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

Short title: Two genes underlying a difference in pupation behavior between \textit{Drosophila} species

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Abstract

The recent boom in genotype-phenotype studies has led to a greater understanding of the genetic architecture of a variety of traits. Among these traits, however, behaviors are still lacking, perhaps because they are complex and environmentally sensitive phenotypes, making them difficult to measure reliably for association studies. Here, we aim to fill this gap in knowledge with the results of a genetic screen for a complex behavioral difference, pupation site choice, between Drosophila melanogaster and D. simulans. In this study, we demonstrate a significant contribution of the X chromosome to the difference in pupation site choice behavior between these species. Using a panel of X-chromosome deletions, we screened the majority of the X chromosome for causal loci, and identified two regions that explain a large proportion of the X-effect. We then used gene disruptions and RNAi to demonstrate the substantial effects of a single gene within each region: Fas2 and tilB. Finally, we show that differences in tilB expression underlie species differences in pupation site choice behavior, and that generally, pupation site choice behavior appears to be correlated with relative expression of this gene. Our results suggest that even complex, environmentally sensitive behaviors may evolve through changes to loci with large phenotypic effects.
Behaviors are complex traits that involve sensory detection, higher level processing, and a coordinated output by the nervous system. This level of processing is highly susceptible to environmentally induced variation. Because of their complexity and sensitivity, behaviors are difficult to study; as a result, we have very little understanding of the genes involved in behavioral variation. In this study, we use common laboratory fruit fly model, *Drosophila*, to address this gap and dissect the genetic underpinnings of an environmentally sensitive behavior that differs between species. We find that a significant amount of the phenotypic difference between species is explained by a single chromosome. We further show that just two genes on this chromosome account for a large majority of its effect, suggesting that the genetic basis of complex behavioral evolution may be simpler than anticipated. For one of these genes, we show that a species-level difference in gene expression is associated with the difference in behavior. Our results contribute to a growing number of studies identifying the genetic components of behavior. Ultimately, we hope to use these data to better predict the number, types, and effects of genetic mutations necessary for complex behaviors to evolve.
**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 (1,2). For example, one subspecies of the yellow fever mosquito, *Aedes aegypti*, prefers to bite humans, while another closely related species prefers other animals (3,4). Understanding insect preferences therefore presents a major inroad to effective disease and pest management (5). 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 (6,7).

Despite the importance of behavioral traits, we know little about the genetic basis of their evolution. GePhe, the most extensive compilation of natural genetic variants associated with trait differences, currently catalogs ~1700 associations, of which only 23 are for behavior (8). From these 23, and others in the literature, it is clear that individual genes can sometimes have large effects on evolved differences in behavior (9–11). It also clear that changes to sensory receptors in the peripheral nervous system can explain dramatic shifts in behavior (3,11,12). 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 (13). 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 (14).

The current lack of genetic studies mapping behavioral variation presumably arises from the fact that behaviors are difficult to measure reliably and repeatedly. Behavioral phenotypes
often integrate multiple cues, are sometimes context dependent, and can be innate or learned, making it difficult to exclude environmentally induced variation. To better understand the genetic basis of behavioral evolution, we therefore need more case studies, with a focused effort on “metamodel” systems with documented behavioral differences between closely related species (15).

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

In the present study, we seek to address the lack of behavioral association studies using the *Drosophila* model system. Here, we investigate the difference in pupation site preference between two *Drosophila* species: *D. melanogaster* and *D. simulans*. When the larval stages of these species are ready to metamorphose into adults, they first enter a pupal stage. The pupa, which lasts for a number of days, is immobile and therefore vulnerable to parasitism, predation, desiccation, and disease (20). Before pupating, larvae enter a "wandering" stage, where they search for an appropriate pupation site (21,22). Depending on the strain and species, larvae vary
from pupating directly on their larval food source to traveling more than 40 cm away from it (23). This behavior is exquisitely sensitive to environmental conditions—individuals alter their behavior in response to light, moisture, pH, the presence of other species, parasitism, and more (24–27). Despite this environmentally induced variation, the effects of genotype on preference are considerable. Within species, strains and populations often differ in how far they travel from their food source before pupating, although the most consistent differences are seen between species (28,29). Interestingly, differences in pupation site choice behavior between species do not correspond to their taxonomic classification (30). For example, *D. melanogaster* and *D. simulans* shared a common ancestor 2-3 million years ago (31), and are extremely similar in terms of their ecology, morphology, and physiology (32). They differ markedly in terms of pupation site choice, however, with *D. simulans* pupating closer to the larval food source, on average (20,29). This is not due to laboratory adaptation, as freshly collected individuals show the same pattern (20,29). These species are frequently collected in the same microhabitats, and their differences in pupation site choice behavior have been postulated to be a form of niche partitioning (20,29). Supporting this hypothesis, pupation site choice responds to density dependent selection in the laboratory (33), and provides a potential increase in competitive ability between species ovipositing in the same media (34).

Here, we investigate the genetic basis of this difference in pupation site choice between *D. melanogaster* and *D. simulans*. Despite substantial reproductive isolation, *D. melanogaster* females can be persuaded to mate with *D. simulans* males in the lab, and vigorous female F1 hybrids result from the cross. Males are usually inviable, but we use a *D. simulans* hybrid male rescue strain (35,36) to circumvent this challenge, and show that a significant proportion of the species difference in pupation behavior can be mapped to the X chromosome, consistent with
findings using other Drosophila species (37). Still, these hybrids remain sterile, so genetic
dissection using a backcross mapping population is not possible. Instead, we use a widely
available set of transgenic D. melanogaster deficiency lines to screen a substantial portion of the
X chromosome (38,39). We use these lines to create hybrid females that lack large, overlapping
portions of the X chromosome from D. melanogaster, and therefore express only D. simulans
alleles in those regions. We identify two loci with large effects on pupation behavior. We then
employ genetic knockouts of candidate loci within these regions to demonstrate their effects, and
use RNAi knockdown to confirm the role of two genes, touch insensitive larvae B (tilB) and
Fasciclin 2 (Fas2). Finally, we use real time RT-PCR to test for species-level differences in gene
expression of tilB in larvae, and show that tilB is more highly expressed in D. melanogaster than
in D. simulans. Our method underscores the potential for the engineered deficiency resource,
which has been widely used to map intrinsic hybrid incompatibilities between species of
Drosophila (40–44), as a powerful tool with which to overcome traditional challenges to
interspecific mapping. Indeed, this method was recently used to identify loci affecting
pheromone differences between these Drosophila species (45), and may prove equally useful for
behavioral differences (46).

