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**Oviposition preferences for ethanol depend on spatial arrangements and differ dramatically
among closely related *Drosophila* Species**

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by

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Abstract

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Recent work on the model fly *Drosophila melanogaster* has reported inconsistencies in that they either do or do not prefer to lay eggs on intermediate concentrations of ethanol. We resolve this discrepancy by showing that this species strongly prefers ovipositing on ethanol when it is close to a non-ethanol substrate, but strongly avoids ethanol when options are farther apart. We also show fluidity of these behaviors among other *Drosophila* species: *D. melanogaster* is more responsive to ethanol than close relatives in that it prefers ethanol more than other species in the close-proximity case, but avoids ethanol more than other species in the distant case. In the close-proximity scenario, the more ethanol-tolerant species generally prefer ethanol more, with the exception of the island endemic *D. santomea*. This species has the lowest tolerance in the clade, but behaves like *D. melanogaster*. We speculate that this could be an adaptation to protect eggs from parasites or predators such as parasitoid wasps, as larvae migrate to non-toxic substrates after hatching. Here we use *D. melanogaster* to dissect the genetic basis of oviposition behavior specifically in the close-proximity case. We identified two QTL using inbred lines from the *Drosophila* Synthetic Population Resource. These loci map to the telomeric

end of chromosome 2 and to the centrosome on chromosome 3. With continued fine mapping using introgression lines, we hope to narrow down the behavior to a few candidate genes. These natural differences both among and within species are an excellent opportunity to study how genes and brains evolve to alter ethanol preferences, and provide an interesting model for genetic variation in preferences in other organisms including humans.

Introduction

Drosophila melanogaster has become a premier model organism for the study of the connections between genes, brain, and behavior. A promising behavioral paradigm is oviposition behavior in females, as it is tightly coupled to fitness and is easy to assay in the lab (Siegal and Hartl, 1999). Because insect larvae have limited mobility relative to their parents, maternal oviposition choices are critical for fitness and have important roles in insect speciation and the evolution of insect pests (Renwick and Chew, 1994; Rundle and Nosil, 2005; Thorsteinson, 1960). Oviposition is a good system to study decisions and preferences, as egg-laying females gather and integrate multiple inputs through continual exploration and sampling before laying each egg (Yang et al., 2008). The numbers of eggs on each substrate is a clear expression of preference which is easy to quantify in a relatively high-throughput manner.

Recent notable discoveries using oviposition as a model system for preferences have found that flies will make contrasting decisions depending on the distance between options (Joseph et al., 2009; Miller et al., 2011; Yang et al., 2008). This was first discovered by allowing flies to choose between sweet and bitter substrates (Yang et al., 2008). Though flies prefer to feed on sweet foods as adults and larvae, they prefer to oviposit on bitter substrate if sweet substrate is nearby. Flies appear to be constantly reassessing the relative positions of the available options, and will alter egg investment depending on the current distances among options (Yang et al., 2008). Similarly, though yeast is a valuable protein source for developing larvae, many genotypes of *D. melanogaster* prefer to oviposit on non-nutritious substrate if a nutritious substrate is nearby (Miller et al., 2011). As the distance between

nutritious and non-nutritious substrates is increased, flies shift from strongly preferring non-nutritious substrate to strongly preferring nutritious substrate, with intermediate preferences at intermediate distances. Responses to acetic acid were found to be similar, with adult flies preferring to position themselves on substrate without acetic acid, but repeatedly venturing onto acetic acid media nearby in order to lay their eggs (Joseph et al., 2009). The adaptive significance of these behaviors remains to be determined, though reasonable hypotheses include choosing to oviposit in a place with fewer toxins, microbes, parasitoids, or large consumers, but which is near enough to the food source that larvae can find it. Regardless, initial investigation into the genes and neural circuits responsible for these behaviors illustrate the great potential to use oviposition as a model to study preferences and decision-making (Gou et al., 2014; Joseph and Heberlein, 2012; Joseph et al., 2009; Yang et al., 2008; Zhu et al., 2014).

Ethanol is particularly interesting as an oviposition cue. It has long been known that ethanol stimulates oviposition in *D. melanogaster* (Adolph, 1920), though this depends on concentration (Azanchi et al., 2013; Ogueta et al., 2010). Despite general agreement that ethanol is attractive to *D. melanogaster* up to concentrations of 10% or more, published results are sometimes contradictory (Eisses, 1997; Hougouto et al., 1982; Jaenike, 1983; McKenzie and Parsons, 1972; Ogueta et al., 2010; Richmond and Gerking, 1979; Siegal and Hartl, 1999; Zhu and Fry, 2015). A comprehensive recent experiment by Azanchi et al. clearly illustrates that ethanol is attractive at least up to 10% when it is close to non-ethanol substrate, at least in the whiteBer strain used (Azanchi et al., 2013). Kacsoh et al., however, found that *D. melanogaster* lay eggs on 0% or 3% ethanol and avoid 6% or higher

concentrations (Kacsoh et al., 2013). In this latter study the substrates were farther apart, which likely explains this discrepancy (see our results below). These two studies also began to explore the neurological basis of oviposition-related ethanol preferences. Azanchi et al. found that subsets of neurons in the dopaminergic system have opposing effects on the valence of ethanol as a cue, and Kacsoh et al. document that neuropeptide-F signaling is also involved. *Drosophila melanogaster* and *D. simulans* were also found to dramatically alter their expressed preference in the presence of ethanol-sensitive parasitoid wasps (Kacsoh et al., 2013). This behavior was seemingly adaptive, as oviposition on ethanol (without nearby non-ethanol food) decreased survival in the absence of wasps but substantially increased offspring survival in the presence of wasps. For these and other reasons we find oviposition with respect to ethanol to be a fascinating system for the exploration of decision-making and preferences. *Drosophila melanogaster* has high ethanol tolerance relative to closely related species, and has likely evolved to utilize ethanol-rich substrates that other *Drosophila* species cannot (McKenzie and Parsons, 1972; McKenzie and Parsons, 1974; Montooth et al., 2006). As such, we hypothesized that the natural genetic differences among species in the *Drosophila* genus may provide a rich substrate for understanding the genetic and neurobiological basis of ethanol-related decisions. Like humans, *D. melanogaster* is from Sub-Saharan Africa (David and Capy, 1988), and colonized the rest of the world Biology Open • Advance article more recently (Lachaise and Silvain, 2004). These newer "cosmopolitan" populations are sometimes considered a different subspecies ("M" subspecies) than their Sub-Saharan relatives ("Z" subspecies) because of partial reproductive isolation between them (Hollocher et al., 1997; Wu et al., 1995). Most Sub-Saharan populations have lower ethanol tolerance than cosmopolitan populations (David et al., 1986;

Montooth et al., 2006), and therefore might show different preferences with respect to ethanol substrates. The sister group to *D. melanogaster* contains three additional species, which are still less tolerant of ethanol: the cosmopolitan *D. simulans* and the island endemics *D. mauritiana* and *D. sechellia* (Mercot et al., 1994; Montooth et al., 2006). This last species is a specialist on *Morinda citrifolia* (Nori) fruit (R’Kha et al., 1991); it shows an extremely low tolerance for ethanol (Mercot et al., 1994). An outgroup to the *melanogaster-simulans* clade includes another broadly distributed African species, *D. yakuba*, and it has a sister species (*D. santomea*) found only at high elevations on the island of São Tomé (Lachaise et al., 2000). Though there are a couple reports concerning the oviposition preferences of *D. simulans* and *D. sechellia* (Kacsoh et al., 2013; Richmond and Gerking, 1979), little is known of the preferences of these species and nothing is known about the others in the subgroup. Here we investigate the preferences of strains from both *D. melanogaster* subspecies, *D. simulans*, *D. mauritiana*, *D. yakuba*, and *D. santomea* and find that there are substantial differences among them. We also find that some, but not all, of these species dramatically alter their decisions depending on the distance between options. All of these species now have reference genomes available, and many will form viable hybrids in the lab, which should facilitate future efforts to characterize how evolutionary changes in genes and nervous systems result in modified ethanol preferences.

Natural populations harbor a tremendous amount of diversity for phenotypic variation, whether it be for physiology, behavior, or disease. This phenotypic diversity is due to the complex interaction of the underlying genetic architecture and its sensitivity to dynamic environmental conditions. Understanding this correlation between genotype and

phenotype will help develop an evolutionary understanding of how populations maintain such a vast amount of variation and how that variation changes in responses to selective pressure. Quantitative trait loci (QTL) mapping is one such tool commonly used in model systems to identify regions of the genome that cosegregate with a given trait. QTL mapping, therefore, is limited to the allelic diversity that results from the segregation between parents of a controlled cross. The resolution of a generated QTL map highly depends on the amount of recombination that occurs and poor resolution may result in confounding effects in tightly linked loci, causing an over or underestimation of effect sizes based on the directionality of their individual effects. QTL mapping has also historically been a poor indicator in modeling alleles with small effects, and the resulting associated loci found in most studies tend to explain only a fraction of the effect size (Ayroles et al., 2009). Despite these shortcomings, QTL mapping has continued to be a powerful and proven method in establishing functional relationships between adaptive traits and underlying linked loci and remains an important tool in association studies. Here, we investigate the possibility of causal loci responsible for the natural variation found in oviposition preference to ethanol in *D. melanogaster*. We find that the preference for ethanol is largely dominant, and are able to map this behavior to two different chromosomes.

