balancer hybrid males show no preference for *D.*
443 *melanogaster* or *D. simulans* females. Thus, there is no clear effect of the loss of *Smr* on male
444 preference when compared to intact balancer hybrid males.
445 In contrast, *Ten-a* and *Pde9* are not held over a balancer, so hybrid crosses yield only
446 knockout males. *Ten-a*$^-$ hybrids court both *D. simulans* and *D. melanogaster* at equal
447 frequencies (p = 1), and with similar effort (p = 1). *Pde9*$^-$ hybrids court *D. melanogaster* at non-

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448 significantly higher frequencies than *D. simulans* females (p = 0.2124), and court both females
449 with equal effort (p = 1). In contrast, melX males with functioning copies of these genes court *D.*
450 *melanogaster* females more frequently (and with non-significantly higher courtship effort).
451 Although these knockout hybrids differ in some aspects from melX males, it is difficult to
452 discern whether these differences are due to the different *D. melanogaster* strains with which
453 these hybrids were made (see Discussion).
454 For *cac* and *Ir11a*, we compared the behavior of *D. melanogaster* RNAi knockdown flies
455 (Figure 6E,F) to their siblings lacking knockdown (balancer). For *Ir11a*, we found that balancer
456 males court *D. simulans* and *D. melanogaster* indiscriminately in terms of courtship frequency
457 (p = 1) and courtship effort (p = 1). We found the same pattern for *cac* balancer males (p = 1 for
458 both CF and CE). While *Ir11a* RNAi males also courted *D. melanogaster* and *D. simulans* females
459 at equal frequencies (p = 0.9032), *cac* RNAi males had significantly lower courtship frequencies
460 with *D. simulans* females than with *D. melanogaster* females (p = 1.34E-05). Interestingly, both
461 *Ir11a* and *cac* RNAi males displayed reduced effort toward *D. simulans* females compared to *D.*
462 *melanogaster* females (p = 8.13E-06 and p = 1.76E-05, respectively).
463 To test if the reduction in courtship frequency and effort of *cac* RNAi males with *D.*
464 *simulans* female is driven by the absence of 7,11-HD on the *D. simulans* cuticle, we also
465 observed *cac* RNAi and balancer males with sham- and 7,11-HD perfumed *D. simulans* (Figure
466 7A,B). We found that 7,11-HD does indeed cause this effect: balancer hybrids court sham
467 perfumed and 7,11-HD perfumed *D. simulans* with equal frequencies (p = 1), and there was no
468 significant difference in courtship effort (p = 0.5876). In contrast, *cac* RNAi hybrids court 7,11-
469 HD perfumed D. simulans at significantly higher frequency and effort than sham perfumed
470 females (p = 3.91E-06 for CF and p = 0.0027 for CE). Thus, *cac* RNAi hybrids require 7,11-HD to
471 stimulate high amounts of courtship.
472
473 ## Discussion
474 ***An important difference in male courtship preference between D. melanogaster and D.***
475 ***simulans partially maps to the X chromosome***

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476 Our observation of two *D. simulans* and two *D. melanogaster* strains confirms a previously
477 described species difference in male courtship preference, where each species dramatically
478 prefers their own females (Manning, 1959). While we did observe some variation in the
479 quantitative amount of courtship among our lines (Figure 2A), the valence of male preference
480 was always consistent within species. Because we also used females from two different lines,
481 this variation in male behavior could be due to individual variation in female CHC quantity
482 (Pardy et al., 2018), variation in other female traits, and/or variation in male preferences
483 (Pischedda et al., 2014). Our results demonstrate that male courtship preferences act as a large
484 reproductive barrier for *D. simulans* and *D. melanogaster*, as has been shown between *D.*
485 *simulans* and *D. sechellia* (Shahandeh et al., 2018). A detailed understanding of the genetic
486 basis of this preference would prove illuminating, not only with respect to the evolution of
487 behavior, but also with respect to the evolution of reproductively isolating barriers.

488 The courtship data we collected using reciprocal *D. melanogaster*/*D. simulans* hybrids
489 (melX and simX males) created in a homogenous background and controlled for cytoplasmic
490 inheritance also confirms the significant role of the X chromosome in male courtship preference
491 differences between these species (Kawanishi et al., 1981). Though we didn't strictly control for
492 an effect of the Y chromosome, it is unlikely to explain our results because hybrids with the *D.*
493 *simulans* Y behave more like *D. melanogaster*, and hybrids with the *D. melanogaster* Y behave
494 more like *D. simulans.* Because we only used reciprocal hybrids to demonstrate the X-effect, we
495 cannot rule out the potential of transgressive autosomal effects that cannot be detected in a
496 hybrid background (Mittleman et al., 2017). Indeed, although the behavior of our hybrids
497 qualitatively mirrors that of the X-donating parent, quantitative differences in both courtship
498 frequency and effort, particularly between LH_M and melX hybrids, suggest an additional role of
499 autosomal loci (likely *D. simulans* dominant, as simX males behave both qualitatively and
500 quantitatively similar to *D. simulans* males). These results mirror findings from a QTL study
501 mapping male courtship frequency differences among *D. simulans* and *D. sechellia*, another
502 species where females express 7,11-HD and males are stimulated to court by it (M.P.
503 Shahandeh & T.L. Turner, in prep). In this case, the *D. simulans* X chromosome contributes to
504 7,11-HD aversion, and autosomal *D. sechellia* loci contribute to 7,11-HD attraction. However, in

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505 *D. simulans-D. sechellia* reciprocal hybrid males, the effects of the autosomal loci are far greater
506 than the X chromosome, as both hybrids behave more similarly to *D. simulans* males. It remains
507 to be seen whether the same loci affect 7,11-HD response between these species, but the
508 effects/interactions of loci are undoubtedly different.
509

510 ***A single segment of the D. melanogaster X chromosome has a large effect on male***
511 ***pheromone preference***

512 We expect all DP(1;Y) hybrid strains to behave like the simX hybrid strain, unless the
513 duplicated *D. melanogaster* X chromosome segment harbors dominant or additive male
514 preference loci, in which case they should behave more like melX hybrids. When paired with *D.*
515 *melanogaster* females, however, we observed no courtship from the 15 DP(1;Y) hybrid strains
516 we observed, which all behaved like simX males in this respect (the 16th unobserved duplication
517 hybrid strain's CF and CE for *D. simulans* and F1 hybrids also follows this trend). This result is
518 consistent with several possibilities, including: (1) the loci for male courtship preference reside
519 in the 20% of the X chromosome not covered by the duplication hybrid strains, (2) *D.*
520 *melanogaster* courtship preference alleles are recessive to, or epistatic with, *D. simulans* alleles,
521 (3) the presence of Y-linked X translocations disrupts male behavior in general by making
522 regions of the X chromosome heterozygous, and/or (4) the *D. melanogaster* allele is not
523 expressed properly due to the genomic environment of the translocation. This last possibility is
524 perhaps unlikely, as the duplications have been shown to rescue the loss of 94% of X-linked
525 mutations (Cook et al., 2010).

