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Understanding How Divergence in Diet Breadth and the Degree of Environmental Variability Contribute to Individual Differences in Decision-Making and Learning

By

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ABSTRACT

Understanding How Divergence in Diet Breadth and the Degree of Environmental Variability Contribute to Individual Differences in Decision-Making and Learning

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The broad focus of my dissertation work centralized on investigating the various selection pressures contributing to the variation we observe in animal cognition, and how that variation evolves. Primarily, I investigated how divergence in habitat breadth contributes to variation in decision-making and learning; more specifically, how environmental variability influences the evolution of these cognitive abilities. Highly variable environments are predicted to favor higher decision-making and learning abilities (because those are the environments where they are hypothesized to be the most adaptive), while uniform environments are predicted to favor more "hardwired", or "rules of thumb", based approaches. To investigate this, we are comparing two closely related species of fruit flies that recently diverged in diet breadth (and thus have been under divergent selection for the degree of environmental variability experienced in nature), *Drosophila simulans* and *Drosophila sechellia*.

Divergence in habitat breadth and the degree of environmental variability experienced in nature are predicted to influence the evolution of general cognitive abilities (i.e., whether you have the general ability to make decisions and learn). However, due to divergent evolutionary histories, not

all stimuli in the environment have equal consequences for everyone. So, when animals begin to diverge in cognition, does this divergence generalize across a broad range of cognitive abilities, stimuli, and contexts? Or is divergence in cognition limited to certain cognitive abilities, stimuli, or contexts? The central focus of my work has been understanding how selection pressures influence the evolution of cognition to produce variation on cognitive outcomes, and whether that divergence generalizes across all cognitive abilities and stimuli, or if divergence is limited to certain cognitive abilities, stimuli, or contexts.

Overall, I found that higher levels of environmental variability were not sufficient for higher generalized cognitive performance to evolve. Our studies found that higher environmental variability was associated with higher decision-making accuracy, but not with higher learning performance. I also found that cognitive performance was significantly influenced by unexpected cognitive biases, such as side bias. Thus, when species diverge in cognition, divergence does not always generalize across cognitive abilities and may be dependent upon the cognitive abilities, stimuli, and contexts involved.

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I am also very grateful to all undergraduate research assistants who assisted with data collection, including Allison Jaffe, Frederick Cavallaro, Omar Pasha, Jessica Nguyen, Erin Harrison, Corina Do, Diana Alvarado, Ginny Qian, Amy Kuriakose, Lynn Pasha, Sriya Kakarla, Anu Ayeni, Yu-Hsuan Chen, Sanjana Krishnan, Emma Every, Janya Ram, Maya Pai, Madeleine O'Brian, Maddie Kendall, Benjamin Johnson, Stephanie Zhou, Oge Anwaegbu, and Sarah Torrensen.

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Table of Contents

Chapter 1

Does Divergence in Habitat Breadth Associate with Species Differences in Decision Making in *Drosophila sechellia* and *Drosophila simulans*?

Abstract: Decision making is involved in many behaviors contributing to fitness, such as habitat choice, mate selection, and foraging. Because of this, high decision-making accuracy (i.e., selecting the option most beneficial for fitness) should be under strong selection. However, decision making is energetically costly, often involving substantial time and energy to survey the environment to obtain high-quality information. Thus, for high decision-making accuracy to evolve, its benefits should outweigh its costs. Inconsistency in the net benefits of decision making across environments is hypothesized to be an important means for maintaining variation in this trait. However, very little is known about how environmental factors influence the evolution of decision making to produce variation among individuals, genotypes, and species. Here, we compared two recently diverged species of *Drosophila* differing substantially in habitat breadth and degree of environmental predictability and variability: *Drosophila sechellia* and *Drosophila simulans*. We found that the species evolving under higher environmental unpredictability and variability showed higher decision-making accuracy, but not higher environmental sampling.

1. Introduction

Decision making is an important behavior involving the evaluation of cues in the environment to select an option among one or more alternatives [1–3].

Decision making is involved in many behaviors contributing to fitness, such as habitat choice, mate selection, and foraging [4]. Because of this, it follows that maximizing the fitness outcomes of decision-making events should be under strong selection, i.e., that individuals should make "accurate" decisions. However, what is actually observed in nature is a great deal of variation in decision-making outcomes [3,5–8]. Animals frequently make decisions failing to result in the most beneficial fitness outcomes (i.e., seemingly "inaccurate" decisions), even under circumstances where the optimal decision appears obvious to human observers, or when making the wrong decision can have severe fitness consequences [9–11]. So why do we observe this variation in decision making, and how is natural selection acting on this trait to influence the evolution of decision making?

Previous work has demonstrated that highly accurate decision making is costly, both in terms of time and energy [12–15]. A decision is considered "highly accurate" if the option most beneficial for fitness is selected above all available alternatives. Maximizing the accuracy of a particular decision often involves surveying the environment to obtain information, a process which takes time and energy, and can expose animals to various risks (such as the risk of predation) [3]. Any time and energy spent on information gathering and processing is no longer available for maximizing other aspects of fitness. Evidence for this cost of decision making has been documented in a speed/accuracy tradeoff observed in the flower foraging strategies of bumblebees [3], where "hasty" individuals maximize foraging efficiency through faster decisions but show lower accuracy in selecting flowers lacking their predator, crab spiders. "Cautious" individuals exhibit higher accuracy in predator avoidance, but have lower foraging efficiency, due to the increased sampling time required to obtain information [3]. Additional evidence for the

cost of decision making has been observed in a speed/accuracy tradeoff in decision making in great tits [7], as well as a tradeoff between developmental stress and female song choice in songbirds, where females raised in small broods with less developmental stress showed significantly stronger preference for male songs compared to females raised with more developmental stress [8].

Because maximizing the fitness outcome of a decision-making event is costly, highly accurate decision making is hypothesized to be favored in unpredictable or risky environments (where there is more variance in potential fitness outcomes), or in environments where the fitness cost of making a wrong decision is particularly high [3,5]. However, very little is currently known about how differences in environmental predictability and variability, often a by-product of species' evolutionary histories, influence the evolution of decision making [3,12,16,17]. In unpredictable or risky environments, or in high-fitness-cost environments, increased information gathering and decision-making accuracy should be favored, as the benefits of collecting and processing information are expected to outweigh the costs [18]. Environmental predictability is lower in environments that are inherently more complex (have a greater breadth of cues or options) and variable (cues or options vary across time or space) [18,19]. In predictable or low-risk environments, less time and energy should be spent on information gathering, and "rules of thumb" or "good enough" approaches to decision making should be favored [20]. Previous work has shown some evidence for this prediction, in that guppies from high-predation environments exhibited slower decision making and higher accuracy, relative to their counterparts from low-predation environments [5]. However, most examples of this trend have been observed between populations of a given species. There is currently a lack of

understanding of how environmental predictability/risk and potential fitness costs contribute to variation in decision making both within and between species, or how species diverge in decision making when they evolve in environments differing in predictability or risk.

To investigate whether divergence in environmental predictability and risk is associated with differences in decision making, we compared two closely related species—one generalist and one specialist—that differ considerably in habitat breadth, and thus the degree of environmental predictability and variability experienced in nature. We predict that generalist species experiencing greater environmental unpredictability and variability should exhibit higher environmental sampling through increased exploratory behavior, and higher decision-making accuracy, relative to their specialist counterparts. Additionally, we predict that the generalist species should have more genotypic variation in exploratory behavior and decision-making accuracy than the specialist species. To address our hypotheses, we compared two closely related species of *Drosophila* recently diverged in habitat use: *Drosophila sechellia* and *Drosophila simulans*, investigating species, sex, and genotypic differences in exploratory behavior (one mechanism of environmental sampling) and habitat choice (an example of decision making).