Materials and Methods

Fly stocks and maintenance

We used two strains of *D. melanogaster*: MelG was collected by the authors in Goleta, California in 2012; Zi237N (referred to throughout as MelZ) was collected by John Pool in Zaire in 2010 (Lack et al., 2015). The two *D. simulans* lines used were ordered from the UCSD Drosophila stock center: Nueva was collected in Nueva, California in 1961 (stock number 14021-251.006) and Oaxaca was collected from a mezcal factory in Rancho Zapata, Oaxaca, Mexico in 2002 (stock number 14021.051.180). We used six lines each for *D. yakuba* and *D. santomea*. *D. yakuba* lines PB 1200.1, 13.6.1, 13.7.1, MF 10.4, MR 4.7 and *D. santomea* lines CAR 1490.3, CAR 1490.17, 1350.14, 1600.4, B 1300.13 were collected and provided by Daniel Ricardo Matute. Yaksyn2005 and Sansyn2005 were each collected by Jerry Coyne on São Tomé in 2005 (Matute et al., 2009). Sansyn2005 was made from several females from the Bom Sucesso field station at ~1150 M elevation, while the Yaksyn2005 strain was made from the females collected at ~800 M at the Pico de São Tomé. The *D. mauritiana* stock used was from the UCSD Drosophila Stock Center and was collected at Le Reduit Mauritius by Maria Margarita Ramos in 2006 (stock number 14021-041.150). We initially included *D. sechellia* in the experiment as well, but this species laid very few eggs in initial assays and was not considered further. All fly strains were grown and maintained in 20 mm vials on standard cornmeal/molasses/yeast media supplemented with live yeast at 25°C on a 12h: 12h light:dark cycle.

Oviposition behavior

Females were collected under CO₂ anesthetization 13 days after eggs were laid by the previous generation and held in groups of five for 24 hours. They were then introduced to the assay chambers described below without anesthetization using an aspirator.

Oviposition substrates consisted of 1% agar (BD #214010), 1% yeast extract (BD #212750), 1% acetic acid (Fischer #144137), 9% Trader Joe's Organic Concord Grape juice, with or without the addition of ethanol (Acros #50530496). The grape juice was first filtered through a paper coffee filter to ensure a homogeneous solution without any lingering fruit bits. Ethanol and acetic acid were added to the media after the grape/yeast/agar solution had cooled to 55°C to minimize loss of volatile fluids through evaporation. Food was then poured into the lids of 35-mm Petri plates (Falcon #351008) and allowed to congeal. For split-patch assays, razorblades were used to divide the Petri plates before pouring the media. These were removed after the media congealed. Controls for the one-patch assays were prepared by placing a small piece of transparency film in between the two substrates.

Oviposition assays were performed within 19.4 cm x 18.5 cm x 12.6 cm plasticware containers (Gladware #819055). Two-patch oviposition assays were made by affixing two Petri plates containing the different substrates 7 cm apart diagonally in a plasticware container. The split-patch assays had a single plate containing both options affixed to the center of the arena. A small cutout was made in the middle of the lid of the plasticware container and lined with mesh to provide venting of ethanol vapors.

Five females were aspirated into each plasticware container and allowed to oviposit undisturbed for 24 hours. Data were collected in 6 blocks. The first four blocks had nearly equal numbers of replicates of each strain in 4 conditions: split-patch data with 6% ethanol,

two-patch data with 6% ethanol, and the same two conditions with 10% ethanol. The final two blocks had nearly equal numbers of each strain in each patch type in 10% ethanol and 15% ethanol. Sample sizes for each strain and treatment combination were not precisely equal in each block because replicates with fewer than 5 total eggs were discarded.

Ethanol tolerance

Females were collected under CO₂ anesthetization 13 days after eggs were laid by the previous generation and held in groups of five for 24 hours in vials. They were then introduced to the assay chambers described below without anesthetization using an aspirator. Each assay chamber was a 20 mm vial, sealed with parafilm, containing half a Fly Plug saturated with a 1 mL solution of water, 0%-20% ethanol, and 3% sucrose (Fischer #BP220-1). Ten females were aspirated into each vial and left for 24 hours undisturbed.

Egg Habitability

Females were collected under CO₂ anesthetization 13 days after eggs were laid by the previous generation and held in groups of five for 24 hours in vials. Single females were then aspirated into plasticware containers and allowed to oviposit for 24 hours. The female was then removed and the container placed back to remain undisturbed for an additional 24 hours. Larvae and eggs were then counted at the end of the allotted time.

Larval behavior

Females were collected under CO₂ anesthetization 13 days after eggs were laid by the previous generation and held in groups of five for 24 hours in vials. Five females from *D. yakuba* and *D. santomea* were aspirated into each plasticware container and allowed to oviposit for 24 hours before their removal from the assay chambers. Females from *D. melanogaster* were allowed to oviposit for only 12 hours before their removal to limit the total number of eggs. Larvae were then counted 30 hours after the start of the assay.

DNA Quantification

We prepared 120 females from the F₂ population with traits ranging from $X < 0.34$ and $X > 0.62$ for DNA extraction. Flies were pulverized using a mortar and pestle, and DNA was extracted using the Qiagen DNeasy Blood & Tissue Kit. 1080 uL of Buffer ATL and 120 uL of proteinase K were used to digest the tissue at 56C for 12 hours. The Qiagen extraction protocol was followed exactly, with the exception of using water instead of the provided buffer as an eluate. An Illumina paired-end library was created at the University of California of Davis Genome Center.

Results

We found major differences among strains when females were presented with the choice to oviposit on ethanol-rich or ethanol-free substrate. Pairwise comparisons of ethanol-free food vs. concentrations of 6%, 10%, and 15% ethanol were conducted, but the differences among species were most pronounced at 10%, where we collected the largest data set (figure 1). In split-patch assays, where the two substrates are presented without a gap between them, preferences paralleled what is known about ethanol tolerance with one major exception (detailed below). Published ethanol tolerances of adult flies, from most to least tolerant, are cosmopolitan *D. melanogaster*, African *D. melanogaster*, *D. simulans*, *D. yakuba* and finally *D. mauritiana* (Mercot et al., 1994). The proportion of eggs laid on ethanol here is in the same rank order as ethanol tolerance, with the cosmopolitan strain of *D. melanogaster* laying 72% of eggs on ethanol and *D. mauritiana* laying only 35% on ethanol (figure 1a). *D. santomea*, for which there are no published ethanol tolerance data, deposited 82% of its eggs on ethanol. We were surprised females of this species preferred ethanol more strongly than *D. melanogaster*, as *D. santomea*'s sister species *D. yakuba* has much lower preference and tolerance.

We used the non-parametric Kruskal-Wallis test to verify that preferences varied significantly among the tested strains ($p < 2.2e-16$). We also conducted a binomial test on each strain to test for significant preference for one of the options. For these tests we simply considered the proportion of replicates within a strain with greater or lesser than 50% on ethanol; these tests have lower power because a replicate with 51% of eggs on ethanol is treated the same as one with 100% of eggs on ethanol, and we Bonferonni corrected for 7

parallel tests. None the less, as shown in figure 1a, some strains clearly preferred ethanol while others significantly avoided it.

In the two-patch assays, when the same two oviposition substrates were presented to females with a 7 cm gap between them, the mean number of eggs laid on ethanol decreased for all strains, though some species like *D. simulans*, responded only slightly (figure 1b). The biggest shifts were seen in the two strains that most strongly preferred ethanol in the split-patch scenario. The strain from the most ethanol tolerant species (cosmopolitan *D. melanogaster*) now laid the *fewest* number of eggs on ethanol (18%). The second biggest shift in mean was seen in *D. santomea*, while its sister species *D. yakuba* changed very little. As a result, the large difference between these sister species in the split-patch scenario (Wilcoxon $p=4.08e-11$) disappeared in the two-patch case (Wilcoxon $p=0.34$). The variance among replicates within strains was higher for all strains in the two-patch case, consistent with the hypothesis that flies have a harder time comparing the available options when they are far apart. Despite this higher variance, there was still significant variation in behavior among strains in the two-patch case (Kruskal-Wallis $p=7.38e-07$).

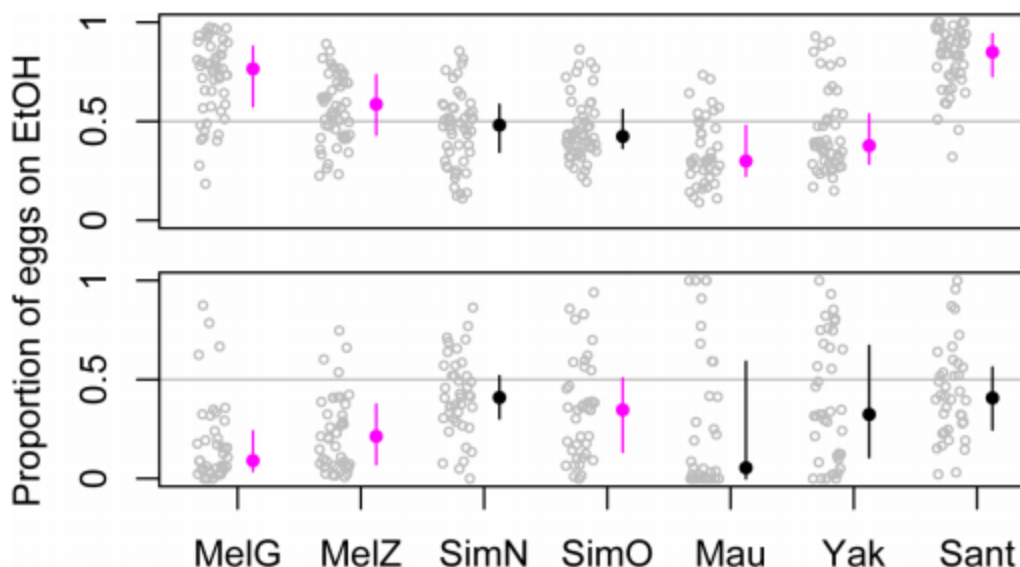


Figure 1. Oviposition on 10% ethanol vs. 0% ethanol. Each strain was presented with the two substrates touching (a split-patch, top figure) or separated by 7 cm (two patches, bottom figure). Each replicate is shown as a grey circle, and the median for each strain is shown as a filled circle with a line spanning the central 50% range. Strains significantly different than random based on a binomial test, Bonferroni corrected for seven parallel tests, are shown in fuchsia.