526 Similar to our duplication hybrids, we never observed any of our *D. simulans* strains
527 courting *D. melanogaster* females, but each courted F1 females at low levels (18% of all *D.*
528 *simulans* males courted F1 females), suggesting they may display intermediate female cues.
529 However, *D. simulans* males still strongly preferred *D. simulans* females over F1 hybrid females,
530 likely due to the presence of 7,11-HD on the F1 female cuticle (Coyne, 1996), which suppresses
531 courtship in *D. simulans* males (Billeter et al., 2009). This is also likely the reason that *D.*
532 *melanogaster* males court F1 females comparably to *D. melanogaster* females. As with the male
533 preference difference between *D. melanogaster* and *D. simulans* females, the preference

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534 difference between *D. simulans* and F1 hybrid females still maps to the X chromosome: simX
535 males behave like *D. simulans* males – courting F1 females infrequently, and significantly less
536 frequently and with less effort, than *D. simulans* females (Figure 2). Likewise, melX males
537 behave like *D. melanogaster*, courting F1 females with a similar frequency as *D. melanogaster*
538 females, and significantly more frequently than *D. simulans* females. Because the overall
539 preference patterns between *D. melanogaster* and *D. simulans* were replicated when we
540 compared melX and simX male courtship towards F1 and *D. simulans* females, we additionally
541 observed each of the 16 DP(1;Y) hybrid strains with F1 hybrid females. Again, we expect the
542 courtship preferences of all DP(1;Y) hybrid strains to resemble the simX hybrid strain unless the
543 duplicated *D. melanogaster* X chromosome segment harbors male preference loci, in which
544 case we expect them to court F1 hybrid females at higher frequencies than *D. simulans* females,
545 as we see for melX males.

546 When we observed hybrid males from the 16 DP(1;Y) genotypes, we found that some
547 males from every strain courted F1 females. Most genotypes courted F1s at low levels, as we
548 saw for simX hybrids. BSC100 hybrids were the only duplication hybrids that displayed higher
549 courtship frequency and effort with F1 hybrids than with *D. simulans* females, replicating the
550 pattern seen in melX hybrid males. However, like simX hybrids, BSC100 hybrids showed no
551 courtship towards *D. melanogaster* females. The fact that BSC100 hybrid males prefer F1
552 females to *D. simulans* females, but are still unwilling to court *D. melanogaster* females,
553 suggests that the *D. melanogaster* variants at this locus are insufficient to completely mask the
554 effects of the *D. simulans* X genome, which is also present in the BSC100 hybrid. We
555 hypothesize that the greater courtship we see towards F1 hybrid females is not seen towards *D.*
556 *melanogaster* females because the multiple, partially redundant cues that influence male
557 courtship in *Drosophila* are more intermediate in F1 females (Arbuthnott et al., 2017). If there
558 are multiple male preferences and female cues that have evolved, the BSC100 duplication may
559 recover the *D. melanogaster* preference towards one signal, but still be insufficient to activate
560 the P1 courtship command neurons because of inhibition on other sensory channels by the *D.*
561 *simulans* genome (Clowney et al., 2015).

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562 This hypothesis is supported by our perfuming data. Although past work has shown that
563 the male preference difference between *D. melanogaster* and *D. simulans* is primarily dictated
564 by female pheromones (Manning, 1959) – specifically 7,11-HD (Billeter et al., 2009) – we could
565 not be sure that that BSC100 hybrid males were responding to this cue, especially because they
566 were unwilling to court *D. melanogaster* females that also express this pheromone. However,
567 we found that BSC100 hybrid males significantly preferred *D. simulans* females perfumed with
568 7,11-HD to sham-perfumed females, and courted them at a frequency and effort comparable to
569 what we saw with F1 hybrid females, which also have 7,11-HD on their cuticle. These results
570 confirm the role of this X region in 7,11-HD response. Taken together, our findings demonstrate
571 that a single 1.35 Mb segment of the X chromosome has a specific effect on the evolved 7,11-
572 HD preference differences between *D. simulans* and *D. melanogaster*.
573
574 ***Subdividing this region for fine-mapping results in the loss of the significant preference***
575 ***difference***
576 In order to further fine map the X chromosome region duplicated in BSC100 hybrids, we
577 created 6 additional hybrid genotypes with partially overlapping duplicated segments (Table
578 1B). When we observed these overlapping duplication hybrid strains, none showed the same
579 pattern we observed for BSC100. Five of these had higher courtship frequencies with *D.*
580 *simulans* females than with F1 females, just like simX males, although two of these differences
581 were not significant. One strain, BSC101, did have a marginally higher CF and CE with F1
582 females than *D. simulans* females, albeit non-significantly. This segment has the largest overlap,
583 covering 98% of the segment in BSC100 hybrids (Figure 3C). We hypothesize 2 potential
584 explanations for the loss of significant preference when this region was subdivided.
585 (1) *The genetic architecture of male courtship preference within this region is polygenic.*
586 It is possible that male courtship preference differences are polygenic— even if these genes are
587 constrained within a single 1.35 Mb segment. In this case, subdivision of this locus may reduce
588 the behavioral effect if these loci are additive, or result in its loss altogether if these loci have
589 epistatic interactions. This possibility fits somewhat with the pattern we observe with our
590 overlapping duplications: hybrids with the smaller overlapping segments have lost the

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591 phenotype entirely, while the largest overlap appears to, at least partially, reproduce the
592 phenotype. The simplest model that fits this scenario consists of at least two interacting loci, at
593 either end of BSC100, such that all loci are never captured by any of the overlapping duplication
594 hybrid strains. BSC101 overlaps 98% of BSC100. If this is indeed the case, then at least one locus
595 must reside within the 2% not covered by BSC101. This type of genetic architecture is not
596 uncommon. Many loci contributing to a single phenotype constrained within a single region
597 have similarly been discovered for morphological traits in *Drosophila* and other organisms
598 (Fanara et al., 2002; Harbison et al., 2004; Miller et al., 2014; Peichel & Marques, 2017).
599 (2) *Hybrid males heterozygous for regions of the X-chromosome behave differently than*
600 *typical hybrids.* Overall, we observed reduced levels of courtship among our duplication hybrid
601 strains compared to simX or melX hybrids, suggesting that the duplication hybrids behave
602 differently from typical hybrids. The consistent reduction in courtship by duplication hybrids
603 also reduces our statistical ability to detect a significant effect. We feel that abnormal behavior
604 of duplication hybrids – particularly those carrying smaller subdivisions of the initial 16
605 duplication segments, is the most likely explanation for why our attempts to fine-map the
606 BSC100 region were unsuccessful.
607 Duplication hybrid males may behave differently for a variety of reasons; the most likely
608 are those that stem from the Y-translocated X-duplication segments themselves. Males made
609 heterozygous for regions of the X chromosome that are typically hemizygous may have
610 abnormal behavior due to epistatic interactions between X chromosome loci. In the melX and
611 simX hybrids, *D. melanogaster* and *D. simulans* X loci are not present in the same genetic
612 background, but they are in duplication hybrids. These loci may interact in unpredictable, non-
613 additive ways, having unforeseen effects on behavior. Alternatively, genes translocated from
614 the X to the Y chromosome may have altered expression patterns due to their new genomic
615 environment, producing a similar effect. Smaller segments, like those we used for fine-
616 mapping, may be more susceptible to this problem, as genes contained within smaller
617 translocated segments are more likely to be surrounded by a foreign genomic environment.
618 The panel of Y-linked X duplications we used to create duplication hybrids was also
619 created using irradiation (Cook et al., 2010). In fact, each breakpoint was induced by irradiating