Results:

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

We measured pupation behavior for 11 D. melanogaster and 12 D. simulans strains collected
from various locations throughout the world (Table S1), and found significant variation among
our D. melanogaster lines and among our D. simulans lines in the proportion of individuals that
pupated on the food surface (Fig 1; D. melanogaster Wilcoxon test: $\chi^2 = 42.69$, df = 10, p<0.0001;
D. simulans Wilcoxon test: $\chi^2 = 56.34$, df = 11, p<0.0001). When we tested for a species difference in pupation behavior, we found that our D. simulans lines had significantly more pupae on the food surface compared to our D. melanogaster lines, on average (Fig 1; Wilcoxon test: $\chi^2 = 8.37$, df = 1, p = 0.0038).

Although we controlled egg density to characterize species differences in pupation behavior (see below), there were viability differences among our surveyed lines. As a result, we had significant variation in the total number of pupae in each vial for our D. melanogaster lines (ANOVA: $F_{10,65}=5.58$, p<0.0001) and our D. simulans lines (ANOVA: $F_{11,66}=8.01$, p<0.0001).

Previous studies have found that larval density correlates with pupation height in D. melanogaster (47). To control for differences in density, we also performed the same analyses above using the residuals from a regression between number of pupae and proportion of pupae on the food. None of our findings changed using this analysis (Fig S1), indicating that our results were not affected by variation in larval density.

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

2. Differences in pupation behavior have a significant X effect

To begin mapping these differences in pupation site choice behavior, we created F1 hybrid females (Fig 2A) and F1 hybrid males that had either a D. melanogaster X chromosome (“melX” males; Fig 2A), or a D. simulans X chromosome (“simX” males; Fig 2B). Both of these males
inherit their cytoplasm from the *D. melanogaster* parent strain, so any differences we observe are directly attributable to the sex chromosomes. We found significant differences among F1 hybrid genotypes when we screened hybrids alongside their parental strains (Fig 3; Full model Wilcoxon test: $\chi^2 = 144.57$, df= 6, $p<0.0001$). Specifically, a significantly higher proportion of F1 hybrid males pupated on the food when they had inherited a *D. simulans* X chromosome (simX males) compared to a *D. melanogaster* X chromosome (melX males), indicating that this species divergence in pupation behavior has a significant X effect ($p<0.0001$ after correcting for multiple comparisons; Fig 3). This is further supported by the fact that the proportion of pupae on the food was not significantly different between melX hybrid males and *D. melanogaster* males, or between simX hybrid males and *D. simulans* males. All of these findings are unchanged when we control for density effects (Fig S2).

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

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

Because we found a significant effect of the X chromosome on the difference in pupation site choice behavior between *D. simulans* and *D. melanogaster*, we devised a crossing scheme using molecularly engineered chromosomal deficiencies (38) to screen the X chromosome for loci
contributing to this difference (Fig 2C). We assayed a total of 90 deficiency strains, covering 87% of the X chromosome (Table S2). These crosses produced two types of hybrid female, both heterozygous at all autosomes (Fig 2C). The deficiency hybrid females contained the *D. melanogaster* deficiency X chromosome and a *D. simulans* X chromosome, making them hemizygous for a segment of the X chromosome. The balancer hybrid females contained the *D. melanogaster* balancer X chromosome and a *D. simulans* X chromosome, so they are heterozygous for *D. melanogaster/D. simulans* over the entirety of the X chromosome. The balancer hybrids provide an excellent experimental control, as they developed in the same environment as our experimental deficiency flies. As a result, we calculated the proportion of deficiency hybrid females that pupated on the food and the proportion of balancer hybrid females that pupated on the food, and used these measures to calculate a “pupation index” (the proportion of deficiency hybrids pupating on the food divided by the proportion of balancer females pupating on the food).

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

To ensure that the deficient region actually reveals D. simulans variation contributing to pupation site choice behavior, rather than creating lines that behave abnormally due to the extended hemizygosity within the deficiency region, we crossed each of the six significant deficiencies listed above to a wild-type D. melanogaster strain, T.4. For two of the six deficiency strains, Df(1)ED411 and Df(1)BSC530, we found no difference in the pupation index when crossed to D. melanogaster compared to the pupation index when crossed to the Lhr strain (Wilcoxon tests; Df(1)ED411: n=29-36, $\chi^2=1.74$, p=0.19, Fig S3A; Df(1)BSC530: n=18-22, $\chi^2=1.47$, p=0.23, Fig S3C), and Df(1)ED6906 had a significantly higher pupation index when crossed to D. melanogaster (n=26-33, $\chi^2=9.42$, p=0.002; Fig S3B). These results suggest that the phenotypes of these three lines are a result of their hemizygosity within the deficiency region, as the deficient D. melanogaster females still pupate on the food more often than the balancer females. When we crossed the remaining three deficiency strains, Df(1)BSC869, Df(1)ED6720, and Df(1)Exel6255, to D. melanogaster, we found a pupation index significantly lower than the index we calculated when crossing to Lhr (Wilcoxon tests; Df(1)BSC869: n=51, $\chi^2=4.35$, p=0.037; Df(1)BSC6720: n=30-35, $\chi^2=7.90$, p=0.0049; Df(1)Exel6255: n=55-57, $\chi^2=4.68$, p=0.0306; Fig 4B), and no different than one (Df(1)BSC869: p=0.46; Df(1)BSC6720: p=0.10; Df(1)Exel6255: p=0.50). Two of these three deficiencies overlap: Df(1)BSC869 and Df(1)ED6720 (Fig 4A). To further confirm that this pattern is not unique to these specific D.
melanogaster and D. simulans strains, we crossed one of the overlapping deficiency strains, Df(1)BSC869, and Df(1)Exel6255 to another D. melanogaster wild-type strain (T.1) and another D. simulans wild-type strain (Mex180). The pattern remained consistent for both of these deficiencies: when crossed to D. melanogaster, the pupation index was significantly lower than when crossed to D. simulans (Fig 4B; Wilcoxon tests: Df(1)BSC869: n=50-51, $\chi^2=7.78$, p=0.0053; Df(1)Exel6255: n= 23, $\chi^2=7.74$, p=0.0054).

One deficiency strain, Df(1)ED7265, had a pupation index significantly lower than the grand mean of all the lines (0.88) following Bonferroni correction for multiple comparisons. This suggests the potential for a region of the D. simulans X chromosome with transgressive effects—that is, a locus that causes D. simulans larvae to pupate farther from the food surface. While this is certainly interesting, we did not pursue this region because its effect is contrary to the species-wide difference we found. This locus may be interesting for further investigation, however, as it could contribute to the significant variation in pupation behavior we observed among D. simulans strains (Fig 1).

