The loci of behavioral evolution: *Fas2* and *tilB* underlie differences in pupation site choice behavior between *Drosophila melanogaster* and *D. simulans*

Short title: Two genes underlying a difference in pupation behavior between *Drosophila* species

Alison Pischedda[+,*,1], Michael P. Shahandeh[+,*,2], and Thomas L. Turner[2]

*Corresponding authors: apischedda@barnard.edu; mshahandeh@ucsb.edu

[1]Department of Biology, Barnard College, Columbia University, New York NY 10027

[2]Dept. of Ecology, Evolution, and Marine Biology, University of California Santa Barbara

[+]Denotes co-first authors

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Abstract

The recent boom in genotype-phenotype studies has led to a greater understanding of the genetic architecture of a variety of traits. Among these traits, however, behaviors are still lacking, perhaps because they are complex and environmentally sensitive phenotypes, making them difficult to measure reliably for association studies. Here, we aim to fill this gap in knowledge with the results of a genetic screen for a complex behavioral difference, pupation site choice, between *Drosophila melanogaster* and *D. simulans*. In this study, we demonstrate a significant contribution of the X chromosome to the difference in pupation site choice behavior between these species. Using a panel of X-chromosome deletions, we screened the majority of the X chromosome for causal loci, and identified two regions that explain a large proportion of the X-effect. We then used gene disruptions and RNAi to demonstrate the substantial effects of a single gene within each region: *Fas2* and *tilB*. Finally, we show that differences in *tilB* expression underlie species differences in pupation site choice behavior, and that generally, pupation site choice behavior appears to be correlated with relative expression of this gene. Our results suggest that even complex, environmentally sensitive behaviors may evolve through changes to loci with large phenotypic effects.

Author summary

Behaviors are complex traits that involve sensory detection, higher level processing, and a coordinated output by the nervous system. This level of processing is highly susceptible to environmentally induced variation. Because of their complexity and sensitivity, behaviors are difficult to study; as a result, we have very little understanding of the genes involved in behavioral variation. In this study, we use common laboratory fruit fly model, *Drosophila*, to address this gap and dissect the genetic underpinnings of an environmentally sensitive behavior that differs between species. We find that a significant amount of the phenotypic difference between species is explained by a single chromosome. We further show that just two genes on this chromosome account for a large majority of its effect, suggesting that the genetic basis of complex behavioral evolution may be simpler than anticipated. For one of these genes, we show that a species-level difference in gene expression is associated with the difference in behavior. Our results contribute to a growing number of studies identifying the genetic components of behavior. Ultimately, we hope to use these data to better predict the number, types, and effects of genetic mutations necessary for complex behaviors to evolve.

51

52 # Introduction

53 The behaviors of closely related species can be remarkably different, and these differences can
54 have important biological consequences. Behavioral evolution in insects has major impacts on
55 crop decimation and disease vectoring (1,2). For example, one subspecies of the yellow fever
56 mosquito, *Aedes aegypti*, prefers to bite humans, while another closely related species prefers
57 other animals (3,4). Understanding insect preferences therefore presents a major inroad to
58 effective disease and pest management (5). Behaviors are also critical to the creation and
59 maintenance of biodiversity, as host, habitat, and mating preference behaviors are often key
60 players in speciation and local adaptation (6,7).

61 Despite the importance of behavioral traits, we know little about the genetic basis of their
62 evolution. GePhe, the most extensive compilation of natural genetic variants associated with trait
63 differences, currently catalogs ~1700 associations, of which only 23 are for behavior (8). From
64 these 23, and others in the literature, it is clear that individual genes can sometimes have large
65 effects on evolved differences in behavior (9–11). It also clear that changes to sensory receptors
66 in the peripheral nervous system can explain dramatic shifts in behavior (3,11,12). With so few
67 studies, however, it is difficult to conclude how frequently we expect single loci to have large
68 effects or how often sensory receptors explain species differences, due to pervasive
69 ascertainment bias (13). Indeed, a recent study discovered an exception to this emerging pattern:
70 the evolution of a central neural circuit, rather than a peripheral sensory neuron, explains
71 differences in mating behavior in two *Drosophila* species (14).

72 The current lack of genetic studies mapping behavioral variation presumably arises from
73 the fact that behaviors are difficult to measure reliably and repeatedly. Behavioral phenotypes

74 often integrate multiple cues, are sometimes context dependent, and can be innate or learned,
75 making it difficult to exclude environmentally induced variation. To better understand the
76 genetic basis of behavioral evolution, we therefore need more case studies, with a focused effort
77 on “metamodel” systems with documented behavioral differences between closely related
78 species (15).

79 The *Drosophila* group is well poised to address these challenges. In *Drosophila*,
80 hundreds of genetically identical individuals from variable wild-caught strains can be reared in a
81 common environment, isolated at the beginning of their adult life stage, and repeatedly assayed
82 for a trait of interest. Such a design significantly reduces the potential for environmentally
83 induced variation to obscure genetic differences in behavior. Additionally, there are many
84 *Drosophila* species with large, characterized differences in a variety of complex behaviors (16).
85 Undeniably, work comparing the repeated evolution of morphological traits across closely
86 related species of *Drosophila* has significantly advanced our understanding of the general
87 patterns linking genotype and phenotype for developmental traits (17–19). Studies that
88 investigate genetic complexity in *Drosophila*, where fine-mapping and functional follow-up are
89 possible, should make similar progress for behaviors.

90 In the present study, we seek to address the lack of behavioral association studies using
91 the *Drosophila* model system. Here, we investigate the difference in pupation site preference
92 between two *Drosophila* species: *D. melanogaster* and *D. simulans*. When the larval stages of
93 these species are ready to metamorphose into adults, they first enter a pupal stage. The pupa,
94 which lasts for a number of days, is immobile and therefore vulnerable to parasitism, predation,
95 desiccation, and disease (20). Before pupating, larvae enter a "wandering" stage, where they
96 search for an appropriate pupation site (21,22). Depending on the strain and species, larvae vary

97 from pupating directly on their larval food source to traveling more than 40 cm away from it (23).
98 This behavior is exquisitely sensitive to environmental conditions— individuals alter their
99 behavior in response to light, moisture, pH, the presence of other species, parasitism, and more
100 (24–27). Despite this environmentally induced variation, the effects of genotype on preference
101 are considerable. Within species, strains and populations often differ in how far they travel from
102 their food source before pupating, although the most consistent differences are seen between
103 species (28,29). Interestingly, differences in pupation site choice behavior between species do
104 not correspond to their taxonomic classification (30). For example, *D. melanogaster* and *D.*
105 *simulans* shared a common ancestor 2-3 million years ago (31), and are extremely similar in
106 terms of their ecology, morphology, and physiology (32). They differ markedly in terms of
107 pupation site choice, however, with *D. simulans* pupating closer to the larval food source, on
108 average (20,29). This is not due to laboratory adaptation, as freshly collected individuals show
109 the same pattern (20,29). These species are frequently collected in the same microhabitats, and
110 their differences in pupation site choice behavior have been postulated to be a form of niche
111 partitioning (20,29). Supporting this hypothesis, pupation site choice responds to density
112 dependent selection in the laboratory (33), and provides a potential increase in competitive
113 ability between species ovipositing in the same media (34).
114 Here, we investigate the genetic basis of this difference in pupation site choice between *D.*
115 *melanogaster* and *D. simulans*. Despite substantial reproductive isolation, *D. melanogaster*
116 females can be persuaded to mate with *D. simulans* males in the lab, and vigorous female F1
117 hybrids result from the cross. Males are usually inviable, but we use a *D. simulans* hybrid male
118 rescue strain (35,36) to circumvent this challenge, and show that a significant proportion of the
119 species difference in pupation behavior can be mapped to the X chromosome, consistent with

120 findings using other *Drosophila* species (37). Still, these hybrids remain sterile, so genetic
121 dissection using a backcross mapping population is not possible. Instead, we use a widely
122 available set of transgenic *D. melanogaster* deficiency lines to screen a substantial portion of the
123 X chromosome (38,39). We use these lines to create hybrid females that lack large, overlapping
124 portions of the X chromosome from *D. melanogaster*, and therefore express only *D. simulans*
125 alleles in those regions. We identify two loci with large effects on pupation behavior. We then
126 employ genetic knockouts of candidate loci within these regions to demonstrate their effects, and
127 use RNAi knockdown to confirm the role of two genes, *touch insensitive larvae B* (*tilB*) and
128 *Fasciclin 2* (*Fas2*). Finally, we use real time RT-PCR to test for species-level differences in gene
129 expression of *tilB* in larvae, and show that *tilB* is more highly expressed in *D. melanogaster* than
130 in *D. simulans*. Our method underscores the potential for the engineered deficiency resource,
131 which has been widely used to map intrinsic hybrid incompatibilities between species of
132 *Drosophila* (40–44), as a powerful tool with which to overcome traditional challenges to
133 interspecific mapping. Indeed, this method was recently used to identify loci affecting
134 pheromone differences between these *Drosophila* species (45), and may prove equally useful for
135 behavioral differences (46).
136

137 # Results:

138 **1. Differences in pupation behavior between *D. melanogaster* and *D. simulans***

139 We measured pupation behavior for 11 *D. melanogaster* and 12 *D. simulans* strains collected
140 from various locations throughout the world (Table S1), and found significant variation among
141 our *D. melanogaster* lines and among our *D. simulans* lines in the proportion of individuals that
142 pupated on the food surface (Fig 1; *D. melanogaster* Wilcoxon test: χ^2= 42.69, df = 10, p<0.0001;

143 *D. simulans* Wilcoxon test: χ^2= 56.34, df = 11, p<0.0001). When we tested for a species
144 difference in pupation behavior, we found that our *D. simulans* lines had significantly more
145 pupae on the food surface compared to our *D. melanogaster* lines, on average (Fig 1; Wilcoxon
146 test: χ^2= 8.37, df = 1, p = 0.0038).

147 Although we controlled egg density to characterize species differences in pupation
148 behavior (see below), there were viability differences among our surveyed lines. As a result, we
149 had significant variation in the total number of pupae in each vial for our *D. melanogaster* lines
150 (ANOVA: $F_{10,65}$=5.58, p<0.0001) and our *D. simulans* lines (ANOVA: $F_{11,66}$=8.01, p<0.0001).
151 Previous studies have found that larval density correlates with pupation height in *D.*
152 *melanogaster* (47). To control for differences in density, we also performed the same analyses
153 above using the residuals from a regression between number of pupae and proportion of pupae
154 on the food. None of our findings changed using this analysis (Fig S1), indicating that our results
155 were not affected by variation in larval density.

156 Although most of our *D. melanogaster* lines have been in the lab since the 1950s/1960s,
157 we found no difference in the proportion of pupae on the food between *D. simulans* strains
158 collected in the 1950s/1960s and those collected in the 2000s (Table S1; Wilcoxon test: χ^2= 0.24,
159 df=1, p=0.62), indicating that the species difference we report here is unlikely to be an artifact of
160 laboratory adaptation in our surveyed strains.

161

162 **2. Differences in pupation behavior have a significant X effect**

163 To begin mapping these differences in pupation site choice behavior, we created F1 hybrid
164 females (Fig 2A) and F1 hybrid males that had either a *D. melanogaster* X chromosome ("melX"
165 males; Fig 2A), or a *D. simulans* X chromosome ("simX" males; Fig 2B). Both of these males

166 inherit their cytoplasm from the *D. melanogaster* parent strain, so any differences we observe are
167 directly attributable to the sex chromosomes. We found significant differences among F1 hybrid
168 genotypes when we screened hybrids alongside their parental strains (Fig 3; Full model
169 Wilcoxon test: χ^2= 144.57, df= 6, p<0.0001). Specifically, a significantly higher proportion of F1
170 hybrid males pupated on the food when they had inherited a *D. simulans* X chromosome (simX
171 males) compared to a *D. melanogaster* X chromosome (melX males), indicating that this species
172 divergence in pupation behavior has a significant X effect (p<0.0001 after correcting for multiple
173 comparisons; Fig 3). This is further supported by the fact that the proportion of pupae on the
174 food was not significantly different between melX hybrid males and *D. melanogaster* males, or
175 between simX hybrid males and *D. simulans* males. All of these findings are unchanged when
176 we control for density effects (Fig S2).
177 Additionally, we found no difference in the proportion of pupae on the food for F1 hybrid
178 females and melX hybrid males (p=0.70), while F1 hybrid females had significantly fewer pupae
179 on the food compared to simX hybrid males (p<0.0001 after correcting for multiple comparisons;
180 Fig 3). The fact that F1 hybrid females behave identically to melX hybrid males indicates that
181 the variation in pupation behavior on the *D. melanogaster* X chromosome (i.e. fewer pupae on
182 the food) is dominant to the pupation behavior on the *D. simulans* X chromosome (i.e. more
183 pupae on the food), because F1 hybrid females have one X chromosome from each species.
184
185 **3. A deficiency screen of the X chromosome identifies two regions of interest**
186 Because we found a significant effect of the X chromosome on the difference in pupation site
187 choice behavior between *D. simulans* and *D. melanogaster*, we devised a crossing scheme using
188 molecularly engineered chromosomal deficiencies (38) to screen the X chromosome for loci

189 contributing to this difference (Fig 2C). We assayed a total of 90 deficiency strains, covering
190 87% of the X chromosome (Table S2). These crosses produced two types of hybrid female, both
191 heterozygous at all autosomes (Fig 2C). The deficiency hybrid females contained the *D.*
192 *melanogaster* deficiency X chromosome and a *D. simulans* X chromosome, making them
193 hemizygous for a segment of the X chromosome. The balancer hybrid females contained the *D.*
194 *melanogaster* balancer X chromosome and a *D. simulans* X chromosome, so they are
195 heterozygous for *D. melanogaster*/*D. simulans* over the entirety of the X chromosome. The
196 balancer hybrids provide an excellent experimental control, as they developed in the same
197 environment as our experimental deficiency flies. As a result, we calculated the proportion of
198 deficiency hybrid females that pupated on the food and the proportion of balancer hybrid females
199 that pupated on the food, and used these measures to calculate a "pupation index" (the proportion
200 of deficiency hybrids pupating on the food divided by the proportion of balancer females
201 pupating on the food).
202 We found significant variation in pupation index among the deficiency hybrid crosses
203 (Kruskal-Wallis Test: χ^2=336.90, df=89, p<0.0001; Fig 4A). A pupation index greater than 1
204 indicates that more deficiency hybrids pupated on the food than balancer hybrids. This suggests
205 that the *D. melanogaster* deficiency region may be revealing recessive *D. simulans* genetic
206 variation that causes the deficiency hybrids to pupate on the food surface. However, the average
207 pupation index across all 90 deficiency hybrid crosses was 0.88 (Fig 4A), which was
208 significantly lower that our expected mean of 1 (Wilcoxon test: p<0.0001). As a result, we
209 compared the pupation index for all deficiencies to our expected value of 1 and to the grand
210 mean pupation index for these lines (0.88). Six deficiencies had pupation indices significantly
211 greater than one: (Df(1)ED411, Df(1)BSC869, Df(1)ED6720, Df(1)ED6906, Df(1)BSC530, and

212 Df(1)Exel6255). Three of these, (Df(1)BSC869, Df(1)ED6906, and Df(1)Exel6255), remained
213 significant after sequential Bonferroni correction for multiple comparisons (Fig 4A; Table S2).
214 Because the other 3 deficiencies, (Df(1)ED411, Df(1)ED6720, and Df(1)BSC530), had pupation
215 indices significantly greater than 0.88 after sequential Bonferroni correction (Table S2), we
216 included them in our list of potential deficiencies of interest.