2. Materials and Methods

2.1. Drosophila Sechellia and Drosophila Simulans: Closely Related Fruit Fly Species that Differ Substantially in Habitat Breadth

D. sechellia and *D. simulans* are recently diverged, with previous work estimating divergence times ranging from 250,000 to 413,000 years ago [21–

23]. Being so closely related, these sister species can produce viable/sterile male F1 offspring and viable/fertile female F1 offspring [24–26].

While closely related, these species differ substantially in the degree of environmental variability experienced in nature. *Drosophila* habitat and food are synonymous, in that the ephemeral rotting fruit patches these animals live on serve as both their source of food as well as where they mate and lay eggs and spend most of their time [27]. As a habitat generalist, *D. simulans* has a broad potential range of habitat options across time and space (depending on the region or time of year), and experiences greater environmental variability relative to *D. sechellia*, a habitat specialist that has evolved to feed and breed preferentially on the *Morinda citrifolia* (noni) fruit [23,24,26]. While *D. sechellia* has specialized on the noni fruit, which is ubiquitous in the Seychelles and present year-round, noni is toxic to other species of *Drosophila* (including *D. simulans*) [24]. Therefore, these species have evolved distinct habitat preferences; the "accurate" habitat choice in regard to fitness outcome differs for each species. For the specialist, *D. sechellia*, noni is the "accurate" choice, while for the generalist, *D. simulans*, "plain" fruit (i.e., not noni) is the "accurate" choice. Additionally, because *D. sechellia* is an island specialist diverged from *D. simulans*, genotypic variation is predicted to be higher in *D. simulans* than in *D. sechellia*.

2.2. Genotypes

In addition to species comparisons, we also investigated genotypic variation and sex differences in exploration and decision making within species. Isofemale lines (hereafter "genotypes") of *D. sechellia* and *D. simulans* were graciously provided by D. Matute in 2016. The *D. sechellia* genotypes (13 total) were collected from various locations across the

Seychelles, while the *D. simulans* genotypes (11 total) were collected from varied locations across central and southern Africa (Nairobi, Namibia, and Zambia) and Madagascar [23,28, pers.comm.]. Each genotype was established using a single wild female and inbred thereafter; therefore, individuals of the same genotype are more genetically similar to one another than to other genotypes. Thus, these genotypes collected from a range of wild populations represent a broad range of natural genetic diversity.

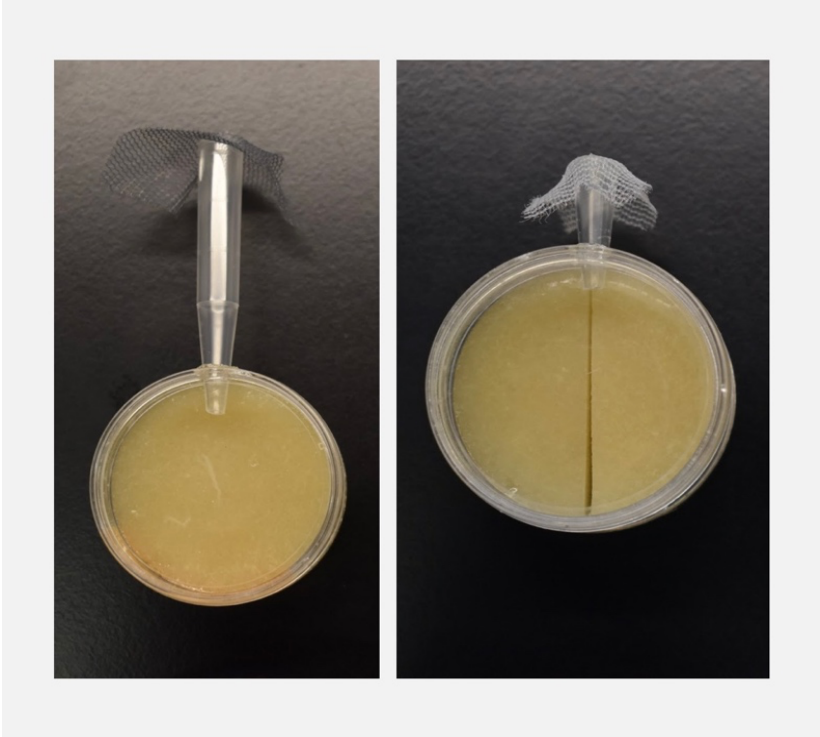
2.3. Rearing

To rear flies for experiments, 10 virgin females were mated to 10 males of the same species and genotype and placed in vials containing standard fly medium (composed of cornmeal, corn syrup, malt sugar, dead yeast, soy flour, tegosept (methyl paraben), proprionic acid, and phosphoric acid). Newly-eclosed virgin male and female F1 offspring were collected under light CO2 anesthesia on day 15, and then housed individually in vials containing standard fly medium. These experimental flies were allowed to recover from anesthesia for three days prior to beginning the exploratory behavior and habitat choice assays. Flies were not deprived of food prior to trials due to concerns that starvation could influence energy levels, exploratory behavior, or habitat choice, as some previous work has shown that flies with less available search time prior to starvation are more likely to settle for less preferred habitats [29].

2.4. Measuring Environmental Sampling (Exploratory Behavior)

To compare variation in environmental sampling between *D. sechellia* and *D. simulans*, we investigated differences in exploratory behavior, i.e., the amount of time it took for flies to emerge from a shelter into a novel

environment. Willingness to emerge from a shelter into a novel environment has previously been considered a reliable measure of exploratory behavior [30–33], with greater time to enter the new environment interpreted as low exploratory behavior. Individual flies were gently aspirated into a 1 mL pipette tip (the shelter) and allowed to emerge on their own accord into a petri dish arena (the novel environment). The arenas consisted of one petri dish containing plain fly food medium (consisting of a standard recipe of agar, malt sugar, inactive dry yeast, and deionized water) covered by a second petri dish (acting as a lid), sealed together with tape to create a self-contained arena (Figure 1a). To prevent escape of the fly during the observation period, the large end of the pipette tip was covered in mesh. During the trial, each fly had the option of either remaining in the pipette tip shelter (which lacked food) or emerging from the shelter into the novel arena (with food). Emergence time was measured in seconds over a 5 h time period. At the conclusion of the 5 h observation period, any flies that had not yet emerged were gently moved from the pipette tips into the arenas, and their recorded emergence time was capped at 18,000 sec.



(a) **(b)**

Figure 1. Environmental sampling (exploratory behavior) assay (**a**) and decision-making (habitat choice) assay (**b**): single flies were aspirated into the pipette tips, which were fitted into a small hole in the arena with plain fly food. A mesh barrier prevented each fly from escaping the pipette tip in the other direction. For the environmental sampling assay, each fly had the option to emerge into the arena or remain in the pipette tip. Each fly's latency to emerge into the arena (in seconds) was recorded. For the decision-making assay, flies were allowed to emerge into the arena on their own accord and were then scan sampled every ten minutes over two hours, and food substrate choice of each fly was recorded.

