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

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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 correlate with the differences in pupation site choice behavior between species. Our results suggest that even complex, environmentally sensitive behaviors may evolve through changes to loci with large phenotypic effects.

Introduction

The behaviors of closely related species can be remarkably different, and these differences can have important biological consequences. Behavioral evolution in insects has major impacts on crop decimation and disease vectoring (Karageorgi et al., 2017; McElhany et al., 1995). For example, one subspecies of the yellow fever mosquito, *Aedes aegypti*, prefers to bite humans, while another closely related species prefers other animals (McBride et al., 2014; Peterson, 1977). Understanding insect preferences therefore presents a major inroad to effective disease and pest management (Zhou et al., 2010). Behaviors are also critical to the creation and maintenance of biodiversity, as host, habitat, and mating preference behaviors are often key players in speciation and local adaptation (Coyne & Orr, 1997, 2004).

Despite the importance of behavioral traits, we know little about the genetic basis of their evolution. GePheBase, the most extensive compilation of natural genetic variants associated with trait differences, currently catalogs over 1800 associations, of which only 22 are for behavior (Martin & Orgogozo, 2013). From these 22, and others in the literature, it is clear that individual genes can sometimes have large effects on evolved differences in behavior (Leary et al., 2012; McGrath et al., 2011; Prince et al., 2017). It also clear that changes to sensory receptors in the peripheral nervous system can explain dramatic shifts in behavior (Cande et al., 2013; Leary et al., 2012; McBride et al., 2014). With so few studies, however, it is difficult to conclude how frequently we expect single loci to have large effects or how often sensory receptors explain species differences, due to pervasive ascertainment bias (Rockman, 2012). Indeed, a recent study discovered an exception to this emerging pattern: the evolution of a central neural circuit, rather than a peripheral sensory neuron, explains differences in mating behavior in two *Drosophila* species (Seeholzer et al., 2018).

67 The current lack of genetic studies mapping behavioral variation presumably arises from
68 the fact that behaviors are difficult to measure reliably and repeatedly. Behavioral phenotypes
69 often integrate multiple cues, are sometimes context dependent, and can be innate or learned,
70 making it difficult to exclude environmentally induced variation. To better understand the
71 genetic basis of behavioral evolution, we therefore need more case studies, with a focused effort
72 on "metamodel" systems with documented behavioral differences between closely related
73 species (Kopp, 2009).

74 Flies in the genus *Drosophila* are well poised to address these challenges. In *Drosophila*,
75 hundreds of genetically identical individuals from variable wild-caught strains can be reared in a
76 common environment, isolated at the beginning of their adult life stage, and repeatedly assayed
77 for a trait of interest. Such a design significantly reduces the potential for environmentally
78 induced variation to obscure genetic differences in behavior. Additionally, there are many
79 *Drosophila* species with large, characterized differences in a variety of complex behaviors
80 (Orgogozo & Stern, 2009). Undeniably, work comparing the repeated evolution of
81 morphological traits across closely related species of *Drosophila* has significantly advanced our
82 understanding of the general patterns linking genotype and phenotype for developmental traits
83 (Kittelmann et al., 2017; Rebeiz & Williams, 2017; Sucena et al., 2003). Studies that investigate
84 genetic complexity in *Drosophila*, where fine-mapping and functional follow-up are possible,
85 should make similar progress for behaviors.

86 In the present study, we seek to address the lack of behavioral association studies using
87 the *Drosophila* model system. Here, we investigate the difference in pupation site preference
88 between two *Drosophila* species: *D. melanogaster* and *D. simulans*. When the larval stages of
89 these species are ready to metamorphose into adults, they first enter a pupal stage. The pupa,

90 which lasts for a number of days, is immobile and therefore vulnerable to parasitism, predation,
91 desiccation, and disease (Markow, 1981a). Before pupating, larvae enter a "wandering" stage,
92 where they search for an appropriate pupation site (Riedl et al., 2007; Sokolowski et al., 1984).
93 Depending on the strain and species, larvae vary from pupating directly on their larval food
94 source to traveling more than 40 cm away from it (Stamps et al., 2005). This behavior has been
95 extensively studied, and is exquisitely sensitive to environmental conditions— individuals alter
96 their behavior in response to light, moisture, pH, the presence of other species, parasitism, and
97 more (Hodge & Caslaw, 1998; Markow, 1981b; Sameoto & Miller, 1968a; Seyahooei et al.,
98 2009). Despite this environmentally induced variation, the effects of genotype on preference are
99 considerable. Within species, strains and populations often differ in how far they travel from
100 their food source before pupating, although the most consistent experimentally demonstrated
101 differences are between species (Markow, 1979; Vandal et al., 2008). Interestingly, differences
102 in pupation site choice behavior between species do not correspond to their taxonomic
103 classification (Shivanna et al., 1996). For example, *D. melanogaster* and *D. simulans* shared a
104 common ancestor 2-3 million years ago (Lachaise & Silvain, 2004), and are extremely similar in
105 terms of their ecology, morphology, and physiology (Parsons, 1975). Previous work shows,
106 however, that they differ markedly in terms of pupation site choice, with *D. simulans* pupating
107 closer to the larval food source, on average (Markow, 1979, 1981a). This difference is not due to
108 laboratory adaptation, as freshly collected individuals show the same pattern (Markow, 1979,
109 1981a). These species are frequently collected in the same microhabitats, and their differences in
110 pupation site choice behavior have been postulated to be a form of niche partitioning (Markow,
111 1979, 1981a). Supporting this hypothesis, pupation site choice responds to density dependent

112 selection in the laboratory (Mueller & Sweet, 1986), and provides a potential increase in
113 competitive ability between species ovipositing in the same media (Arthur & Middlecote, 1984).
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 (Brideau et al., 2006a; Watanabe, 1979) to circumvent this challenge, and show that
119 a significant proportion of the species difference in pupation behavior can be mapped to the X
120 chromosome, consistent with findings using other *Drosophila* species (Erezyilmaz & Stern,
121 2013). Still, these hybrids remain sterile, so genetic dissection using a backcross mapping
122 population is not possible. Instead, we use a widely available set of transgenic *D. melanogaster*
123 deficiency lines to screen a substantial portion of the X chromosome (Cook et al., 2012; Ryder et
124 al., 2004). We use these lines to create hybrid females that lack large, overlapping portions of the
125 X chromosome from *D. melanogaster*, and therefore express only *D. simulans* alleles in those
126 regions. We identify two loci with large effects on pupation behavior. We then employ genetic
127 knockouts of candidate loci within these regions to demonstrate their effects, and use RNAi
128 knockdown to further test the role of two genes, *touch insensitive larvae B* (*tilB*) and *Fasciclin 2*
129 (*Fas2*). Finally, we use real time qRT-PCR to test for species-level differences in gene
130 expression of *tilB* in larvae, and show that *tilB* is more highly expressed in *D. melanogaster* than
131 in *D. simulans*.
132
133
134

135 # Methods

136

137 **General fly maintenance**

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

147

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

149 We measured pupation behavior for 11 *D. melanogaster* and 12 *D. simulans* strains collected
150 from various locations throughout the world (Table S1). The 11 *D. melanogaster* strains included
151 10 of the "founder" wild-type inbred lines of the Drosophila Synthetic Population Resource
152 (King et al., 2012; flyrils.org), and a single wild-type line created from the LH_M laboratory-
153 adapted population (Rice et al., 2005). The 12 *D. simulans* strains included 11 wild-type strains
154 and a single strain carrying *Lethal hybrid rescue* (*Lhr*), a mutation that restores viability in *D.*
155 *melanogaster*/*D. simulans* hybrid males.

156 To measure pupation behavior, we placed 10 males and 10 females from a specific line
157 (both 3-5 days old) into half pint bottles and allowed females to oviposit overnight on a 10 mm

158 diameter petri dish filled with food medium that was placed in the opening of the bottle. In total,
159 we set up 5 bottles for each line. The following morning, we transferred 100 eggs from the petri
160 dishes into vials containing food medium that were lined with an acetate sleeve on which the
161 larvae could pupate. In total, we set up 5-8 vials per line. Vials were held at 25°C for 8 days, at
162 which time the liner was removed and the locations of the pupae were recorded (8 days was long
163 enough for almost all larvae to pupate without any flies eclosing). A pupa was considered "on"
164 the food if it was within 1 cm of the food surface, while all pupae that were further than 1 cm
165 from the food surface were considered "off" the food. For each vial, we calculated the proportion
166 of pupae on the food surface. For comparisons between species, our unit of replication was the
167 mean proportion of pupae on the food surface from each line (i.e. N=11 for *D. melanogaster* and
168 N=12 for *D. simulans*).
169
170 **Crossing *D. melanogaster* with *D. simulans***
171 For all crosses below, we created F1 hybrids between *D. melanogaster* and *D. simulans* using the
172 following protocol. *D. simulans* males were collected as virgins within 6 hours of eclosion and
173 held at room temperature in groups of 20 in vials containing food medium for 3-4 days. To set up
174 crosses, we collected young *D. melanogaster* virgin females within 2-3 hours of eclosion, and
175 combined 8-12 of these females with 20 *D. simulans* males in vials containing food medium
176 supplemented with an *ad lib* amount of live yeast on the surface. We then pushed a long foam
177 plug down into the vial, leaving approximately 1 cm of space above the food surface. We held
178 flies under these conditions for 3 days, at which time they were transferred from these "cross
179 vials" into "pupation vials" that contained food medium with no added yeast, and were lined
180 with an acetate sleeve on which the larvae could pupate. We always set up crosses using *D.*

181 *melanogaster* females and *D. simulans* males, because crosses in the opposite direction were
182 never successful.
183
184 **Measuring pupation behavior in F1 hybrids**
185 To create F1 hybrid males and females, we used *D. simulans* males from the *Lethal hybrid*
186 *rescue* (*Lhr*) strain (Watanabe, 1979). The *Lhr* mutation restores viability in F1 hybrid males,
187 which are usually lethal (Brideau et al., 2006b). To create F1 hybrid females and F1 males with a
188 *D. melanogaster* X chromosome ("melX" males), we crossed wild-type females from our LH_M
189 strain (provided by William Rice) to *Lhr D. simulans* males (Fig 1A). Because we were unable to
190 successfully cross *D. simulans* females to *D. melanogaster* males, we created F1 hybrid males
191 with the *D. simulans* X chromosome ("simX" males) by crossing *D. melanogaster* LH_M females
192 that carry a compound X chromosome (*C(1)DX y f*) (Rice et al., 2005) to *D. simulans Lhr* males.
193 The background of this strain is identical to that of LH_M, with the exception of the compound X
194 chromosome. The compound X in these females ensured that the X chromosome was transmitted
195 from *D. simulans* fathers to their F1 hybrid sons (Fig 1B). For each direction of the cross, we
196 combined *D. melanogaster* females with *D. simulans* males, as described above. This crossing
197 scheme ensures that all maternal inheritance (cytoplasmic and mitochondrial) in the reciprocal
198 male hybrid crosses originates from the *D. melanogaster* parent. Thus, these hybrids have an
199 identical background with the exception of the sex chromosomes, and any differences we
200 observe between melX and simX males are directly attributable to their different sex
201 chromosomes.
202 After 3 days in the cross vial, we transferred males and females into pupation vials for 24
203 hours, at which time the flies were removed. While screening hybrid pupation behavior, we also

204 concurrently screened pupation behavior for the parental *D. melanogaster* strain (LH_M) and the
205 parental *D. simulans* strain (*Lhr*) for comparison. Parental strain cross vials contained only a
206 moderate amount of yeast, were set up with only 5 males and 5 females (pure species crosses
207 produce more offspring), and did not have a plug pushed down into the vial, but were otherwise
208 treated identically to the hybrid crosses. In total, we set up 30-33 vials per treatment.

209 All pupation vials were held at 25°C for 8 days, at which time the liner was removed. We
210 removed any remaining larvae, and cut the liner at a point 1 cm above the food surface. The
211 portion of the liner that contained pupae within 1 cm of the food surface was returned to the
212 original vial (the "on vial"), while the portion of the liner with pupae further off of the food
213 surface was placed in another vial containing food medium (the "off vial"). The flies that eclosed
214 were sexed and counted 7 days later (15 days post-egg); all flies that eclosed in the "on vial"
215 were considered flies that pupated on the food surface, while all flies that eclosed in the "off vial"
216 were considered flies that pupated off the food surface. We then calculated the proportion of
217 individuals that pupated on the food for each type of individual (genotype and sex).

218 To assess the validity of using hybrid behavior to map interspecific differences, we set up
219 an additional experiment to ensure that hybrids behaved typically with respect to pupation site
220 choice behavior. This is a potential concern, as hybrids between *D. melanogaster* and *D.*
221 *simulans* are known to differ from either parent in a variety of traits (Barbash & Ashburner, 2003;
222 Sturtevant, 1920; Takano, 1998). For a subset of pupation vials, we calculated the average
223 pupation height of males and females from each strain as follows. Instead of dividing the
224 pupation liner into "on" food and "off" food sections, we cut the liner at 8 intervals, each spaced
225 1 cm from the last, starting at the food surface. These portions of the liner were ranked from 1
226 (on the food surface) to 8 (the furthest from the food surface), and were transferred to separate

227 vials containing food medium. 7 days later the flies that eclosed were counted, and we used the
228 rankings to calculate a mean pupation height, such that a higher value indicates a farther distance
229 from the food. We performed this experiment for our *D. simulans* strain (*Lhr*), our reciprocal
230 hybrid crosses (melX and simX in an LH_M background), the *D. melanogaster* strain w^{1118}, and
231 melX hybrids in a w^{1118} background (w^{1118} is the background strain for the majority of
232 deficiencies we used in our screen).
233
234

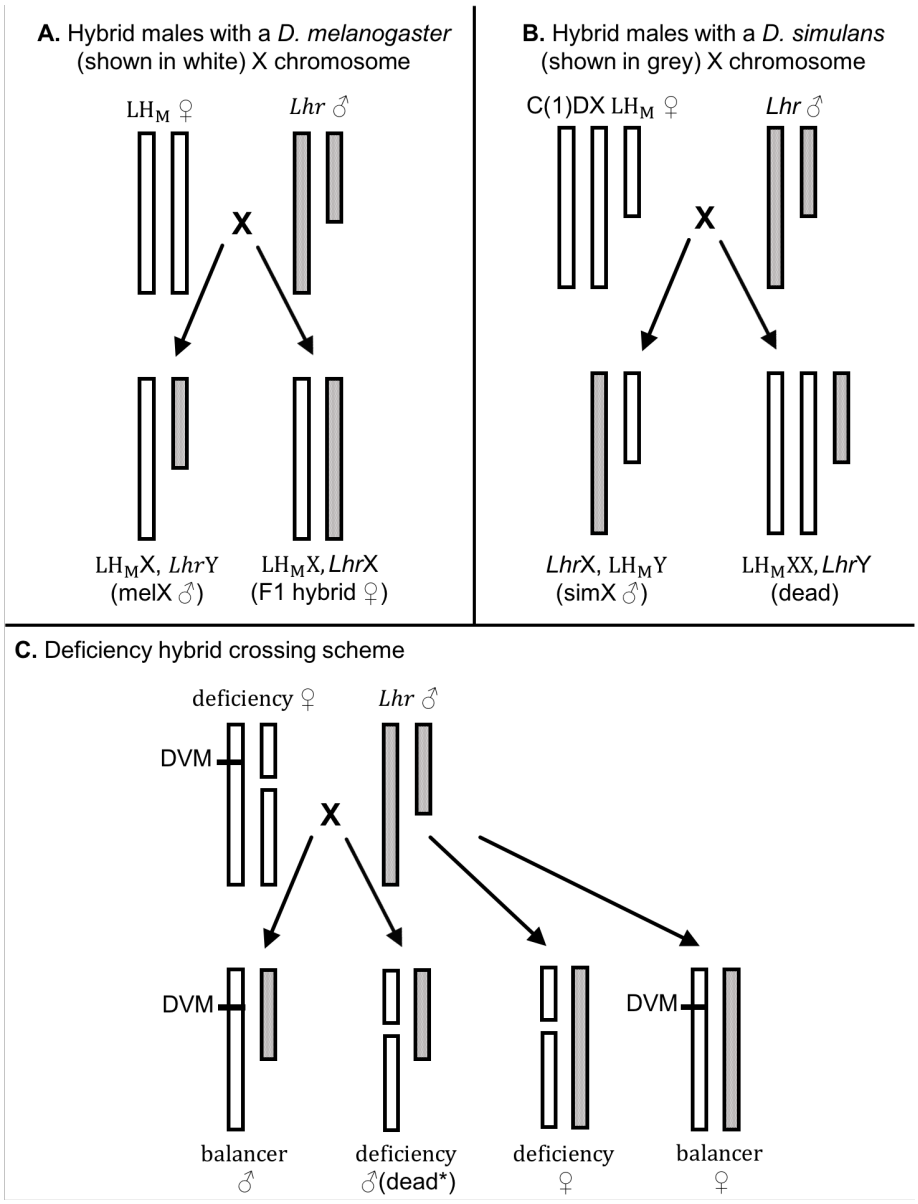


Fig 1. Crossing schemes to generate reciprocal X chromosome hybrid males and deficiency/balancer hybrid females. A. Crossing wild-type *D. melanogaster* females (LH_M, shown in white) to *D. simulans* males (*Lhr*, shown in grey) produces hybrid males with a *D. melanogaster* X chromosome (melX) and hybrid females. **B.** Crossing a compound X C(1)DX LH_M female to *Lhr* males produces hybrid males with a *D. simulans* X chromosome (simX). Females of this cross would inherit two *D. melanogaster* X chromosomes and a *D. simulans* Y chromosome, but are inviable. **A and B.** Note that the background of the reciprocal male hybrids resulting from each cross (melX and simX) is an identical combination of *Lhr* and LH_M with the exception of the sex chromosomes. **C.** Crossing *D. melanogaster* X chromosome deficiency lines, which have a balancer X chromosome with a dominant visible marker (DVM) and an X chromosome with a large deletion, to *Lhr* produces deficiency hybrid females, balancer females, balancer males, and deficiency males (*mostly dead due to large deletions on a hemizygous chromosome with some deficiency lines being exceptions).