217 To ensure that the deficient region actually reveals *D. simulans* variation contributing to
218 pupation site choice behavior, rather than creating lines that behave abnormally due to the
219 extended hemizygosity within the deficiency region, we crossed each of the six significant
220 deficiencies listed above to a wild-type *D. melanogaster* strain, T.4. For two of the six deficiency
221 strains, Df(1)ED411 and Df(1)BSC530, we found no difference in the pupation index when
222 crossed to *D. melanogaster* compared to the pupation index when crossed to the *Lhr* strain
223 (Wilcoxon tests; Df(1)ED411: n= 29-36, χ^2=1.74, p=0.19, Fig S3A; Df(1)BSC530: n=18-22,
224 χ^2=1.47, p=0.23, Fig S3C), and Df(1)ED6906 had a significantly higher pupation index when
225 crossed to *D. melanogaster* (n= 26-33, χ^2=9.42, p=0.002; Fig S3B). These results suggest that
226 the phenotypes of these three lines are a result of their hemizygosity within the deficiency region,
227 as the deficient *D. melanogaster* females still pupate on the food more often than the balancer
228 females. When we crossed the remaining three deficiency strains, Df(1)BSC869, Df(1)ED6720,
229 and Df(1)Exel6255, to *D. melanogaster*, we found a pupation index significantly lower than the
230 index we calculated when crossing to *Lhr* (Wilcoxon tests; Df(1)BSC869: n= 51, χ^2=4.35,
231 p=0.037; Df(1)BSC6720: n= 30-35, χ^2=7.90, p=0.0049; Df(1)Exel6255: n= 55-57, χ^2=4.68,
232 p=0.0306; Fig 4B), and no different than one (Df(1)BSC869: p=0.46; Df(1)BSC6720: p=0.10;
233 Df(1)Exel6255: p=0.50). Two of these three deficiencies overlap: Df(1)BSC869 and
234 Df(1)ED6720 (Fig 4A). To further confirm that this pattern is not unique to these specific *D.*

235 *melanogaster* and *D. simulans* strains, we crossed one of the overlapping deficiency strains,
236 Df(1)BSC869, and Df(1)Exel6255 to another *D. melanogaster* wild-type strain (T.1) and another
237 *D. simulans* wild-type strain (Mex180). The pattern remained consistent for both of these
238 deficiencies: when crossed to *D. melanogaster,* the pupation index was significantly lower than
239 when crossed to *D. simulans* (Fig 4B; Wilcoxon tests: Df(1)BSC869: n=50-51, χ^2=7.78,
240 p=0.0053; Df(1)Exel6255: n= 23, χ^2=7.74, p=0.0054).
241 One deficiency strain, Df(1)ED7265, had a pupation index significantly lower than the
242 grand mean of all the lines (0.88) following Bonferroni correction for multiple comparisons. This
243 suggests the potential for a region of the *D. simulans* X chromosome with transgressive effects—
244 that is, a locus that causes *D. simulans* larvae to pupate farther from the food surface. While this
245 is certainly interesting, we did not pursue this region because its effect is contrary to the species-
246 wide difference we found. This locus may be interesting for further investigation, however, as it
247 could contribute to the significant variation in pupation behavior we observed among *D.*
248 *simulans* strains (Fig 1).
249
250 **4. Gene knockouts and RNAi knockdown suggest that *Fas2* is involved in divergent**
251 **pupation behavior**
252 Our deficiency screen identified two regions of interest. The first is the overlap of Df(1)BSC869
253 and DF(1)ED6720, excluding the region covered by DF(1)ED6727, which did not have a
254 pupation index greater than 1 (Fig 4A, Table S2). The resulting region of interest spans
255 X:4,204,351 - 4,325,174 (Fig 4C). Within this region there are 23 genes, of which 20 are protein
256 coding (Table S3A). According to modENCODE expression data, 15 of those 20 protein coding
257 genes are expressed in larvae, while only 6 are also expressed in the larval nervous system,

258 which we would expect for genes regulating behavior (48). Five of these six genes are well
259 described. At the time of assay, only two of the five characterized genes within this region had
260 non-lethal verified loss-of-function alleles available: *Fas2* (Fasciclin2) and *mei9* (meiotic 9). We
261 tested knockouts of each for an effect on pupation site choice behavior. We found no significant
262 difference in the pupation index obtained when we crossed the *mei-9*^{A1} mutant allele to the *Lhr D.*
263 *simulans* strain and the T.4 *D. melanogaster* strain (Wilcoxon test: n= 51-52, χ^2=0.37, p=0.54;
264 Fig S4B), indicating that *mei9* is unlikely to be involved in pupation site choice. In contrast,
265 when we crossed the mutant allele *Fas2*^{eb112} to *D. simulans* (*Lhr*) and *D. melanogaster* (T.4), we
266 found that *Fas2*^{eb112} hybrids had a significantly higher pupation index than the *D. melanogaster*
267 knockouts (Wilcoxon test: n= 50-51, χ^2= 6.97, p= 0.0083; Fig 5A), suggesting that *Fas2* may be
268 involved in pupation site choice. To ensure this pattern is not unique to these strains, we crossed
269 *Fas2*^{eb112} to additional *D. simulans* (Mex180) and *D. melanogaster* (T.1) wild-type strains. We
270 again found the same pattern: the pupation index for knockout hybrids was significantly higher
271 than for *D. melanogaster* knockouts (Wilcoxon test: n= 52-53, χ^2= 27.2, p<0.0001; Fig 5A). As
272 further verification, we tested a second *Fas2* strain: a p-element insertion allele, *Fas2*^{G0293}, and
273 similarly found that the pupation index for *Fas2*^{G0293} hybrids (crossed to *Lhr*) was significantly
274 higher than that for *D. melanogaster* knockouts (crossed to T.4; Wilcoxon test: n= 52-53, χ^2=
275 9.73, p=0.0018; Fig 5B). When we used the combined consensus p-value test (49) to look at the
276 overall pattern for *Fas2*^{eb112}, and *Fas2* as a whole (i.e. including results from both *Fas2*^{eb112} and
277 *Fas2*^{G0293}), we found a strongly significant pattern of higher pupation indices for hybrid crosses
278 compared to *D. melanogaster* crosses (*Fas2*^{eb112}: p= 3.59 x 10^{-6}; *Fas2*: p= 2.35 x 10^{-8}).
279 Next, we used RNAi with the elav-Gal4 driver to reduce expression of *Fas2* throughout
280 the nervous system in *D. melanogaster*. We compared the proportion of experimental flies that

281 pupated on the food for the RNAi cross (UAS-*Fas2* x elav-Gal4) to that of two controls (both
282 crossed to elav-Gal4): the background stock in which the RNAi lines were created (*y v; attP2,*
283 *y+*) and the Gal4-1 stock, which has a hairpin targeting Gal4 in VALIUM20 to control for Gal4
284 effects. We found that a significantly higher proportion of RNAi flies pupated on the food
285 compared to the control flies from either the background (Wilcoxon test: n= 42-48, $p<0.0001$
286 after sequential Bonferroni correction) or Gal4-1 cross (Wilcoxon test: n= 42-43, $p<0.0001$ after
287 sequential Bonferroni correction; Fig 5C). These results are unchanged when we control for
288 density effects (Fig S5A), providing further evidence for *Fas2*'s role in pupation site choice.
289

290 **5. Gene knockouts and RNAi knockdown suggest that *tilB* is involved in divergent pupation**
291 **behavior**

292 The second region of interest identified by our deficiency screen was the region deleted by
293 DF(1)Exel6255 (X:21,519,203 – 22,517,665; Fig 4D). Within this region are 28 genes, of which
294 22 are protein coding (Table S3B). Of the 22 protein coding genes, 14 are expressed in larvae –
295 13 of which have some expression in the larval nervous system (48). Of these, 7 are described.
296 We obtained knockout strains for both of the characterized genes expressed in the larval nervous
297 system that had verified loss-of-function alleles available at the time: *tilB* (touch insensitive larva
298 B) and *wap* (wings apart). We found no significant difference in the pupation index obtained
299 when we crossed the wap^2 mutant allele to the *Lhr D. simulans* strain and the T.4 *D.*
300 *melanogaster* strain (Wilcoxon test: n= 51-55, $\chi^2=0.32$, $p=0.57$; Fig S4A), indicating that *wap* is
301 unlikely to be involved in pupation site choice. In contrast, when we crossed the $tilB^1$ and $tilB^2$
302 mutant alleles to *D. simulans* (*Lhr*) and *D. melanogaster* (T.4), we found that the *tilB* knockout
303 hybrids had significantly higher pupation indices than the *D. melanogaster* knockouts for both

304 alleles (Wilcoxon tests; *tilB*^{1}: n= 56, χ^2= 6.61, p= 0.0101; Fig 6A; *tilB*^{2}: n= 57, χ^2= 6.61, p=
305 0.0101; Fig 6B). To test whether this difference is consistent in other backgrounds, we crossed
306 both the *tilB*^{1} and *tilB*^{2} mutant alleles to additional *D. simulans* (Mex180) and *D. melanogaster*
307 (T.1) wild-type strains. We had a difficult time crossing our *tilB* strains to the Mex180 strain, so
308 our sample sizes for these crosses are smaller, but there is a nonsignificant trend towards a higher
309 pupation index for the knockout hybrids compared to the *D. melanogaster* hybrids for both
310 alleles (Wilcoxon tests; *tilB*^{1}: n=36-37, χ^2= 2.80, p= 0.0943, Fig 6A; *tilB*^{2}: n=12-18, χ^2= 3.44, p=
311 0.0638, Fig 6B). We used the combined consensus p-value test (49) to look at the overall pattern
312 for *tilB*^{1}, *tilB*^{2}, and *tilB* as a whole (i.e. including results from both *tilB*^{1} and *tilB*^{2}), and found a
313 strongly significant pattern of higher pupation indices for hybrid crosses compared to *D.*
314 *melanogaster* crosses (*tilB*^{1}: p= 0.0022; *tilB*^{2}: p= 0.0037; *tilB*: p= 2.47 x 10^{-5}).
315 As for *Fas2* above, we then used RNAi with the elav-Gal4 driver to reduce expression of
316 *tilB* throughout the nervous system in *D. melanogaster*. We found that a significantly higher
317 proportion of RNAi flies pupated on the food compared to the control flies from either the
318 background (Wilcoxon test: n= 46-47, p<0.01 after sequential Bonferroni correction) or Gal4-1
319 cross (Wilcoxon test: n= 41-46, p<0.01 after sequential Bonferroni correction; Fig 6C), These
320 findings are consistent when we control for density effects (Fig S5B), providing additional
321 support for *tilB*'s role in pupation site choice.
322
323 **6. *tilB* is expressed more highly in *D. melanogaster* strains**
324 We performed RT-qPCR to quantify relative *tilB* transcript abundance for two strains of *D.*
325 *simulans* (Per005 and Geo288) and two strains of *D. melanogaster* (CA1 and T.4). For the two *D.*
326 *simulans* strains and the Geo288 *D. melanogaster* strain, we collected larvae from two stages of

327 larval development (96 and 120 hours following oviposition). For the other *D. melanogaster*
328 strain (T.4), we were only able to obtain enough larval tissue at 96 hours following oviposition,
329 due to low fecundity. Because the relative transcript abundance data had a skewed distribution,
330 we used the reciprocal root transformation to normalize the species and overall distributions
331 (Shapiro-Wilk tests for normality: all $p > 0.34$). We then analyzed the relative transcript
332 abundance of *tilB* using a nested ANOVA with the following factors: species, strain nested
333 within species, larval age (96 and 120 hours), and the interaction between species and larval age.
334 The interaction term between species and larval age was not significant ($p=0.41$), so we removed
335 it from the model. We found that larvae from the 120-hour sampling period had significantly
336 lower *tilB* expression than 96-hour larvae ($F_{1,14}= 6.16$, $p= 0.026$). While we did not detect any
337 significant differences between the 2 strains from the same species ($F_{2,14}=2.39$, $p=0.13$), we
338 found a significantly higher average relative amount of *tilB* transcript in *D. melanogaster* larvae
339 compared to *D. simulans* larvae ($F_{1,14}= 9.74$, $p= 0.0075$; Fig 7).
340 Because we performed rt-PCR on an extreme strain and a strain closer to the average for
341 each species, these four lines represent a continuum of pupation site choice behavior, with T.4 (*D.*
342 *melanogaster*) having the lowest proportion of pupae on the food, followed by CA1 (*D.*
343 *melanogaster*), then Per005 (*D. simulans*) and Geo288 (*D. simulans*) having the highest
344 proportion of pupae on the food (Fig 1). These four strains follow an identical pattern for *tilB*
345 gene expression, with T.4 having the highest relative transcript abundance, and Geo288 having
346 the lowest (Fig 7). Although it is not possible to detect a significant effect with a sample size of 4,
347 this suggests that *tilB* gene expression may be negatively correlated with the proportion of pupae
348 on the food (Spearman's rank correlation: $r_s = -1$, $p< 0.10$).
349

350 **7. Effect sizes**

351 We used the results above to estimate how much of the difference in pupation site preference
352 between *D. melanogaster* and *D. simulans* can be attributed to: *i*) the X chromosome, *ii*) our
353 deficiencies of interest (Df(1)BSC869/DF(1)ED6720 and Df(1)Exel6255), and *iii*) *Fas2* and *tilB*.
354 We first calculated the "species difference ratio" (using the data from our parental/hybrid screen
355 in Fig 3) by dividing the median proportion of males on the food for *D. simulans* by the median
356 proportion of males on the food for *D. melanogaster* males (species difference ratio = 5.27). To
357 determine how much of this species difference ratio can be attribute to the X chromosome, we
358 calculated an "X effect ratio" by dividing the median proportion of simX males on the food by
359 the median proportion of melX males on the food. By then dividing the "X effect ratio" by the
360 "species difference ratio", we found that the X chromosome accounts for approximately 55.6%
361 (95% CI= 31.4%-80.2%) of the difference in pupation site preference between *D. melanogaster*
362 and *D. simulans*. It is important to note, however, that this may be an overestimate, as calculating
363 effect sizes using only reciprocal hybrids does not account for potential transgressive autosomal
364 effects (50).