2.5. Measuring Decision Making (Habitat Choice)

To compare decision-making accuracy between *D. sechellia* and *D. simulans*, the same individual flies evaluated for exploratory behavior were gently aspirated into a shortened pipette tip and allowed to emerge on their own accord into a small petri dish arena (Figure 1b) The arena contained two

ecologically relevant food substrate options: plain fly food medium (consisting of a standard recipe of agar, malt sugar, inactive dry yeast, and deionized water), and imitation *M. citrifolia* (noni) fruit medium. Imitation noni food medium was created by adding both octanoic and hexanoic acids to the plain fly food medium, based on a recipe developed by I. Dworkin and C. Jones [34]. Previous work has extensively demonstrated that both octanoic and hexanoic acids are responsible for the toxicity of noni and are involved both in attracting *D. sechellia* and repelling *D. simulans*, thereby providing a good proxy for noni [24,35–39]. Plain fly food medium and imitation noni medium were used in lieu of real fruit patches to ensure that every fly was faced with an identical choice. Previous work has demonstrated that the levels of octanoic and hexanoic acids in noni can vary substantially depending on fruit ripeness, so use of imitation food substrates allowed us to carefully reproduce foods with a molecularly defined composition [24,35].

Shortened pipette tips were used for introducing the flies into the habitat choice arenas in this stage of the experiment because they significantly reduce the emergence time of the flies, relative to the 1 mL pipette tips used during the exploratory behavior stage. Habitat choice arenas consisted of one petri dish containing the two food substrate halves, which were cut to be flush and covered by a second petri dish (acting as a lid), sealed together with tape (Figure 1b). The relative location of each of the food substrate halves was varied, such that there was a 1:1 ratio of habitat choice arenas with imitation noni on the left-hand side and plain on the right-hand side, and imitation noni on the right-hand side and plain on the left-hand side. Individuals were assigned randomly to habitat choice arenas. Following emergence into the arena, immediate food choice was recorded. Flies were then scan sampled, with food choice recorded for each fly every 10 minutes over the course of a

2 h observation period. In total, we collected 13 habitat choice observations for each individual fly. Some previous work has demonstrated that animals often exhibit higher discrimination accuracy in free choice tests (which offer the option to opt out of making a choice) than in forced choice tests (which lack the option to opt out, and thus force a choice) [40–43]. However, previous work has also shown that the importance of free choice in discrimination accuracy increases with task difficulty [41]. Because of the simplicity of our habitat choice assay and the substantial ecological relevance of our stimuli, we opted for a forced choice test.

2.6. Replication

A total sample size of 526 individuals were measured for both the exploratory behavior and habitat choice experiments. For each of the 24 genotypes, a range of 5 to 22 males and a range of 5 to 16 females were measured. The number of replicates varied between genotypes because of variation in the availability of flies on the day of testing. For exploratory behavior via emergence time, each fly ($N = 526$) was observed for a 5 h time block, totaling in 2630 h of observation. For decision making via habitat choice, each fly ($N = 526$) was observed for a 2 h time block, totaling 1052 hours of observation.

2.7. Analysis

All analyses were conducted in R version 3.6.1 (Vienna, Australia, R core team 2019).

Exploratory behavior

For exploratory behavior, our goal was to investigate species, sex, and genotype differences in willingness to emerge from a shelter (pipette tip) into a novel environment (petri dish arena containing food). To do so, we used a mixed-effect proportional hazards model in the coxme package [44]. Cox proportional hazards models are often used to investigate how various fixed and random predictor variables modify an underlying hazard function [45] and are particularly useful for censored data. Because our emergence trials were capped at 5 h, any individuals that had not yet emerged by the end of the trial were assigned an emergence time of 18,000 sec, meaning the data are right censored. Cox proportional hazards models allowed us to test how species and sex affected the "hazard" of leaving the shelter to emerge into the novel environment, while also taking into consideration the right-censoring of our data set.

Species and sex were included as fixed predictor variables and genotype and trial date were included as random predictor variables. Censoring was modeled using the survival package in R [44] to include a "status" variable indicating whether each measurement was right censored or not. Initial models showed no evidence of non-proportional hazards (global p-value = 1.00), indicating that our data set met the assumptions of cox proportional hazards models. Cox proportional hazards models were run for both a main effects-only model (Species + Sex) and a two-way fixed-effects interaction model (Species x Sex). The AIC model comparison indicated that the best summary model was the one with main effects only (Species + Sex), with a delta AIC of 2.0 between the first- and second-best models. This final model (Species + Sex) was used for estimating the effect of genotype. To calculate

p-values for the fixed effects, we used Type III Wald Chi-square tests implemented in the car package [46]. To test the significance of genotype, we used a likelihood ratio test. To further investigate genotypic variation within species, we ran additional follow-up models separately for each species (with sex included as the fixed predictor variable and genotype and trial date included as the random predictor variables) and tested the significance of genotype using a likelihood ratio test.

2.8. Decision Making

Our goal was to investigate whether species, sexes, and/or genotypes differed in habitat choice when presented with the choice between two ecologically relevant food substrates: plain fruit medium or imitation noni medium.

2.9. Measuring and Calculating Habitat Choice

Habitat choice measurements were calculated by averaging the proportion of time each fly spent on the imitation noni medium (with a plain fruit medium observation arbitrarily given a value of 0, while an observation on imitation noni was given a value of 1). For observations where the fly was located in the middle of the two options and failed to make a clear decision between the mediums, no decision was indicated, and the observation was not included in the final habitat choice calculation. This situation was rare—out of 6659 total observations, 261 observations were removed because of failure to choose, resulting in a final total of 6398 observations used for the analysis.

Thus, to generate a habitat choice measurement for each individual, the number of observations on noni were totaled and then divided by the total number of 13 observations (minus any failures to choose between the two

mediums). Habitat choice values at or closer to 0 indicated higher preference for plain medium, while values at or closer to 1 indicated higher preference for the noni medium. Habitat preference values of 0.5 indicated no observable preference for either the plain or noni substrates.

2.10. Decision Making: Species and Sex Differences in Habitat Choice

To investigate whether species differed significantly in habitat choice, we ran a generalized linear mixed model in the lme4 package in R [47], testing the effect of species on habitat choice. Generalized linear mixed models provide a flexible approach to analyzing non-normal data when random effects are present [47].

Species, sex, and arena food orientation (whether imitation noni was on the right or left-hand side) were included as fixed effects. Random effects were included to account for the non-independence of genotype as well as the non-independence of the 13 habitat choice observations taken for each individual fly. A random effect was also included for the trial date to indicate which flies were tested on the same day. Model slopes were fitted to a binomial distribution. Generalized linear mixed models were run for all two-way fixed-effects interactions (Species x Sex; Species x Arena Orientation; Sex x Arena Orientation), a three-way fixed-effects interaction (Species x Sex x Arena Orientation), and no interactions (Species + Sex + Arena Orientation). Using AIC model comparison, the best summary model included (Species x Sex + Sex x Arena Orientation), with a delta AIC of 2.2 between the first- and second-best models. The best summary model (Species x Sex + Sex x Arena Orientation) was also used for estimating the effect of genotype. To calculate p-values for the fixed effects, we used Type III Wald Chi-square tests implemented in the car package [46]. In the final model, we tested the

significance of genotype using a likelihood ratio test. To further investigate genotypic variation within species, we ran additional follow-up models separately for each species (with sex and arena orientation included as the fixed predictor variables, and genotype and trial date included as the random predictor variables) and tested the significance of genotype using a likelihood ratio test.