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620 males, originally creating strains that contained large subdivisions of the X chromosome, like
621 BSC100. Further subdivision of these regions (to create strains like BSC101 and all of the smaller
622 subdivisions that we used for fine-mapping) required additional irradiation. This additional
623 irradiation likely introduced new mutations to the genetic background of these flies, making
624 comparisons between BSC100 and its subdividing strains, like BSC101, imperfect.
625 Finally, these duplication segments are marked with a dominant visible eye mutation,
626 *Bar*, that substantially reduces the shape of the eye to a small sliver in males. These males likely
627 have restricted fields of vision, and may have difficulty tracking females in the courtship arena,
628 as has been shown for mutations affecting eye pigmentation (Connolly & Cook, 1973; Spiess &
629 Schwer, 1978). Indeed, our data collectors noted during courtship observations that males
630 often seemed to lose track of the females they were courting, and courtship would cease. This,
631 too, likely contributed to the reduced courtship frequency and effort we observed, but is
632 constant across all duplication hybrids. Regardless of the cause(s) of atypical behavior in our
633 duplication hybrids, our failure to fine-map the BSC100 region must be considered in light of
634 the above caveats.
635
636 ***Testing five candidate genes yields inconclusive results***
637 We were able to test five candidate genes we identified within the BSC100 region,
638 either through the use of gene aberrations or RNAi knockdown. Qualitatively, our experiments
639 using an *Smr* gene aberration suggest that the loss of *Smr* expression in a melX hybrid
640 background has no effect on courtship behavior, as *Smr*$^-$ hybrids behaved like *Smr* balancer
641 hybrids in that they court both *D. simulans* and *D. melanogaster* females at equal frequency
642 and with equal effort. Quantitatively, however, *Smr*$^-$ hybrids showed higher overall courtship
643 frequencies and efforts towards both females compared to balancer hybrids. This result
644 suggests that males harboring an X chromosome balancer are less vigorous, and may behave
645 atypically due to the presences of large inversions on a hemizygous sex chromosome. Thus,
646 balancer hybrids are not an ideal comparison.
647 The results of our comparisons of *Ten-a*$^-$ and *Pde9*$^-$ hybrids are congruent with that of
648 *Smr*$^-$ hybrids. *Ten-a*$^-$ and *Pde9*$^-$ hybrids also court both female types with equal frequency and

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649 effort. There are, however, quantitative differences in the courtship frequencies of each hybrid,
650 suggesting that *D. melanogaster* genetic background also influences male behavior (each has
651 the same *D. simulans* background), making comparisons between strains imperfect.
652 Nonetheless, in courting indiscriminately, all three of these strains display a different
653 overall pattern of courtship than intact melX males, which court *D. melanogaster* females at
654 significantly higher frequencies than *D. simulans* females (melX males also display non-
655 significantly higher efforts with *D. melanogaster* females). This may simply be because melX
656 males were created using a different *D. melanogaster* background (LH_M), and other *D.*
657 *melanogaster* backgrounds may not discriminate as strongly (consistent with the *Smr* balancer
658 hybrid data and RNAi/balancer data discussed below). In the case of *Ten-a*$^-$ hybrids, the *D.*
659 *melanogaster* background is Canton-S, which also courts *D. melanogaster* more frequently than
660 *D. simulans* females, and does not differ from LH_M in this respect (Figure 2A). Thus, it may also
661 be because each of these genes plays a small roll in reducing male preference for *D.*
662 *melanogaster* females, and additively produce a much larger effect, like that seen with BSC100
663 hybrids.
664 The results of our RNAi knockdown screen did identify one gene that, when knocked
665 down, significantly changed male behavior: *cac*. Although there was a general effect of RNAi
666 knockdown on courtship effort overall, only *cac* RNAi males showed reduced courtship
667 frequency towards *D. simulans* females, while *Ir11a* RNAi males and both *cac* and *Ir11a*
668 balancer males displayed high courtship frequencies to both species. Further, we've shown that
669 this effect is driven by the lack of 7,11-HD on the *D. simulans* female cuticle, because when we
670 add 7,11-HD synthetically, *cac* RNAi male courtship frequency and effort with *D. simulans*
671 females return to high levels. This result is, at first, not entirely intuitive; the loss of expression
672 of a *D. melanogaster* allele makes males behave comparatively *more* like *D. melanogaster*. We
673 take this result to imply that *cac*, a calcium channel subunit expressed in neurons, is important
674 to general signal transduction in the male CNS. Thus, the loss of *cac* expression results in
675 difficulty activating P1 courtship neurons in the absence of multiple attractive stimuli, like 7,11-
676 HD, that activate different pathways converging on P1 courtship neurons (Clowney et al., 2015).
677 If anything, this result suggests that *cac* is neither necessary nor sufficient for 7,11-HD

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678 response.

679 Ultimately, these results are difficult to interpret, because it is unclear what phenotypes
680 to expect from a hemizygous male harboring a gene disruption. The only case where this test
681 should yield a clear result is where the difference in behavior is attributable to a loss of function
682 or expression in *D. simulans*. However, differences in phenotype can also be due to coding
683 differences among genes, or differences in the amount, location, or timing of gene expression.
684 In these cases, it is unclear what phenotypic change to expect from males completely lacking a
685 gene altogether. While comparing gene aberrations from a *D. simulans* male may provide the
686 reciprocal test (if phenotypic change is due to a loss in *D. melanogaster*), this test is still subject
687 to all of the same problems discussed above. Additionally, it would require significant time and
688 effort to create these aberrations, as none are currently available in *D. simulans* strains. This
689 difficulty is specific to mapping male phenotypes on the X chromosome, as quantitative
690 complementation (Turner, 2014) and reciprocal hemizygosity tests are not possible (Stern,
691 2014).

692 It is important to note that we only tested five candidate genes. We selected these
693 genes because they met specific criteria (see methods) that we believed made them likely
694 candidates, and because screening 159 knockouts is too large an undertaking (we observed
695 nearly 500 pairs of courting flies to test our 5 candidates). It is quite possible, then, that the
696 gene(s) responsible are among those that did not meet our strict criteria. For instance, perhaps
697 the gene(s) are expressed in the developing larvae, rather than the adult CNS. Alternatively,
698 perhaps the gene(s) are not specific to the nervous system, and instead have a more general
699 function. Finally, the gene(s) may not directly interact with *Fru*, and instead act downstream or
700 independently of *Fru*.

701

702 ***Conclusions***

703 Our results demonstrate that male courtship preference differences between *D. melanogaster*
704 and *D. simulans* is at least partially explained by 1.35 Mb region of the X chromosome. We
705 further show that this region responds to the presence of the *D. melanogaster* cuticular
706 hydrocarbon pheromone, 7,11-HD. Unfortunately, attempts to fine-map this region were

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707 unsuccessful using the DP(1;Y) hybrid method and a candidate gene approach. Because hybrid
708 offspring of these species are sterile, we cannot pursue other avenues to map the causal loci,
709 such as QTL mapping. Similarly, because males are hemizygous for the X chromosome, we
710 cannot use large engineered chromosomal deletions (as in Laturney & Moehring, 2012; Pardy
711 et al., 2018).