365 We then estimated the effect size of our deficiencies by calculating "deficiency effect
366 ratios" (the median pupation index when the deficiency was crossed to the *D. simulans Lhr*
367 strain/the median pupation index when the deficiency was crossed to the *D. melanogaster* T.4
368 strain). Because Df(1)BSC869 and DF(1)ED6720 overlap, we used their mean deficiency effect
369 ratio to estimate the effect size of the overlapping region, and found that it explains
370 approximately 44.1% (95% CI= 33.5%-74/5%) of the X chromosome effect. Similarly, we found
371 that Df(1)Exel6255 explains approximately 52.3% (95% CI= 33.7%-85.9%) of the X
372 chromosome effect.

373 Finally, we estimated the effect size of our two identified candidate genes, *Fas2* and *tilB*.
374 For each allele we tested, we calculated a "knockout effect ratio" (the median pupation index
375 when the knockout was crossed to the *D. simulans Lhr* strain/the median pupation index when
376 the knockout was crossed to the *D. melanogaster* T.4 strain) and then we used the average
377 knockout effect ratio of the two alleles for each gene (i.e. *Fas2*: average of $Fas2^{eb112}$ and
378 $Fas2^{G0293}$; *tilB*: average of $tilB^{1}$ and $tilB^{2}$) to estimate the effect size. We found that *Fas2*
379 explains approximately 93% (95% CI= 78.5%-134.9%) of the pupation difference attributed to
380 the overlap between Df(1)BSC869 and DF(1)ED6720, and 41% (95% CI= 33.8%-76.5%) of the
381 pupation difference attributed to the X chromosome. Similarly, we found the *tilB* explains
382 approximately 87.2% (95% CI= 66.2%-135.9%) of the pupation difference attributed to
383 Df(1)Exel6255, and 45.6% (95% CI= 35.5%-76.1%) of the pupation difference attributed to the
384 X chromosome. While our calculation of the effect size of the X chromosome may be an
385 overestimate (see above), we calculated the effect size of the deficiencies and individual gene
386 knockouts in similar hybrid backgrounds, so their contributions to the overall X-effect (and the
387 contribution of each knockout to the deficiency effect size) are likely more accurate. Still, these
388 estimates could be affected by variation in the different *D. melanogaster* backgrounds in which
389 the deficiencies and knockout strains were made.
390

391 # Discussion

392 **A species-level difference in pupation site choice behavior**

393 Our initial survey of pupation site choice behavior in *D. melanogaster* and *D. simulans* expands
394 upon a previously reported interspecific difference (29). Consistent with these previous results,
395 on average, our *D. simulans* strains had a greater proportion of flies pupating on the food surface.

396 However, we used 11 *D. melanogaster* and 12 *D. simulans* strains sourced from around the globe
397 to demonstrate this difference. While the species difference holds when comparing the grand
398 mean of all strains for each species, there is substantial variation within species. This variation is
399 so significant that the species' distributions overlap, with some *D. simulans* strains, like Mex180
400 and Cal006, more closely resembling *D. melanogaster* strains in pupation behavior (Fig 1). To
401 our knowledge, our study is the first to extensively record this variation, which presents a useful
402 tool for better understanding the evolution of pupation site choice behavior. These documented
403 differences in pupation behavior among *Drosophila* species, in combination with our
404 understanding of the environmental variables that affect this behavior within species
405 (24,25,47,51), will be useful in identifying the selection pressures (if any) that affect the
406 evolution of this trait. Differences in pupation site choice behavior may be a form a niche
407 partitioning where species co-occur, as has been previously suggested (34). Alternatively,
408 pupation site choice may be an adaptive response to parasite or parasitoid presence (52). A
409 globally sourced panel of lines with significant variation, such as our own, provides an inroad for
410 studies comparing pupation behavior to differences in the ecology of each collection site, such
411 that we can better understand the ultimate causes of this behavioral evolution.
412

413 **The genetic architecture of pupation site choice behavioral evolution**

414 Using hybrid crosses in the same background that controlled for maternal inheritance, we were
415 able to estimate the effect of the X chromosome on pupation behavior. We found a significant
416 effect of the X, in that reciprocal hybrid males pupate in similar locations as the X-donating
417 parent. We calculate that this chromosome explains 55.6% (95% CI= 31.4%-80.2%) of the total
418 phenotypic difference between parent strains. Although the exact contribution of the X

419 chromosome reported here may be an overestimate due to transgressive autosomal effects that
420 cannot be detected in a hybrid background (50), a similar X-effect has been detected for pupation
421 behavior when comparing *D. simulans* and *D. sechellia* (37).

422 We also found that hybrid females, which inherit one X chromosome from each parent,
423 pupate more like the *D. melanogaster* parent strain. This suggests that *D. melanogaster* alleles
424 are dominant to *D. simulans* alleles. The dominance of *D. melanogaster* alleles makes it possible
425 to use engineered deletions, available in *D. melanogaster* strains, to map regions containing
426 recessive *D. simulans* variation affecting pupation behavior (38). This technique has long been
427 employed to map morphological traits, physiological traits, and hybrid incompatibility loci,
428 (42,45,53–57), but less so for behaviors (46,58,59).

429 The results of our deficiency screen identify two regions of the X chromosome with
430 detectable effects on pupation behavior: one spans X:4,204,351 – 4,325,174 and explains
431 ~44.1% of the X-effect, and the other spans X:21,519,203 – 22,517,665 and explains ~52.3% of
432 the X-effect. These regions contain 23 and 28 genes, respectively. Our analysis of the available
433 gene knockouts within these regions points to two genes: *tilB* and *Fas2* (see below). We
434 calculate that *tilB* and *Fas2* explain the majority of the effect of their respective deficiency
435 regions (point estimate = 87.2%, 95% CI= 66.2%-135.9%; point estimate = 93%, 95% CI=
436 78.5%-134.9% respectively). Taken together, our results suggest that a substantial share of the
437 difference in pupation behavior between *D. simulans* and *D. melanogaster* can be attributed to
438 just two genes.

439

440 ***tilB* and *Fas2*: loci of evolution for divergent pupation behavior between *D. simulans* and *D.***
441 ***melanogaster***

442 We have presented substantial evidence for a role of both *tilB* (*touch insensitive larva B*) and
443 *Fas2* (*Fasciclin* 2) in the divergence of pupation behavior among these species. For each locus,
444 we have shown that two independent knockouts replicate the pattern of the regions identified by
445 the deficiency screen: hybrid females hemizygous for the *D. simulans* locus pupate on the food
446 surface significantly more often than females with both the *D. simulans* and *D. melanogaster* loci.
447 Further, we show that this is not simply an effect of hemizygosity within this region in two ways.
448 First, we demonstrate that this effect is lost when we crossed these knockout strains to *D.*
449 *melanogaster* strains, suggesting that the pattern we observe in the hybrid knockouts is in fact a
450 result of revealing the recessive *D. simulans* alleles. Second, knockouts for one other gene within
451 each region, *wap* and *mei9*, did not replicate this pattern when crossed to a *D. simulans* strain. In
452 addition, we crossed *tilB* and *Fas2* knockouts to multiple *D. melanogaster* and *D. simulans*
453 strains, demonstrating that this is unlikely to be a strain-specific effect, and rather is a species-
454 level difference.

455 We then used RNAi knockdown of each gene in *D. melanogaster* to show that reduced
456 expression of *tilB* and *Fas2* transcripts results in a more *D. simulans*-like pupation site choice
457 behavior. To directly test for differences in expression of *tilB*, we performed RT-PCR during two
458 larval developmental time points. Congruent with our RNAi knock-down results, we find that
459 mean relative transcript abundance is significantly higher in *D. melanogaster* larvae than in *D.*
460 *simulans* larvae. Interestingly, we surveyed two lines per species, representing a continuum of
461 pupation site choice behavior, and found that the mean proportion of pupae on the food surface
462 for these lines was perfectly negatively correlated with *tilB* gene expression. Although these
463 results are only for 4 lines, they suggest that lower *tilB* gene expression may be associated with a
464 higher proportion of larvae pupating on the food surface.

465 While our present study does not present a functional analysis of the *D. melanogaster* or
466 *D. simulans Fas2* or *tilB* alleles, we can use the *D. melanogaster* annotation of each gene to
467 speculate about their role in the evolution of pupation behavior. *Fas2* is a large gene, spanning
468 over 70,000 base pairs, with expression peaking during the larval wandering stage (L3) (48). It is
469 also complex, with seven transcripts composed of various combinations of sixteen exons.
470 Broadly, *Fas2* functions as a neuronal recognition molecule, and is involved in patterning the
471 larval nervous system (60). Expression of *Fas2* is critical for synapse formation and growth at
472 the larval neuromuscular junction (61,62) and is also important for patterning of the larval
473 mushroom body (63). With expression in both the central and peripheral nervous system, it is
474 possible that differences at the *Fas2* locus differentially wire the *D. melanogaster* and *D.*
475 *simulans* brains, altering how larvae perceive or interpret stimuli. Whether these differences are a
476 result of evolution of the protein sequence, and/or spatial or temporal differences in transcript
477 expression remains to be determined.
478 Unlike *Fas2*, *tilB* is a short gene, spanning just over 1,700 base pairs, with a single
479 transcript composed of 5 exons. *tilB* is also expressed in wandering larvae and pupae, though it
480 shows higher expression in testes of adult males, due to its role in developing sperm flagella (48).
481 In fact, *tilB* is associated with ciliary motility (64), and is a part of the mechanosensory
482 transduction machinery (65). Mutant *tilB* larvae display normal locomotor activity, but have a
483 reduced withdrawal response to physical disturbance (66). Consistent with this finding, our data
484 suggest that changes in *tilB* expression could potentially result in differences in peripheral
485 sensory perception between *D. melanogaster* and *D. simulans* larvae, ultimately influencing
486 larval pupation site choice behavior. A more precise functional analysis of *tilB* is necessary to
487 test this hypothesis.

488

489 **Conclusions**

490 Here we show that a substantial amount of the difference in pupation site choice behavior
491 between *D. melanogaster* and *D. simulans* is attributed to the X chromosome. This difference is
492 not, however, entirely explained by the X, and further study of the autosomal genome using the
493 same approach may reveal additional genes with substantial effects.

494 Using engineered chromosomal deletions, we identify two regions on the X chromosome
495 associated with pupation site choice behavior. We then use gene knockouts and RNAi to show
496 that the large majority (if not entire) effect of each of these regions is explained by a single gene.
497 Overall, this indicates that individual genes can have substantial effects on behavioral differences.

498 Our results highlight two main areas for further research. First, while we now have a
499 better understanding of the genetic underpinnings of pupation site choice behavior evolution, we
500 have little idea of why differences evolved in the first place. The variation we recorded among
501 globally sourced *D. melanogaster* and *D. simulans* strains provides a valuable tool with which to
502 pursue this line of inquiry. Second, while we have identified specific genes involved in the
503 evolution of this phenotype, we have little understanding of their function in pupation site choice
504 behavior, or how exactly these functions have evolved. The results of our expression study
505 suggest a role of expression differences for *tilB*, but further functional follow-up is necessary to
506 identify the precise molecular underpinnings of pupation site choice preference.

507

508 # Methods

509

510 **General fly maintenance**

511 Unless otherwise stated, we maintained all fly strains for these experiments in 20 mm diameter
512 vials containing standard cornmeal-molasses-yeast medium at 25°C under a 12h:12h light/dark
513 cycle at 50% relative humidity. Under these conditions, we established non-overlapping two-
514 week lifecycles as follows. For all stocks, except LH_M and *Lhr* (see below), we transferred male
515 and female adult flies into fresh vials containing food media supplemented with live yeast on the
516 surface for 1-3 days, at which point the flies were discarded. 14 days later (after all progeny had
517 eclosed), we again transferred adult flies into fresh vials for 1-3 days to begin the next generation.
518 We maintained LH_M and *Lhr* identically, except we additionally regulated density by transferring
519 only 10 males and 10 females to begin the next generation.
520

521 **Characterizing pupation behavior for *D. melanogaster* and *D. simulans***

522 We measured pupation behavior for 11 *D. melanogaster* and 12 *D. simulans* strains collected
523 from various locations throughout the world (Table S1). The 11 *D. melanogaster* strains included
524 10 of the "founder" wild-type inbred lines of the Drosophila Synthetic Population Resource
525 (King et al., 2012; flyrils.org), and a single wild-type line created from the LH_M laboratory-
526 adapted population (68). The 12 *D. simulans* strains included 11 wild-type strains and a single
527 strain carrying *Lethal hybrid rescue* (*Lhr*), a mutation that restores viability in *D.*
528 *melanogaster*/*D. simulans* hybrid males.