249 **Mapping hybrid pupation behavior using the Bloomington Deficiency Kit**

250 Because we found a significant effect of the X chromosome on the difference in pupation site
251 choice behavior between *D. simulans* and *D. melanogaster*, we devised a crossing scheme using
252 molecularly engineered chromosomal deficiencies to screen the X chromosome for loci
253 contributing to this difference. These deficiencies are part of the Bloomington Deficiency Kit
254 (Cook et al., 2012), available from the Bloomington Drosophila Stock Center (BDSC). We
255 assayed a total of 90 deficiency strains covering 87% of the X chromosome (Table S2). We
256 restricted our deficiency screen to lines from the BSC, Exelixis, and DrosDel sets to control for
257 strain background effects while also maximizing chromosome coverage.

258 To set up crosses, we collected deficiency females as young virgins (2-3 hours after
259 eclosing) and crossed them to *D. simulans* males from the *Lhr* strain. After 3 days in the cross
260 vial, we transferred males and females into pupation vials for 24-48 h, at which time the flies
261 were removed. We then divided the pupations vials into "on" and "off" vials as we did for our F1
262 hybrids (above).

263 These crosses produced two types of hybrid female that were heterozygous for *D.*
264 *melanogaster*/*D. simulans* at each autosome (Fig 1C). The deficiency hybrid females contain the
265 *D. melanogaster* deficiency X chromosome and a *D. simulans* X chromosome, making them
266 hemizygous for a segment of the X chromosome. At this locus, these hybrid females only
267 express *D. simulans* alleles. The balancer hybrid females contained the *D. melanogaster* balancer
268 X chromosome (marked with a dominant visible marker) and a *D. simulans* X chromosome.
269 These females are heterozygous for *D. melanogaster*/*D. simulans* over the entirety of the X
270 chromosome, and thus express both *D. simulans* and *D. melanogaster* alleles. Although the
271 deficiency hybrids are our flies of interest, the balancer hybrids provide an experimental control,

272 as these females developed in the same environment as our experimental flies. As a result, we
273 calculated the proportion of deficiency hybrid females that pupated on the food and the
274 proportion of balancer hybrid females that pupated on the food. We then used these measures to
275 calculate a "pupation index" as the proportion of deficiency hybrids pupating on the food divided
276 by the proportion of balancer females pupating on the food. To increase the accuracy of our
277 estimates, we only included pupation vials in our analysis that yielded at least 10 of each type of
278 female. For each deficiency hybrid strain we measured, we report the median pupation index of
279 all replicates, because there were often high-scoring outliers that significantly skewed the mean
280 pupation index. These outliers almost always had abnormally high pupation indices, so focusing
281 on median values makes our findings more conservative.

282 Any deficiency hybrid cross with a median pupation index greater than 1 indicates that
283 more deficiency females pupated on the food compared to balancer females, potentially because
284 the deficiency includes *D. melanogaster* genetic variation that is involved in pupation site choice
285 behavior. Alternatively, simply creating flies that are hemizygous at a locus on the X
286 chromosome may result in a variety of pleiotropic effects that make larvae less likely to climb up
287 the vial. To test for this, when a deficiency hybrid cross showed a pupation index significantly
288 greater than 1 (Table S2), we crossed that *D. melanogaster* deficiency strain to a *D.*
289 *melanogaster* wild-type strain (T.4). If these *D. melanogaster* deficiency crosses displayed the
290 same pattern, we considered the effect of the deficiency on pupation behavior to be a byproduct
291 of deleting a large portion of the X chromosome, rather than revealing recessive *D. simulans*
292 variation, and discarded them. If instead the pupation index for the *D. melanogaster* cross was
293 significantly lower than the pupation index for the *D. simulans* cross, we pursued that deficiency
294 for further validation. To ensure that this pattern is not a result of epistasis from the hemizygous

295 region in a hybrid background, we further crossed these deficiencies to an additional *D.*
296 *melanogaster* (T.1) and *D. simulans* (Mex180) strain to test for background-specific effects.
297
298 **Testing candidate genes in deficiency regions using gene knockouts and RNAi knockdown**
299 For regions of interest identified by our deficiency screen, we ordered transgenic knockouts for
300 any genes available within the region at the time. Our deficiency screen identified two regions of
301 interest. The first is the overlap of Df(1)BSC869 and DF(1)ED6720, excluding the region
302 covered by DF(1)ED6727, which did not have a pupation index greater than 1 (Fig 4A, Table
303 S2). The resulting region of interest spans X:4,204,351 - 4,325,174 (Fig 4C). Within the first
304 region, there are 23 genes, of which 20 are protein coding (Table S3A). According to
305 modENCODE expression data, 15 of those 20 protein coding genes are expressed in larvae,
306 while only 6 are also expressed in the larval nervous system, which we would expect for genes
307 regulating behavior (Graveley et al., 2011). Five of these six genes are well described. At the
308 time of assay, only three of the five characterized genes within this region had non-lethal verified
309 loss-of-function alleles available: *Fas2* (Fasciclin2), *mei9* (meiotic 9), and *norpA* (no receptor
310 potential A). We tested knockouts of each for an effect on pupation site choice behavior. We
311 screened two *Fas2* knockouts, the mutant allele $Fas2^{eb112}$ ($Fas2^{eb112}$/FM7c; Grenningloh et al.,
312 1991; provided by Brian McCabe), and a p-element insertion allele, $Fas2^{G0293}$ (former BDSC
313 Stock 11850; full genotype: $w^{67c23}P\{lacW\}fas2^{G0293}$/FM7c). It is important to note that the nature
314 of the lesion is uncharacterized for $Fas2^{G0293}$ (although our behavioral data below suggest that it
315 is indeed a loss of function allele, as it behaves indistinguishably from the verified knockout
316 $Fas2^{eb112}$). We additionally screened the $mei\text{-}9^{A1}$ mutant allele (w^{1} $mei\text{-}9^{A1}$/FM7h; BDSC stock
317 #6792) and the $norpA^{36}$ mutant allele (w^{*} $norpA^{36}$; BDSC stock #6792). Because $norpA^{36}$ is not

318 held over a balancer, we also set up crosses using a *norpA* rescue strain created in the same
319 background (*w* norpA*^{36}; *P{w[+mC]=ninaE.norpA.E}2*; BDSC stock # 52276) as a control. It is
320 worth noting that while *norpA* is expressed in the larval nervous system, it is only partially
321 contained within this region, and is also largely deleted by another deficiency (Df(1)ED6727;
322 Table S2) that did not display a pupation index significantly greater than 1 or 0.88 (the grand
323 mean for all deficiency hybrids).

324 The second region of interest identified by our deficiency screen was the region deleted
325 by DF(1)Exel6255 (X:21,519,203 – 22,517,665; Fig 4D). Within this region are 28 genes, of
326 which 22 are protein coding (Table S3B). Of the 22 protein coding genes, 14 are expressed in
327 larvae – 13 of which have some expression in the larval nervous system (Graveley et al., 2011).
328 Of these, 7 are described. We obtained knockout strains for both of the characterized genes
329 expressed in the larval nervous system that had verified loss-of-function alleles available at the
330 time: *tilB* (touch insensitive larva B) and *wap* (wings apart). We screened two *tilB* mutant alleles,
331 *tilB*^{1} and *tilB*^{2} (*y w tilB*$^{1/2}$ /FM4; Kernan et al., 1994; provided by Daniel Eberl), and the *wap*^{2}
332 mutant allele (*wap*^{2}/FM6; BDSC stock # 8133).

333 Like the deficiency strains, each of our gene disruptions (with the exception of *norpA*) is
334 held over a balancer chromosome with a visible marker. To measure the pupation behavior of
335 hybrids containing knockout copies of these *D. melanogaster* genes, we crossed each *D.*
336 *melanogaster* knockout strain to *Lhr* using the previously described methods, and calculated the
337 pupation index as the proportion of knockout females on food / the proportion of balancer
338 females on the food. As for our deficiency screen, any hybrid knockouts with a median PI greater
339 than 1 for the hybrid cross suggest that the knockout gene may be involved in pupation site
340 choice. We also crossed each *D. melanogaster* knockout strain to a wild-type *D. melanogaster*

341 (T.4) strain to ensure that hybrid knockouts with a pupation index greater than 1 are not simply
342 an artifact of being hemizygous for this particular gene. We crossed knockout strains that
343 displayed the pattern we expect for a gene involved in pupation site choice (i.e. a pupation index
344 significantly greater than 1 when crossed to *D. simulans*, which is also significantly greater than
345 the pupation index when crossed to *D. melanogaster*) to an additional *D. simulans* (Mex180) and
346 *D. melanogaster* (T.1) wild-type strain for verification. For *norpA*, we crossed the mutant and
347 control strains to *Lhr*, and compared hybrid *norpA* mutant females to hybrid *norpA* rescue
348 females. We additionally crossed these strains to the *D. melanogaster* T.4 strain.

349 Our knockout screen identified two genes that appear to be involved in pupation site
350 choice: *tilB* and *Fas2*. We further tested the effects of these genes on pupation behavior using
351 RNAi knockdown in *D. melanogaster*. We used the elav-Gal4 driver (P{w[+mC]=GAL4-
352 elav.L}2/CyO; BDSC #8765), which expresses Gal4 throughout the nervous system. We drove
353 down the expression of *tilB* and *Fas2* throughout the nervous system by crossing elav-Gal4
354 virgin females to UAS-*tilB* (BDSC #29391: y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF03324}attP2)
355 and UAS-*Fas2* (BDSC #34084: y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01098}attP2)
356 males, respectively. The resulting flies express a gene-specific hairpin RNA throughout the
357 nervous system, causing the degradation of mRNA, and thus, reduced expression of that gene
358 (Perkins et al., 2015; Zeng et al., 2015). As experimental controls for each RNAi cross, we also
359 crossed elav-Gal4 virgin females to the RNAi background stock (*y v; attP2, y+* (y^1 v^1 ;
360 P{y[+t7.7]=CaryP}attP2; BDSC stock # 36303) and the Gal4-1 stock (containing a hairpin RNA
361 targeting Gal4 in VALIUM20; BDSC stock # 35784). Together, these controls allow us to
362 account for the effect of both the Gal4 mutation and general expression of hairpin RNA
363 throughout the nervous system. Any differences we detect between these controls and our RNAi

364 crosses must therefore be due to the expression of the gene-specific (*tilB* or *Fas2*) hairpin RNA.
365 We set up pupation vials using the methods described above, and for each cross, we calculated
366 the proportion of RNAi (or control) flies on the food (removing any data points with fewer than
367 20 experimental flies). If more RNAi flies pupate on the food in the experimental cross (in which
368 the expression of the gene is driven down) compared to the control crosses (in which gene
369 expression is unaffected), this provides further support for that gene's involvement in pupation
370 site choice. For these vials, we additionally determined the average pupation height of flies from
371 each cross using the methods described above for F1 hybrids; if RNAi flies pupate closer to the
372 food than flies from the control crosses, this suggests that gene may be involved in pupation site
373 choice.
374
375 **Testing *tilB* for species-specific differences in larval transcript expression**
376 We selected two each of our 11 *D. melanogaster* and 12 *D. simulans* strains to test for larval
377 stage-specific expression differences of candidate genes using real time qRT-PCR – one extreme
378 and one average. For *D. simulans*, we selected Geo288 and Per005 (Table S1), because Geo288
379 has the highest proportion of pupae on the food of the *D. simulans* strains and Per005 is closest
380 to the species mean (Fig 2). For *D. melanogaster*, we selected CA1 and T.4 (Table S1) because
381 T.4 has the lowest proportion of pupae on the food of the *D. melanogaster* strains, and CA1 is
382 closer to the species mean (Fig 2).
383 To harvest larvae from these strains, we allowed adult females to oviposit in standard
384 vials containing food media between the hours of 8 AM and 12 PM over two consecutive days.
385 120 hours after the final oviposition day, we floated larvae out of the food media using a 20%
386 sucrose in water solution, sucked them up using a transfer pipet, briefly rinsed them with DI

387 water on cheesecloth, and snap froze them using liquid nitrogen (Nichols et al., 2012). In this
388 way, we collected 20-30 mg of larvae from two developmental time points: 96 and 120 hours
389 following oviposition. These time points approximate early wandering and late wandering larval
390 stages. We chose these time points because they are presumably when pupation site choice
391 occurs, and because the larvae are large enough for many to be harvested at once using the above
392 methods. We extracted mRNA using the Qiagen RNeasy Plus Mini Kit, and prepared cDNA
393 using the Promega Verso kit.

394 To quantify transcript abundance, we designed primers that span a single intron near the
395 3' end of *tilB* (Huggett et al., 2005). Additionally, we used primers for the gene *RpL32* as an
396 internal control (Al-Atia et al., 1985; Chertemps et al., 2007). *Fas2* is a complex gene with
397 multiple splice forms, so we were unsuccessful in designing general primers that would amplify
398 all transcripts in both species. For this reason, we did not include *Fas2* in these experiments. A
399 full list of primers and transcripts can be found in Table S4A. For each stage and strain, we
400 prepared two to three biological replicates, which we then amplified in two technical replicates
401 for 40 rounds of qPCR. Using *RpL32* transcript number as an internal control, we calculated
402 relative transcript abundance while correcting for species differences in primer efficiency. We
403 estimated primer efficiency differences by serially diluting gDNA from each of our *D.*
404 *melanogaster* and *D. simulans* strains, performing qPCR, and using a standard curve to calculate
405 adjusted amplification factors (Table S4B). To ensure that we were amplifying cDNA made from
406 RNA, and not gDNA contamination, we performed gel electrophoresis on our cDNA samples to
407 ensure we only visualized the short, intron-less, band.
408
409

410 **Estimating effect sizes**

411 We used our results to estimate how much of the difference in pupation site preference
412 between *D. melanogaster* and *D. simulans* can be attributed to: *i*) the X chromosome, *ii*) our
413 deficiencies of interest (Df(1)BSC869/DF(1)ED6720 and Df(1)Exel6255), and *iii*) *Fas2* and *tilB*.
414 We first calculated the "species difference ratio" (using the data from our parental/hybrid screen
415 in Fig 3) by dividing the median proportion of males on the food for *D. simulans* by the median
416 proportion of males on the food for *D. melanogaster* males (species difference ratio = 5.27). We
417 then calculated an "X effect ratio" by dividing the median proportion of simX males on the food
418 by the median proportion of melX males on the food. To determine how much of this species
419 difference can be attributed to the X chromosome, we divided the "X effect ratio" by the
420 "species difference ratio".