712 Still, our findings contribute to our understanding of the 7,11-HD preference phenotype.
713 Although the neuronal circuitry required for 7,11-HD response in *D. melanogaster* has been
714 known for some time (Clowney et al., 2015), it was just recently found that the same circuitry
715 detects and responds to 7,11-HD in *D. simulans* (Seeholzer et al., 2018). While the anatomy of
716 this circuit has remained constant during the divergence of these species, the valence of male
717 response has undoubtedly changed – in large part due to changes in the interactions between
718 these neurons, rather than their physical connections. It is still unclear what genetic changes
719 are required to modify the interactions of these neurons, but our results provide a narrowed
720 region of the genome with which to identify and continue to test candidates. Our results also
721 highlight the difficulty of dissecting such a complex phenotype using a purely mapping
722 approach. It is our hope that these data, when paired with functional dissection of the nervous
723 system, can contribute to the identification of alleles explaining behavioral evolution. This is a
724 necessary goal if we wish to understand the common patterns of genetic change underlying
725 behavioral divergence.

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Table 1. DP(1;Y) *D. melanogaster* stocks. 1A. The 16 Y-linked X duplication strains used to create duplication hybrids and the reported breakpoints of their Y-linked X duplication segment (if multiple segments, base pairs are indicated for each). Ranges are shown for breakpoints where precise estimation is not available. **1B.** The 6 Y-linked X duplication strains used to create duplication hybrids overlapping the region covered by BSC100 and the reported breakpoints of their Y-linked X duplication segment. Note, all translocations contain a basal segment of the X chromosome (X:1;X:493529), and many also contain a region from the end of the X chromosome (ex: X:21572099-22456281). Coordinates of translocated segments are from *D. melanogaster* genome release 6 (Dos Santos et al., 2015).

Table 1A DP(1;Y) strains and breakpoints

Duplication	DP(X:Start;X:Finish)
BSC297	X:1;X:1947870-2009846
BSC74	X:1922620-2009846;X:3689139 & X:1;X:493529
BSC158	X:3842645-3949866;X:4931440-4931826 & X:1;X:493529
BSC277	X:4919037-5430041;X:6695007 & X:1;X:523278
BSC33	X:8129732-8192725;X:9030055 & X:1;X:493529
BSC170	X:8589125-8688160;X:9686653 & X:1;X:493529
BSC58	X:9355691-9500067;X:10744934 & X:1;X:493529
BSC264	X:11136887-11168928;X:11453958 & X:21572099-22456281;h28-h29 & X:1;X:493529
BSC100	X:11453063-11557017;X:12903175 & X:21572099-22456281;h28-h29 & X:1;X:493529
BSC267	X:13762300-13830297;X:15498953 & X:1;X:493529
BSC266	X:15484325-15569994;X:16091666 & X:1;X:493529
BSC228	X:15982454-16005803;X:16655817 & X:1;X:493529
BSC200	X:16533041-16610393;X:17682814 & X:1;X:493529
BSC68	X:17698398-17842622;X:18506941 & X:1;X:493529
BSC129	X:18506719-18632082;X:19887155 & X:1;X:493529
BSC276	X:20393272-20429518;h28-h29 & X:1;X:361245-493529

Table 1B Overlap BSC100

Duplication	DP(X:Start;X:Finish)
BSC47	X:11325824-11349993;X:12007087 & X:21318903-21382540;h28-h29 & X:1;X:493529
BSC49	X:11409964-11453063;X:12007087 & X:1;X:493529
BSC54	X:11580188-11706436;X:12007087 & X:21572099-22456281;h28-h29 & X:1;X:493529
BSC101	X:11557017-11580188;X:12903175 & X:1;X:493529
BBSC315	X:11839318-11881910;X:13284291 & X:1;X:493529
BSC317	X:12006937-12107815;X:13284291 & X:1;X:493529

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Table 2. A. A list of the available Bloomington *Drosophila* Stock Center gene aberration strains used to test candidate genes. **B.** A list of UAS-hairpin RNAi and pan-neuronal Gal4 drivers used to knockdown expression of genes when aberrations were available, or when aberrations were male lethal.

Table 2A. Gene aberrations for candidate genes

BDSC #	Gene target	genotype
57114	*papillote* (*pot*)	y[1] w[*] pot[D] P{ry[+t7.2]=neoFRT}19A/FM7c, P{w[+mC]=GAL4-Kr.C}DC1, P{w[+mC]=UAS-GFP.S65T}DC5, sn[+]
13116	*Smrtr* (*Smr*)	w[1118] P{w[+mGT]=GT1}Smr[BG01648]/FM7a
42195	*Phosphodiesterase 9* (*Pde9*)	y[1] w[*]; Mi{y[+mDint2]=MIC}Pde9[MI06972]
35826	*Tenascin-a* (*Ten-a*)	Ten-a[cbd-KS171] introgressed into a CantonS background

Table 2B. Strains used for RNAi knockdown

BDSC #	Gene target	genotype
27244	*cacophony* (*cac*)	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02572}attP2
61898	*Ionotropic receptor 11a* (*Ir11a*)	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMJ23453}attP40
8765	Elav-Gal4	P{w[+mC]=GAL4-elav.L}2/CyO

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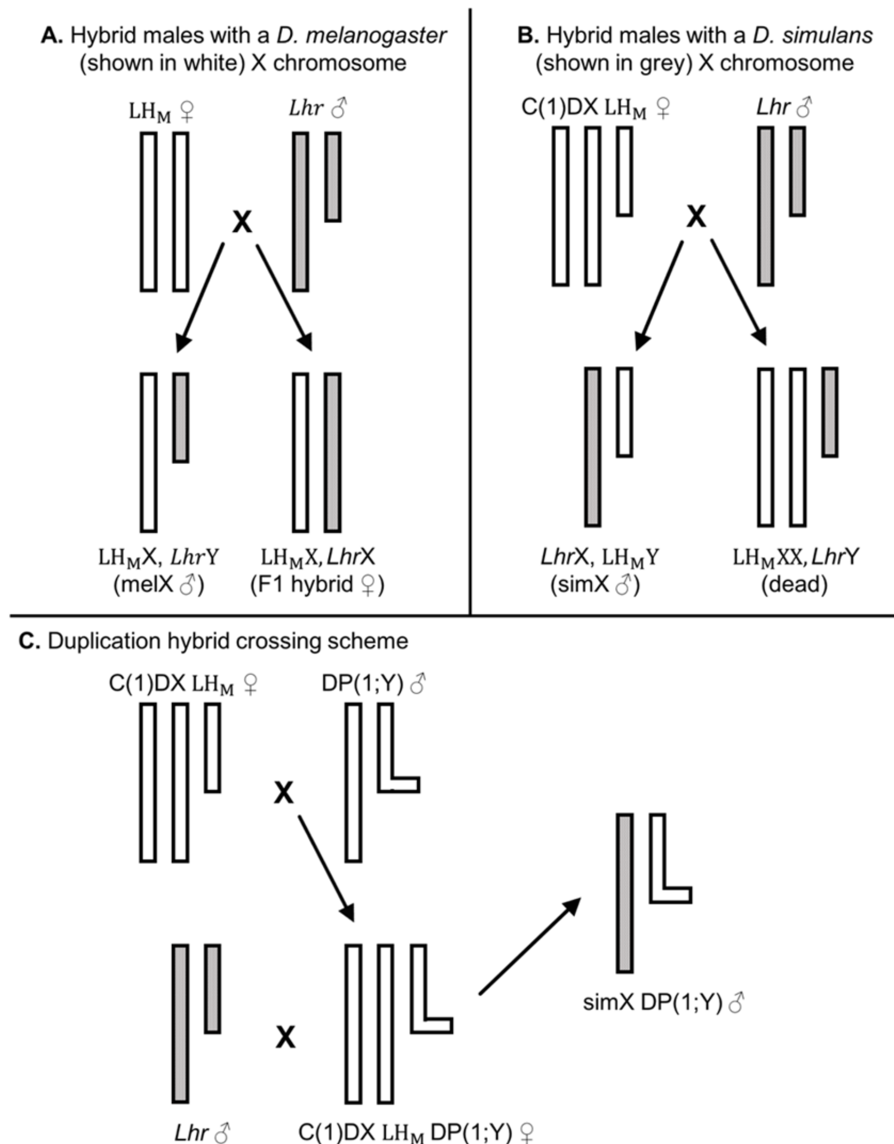