529 To measure pupation behavior, we placed 10 males and 10 females from a specific line
530 (both 3-5 days old) into half pint bottles and allowed females to oviposit overnight on a 10 mm
531 diameter petri dish filled with food medium that was placed in the opening of the bottle. In total,
532 we set up 5 bottles for each line. The following morning, we transferred 100 eggs from the petri
533 dishes into vials containing food medium that were lined with an acetate sleeve on which the

534 larvae could pupate. In total, we set up 5-8 vials per line. Vials were held at 25°C for 8 days, at
535 which time the liner was removed and the locations of the pupae were recorded (8 days was long
536 enough for almost all larvae to pupate without any flies eclosing). A pupa was considered "on"
537 the food if it was within 1 cm of the food surface, while all pupae that were further than 1 cm
538 from the food surface were considered "off" the food. For each vial, we calculated the proportion
539 of pupae on the food surface. For comparisons between species, our unit of replication was the
540 mean proportion of pupae on the food surface from each line (i.e. N=11 for *D. melanogaster* and
541 N=12 for *D. simulans*).
542
543 **Crossing *D. melanogaster* with *D. simulans***
544 For all crosses below, we created F1 hybrids between *D. melanogaster* and *D. simulans* using the
545 following protocol. *D. simulans* males were collected as virgins within 6 hours of eclosion and
546 held at room temperature in groups of 20 in vials containing food medium for 3-4 days. To set up
547 crosses, we collected young *D. melanogaster* virgin females within 2-3 hours of eclosion, and
548 combined 8-12 of these females with 20 *D. simulans* male in vials containing food medium
549 supplemented with an *ad lib* amount of live yeast on the surface. We then pushed a long foam
550 plug down into the vial, leaving approximately 1 cm of space above the food surface. We held
551 flies under these conditions for 3 days, at which time they were transferred from these "cross
552 vials" into "pupation vials" that contained food medium with no added yeast, and were lined
553 with an acetate sleeve on which the larvae could pupate. We always set up crosses using *D.*
554 *melanogaster* females and *D. simulans* males, because crosses in the opposite direction were
555 never successful.
556

557 **Measuring pupation behavior in F1 hybrids**

558 To create F1 hybrid males and females, we used *D. simulans* males from the *Lethal hybrid*
559 *rescue* (*Lhr*) strain (36). The *Lhr* mutation restores viability in F1 hybrid males, which are
560 usually lethal (69). To create F1 hybrid females and F1 males with a *D. melanogaster* X
561 chromosome ("melX" males), we crossed wild-type females from our LH_M strain (provided by
562 Dr. William Rice) to *Lhr D. simulans* males (Fig 2A). Because we were unable to successfully
563 cross *D. simulans* females to *D. melanogaster* males, we created F1 hybrid males with the *D.*
564 *simulans* X chromosome ("simX" males) by crossing *D. melanogaster* LH_M females that carry a
565 compound X chromosome (*C(1)DX y f*) (68) to *D. simulans Lhr* males. The compound X in these
566 females ensured that the X chromosome was transmitted from *D. simulans* fathers to their F1
567 hybrid sons (Fig 2B). Thus, for each direction of the cross, we combined *D. melanogaster*
568 females with *D. simulans* males, as described above. This crossing scheme ensures that all
569 maternal inheritance (cytoplasmic and mitochondrial) in the reciprocal male hybrid crosses
570 originates from the *D. melanogaster* parent. Thus, any differences we observe between melX and
571 simX males are directly attributable to their different X chromosomes.

572 After 3 days in the cross vial, we transferred males and females into pupation vials for 24
573 hours, at which time the flies were removed. While screening hybrid pupation behavior, we also
574 concurrently screened pupation behavior for the parental *D. melanogaster* strain (LH_M) and the
575 parental *D. simulans* strain (*Lhr*) for comparison. Parental strain cross vials contained only a
576 moderate amount of yeast, were set up with only 5 males and 5 females (pure species crosses
577 produce more offspring), and did not have a plug pushed down into the vial, but were otherwise
578 treated identically to the hybrid crosses. In total, we set up 30-33 vials per treatment.

579 All pupation vials were held at 25°C for 8 days, at which time the liner was removed. We
580 removed any remaining larvae, and cut the liner at a point 1 cm above the food surface. The
581 portion of the liner that contained pupae within 1 cm of the food surface was returned to the
582 original vial (the "on vial"), while the portion of the liner with pupae further off of the food
583 surface was placed in another vial containing food medium (the "off vial"). The flies that eclosed
584 were sexed and counted 7 days later (15 days post-egg); all flies that eclosed in the "on vial"
585 were considered flies that pupated on the food surface, while all flies that eclosed in the "off
586 vial" were considered flies that pupated off the food surface. We then calculated the proportion
587 of individuals that pupated on the food for each type of individual (genotype and sex).
588
589 **Mapping hybrid pupation behavior using the Bloomington Deficiency Kit**
590 Because we found a significant effect of the X chromosome on the difference in pupation site
591 choice behavior between *D. simulans* and *D. melanogaster*, we devised a crossing scheme using
592 molecularly engineered chromosomal deficiencies to screen the X chromosome for loci
593 contributing to this difference. These deficiencies are part of the Bloomington Deficiency Kit
594 (38), available from the Bloomington Drosophila Stock Center (BDSC). We assayed a total of 90
595 deficiency strains covering 87% of the X chromosome (Table S2). We restricted our deficiency
596 screen to lines from the BSC, Exelixis, and DrosDel sets to control for strain background effects
597 while also maximizing chromosome coverage.
598 To set up crosses, we collected deficiency females as young virgins (2-3 hours after
599 eclosing) and crossed them to *D. simulans* males from the *Lhr* strain. After 3 days in the cross
600 vial, we transferred males and females into pupation vials for 24-48 h, at which time the flies

601 were removed. We then divided the pupations vials into “on” and “off” vials as we did for our F1
602 hybrids (above).
603 These crosses produced two types of hybrid female that were heterozygous for *D.*
604 *melanogaster*/*D. simulans* at each autosome (Fig 2C). The deficiency hybrid females contain the
605 *D. melanogaster* deficiency X chromosome and a *D. simulans* X chromosome, making them
606 hemizygous for a segment of the X chromosome. At this locus, these hybrid females only
607 express *D. simulans* alleles. The balancer hybrid females contained the *D. melanogaster* balancer
608 X chromosome (marked with a dominant visible marker) and a *D. simulans* X chromosome.
609 These females are heterozygous for *D. melanogaster*/*D. simulans* over the entirety of the X
610 chromosome, and thus express both *D. simulans* and *D. melanogaster* alleles. Although the
611 deficiency hybrids are our flies of interest, the balancer hybrids provide an excellent
612 experimental control, as these females developed in the same environment as our experimental
613 flies. As a result, we calculated the proportion of deficiency hybrid females that pupated on the
614 food and the proportion of balancer hybrid females that pupated on the food. We then used these
615 measures to calculate a “pupation index” as the proportion of deficiency hybrids pupating on the
616 food divided by the proportion of balancer females pupating on the food. To increase the
617 accuracy of our estimates, we only included pupation vials in our analysis that yielded at least 10
618 of each type of female. For each deficiency hybrid strain we measured, we report the median
619 pupation index of all replicates, because there were often high-scoring outliers that significantly
620 skewed the mean pupation index. These outliers almost always had abnormally high pupation
621 indices, so focusing on median values makes our findings more conservative.
622 Any deficiency hybrid cross with a median pupation index greater than 1 indicates that
623 more deficiency females pupated on the food compared to balancer females, potentially because

624 the deficiency includes *D. melanogaster* genetic variation that is involved in pupation site choice
625 behavior. Alternatively, simply creating flies that are hemizygous at a locus on the X
626 chromosome may result in a variety of pleiotropic effects that make larvae less likely to climb up
627 the vial. To test for this, when a deficiency hybrid cross showed a pupation index significantly
628 greater than 1 (Table S2), we crossed that *D. melanogaster* deficiency strain to at least one other
629 *D. melanogaster* wild-type strain. If these *D. melanogaster* deficiency crosses displayed the
630 same pattern, we considered the effect of the deficiency on pupation behavior to be a byproduct
631 of deleting a large portion of the X chromosome, rather than revealing recessive *D. simulans*
632 variation, and discarded them. If instead the pupation index for the *D. melanogaster* crosses was
633 significantly lower than the pupation index for the *D. simulans* crosses, we pursued that
634 deficiency for further validation. To ensure that this pattern is not a result of epistasis from the
635 hemizygous region in a hybrid background, we further crossed these deficiencies to an additional
636 *D. melanogaster* and *D. simulans* strain, to test for background-specific effects.
637
638 **Testing candidate genes in deficiency regions using gene knockouts and RNAi knockdown**
639 For regions of interest identified by our deficiency screen, we ordered transgenic knockouts for
640 any genes available within the region at the time. We focused on two regions: the significant
641 overlap of Df(1)BSC869 and DF(1)ED6720 (spanning X:4,204,351-X:4,325,174), and the region
642 covered by DF(1)Exel6255 (X:21,519,203;X:22,517,665). For the first region, we obtained
643 knockouts for *Fas2* and *mei9*. We screened two *Fas2* knockouts, the mutant allele $Fas2^{eb112}$
644 ($Fas2^{eb112}$/FM7c; Grenningloh et al., 1991; provided by Brian McCabe), and a p-element
645 insertion allele, $Fas2^{G0293}$ (former BDSC Stock 11850; full genotype: w^{67c23}
646 $P\{lacW\}fas2^{G0293}$/FM7c), and the $mei\text{-}9^{A1}$ mutant allele (w^{1} $mei\text{-}9^{A1}$/FM7h; BDSC stock #6792).

647 For the second region, we obtained knockouts for two genes: *tilB* and *wap*. We screened two *tilB*
648 mutant alleles, $tilB^{1}$ and $tilB^{2}$ (*y w* $tilB^{1/2}$ /FM4; Kernan et al., 1994; provided by Daniel Eberl),
649 and the wap^{2} mutant allele (wap^{2}/FM6; DSC stock # 8133). Like the deficiency strains, each of
650 our gene disruptions is held over a balancer chromosome with a visible marker. To measure the
651 pupation behavior of hybrids containing knockout copies of these *D. melanogaster* genes, we
652 crossed each *D. melanogaster* knockout strain to *Lhr* using the previously described methods,
653 and calculated the pupation index as the proportion of knockout females on food / the proportion
654 of balancer females on the food. As for our deficiency screen, any hybrid knockouts with a
655 median PI greater than 1 for the hybrid cross suggest that the knockout gene may be involved in
656 pupation site choice. We also crossed each *D. melanogaster* knockout strain to a wild-type *D.*
657 *melanogaster* (T.4) strain to ensure that hybrid knockouts with a pupation index greater than 1
658 are not simply an artifact of being hemizygous for this particular gene. We crossed knockout
659 strains that displayed the pattern we expect for a gene involved in pupation site choice (i.e. a
660 pupation index significantly greater than 1 when crossed to *D. simulans*, which is also
661 significantly greater than the pupation index when crossed to *D. melanogaster*) to an additional
662 *D. simulans* (Mex180) and *D. melanogaster* (T.1) wild-type strain for verification.
663 Our knockout screen identified two genes that appear to be involved in pupation site
664 choice: *tilB* and *Fas2*. We further tested the effects of these genes on pupation behavior using
665 RNAi knockdown in *D. melanogaster*. We used the elav-Gal4 driver (P{w[+mC]=GAL4-
666 elav.L}2/CyO; BDSC #8765), which expresses Gal4 throughout the nervous system. We drove
667 down the expression of *tilB* and *Fas2* throughout the nervous system by crossing elav-Gal4
668 virgin females to UAS-*tilB* (BDSC #29391: y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF03324}attP2)
669 and UAS-*Fas2* (BDSC #34084: y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01098}attP2)

670 males, respectively. The resulting flies express a gene-specific hairpin RNA throughout the
671 nervous system, causing the degradation of mRNA, and thus, reduced expression of that gene
672 (70,71). As experimental controls for each RNAi cross, we also crossed elav-Gal4 virgin females
673 to the RNAi background stock (*y v; attP2, y+* (y^1 v^1 ; P{y[+t7.7]=CaryP}attP2; BDSC stock #
674 36303) and the Gal4-1 stock (containing a hairpin RNA targeting Gal4 in VALIUM20; BDSC
675 stock # 35784). Together, these controls allow us to account for the effect of both the Gal4
676 mutation and general expression of hairpin RNA throughout the nervous system. Any differences
677 we detect between these controls and our RNAi crosses must therefore be due to the expression
678 of the gene-specific (*tilB* or *Fas2*) hairpin RNA. We set up pupation vials using the methods
679 described above, and for each cross, we calculated the proportion of RNAi (or control) flies on
680 the food (removing any data points with fewer than 20 experimental flies). If more RNAi flies
681 pupate on the food in the experimental cross (in which the expression of the gene is driven down)
682 compared to the control crosses (in which gene expression is unaffected), this provides further
683 support for that gene's involvement in pupation site choice.
684
685 **Testing candidate genes for species-specific differences in larval transcript expression**
686 We selected two each of our 11 *D. melanogaster* and 12 *D. simulans* strains to test for larval
687 stage-specific expression differences of candidate genes using real time RT-PCR – one extreme
688 and one average. For *D. simulans*, we selected Geo288 and Per005 (Table S1), because Geo288
689 has the highest pupation index of any *D. simulans* strain and Per005 is closest to the species
690 mean (Fig 1). For *D. melanogaster*, we selected CA1 and T.4 (Table S1) because T.4 has the
691 lowest pupation index of any *D. melanogaster* strain, and CA1 is closer to the species mean (Fig
692 1).