The additional data collected comparing ethanol-free substrates to 6% ethanol were similar to that of the 10% ethanol experiment (Figure 3). The rank order among strains was the same in the split-patch case, with the exception of cosmopolitan *D. melanogaster*, which laid slightly more eggs on ethanol (72%) than *D. santomea* (64%). Note that *D. santomea* laid *more* eggs on ethanol when ethanol concentrations were higher, while *D. melanogaster* did not change their behavior with increasing concentrations (mean = 72% in both cases). Although differences between strains were significant in the split-plate case at 6% ethanol

(Kruskal-Wallis $p=1.51e-06$), this was not the case in the two-patch assays at the same concentration (Kruskal-Wallis $p=0.11$). Just like in the 10% case, the strains that preferred ethanol most strongly in the split-patch case changed the most when these choices were placed farther apart, so that *D. santomea* and cosmopolitan *D. melanogaster* laid the fewest and the second fewest eggs on ethanol, respectively. Although sample sizes are too low for statistical significance at 15% ethanol, the rank order of strains in the split plate case was nearly the same, with *D. melanogaster* and *D. santomea* still laying over 60% of eggs on this very high ethanol concentration, while all other strains laying below 50% (Figure 2). When these two options were presented with a 7 cm gap between them, all strains strongly avoided the substrate with 15% ethanol (Figure 2).

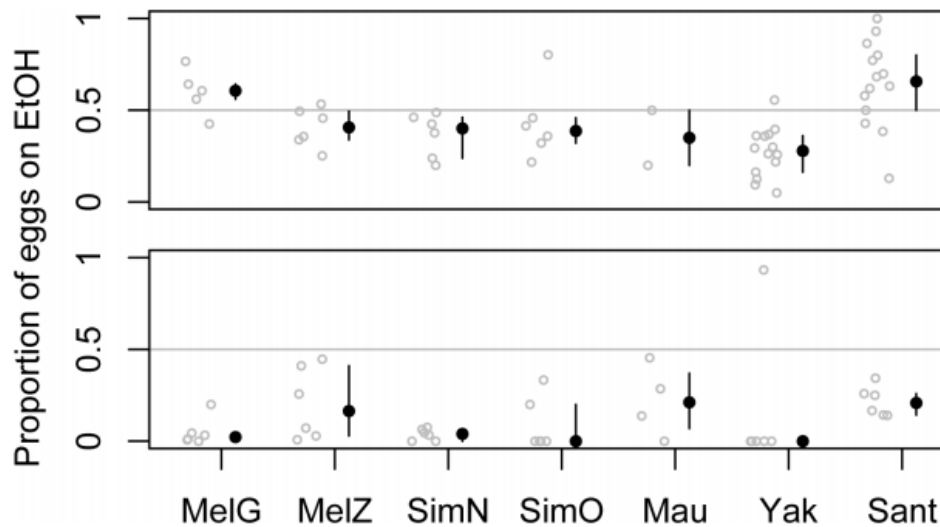


Figure 2. Oviposition on 15% ethanol vs. 0% ethanol. Each strain was presented with the two substrates touching (a split-patch, top figure) or separated by 7 cm (two patches, bottom figure). Each replicate is shown as a grey circle, and the median for each strain is shown as a filled circle with a line spanning the central 50% range.

Diffusion could create a gradient in ethanol concentration when substrates are touching, and this might affect preferences (Schwartz et al., 2012). Diffusion is unlikely to be responsible for the proximity effect seen here for several reasons, including the quantitative effect of increasing distance seen in previous work (Miller et al., 2011). To ensure that the proximity effect seen here was due to distance itself rather than diffusion, we set up two additional assays. We created a split-patch assay with a piece of transparency film placed between options to eliminate diffusion. Using the cosmopolitan *D. melanogaster* strain, we found no significant differences between the preferences within the split-patch assays with or without the transparency film (Wilcoxon $p=0.81$, Figure 4). We also collected data for the two-patch case with only 0.5 cm between options, rather than 7 cm as above. We found that *D. melanogaster* females shifted their preference significantly when options were not touching, even if they were within 0.5 cm (Wilcoxon $p=4.4e-5$). Preferences at 0.5 cm separation were intermediate to 0 cm and 7 cm, illustrating that distance has a quantitative effect on preference (Figure 4).

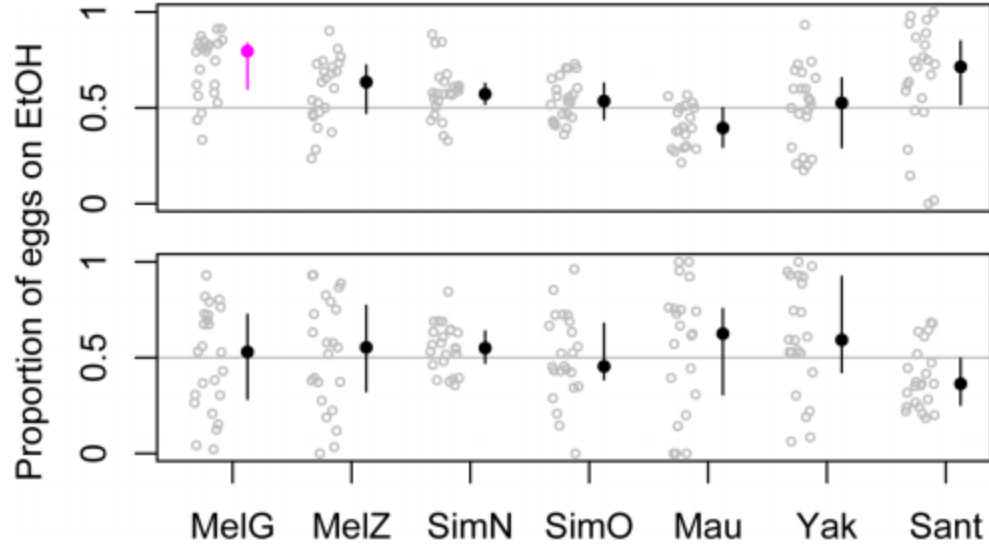


Figure 3. Oviposition on 6% ethanol vs. 0% ethanol. Each strain was presented with the two substrates touching (a split-patch, top figure) or separated by 7 cm (two patches, bottom figure). Each replicate is shown as a grey circle, and the median for each strain is shown as a filled circle with a line spanning the central 50% range. Strains significantly different than random based on a binomial test, Bonferroni corrected for seven parallel tests, are shown in fuchsia.

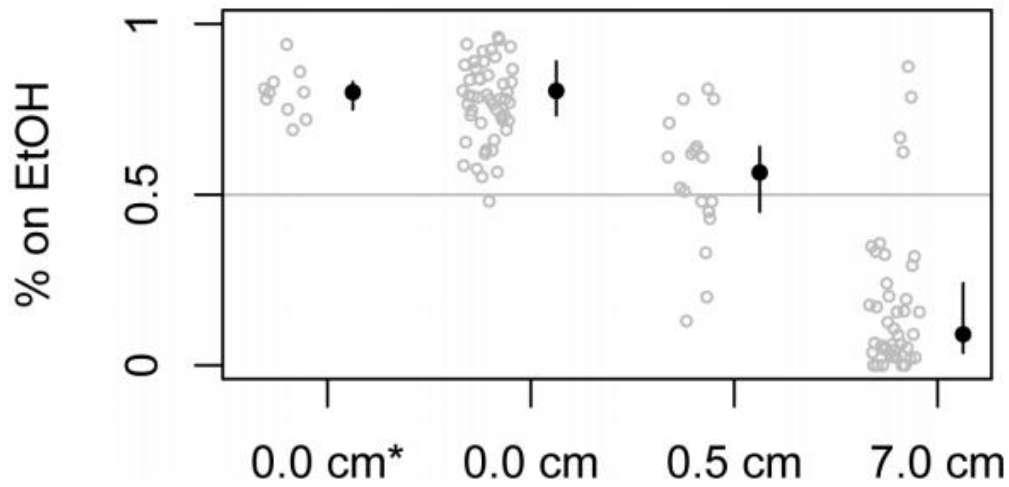


Figure 4. Testing the role of diffusion. We compared the data for Cosmopolitan *D. melanogaster* to two additional controls: a split-patch case where the options are touching except for a slice of transparency paper between them (0.0 cm*), and a two-patch case where the options were much closer (0.5 cm). Split-patch data (0.0 cm) and two-patch data (7.0 cm) are data from figure 1 with additional replicates run at the same time as the new assays. All data were 0% vs. 10% ethanol.

Ethanol preferences are decoupled from ethanol tolerance in *D. santomea*

Drosophila santomea is the only species in our experiment with no published data on ethanol tolerance. Because this species behaved like the highly tolerant *D. melanogaster*, we predicted that it may have also evolved high ethanol tolerance. We measured ethanol tolerance by placing 10 flies in a vial with a water-sucrose solution and various concentrations of ethanol; Figure 5 shows the proportion of flies dead after 24 hours. We compared *D. santomea* to its sister species *D. yakuba*, to cosmopolitan *D. melanogaster* (expected to have high tolerance) and finally to *D. sechellia*, which is reported to have the lowest tolerance in the species group. While *D. melanogaster* suffered no mortality at any ethanol concentration tested (5%-25%), *D. santomea* suffered 93% mortality at only 10%. Indeed, *D. santomea* was similar to, and possibly even less tolerant than the sensitive species *D. sechellia*. To determine if *D. santomea* had significantly lower tolerance than that of *D. yakuba*, we used Wilcoxon rank-sum tests to compare mortality at each concentration. Except at the highest concentration, which is fatal to both species, and lowest concentration, these species were significantly different at all concentrations tested ($p=0.04$ at 20% EtOH, $p\leq 0.006$ at all other concentrations).