Figure 1. Hybrid crossing schemes. Each diagram shows only the sex chromosomes. Y chromosomes are depicted as shorter, while Y chromosomes with X duplications are depicted as an "L". *D. melanogaster* (LH_M) chromosomes are shown in white, and *D. simulans* (*Lhr*) chromosomes are shown in grey. **A.** To create melX hybrids, we crossed LH_M females to *Lhr* males, resulting in hybrid males with the *D. melanogaster* X chromosome and F1 hybrid females. **B.** To create simX hybrids, we crossed C(1)DX-LH_M females to *Lhr* males, resulting in hybrid males with the *D. simulans* X chromosome. Females of this cross are inviable. **C.** To create duplication hybrids, we first crossed males from DP(1;Y) strains to C(1)DXLH_M females. We took the female offspring of this first cross and crossed them to *Lhr* males. The male offspring of this second cross inherit the *D. simulans* X chromosome in addition to a segment of the *D. melanogaster* X chromosome (depicted by the small horizontal bar) attached to the *D. melanogaster* Y chromosome.

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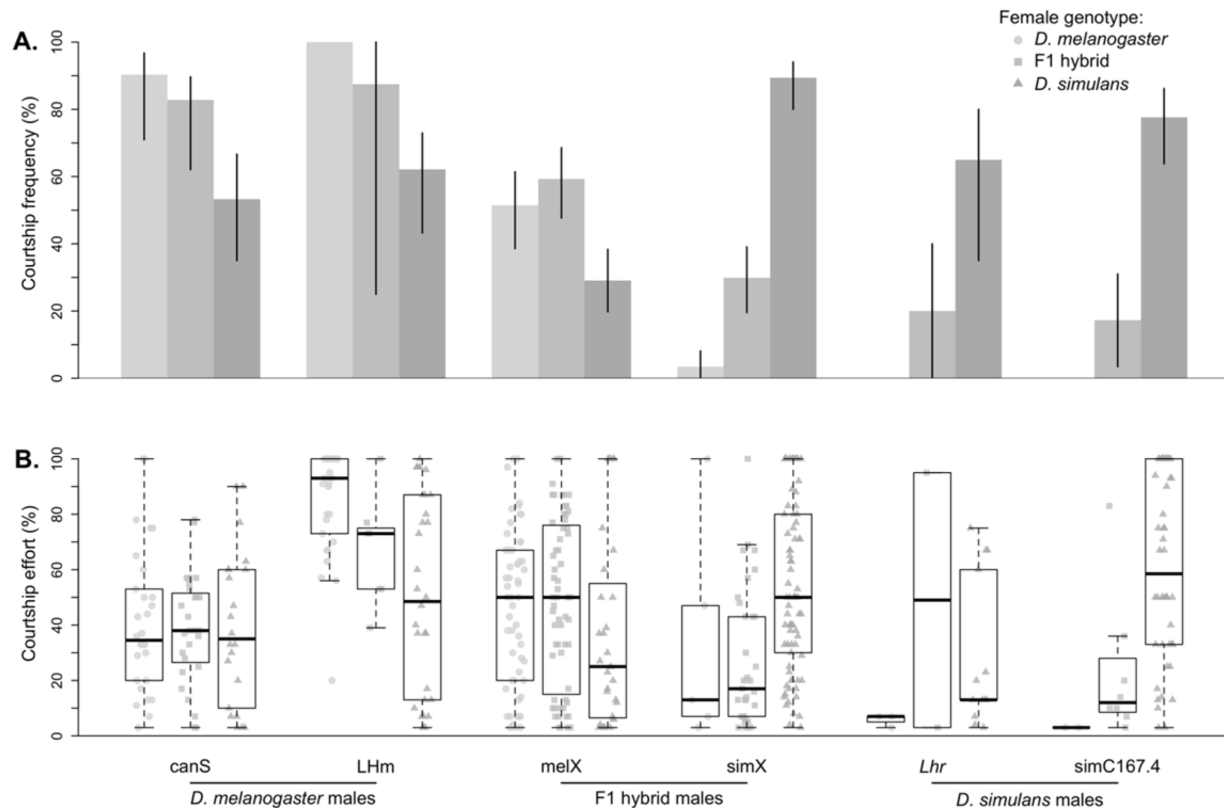

Figure 2. Courtship behaviors of *D. melanogaster* males, *D. simulans* males, and their hybrids. **A.** Courtship frequencies are shown for two strains of *D. melanogaster* (Canton-S and LH_M), two strains of *D. simulans* (*Lhr* and simC167.4), and their reciprocal hybrids (melX and simX) toward three female types: *D. melanogaster* (light grey), *D. simulans* (grey), and F1 hybrids (dark grey). Whiskers represent 95% bias corrected and accelerated bootstrapped confidence intervals. **B.** Courtship effort is shown for the same male and female combinations. Courtship effort is calculated as the percent of time that males spent courting (only males that courted were included in these calculations). Boxplots show the median (bold black line), interquartile range (box) and full extent of the data excluding outliers (whiskers).