3. Results

3.1. Sexes, but not Species, Differ in Exploratory Behavior

We found support for sex differences in exploratory behavior, represented by the amount of time (in seconds) taken to emerge from a shelter into a novel environment. We found a significant effect of sex on emergence time (X^2 = 4.06, degrees of freedom = 1, p = 0.04), with males showing significantly shorter emergence time than females (Figure 2). We found no effect of species on emergence time ($X^2 = 1.97$, degrees of freedom = $1, p = 0.16$), nor an effect of a two-way interaction between Species and Sex (X^2 = 0.05, degrees of freedom = $1, p = 0.83$). However, we did find evidence that genotypes differed significantly in emergence time based on the results of our likelihood ratio test, as including genotype as a random effect significantly improved model fit (likelihood ratio = 18.25, degrees of freedom = 1, $p < 0.001$). Additional follow-up models investigating the effect of genotype separately for each species indicated that the *D. simulans* (generalist) genotypes differed significantly in emergence time. A likelihood ratio test indicated that including genotype as a random effect significantly improved model fit for *D. simulans* ($X^2 = 19.12$, degrees of freedom = $1, p < 0.0001$). However, this was

not the case for the *D. sechellia* (specialist) genotypes ($X^2 = 0.0005$, degrees of freedom = 1, $p = 0.98$) (Figure 3).

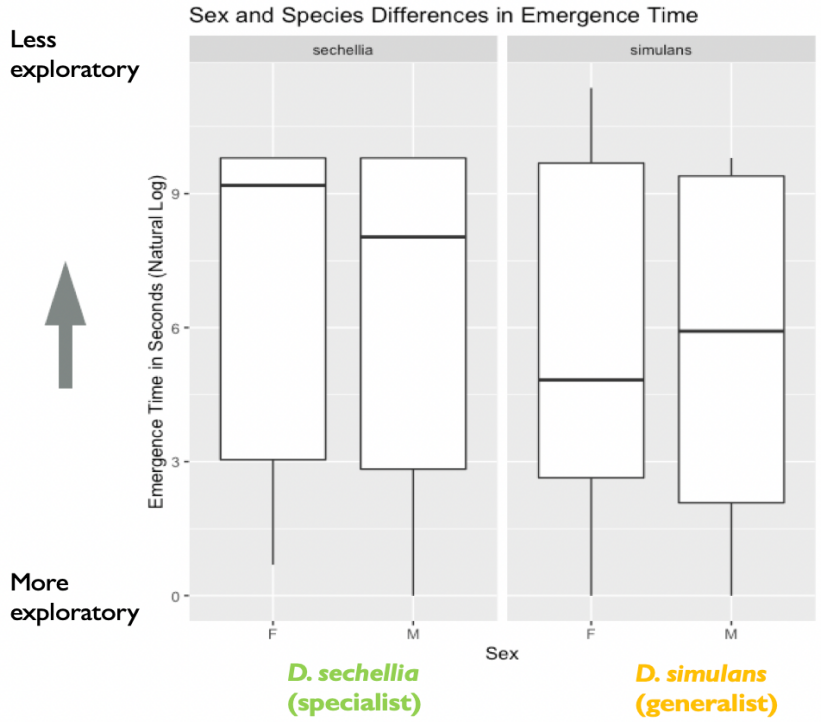


Figure 2. Sex and species differences in emergence time (in seconds): the plot represents species and sex differences in emergence time (in seconds), as noted in the main text. Data are from the first experiment measuring exploratory behavior as a mechanism for environmental sampling. The y-axis represents the natural log of emergence time in seconds. A lower emergence time indicates higher exploratory behavior, while a higher emergence time represents lower exploratory behavior (N = 526 flies). The box in the plot represents the inter-quartile range of scores, including the middle 50% of scores for the group, with the median score represented by the middle quartile mark (or line). The whiskers represent the score ranges outside the inter-quartile range, including the top 25% of scores for the group in the upper whisker, and the bottom 25% of scores in the lower whisker. Our findings indicated significant sex, but not species, differences in exploratory behavior, with males showing significantly shorter emergence time than females.

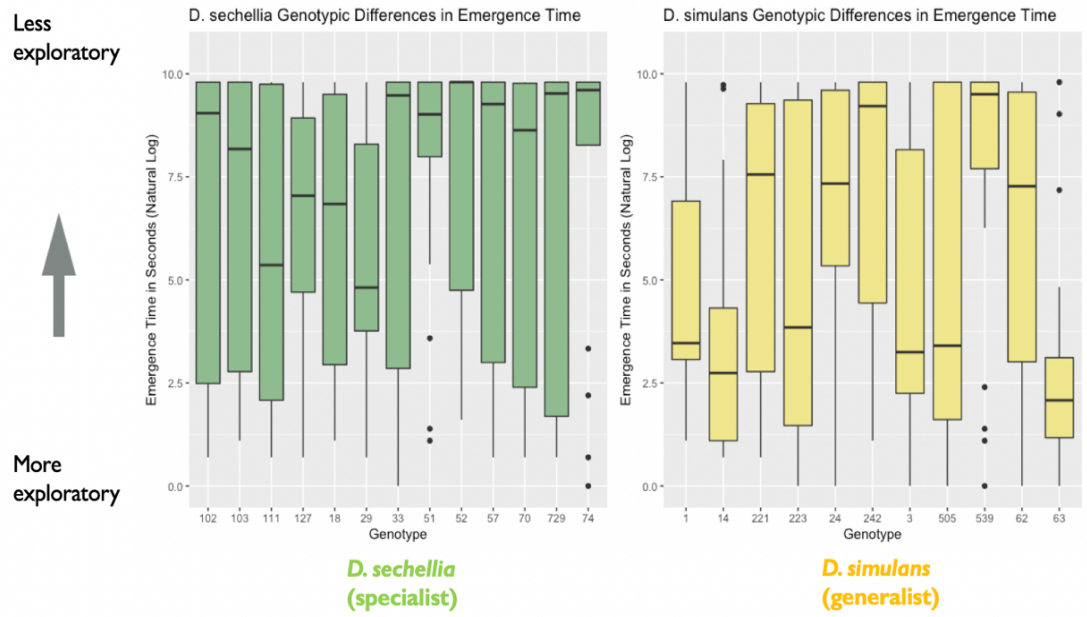


Figure 3. Genotypic differences in emergence time (in seconds): the plot represents genotypic differences in emergence time (in seconds), as noted in the main text. Data are from the first experiment measuring exploratory behavior. The y-axis represents the natural log of emergence time in seconds. A lower emergence time indicates higher exploratory behavior, while a higher emergence time represents lower exploratory behavior (N = 526 flies). The box in the plot represents the inter-quartile range of scores, including the middle 50% of scores for the group, with the median score represented by the middle quartile mark (or line). The whiskers represent the score ranges outside the inter-quartile range, including the top 25% of scores for the group in the upper whisker, and the bottom 25% of scores in the lower whisker. Our findings indicated significant genotypic differences in exploratory behavior.

3.2. Decision Making: Habitat Choice

3.2.1. Species and Genotypes Differ in Habitat Choice

The majority of flies sampled both substrates at least once: the majority of the mean preference scores (73%, $N = 382$) were greater than 0 and less than 1. A total of 144 (27%) of the mean scores were either 0 ($N = 104$) or 1 ($N = 40$), indicating that we observed the fly sampling only one of the food types.

We found support for species differences in habitat choice between *D. sechellia* and *D. simulans*. We observed a significant effect of species on habitat choice ($X^2 = 54.55$, degrees of freedom = 1, $p < 0.0001$), with each species showing the hypothesized preference for their expected host (*D. simulans*/plain and *D. sechellia*/noni). *D. simulans*, the habitat generalist, showed stronger habitat choice preference for the plain fruit substrate than *D. sechellia,* the habitat specialist, showed for the imitation noni fruit substrate (Figure 4). Additionally, we found evidence that genotypes differed significantly in habitat choice, based on the results of the likelihood ratio test, as including genotype as a random effect significantly improved the fit of the final model (likelihood ratio = 10.29, degrees of freedom = 1, $p = 0.001$). Additional follow-up models investigating the effect of genotype separately for each species indicated that *D. simulans* genotypes differed significantly in habitat choice. A likelihood ratio test indicated that including genotype as a random effect significantly improved model fit for *D. simulans* ($X^2 = 34.26$, degrees of freedom = 1, $p < 0.0001$). However, this was not the case for *D. sechellia* genotypes ($X^2 = 0$, degrees of freedom = 1, $p = 1$) (Figure 5).