4. Gene knockouts and RNAi knockdown suggest that Fas2 is involved in divergent pupation behavior

Our deficiency screen identified two regions of interest. The first is the overlap of Df(1)BSC869 and DF(1)ED6720, excluding the region covered by DF(1)ED6727, which did not have a pupation index greater than 1 (Fig 4A, Table S2). The resulting region of interest spans X:4,204,351 - 4,325,174 (Fig 4C). Within this region there are 23 genes, of which 20 are protein coding (Table S3A). According to modENCODE expression data, 15 of those 20 protein coding genes are expressed in larvae, while only 6 are also expressed in the larval nervous system,
which we would expect for genes regulating behavior (48). Five of these six genes are well
described. At the time of assay, only two of the five characterized genes within this region had
non-lethal verified loss-of-function alleles available: Fas2 (Fasciclin2) and mei9 (meiotic 9). We
tested knockouts of each for an effect on pupation site choice behavior. We found no significant
difference in the pupation index obtained when we crossed the mei-9A1 mutant allele to the Lhr D.
simulans strain and the T.4 D. melanogaster strain (Wilcoxon test: n= 51-52, \( \chi^2=0.37, p=0.54 \);
Fig S4B), indicating that mei9 is unlikely to be involved in pupation site choice. In contrast,
when we crossed the mutant allele Fas2eb112 to D. simulans (Lhr) and D. melanogaster (T.4), we
found that Fas2eb112 hybrids had a significantly higher pupation index than the D. melanogaster
knockouts (Wilcoxon test: n= 50-51, \( \chi^2= 6.97, p=0.0083 \); Fig 5A), suggesting that Fas2 may be
involved in pupation site choice. To ensure this pattern is not unique to these strains, we crossed
Fas2eb112 to additional D. simulans (Mex180) and D. melanogaster (T.1) wild-type strains. We
again found the same pattern: the pupation index for knockout hybrids was significantly higher
than for D. melanogaster knockouts (Wilcoxon test: n= 52-53, \( \chi^2= 27.2, p<0.0001 \); Fig 5A). As
further verification, we tested a second Fas2 strain: a p-element insertion allele, Fas2G0293, and
similarly found that the pupation index for Fas2G0293 hybrids (crossed to Lhr) was significantly
higher than that for D. melanogaster knockouts (crossed to T.4; Wilcoxon test: n= 52-53, \( \chi^2=
9.73, p=0.0018 \); Fig 5B). When we used the combined consensus p-value test (49) to look at the
overall pattern for Fas2eb112, and Fas2 as a whole (i.e. including results from both Fas2eb112 and
Fas2G0293), we found a strongly significant pattern of higher pupation indices for hybrid crosses
compared to D. melanogaster crosses (Fas2eb112: \( p= 3.59 \times 10^{-6}; \) Fas2: \( p= 2.35 \times 10^{-8} \)).

Next, we used RNAi with the elav-Gal4 driver to reduce expression of Fas2 throughout
the nervous system in D. melanogaster. We compared the proportion of experimental flies that
pupated on the food for the RNAi cross (UAS-Fas2 x elav-Gal4) to that of two controls (both
crossed to elav-Gal4): the background stock in which the RNAi lines were created (y v; attP2,
y+) and the Gal4-1 stock, which has a hairpin targeting Gal4 in VALIUM20 to control for Gal4
effects. We found that a significantly higher proportion of RNAi flies pupated on the food
compared to the control flies from either the background (Wilcoxon test: n= 42-48, p<0.0001
after sequential Bonferroni correction) or Gal4-1 cross (Wilcoxon test: n= 42-43, p<0.0001 after
sequential Bonferroni correction; Fig 5C). These results are unchanged when we control for
density effects (Fig S5A), providing further evidence for Fas2’s role in pupation site choice.

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

The second region of interest identified by our deficiency screen was the region deleted by
DF(1)Exel6255 (X:21,519,203 – 22,517,665; Fig 4D). Within this region are 28 genes, of which
22 are protein coding (Table S3B). Of the 22 protein coding genes, 14 are expressed in larvae –
13 of which have some expression in the larval nervous system (48). Of these, 7 are described.
We obtained knockout strains for both of the characterized genes expressed in the larval nervous
system that had verified loss-of-function alleles available at the time: tilB (touch insensitive larva
B) and wap (wings apart). We found no significant difference in the pupation index obtained
when we crossed the wap² mutant allele to the Lhr D. simulans strain and the T.4 D.
melanogaster strain (Wilcoxon test: n= 51-55, χ²=0.32, p=0.57; Fig S4A), indicating that wap is
unlikely to be involved in pupation site choice. In contrast, when we crossed the tilB¹ and tilB²
mutant alleles to D. simulans (Lhr) and D. melanogaster (T.4), we found that the tilB knockout
hybrids had significantly higher pupation indices than the D. melanogaster knockouts for both
alleles (Wilcoxon tests; tilB\(^1\): n= 56, \(\chi^2= 6.61\), p= 0.0101; Fig 6A; tilB\(^2\): n= 57, \(\chi^2= 6.61\), p= 0.0101; Fig 6B). To test whether this difference is consistent in other backgrounds, we crossed both the tilB\(^1\) and tilB\(^2\) mutant alleles to additional \(D.\) sim\(ul\)\(ans\) (Mex180) and \(D.\) melanogaster (T.1) wild-type strains. We had a difficult time crossing our tilB strains to the Mex180 strain, so our sample sizes for these crosses are smaller, but there is a nonsignificant trend towards a higher pupation index for the knockout hybrids compared to the \(D.\) melanogaster hybrids for both alleles (Wilcoxon tests; tilB\(^1\): n=36-37, \(\chi^2= 2.80\), p= 0.0943, Fig 6A; tilB\(^2\): n=12-18, \(\chi^2= 3.44\), p= 0.0638, Fig 6B). We used the combined consensus p-value test (49) to look at the overall pattern for tilB\(^1\), tilB\(^2\), and tilB as a whole (i.e. including results from both tilB\(^1\) and tilB\(^2\)), and found a strongly significant pattern of higher pupation indices for hybrid crosses compared to \(D.\) melanogaster crosses (tilB\(^1\): p= 0.0022; tilB\(^2\): p= 0.0037; tilB: p= 2.47 \times 10^{-5}).

As for Fas2 above, we then used RNAi with the elav-Gal4 driver to reduce expression of tilB throughout the nervous system in \(D.\) melanogaster. We found that a significantly higher proportion of RNAi flies pupated on the food compared to the control flies from either the background (Wilcoxon test: n= 46-47, p<0.01 after sequential Bonferroni correction) or Gal4-1 cross (Wilcoxon test: n= 41-46, p<0.01 after sequential Bonferroni correction; Fig 6C). These findings are consistent when we control for density effects (Fig S5B), providing additional support for tilB’s role in pupation site choice.