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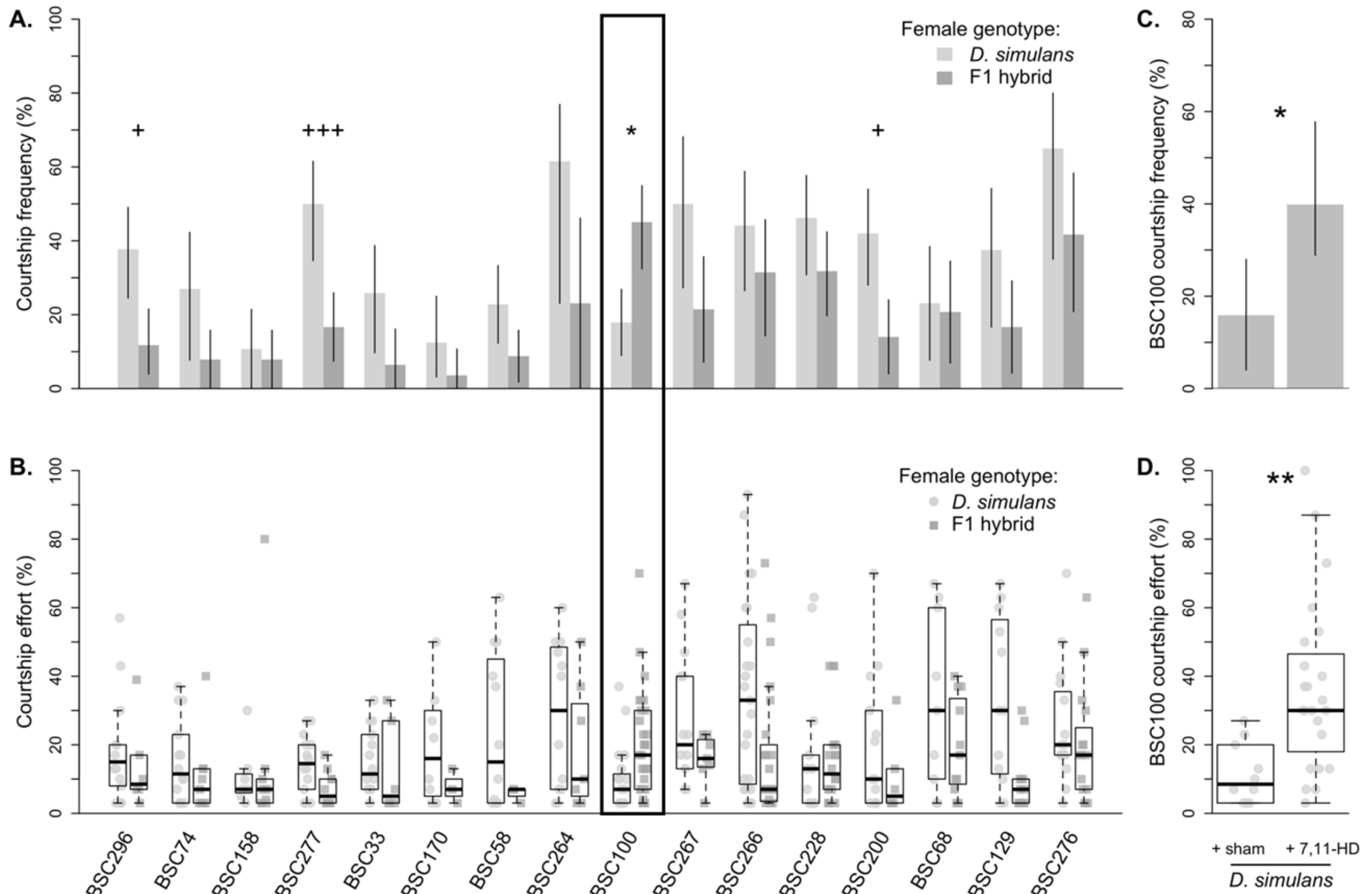

Figure 3. Courtship behaviors of duplication hybrid males. A. The courtship frequencies for each of the 16 duplication hybrids when paired with both *D. simulans* females (light grey) and F1 females (dark grey). **B.** The courtship effort for each of the 16 duplication hybrids when paired with *D. simulans* females (light grey circles, left) and F1 females (dark grey squares, right).BSC100, the only duplication to display greater courtship frequency and effort towards F1 hybrids over *D. simulans* females is enclosed within the black box. **C.** The frequency of BSC100 males that courted sham-perfumed *D. simulans* females (left), and *D. simulans* females perfumed with synthetic 7,11-HD (right). **D.** The courtship effort of BSC100 males that courted sham-perfumed *D. simulans* females (light grey circles, left), and *D. simulans* females perfumed with synthetic 7,11-HD (dark grey squares, right). For all, symbols denote degree of significance after correction for multiple comparisons (* = p< 0.05, ** = p < 0.01, and *** = p < 0.001). Asterisks mark duplication hybrid lines that court F1 hybrid females significantly more. Plus signs mark duplication hybrid lines that court *D. simulans* females more. For A. and C., whiskers represent 95% bias corrected and accelerated bootstrapped confidence intervals. For B. and D., boxplots show the median (bold black line), interquartile range (box) and full extent of the data excluding outliers (whiskers).

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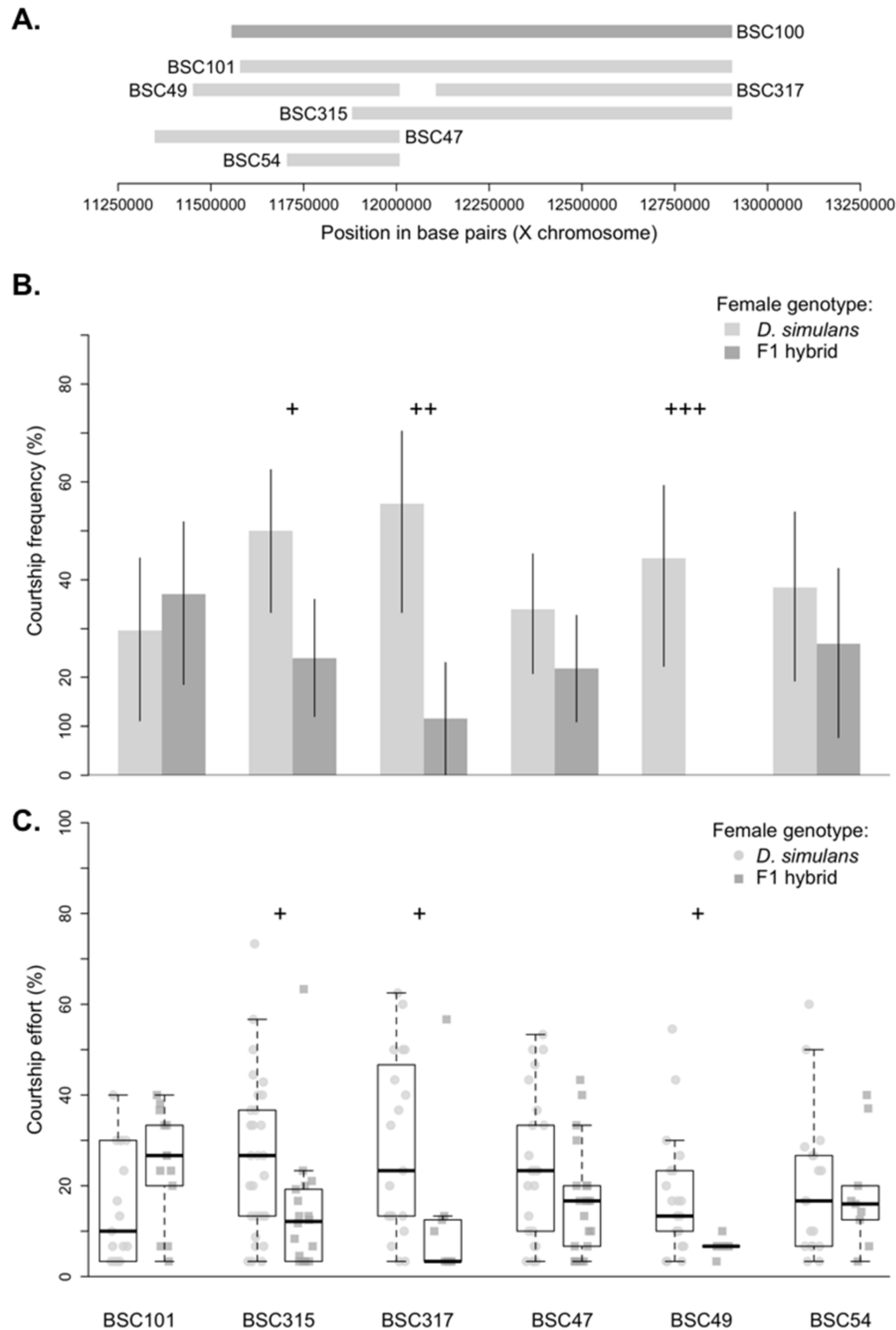