693 To harvest larvae from these strains, we allowed adult females to oviposit in standard
694 vials containing food media between the hours of 8 AM and 12 PM over two consecutive days.
695 120 hours after the final oviposition day, we floated larvae out of the food media using a 20%
696 sucrose in water solution, sucked them up using a transfer pipet, briefly rinsed them with DI
697 water on cheesecloth, and snap froze them using liquid nitrogen (72). In this way, we collected
698 20-30 mg of larvae from two developmental time points: 96, and 120 hours following
699 oviposition. These time points approximate early wandering and late wandering larval stages.
700 We chose these time points because they are presumably when pupation site choice occurs, and
701 because larvae are large enough for many to be harvested at once using the above methods. We
702 extracted mRNA using the Qiagen RNeasy Plus Mini Kit, and prepared cDNA using the
703 Promega Verso kit.
704 To quantify transcript abundance, we designed primers that span a single intron near the
705 3' end of *tilB* (73). Additionally, we used primers for the gene *RpL32* as an internal control
706 (74,75). *Fas2* is a complex gene with multiple splice forms, so we were unsuccessful in
707 designing general primers that would amplify all transcripts in both species. For this reason, we
708 did not include *Fas2* in these experiments. A full list of primers and transcript lengths can be
709 found in Table S4A. For each stage and strain, we prepared two to three biological replicates,
710 which we then amplified in two technical replicates for 40 rounds of qPCR. Using *RpL32*
711 transcript number as an internal control, we calculated relative transcript abundance while
712 correcting for species differences in primer efficiency. We estimated primer efficiency
713 differences by serially diluting gDNA from each of our *D. melanogaster* and *D. simulans* strains,
714 performing qPCR, and using a standard curve to calculate adjusted amplification factors (Table
715 S4B). To ensure that we were amplifying cDNA made from RNA, and not gDNA contamination,

716 we performed gel electrophoresis on our cDNA samples to ensure we only visualized the short,
717 intron-less, band.
718

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726

727 Author contributions

728 **Alison Pischedda:**
729 Conceptualization; formal analysis; investigation; methodology; project administration;
730 visualization; writing – original draft preparation
731 **Michael P Shahandeh:**
732 Conceptualization; formal analysis; investigation; methodology; project administration;
733 visualization; writing – original draft preparation
734 **Thomas L Turner:**
735 Conceptualization; funding acquisition; methodology; supervision; writing – review and editing

736
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1023
1024 **Figure Captions:**

1025

1026 **Fig 1. Pupation behavior differences between *D. melanogaster* and *D. simulans*.**
1027 The mean proportion of individuals in a vial that pupated on the surface of the food for 11 *D.*
1028 *melanogaster* lines and 12 *D. simulans* lines described in Table S1. Error bars denote the 95%
1029 confidence interval around each individual mean (N = 5-8). The dashed horizontal lines indicate
1030 the grand mean for each species. The boxes surrounding the dashed lines denote the 95%
1031 confidence interval around the grand mean.

1032

1033 **Fig 2. Crossing schemes to generate reciprocal X chromosome hybrid males and**
1034 **deficiency/balancer hybrid females. A.** Crossing wild-type *D. melanogaster* females (LH_M,
1035 shown in white) to *D. simulans* males (*Lhr*, shown in grey) produces hybrid males with a *D.*
1036 *melanogaster* X chromosome (melX) and hybrid females. **B.** Crossing a compound X C(1)DX
1037 LH_M female to *Lhr* males produces hybrid males with a *D. simulans* X chromosome (simX).
1038 Females of this cross would inherit two *D. melanogaster* X chromosomes and a *D. simulans* Y
1039 chromosome, but are inviable. **C.** Crossing *D. melanogaster* X chromosome deficiency lines,
1040 which have a balancer X chromosome with a dominant visible marker (DVM) and an X
1041 chromosome with a large deletion, to *Lhr* produces deficiency hybrid females, balancer females,
1042 balancer males, and deficiency males (*mostly dead due to large deletions on a hemizygous
1043 chromosome with some deficiency lines being exceptions).

1044

1045 **Fig 3. Pupation site choice behavior for *D. melanogaster*, *D. simulans*, and their F1 hybrids.**
1046 The mean proportion of individuals that pupated on the food surface for males and females from

1047 both species and their F1 hybrids. *D. melanogaster* males and females were taken from the LH_M
1048 strain, while *D. simulans* males and females were taken from the *Lhr* strain. F1 hybrids resulted
1049 from a cross between these two strains. The "melX" hybrid males have the *D. melanogaster* X
1050 chromosome, and the "simX" hybrid males have the *D. simulans* X chromosome. Both hybrids
1051 have *D. melanogaster* cytoplasmic inheritance. Columns labeled with different letters are
1052 significantly different from one another after sequential Bonferroni correction for multiple
1053 comparisons ($p < 0.0001$ after correction, N = 26-33). Error bars indicate the 95% confidence
1054 interval surrounding the mean.
1055
1056 **Fig 4. Hybrid deficiency screen of the X chromosome identifies two regions of interest. A.**
1057 The median pupation index for each of the 90 deficiency hybrid crosses (y-axis) is plotted by the
1058 physical map distance each engineered deletion spans along the X chromosome (x-axis).
1059 Deficiencies with a pupation index significantly greater than 1 (solid line) after correction for
1060 multiple comparisons are denoted by an asterisk (significance levels for all deficiencies are listed
1061 in Table S2). Deficiencies with a pupation index significantly greater than 0.88 (the grand mean,
1062 dashed line) after correction for multiple comparisons are denoted with a cross. All of these lines
1063 also had pupation indices significantly greater than 1 before correcting for multiple comparisons.
1064 The two regions we pursued for candidate gene validation are highlighted in light grey. Note, we
1065 did not pursue the remaining three significant deficiency strains because they showed similar
1066 pupation indices when crossed to *D. melanogaster* (Fig S3). **B.** The pupation indices of the
1067 deficiencies from the grey highlighted areas in Part A are shown for the original hybrid cross
1068 (*Lhr*) and for a cross to the T.4 *D. melanogaster* strain. BSC869 and Exel6255 were additionally
1069 crossed to *D. simulans* strain Mex180 and *D. melanogaster* strain T.1. Asterisks denote a

1070 significant difference between pupation indices of deficiency strains crossed to *D. melanogaster*
1071 and *D. simulans* (* = $p < 0.05$, ** = $p < 0.01$). Error bars are 95% confidence intervals calculated
1072 from 10,000 bootstraps of the median. **C.** The region uncovered by the overlap of deficiencies
1073 BSC869 and ED6720 and the 23 genes contained within it. Direction of the gene transcript is
1074 denoted with an arrow. The two genes with available disruption strains *(Fas2* and *mei9*) are
1075 labelled. **D.** The region uncovered by Exel6255 and the 28 genes contained within it. Direction
1076 of the gene transcript is denoted with an arrow. The two genes with available disruption strains
1077 *(tilB* and *wap*) are labelled.
1078
1079 **Fig 5. Knockouts and RNAi knockdown confirm the role of *Fas2* in evolved differences in**
1080 **pupation site choice. A.** The median pupation indices of the $Fas2^{eb112}$ gene disruption are shown
1081 for the comparison between the original hybrid cross (*Lhr*) and a cross to the *D. melanogaster*
1082 T.4 strain. Also shown is the comparison for crosses to the *D. simulans* strain Mex180 and the *D.*
1083 *melanogaster* strain T.1. **B.** The median pupation indices for a second gene disruption, $Fas2^{G0293}$,
1084 are shown for the original hybrid cross (*Lhr*) and a *D. melanogaster* strain (T.4). For A and B,
1085 asterisks denote significance (** = $p < 0.01$, **** = $p<0.0001$; N = 50-53). **C.** The results of
1086 pan-neuronal knock down of the *Fas2* transcript via RNAi. The mean proportion of
1087 RNAi/control individuals found on the food are shown for the control cross (elav-Gal4 driver
1088 crossed to the RNAi background stock), the Gal-4 hairpin RNA cross (Gal-4), and *Fas2* RNAi
1089 cross. For C, asterisks denote significance after correcting for multiple comparisons (**** =
1090 $p<0.0001$; N = 42-48). Error bars for all are 95% confidence intervals calculated from 10,000
1091 bootstraps of the median.
1092

1093 **Fig 6. Knockouts and RNAi knockdown confirm the role of *tilB* in evolved differences in**
1094 **pupation site choice. A.** The median pupation indices of the *tilB*[1] gene disruption are shown for
1095 the comparison between the original hybrid cross (*Lhr*) and a cross to the *D. melanogaster* T.4
1096 strain. Also shown is the comparison for crosses to the *D. simulans* strain Mex180 and the *D.*
1097 *melanogaster* strain T.1. **B.** The median pupation indices for a second gene disruption, *tilB*[2], are
1098 shown for the original hybrid cross (*Lhr*) and the cross to *D. melanogaster* strain T.4, and for
1099 crosses to the *D. simulans* strain Mex180, and the *D. melanogaster* strain T.1. For A and B,
1100 asterisks denote significance (* = $p < 0.05$, ** = $p = 0.01$; N = 41-47). **C.** The results of pan-
1101 neuronal knock down of the *tilB* transcript via RNAi. The mean proportion of RNAi/control
1102 individuals found on the food are shown for the control cross (elav-Gal4 driver crossed to the
1103 RNAi background stock), the Gal-4 hairpin RNA cross (Gal-4), and *tilB* RNAi cross. For C,
1104 asterisks denote significance after correcting for multiple comparisons (* = $p < 0.05$, ** = $p <$
1105 0.01; N = 41-47). Error bars for all are 95% confidence intervals calculated from 10,000
1106 bootstraps of the median.
1107
1108 **Fig 7. Relative differences in *tilB* transcript abundance between species.** The relative
1109 abundance of *tilB* transcript detected by RT-qPCR in *D. melanogaster* and *D. simulans*. Each
1110 data point represents the average of two technical replicates for a single biological replicate.
1111 Relative transcript abundance is significantly higher in *D. melanogaster* strains on average ($p <$
1112 0.01). Light grey squares (T.4) and triangles (CA1) represent the two *D. melanogaster* strains
1113 measured, while dark grey diamonds (Per005) and circles (Geo288) represent the two *D.*
1114 *simulans* strains. The dashed lines depict the average expression across both time points for each
1115 strain. The black circles represent the species-wide mean, and error bars depict the 95%

1116 confidence interval surrounding the mean. Note that the exact rank of relative *tilB* expression is
1117 perfectly inversely correlated to the pupation indices of each strain (i.e., the strain with the
1118 highest *tilB* expression has the lowest pupation index, and so on).
1119
1120 **Fig S1. Pupation behavior differences between *D. melanogaster* and *D. simulans* after**
1121 **controlling for density.**
1122 The mean residuals from a regression between the proportion of individuals in a vial that pupated
1123 on the surface of the food and the total number of pupae in the vial for 11 *D. melanogaster* lines
1124 and 12 *D. simulans* lines described in Table S1. Error bars denote the 95% confidence interval
1125 around each individual mean (N = 5-8). The dashed horizontal lines indicate the grand mean for
1126 each species. The boxes surrounding the dashed lines denote the 95% confidence interval around
1127 the grand mean.
1128
1129 **Fig S2. Pupation behavior for *D. melanogaster*, *D. simulans*, and their F1 hybrids after**
1130 **controlling for density.** The mean residual proportion of individuals that pupated on the food
1131 surface are shown for males and females from both species and their F1 hybrids. *D.*
1132 *melanogaster* males and females were taken from the LH_M strain, while *D. simulans* males and
1133 females were taken from the *Lhr* strain. F1 hybrids resulted from a cross between these two
1134 strains. The "melX" hybrid males have the *D. melanogaster* X chromosome, and the "simX"
1135 hybrid males have the *D. simulans* X chromosome. Both hybrids have *D. melanogaster*
1136 cytoplasmic inheritance. Columns labeled with different letters are significantly different from
1137 one another after sequential Bonferroni adjustment for multiple comparisons ($p < 0.0001$, N =
1138 26-33). Error bars indicate the 95% confidence interval surrounding the mean.

1139

1140 **Fig S3. Pupation site choice behavior for additional significant hybrid deficiencies.** Median
1141 pupation indices for **A.** Df(1)ED411 (N=29-36), **B.** Df(1)ED6906 (N=26-33), and **C.**
1142 Df(1)BSC530 (N=18-22). Each of the three deficiencies displayed a median pupation index
1143 significantly greater than the average (0.88) when crossed to *D. simulans* (*Lhr*) after correcting
1144 for multiple comparisons. However, when each was crossed to *D. melanogaster* (T.4), the
1145 pupation index was still significantly greater than average, indicating that the behavior of these
1146 flies is impacted by the deficient region in general, rather than the *Lhr* genotype it reveals.

1147

1148 **Fig S4. Pupation behavior for knockout strains crossed to both *D. melanogaster* and *D.***
1149 ***simulans*. A.** The median pupation index of a *wap* gene disruption crossed to *D. melanogaster*
1150 (T.4, N = 51) and *D. simulans* (*Lhr*, N = 55). **B.** The median pupation index of a *mei9* gene
1151 disruption crossed to *D. melanogaster* (T.4, N = 52) and *D. simulans* (*Lhr*, N = 51).

1152

1153 **Fig S5. Pupation site choice behavior for *Fas2* and *tilB* RNAi crosses after controlling for**
1154 **density. A.** The results of pan-neuronal knock down of the *Fas2* transcript via RNAi. The mean
1155 residual proportion of RNAi/control individuals found on the food are shown for the control
1156 cross, the Gal-4 line, and RNA interference cross. Asterisks denote significance after correcting
1157 for multiple comparisons (* = $p < 0.05$, ** = $p < 0.01$, ** = $p<0.001$; N = 42-48). **B.** The results
1158 of pan-neuronal knock down of the *tilB* transcript via RNAi. The mean residual proportion of
1159 RNAi/control individuals found on the food are shown for the control cross, the Gal-4 line, and
1160 RNA interference cross. Asterisks denote significance after correcting for multiple comparisons
1161 (* = $p < 0.05$, ** = $p < 0.01$; N = 41-47).

1162 **Table Captions:**

1163

1164 **Table S1: Strains used to characterize pupation behavior in *D. melanogaster* and *D.***

1165 ***simulans.*** With the exception of LH_M, the *D. melanogaster* (mel) lines were the founders of the

1166 Drosophila Synthetic Population Resource (flyrils.org). The *D. simulans (sim)* lines were

1167 obtained from the National Drosophila Species Stock Center.

1168

1169 **Table S2: X deficiencies used to map pupation behavior between *D. melanogaster* and *D.***

1170 ***simulans*.** All deficiencies were obtained from the Bloomington Drosophila Stock Center

1171 (BDSC). The median pupation index (PI) is listed for each deficiency. Deficiencies with a PI > 1

1172 indicate that more deficiency hybrids pupated on the food compared to balancer hybrids.