421 We then estimated the effect size of our deficiencies by calculating "deficiency effect
422 ratios" (the median pupation index when the deficiency was crossed to the *D. simulans Lhr*
423 strain/the median pupation index when the deficiency was crossed to the *D. melanogaster* T.4
424 strain). Because Df(1)BSC869 and DF(1)ED6720 overlap, we used their mean deficiency effect
425 ratio to estimate the effect size of the overlapping region. To determine how much of the X effect
426 difference can be attributed to each deficiency region, we divided the "deficiency effect ratio" by
427 the "X effect ratio".

428 Finally, we estimated the effect size of our two identified candidate genes, *Fas2* and *tilB*.
429 For each allele we tested, we calculated a "knockout effect ratio" (the median pupation index
430 when the knockout was crossed to the *D. simulans Lhr* strain/the median pupation index when
431 the knockout was crossed to the *D. melanogaster* T.4 strain), and then we used the average
432 knockout effect ratio of the two alleles for each gene (i.e. *Fas2*: average of $Fas2^{eb112}$ and

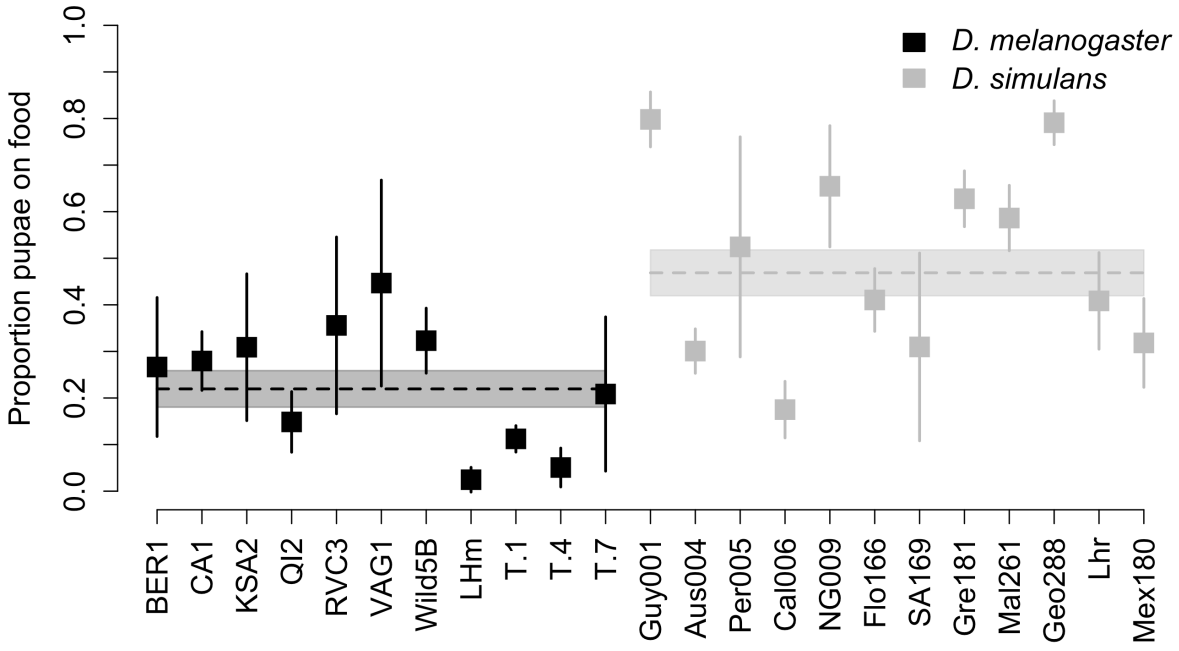
433 *Fas2*^{G0293}; *tilB*: average of *tilB*^{1} and *tilB*^{2}). We then determined the contribution of each gene to
434 its deficiency effect size, and to the overall X effect, by dividing the "knockout effect ratio" by
435 the "deficiency effect ratio" and the "X effect ratio", respectively.
436 For all effect size estimates, we calculated bootstrapped 95% confidence intervals using
437 100,000 bootstraps.
438
439 **Data availability**
440 All relevant data supporting the findings of this study are represented in the figures and
441 supplementary tables.
442

443 Results:

444 **1. Differences in pupation behavior between *D. melanogaster* and *D. simulans***
445 We found significant variation among the 11 *D. melanogaster* and 12 *D. simulans* strains in the
446 proportion of individuals that pupated on the food surface (Fig 2; *D. melanogaster* Wilcoxon test:
447 χ^2= 42.69, df = 10, p<0.0001; *D. simulans* Wilcoxon test: χ^2= 56.34, df = 11, p<0.0001). When
448 we tested for a species difference in pupation behavior, we found that our *D. simulans* lines had
449 significantly more pupae on the food surface compared to our *D. melanogaster* lines, on average
450 (Fig 2; Wilcoxon test: χ^2= 8.37, df = 1, p = 0.0038).
451 Although we controlled egg density to characterize species differences in pupation
452 behavior, there were viability differences among our surveyed lines. As a result, we had
453 significant variation in the total number of pupae in each vial for our *D. melanogaster* lines
454 (ANOVA: $F_{10,65}$=5.58, p<0.0001) and our *D. simulans* lines (ANOVA: $F_{11,66}$=8.01, p<0.0001).
455 Previous studies have found that larval density correlates with pupation height in *D.*

456 *melanogaster* (Sokal et al., 1960). To control for differences in density, we also performed the
457 same analyses above using the residuals from a regression between number of pupae in the vial
458 and proportion of pupae on the food. None of our findings changed using this analysis (Fig S1),
459 indicating that our results were not affected by variation in larval density.

460 Although most of our *D. melanogaster* lines have been in the lab since the 1950s and
461 1960s, we found no difference in the proportion of pupae on the food between *D. simulans*
462 strains collected in the 1950s/1960s and those collected in the 2000s (Table S1; Wilcoxon test:
463 χ^2= 0.24, df=1, p=0.62), indicating that the species difference we report here is unlikely to be an
464 artifact of laboratory adaptation in our surveyed strains.



465

466 **Fig 2. Pupation behavior differences between *D. melanogaster* and *D. simulans*.**
467 The mean proportion of individuals in a vial that pupated on the surface of the food for 11 *D.*
468 *melanogaster* lines and 12 *D. simulans* lines described in Table S1. Error bars denote the 95%
469 confidence interval around each individual mean (N = 5-8). The dashed horizontal lines indicate the grand
470 mean for each species. The boxes surrounding the dashed lines denote the 95% confidence interval
471 around the grand mean.
472

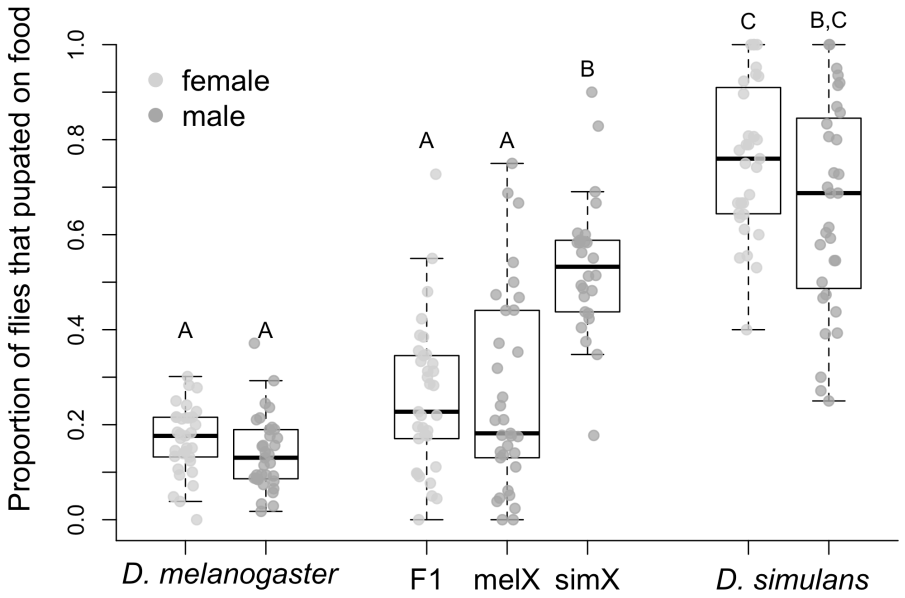
473 **2. Differences in pupation behavior have a significant X effect**

474 We found significant differences among F1 hybrid genotypes when we screened hybrids
475 alongside their parental strains (Fig 3; Full model Wilcoxon test: χ^2= 144.57, df= 6, p<0.0001).
476 Specifically, a significantly higher proportion of F1 hybrid males pupated on the food when they
477 had inherited a *D. simulans* X chromosome (simX males) compared to a *D. melanogaster* X
478 chromosome (melX males), indicating that this species divergence in pupation behavior has a
479 significant X effect (p<0.0001 after correcting for multiple comparisons; Fig 3). This is
480 supported by the fact that the proportion of individuals that pupated on the food was not
481 significantly different between melX hybrid males and *D. melanogaster* males, or between simX
482 hybrid males and *D. simulans* males. All of these findings are unchanged when we control for
483 density effects (Fig S2).

484 Additionally, we found no difference in the proportion of F1 hybrid females and melX
485 hybrid males (p=0.70) that pupated on the food, while F1 hybrid females pupated on the food
486 significantly less often compared to simX hybrid males (p<0.0001 after correcting for multiple
487 comparisons; Fig 3). The fact that F1 hybrid females behave identically to melX hybrid males
488 indicates that the variation in pupation behavior on the *D. melanogaster* X chromosome (i.e.
489 fewer pupae on the food) is dominant to the pupation behavior on the *D. simulans* X
490 chromosome (i.e. more pupae on the food), because F1 hybrid females have one X chromosome
491 from each species.

492 The fact that hybrid males and females with a *D. melanogaster* X chromosome behave
493 indistinguishably from their *D. melanogaster* parent strain, and hybrid males with a *D. simulans*
494 X chromosome behave indistinguishably from their *D. simulans* parent strain (Fig 3), suggests
495 that hybrid pupation site choice behavior falls well within the typical range exhibited by either

496 parent strain. We found a similar pattern when we compared the average pupation height of F1
497 hybrids and their parents: F1 females and melX males did not have significantly different
498 pupation heights compared to their *D. melanogaster* parents, and simX males did not differ from
499 their *D. simulans* parental strain (Fig S3, Table S5). This is important to note, as it implies that
500 hybrids, particularly simX hybrids, are not just unable to climb the vial walls due to
501 developmental inconsistencies caused by intrinsic incompatibilities. If anything, simX hybrids
502 displayed slightly higher proportions of individuals climbing the vial walls (and pupated slightly
503 further from the food) compared to their parent strain, underscoring their vigor.



504

505 **Fig 3. Pupation site choice behavior for *D. melanogaster*, *D. simulans*, and their F1 hybrids.** The
506 proportion of individuals that pupated on the food surface for males and females from both species and
507 their F1 hybrids. *D. melanogaster* males and females were taken from the LH_M strain, while *D. simulans*
508 males and females were taken from the *Lhr* strain. F1 hybrids resulted from a cross between these two
509 strains. The "melX" hybrid males have the *D. melanogaster* X chromosome, and the "simX" hybrid males
510 have the *D. simulans* X chromosome. Both hybrids have *D. melanogaster* cytoplasmic inheritance. Box
511 plots display the median (bold bar), interquartile range (box), and full extent of the data set excluding
512 outliers (whiskers). Bars labeled with different letters are significantly different from one another after
513 sequential Bonferroni correction for multiple comparisons ($p < 0.0001$ after correction, N = 26-33).

514 **3. A deficiency screen of the X chromosome identifies two regions of interest**

515 We found significant variation in pupation index among the deficiency hybrid crosses
516 (Kruskal-Wallis Test: χ^2=336.90, df=89, p<0.0001; Fig 4A). A pupation index greater than 1
517 indicates that more deficiency hybrids pupated on the food than balancer hybrids. This suggests
518 that the *D. melanogaster* deficiency region may be revealing recessive *D. simulans* genetic
519 variation that causes the deficiency hybrids to pupate on the food surface. However, the average
520 pupation index across all 90 deficiency hybrid crosses was 0.88 (Fig 4A), which was
521 significantly lower that our expected mean of 1 (Wilcoxon test: p<0.0001). As a result, we
522 compared the pupation index for all deficiencies to our expected value of 1 and to the grand
523 mean pupation index for these lines (0.88). Six deficiencies had pupation indices significantly
524 greater than one: (Df(1)ED411, Df(1)BSC869, Df(1)ED6720, Df(1)ED6906, Df(1)BSC530, and
525 Df(1)Exel6255). Three of these, (Df(1)BSC869, Df(1)ED6906, and Df(1)Exel6255), remained
526 significant after sequential Bonferroni correction for multiple comparisons (Fig 4A; Table S2).
527 Because the other 3 deficiencies, (Df(1)ED411, Df(1)ED6720, and Df(1)BSC530), had pupation
528 indices significantly greater than 0.88 after sequential Bonferroni correction (Table S2), we
529 included them in our list of potential deficiencies of interest.

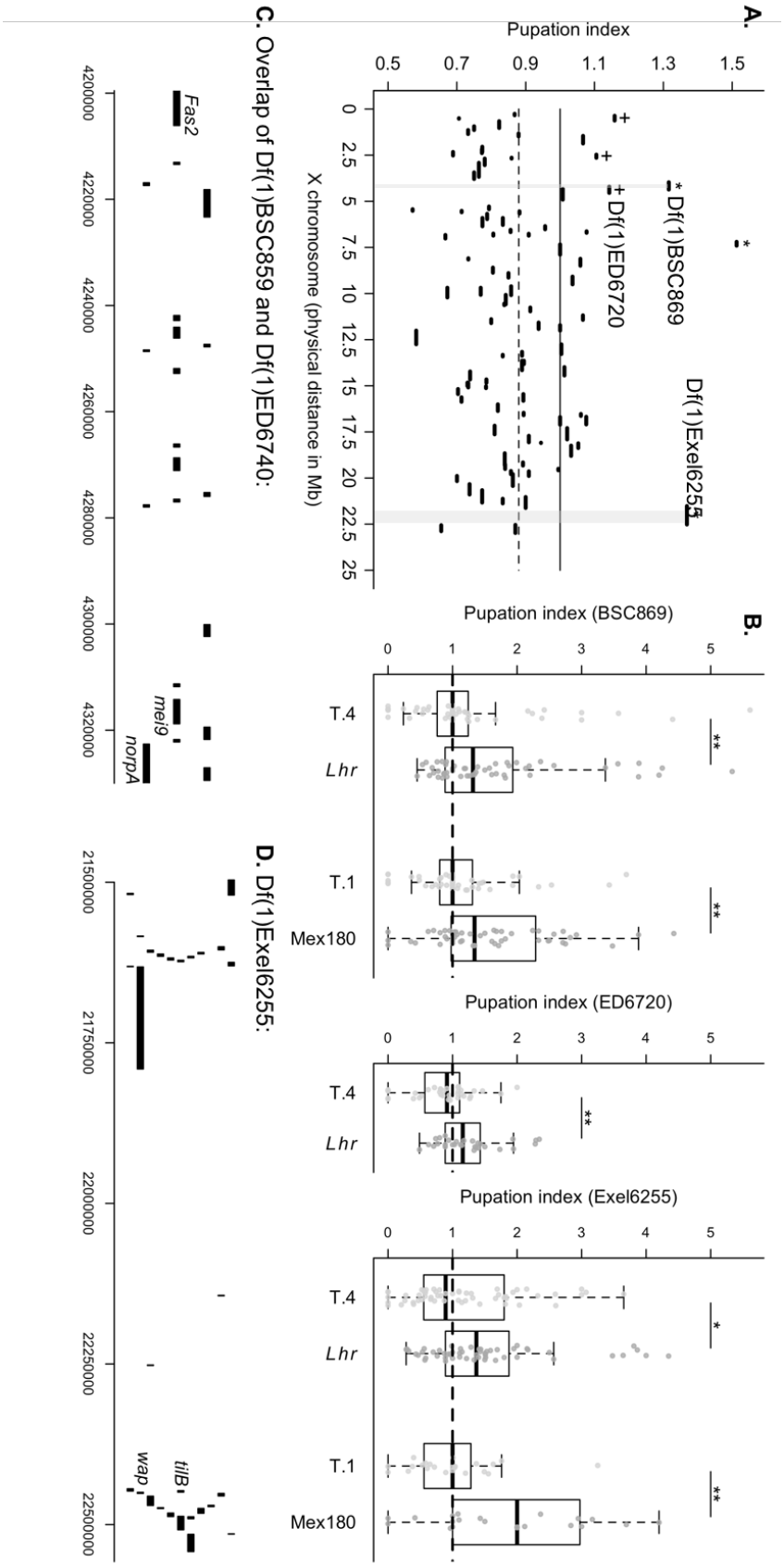
530 To ensure that the deficient region actually reveals *D. simulans* variation contributing to
531 pupation site choice behavior, rather than creating lines that behave abnormally due to the
532 extended hemizygosity within the deficiency region, we crossed each of the six significant
533 deficiencies listed above to the T.4 wild-type *D. melanogaster* strain. For two of the six
534 deficiency strains, Df(1)ED411 and Df(1)BSC530, we found no difference in the pupation index
535 when crossed to *D. melanogaster* compared to the pupation index when crossed to the *Lhr* strain
536 (Wilcoxon tests; Df(1)ED411: n= 29-36, χ^2=1.74, p=0.19, Fig S4A; Df(1)BSC530: n=18-22,

537 χ^2=1.47, p=0.23, Fig S4C), and Df(1)ED6906 had a significantly higher pupation index when
538 crossed to *D. melanogaster* (n= 26-33, χ^2=9.42, p=0.002; Fig S4B). These results suggest that
539 the phenotypes of these three lines are a result of their hemizygosity within the deficiency region,
540 as the deficient *D. melanogaster* females still pupate on the food more often than the balancer
541 females. Note, this effect is unlikely to be driven by the balancer females, as the same balancers
542 occur in many of the other deficiency lines we screened.