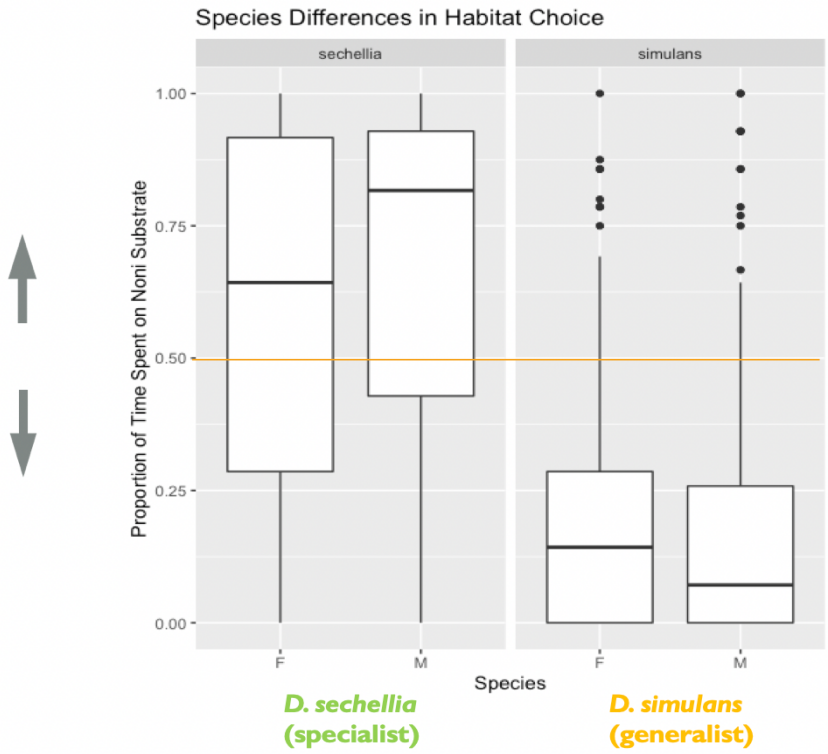


Figure 4. Species and sex differences in habitat choice: the plot represents species and sex differences in habitat (food substrate) choice, as noted in the main text. Data are from the second experiment measuring habitat choice as a representation of decision making. The y-axis represents the proportion of time spent on the imitation noni substrate. Lower values closer to 0 indicate a habitat preference for the plain substrate, while higher values closer to 1 indicate a habitat preference for the imitation noni substrate. A value on or near 0.5 (marked by the orange line) indicates no demonstrable habitat preference for either the plain or imitation noni substrates (N = 526 flies). The box in the plot represents the inter-quartile range of scores, including the middle 50% of scores for the group, with the median score represented by the middle quartile mark (or line). The whiskers represent the score ranges outside the inter-quartile range, including the top 25% of scores for the group in the upper whisker, and the bottom 25% of scores in the lower whisker. The dots beyond the whiskers represent outlier values. Our findings indicated that species differed significantly in habitat choice. Additionally, sex significantly influenced habitat choice, with males showing stronger habitat choice preference than females.

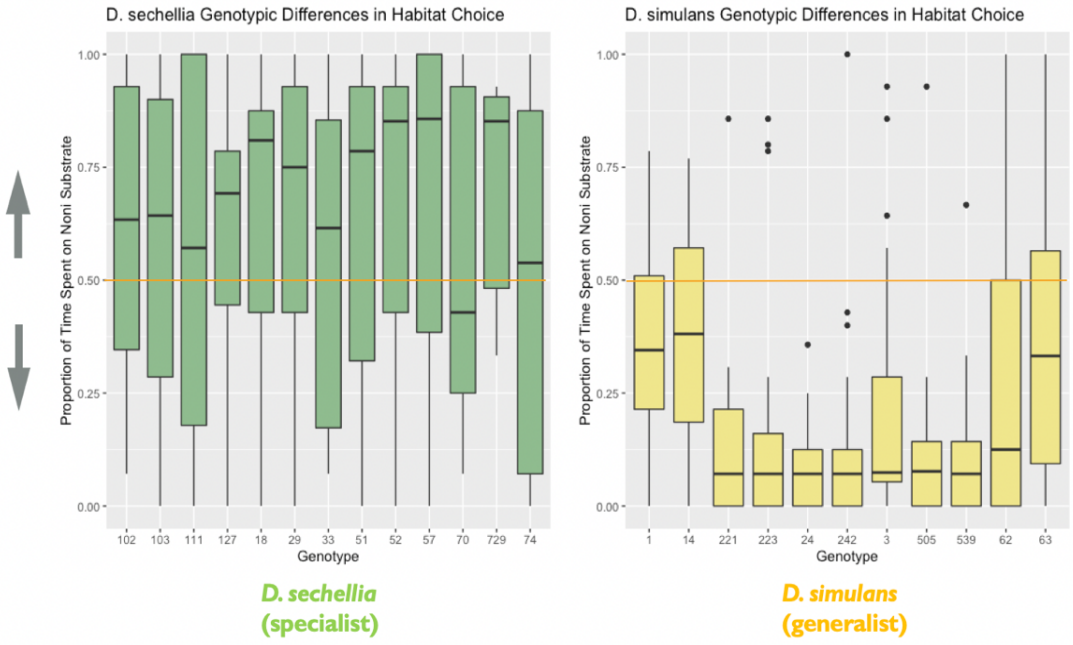


Figure 5. Genotypic differences in habitat choice: the plot represents genotypic differences in habitat (food substrate) choice, as noted in the main text. Data are from the second experiment measuring habitat choice. The y-axis represents the proportion of time spent on the imitation noni substrate. Lower values closer to 0 indicate a habitat preference for the plain substrate, while higher values closer to 1 indicate a habitat preference for the imitation noni substrate. A value on or near 0.5 (marked by the orange line) indicates no demonstrable habitat preference for either the plain or imitation noni substrates (*N* = 526 flies). The box in the plot represents the inter-quartile range of scores, including the middle 50% of scores for the group, with the median score represented by the middle quartile mark (or line). The whiskers represent the score ranges outside the inter-quartile range, including the top 25% of scores for the group in the upper whisker, and the bottom 25% of scores in the lower whisker. The dots beyond the whiskers represent outlier values. Our findings indicated that genotypes differed significantly in habitat choice.

3.2.2. Sex Influences Habitat Choice, with Males Showing Stronger Habitat Choice than Females

We observed a significant effect of sex on habitat choice ($X^2 = 10.13$, degrees of freedom = 1, $p = 0.002$), as well as a significant two-way interaction between species and sex on habitat choice ($X^2 = 7.77$, degrees of freedom = 1, $p = 0.005$). These results indicate that the males show a significantly stronger preference for their expected habitats, relative to their female counterparts (Figure 6), with *D. sechellia* (the specialist) showing a larger sex difference in habitat choice preference than *D. simulans* (the generalist).