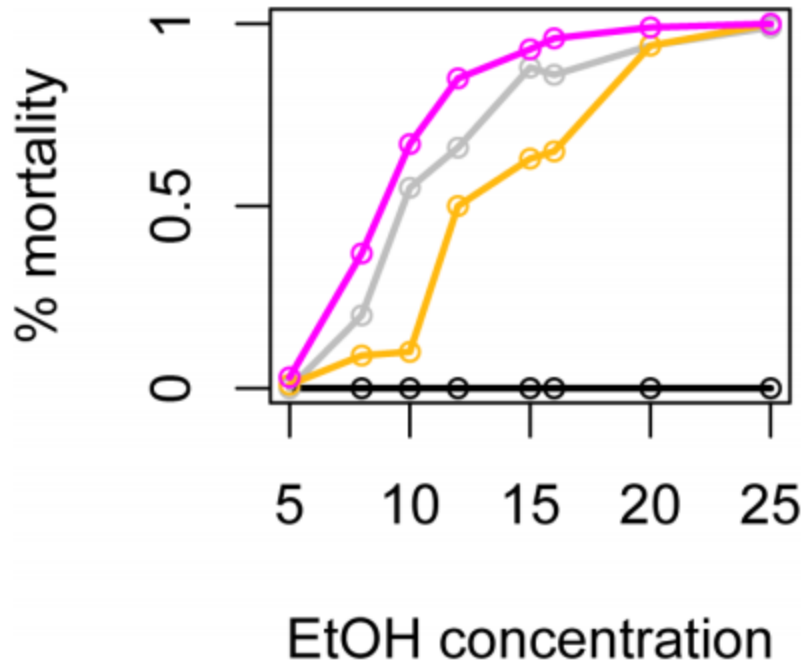


Figure 5. Ethanol tolerance of adults. The proportion of females surviving after 24 hours of ethanol exposure was measured for four species: *D. santomea* (fuchsia), *D. sechellia* (grey), *D. yakuba* (gold), and *D. melanogaster* (black). Only medians are shown for simplicity; sample sizes for each strain at each concentration varied from 3-16 replicate vials of ten females each.

***Drosophila santomea* and *D. yakuba* differ at the species level**

The big difference in preference between the sister species *D. santomea* and *D. yakuba* was surprising, as these species split only ~400,000 years ago and still share lots of genetic variation (Llopart et al., 2002). We measured five additional genotypes per species in order to confirm that this difference is a species level property, rather than something particular to the strains used. All six of the *D. santomea* strains investigated averaged more than 50% of their eggs on ethanol, while all six of the *D. yakuba* strains averaged less than 50% of their eggs on ethanol (Figure 6). A Wilcoxon Rank-Sum test comparing the means of the six *D. yakuba* strains to the means of the six *D. santomea* strains is significant ($p=0.008$). These data support the conclusion of a major species-level difference in preference, though there appears to also be variation among strains within a species (Figure 6).

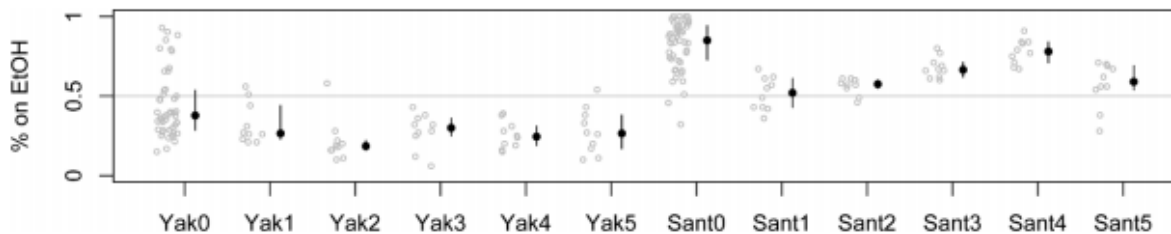


Figure 6. *D. yakuba* and *D. santomea* are different at the species level. Multiple strains of each species were presented with the two substrates touching (a split-patch, top figure) or separated by 7 cm (two patches, bottom figure). Each replicate is shown as a grey circle, and the median for each strain is shown as a filled circle with a line spanning the central 50% range. Yak0 and Sant0 are the data from figure 1.

***Drosophila santomea* behavior is not inherently maladaptive**

It is surprising that *D. santomea* would prefer to lay eggs on ethanol-containing substrate but have a low tolerance for ethanol as adults. One possibility is that the lab assay is unnatural enough that they are making a maladaptive choice which they would not make under natural circumstances. However, because they only exhibit this behavior when non-ethanol substrates are nearby, it is possible that it reproduces their natural behavior and is adaptive. If eggs laid on ethanol develop without problems, and larvae travel to non-ethanol substrates after hatching, there may be little to no cost in choosing ethanol substrates. Laying eggs in ethanol might then be adaptive in response to parasitoids, predators, or pathogens in nature. To begin to address this question we first determined if *D. santomea* eggs hatch equally well on ethanol and non-ethanol substrates; *D. yakuba* and Cosmopolitan *D. melanogaster* were included for comparison. We saw no evidence that ethanol substrate reduced the proportion of eggs hatched for any species (Figure 8). For *D. santomea*, 65% and 61.5% of eggs hatched after 24 hours on ethanol-free substrate and 10% ethanol, respectively (Wilcoxon $p=0.39$).

We also tested whether larvae of *D. santomea* leave ethanol substrate after hatching, and again included *D. yakuba* and *D. melanogaster* for comparison. We allowed females of each species to oviposit on split-patch assays, then checked the positions of the larvae after 30 hours (Figure 7). As before, *D. melanogaster* and *D. yakuba* laid a majority of their eggs on ethanol while *D. santomea* did not, and variation among strains in oviposition preference was again significant (Kruskal-Wallis $p=4.52e-06$). After hatching, however, the larvae of all three species significantly avoided ethanol (Binomial $p < 0.05$ for each species after correction for multiple tests). Larvae were clearly moving away from ethanol relative to where they hatched for each species, as Wilcoxon tests comparing the oviposition preference

to larval preference was significant for all three species ($p < 0.001$ in all cases). In striking contrast to the differences among species in oviposition preferences, there were no differences among species in larval preferences (Kruskal-Wallis $p = 0.12$).

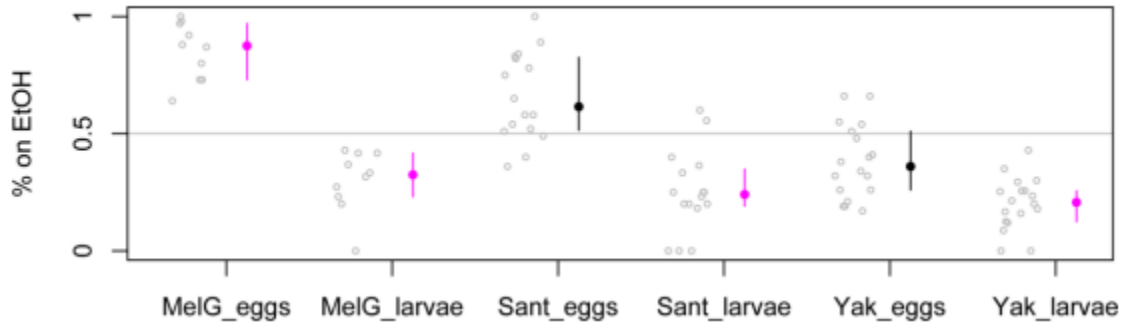


Figure 7. Larval preferences for ethanol. Each strain was presented with the two substrates touching (a split-patch) and allowed to lay eggs. The positions of all larvae hatched after 24 hours were then recorded. Each replicate is shown as a grey circle, and the median for each strain is shown as a filled circle with a line spanning the central 50% range. Strains significantly different than random based on a binomial test, Bonferroni corrected for three parallel tests, are shown in fuchsia.

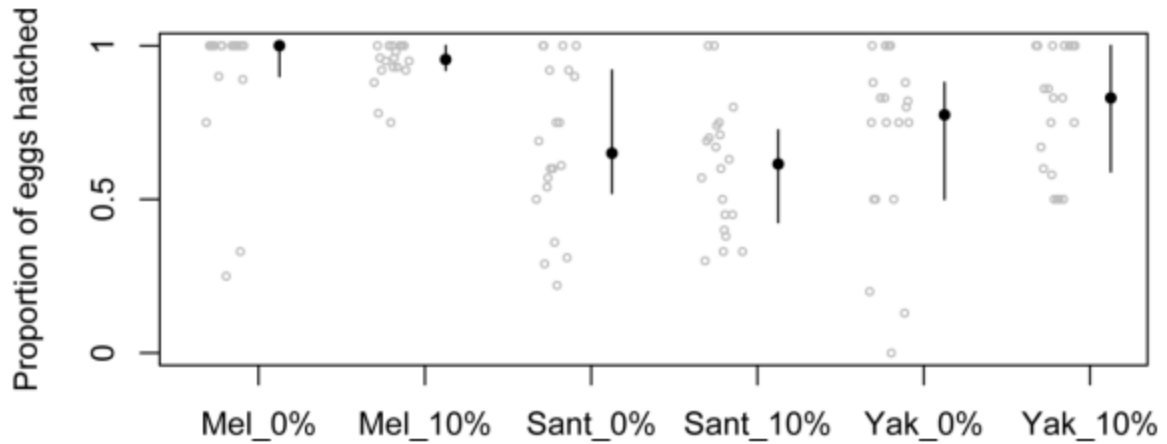


Figure 8. Eggs hatch equally well on ethanol. We compared the proportion of eggs hatching after 24 hours on 0% and 10% ethanol for three species: *D. melanogaster*, *D. yakuba*, and *D. santomea*. The proportion of eggs hatching did not differ between concentrations within a species.