543 When we crossed the remaining three deficiency strains, Df(1)BSC869, Df(1)ED6720,
544 and Df(1)Exel6255, to the T.4 *D. melanogaster* strain, we found a pupation index significantly
545 lower than the index we calculated when crossing to *Lhr* (Wilcoxon tests; Df(1)BSC869: n= 51,
546 χ^2=4.35, p=0.037; Df(1)BSC6720: n= 30-35, χ^2=7.90, p=0.0049; Df(1)Exel6255: n= 55-57,
547 χ^2=4.68, p=0.0306; Fig 4B), and no different than one (Df(1)BSC869: p=0.46; Df(1)BSC6720:
548 p=0.10; Df(1)Exel6255: p=0.50). This result suggests that these deficient regions do reveal
549 recessive *D. simulans* variation affecting pupation site choice behavior in hybrids, but have no
550 effect when made hemizygous in *D. melanogaster.* Two of these three deficiencies overlap:
551 Df(1)BSC869 and Df(1)ED6720 (Fig 4A). To further confirm that this pattern is not unique to
552 these specific *D. melanogaster* and *D. simulans* wild-type strains, we crossed one of the
553 overlapping deficiency strains, Df(1)BSC869, and Df(1)Exel6255 to another *D. melanogaster*
554 wild-type strain (T.1) and another *D. simulans* wild-type strain (Mex180). The pattern remained
555 consistent for both of these deficiencies: when crossed to *D. melanogaster,* the pupation index
556 was significantly lower than when crossed to *D. simulans* (Fig 4B; Wilcoxon tests: Df(1)BSC869:
557 n=50-51, χ^2=7.78, p=0.0053; Df(1)Exel6255: n= 23, χ^2=7.74, p=0.0054).

558 One deficiency strain, Df(1)ED7265, had a pupation index significantly lower than the
559 grand mean of all the lines (0.88) following Bonferroni correction for multiple comparisons

560 (Table S2). This suggests the potential for a region of the *D. simulans* X chromosome with
561 transgressive effects—that is, a locus that causes *D. simulans* larvae to pupate farther from the
562 food surface. While this is certainly interesting, we did not pursue this region because its effect is
563 contrary to the species-wide difference we found. This locus may be interesting for further
564 investigation, however, as it could contribute to the significant variation in pupation behavior we
565 observed among *D. simulans* strains (Fig 2).


A.
Pupation index
0.5
0.7
0.9
1.1
1.3
1.5
Df(1)BSC869
Df(1)ED6720
Df(1)Exel6255
X chromosome (physical distance in Mb)
0
2.5
5
7.5
10
12.5
15
17.5
20
22.5
25
B.
Pupation index (BSC869)
Pupation index (ED6720)
Pupation index (Exel6255)
T.4
Lhr
T.1
Mex180
C. Overlap of Df(1)BSC859 and Df(1)ED6740:
Fas2
mei9
norpA
4200000
4220000
4240000
4260000
4280000
4300000
4320000
D. Df(1)Exel6255:
wap
tilB
21500000
21750000
22000000
22250000
22500000

566

567 **Fig 4. Hybrid deficiency screen of the X chromosome identifies two regions of interest. A.** The
568 median pupation index for each of the 90 deficiency hybrid crosses (y-axis) is plotted by the physical map
569 distance each engineered deletion spans along the X chromosome (x-axis). Deficiencies with a pupation
570 index significantly greater than 1 (solid line) after correction for multiple comparisons are denoted by an
571 asterisk (significance levels for all deficiencies are listed in Table S2). Deficiencies with a median
572 pupation index significantly greater than 0.88 (the grand mean, dashed line) after correction for multiple
573 comparisons are denoted with a cross. All of these lines also had pupation indices significantly greater
574 than 1 before correcting for multiple comparisons. The two regions we pursued for candidate gene
575 validation are highlighted in light grey. Note, we did not pursue the remaining three significant deficiency
576 strains because they showed similar pupation indices when crossed to *D. melanogaster* (Fig S4). **B.** The
577 pupation indices of the deficiencies from the grey highlighted areas in Part A are shown for the original
578 hybrid cross (*Lhr*) and for a cross to the T.4 *D. melanogaster* strain. BSC869 and Exel6255 were
579 additionally crossed to *D. simulans* strain Mex180 and *D. melanogaster* strain T.1. Asterisks denote a
580 significant difference between pupation indices of deficiency strains crossed to *D. melanogaster* and *D.*
581 *simulans* (* = $p < 0.05$, ** = $p < 0.01$). **C.** The region uncovered by the overlap of deficiencies BSC869
582 and ED6720 and the 23 genes contained within it. Direction of the gene transcript is denoted with an
583 arrow. The three genes with available disruption strains *(Fas2*, *mei9,* and *norpA*) are labelled. **D.** The
584 region uncovered by Exel6255 and the 28 genes contained within it. Direction of the gene transcript is
585 denoted with an arrow. The two genes with available disruption strains *(tilB* and *wap*) are labelled.
586

587 **4. Gene knockouts and RNAi knockdown suggest that *Fas2* is involved in divergent**

588 **pupation behavior**

589 We tested knockouts of three candidate genes in our first region of interest: the overlap of
590 Df(1)BSC869 and DF(1)ED6720, excluding the region covered by DF(1)ED6727 (Fig 4C). We
591 found no significant difference in the pupation index obtained when we crossed the *mei-9*^{A1}
592 mutant allele to the *Lhr D. simulans* strain and the T.4 *D. melanogaster* strain (Wilcoxon test: n=
593 51-52, χ^2=0.37, p=0.54; Fig S5A), indicating that *mei9* is unlikely to be involved in pupation site
594 choice. Similarly, we found no significant differences in the pupation behavior of female hybrids
595 containing the *norpA*^{36} mutant allele and female hybrids containing a *norpA* rescue allele
596 (Wilcoxon test: n= 22-24, χ^2=3.10, p=0.08 Fig S6), with the knockout hybrids actually having a
597 slightly lower proportion of flies pupating on the food. These data suggest that gene knockouts
598 with expression in the larval nervous system do not, in general, increase the number of hybrids
599 pupating on the food.

600 In contrast, when we crossed the mutant allele *Fas2*^{eb112} to *D. simulans* (*Lhr*) and *D.*
601 *melanogaster* (T.4), we found that *Fas2*^{eb112} hybrids had a significantly higher pupation index
602 than the *D. melanogaster* knockouts (Wilcoxon test: n= 50-51, χ^2= 6.97, p= 0.0083; Fig 5A),
603 suggesting that *Fas2* may be involved in pupation site choice. To ensure this pattern is not
604 unique to these strains, we crossed *Fas2*^{eb112} to additional *D. simulans* (Mex180) and *D.*
605 *melanogaster* (T.1) wild-type strains. We again found the same pattern: the pupation index for
606 knockout hybrids was significantly higher than for *D. melanogaster* knockouts (Wilcoxon test:
607 n= 52-53, χ^2= 27.2, p<0.0001; Fig 5A). As further verification, we tested a second *Fas2* strain: a
608 p-element insertion allele, *Fas2*^{G0293}, and similarly found that the pupation index for *Fas2*^{G0293}
609 hybrids (crossed to *Lhr*) was significantly higher than that for *D. melanogaster* knockouts
610 (crossed to T.4; Wilcoxon test: n= 52-53, χ^2= 9.73, p=0.0018; Fig 5B). Although the nature of
611 the lesion is uncharacterized for *Fas2*^{G0293}, these results suggest that it is indeed a loss of
612 function allele, as it behaves indistinguishably from the verified knockout *Fas2*^{eb112}. We used the
613 consensus combined p-value test (Rice, 1990), which tests the combined effect of independent
614 tests of the same hypothesis, to look at the overall pattern for *Fas2*^{eb112}, and *Fas2* as a whole (i.e.
615 including results from both *Fas2*^{eb112} and *Fas2*^{G0293}), and found a strongly significant pattern of
616 higher pupation indices for hybrid crosses compared to *D. melanogaster* crosses (*Fas2*^{eb112}: p=
617 3.59 x 10^{-6}; *Fas2*: p= 2.35 x 10^{-8}).
618 Next, we used RNAi with the elav-Gal4 driver to reduce expression of *Fas2* throughout
619 the nervous system in *D. melanogaster*. We found that a significantly higher proportion of *Fas2*
620 RNAi flies pupated on the food compared to the control flies from either the background
621 (Wilcoxon test: n= 42-48, p<0.0001 after sequential Bonferroni correction) or Gal4-1 cross
622 (Wilcoxon test: n= 42-43, p<0.0001 after sequential Bonferroni correction; Fig 5C); these results

623 are unchanged when we control for density effects (Fig S7A). Similarly, when we compared
624 pupation height, we found that RNAi flies pupated significantly closer to the food compared to
625 both the background (Wilcoxon test: n=42-48, $p<0.0001$ after sequential Bonferroni correction)
626 and Gal-4 crosses (Wilcoxon test: n= 42-43, $p<0.0001$ after sequential Bonferroni correction; Fig
627 S8A), providing further evidence for *Fas2*'s role in pupation site choice.
628

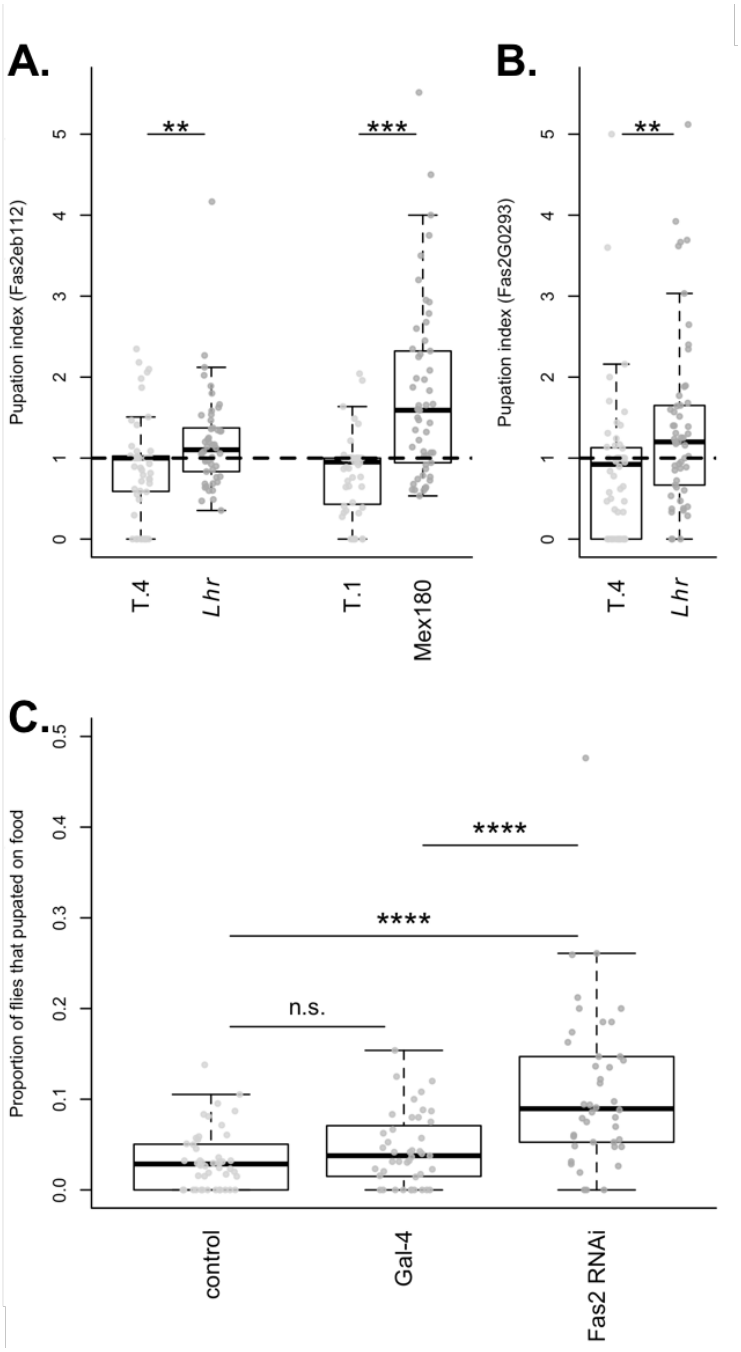


Fig 5. Knockouts and RNAi knockdown confirm the role of *Fas2* in evolved differences in pupation site choice. A. The pupation indices of the *Fas2*^{eb112} gene disruption are shown for the comparison between the original hybrid cross (*Lhr*) and a cross to the *D. melanogaster* T.4 strain. Also shown is the comparison for crosses to the *D. simulans* strain Mex180 and the *D. melanogaster* strain T.1. **B.** The pupation indices for a second gene disruption, *Fas2*^{G0293}, are shown for the original hybrid cross (*Lhr*) and a *D. melanogaster* strain (T.4). For A and B, asterisks denote significance (** = $p < 0.01$, **** = $p<0.0001$; N = 50-53). **C.** The results of pan-neuronal knockdown of the *Fas2* transcript via RNAi. The proportion of RNAi/control individuals found on the food are shown for the control cross (elav-Gal4 driver crossed to the RNAi background stock), the Gal-4 hairpin RNA cross (Gal-4), and *Fas2* RNAi cross. For C, asterisks denote significance after correcting for multiple comparisons (**** = $p<0.0001$; N = 42-48).