### 6. tilB is expressed more highly in \(D.\) melanogaster strains

We performed RT-qPCR to quantify relative tilB transcript abundance for two strains of \(D.\) sim\(ul\)\(ans\) (Per005 and Geo288) and two strains of \(D.\) melanogaster (CA1 and T.4). For the two \(D.\) sim\(ul\)\(ans\) strains and the Geo288 \(D.\) melanogaster strain, we collected larvae from two stages of
larval development (96 and 120 hours following oviposition). For the other *D. melanogaster*
strain (T.4), we were only able to obtain enough larval tissue at 96 hours following oviposition,
due to low fecundity. Because the relative transcript abundance data had a skewed distribution,
we used the reciprocal root transformation to normalize the species and overall distributions
(Shapiro-Wilk tests for normality: all p >0.34). We then analyzed the relative transcript
abundance of *tilB* using a nested ANOVA with the following factors: species, strain nested
within species, larval age (96 and 120 hours), and the interaction between species and larval age.
The interaction term between species and larval age was not significant (p=0.41), so we removed
it from the model. We found that larvae from the 120-hour sampling period had significantly
lower *tilB* expression than 96-hour larvae (F\(_1,14\)= 6.16, p= 0.026). While we did not detect any
significant differences between the 2 strains from the same species (F\(_2,14\)=2.39, p=0.13), we
found a significantly higher average relative amount of *tilB* transcript in *D. melanogaster* larvae
compared to *D. simulans* larvae (F\(_1,14\)= 9.74, p= 0.0075; Fig 7).

Because we performed rt-PCR on an extreme strain and a strain closer to the average for
each species, these four lines represent a continuum of pupation site choice behavior, with T.4 (*D.
melanogaster*) having the lowest proportion of pupae on the food, followed by CA1 (*D.
melanogaster*), then Per005 (*D. simulans*) and Geo288 (*D. simulans*) having the highest
proportion of pupae on the food (Fig 1). These four strains follow an identical pattern for *tilB*
gene expression, with T.4 having the highest relative transcript abundance, and Geo288 having
the lowest (Fig 7). Although it is not possible to detect a significant effect with a sample size of 4,
this suggests that *tilB* gene expression may be negatively correlated with the proportion of pupae
on the food (Spearman’s rank correlation: \( r_s = -1, p< 0.10 \)).
7. Effect sizes

We used the results above to estimate how much of the difference in pupation site preference between *D. melanogaster* and *D. simulans* can be attributed to: *i*) the X chromosome, *ii*) our deficiencies of interest (Df(1)BSC869/DF(1)ED6720 and Df(1)Exel6255), and *iii*) *Fas2* and *tilB*.

We first calculated the “species difference ratio” (using the data from our parental/hybrid screen in Fig 3) by dividing the median proportion of males on the food for *D. simulans* by the median proportion of males on the food for *D. melanogaster* males (species difference ratio = 5.27). To determine how much of this species difference ratio can be attribute to the X chromosome, we calculated an “X effect ratio” by dividing the median proportion of simX males on the food by the median proportion of melX males on the food. By then dividing the “X effect ratio” by the “species difference ratio”, we found that the X chromosome accounts for approximately 55.6% (95% CI= 31.4%-80.2%) of the difference in pupation site preference between *D. melanogaster* and *D. simulans*. It is important to note, however, that this may be an overestimate, as calculating effect sizes using only reciprocal hybrids does not account for potential transgressive autosomal effects (50).

We then estimated the effect size of our deficiencies by calculating “deficiency effect ratios” (the median pupation index when the deficiency was crossed to the *D. simulans Lhr* strain/the median pupation index when the deficiency was crossed to the *D. melanogaster T.4* strain). Because Df(1)BSC869 and DF(1)ED6720 overlap, we used their mean deficiency effect ratio to estimate the effect size of the overlapping region, and found that it explains approximately 44.1% (95% CI= 33.5%-74/5%) of the X chromosome effect. Similarly, we found that Df(1)Exel6255 explains approximately 52.3% (95% CI= 33.7%-85.9%) of the X chromosome effect.
Finally, we estimated the effect size of our two identified candidate genes, *Fas2* and *tilB*.

For each allele we tested, we calculated a “knockout effect ratio” (the median pupation index when the knockout was crossed to the *D. simulans* *Lhr* strain/the median pupation index when the knockout was crossed to the *D. melanogaster* T.4 strain) and then we used the average knockout effect ratio of the two alleles for each gene (i.e. *Fas2*: average of *Fas2*<sup>eb11</sup> and *Fas2*<sup>G0293</sup>; *tilB*: average of *tilB*<sup>1</sup> and *tilB*<sup>2</sup>) to estimate the effect size. We found that *Fas2* explains approximately 93% (95% CI= 78.5%-134.9%) of the pupation difference attributed to the overlap between Df(1)BSC869 and DF(1)ED6720, and 41% (95% CI= 33.8%-76.5%) of the pupation difference attributed to the X chromosome. Similarly, we found the *tilB* explains approximately 87.2% (95% CI= 66.2%-135.9%) of the pupation difference attributed to Df(1)Exel6255, and 45.6% (95% CI= 35.5%-76.1%) of the pupation difference attributed to the X chromosome. While our calculation of the effect size of the X chromosome may be an overestimate (see above), we calculated the effect size of the deficiencies and individual gene knockouts in similar hybrid backgrounds, so their contributions to the overall X-effect (and the contribution of each knockout to the deficiency effect size) are likely more accurate. Still, these estimates could be affected by variation in the different *D. melanogaster* backgrounds in which the deficiencies and knockout strains were made.

**Discussion**

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

Our initial survey of pupation site choice behavior in *D. melanogaster* and *D. simulans* expands upon a previously reported interspecific difference (29). Consistent with these previous results, on average, our *D. simulans* strains had a greater proportion of flies pupating on the food surface.
However, we used 11 *D. melanogaster* and 12 *D. simulans* strains sourced from around the globe to demonstrate this difference. While the species difference holds when comparing the grand mean of all strains for each species, there is substantial variation within species. This variation is so significant that the species’ distributions overlap, with some *D. simulans* strains, like Mex180 and Cal006, more closely resembling *D. melanogaster* strains in pupation behavior (Fig 1). To our knowledge, our study is the first to extensively record this variation, which presents a useful tool for better understanding the evolution of pupation site choice behavior. These documented differences in pupation behavior among *Drosophila* species, in combination with our understanding of the environmental variables that affect this behavior within species (24,25,47,51), will be useful in identifying the selection pressures (if any) that affect the evolution of this trait. Differences in pupation site choice behavior may be a form of niche partitioning where species co-occur, as has been previously suggested (34). Alternatively, pupation site choice may be an adaptive response to parasite or parasitoid presence (52). A globally sourced panel of lines with significant variation, such as our own, provides an inroad for studies comparing pupation behavior to differences in the ecology of each collection site, such that we can better understand the ultimate causes of this behavioral evolution.