Oviposition preference variation within *D. melanogaster* maps to 2 QTLs

We are interested in identifying natural genetic differences that affect these oviposition preferences. The differences we found among species could potentially be mapped to the gene level, but we first decided to further investigate diversity within *D. melanogaster*. If there are strains within this model species with different preferences, mapping these intra-species differences could be a faster way to find causal alleles.

We found considerable variation among strains of *D. melanogaster* when females were presented with the choice to oviposit on ethanol-rich or ethanol-free substrate on split-patch assays (Figure 9). We assayed 11 highly inbred founder strains from the *Drosophila*

Synthetic Population Resource (DSPR). These strains were collected from diverse locations in the 1950s and 1960s and serve to represent the global genetic variation (Figure 10). We chose to test preferences using 10% ethanol, as the differences were most pronounced at this concentration in previous experiments (Figures 1-3). We used the non-parametric Kruskal-Wallis test to verify that preferences varied significantly among the tested strains ($p < 2.2 \times 10^{-16}$).

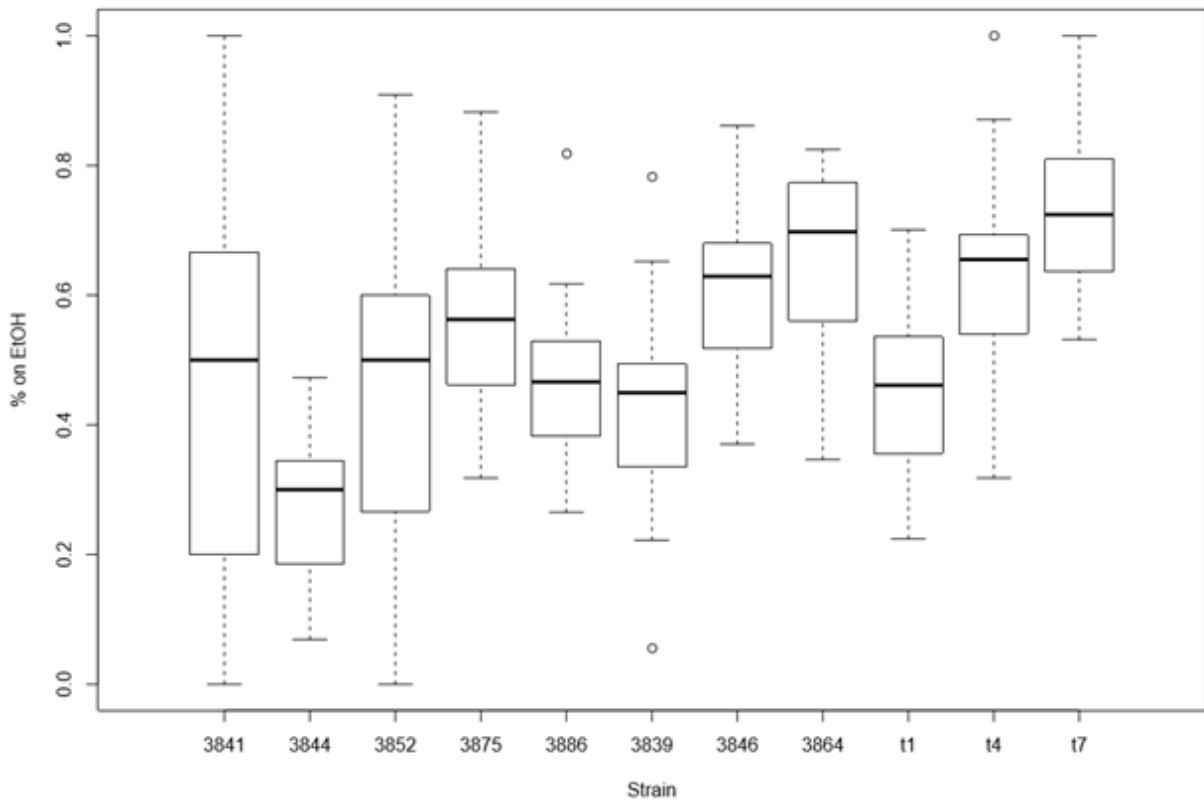


Figure 9: Oviposition on 10% ethanol vs. 0% ethanol. Each strain was presented with two substrates touching (a split-patch). The data is presented as a box and whisker plot, with the bisecting line representing the median, the box showing the central quartiles, the whiskers

showing the outer quartiles, and statistical outliers plotted as circles. Minimum sample size per line = 6 replicates, mean = 21 replicates.

Founder	Vial Code	Stock Center	Stock Number	Stock Name	Collection Site	Collection Year
A2	3841	Bloomington	3841	BOG1	Bogata, Columbia	1962
A3	3844	Bloomington	3844	BS1	Barcelona, Spain	1954
A4	3852	Bloomington	3852	KSA2	Koriba Dam, South Africa	1963
A5	3875	Bloomington	3875	VAG1	Athens, Greece	1965
A6	3886	Bloomington	3886	wild5B	Red Top montain, Georgia, USA	1966
A7	t7	Tuscon	14021-0231.7	n/a	Ken-ting, Taiwain	1968
B1	3839	Bloomington	3839	BER1	Bermuda	1954
B2	3846	Bloomington	3846	CA1	Capetown, South Africa	1954
B3	3864	Bloomington	3864	QI2	Israel	1954
B6	t1	Tuscon	14021-0231.1	n/a	Ica, Peru	1956
B7	t4	Tuscon	14021-0231.4	n/a	Kuala Lumpur, Malaysia	1962

Figure 10: Information on strains used in this section. Information for each strain is listed here, along with collection date and location. All strains were inbred for >18 generations upon arrival from the stock center.

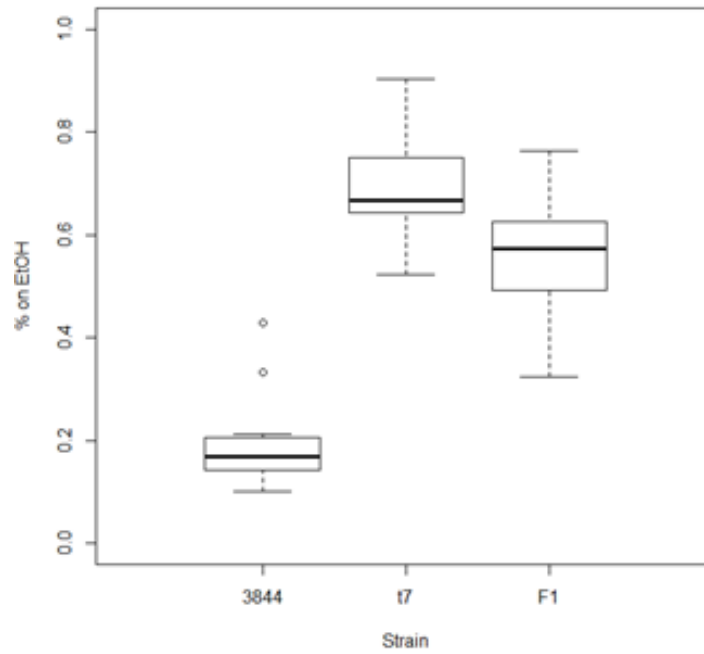


Figure 11. Preference for ethanol is largely dominant. Oviposition on 10% ethanol vs. 0% ethanol. Each strain was presented with two substrates touching (a split-patch). The data is presented as a box and whisker plot, with the bisecting line representing the median, the box showing the central quartiles, the whiskers showing the outer quartiles, and statistical outliers plotted as circles. Minimum sample size per line = 19 replicates, mean = 20 replicates.

To further characterize this variation, we crossed two strains with the most divergent phenotypes and measured the preference of the F1 hybrids alongside the parents. The 3844 line averaged only 17% of its eggs on the ethanol-rich substrate, while the t7 line averaged 67%. Note that these medians differ slightly from the ones in figure 9, as the data here are collected at a later date. Indeed, we found that these preferences varied across experiments, presumably due to incredible sensitivity to environmental effects. None the less, the differences between parent strains were large and consistent, with one strain preferring ethanol and another avoiding it.

Trait values of F1 genotypes reveal that oviposition preferences for alcoholic substrate is largely dominant, with 57% of F1 individuals preferring to oviposit on ethanol. This preference does not show complete dominance as the F1 distribution is significantly different from that of t7 (Wilcoxin $p = 6.9e-4$).