640

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

642 **behavior**

643 We tested knockouts of two candidate genes in our second region of interest: DF(1)Exel6255

644 (Fig 4D). We found no significant difference in the pupation index obtained when we crossed the

645 *wap*^{2} mutant allele to the *Lhr D. simulans* strain and the T.4 *D. melanogaster* strain (Wilcoxon

646 test: n= 51-55, χ^2=0.32, p=0.57; Fig S5B), indicating that *wap* is unlikely to be involved in

647 pupation site choice. In contrast, when we crossed the *tilB*^{1} and *tilB*^{2} mutant alleles to *D.*

648 *simulans* (*Lhr*) and *D. melanogaster* (T.4), we found that the *tilB* knockout hybrids had

649 significantly higher pupation indices than the *D. melanogaster* knockouts for both alleles

650 (Wilcoxon tests; *tilB*^{1}: n= 56, χ^2= 6.61, p= 0.0101; Fig 6A; *tilB*^{2}: n= 57, χ^2= 6.61, p= 0.0101; Fig

651 6B). To test whether this difference is consistent in other backgrounds, we crossed both the *tilB*^{1}

652 and *tilB*^{2} mutant alleles to additional *D. simulans* (Mex180) and *D. melanogaster* (T.1) wild-type

653 strains. We had difficulty crossing our *tilB* strains to the Mex180 strain, so our sample sizes for

654 these crosses are smaller, but there is a nonsignificant trend towards a higher pupation index for

655 the knockout hybrids compared to the *D. melanogaster* hybrids for both alleles (Wilcoxon tests;

656 *tilB*^{1}: n=36-37, χ^2= 2.80, p= 0.0943, Fig 6A; *tilB*^{2}: n=12-18, χ^2= 3.44, p= 0.0638, Fig 6B). We

657 used the combined consensus p-value test (Rice, 1990) to look at the overall pattern for *tilB*^{1},

658 *tilB*^{2}, and *tilB* as a whole (i.e. including results from both *tilB*^{1} and *tilB*^{2}), and found a strongly

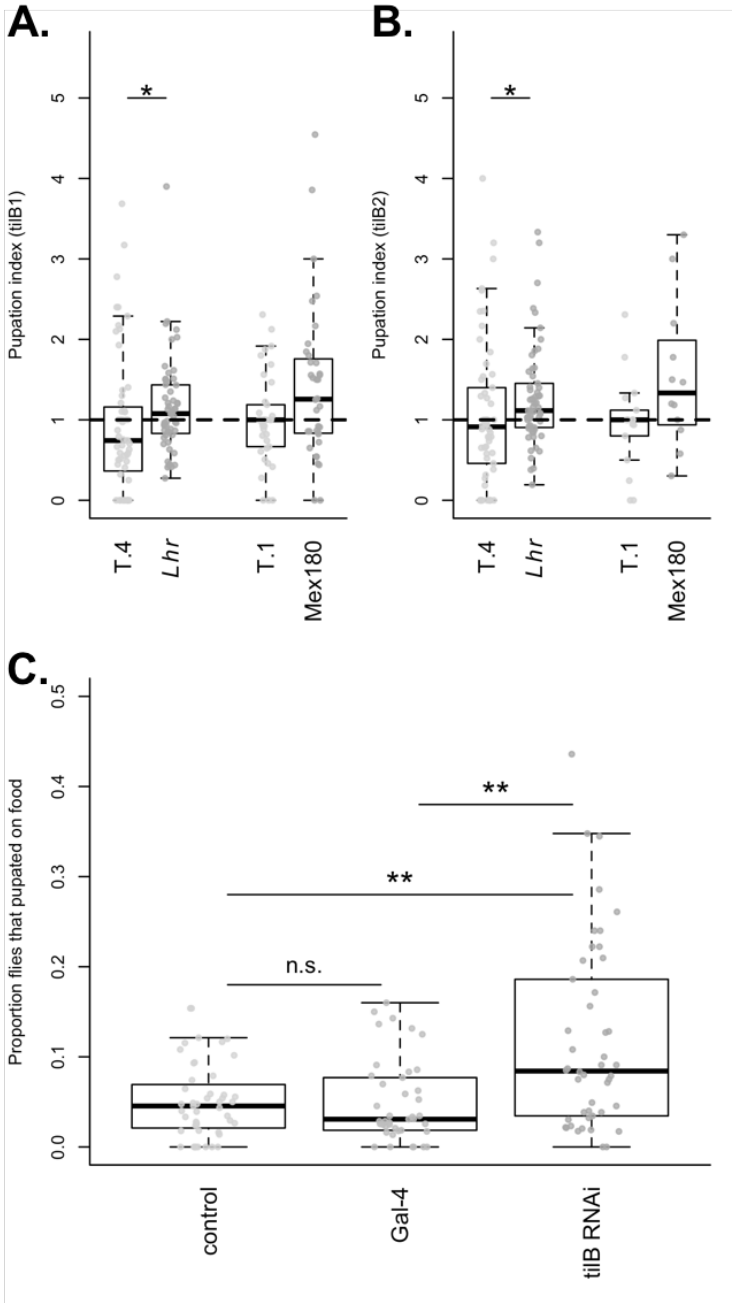
659 significant pattern of higher pupation indices for hybrid crosses compared to *D. melanogaster*

660 crosses (*tilB*^{1}: p= 0.0022; *tilB*^{2}: p= 0.0037; *tilB*: p= 2.47 x 10^{-5}).

661 As for *Fas2* above, we then used RNAi with the elav-Gal4 driver to reduce expression of

662 *tilB* throughout the nervous system in *D. melanogaster*. We found that a significantly higher

663 proportion of RNAi flies pupated on the food compared to the control flies from either the
664 background (Wilcoxon test: n= 46-47, $p<0.01$ after sequential Bonferroni correction) or Gal4-1
665 cross (Wilcoxon test: n= 41-46, $p<0.01$ after sequential Bonferroni correction; Fig 6C); these
666 findings are consistent when we control for density effects (Fig S7B). In addition, when we
667 compared pupation height, we found that RNAi flies pupated significantly closer to the food
668 compared to both the background (Wilcoxon test: n=46-47, $p<0.0001$ after sequential Bonferroni
669 correction) and Gal-4 crosses (Wilcoxon test: n= 46-47, $p<0.0001$ after sequential Bonferroni
670 correction; Fig S8B), providing additional support for *tilB*'s role in pupation site choice.



671

672 **Fig 6. Knockouts and RNAi knockdown confirm the role of *tilB* in evolved differences in pupation**
673 **site choice. A.** The pupation indices of the *tilB*[1] gene disruption are shown for the comparison between
674 the original hybrid cross (*Lhr*) and a cross to the *D. melanogaster* T.4 strain. Also shown is the
675 comparison for crosses to the *D. simulans* strain Mex180 and the *D. melanogaster* strain T.1. **B.** The
676 pupation indices for a second gene disruption, *tilB*[2], are shown for the original hybrid cross (*Lhr*) and the
677 cross to *D. melanogaster* strain T.4, and for crosses to the *D. simulans* strain Mex180, and the *D.*
678 *melanogaster* strain T.1. For A and B, asterisks denote significance (* = $p < 0.05$, ** = $p = 0.01$; N = 41-
679 47). **C.** The results of pan-neuronal knockdown of the *tilB* transcript via RNAi. The proportion of
680 RNAi/control individuals found on the food are shown for the control cross (elav-Gal4 driver crossed to
681 the RNAi background stock), the Gal-4 hairpin RNA cross (Gal-4), and *tilB* RNAi cross. For C, asterisks
682 denote significance after correcting for multiple comparisons (* = $p < 0.05$, ** = $p < 0.01$; N = 41-47).

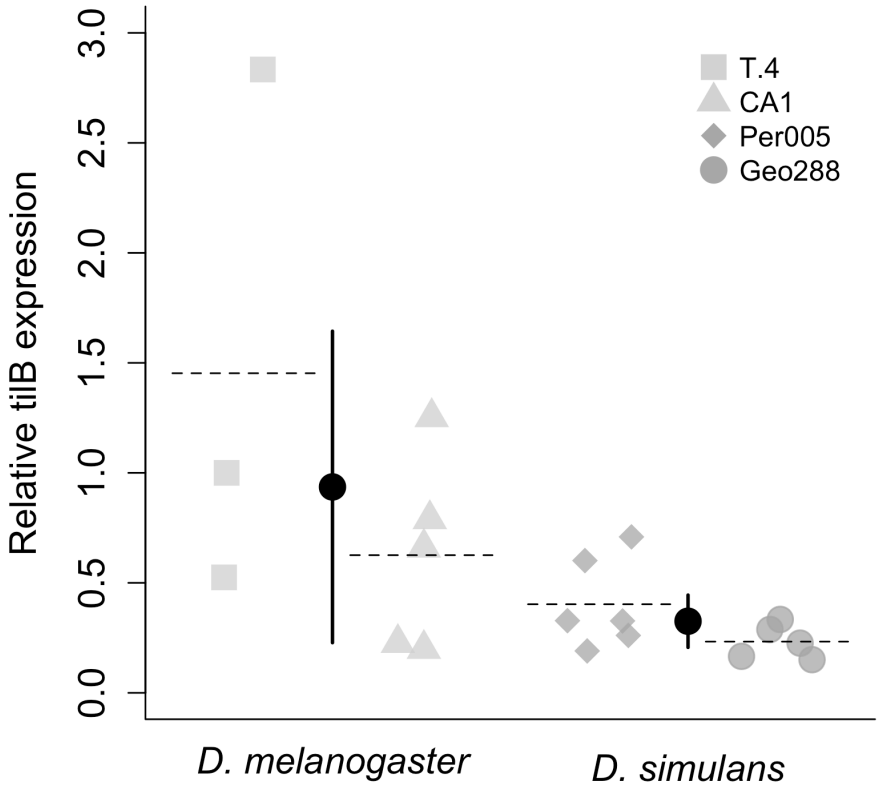
683

684 **6. *tilB* is expressed more highly in *D. melanogaster* strains**

685 We performed qRT-PCR to quantify relative *tilB* transcript abundance for two strains of *D.*
686 *simulans* (Per005 and Geo288) and two strains of *D. melanogaster* (CA1 and T.4). For the two *D.*
687 *simulans* strains and the Geo288 *D. melanogaster* strain, we collected larvae from two stages of
688 larval development (96 and 120 hours following oviposition). For the other *D. melanogaster*
689 strain (T.4), we were only able to obtain enough larval tissue at 96 hours following oviposition,
690 due to low fecundity. Because the relative transcript abundance data had a skewed distribution,
691 we used the reciprocal root transformation to normalize the species and overall distributions
692 (Shapiro-Wilk tests for normality: all $p > 0.34$). We then analyzed the relative transcript
693 abundance of *tilB* using a nested ANOVA with the following factors: species, strain nested
694 within species, larval age (96 and 120 hours), and the interaction between species and larval age.
695 The interaction term between species and larval age was not significant ($p=0.41$), so we removed
696 it from the model. We found that larvae from the 120-hour sampling period had significantly
697 lower *tilB* expression than 96-hour larvae ($F_{1,14}= 6.16$, $p= 0.026$). While we did not detect any
698 significant differences between the 2 strains from the same species ($F_{2,14}=2.39$, $p=0.13$), we
699 found a significantly higher average relative amount of *tilB* transcript in *D. melanogaster* larvae
700 compared to *D. simulans* larvae ($F_{1,14}= 9.74$, $p= 0.0075$; Fig 7).

701 Because we performed qRT-PCR on an extreme strain and a strain closer to the average
702 for each species, these four lines represent a continuum of pupation site choice behavior, with
703 T.4 (*D. melanogaster*) having the lowest proportion of pupae on the food, followed by CA1 (*D.*
704 *melanogaster*), then Per005 (*D. simulans*), and last, Geo288 (*D. simulans*) having the highest
705 proportion of pupae on the food (Fig 2). These four strains follow an identical pattern for *tilB*

706 gene expression, with T.4 having the highest relative transcript abundance, and Geo288 having
707 the lowest (Fig 7). Although it is not possible to detect a significant effect with a sample size of 4,
708 this suggests that *tilB* gene expression may be negatively correlated with the proportion of pupae
709 on the food (Spearman's rank correlation: $r_s = -1$, $p < 0.10$).



710

711 **Fig 7. Relative differences in *tilB* transcript abundance between species.** The relative abundance of
712 *tilB* transcript detected by qRT-PCR in *D. melanogaster* and *D. simulans*. Each data point represents the
713 average of two technical replicates for a single biological replicate. Relative transcript abundance is
714 significantly higher in *D. melanogaster* strains on average ($p < 0.01$). Light grey squares (T.4) and
715 triangles (CA1) represent the two *D. melanogaster* strains measured, while dark grey diamonds (Per005)
716 and circles (Geo288) represent the two *D. simulans* strains. The dashed lines depict the average
717 expression across both time points for each strain. The black circles represent the species-wide mean,
718 and error bars depict the 95% confidence interval surrounding the mean. Note that the exact rank of
719 relative *tilB* expression is perfectly inversely correlated to the pupation indices of each strain (i.e., the
720 strain with the highest *tilB* expression has the lowest proportion of pupae on the food, and so on).
721

722 **7. Effect sizes**

723 We used the results above to estimate how much of the difference in pupation site preference
724 between *D. melanogaster* and *D. simulans* can be attributed to the X chromosome, and found
725 that the X accounts for approximately 55.6% (95% CI= 31.4%-80.2%) of the difference. We then
726 estimated the effect size of our deficiencies of interest, and found that the overlap of
727 Df(1)BSC869 and DF(1)ED6720 explains approximately 44.1% (95% CI= 33.5%-74/5%) of the
728 X chromosome effect, and Df(1)Exel6255 explains approximately 52.3% (95% CI= 33.7%-
729 85.9%) of the X effect. Finally, we estimated the effect size of our two identified candidate genes,
730 *Fas2* and *tilB*. We found that *Fas2* explains approximately 93% (95% CI= 78.5%-134.9%) of the
731 pupation difference attributed to the overlap between_Df(1)BSC869 and DF(1)ED6720, and 41%
732 (95% CI= 33.8%-76.5%) of the pupation difference attributed to the X chromosome. Similarly,
733 we found the *tilB* explains approximately 87.2% (95% CI= 66.2%-135.9%) of the pupation
734 difference attributed to Df(1)Exel6255, and 45.6% (95% CI= 35.5%-76.1%) of the pupation
735 difference attributed to the X chromosome.

736 It is important to note that our effect size estimate for the X chromosome may be an
737 overestimate, as calculating effect sizes using only reciprocal hybrids does not account for
738 potential transgressive autosomal effects (Mittleman et al., 2017). While subject to the same
739 potential effects, the effect size of the deficiencies and individual gene knockouts were
740 calculated as proportions of the total X effect (and also of the deficiency effect for gene
741 knockouts), and thus represent a more accurate estimate of their overall contribution, regardless
742 of the total X effect. Still, these estimates could be affected by variation in the different *D.*
743 *melanogaster* backgrounds in which the deficiencies and knockout strains were made.
744

745 # Discussion

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

747 Our initial survey of pupation site choice behavior in *D. melanogaster* and *D. simulans* expands
748 upon a previously reported interspecific difference (Markow, 1979). Consistent with these
749 previous results, on average, *D. simulans* strains had a greater proportion of flies pupating on the
750 food surface. However, we used 11 *D. melanogaster* and 12 *D. simulans* strains sourced from
751 around the globe to demonstrate this difference. While the species difference holds when
752 comparing the grand mean of all strains for each species, there is substantial variation within
753 species. This variation is so significant that the species' distributions overlap, with some *D.*
754 *simulans* strains, like Mex180 and Cal006, more closely resembling *D. melanogaster* strains in
755 pupation behavior (Fig 2). These documented differences in pupation behavior among
756 *Drosophila* species, in combination with our understanding of the environmental variables that
757 affect this behavior within species (Hodge & Caslaw, 1998; Sameoto & Miller, 1968b;
758 Seyahooei et al., 2009; Sokal et al., 1960), will be useful in identifying the selection pressures (if
759 any) that affect the evolution of this trait. Differences in pupation site choice behavior may be a
760 form a niche partitioning where species co-occur, as has been previously suggested (Arthur &
761 Middlecote, 1984). Alternatively, pupation site choice may be an adaptive response to parasite or
762 parasitoid presence (Kraaijeveld & Godfray, 2003). A globally sourced panel of lines with
763 significant variation, such as we describe, provides an inroad for studies comparing pupation
764 behavior to differences in the ecology of each collection site, such that we can better understand
765 the ultimate causes of this behavioral evolution.