The genetic architecture of pupation site choice behavioral evolution

Using hybrid crosses in the same background that controlled for maternal inheritance, we were able to estimate the effect of the X chromosome on pupation behavior. We found a significant effect of the X, in that reciprocal hybrid males pupate in similar locations as the X-donating parent. We calculate that this chromosome explains 55.6% (95% CI= 31.4%-80.2%) of the total phenotypic difference between parent strains. Although the exact contribution of the X
chromosome reported here may be an overestimate due to transgressive autosomal effects that cannot be detected in a hybrid background (50), a similar X-effect has been detected for pupation behavior when comparing D. simulans and D. sechellia (37).

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

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

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

We then used RNAi knockdown of each gene in D. melanogaster to show that reduced expression of tilB and Fas2 transcripts results in a more D. simulans-like pupation site choice behavior. To directly test for differences in expression of tilB, we performed RT-PCR during two larval developmental time points. Congruent with our RNAi knock-down results, we find that mean relative transcript abundance is significantly higher in D. melanogaster larvae than in D. simulans larvae. Interestingly, we surveyed two lines per species, representing a continuum of pupation site choice behavior, and found that the mean proportion of pupae on the food surface for these lines was perfectly negatively correlated with tilB gene expression. Although these results are only for 4 lines, they suggest that lower tilB gene expression may be associated with a higher proportion of larvae pupating on the food surface.
While our present study does not present a functional analysis of the *D. melanogaster* or *D. simulans* *Fas2* or *tilB* alleles, we can use the *D. melanogaster* annotation of each gene to speculate about their role in the evolution of pupation behavior. *Fas2* is a large gene, spanning over 70,000 base pairs, with expression peaking during the larval wandering stage (L3) (48). It is also complex, with seven transcripts composed of various combinations of sixteen exons. Broadly, *Fas2* functions as a neuronal recognition molecule, and is involved in patterning the larval nervous system (60). Expression of *Fas2* is critical for synapse formation and growth at the larval neuromuscular junction (61,62) and is also important for patterning of the larval mushroom body (63). With expression in both the central and peripheral nervous system, it is possible that differences at the *Fas2* locus differentially wire the *D. melanogaster* and *D. simulans* brains, altering how larvae perceive or interpret stimuli. Whether these differences are a result of evolution of the protein sequence, and/or spatial or temporal differences in transcript expression remains to be determined.

Unlike *Fas2*, *tilB* is a short gene, spanning just over 1,700 base pairs, with a single transcript composed of 5 exons. *tilB* is also expressed in wandering larvae and pupae, though it shows higher expression in testes of adult males, due to its role in developing sperm flagella (48). In fact, *tilB* is associated with ciliary motility (64), and is a part of the mechanosensory transduction machinery (65). Mutant *tilB* larvae display normal locomotor activity, but have a reduced withdrawal response to physical disturbance (66). Consistent with this finding, our data suggest that changes in *tilB* expression could potentially result in differences in peripheral sensory perception between *D. melanogaster* and *D. simulans* larvae, ultimately influencing larval pupation site choice behavior. A more precise functional analysis of *tilB* is necessary to test this hypothesis.
Conclusions

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

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

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

Methods

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

Characterizing pupation behavior for D. melanogaster and D. simulans

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

To measure pupation behavior, we placed 10 males and 10 females from a specific line (both 3-5 days old) into half pint bottles and allowed females to oviposit overnight on a 10 mm diameter petri dish filled with food medium that was placed in the opening of the bottle. In total, we set up 5 bottles for each line. The following morning, we transferred 100 eggs from the petri dishes into vials containing food medium that were lined with an acetate sleeve on which the
larvae could pupate. In total, we set up 5-8 vials per line. Vials were held at 25°C for 8 days, at which time the liner was removed and the locations of the pupae were recorded (8 days was long enough for almost all larvae to pupate without any flies eclosing). A pupa was considered “on” the food if it was within 1 cm of the food surface, while all pupae that were further than 1 cm from the food surface were considered “off” the food. For each vial, we calculated the proportion of pupae on the food surface. For comparisons between species, our unit of replication was the mean proportion of pupae on the food surface from each line (i.e. N=11 for *D. melanogaster* and N=12 for *D. simulans*).

**Crossing *D. melanogaster* with *D. simulans***

For all crosses below, we created F1 hybrids between *D. melanogaster* and *D. simulans* using the following protocol. *D. simulans* males were collected as virgins within 6 hours of eclosion and held at room temperature in groups of 20 in vials containing food medium for 3-4 days. To set up crosses, we collected young *D. melanogaster* virgin females within 2-3 hours of eclosion, and combined 8-12 of these females with 20 *D. simulans* male in vials containing food medium supplemented with an *ad lib* amount of live yeast on the surface. We then pushed a long foam plug down into the vial, leaving approximately 1 cm of space above the food surface. We held flies under these conditions for 3 days, at which time they were transferred from these “cross vials” into “pupation vials” that contained food medium with no added yeast, and were lined with an acetate sleeve on which the larvae could pupate. We always set up crosses using *D. melanogaster* females and *D. simulans* males, because crosses in the opposite direction were never successful.
Measuring pupation behavior in F1 hybrids

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

After 3 days in the cross vial, we transferred males and females into pupation vials for 24 hours, at which time the flies were removed. While screening hybrid pupation behavior, we also concurrently screened pupation behavior for the parental *D. melanogaster* strain (LHM) and the parental *D. simulans* strain (*Lhr*) for comparison. Parental strain cross vials contained only a moderate amount of yeast, were set up with only 5 males and 5 females (pure species crosses produce more offspring), and did not have a plug pushed down into the vial, but were otherwise treated identically to the hybrid crosses. In total, we set up 30-33 vials per treatment.
All pupation vials were held at 25°C for 8 days, at which time the liner was removed. We removed any remaining larvae, and cut the liner at a point 1 cm above the food surface. The portion of the liner that contained pupae within 1 cm of the food surface was returned to the original vial (the “on vial”), while the portion of the liner with pupae further off of the food surface was placed in another vial containing food medium (the “off vial”). The flies that eclosed were sexed and counted 7 days later (15 days post-egg); all flies that eclosed in the “on vial” were considered flies that pupated on the food surface, while all flies that eclosed in the “off vial” were considered flies that pupated off the food surface. We then calculated the proportion of individuals that pupated on the food for each type of individual (genotype and sex).

Mapping hybrid pupation behavior using the Bloomington Deficiency Kit

Because we found a significant effect of the X chromosome on the difference in pupation site choice behavior between *D. simulans* and *D. melanogaster*, we devised a crossing scheme using molecularly engineered chromosomal deficiencies to screen the X chromosome for loci contributing to this difference. These deficiencies are part of the Bloomington Deficiency Kit (38), available from the Bloomington Drosophila Stock Center (BDSC). We assayed a total of 90 deficiency strains covering 87% of the X chromosome (Table S2). We restricted our deficiency screen to lines from the BSC, Exelixis, and DrosDel sets to control for strain background effects while also maximizing chromosome coverage.