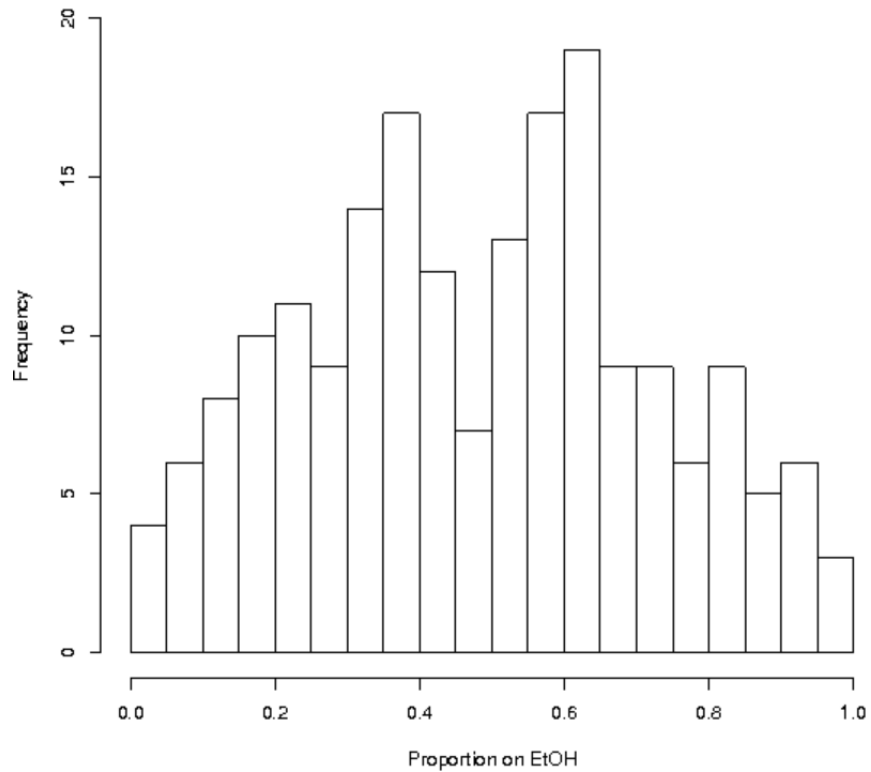


Figure 12: Distribution of F2 preferences. Each female was presented with two substrates, 0% and 10% ethanol, touching (a split-patch).

We then intercrossed the F1 generation to produce F2 progeny. The preferences of 194 individuals from the F2 population were quantified in order to create the distribution seen in figure 12. In this F2 distribution there is a break at 0.45 suggestive of a bimodal distribution, hinting at the operation of a major locus.

During quantification of preference in these oviposition assays, we discarded data from females that laid fewer than 4 eggs per assay. Such replicates were considered to be too difficult to derive any accurate quantifiable preference. Female preference for ethanol were compared to the number of eggs laid per replicate in the F2 population used for quantification (Figure 13). Though there were variation in the amount of eggs laid per replicate, this did not seem to affect the females' behavior of choice (Pearson $p = 0.47$).

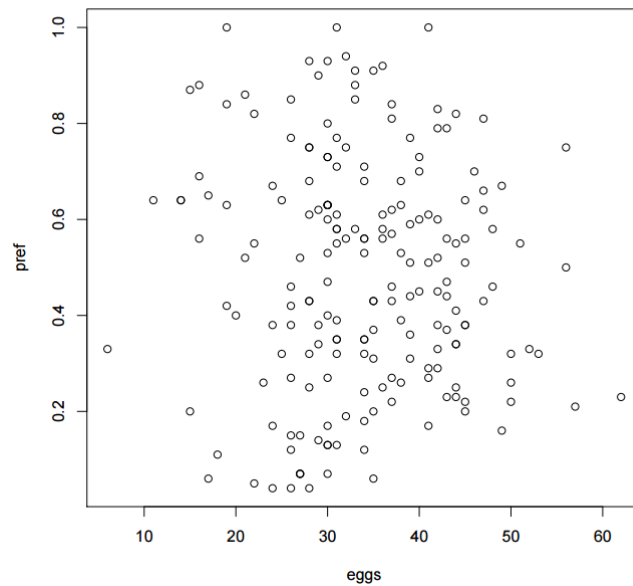


Figure 13: Egg number is not correlated with oviposition preference. The data are presented in a scatterplot to visually outline the relationship between number of eggs per assay and the preference per assay. Each female was presented with two substrates, 0% and 10% ethanol, touching (a split-patch).

We carried out F2 QTL mapping to provide a coarse map of loci contributing to behavioral variation between 3844 (avoids ethanol) and t7 (prefers ethanol). To maximize power, only flies with preferences ranging from $X < 0.34$ and $X > 0.62$ were sequenced due to limited resources. Using interval mapping on 120 genotyped individuals from the F2 population, we identified two QTLs, one on the telomeric end of chromosome 2, and the other closer to the centromere on chromosome 3 (Figure 14).

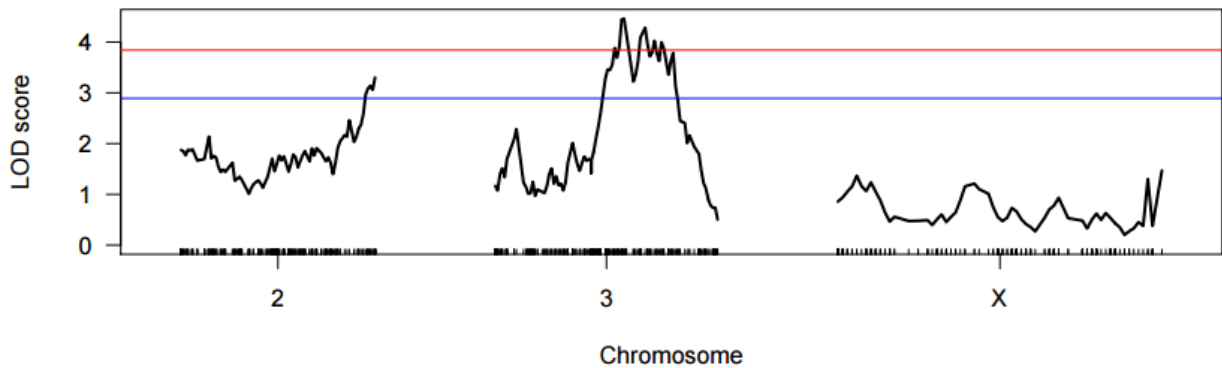


Figure 14: QTL Map. The blue line represents a threshold LOD score of 2.89, 5% FDR and the red line represents a threshold LOD score of 3.84, or a 1% FDR.

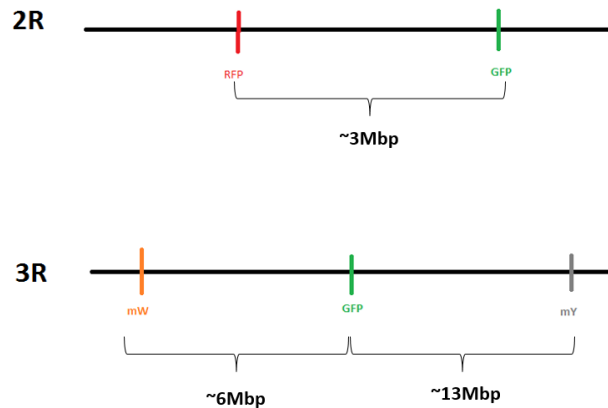


Figure 15: Fine mapping markers. Shown here are the markers introgressed into both 3844 and t7 backgrounds. Distances between each marker are shown in million base pairs (Mbps).

Stock Number	Details of stock
24484	24484 y[1] M{vas-int.Dm}ZH-2A w[*]; M{3xP3-RFP.attP'}ZH-58A
24456	w1118; Mi{ET1}MB02863
6309	y[1] w[*]; CxD/TM3, Sb[1] Ser[1]
3628	y[1] w[*]; nub[2] b[1] sna[Sco] pr[1] cn[1]/CyO
23812	w1118; Mi{ET1}CG6006MB02490
18620	w[1118]; PBac{w[+mC]=WH}CG14693[f03110]
37973	y1 w*; Mi{MIC}CG34375MI04673

Figure 16: Information on strains used to create introgression lines. Information for each strain is listed here. All stocks used were ordered from the Bloomington Stock Center.

QTL mapping of intercrosses between phenotypically divergent lines is a common way to identify loci that influence complex traits. This method is a powerful way to identify the loci responsible for the difference between the parental lines. Due to the limited amount of recombination, however, loci are usually poorly resolved and the predicted effects of the QTL are limited to the intercross background. To resolve our region of interest, we introgressed the QTL region from a multimarker line of unknown genetic background into our near-isogenic inbred DSPR parental lines. We intended to create a panel of introgression lines from successive generations of recombination to narrow the QTL down to a small genomic interval (Figure 16). Because the creation of such lines involve extensive work, we decided to first phenotype introgression lines with intervals spanning the entire lengths of our regions of interest (Figure 15). For the QTL in the 2nd chromosome, this length is 3 Mbps wide and bounded by two benign fluorescent markers. We compared these doubly marked heterozygote recombinants to their nonmarked sister to control for any effect of rearing environment. To account for potential additive or dominant allelic effects, we also created and assayed homozygous lines within the same block. If the QTL acts in an additive manner, the heterozygotes will show intermediate preference and be closer to the mean. If the QTL acts in a dominant manner, we should see similar preferences in the heterozygotes and the homozygotes. We acknowledge that the homozygous lines are limited in that vial effects cannot be controlled for in comparison of marker inserted lines to their wild-type controls. Density and environmental effects were kept as consistent as possible between replicates to minimize any confounding variables. We found no discernable differences between the preferences in the heterozygous and homozygous introgression lines when compared to the inbred DSPR parents in both the 3844 background (Kruskal-Wallis $p = 0.99$) and the t7

background (Kruskal-Wallis $p = 0.97$) (Figure 17). Because of this, we conclude that the QTL peak seen on chromosome 2 may have been a false discovery.