Figure 4. Courtship behaviors of BSC100 overlapping duplication hybrid males. A. The physical positions of the original duplication hybrid (BSC100), and the six partially overlapping strains we assayed to fine-map the region. **B.** The courtship frequencies for each of the 6 overlapping duplication hybrids when paired with both *D. simulans* females (light grey) and F1 females (dark grey). Whiskers represent 95% bias corrected and accelerated bootstrapped confidence intervals. **C.** The courtship effort for each of the 6 overlapping duplication hybrids when paired

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with *D. simulans* females (light grey circles, left) and F1 females (dark grey squares, right). Boxplots show the median (bold black line), interquartile range (box) and full extent of the data excluding outliers (whiskers). For all, plus signs mark duplication hybrid lines that court *D. simulans* females more, and denote degree of significance after correction for multiple comparisons (+ = p< 0.05, ++ = p < 0.01, and +++ = p < 0.001).

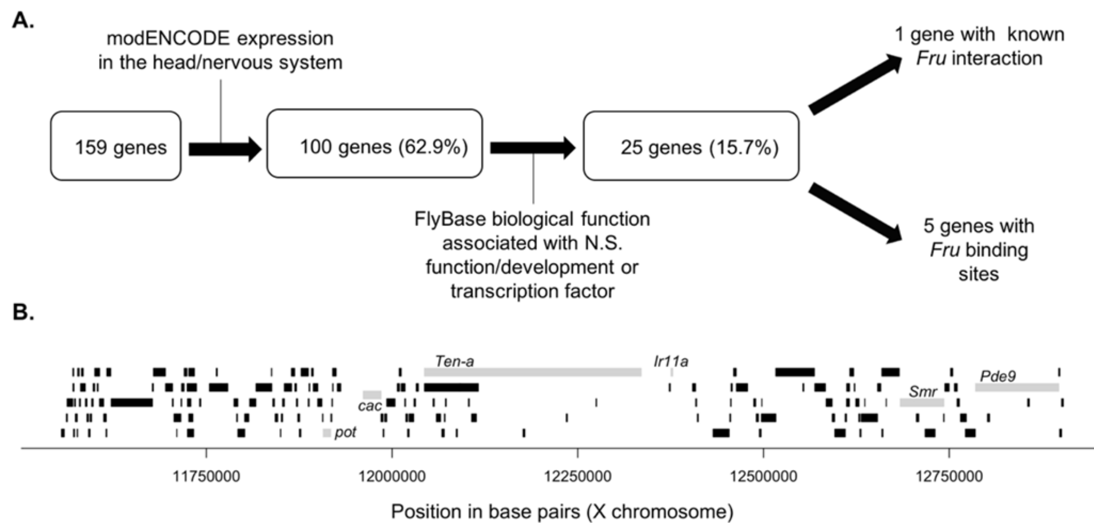

Figure 5. The 159 genes uncovered by BSC100. A. A pipeline for identifying relevant candidate genes among the 159 covered by BSC100. We expect genes to be expressed in the central nervous system and to have nervous system functions (or act as transcription factors for loci with such functions). Because *Fruitless* is important to wiring the male nervous system, we also looked for specific genes that interact with *Fru* as particularly strong candidates. **B.** A schematic of the 150 genes. The 6 genes that meet our filtering criteria are relatively evenly distributed across the region and shown in grey.

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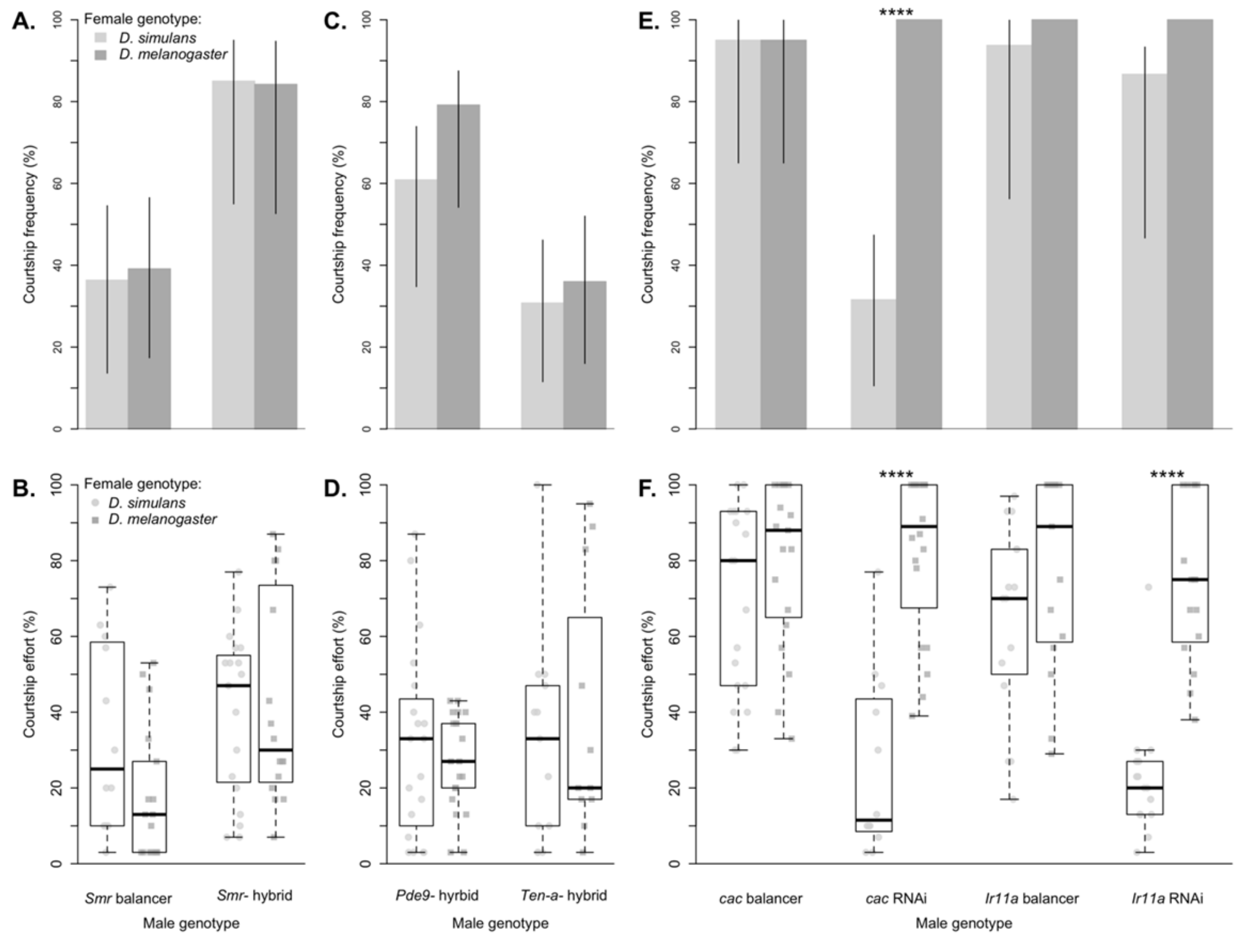