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

These crosses produced two types of hybrid female that were heterozygous for *D. melanogaster/D. simulans* at each autosome (Fig 2C). The deficiency hybrid females contain the *D. melanogaster* deficiency X chromosome and a *D. simulans* X chromosome, making them hemizygous for a segment of the X chromosome. At this locus, these hybrid females only express *D. simulans* alleles. The balancer hybrid females contained the *D. melanogaster* balancer X chromosome (marked with a dominant visible marker) and a *D. simulans* X chromosome. These females are heterozygous for *D. melanogaster/D. simulans* over the entirety of the X chromosome, and thus express both *D. simulans* and *D. melanogaster* alleles. Although the deficiency hybrids are our flies of interest, the balancer hybrids provide an excellent experimental control, as these females developed in the same environment as our experimental flies. As a result, we calculated the proportion of deficiency hybrid females that pupated on the food and the proportion of balancer hybrid females that pupated on the food. We then used these measures to calculate a “pupation index” as the proportion of deficiency hybrids pupating on the food divided by the proportion of balancer females pupating on the food. To increase the accuracy of our estimates, we only included pupation vials in our analysis that yielded at least 10 of each type of female. For each deficiency hybrid strain we measured, we report the median pupation index of all replicates, because there were often high-scoring outliers that significantly skewed the mean pupation index. These outliers almost always had abnormally high pupation indices, so focusing on median values makes our findings more conservative.

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

**Testing candidate genes in deficiency regions using gene knockouts and RNAi knockdown**

For regions of interest identified by our deficiency screen, we ordered transgenic knockouts for any genes available within the region at the time. We focused on two regions: the significant overlap of Df(1)BSC869 and DF(1)ED6720 (spanning X:4,204,351-X:4,325,174), and the region covered by DF(1)Exel6255 (X:21,519,203;X:22,517,665). For the first region, we obtained knockouts for *Fas2* and *mei9*. We screened two *Fas2* knockouts, the mutant allele *Fas2*eb112 (*Fas2*eb112/FM7c; *Grenningloh et al., 1991*; provided by Brian McCabe), and a p-element insertion allele, *Fas2*G0293 (former BDSC Stock 11850; full genotype: *w*67c23 *P{lacW}fas2*G0293/FM7c), and the *mei-9*41 mutant allele (*w* mei-9*41/FM7h; BDSC stock #6792).
For the second region, we obtained knockouts for two genes: \textit{tilB} and \textit{wap}. We screened two \textit{tilB} mutant alleles, \textit{tilB}^1 and \textit{tilB}^2 (\textit{y w tilB}^{1/2}/FM4; Kernan et al., 1994; provided by Daniel Eberl), and the \textit{wap}^2 mutant allele (\textit{wap}^2/FM6; DSC stock # 8133). Like the deficiency strains, each of our gene disruptions is held over a balancer chromosome with a visible marker. To measure the pupation behavior of hybrids containing knockout copies of these \textit{D. melanogaster} genes, we crossed each \textit{D. melanogaster} knockout strain to \textit{Lhr} using the previously described methods, and calculated the pupation index as the proportion of knockout females on food / the proportion of balancer females on the food. As for our deficiency screen, any hybrid knockouts with a median PI greater than 1 for the hybrid cross suggest that the knockout gene may be involved in pupation site choice. We also crossed each \textit{D. melanogaster} knockout strain to a wild-type \textit{D. melanogaster} (T.4) strain to ensure that hybrid knockouts with a pupation index greater than 1 are not simply an artifact of being hemizygous for this particular gene. We crossed knockout strains that displayed the pattern we expect for a gene involved in pupation site choice (i.e. a pupation index significantly greater than 1 when crossed to \textit{D. simulans}, which is also significantly greater than the pupation index when crossed to \textit{D. melanogaster}) to an additional \textit{D. simulans} (Mex180) and \textit{D. melanogaster} (T.1) wild-type strain for verification.

Our knockout screen identified two genes that appear to be involved in pupation site choice: \textit{tilB} and \textit{Fas2}. We further tested the effects of these genes on pupation behavior using RNAi knockdown in \textit{D. melanogaster}. We used the elav-Gal4 driver (P\{w[+mC]=GAL4-elav.L\}2/CyO; BDSC #8765), which expresses Gal4 throughout the nervous system. We drove down the expression of \textit{tilB} and \textit{Fas2} throughout the nervous system by crossing elav-Gal4 virgin females to UAS-\textit{tilB} (BDSC #29391: \textit{y[1] v[1]}; P\{y[+t7.7] v[+t1.8]=TRiP.JF03324\}attP2) and UAS-\textit{Fas2} (BDSC #34084: \textit{y[1] sc[*] v[1]}; P\{y[+t7.7] v[+t1.8]=TRiP.HMS01098\}attP2).
males, respectively. The resulting flies express a gene-specific hairpin RNA throughout the nervous system, causing the degradation of mRNA, and thus, reduced expression of that gene (70,71). As experimental controls for each RNAi cross, we also crossed elav-Gal4 virgin females to the RNAi background stock (y v; attP2, y+ (y1 v1 ; P{y[+t7.7]=CaryP}attP2; BDSC stock #36303) and the Gal4-1 stock (containing a hairpin RNA targeting Gal4 in VALIUM20; BDSC stock #35784). Together, these controls allow us to account for the effect of both the Gal4 mutation and general expression of hairpin RNA throughout the nervous system. Any differences we detect between these controls and our RNAi crosses must therefore be due to the expression of the gene-specific (tilB or Fas2) hairpin RNA. We set up pupation vials using the methods described above, and for each cross, we calculated the proportion of RNAi (or control) flies on the food (removing any data points with fewer than 20 experimental flies). If more RNAi flies pupate on the food in the experimental cross (in which the expression of the gene is driven down) compared to the control crosses (in which gene expression is unaffected), this provides further support for that gene’s involvement in pupation site choice.

**Testing candidate genes for species-specific differences in larval transcript expression**

We selected two each of our 11 *D. melanogaster* and 12 *D. simulans* strains to test for larval stage-specific expression differences of candidate genes using real time RT-PCR – one extreme and one average. For *D. simulans*, we selected Geo288 and Per005 (Table S1), because Geo288 has the highest pupation index of any *D. simulans* strain and Per005 is closest to the species mean (Fig 1). For *D. melanogaster*, we selected CA1 and T.4 (Table S1) because T.4 has the lowest pupation index of any *D. melanogaster* strain, and CA1 is closer to the species mean (Fig 1).
To harvest larvae from these strains, we allowed adult females to oviposit in standard vials containing food media between the hours of 8 AM and 12 PM over two consecutive days. 120 hours after the final oviposition day, we floated larvae out of the food media using a 20% sucrose in water solution, sucked them up using a transfer pipet, briefly rinsed them with DI water on cheesecloth, and snap froze them using liquid nitrogen (72). In this way, we collected 20-30 mg of larvae from two developmental time points: 96, and 120 hours following oviposition. These time points approximate early wandering and late wandering larval stages. We chose these time points because they are presumably when pupation site choice occurs, and because larvae are large enough for many to be harvested at once using the above methods. We extracted mRNA using the Qiagen RNeasy Plus Mini Kit, and prepared cDNA using the Promega Verso kit.