3.2.3. A left-side bias influences habitat choice, particularly for females

We also found evidence that the orientation of the food options within the habitat choice arena (whether imitation noni was on the left or right-hand side) significantly influenced habitat choice ($X^2 = 20.97$, degrees of freedom = 1, $p < 0.001$). We observed evidence of a significant left-side bias in both species and sexes, which impacted habitat choice. This bias towards the left either facilitated or conflicted with habitat choice, depending on arena orientation. When the preferred habitat substrate was on the left, habitat choice was more accurate than when the preferred habitat substrate was on the right (Figure 6). These results indicate that a spatial bias can interfere with the outcome of this seemingly simple decision-making process. We also observed a significant two-way interaction between sex and habitat choice arena orientation on habitat choice ($X^2 = 4.52$, degrees of freedom = 1, $p = 0.03$), with females showing a significantly stronger left-side bias than males (Figure 6).

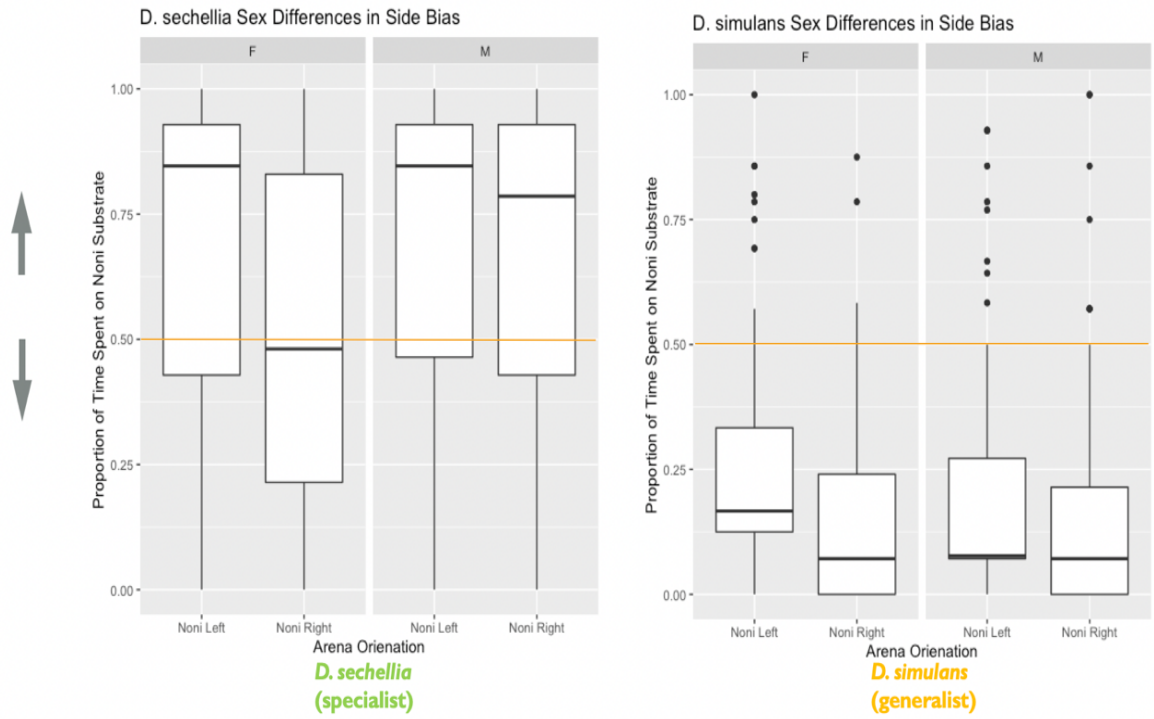


Figure 6. Species and sex differences in the effect of side bias on habitat choice: the plot represents species and sex differences in the effect of side bias on habitat choice, as described in the main text. Data are from the second experiment measuring habitat choice as a representation of decision making. The y-axis represents the proportion of time spent on the imitation noni substrate. Lower values closer to 0 indicate a habitat preference for the plain substrate, while higher values closer to 1 indicate a habitat preference for the imitation noni substrate. A value on or near 0.5 (marked by the orange line) indicates no demonstrable habitat preference for either the plain or imitation noni substrates. The x-axis is divided both by sex, as well as the arena orientation i.e., whether imitation noni substrate was on the left or right (N = 526 flies). The box in the plot represents the inter-quartile range of scores, including the middle 50% of scores for the group, with the median score represented by the middle quartile mark (or line). The whiskers represent the score ranges outside the inter-quartile range, including the top 25% of scores for the group in the upper whisker, and the bottom 25% of scores in the lower whisker. The dots beyond the whiskers represent outlier values. Our findings indicated that species and sexes differed significantly in habitat

choice. Additionally, a left-side bias significantly influenced habitat choice, with females showing a stronger effect of left-side bias on habitat choice than males.

4. Discussion and Conclusions

Understanding how environmental factors influence decision making to produce variation in outcomes is important for understanding how decision making evolves, and for informing a more complete understanding of behavioral variation. Currently, there is a lack of understanding in what determines whether the fitness benefits of maximizing decision-making accuracy will outweigh the associated costs, and what is generating the variation in decision making observed in nature. In this study, we investigated whether differences in the degree of environmental predictability and variability experienced in nature were associated with differences in decision-making outcomes. In our species comparison of *D. sechellia* and *D. simulans*, we found that higher environmental unpredictability (in the form of higher habitat variability and wider diet breadth) was associated with higher habitat choice accuracy, but not with higher exploratory behavior. *D. simulans*, the generalist, showed higher habitat choice accuracy relative to *D. sechellia,* the specialist. While differences in environmental predictability were not associated with the predicted differences in exploratory behavior between these species, we did find that sex had a significant effect on exploratory behavior, with the males of both species being more exploratory than females. These findings are congruent with our hypothesis that higher environmental unpredictability should favor higher decision-making accuracy, but not our hypothesis that high environmental unpredictability should be associated with increased exploratory behavior and environmental sampling. Additionally, we found evidence that there was a significant effect of genotype on both

exploratory behavior and habitat choice for *D. simulans*, but not *D. sechellia*. These findings indicate that *D. simulans* genotypes showed significant variation in exploratory behavior and habitat choice, while *D. sechellia* genotypes did not.

Additionally, we found that females showed lower habitat choice accuracy, relative to their male counterparts, and this effect was most pronounced in *D. sechellia* (the specialist). This finding was unexpected, particularly because female *Drosophila* must select habitats both for foraging and laying eggs and are therefore expected to be under strong selection to choose correctly. Female *D. sechellia* in particular gain fertility benefits from their noni host [24].

We also observed a significant effect of left-side bias on habitat choice for both species and sexes, reinforcing the accurate habitat choice when the accurate substrate was on the left and conflicting with the accurate habitat choice when the accurate substrate was on the right. The effect of left-side bias on habitat choice was most pronounced in females, particularly *D. sechellia* (the specialist). The stronger effect of left-side bias observed in females appeared to be a contributing factor to the sex differences in habitat choice accuracy, as females showed a stronger influence of side bias on their habitat choice, exhibiting significantly lower habitat choice accuracy when the preferred substrate was on the right.

While the predicted association between high habitat choice accuracy and high exploratory behavior was not observed between the two species, an association was observed for the males of each species, which showed both higher habitat choice accuracy and higher exploratory behavior, relative to their female counterparts. These sex differences were unexpected; but given that virgin females were used for this experiment, it is possible that the

females were less motivated to both locate and choose the correct habitat. Additionally, male *Drosophila* are known to defend territories for mating purposes [48,49], perhaps leading to a higher motivation to both locate and select the ideal habitat. Future experiments measuring both virgin and mated flies could help elucidate whether differences in motivation (based on mated status) are a contributing factor to the observed differences in exploratory behavior and habitat choice accuracy between males and females.