766

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

768 Using hybrid crosses in the same background that controlled for maternal inheritance, we were
769 able to estimate the effect of the X chromosome on pupation behavior. We found no evidence
770 that hybrid behavior was atypical relative to either parent strain, suggesting these measurements
771 are indeed reliable. We found a significant effect of the X, in that reciprocal hybrid males pupate
772 in similar locations as the X-donating parent. We calculate that this chromosome explains 55.6%
773 (95% CI= 31.4%-80.2%) of the total phenotypic difference between parent strains. Although the
774 exact contribution of the X chromosome reported here may be an overestimate due to
775 transgressive autosomal effects that cannot be detected in a hybrid background (Mittleman et al.,
776 2017), a similar X-effect has been detected for pupation behavior when comparing *D. simulans*
777 and *D. sechellia* (Erezyilmaz & Stern, 2013).

778 We also found that hybrid females, which inherit one X chromosome from each parent,
779 pupate like the *D. melanogaster* parent strain. This suggests that *D. melanogaster* alleles are
780 dominant to *D. simulans* alleles. The dominance of *D. melanogaster* alleles makes it possible to
781 use engineered deletions, available in *D. melanogaster* strains, to map regions containing
782 recessive *D. simulans* variation affecting pupation behavior (Cook et al., 2012).

783 The three significant deficiencies identify two regions of the X chromosome with
784 detectable effects on pupation behavior: one spans X:4,204,351 – 4,325,174 and explains ~44.1%
785 of the X-effect, and the other spans X:21,519,203 – 22,517,665 and explains ~52.3% of the X-
786 effect. These regions contain 23 and 28 genes, respectively. Our analysis of the available gene
787 knockouts within these regions points to two genes: *tilB* and *Fas2* (see below). We calculate that
788 *tilB* and *Fas2* explain the majority of the effect of their respective deficiency regions (point
789 estimate = 87.2%, 95% CI= 66.2%-135.9%; point estimate = 93%, 95% CI= 78.5%-134.9%,

790 respectively). Taken together, our results suggest that a substantial share of the difference in
791 pupation behavior between *D. simulans* and *D. melanogaster* can be attributed to just two genes.
792
793 ***tilB* and *Fas2*: loci of evolution for divergent pupation behavior between *D. simulans* and *D.***
794 ***melanogaster***
795 We have presented substantial evidence for a role of both *tilB* (*touch insensitive larva B*) and
796 *Fas2* (*Fasciclin* 2) in the divergence of pupation behavior among these species. For each locus,
797 we have shown that two independent knockouts replicate the pattern of the regions identified by
798 the deficiency screen: hybrid females hemizygous for the *D. simulans* locus pupate on the food
799 surface significantly more often than females with both the *D. simulans* and *D. melanogaster* loci.
800 We then used RNAi knockdown of each gene in *D. melanogaster* to show that reduced
801 expression of *tilB* and *Fas2* transcripts results in a more *D. simulans*-like pupation site choice
802 behavior. To directly test for differences in expression of *tilB*, we performed qRT-PCR during
803 two larval developmental time points. Congruent with our RNAi knockdown results, we find that
804 mean relative transcript abundance is significantly higher in *D. melanogaster* larvae than in *D.*
805 *simulans* larvae. Interestingly, we surveyed two lines per species, representing a continuum of
806 pupation site choice behavior, and found that the mean proportion of pupae on the food surface
807 for these lines was perfectly negatively correlated with *tilB* gene expression. Although these
808 results are only for 4 lines, they suggest that lower *tilB* gene expression may be associated with a
809 higher proportion of larvae pupating on the food surface.
810 While our present study does not present a functional analysis of the *D. melanogaster* or
811 *D. simulans Fas2* or *tilB* alleles, we can use the *D. melanogaster* annotation of each gene to
812 speculate about their role in the evolution of pupation behavior. *Fas2* is a large gene, spanning

813 over 70,000 base pairs, with expression peaking during the larval wandering stage (L3)
814 (Graveley et al., 2011). It is also complex, with seven transcripts composed of various
815 combinations of 16 exons. Broadly, *Fas2* functions as a neuronal recognition molecule, and is
816 involved in patterning the larval nervous system (Grenningloh et al., 1991). Expression of *Fas2*
817 is critical for synapse formation and growth at the larval neuromuscular junction (Davis et al.,
818 1997; Schuster et al., 1996) and is also important for patterning of the larval mushroom body
819 (Kurusu et al., 2002). With expression in both the central and peripheral nervous system, it is
820 possible that differences at the *Fas2* locus differentially wire the *D. melanogaster* and *D.*
821 *simulans* brains, altering how larvae perceive or interpret stimuli. Whether these differences are a
822 result of evolution of the protein sequence, and/or spatial or temporal differences in transcript
823 expression remains to be determined.
824 Unlike *Fas2*, *tilB* is a short gene, spanning just over 1,700 base pairs, with a single
825 transcript composed of 5 exons. *tilB* is also expressed in wandering larvae and pupae, though it
826 shows higher expression in testes of adult males, due to its role in developing sperm flagella
827 (Graveley et al., 2011). In fact, *tilB* is associated with ciliary motility (Kavlie et al., 2010), and is
828 a part of the mechanosensory transduction machinery (Göpfert & Robert, 2003). Mutant *tilB*
829 larvae display normal locomotor activity, but have a reduced withdrawal response to physical
830 disturbance (Kernan et al., 1994). Consistent with this finding, our data suggest that changes in
831 *tilB* expression could potentially result in differences in peripheral sensory perception between *D.*
832 *melanogaster* and *D. simulans* larvae, ultimately influencing larval pupation site choice behavior.
833 A more precise functional analysis of *tilB* is necessary to test this hypothesis.
834
835 **Standards of evidence and the challenges of interspecific mapping**

836 Above, we have discussed the results of our interspecific deficiency screen of the
837 *Drosophila* X chromosome. This technique has long been employed to map morphological traits,
838 physiological traits, hybrid incompatibility loci, (Barbash et al., 2003; Bour et al., 2000; Cattani
839 & Presgraves, 2012; Cote et al., 1986; Konopka & Benzer, 1971; Pardy et al., 2018; Sawamura
840 et al., 2004), and behaviors (Fanara et al., 2002; Laturney & Moehring, 2012; Moehring &
841 Mackay, 2004). Nonetheless, these screens have been criticized due to their susceptibility to
842 epistatic interactions that can produce false positives (Anholt & Mackay, 2004) and for the
843 imperfect comparison of deficiency chromosomes to balancer chromosomes (Stern, 2014). These
844 problems may be exacerbated in a hybrid background. These issues are potentially reflected by
845 the global average pupation index for these deficiencies being 0.88, rather than 1 (Fig 4A),
846 indicating that, on average, more balancer hybrids pupated on the food compared to deficiency
847 hybrids.
848 We attempted to exclude the possibility of false positives in our initial deficiency screen
849 in two ways. First, we crossed each deficiency with a significant pupation index to *D.*
850 *melanogaster* to test for deleterious effects of the deficiencies themselves. Indeed, 3 of 6
851 significant deficiencies produced similar results when crossed to *D. melanogaster*, and were
852 discarded. Next, to ensure the patterns shown by the remaining three were not due to epistasis
853 among strains, we crossed each to a second *D. simulans* and *D. melanogaster* strain and found
854 consistent results. Taken together, these results suggest that these three deficiencies are likely
855 revealing recessive *D. simulans* variation, rather than background epistatic interactions. It should
856 be noted, however, that species-wide epistasis cannot be conclusively ruled out; nonetheless,
857 such a result is still biologically relevant and meaningful.

858 The results of our gene disruption tests further enforce our deficiency screen results,
859 again, with multiple controls to limit the possibility of false positives and/or strain-specific
860 epistatic effects. We tested *D. melanogaster* gene disruption strains in a hybrid background for
861 two or three potential candidate genes that are expressed in the larval nervous system from each
862 region identified by our deficiency screen. For each region, only a single candidate gene (*Fas2*
863 and *tilB*) replicated the deficiency effect, suggesting that disrupting larval nervous system genes
864 in a hybrid background does not generally interfere with pupation behavior. Further, we crossed
865 our *tilB* and *Fas2* gene disruption strains to multiple *D. melanogaster* and *D. simulans* strains,
866 and found no evidence of strain-specific epistatic interactions. Finally, we tested two knockouts
867 of each candidate gene to ensure that our results were not specific to the gene aberration.
868 In addition to our hybrid mapping experiments, we have shown that a reduction in *Fas2*
869 and *tilB* gene expression via RNAi knockdown in a *D. melanogaster* background leads to a more
870 *D. simulans*-like phenotype. This test is performed in a pure *D. melanogaster* background,
871 eliminating the potential for hybrid background epistasis, and the results are consistent with our
872 gene disruption screen. Additionally, these results hold true when calculating both the proportion
873 of flies pupating on the food surface, and the average pupation height. This result is even further
874 corroborated for *tilB*, which shows higher relative expression in *D. melanogaster* compared to *D.*
875 *simulans* strains (Figure 7). Taken together, our hybrid gene disruption screens and gene
876 expression studies in *D. melanogaster* make an excellent case for a role of *tilB* and *Fas2* in
877 divergent pupation site choice behavior.
878 Higher standards of evidence have been suggested for mapping in *Drosophila* – the
879 reciprocal hemizygosity test being the gold standard (Stern, 2014). In this study, we performed
880 one half of this test (hybrid females with a disrupted *D. melanogaster* X chromosome and an

881 intact *D. simulans* X chromosome). Unfortunately, the reciprocal half of the test (hybrid females
882 with a disrupted *D. simulans* X chromosome and an intact *D. melanogaster* X chromosome) is
883 not possible in this case for several reasons. The hybrid cross is only successful in one direction
884 (*D. simulans* males crossed to *D. melanogaster* females), so we would need to use *D. simulans*
885 gene disruption males to create the reciprocal cross. This is not an option, however, as *tilB*
886 disruptions are male sterile, and *Fas2* disruptions are male lethal.

887 A test that could potentially support our findings would be the addition of dominant *D.*
888 *melanogaster Fas2 and tilB* alleles to a *D. simulans* X background to recover the *D.*
889 *melanogaster* behavior of pupating off the food surface. We previously performed a similar
890 experiment in males using *D. melanogaster* Y-linked X duplication strains (Table S6) (Cook et
891 al., 2010). Unfortunately, making hybrid males that have two broad segments of the X
892 chromosome created flies that pupated almost entirely on the surface of the food (Fig S9). A
893 similar experiment could be performed in females using third chromosome-linked X duplication
894 strains (Venken et al., 2010), however this is likely to produce a similar effect. Further, these
895 experiments are prone to the same potential epistatic interactions as the deficiencies.

896 While we have presented multiple lines of evidence, all consistently supporting a role for
897 *tilB* and *Fas2* in divergent pupation site choice behavior, these results must be considered in light
898 of the above caveats. We have taken measures to address these caveats as completely as possible,
899 and present our results with a high standard of evidence. Our study highlights the difficulty of
900 interspecific mapping in producing conclusive results, and underscores a need for transgenic
901 tools to be developed in non-model *Drosophila* species.

902

903 **Areas for future research**

904 Our results highlight two main areas for further research. First, while we now have a
905 better understanding of the genetic underpinnings of pupation site choice behavior evolution, it is
906 unclear why these differences evolved in the first place. The variation we recorded among
907 globally sourced *D. melanogaster* and *D. simulans* strains provides a valuable tool with which to
908 pursue this line of inquiry. Second, while we have provided evidence for two specific genes
909 involved in the evolution of this phenotype, we have little understanding of their function in
910 pupation site choice behavior, or how exactly these functions have evolved. The results of our
911 expression study suggest a role of expression differences for *tilB*, but further functional follow-
912 up is necessary to identify the precise molecular underpinnings of pupation site choice
913 preference.
914

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924
925

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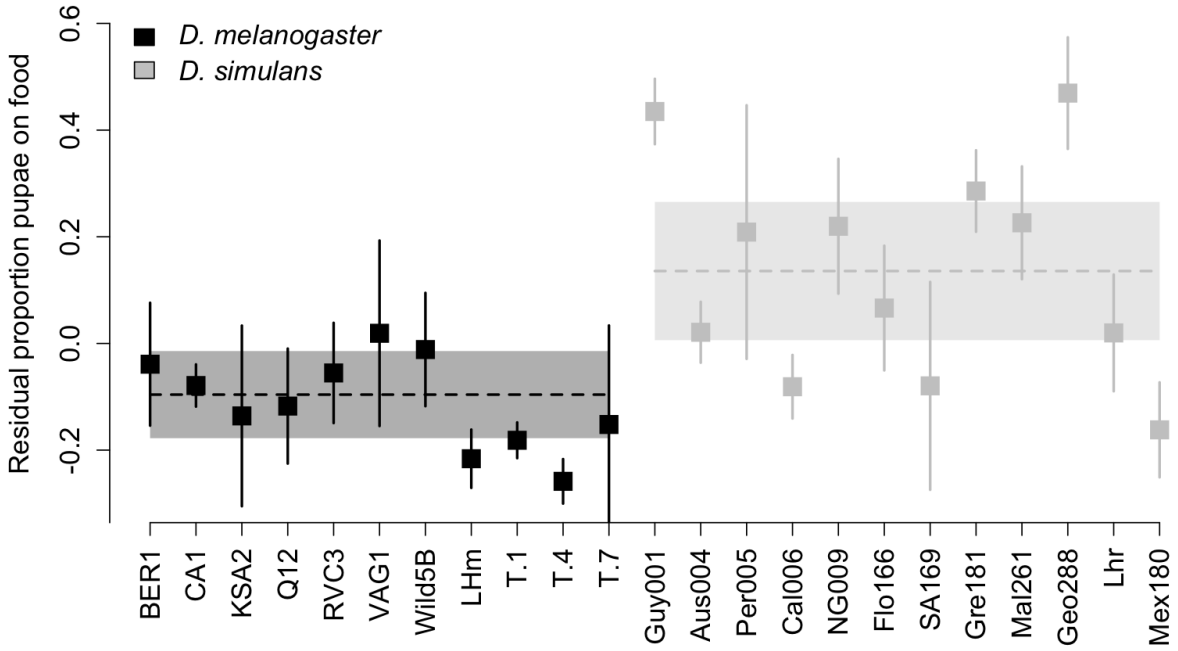
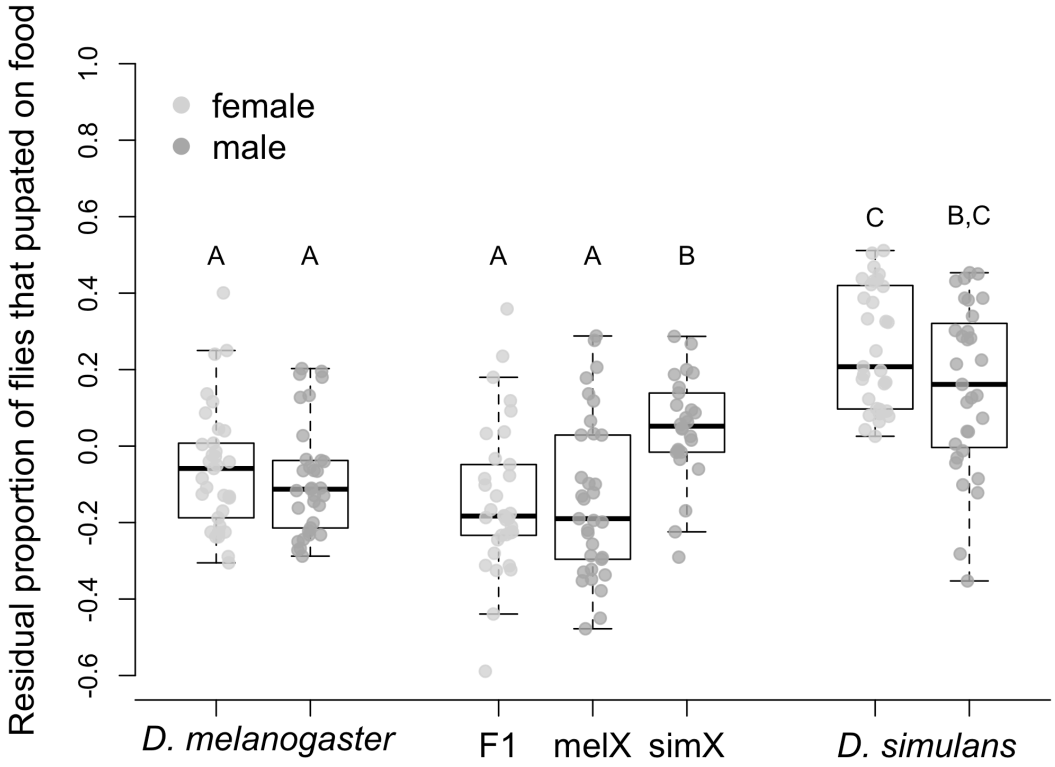
1172 # Supplementary Figures and Captions:



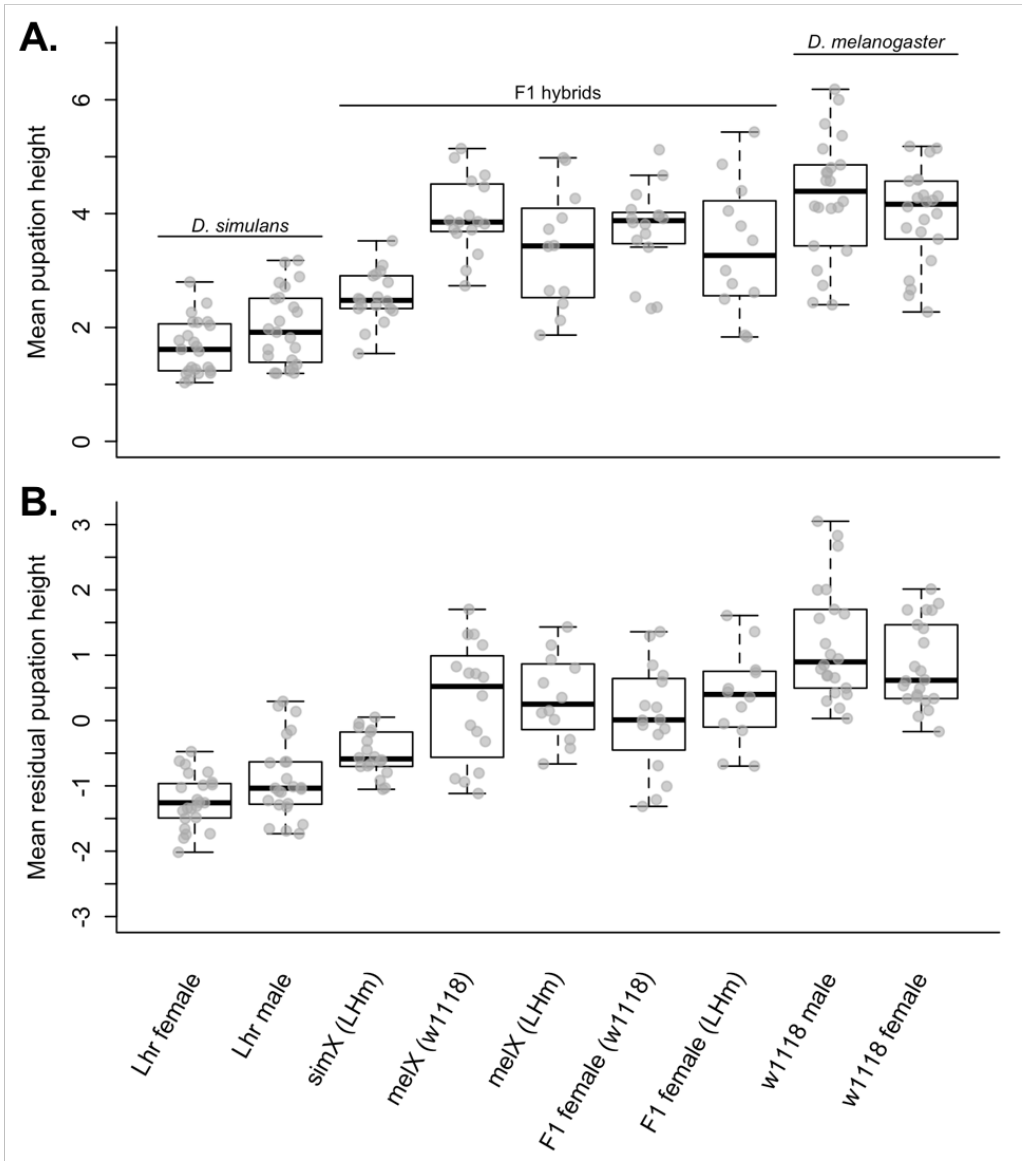
1173

1174 **Fig S1. Pupation behavior differences between *D. melanogaster* and *D. simulans* after controlling**
1175 **for density.** The mean residuals from a regression between the proportion of pupae on the surface of the
1176 food and the total number of pupae in the vial for 11 *D. melanogaster* lines and 12 *D. simulans* lines
1177 described in Table S1. Error bars denote the 95% confidence interval around each individual mean (N =
1178 5-8). The dashed horizontal lines indicate the grand mean for each species. The boxes surrounding the
1179 dashed lines denote the 95% confidence interval around the grand mean.



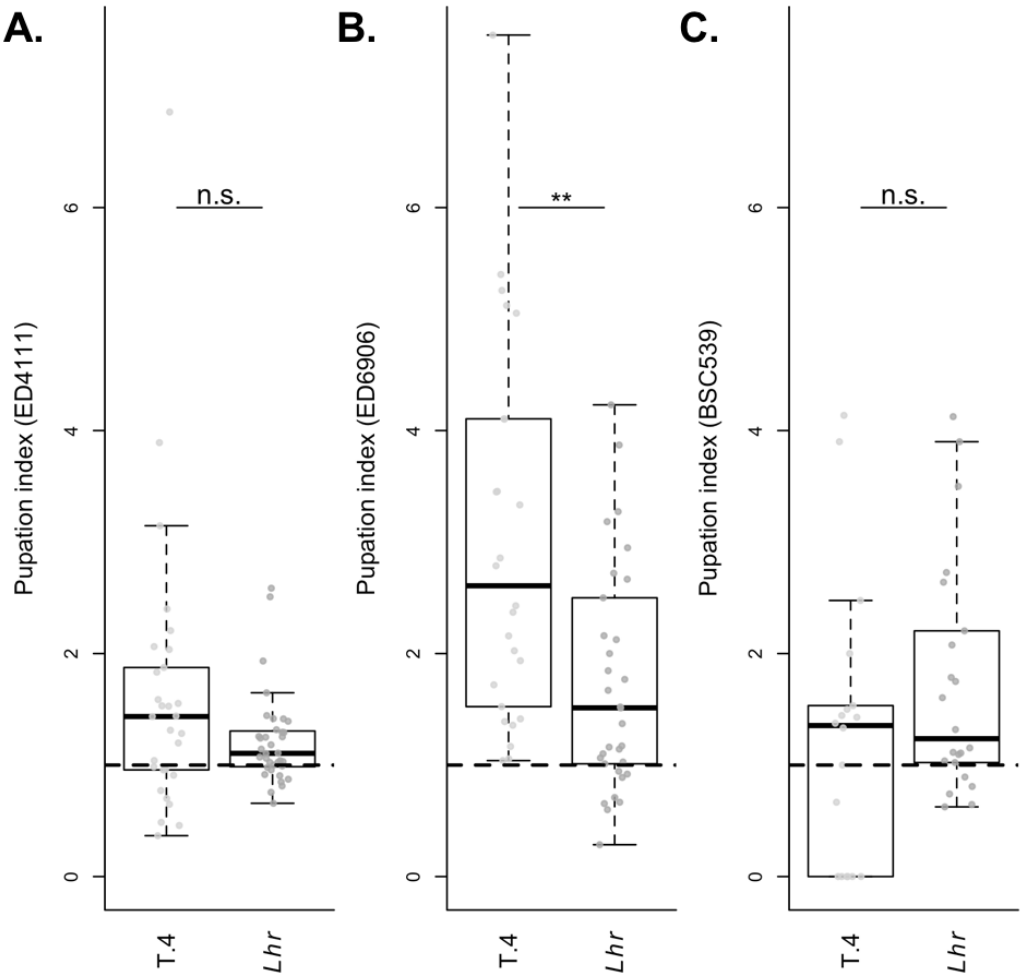
1180

1181 **Fig S2. Pupation behavior for *D. melanogaster*, *D. simulans*, and their F1 hybrids after controlling**
1182 **for density.** The mean residuals from a regression between the proportion of individuals in a vial that
1183 pupated on the surface of the food and the total number of individuals in the vial are shown for males and
1184 females from both species and their F1 hybrids. *D. melanogaster* males and females were taken from the
1185 LH_M strain, while *D. simulans* males and females were taken from the *Lhr* strain. F1 hybrids resulted from
1186 a cross between these two strains. The "melX" hybrid males have the *D. melanogaster* X chromosome,
1187 and the "simX" hybrid males have the *D. simulans* X chromosome. Both hybrids have *D. melanogaster*
1188 cytoplasmic inheritance. Box plots labeled with different letters are significantly different from one another
1189 after sequential Bonferroni adjustment for multiple comparisons ($p < 0.0001$, N = 26-33).



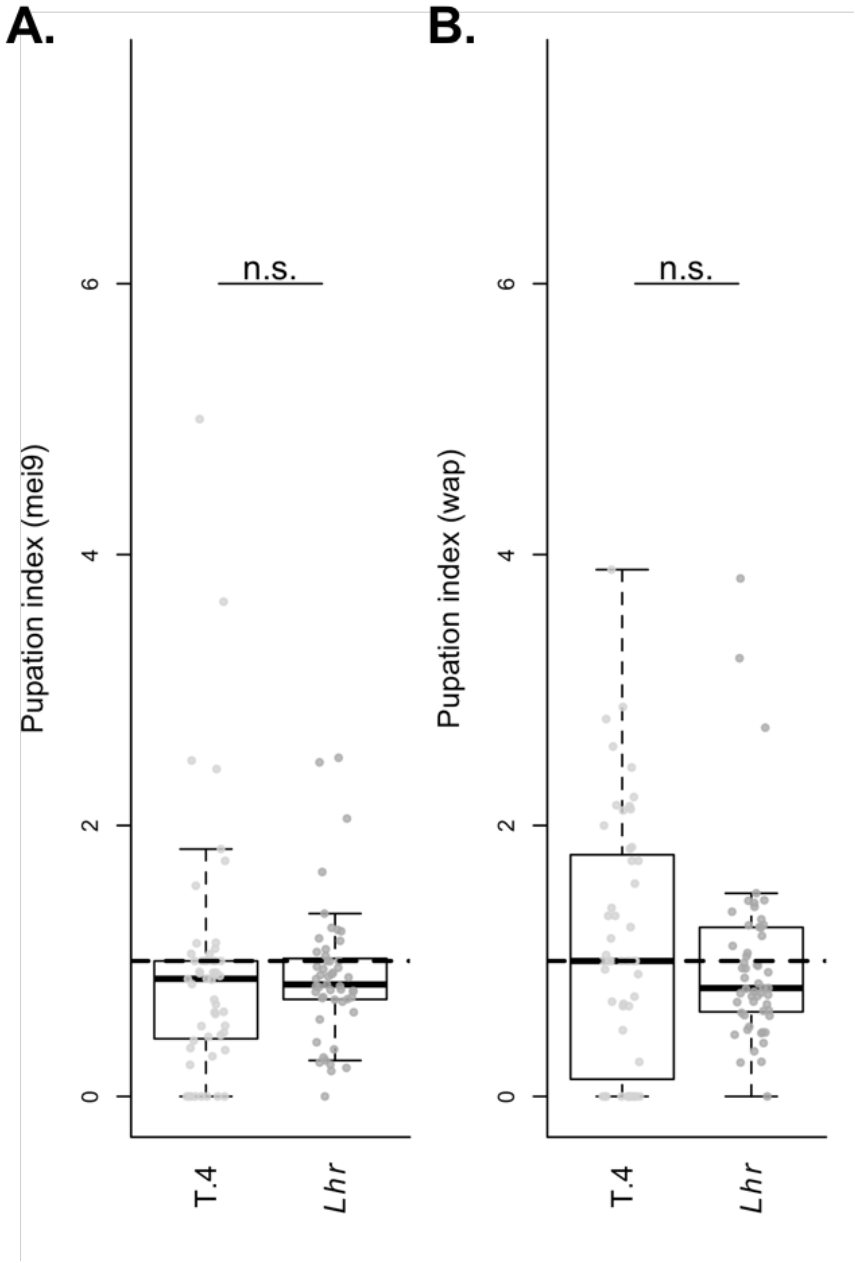
1190

1191 **Fig S3. Pupation behavior for *D. melanogaster*, *D. simulans*, and their F1 hybrids, measured as**
1192 **average pupation height within a vial. A.** The average pupation height is shown for males and females
1193 from both species and their F1 hybrids. Higher pupation heights indicate individuals pupated further from
1194 the food. **B.** The residuals from a regression between the average pupation height of individuals in a vial
1195 and the total number of individuals in that vial are shown for males and females from both species and
1196 their F1 hybrids. The results from pairwise statistical tests can be found in Table S5.



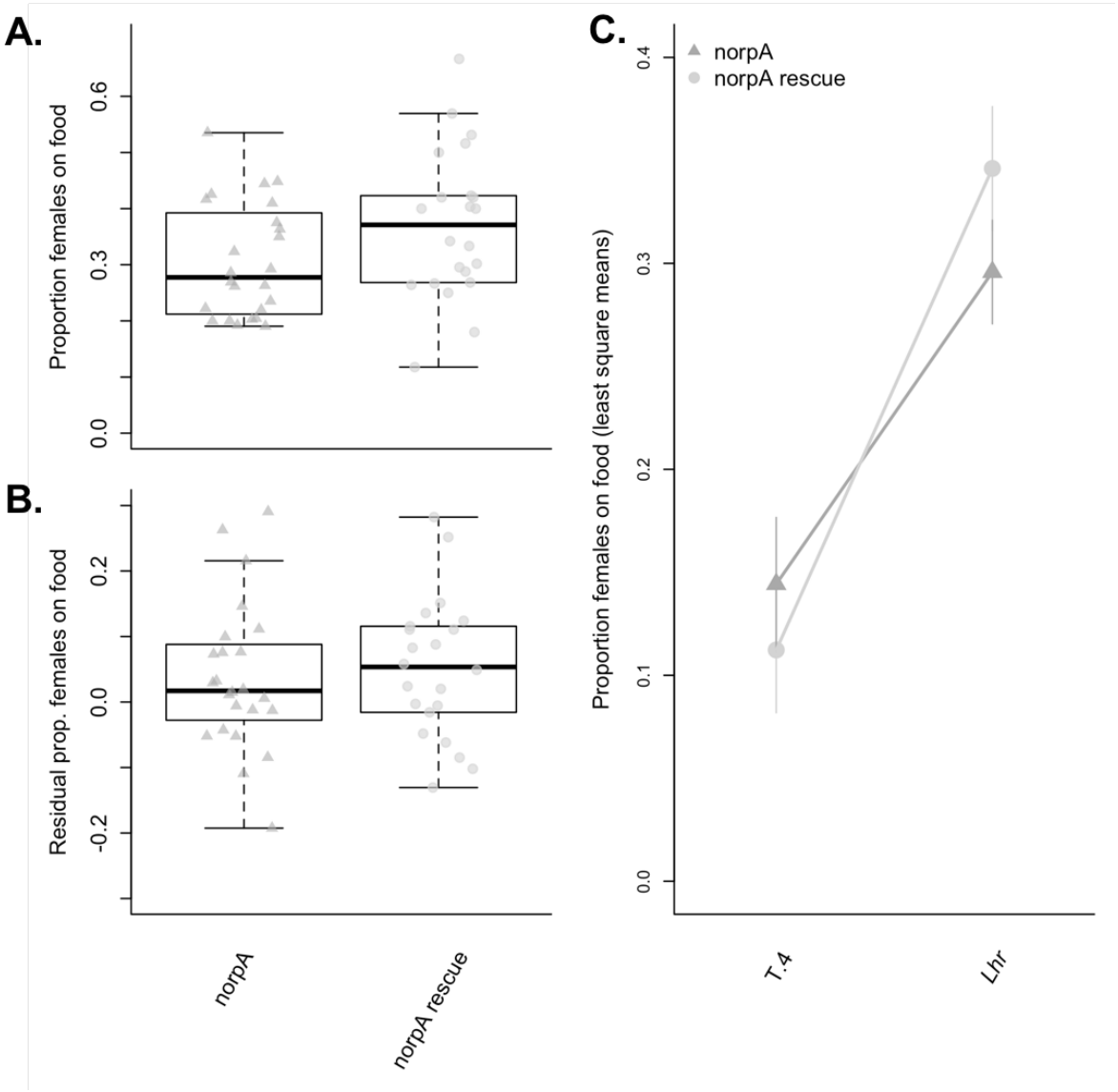
1197

1198 **Fig S4. Pupation site choice behavior for additional significant hybrid deficiencies.** Pupation
1199 indices for **A.** Df(1)ED411 (N=29-36), **B.** Df(1)ED6906 (N=26-33), and **C.** Df(1)BSC530 (N=18-22). Each
1200 of the three deficiencies displayed a median pupation index significantly greater than the average (0.88)
1201 when crossed to *D. simulans* (*Lhr*) after correcting for multiple comparisons. However, when each was
1202 crossed to *D. melanogaster* (T.4), the pupation index was still significantly elevated, indicating that the
1203 behavior of these flies is impacted by the deficient region in general, rather than the *Lhr* genotype it
1204 reveals.