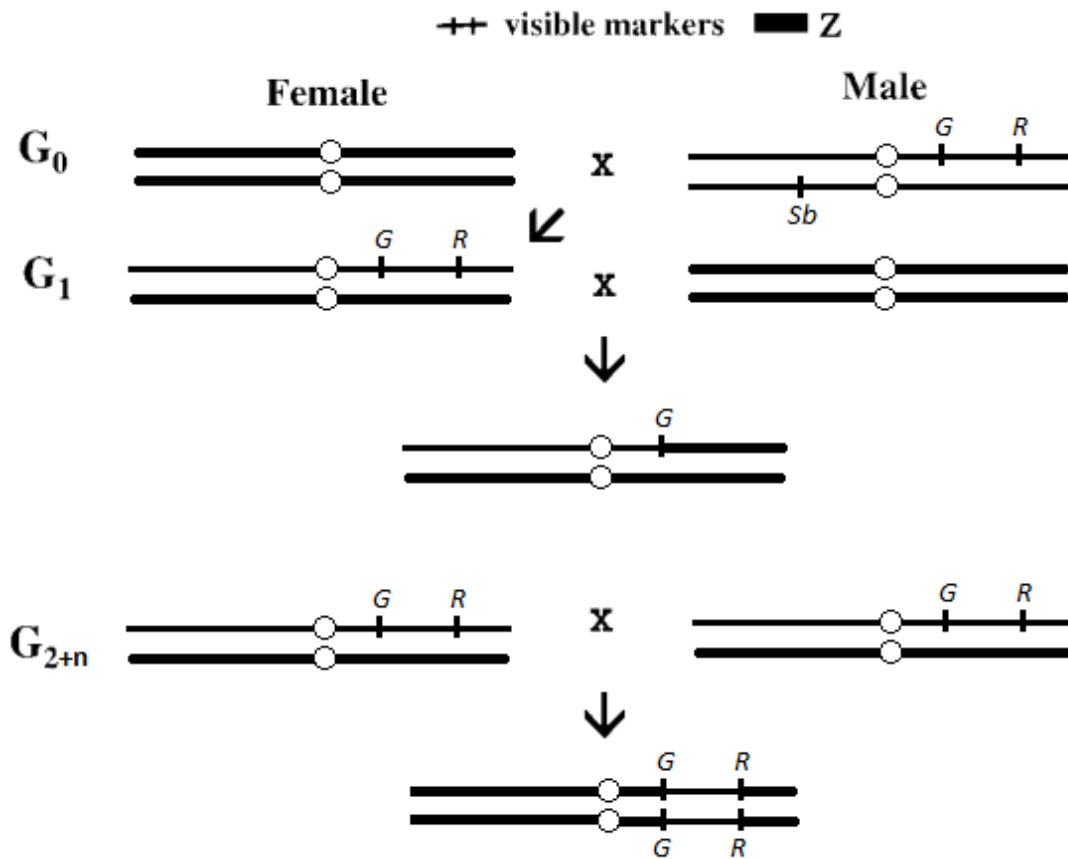


Figure 17: Second Chromosome Introgression Crosses. An example of the crosses performed to construct the second chromosome recombinant lines between an inbred background (Z), 3844 or t7, and the multimarker line. Thick bars indicate the Z chromosomes and the thin bars with ticks indicate the marker chromosomes. Introgression stocks are maintained without recombination by backcrossing the heterozygous introgression males to inbred DSPR females.

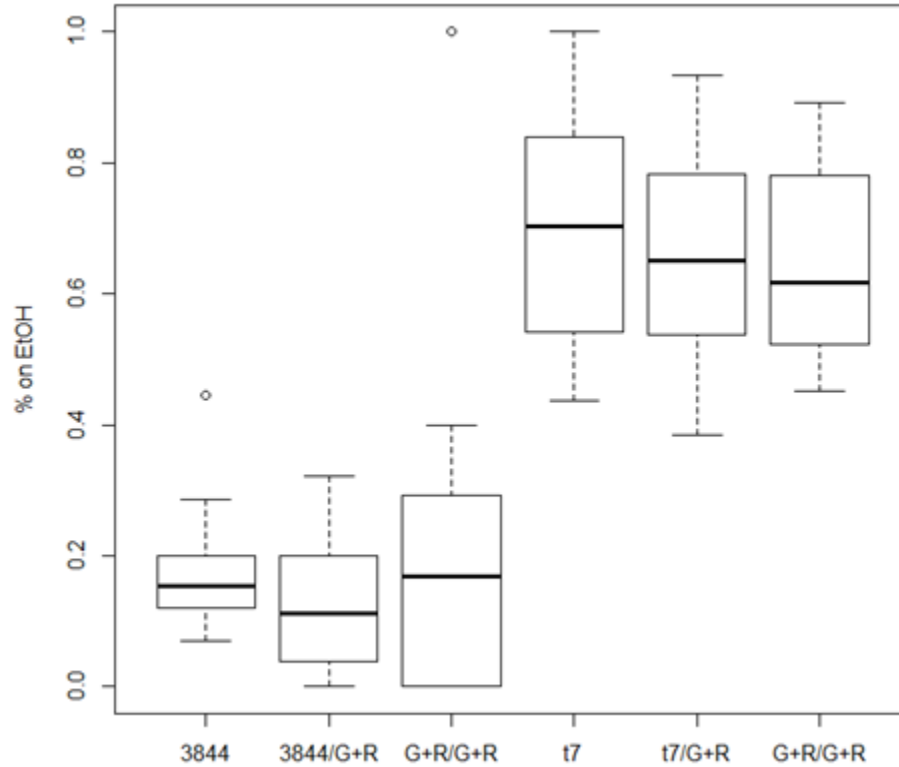


Figure 18. Oviposition on 10% ethanol vs. 0% ethanol. Each strain was presented with two substrates touching (a split-patch). G+R denotes the two benign markers, GFP and RFP, flanking the 3 Mbp region of interest. The data is presented as a box and whisker plot, with the bisecting line representing the median, the box showing the central quartiles, the whiskers showing the outer quartiles, and statistical outliers plotted as circles. Minimum sample size per line = 12 replicates, mean = 27 replicates.

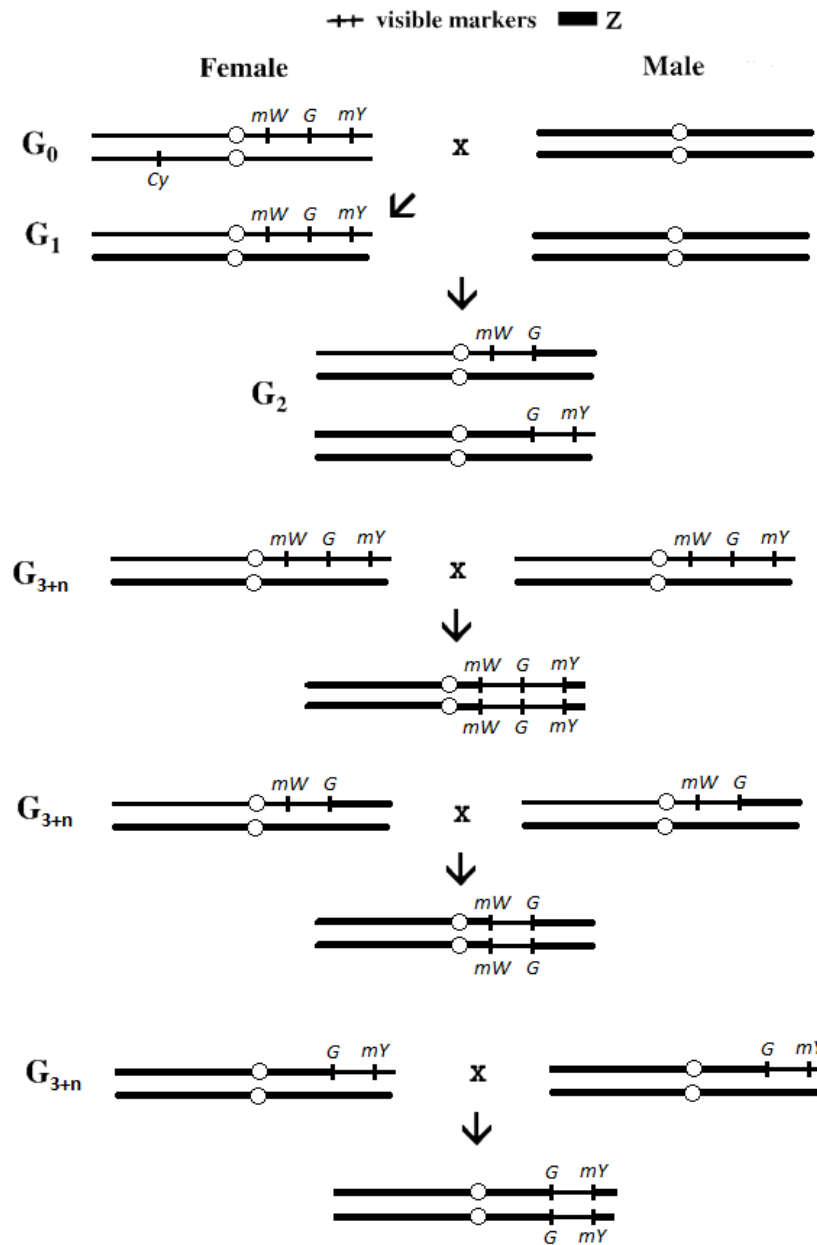


Figure 19: Third Chromosome Introgression Crosses. An example of the crosses performed to construct the third chromosome recombinant line between the 3844 DSPR inbred background (Z), and the multimarker line. Thick bars indicate the Z chromosomes and the thin bars with ticks indicate the marker chromosomes. Introgression stocks are

maintained without recombination by backcrossing the heterozygous introgression males to inbred DSPR females.