Additionally, these findings indicate that certain cognitive biases, such as side bias, can be significantly influential in the decision-making outcomes of even seemingly simple decisions. This is surprising, given the simplicity of the choice (a two-choice assay in a small arena) and the ecological relevance of the habitat stimuli (particularly that the noni is toxic to *D. simulans*). Side bias, or "handedness", has been observed in many other species [6,50–56], and previous work has indicated that it can influence the outcomes of various cognitive processes, such as foraging decisions and preference [6], spatial processing [57], and learning [50,58–60]. One proposed explanation for the existence of side bias is brain lateralization, which is the specialization of one hemisphere for a particular function, leaving the other hemisphere free to perform other or additional functions [61] Thus, the presence of a side bias could be indicative of a "good enough" approach to decision making, possibly providing a means for lowering the costs of decision making or managing errors under evolutionary constraints [20]. However, it is important to note that the observed side bias is likely not due to a true side, or space-use, bias, but a response to subtle differences in the environment that are not readily apparent to human observers, such as light or magnetic field. This is particularly because "side" is relative to an individual's position or orientation within an environment, and subject to change as the individual moves.

However, whether due to a true side bias or subtle environmental factors, the result remains surprising: when faced with a choice between two habitats, imperceptible (to humans) variation in seemingly unimportant environmental factors can dramatically influence choice. For example, *D. simulans* flies were apparently willing to spend time on food that is toxic to them (imitation noni), so long as that food was on the left. The mechanisms and evolution of this unexpected bias require further investigation.

This study, while powerful, was limited in a number of ways. First, the comparison was limited to only two species—one generalist and one specialist species. *D. sechellia* and *D. simulans* provide a strong model for investigating how recent divergence in habitat breadth influences the evolution of decision making, given that they are recently diverged and crossable, but occupy substantially different habitats. However, a range of physiological adaptations have accompanied the specialization of *D. sechellia* on noni, such as resistance to octanoic acid and hexanoic acid toxicity, changes in oviposition preference and olfaction, reduced ovariole number and egg production, and changes in larval morphology [24,37]. Additionally, differences in the potential costs associated with making an "incorrect" decision may have contributed to variation in decision-making accuracy between these species. *D. sechellia* can live on either noni or plain food (under laboratory conditions), but noni is poison to *D. simulans*. Therefore, it is possible that the observed differences in decision making between these species are due to something other than the differences in habitat breadth. Future work incorporating additional specialist/generalist comparisons within the *D. melanogaster* subgroup would help further demonstrate whether our findings are unique to the *D. simulans*/*D. sechellia* pair or are indicative of a larger pattern. Second, our measurement of exploratory behavior was limited to one measurement of

emergence time and may not be the appropriate proxy for environmental sampling. The exploratory behavior observation period was capped at 5 h, with many individuals taking longer than 5 h to emerge. This possibly indicates a need for a longer experimental observation period, or a different measurement of exploratory behavior/environmental sampling. Additional information on exploratory behavior and environmental sampling could be obtained in future experiments by recording flies in the habitat choice arenas and measuring the amount of movement in addition to the amount of time spent on each of the habitat substrates. Third, while the plain fly medium recipe used for the assays differed from the plain fly medium these species were raised on, the plain fly food used for the trials was more similar to the rearing environment than the imitation noni substrate. Therefore, it is possible that the observed differences in habitat choice accuracy between these species are due, in part, to a familiarity effect. It is also possible that *D. sechellia* (the specialist) had decreased energy or motivation from not being raised on their preferred noni host. Future experiments raising flies on both plain and imitation noni substrates would help elucidate how rearing environment and prior experience influences habitat choice in these species.

In regard to our species comparison of *D. sechellia* and *D. simulans*, these findings support our hypothesis that higher environmental unpredictability should be associated with higher decision-making accuracy, but not our hypothesis that high environmental unpredictability should be associated with increased exploratory behavior and environmental sampling. Additionally, these findings demonstrate that cognitive biases, such as a side bias, can significantly influence the outcomes of even seemingly simple decisions. This study serves as an early step in investigating the environmental factors

influencing the evolution of decision making, as well as informing a fuller understanding of why we observe behavioral variation among animals.

Chapter 2

Evolutionary changes in gustatory but not visual learning in Two Recently Diverged Species of *Drosophila*

Abstract: Learning is hypothesized to confer greater fitness benefits in environments where the amount of new information is expected to be high; thus, learning is predicted to evolve in environments that are more variable across time and space. Despite these long-standing predictions, we currently lack empirical studies investigating how environmental variability is contributing to evolutionary divergence in learning, which are needed to test this hypothesis. In this study, we investigated how differences in the degree of environmental variability experienced in nature contribute to divergence in learning. Additionally, we investigated *how* learning evolves - more specifically, if divergence in learning acts through "general" learning ability, with the responses correlated across a wide range of stimuli and contexts, or if divergence in learning is limited to certain stimuli or contexts. To do so, we compared two closely related species of *Drosophila* that experience markedly different degrees of environmental variability in nature: one dietary generalist and one dietary specialist species. We conducted two associative learning experiments, one learning trial using gustatory stimuli, and one learning trial using visual stimuli. We did not find support for the hypothesis that species evolving in highly variable environments should also evolve higher learning performance; indeed, we saw that the specialist species had greater learning performance. Moreover, these species differences were limited to the gustatory context. Our results suggest that the existing theory on the evolution of learning may not be relevant to all species pairs.

Introduction

Learning, defined as the modification of behavior following an informative event or experience (Thompson 1986) is a cognitive process involved in foraging, mate choice, predator avoidance, and many other important behaviors impacting fitness (Shettleworth 2001). As such, many previous studies have aimed to elucidate why we observe variation in learning, primarily focusing on the various selection pressures that may demand higher cognitive abilities for individuals to survive and succeed in a given environment (Buchanan et al. 2013, Lambert & Guillette 2021), as well as exploring how the potential fitness benefits of learning compare to the associated energy costs (Burger et al. 2008; Chittka et al. 2009; Laughlin 1998; Mery & Kawecki 2004; Ricklefs 2004; Snell-Rood et al. 2011; Yirmiya 2011). Past studies have found several positive associations between learning performance and various environmental pressures (such as the severity of seasonal, temperature, and weather fluctuations, complexity of the social environment, predation pressures, etc.), contributing to our understanding of the types of environments that favor learning (Buchanan et al. 2013; Kotrschal & Taborsky 2010; Roth et al. 2010; Roth et al. 2012). For example, one of the most popular examples of this is referred to as the Social Brain Hypothesis, where larger and more complex social groups have been found to be associated with larger brains and higher learning performance (Cummings & Ramsey 2015; Dunbar 1998; Ashton et al. 2018). However, we still currently lack a full understanding of which types of environments favor cognition, and how the unique selection pressures of a particular environment may be contributing to the variation we observe in cognitive performance.

The potential fitness benefits of learning are hypothesized to vary across environments. Specifically, because learning involves the modification of behavior in response to new information (Thompson 1986), learning is hypothesized to confer greater fitness benefits in environments where the amount of new information encountered is expected to be high (Kalan et al. 2020; Schuck-Paim et al. 2008; Sol et al. 2005; Sampedro-Piquero et L. 2018; Lambert & Guillette 2021). Novel information is expected to be more common in environments that exhibit higher levels of variability across time and space. The degree of environmental variability can be influenced by several factors, including the range and severity of seasonal, temperature, or weather fluctuations (Buchanan et al. 2013, Roth et al. 2010; Roth et al. 2012), the availability and distribution of food and resources (Kotrschal & Taborsky 2010), etc. Thus, it follows that learning should be favored in environments that are more variable across time and space. Indeed, many previous studies have found positive correlations between the degree of environmental variability and learning (Buchanan et al. 2013). For example, one previous study found that cichlid fish raised on unpredictable diets in temporally variable environments demonstrated higher learning performance, relative to conspecifics raised in more constant environments (Kotrschal & Taborsky 2010). Additional studies have also found associations between higher severity of environmental perturbations and higher learning performance in black capped chickadees (Roth et al. 2010; Roth et al. 2012). However, most previous studies investigating how environmental variability contributes to variation in learning have focused on the plasticity of learning within an individual's lifetime (Buchanan et al. 2013; Kotrschal & Taborsky 2010; Roth et al. 2010; Roth et al. 2012). We

currently lack understanding of how environmental variability is contributing to evolutionary divergence in learning.