To quantify transcript abundance, we designed primers that span a single intron near the 3’ end of tilB (73). Additionally, we used primers for the gene RpL32 as an internal control (74,75). Fas2 is a complex gene with multiple splice forms, so we were unsuccessful in designing general primers that would amplify all transcripts in both species. For this reason, we did not include Fas2 in these experiments. A full list of primers and transcript lengths can be found in Table S4A. For each stage and strain, we prepared two to three biological replicates, which we then amplified in two technical replicates for 40 rounds of qPCR. Using RpL32 transcript number as an internal control, we calculated relative transcript abundance while correcting for species differences in primer efficiency. We estimated primer efficiency differences by serially diluting gDNA from each of our D. melanogaster and D. simulans strains, performing qPCR, and using a standard curve to calculate adjusted amplification factors (Table S4B). To ensure that we were amplifying cDNA made from RNA, and not gDNA contamination,
we performed gel electrophoresis on our cDNA samples to ensure we only visualized the short, intron-less, band.

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Figure Captions:

Fig 1. Pupation behavior differences between *D. melanogaster* and *D. simulans*.

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

Fig 2. Crossing schemes to generate reciprocal X chromosome hybrid males and deficiency/balancer hybrid females. A. Crossing wild-type *D. melanogaster* females (LHM, 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<sub>M</sub> 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. 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*).

Fig 3. Pupation site choice behavior for *D. melanogaster, D. simulans*, and their F1 hybrids.

The mean proportion of individuals that pupated on the food surface for males and females from
both species and their F1 hybrids. *D. melanogaster* males and females were taken from the LH
strain, while *D. simulans* males and females were taken from the *Lhr* strain. F1 hybrids resulted
from a cross between these two strains. The “melX” hybrid males have the *D. melanogaster* X
chromosome, and the “simX” hybrid males have the *D. simulans* X chromosome. Both hybrids
have *D. melanogaster* cytoplasmic inheritance. Columns labeled with different letters are
significantly different from one another after sequential Bonferroni correction for multiple
comparisons (p < 0.0001 after correction, N = 26-33). Error bars indicate the 95% confidence
interval surrounding the mean.

**Fig 4. Hybrid deficiency screen of the X chromosome identifies two regions of interest.**

A. The median pupation index for each of the 90 deficiency hybrid crosses (y-axis) is plotted by the
physical map distance each engineered deletion spans along the X chromosome (x-axis).
Deficiencies with a pupation index significantly greater than 1 (solid line) after correction for
multiple comparisons are denoted by an asterisk (significance levels for all deficiencies are listed
in Table S2). Deficiencies with a pupation index significantly greater than 0.88 (the grand mean,
dashed line) after correction for multiple comparisons are denoted with a cross. All of these lines
also had pupation indices significantly greater than 1 before correcting for multiple comparisons.
The two regions we pursued for candidate gene validation are highlighted in light grey. Note, we
did not pursue the remaining three significant deficiency strains because they showed similar
pupation indices when crossed to *D. melanogaster* (Fig S3). B. The pupation indices of the
deficiencies from the grey highlighted areas in Part A are shown for the original hybrid cross
(Lhr) and for a cross to the T.4 *D. melanogaster* strain. BSC869 and Exel6255 were additionally
crossed to *D. simulans* strain Mex180 and *D. melanogaster* strain T.1. Asterisks denote a
significant difference between pupation indices of deficiency strains crossed to *D. melanogaster* and *D. simulans* (* = p < 0.05, ** = p < 0.01). Error bars are 95% confidence intervals calculated from 10,000 bootstraps of the median. C. The region uncovered by the overlap of deficiencies BSC869 and ED6720 and the 23 genes contained within it. Direction of the gene transcript is denoted with an arrow. The two genes with available disruption strains (*Fas2* and *mei9*) are labelled. D. The region uncovered by Exel6255 and the 28 genes contained within it. Direction of the gene transcript is denoted with an arrow. The two genes with available disruption strains (*tilB* and *wap*) are labelled.

**Fig 5.** Knockouts and RNAi knockdown confirm the role of *Fas2* in evolved differences in pupation site choice. A. The median pupation indices of the *Fas2*<sup>eb112</sup> 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 median pupation indices for a second gene disruption, *Fas2*<sup>G0293</sup>, 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 knock down of the *Fas2* transcript via RNAi. The mean 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). Error bars for all are 95% confidence intervals calculated from 10,000 bootstraps of the median.
Fig 6. Knockouts and RNAi knockdown confirm the role of \textit{tilB} in evolved differences in pupation site choice. A. The median pupation indices of the \textit{tilB} \textsuperscript{1} gene disruption are shown for the comparison between the original hybrid cross (\textit{Lhr}) and a cross to the \textit{D. melanogaster} T.4 strain. Also shown is the comparison for crosses to the \textit{D. simulans} strain Mex180 and the \textit{D. melanogaster} strain T.1. B. The median pupation indices for a second gene disruption, \textit{tilB} \textsuperscript{2}, are shown for the original hybrid cross (\textit{Lhr}) and the cross to \textit{D. melanogaster} strain T.4, and for crosses to the \textit{D. simulans} strain Mex180, and the \textit{D. melanogaster} strain T.1. For A and B, asterisks denote significance (* = p < 0.05, ** = p = 0.01; N = 41-47). C. The results of pan-neuronal knock down of the \textit{tilB} transcript via RNAi. The mean 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 \textit{tilB} RNAi cross. For C, asterisks denote significance after correcting for multiple comparisons (* = p < 0.05, ** = p < 0.01; N = 41-47). Error bars for all are 95% confidence intervals calculated from 10,000 bootstraps of the median.

Fig 7. Relative differences in \textit{tilB} transcript abundance between species. The relative abundance of \textit{tilB} transcript detected by RT-qPCR in \textit{D. melanogaster} and \textit{D. simulans}. Each data point represents the average of two technical replicates for a single biological replicate. Relative transcript abundance is significantly higher in \textit{D. melanogaster} strains on average (p < 0.01). Light grey squares (T.4) and triangles (CA1) represent the two \textit{D. melanogaster} strains measured, while dark grey diamonds (Per005) and circles (Geo288) represent the two \textit{D. simulans} strains. The dashed lines depict the average expression across both time points for each strain. The black circles represent the species-wide mean, and error bars depict the 95%
confidence interval surrounding the mean. Note that the exact rank of relative tilB expression is perfectly inversely correlated to the pupation indices of each strain (i.e., the strain with the highest tilB expression has the lowest pupation index, and so on).