1173 Included are the uncorrected p-values from 1-sample Wilcoxon tests for each deficiency against

1174 the predicted PI = 1, and against the grand mean for all deficiencies (PI = 0.88). Uncorrected p-

1175 values that were significantly greater than 1 (or 0.88) are indicated in red, and those that

1176 remained significant after sequential Bonferonni correction for multiple comparisons are

1177 highlighted in yellow, and denoted with asterisks (* = p<0.05, **= p<0.01, ***=p<0.001, ****=

1178 p<0.0001 after correcting for multiple comparisons). Potential deficiencies of interest that were

1179 selected for further investigation are also highlighted in yellow. Only lines with a PI

1180 significantly >1 AND a PI significantly > 0.88 after adjusting for multiple comparisons were

1181 selected.

1182

1183 **Table S3A. The genes within the region uncovered by the overlap of Df(1)BSC869 and**
1184 **DF(1)ED6720**. Listed in the table are physical position, strand, flybase ID, gene name,
1185 expression in larvae, expression in the larval nervous system, and known biological functions.
1186 **Table S3B. The genes within the region uncovered by Df(1)Exel6255.** Listed in the table are
1187 physical position, strand, flybase ID, gene name, expression in larvae, expression in the larval
1188 nervous system, and known biological functions.
1189
1190 **Table S4A. Primers used for RT-PCR of *tilB* and *RpL32*.** Primers selected for amplification
1191 of tilB and RpL32 mRNA transcripts. Left and right primers are listed, along with their genomic
1192 start positions. The length of the fragments amplified from gDNA (including introns) as well as
1193 from mRNA (no introns) are included.
1194 **Table S4B. Standard curve for primer efficiency**
1195
1196

Figure 1

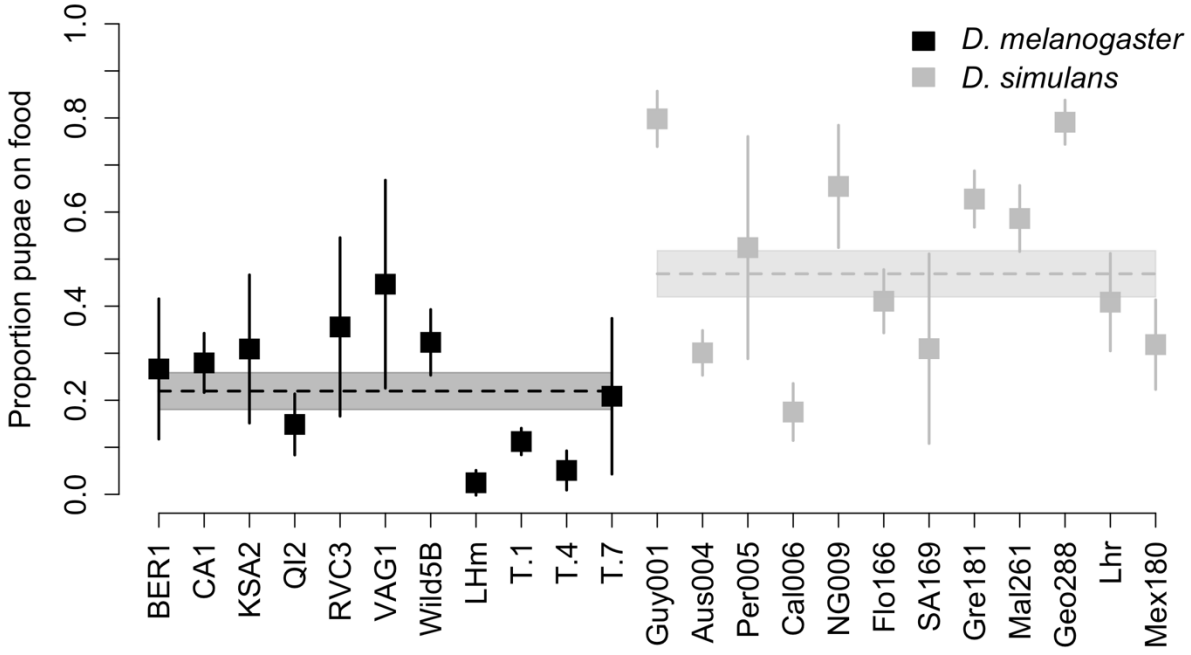

Proportion pupae on food
0.0
0.2
0.4
0.6
0.8
1.0
D. melanogaster
D. simulans
BER1
CA1
KSA2
QI2
RVC3
VAG1
Wild5B
LHm
T.1
T.4
T.7
Guy001
Aus004
Per005
Cal006
NG009
Flo166
SA169
Gre181
Mal261
Geo288
Lhr
Mex180

Figure 2

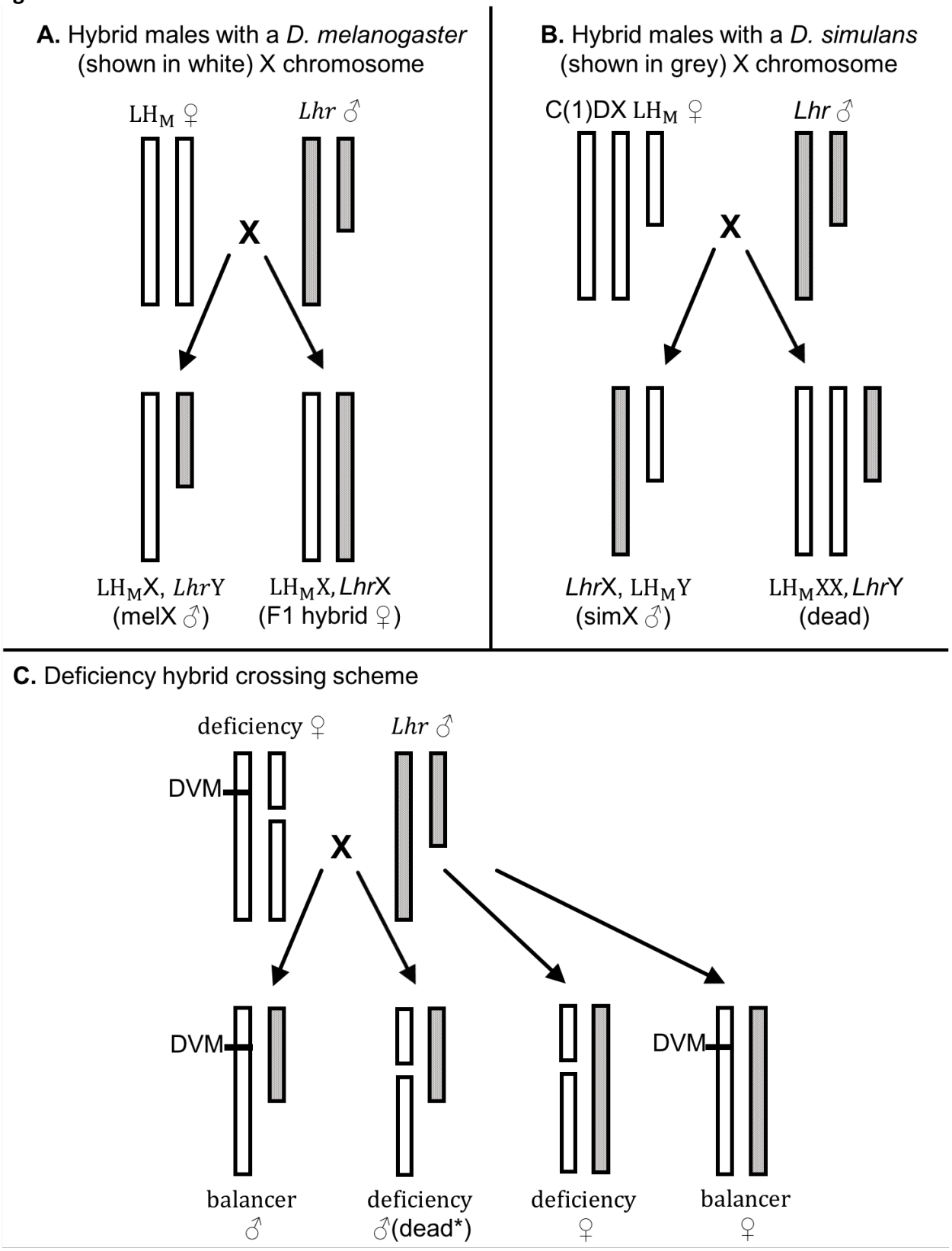

A. Hybrid males with a D. melanogaster (shown in white) X chromosome
LHM ♀
Lhr ♂
X
LHMX, LhrY
(melX ♂)
LHMX, LhrX
(F1 hybrid ♀)
B. Hybrid males with a D. simulans (shown in grey) X chromosome
C(1)DX LHM ♀
Lhr ♂
X
LhrX, LHMY
(simX ♂)
LHMXX, LhrY
(dead)
C. Deficiency hybrid crossing scheme
deficiency ♀
Lhr ♂
DVM
X
DVM
DVM
balancer ♂
deficiency ♂(dead*)
deficiency ♀
balancer ♀

Figure 3

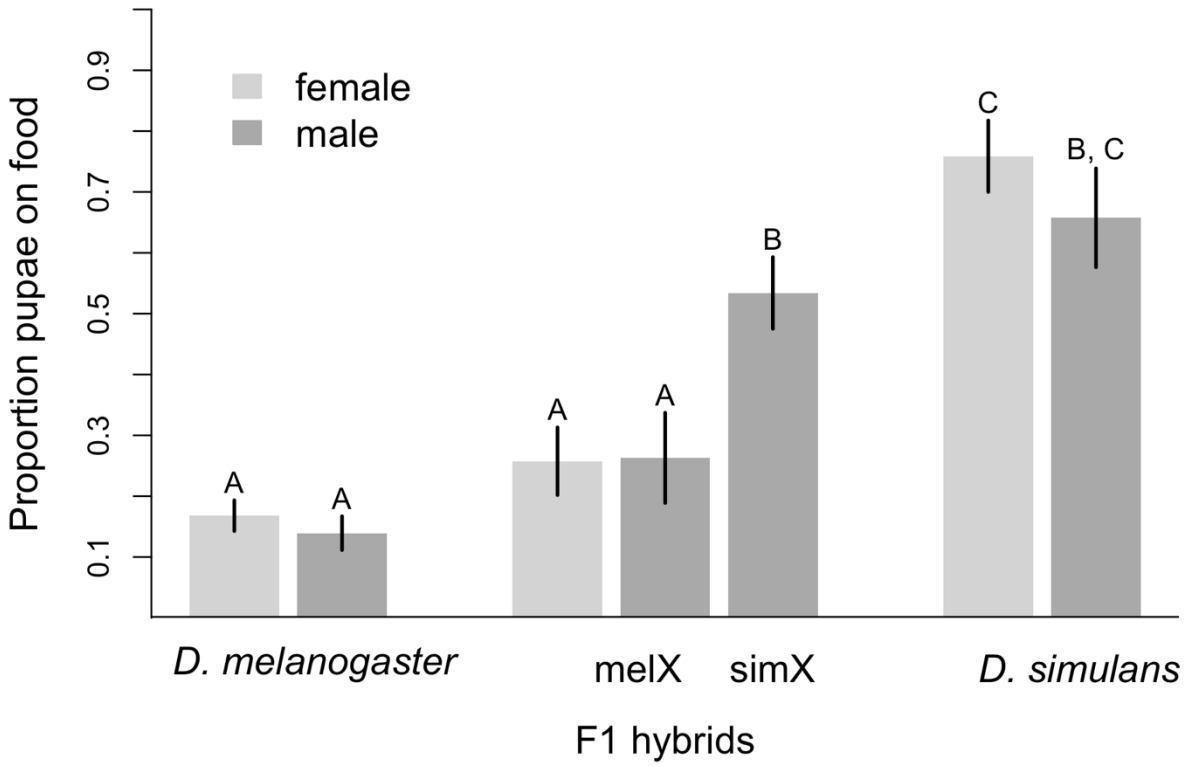
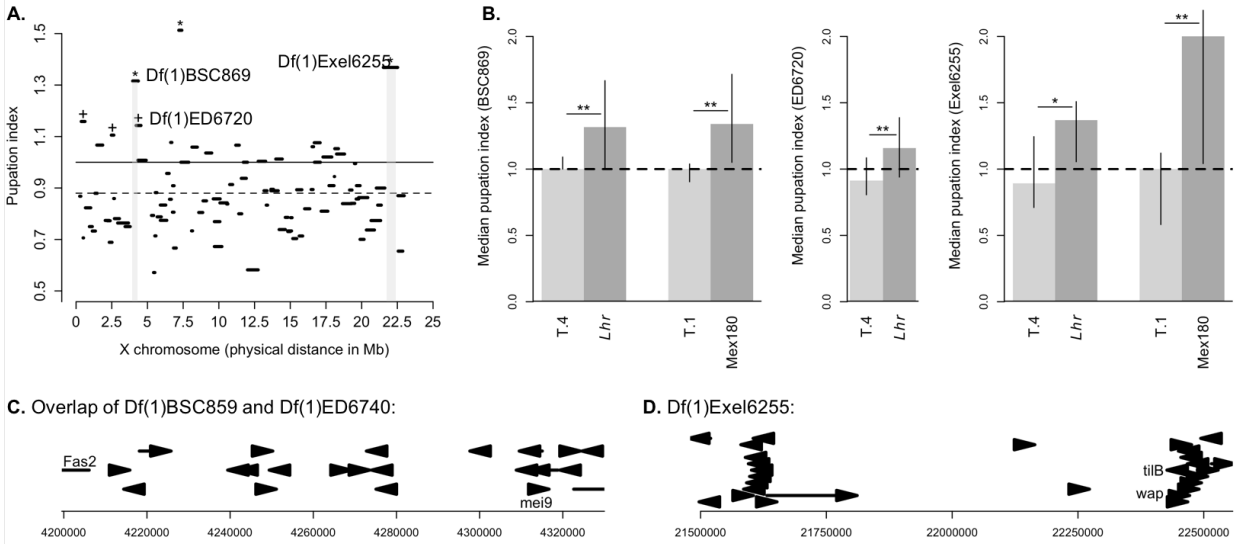