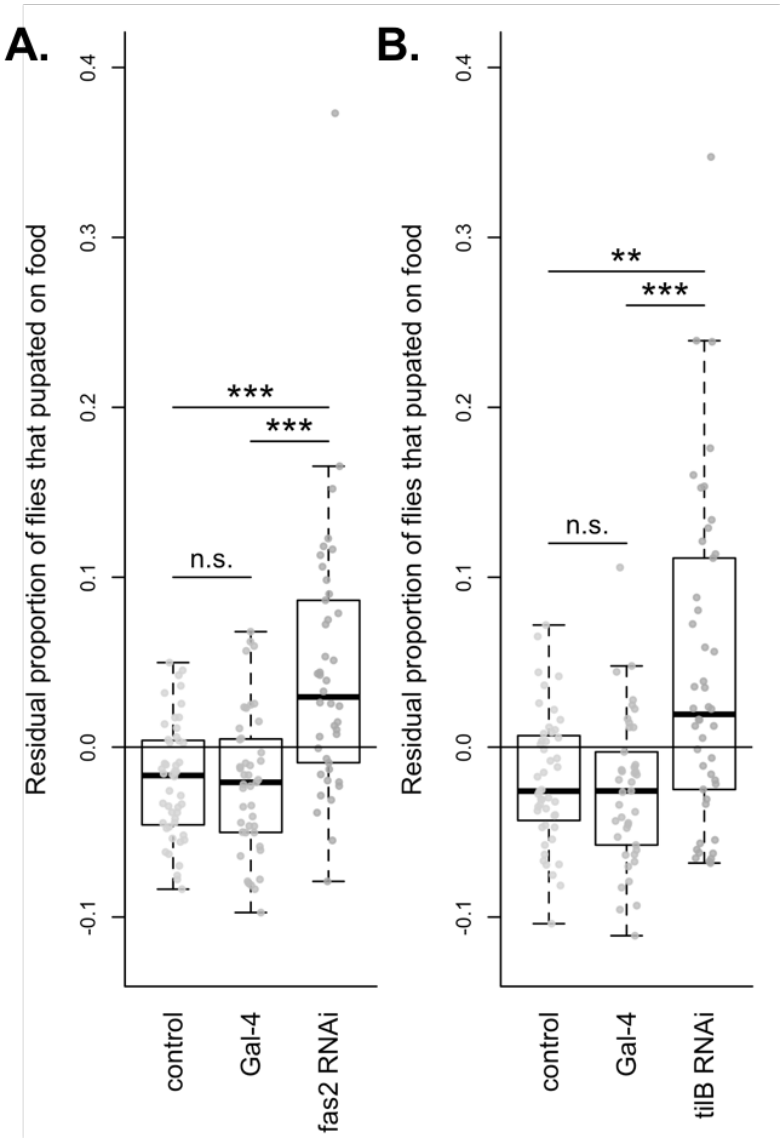
1205

1206 **Fig S5. Pupation behavior for knockout strains crossed to both *D. melanogaster* and *D. simulans*.**
1207 **A.** The pupation indices of a *mei9* gene disruption crossed to *D. melanogaster* (T.4, N = 52) and *D.*
1208 *simulans* (*Lhr*, N = 51). **B.** The pupation indices of a *wap* gene disruption crossed to *D. melanogaster* (T.4,
1209 N = 51) and *D. simulans* (*Lhr*, N = 55).
1210



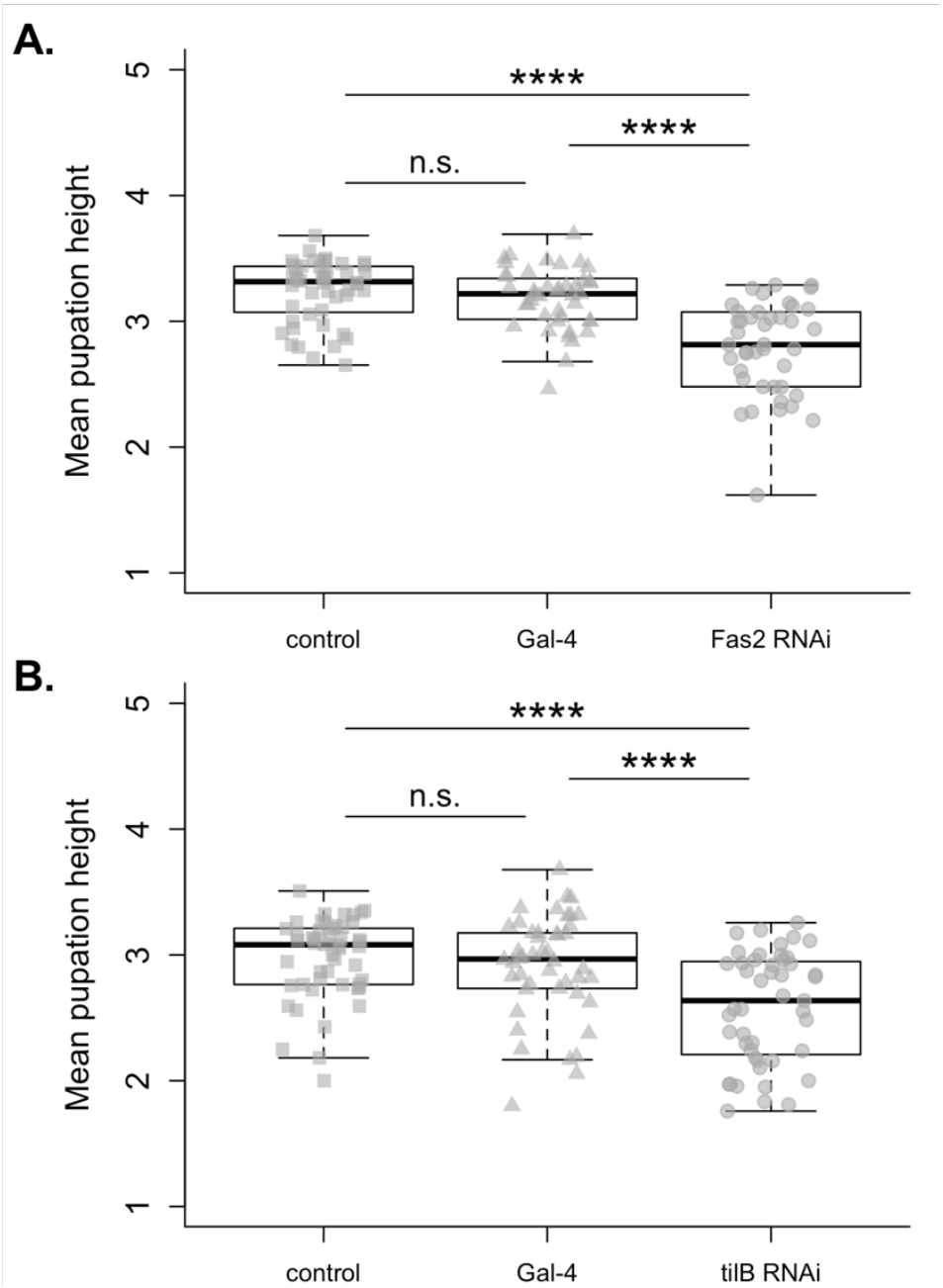
1211

1212 **Fig S6. Pupation behavior for norpA knockouts and control. A.** The proportion of females that
1213 pupated on the food is shown for the *norpA* mutant allele (norpA) and the *norpA* rescue allele (norpA
1214 rescue) when crossed to *D. simulans* (*Lhr*). There was no significant difference in pupation behavior
1215 between the knockout and the rescue allele (p=0.08). **B.** Pupation behavior for *norpA* knockout and *norpA*
1216 rescue allele female hybrids (crossed to *Lhr*) after controlling for density. Vials were adjusted for density
1217 using the residuals from a regression between the proportion of females in a vial that pupated on the
1218 surface of the food and the total number of individuals in the vial. There was no significant difference
1219 between the knockout and rescue allele after controlling for density (p=0.52). **C.** The proportion of
1220 females that pupated on the food when the *norpA* mutant allele (dark grey, triangle) and the *norpA* rescue
1221 allele (light grey, circle) were crossed to *D. melanogaster* (T.4) and *D. simulans* (*Lhr*). Shown are the least
1222 square means (± 1 SE) from an ANOVA with "species", "*norpA* allele" and their interaction as fixed effects.
1223 The actual analysis was conducted using residuals to control for density, and the "*norpA* allele" x "species"
1224 interaction term was removed from the model as it was not significant (p=0.37).



1225

1226 **Fig S7. Pupation site choice behavior for *Fas2* and *tilB* RNAi crosses after controlling for density.**
1227 **A.** The results of pan-neuronal knock down of the *Fas2* transcript via RNAi. Shown are the mean
1228 residuals from a regression between the proportion of individuals that pupated on the surface of the food
1229 and the total number of individuals in the vial for the control cross, the Gal-4 line, and RNA interference
1230 cross. Asterisks denote significance after correcting for multiple comparisons (* = $p < 0.05$, ** = $p < 0.01$,
1231 ** = $p<0.001$; N = 42-48). **B.** The results of pan-neuronal knockdown of the *tilB* transcript via RNAi.
1232 Shown are the mean residuals from a regression between the proportion of individuals that pupated on
1233 the surface of the food and the total number of individuals in the vial for the control cross, the Gal-4 line,
1234 and RNA interference cross. Asterisks denote significance after correcting for multiple comparisons (* = p
1235 < 0.05, ** = $p < 0.01$; N = 41-47).



1236

1237 **Fig S8. Pupation site choice behavior for *Fas2* and *tilB* RNAi crosses, measured as average**
1238 **pupation height within a vial.** Higher pupation heights indicate individuals pupated further from the food.
1239 **A.** The results of pan-neuronal knockdown of the *Fas2* transcript via RNAi. The average pupation height
1240 of individuals found on the food are shown for the control cross, the Gal-4 line, and RNA interference
1241 cross. Asterisks denote significance after correcting for multiple comparisons (**** = p<0.0001; N = 42-48).
1242 **B.** The results of pan-neuronal knockdown of the *tilB* transcript via RNAi. The average pupation height of
1243 individuals found on the food are shown for the control cross, the Gal-4 line, and RNA interference cross.
1244 Asterisks denote significance after correcting for multiple comparisons (*** = p<0.0001; N = 46-45).

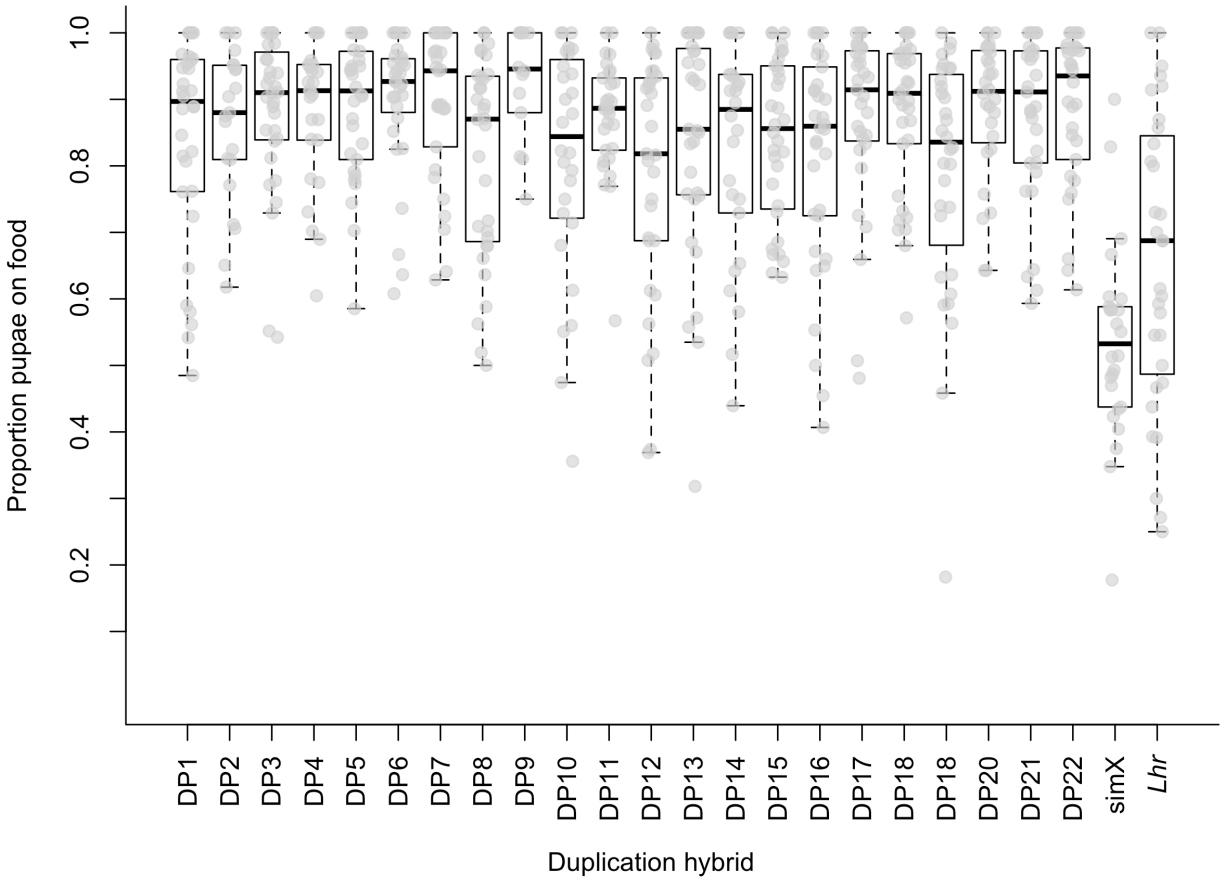


Fig S9. Duplication Screen. The results of a duplication hybrid screen. Each duplication hybrid was created by crossing a DP(1;Y) *D. melanogaster* female, which carries a unique segment of the X chromosome attached to the Y chromosome in an XXY background, to an *Lhr D. simulans* male. The resulting hybrid males have a complete *D. simulans* X chromosome in addition to 1 of 22 segments of the *D. melanogaster* X chromosome translocated to the Y chromosome. Females are largely inviable. In this way, we tested for dominant *D. melanogaster* X loci underlying the interspecific pupation difference. We scored pupation behavior following the same methods used for deficiency and knockout hybrids, and measured the proportion of pupae on the food for each duplication hybrid. Shown for comparison are the values for males from the *D. simulans* parent strain (*Lhr*) and hybrid males with a *D, simulans* X chromosome (simX). Interestingly, all of the duplication hybrids had significantly more males pupating on the food surface than either their *D. simulans* parent strain or typical simX hybrids that are not heterozygous for a region of the X. From this, we conclude that making males heterozygous over broad regions of the X chromosome results in behavioral or developmental inconsistencies that leave larvae unable to climb the vial walls to pupate. Information about these Y-linked X duplications can be found in Table S5.