Figure 6. The courtship behaviors of RNAi knockdown and *D. melanogaster* X chromosome hybrids with individual gene knockouts. A. The courtship frequency of *Smr* knockout hybrid males (*Smr* hybrid) is compared to *Smr* X chromosome balancer males (*Smr* balancer). **B.** The courtship effort of *Smr* knockout hybrid males (*Smr* hybrid) is compared to *Smr* X chromosome balancer males (*Smr* balancer). **C.** The courtship frequency for *Pde9* and *Ten-a* knockout hybrid males. **D.** The courtship effort for *Pde9* and *Ten-a* knockout hybrid males. **E.** The courtship frequency of RNAi knockdown and balancer genotypes for two genes, *cac* and *Ir11a* for males paired with *D. simulans* females (light grey bars) and *D. melanogaster* females (dark grey bars). **F.** The courtship effort of RNAi knockdown and balancer genotypes for two genes, *cac* and *Ir11a* for males paired with *D. simulans* females (light grey points) and *D. melanogaster* females (dark grey points). For A., C., and E., whiskers represent 95% bias corrected and accelerated bootstrapped confidence intervals. For B., D., and F., boxplots show the median (bold black line), interquartile range (box) and full extent of the data excluding outliers (whiskers). Asterisks denote degree of significance after correction for multiple comparisons (**** = $p < 0.0001$).

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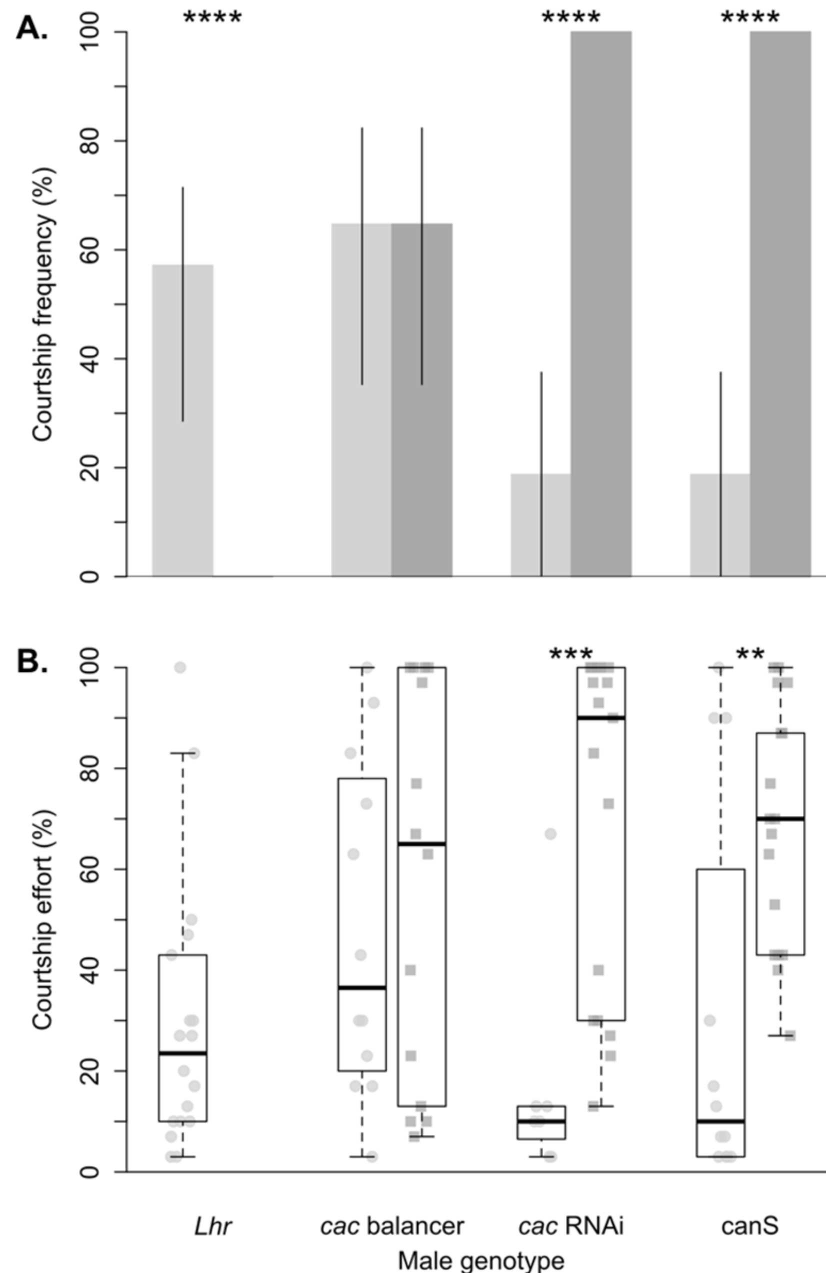

Figure 7. *cac* RNAi male behavior is driven by 7,11-HD. A. The courtship frequency of RNAi knock down and balancer genotypes for *cac, Lhr*, and Canton-S (canS) for males paired with *D. simulans* females (light grey bars) and *D. melanogaster* females (dark grey bars). Whiskers represent 95% bias corrected and accelerated bootstrapped confidence intervals. **B.** The courtship effort of RNAi knockdown and balancer genotypes for *cac, Lhr*, and canS for males paired with *D. simulans* females (light grey circles) and *D. melanogaster* females (dark grey squares). Boxplots show the median (bold black line), interquartile range (box) and full extent of the data excluding outliers (whiskers). For all, asterisks denote degree of significance after correction for multiple comparisons (** = $p < 0.01$, and *** = $p < 0.001$, **** = $p < 0.0001$).

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Supplementary information

Table S3 Crosses of *pot* aberrant *D. melanogaster* to *D. simulans* yields no offspring. We crossed *D. melanogaster pot* aberrant females to *D. simulans Lhr* males using the crossing methods described in the main text three separate times. This table shows the offspring count for female, balancer male, and pot$^-$ male offspring. No cross produced pot$^-$ offspring and only two *pot* balancer males resulted from all three crosses.

Table S3	**Offspring #**		
Cross (female x male)	**females**	***pot*$^-$ males**	***pot* bal. males**
pot x *Lhr* -1	43	0	1
pot x *Lhr* -2	49	0	1
pot x *Lhr* -3	26	0	0