Proportion pupae on food
0.1
0.3
0.5
0.7
0.9
female
male
A
A
A
A
B
C
B, C
D. melanogaster
melX
simX
D. simulans
F1 hybrids

Figure 4



C. Overlap of Df(1)BSC859 and Df(1)ED6740:

D. Df(1)Exel6255:

Figure 5

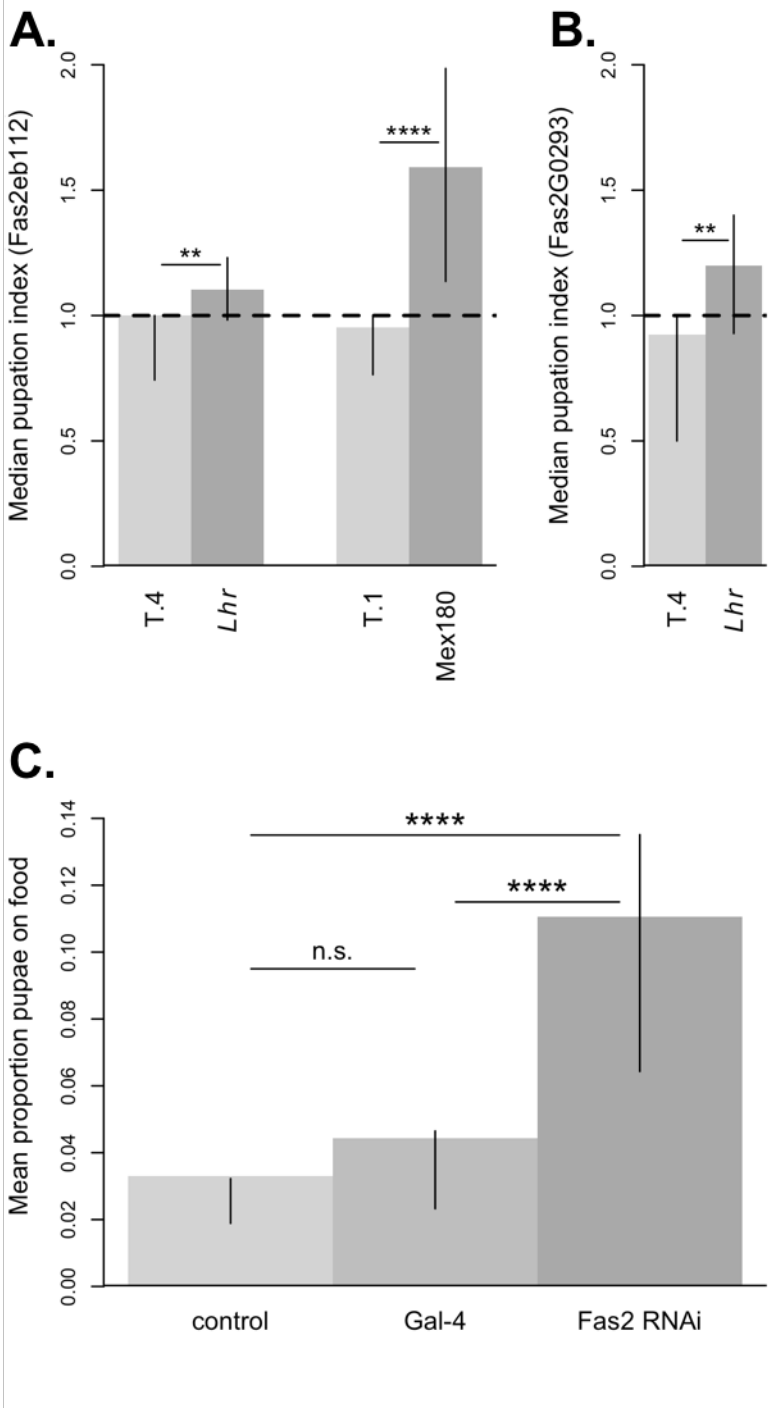

A.
Median pupation index (Fas2eb112)
0.0
0.5
1.0
1.5
2.0
**
****
T.4
Lhr
T.1
Mex180
B.
Median pupation index (Fas2G0293)
0.0
0.5
1.0
1.5
2.0
**
T.4
Lhr
C.
Mean proportion pupae on food
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
****
****
n.s.
control
Gal-4
Fas2 RNAi

Figure 6

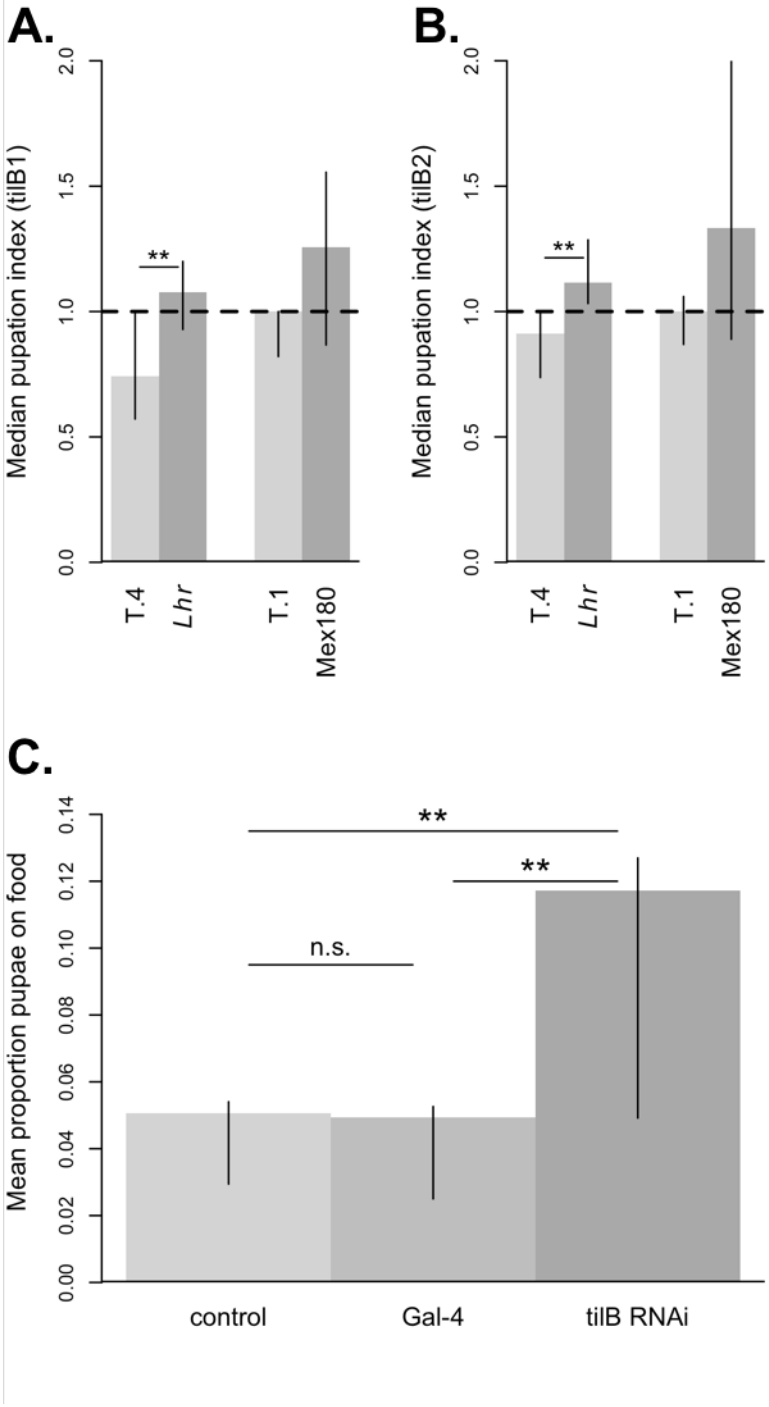

A.
Median pupation index (tilB1)
0.0
0.5
1.0
1.5
2.0
**
T.4
Lhr
T.1
Mex180
B.
Median pupation index (tilB2)
0.0
0.5
1.0
1.5
2.0
**
T.4
Lhr
T.1
Mex180
C.
Mean proportion pupae on food
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
**
**
n.s.
control
Gal-4
tilB RNAi

Figure 7

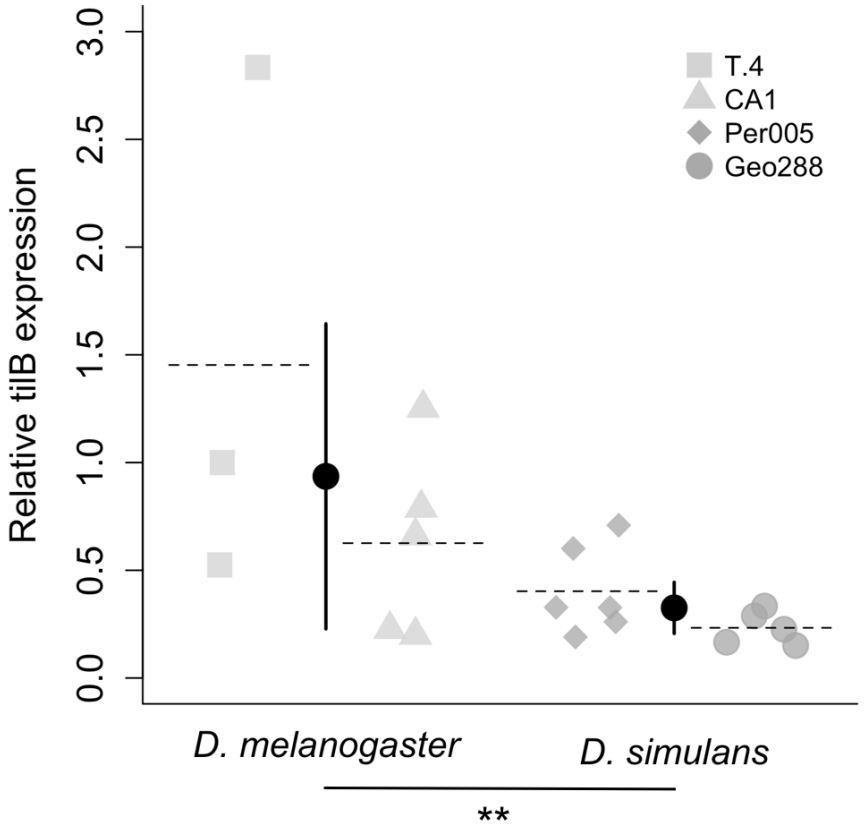

Relative tilB expression
0.0
0.5
1.0
1.5
2.0
2.5
3.0
T.4
CA1
Per005
Geo288
D. melanogaster
D. simulans
**

Figure S1

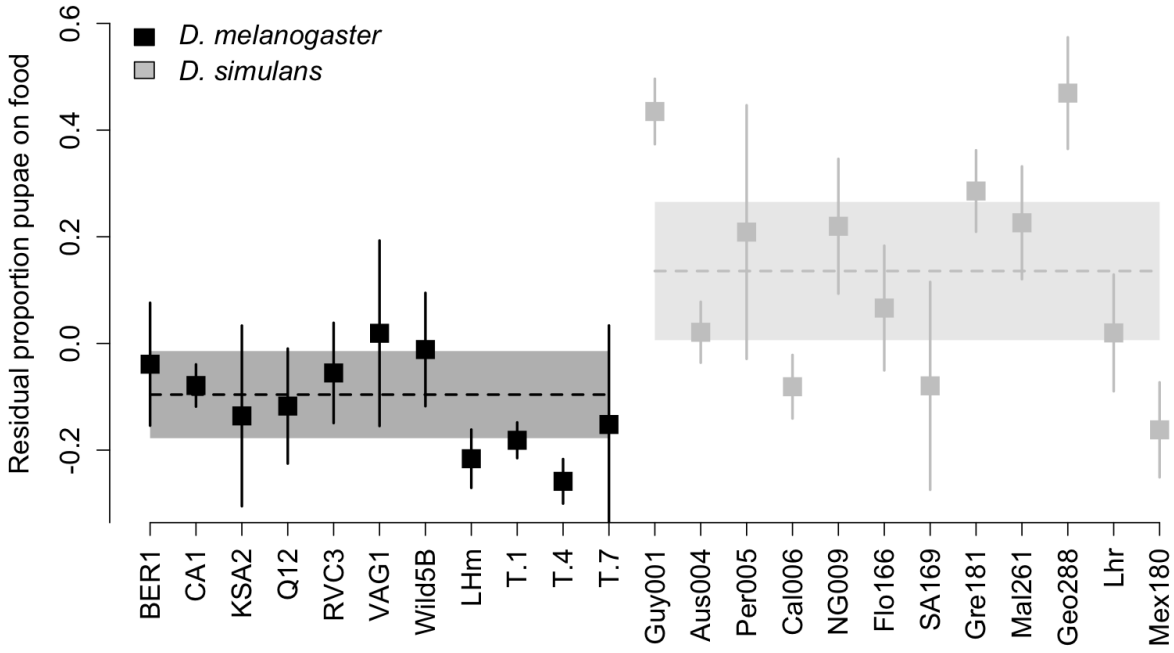

D. melanogaster
D. simulans
Residual proportion pupae on food
0.6
0.4
0.2
0.0
-0.2
BER1
CA1
KSA2
Q12
RVC3
VAG1
Wild5B
LHm
T.1
T.4
T.7
Guy001
Aus004
Per005
Cal006
NG009
Flo166
SA169
Gre181
Mal261
Geo288
Lhr
Mex180