Fig S1. Pupation behavior differences between D. melanogaster and D. simulans after controlling for density.

The mean residuals from a regression between the proportion of individuals in a vial that pupated on the surface of the food and the total number of pupae in the vial for 11 D. melanogaster lines and 12 D. simulans lines described in Table S1. Error bars denote the 95% confidence interval around each individual mean (N = 5-8). The dashed horizontal lines indicate the grand mean for each species. The boxes surrounding the dashed lines denote the 95% confidence interval around the grand mean.

Fig S2. Pupation behavior for D. melanogaster, D. simulans, and their F1 hybrids after controlling for density. The mean residual proportion of individuals that pupated on the food surface are shown for males and females from both species and their F1 hybrids. D. melanogaster males and females were taken from the LH*M strain, while D. simulans males and females were taken from the Lhr strain. F1 hybrids resulted from a cross between these two strains. The “melX” hybrid males have the D. melanogaster X chromosome, and the “simX” hybrid males have the D. simulans X chromosome. Both hybrids have D. melanogaster cytoplasmic inheritance. Columns labeled with different letters are significantly different from one another after sequential Bonferroni adjustment for multiple comparisons (p < 0.0001, N = 26-33). Error bars indicate the 95% confidence interval surrounding the mean.
**Fig S3. Pupation site choice behavior for additional significant hybrid deficiencies.** Median pupation 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 of the three deficiencies displayed a median pupation index significantly greater than the average (0.88) when crossed to *D. simulans (Lhr)* after correcting for multiple comparisons. However, when each was crossed to *D. melanogaster (T.4)*, the pupation index was still significantly greater than average, indicating that the behavior of these flies is impacted by the deficient region in general, rather than the *Lhr* genotype it reveals.

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

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

Table S1: Strains used to characterize pupation behavior in *D. melanogaster* and *D. simulans*. With the exception of LH_M, the *D. melanogaster* (mel) lines were the founders of the Drosophila Synthetic Population Resource (flyrils.org). The *D. simulans* (sim) lines were obtained from the National Drosophila Species Stock Center.

Table S2: X deficiencies used to map pupation behavior between *D. melanogaster* and *D. simulans*. All deficiencies were obtained from the Bloomington Drosophila Stock Center (BDSC). The median pupation index (PI) is listed for each deficiency. Deficiencies with a PI > 1 indicate that more deficiency hybrids pupated on the food compared to balancer hybrids. Included are the uncorrected p-values from 1-sample Wilcoxon tests for each deficiency against the predicted PI = 1, and against the grand mean for all deficiencies (PI = 0.88). Uncorrected p-values that were significantly greater than 1 (or 0.88) are indicated in red, and those that remained significant after sequential Bonferonni correction for multiple comparisons are highlighted in yellow, and denoted with asterisks (* = p<0.05, **= p<0.01, ***=p<0.001, ****=p<0.0001 after correcting for multiple comparisons). Potential deficiencies of interest that were selected for further investigation are also highlighted in yellow. Only lines with a PI significantly >1 AND a PI significantly > 0.88 after adjusting for multiple comparisons were selected.
Table S3A. The genes within the region uncovered by the overlap of Df(1)BSC869 and DF(1)ED6720. Listed in the table are physical position, strand, flybase ID, gene name, expression in larvae, expression in the larval nervous system, and known biological functions.

Table S3B. The genes within the region uncovered by Df(1)Exel6255. Listed in the table are physical position, strand, flybase ID, gene name, expression in larvae, expression in the larval nervous system, and known biological functions.

Table S4A. Primers used for RT-PCR of tilB and RpL32. Primers selected for amplification of tilB and RpL32 mRNA transcripts. Left and right primers are listed, along with their genomic start positions. The length of the fragments amplified from gDNA (including introns) as well as from mRNA (no introns) are included.

Table S4B. Standard curve for primer efficiency
Figure 1
A. Hybrid males with a *D. melanogaster* (shown in white) X chromosome

\[ \text{LH}_M \, \text{♀} \quad \text{Lhr} \, \text{♂} \]

- LH\(_M\)X, LhrY (melX ♀)
- LH\(_M\)X, LhrX (F1 hybrid ♀)

B. Hybrid males with a *D. simulans* (shown in grey) X chromosome

\[ \text{C(1)DX LH}_M \, \text{♀} \quad \text{Lhr} \, \text{♂} \]

- LhrX, LH\(_M\)Y (simX ♂)
- LH\(_M\)XX, LhrY (dead)

C. Deficiency hybrid crossing scheme

- deficiency ♀
- Lhr ♂
- DVM

- balancer ♀
- deficiency ♀ (dead*)
- deficiency ♂
- balancer ♀
Figure 3

Proportion pupae on food

- D. melanogaster
- melX
- simX
- D. simulans

Female
Male

A
A
A
B
C
B, C

F1 hybrids
Figure 4

A. Population index as a function of chromosome position for different alleles.

B. Median population index for different time points (T4, Lhr, T1, Max100).

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

D. Df(1)Exel6255.
Figure 5

A. Median pupation index (Fas2eab1;112)

B. Median pupation index (Fas2G0293)

C. Mean proportion pupae on food

control  Gal-4  Fas2 RNAi
Figure 6

A.

B.

C.

Median pupation index (tIIB1) vs. T.4, Lhr, T.1, Mex180

Median pupation index (tIB2) vs. T.4, Lhr, T.1, Mex180

Mean proportion pupae on food vs. control, Gal-4, tilB RNAi
Figure 7

![Graph showing relative tilB expression in D. melanogaster and D. simulans. Legend includes symbols for T.4, CA1, Per005, and Geo288.](image)
Figure S1

Residual proportion pupae on food

-0.2
0.0
0.2
0.4
0.6

D. melanogaster
D. simulans

BER1 CA1 KSA2 Q12 RVC3 VAG1 Wild5B LHm T-1 T-4 T.7
Guy001 Aus004 Per005 Ca006 NG009 Flo166 SA169 Gre181 Mal261 Gec288 Uhr Mex180
Figure S2

Residual proportion pupae on food

-0.3 -0.2 -0.1 0.0 0.1 0.2 0.3 0.4

D. melanogaster  melX simX  D. simulans

F1 hybrids

- Female
- Male

A, A, A, B, C

B, C
Figure S3

A. Median pupation index (Df(1)ED411) for T4 and Lhr.

B. Median pupation index (Df(1)ED6965) for T4 and Lhr, with ** indicating a significant difference.

C. Median pupation index (Df(1)BSC530) for T4 and Lhr, with n.s. indicating no significant difference.
Figure S4

A. Median pupation index (wap)

B. Median pupation index (mel9)

n.s.