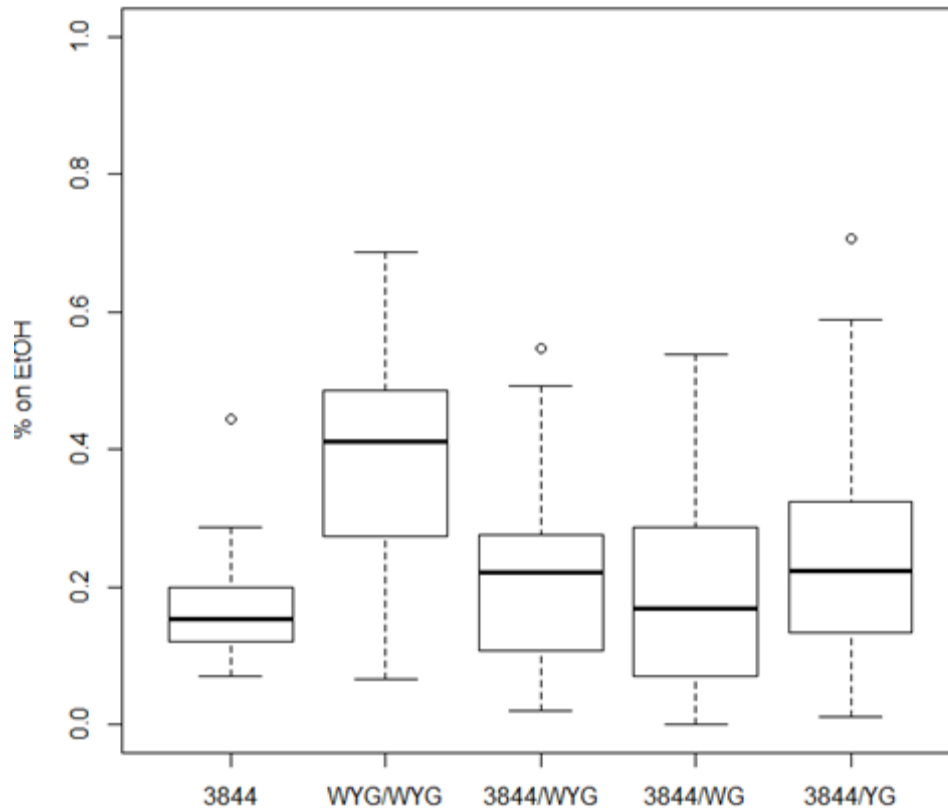


Figure 20. Heterozygous marked lines do not display a shift in preference. Oviposition on 10% ethanol vs. 0% ethanol. Each strain was presented with two substrates touching (a split-patch). WYG denotes the three benign markers, mini-white, mini-yellow, and GFP, spanning the 12 Mbp region of interest. The data is presented as a box and whisker plot, with the bisecting line representing the median, the box showing the central quartiles, the whiskers showing the outer quartiles, and statistical outliers plotted as circles. Minimum sample size per line = 12 replicates, mean = 27 replicates.

We then looked to see if introgressing the QTL on the third chromosome would have any effect on behavior. Again, we created heterozygous and homozygous introgression lines with intervals spanning the entire length of our region of interest, 19 Mbps encompassing 3 different benign markers (Figure 15). Due to time constraints, we only created introgression lines within the 3844 parent background (Figure 19). Although there were no discernable differences between the 3844 parent and the heterozygous introgression line (Wilcoxin, $p = 0.20$), we saw significant differences when the parental line was paired with the homozygous introgression line (Wilcoxin, $p = 3.1e-4$) (Figure 20). This seems to suggest some threshold effect such that one copy of introgressed region is not enough to affect oviposition preference. We also created and assayed doubly marked introgression lines that spanned a smaller genomic interval, 6 Mbps and 13 Mbps. When compared with the inbred parent, none of the heterozygotes showed any difference in preference (Kruskal-Wallis, $p = 0.99$), suggesting the absence of any additive effects.

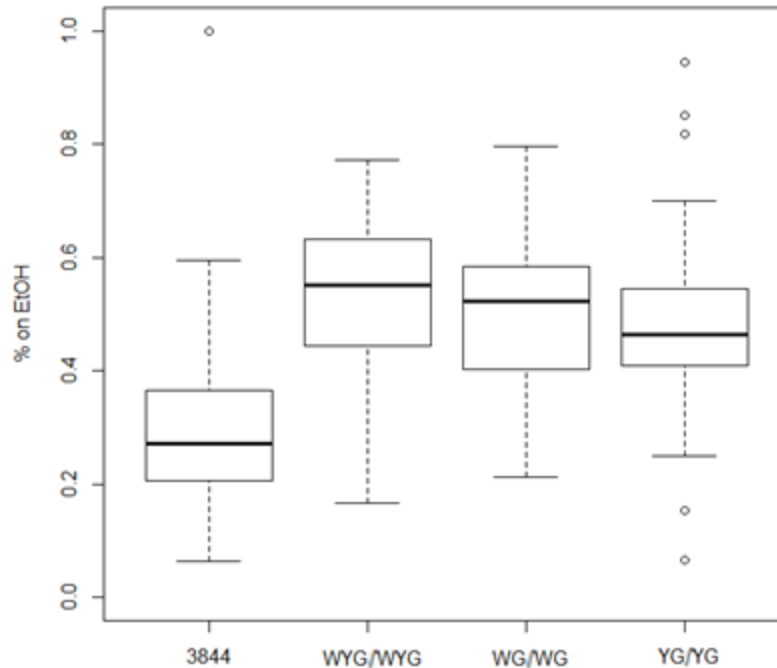


Figure 21. Homozygous marked lines display a shift in preference. Oviposition on 6% ethanol vs. 0% ethanol. Each strain was presented with two substrates touching (a split-patch). The data is presented as a box and whisker plot, with the bisecting line representing the median, the box showing the central quartiles, the whiskers showing the outer quartiles, and statistical outliers plotted as circles. Minimum sample size per line = 37 replicates, mean = 40 replicates.

To further investigate the effect of this marked haplotype, we also created and assayed homozygous lines for the smaller doubly-marked introgressed regions. The concentration of ethanol tested was decreased to 6% from 10% as variation between the lines tested was observed to be more pronounced at this concentration (Figure 21). We observed that all the homozygous introgression lines preferred ethanol more than the 3844 inbred

parent (Wilcoxon $p < 1.8e-7$). However, we also found that there was no difference in preference between the homozygotes (Kruskal-Wallis $p = 0.09$). More work needs to be done to resolve the effect of the marked haplotype. With continued fine mapping using introgression lines, we hope to correlate the behavior to smaller causal loci.

Discussion

Though many studies have investigated how *D. melanogaster* choose among oviposition substrates with respect to ethanol, our work has discovered the important role of space in modulating those preferences. We find that a Cosmopolitan strain of *D. melanogaster* prefers to oviposit on substrates up to at least 15% ethanol as long as they are immediately adjacent to non-ethanol substrate. When options are separated by a gap of only a few centimeters, however, this strain strongly avoids substrates with 10% ethanol or more. This proximity effect was also seen in *D. melanogaster* from Sub-Saharan Africa. Determining the evolutionary significance of this behavior would require additional work, as our assay is designed to be simple, scalable, and replicable rather than ecologically realistic. A reasonable hypothesis might be that female flies prefer to oviposit near a larval food source rather than on it because nutritious food sources like rotting fruit can be rich with bacteria and fungi, attracting other fly larvae and also potential predators like ants and parasitoid wasps. Ethanol may offer some protection from predators and parasites with a lower ethanol tolerance (Kacsoh et al., 2013). In distances too large for larvae to easily transverse this advantage is diminished and females allocate more eggs towards sites promoting larval development. Ethanol might also serve as a cryoprotectant in temperate *D. melanogaster* populations (Zhu and Fry, 2015), though this is unlikely to be relevant in *D. santomea*, which is endemic to tropical São Tomé. Regardless of the fitness consequences, however, this "proximity effect" has now been found for a variety of oviposition cues, and seems likely to be a general phenomenon in this species (Miller et al., 2011; Yang et al., 2008). It is important to note that we have only investigated a single strain of *D. melanogaster* from each subspecies, and there

is sure to be variation in behavior among strains as we have seen for other species here and for other *D. melanogaster* oviposition behaviors (Miller et al., 2011).

In addition to clarifying the preferences of *D. melanogaster*, our results from additional species are interesting for several reasons. First, it is clear that preferences vary substantially among species. Because we raised all species in a common environment, these behavioral differences are likely to be caused by genetic differences. Naturally evolved differences are filtered by natural selection, and this may make them more likely than induced mutations to be found at key control points in the system (Stern, 2013). These genetic differences may therefore prove to be valuable tools for probing the genetic and neural substrates of decision making.

Second, we show that ethanol preference and ethanol tolerance are not completely coupled: the most and least tolerant species, *D. melanogaster* and *D. santomea*, behave in similar ways. We see a positive relationship between tolerance and preference across the other strains, which could result from pleiotropy (if the same genes affect the two traits) or intragenomic coevolution (if species with low tolerance have also evolved to dislike ethanol). Preference differences between *D. yakuba* and *D. santomea*, however, are clearly not a pleiotropic effect of tolerance genes, which makes the identity of these genes especially interesting for understanding decision making.

We also see interesting differences in how species react to the distance between options. *Drosophila santomea* and *D. melanogaster* change egg allocation dramatically with distance, but their sister taxa change very little in each case. The difference between *D. yakuba* and *D. santomea* can be conceptualized as a gene-by-environment interaction, because these species do not differ when options are far apart but do differ when they are

close together (figure 1). The genetic basis of this divergence might therefore be informative about the mechanism underlying this proximity effect.

Finally, we used a phenotypically divergent pair of inbred lines of *D. melanogaster* to create an intercross population for QTL mapping. We identified two QTL peaks on separate chromosomes and try to resolve them by creating recombinant introgression lines spanning parts of our regions of interest. Though we failed to resolve the QTL on chromosome 2, we found that 3rd chromosomal homozygote introgression lines were phenotypically different to their wild-type controls. Because this region spans 19Mbps, phenotyping of additional introgression lines is needed to further resolve this region. We hope to create a panel of introgression lines from successive generations of recombination to narrow the QTL down to a small genomic interval. In the long-term, we believe that combining the study of natural variation in behavior with the neurogenetic tools developed in the *D. melanogaster* model system will lead to major advances in our understanding of behavior and evolution.

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