Figure S2

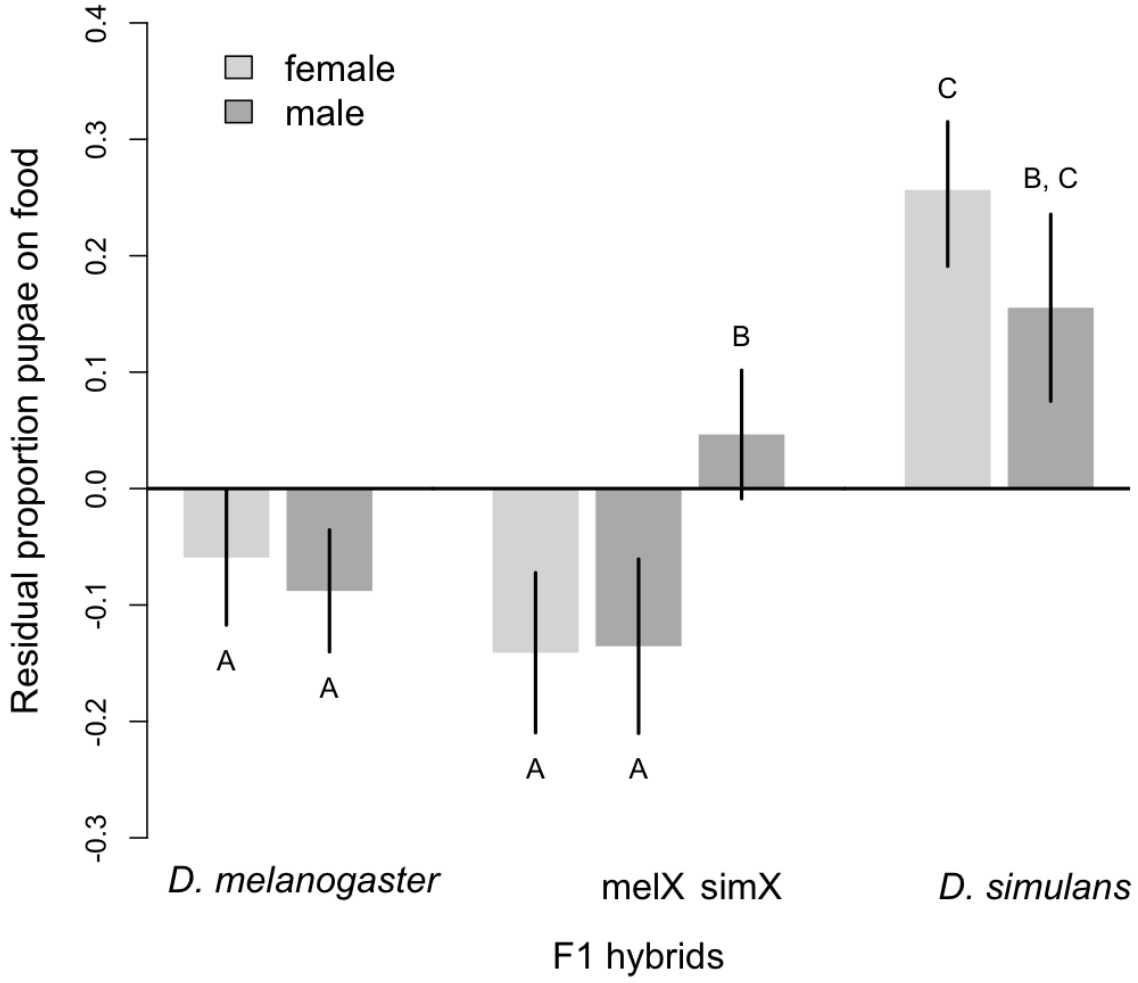

female
male
Residual proportion pupae on food
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
A
A
A
A
B
C
B, C
D. melanogaster
melX simX
D. simulans
F1 hybrids

Figure S3

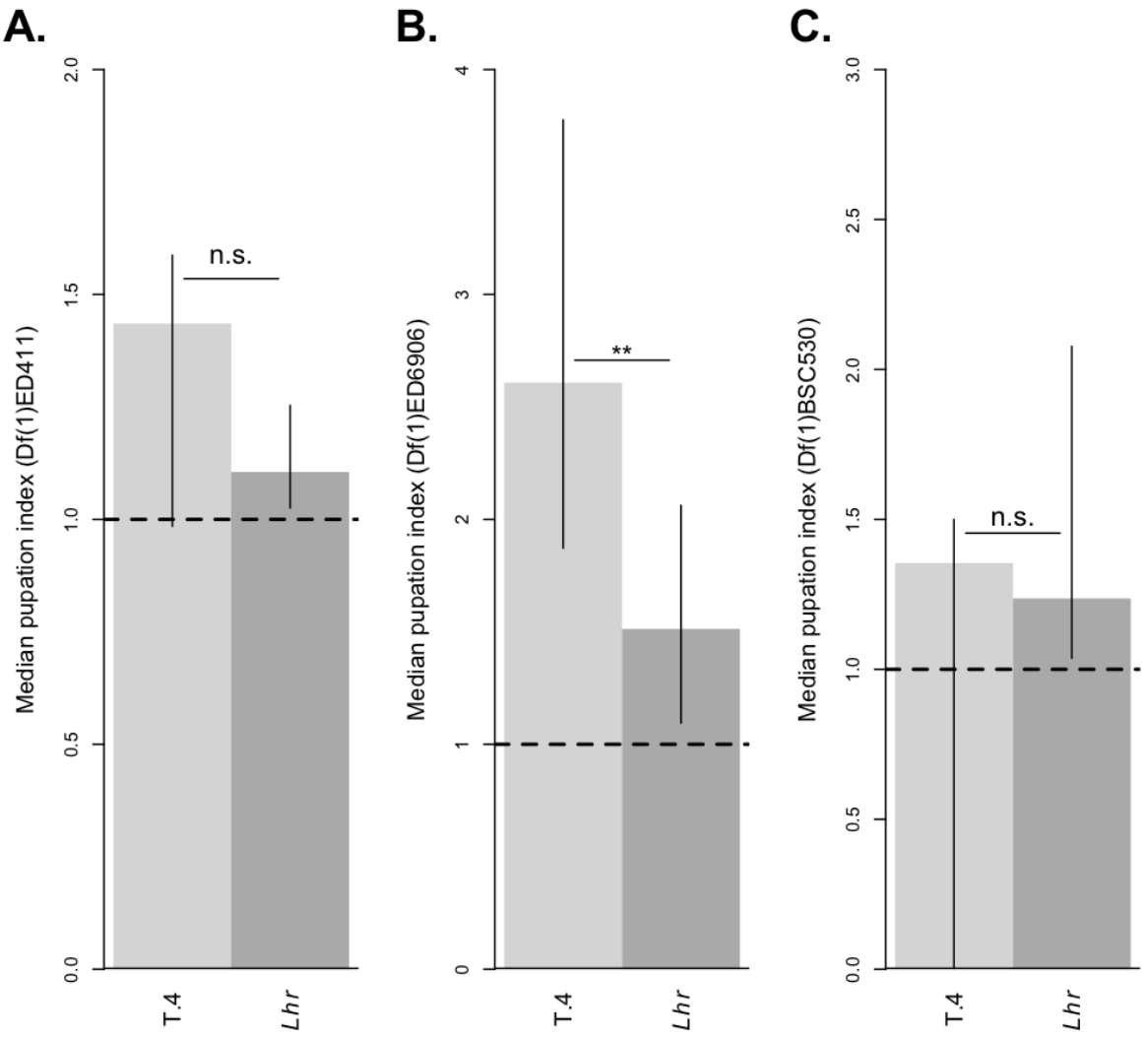

A.
B.
C.
Median pupation index (Df(1)ED411)
Median pupation index (Df(1)ED6906)
Median pupation index (Df(1)BSC530)
n.s.
**
n.s.
T.4
Lhr
T.4
Lhr
T.4
Lhr
0.0
0.5
1.0
1.5
2.0
0
1
2
3
4
0.0
0.5
1.0
1.5
2.0
2.5
3.0

Figure S4

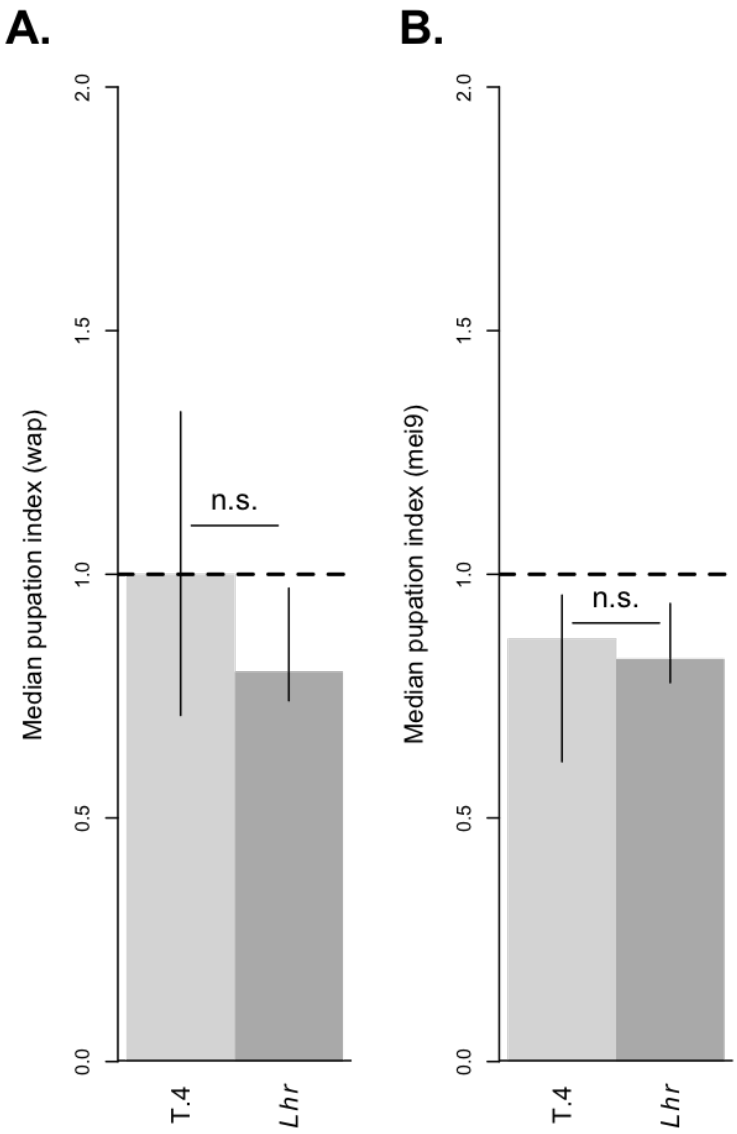
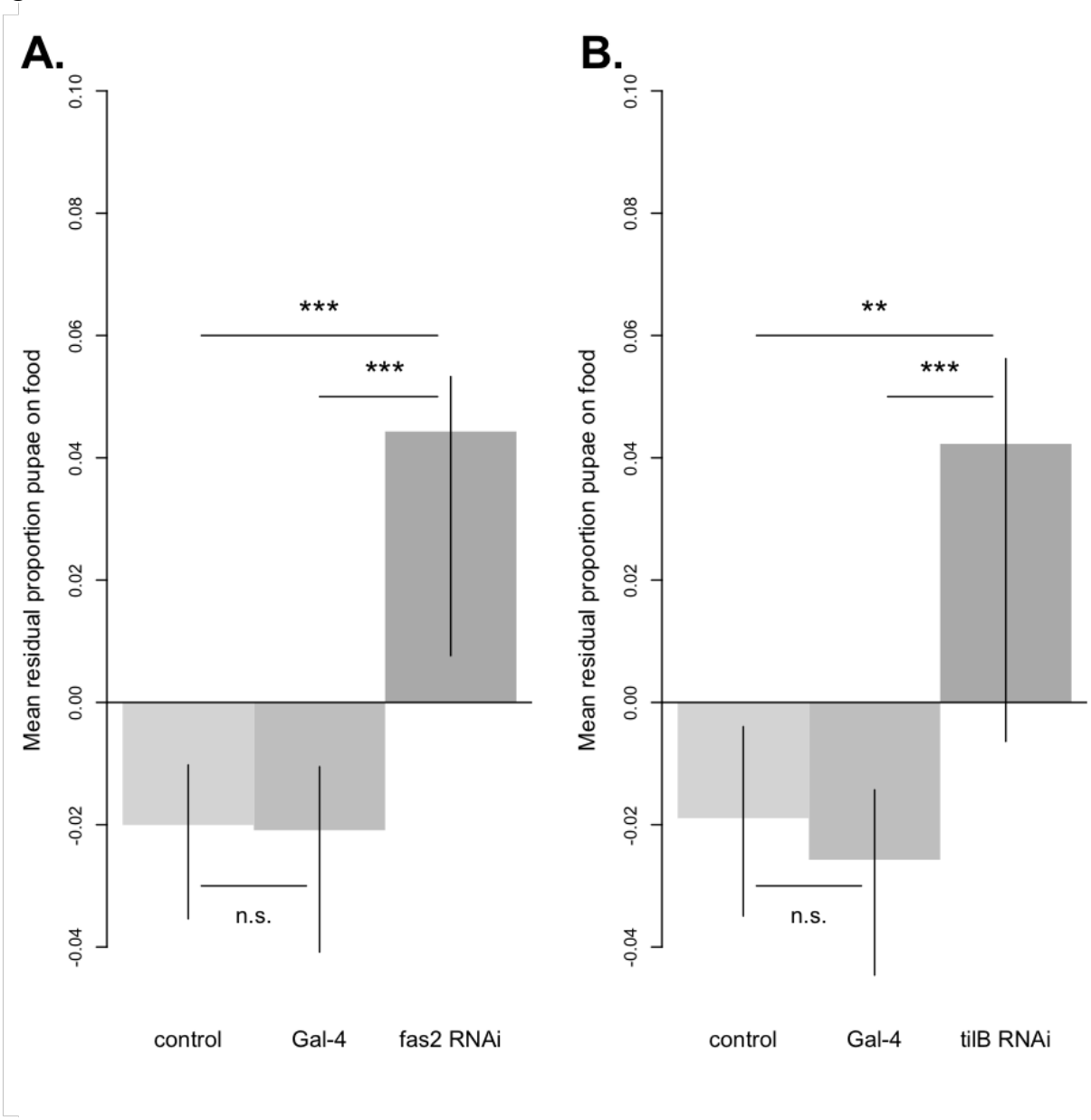

A.
B.
Median pupation index (wap)
Median pupation index (mei9)
2.0
1.5
1.0
0.5
0.0
n.s.
n.s.
T.4
Lhr
T.4
Lhr

Figure S5


A.
B.
Mean residual proportion pupae on food
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
-0.04
***
***
**
***
n.s.
n.s.
control
Gal-4
fas2 RNAi
control
Gal-4
tilB RNAi