In addition to investigating the particular selection pressures that are contributing to evolutionary divergence in learning, it is important to investigate exactly *how* species are diverging in learning when subjected to these selection pressures. One way in which species could possibly be diverging in learning is in terms of their "general cognitive ability". Many previous studies, particularly in the field of psychology, have found positive correlations between cognitive abilities (including learning, memory, problem-solving, etc.), with individuals frequently exhibiting consistent performance across a wide range of cognitive tests and generally maintaining their rank, relative to other members of the test group (Matzel et al. 2003; Plomin 1999; Plomin & Spinath 2002). Indeed, the influence of a general cognitive ability on performance across diverse tests of cognitive abilities has been previously observed in humans and some other animals (Galsworthy et al. 2002; Matzel et al. 2003; Matzel et al. 2020; Plomin 1999; Plomin 2001; Plomin & Spinath 2002; Ashton et al. 2018). Thus, this evidence provided by the literature may predict that there is an underlying general cognitive ability influencing variation in learning performance (as well as other cognitive abilities), and that when animals diverge in learning, a general divergence in all aspects of cognition could be expected.

However, an alternative theory for how species could be diverging in learning is based more in evolutionary theory, which predicts that divergence should only occur for the specific traits under selection. A large body of literature has demonstrated that animals are often better able to learn about or associate some stimuli over others, a phenomenon known as "prepared learning" (Garcia & Koelling 1966; Dunlap & Stephens 2014).

For example, one seminal study found that rats are able to learn some associations (e.g., taste and gastric illness) but not others (e.g. light-sound combinations and gastric illness) (Dunlap & Stephens 2014; Garcia and Koelling 1966). Previous work has also demonstrated that not all learning experiences have equal fitness consequences, supporting the idea that selection favors specific types of learning in specific contexts. For example, one study comparing the associative learning abilities of vampire and fruit bats demonstrated that unlike fruit bats, vampire bats are unable to form taste aversions (Ratcliffe et al. 2003). Considering that vampire bats are dietary specialists (unlike fruit bats, which are dietary generalists), this makes sense evolutionarily, since learning a taste aversion for the one food item that one has specialized on would likely have very negative fitness consequences. However, to our knowledge it is currently unknown whether this evolutionary reduction in learning capabilities is specific to taste aversion or generalizes across contexts. In sum, we currently lack an understanding of the conditions under which learning evolves, and whether selection on learning results in changes to general learning ability, or in changes specific to the context in which selection has occurred.

To investigate if divergence in environmental variability is associated with evolutionary divergence in learning performance, and whether these differences generalize across stimuli and contexts, we compared two closely related species that experience markedly different degrees of environmental variability in nature: one dietary generalist and one dietary specialist species. We conducted two associative learning experiments, one learning trial using gustatory stimuli, and one learning trial using visual stimuli. Previous work supports the idea that these species have been under divergent selection for their response to gustatory stimuli (Dworkin & Jones 2009), whereas this is

not expected to be the case for visual stimuli. We hypothesize that higher levels of environmental variability should favor higher learning performance in the generalist, but not the specialist. If environmental variability selects for high "general learning ability", we should observe species differences in learning for both the gustatory and visual stimuli, with no significant differences in learning performance between stimuli. If environmental variability selects for evolutionary changes in learning for only the specific stimuli most relevant in terms of fitness consequences (i.e., food and resources), we should observe species differences in learning for the gustatory, but not the visual, stimuli.

Materials and Methods

Study System: A comparative approach using D. sechellia and D. simulans

We compared two closely related species of *Drosophila* that have historically experienced extreme differences in habitat and diet breadth: *D. sechellia* and *D. simulans*. Previous work has estimated recent divergence times for these species, ranging from 250,000 to 413,000 years ago (Garrigan et al. 2012; Kliman et al. 2000; Schrider et al. 2018). *Drosophila* habitats consist of ephemeral rotting fruit patches where flies eat, mate, lay eggs, and spend the majority of their time (Powell 1997). As a habitat generalist, *D. simulans* has a broad range of habitat (i.e., fruit) options that, depending on the region and time of year, can vary substantially across time and space. This is in direct contrast to *D. sechellia*, a habitat specialist that has evolved to feed and breed preferentially on *M. citrifolia*, or "noni", fruit, which is ubiquitous in the Seychelles, present year-round, and toxic to other species of *Drosophila* (including *D. simulans*) (Schrider et al. 2018; Jones

2005; R'Kha et al. 1990). Thus, these species have diverged substantially in the amount of environmental variability historically experienced in nature. Previous work has supported the idea that *D. sechellia* has adapted behaviorally to their noni host; for example, *D. sechellia* prefer noni fruit over all other fruits, whereas *D. simulans* shows the opposite behavior (Burns et al 2020; Dworkin & Jones 2009).

Genotypes

In addition to species differences, we also investigated genotypic variation. Isofemale lines, hereafter "genotypes", were selected randomly from a range of genotypes generously provided by D. Matute in 2016 (Burns et al. 2020). *D. sechellia* genotypes (specifically NF 18, NF 33, NF 74, and NF 111) were collected from various locations across the Seychelles, while the *D. simulans* genotypes were collected across various locations in central and southern Africa (specifically NMB-014 collected in Namibia, NS-39 collected in Nairobi, LNP 15-063 collected in the Luwanga National Park, Zambia, and LZV 15-003 collected in the Lower Zambieze Valley, Zambia) (Matute et al. 2014; Schrider et al. 2018; pers.comm.). Each genotype was established by inbreeding a single wild female; therefore, individuals of the same genotype are more genetically similar to one another than they are to individuals of other genotypes. Thus, these genotypes represent a sample of natural genetic diversity of these species.

Rearing

To rear flies for the trials, 10 virgin females were placed with 10 males of the same species and genotype and housed in vials containing a standard fly medium, which consisted of corn meal, corn syrup, malt sugar, dead yeast, soy flour, tegosept (methyl paraben), propionic acid, and phosphoric acid. The parents were allowed to mate and lay eggs for 14 days and newly eclosed virgin offspring were collected under light CO_2 anesthesia on day 15. Collected flies were housed individually in vials containing standard fly medium and allowed to recover from the CO_2 anesthesia for 3 days prior to beginning trials. Flies were not starved prior to trials due to concerns that food deprivation could influence energy levels or habitat choice, as previous work has demonstrated that flies subjected to starvation are more likely to settle for less preferred habitats (Davis 2007).

Quinine avoidance

Quinine has been used extensively as an aversive gustatory stimulus in aversive conditioning experiments in *D. melanogaster*. Previous work has demonstrated that quinine, a bitter tasting substance, is noxious to *Drosophila melanogaster*, a species closely related to *D. sechellia* and *D. simulans*. In *D. melanogaster,* flies show strong avoidance of quinine that does not lessen over time (i.e., flies do not habituate to quinine) (Mery and Kawecki 2002). To test whether the closely related *D. simulans* and *D. sechellia* also avoid quinine, we allowed flies from the different species to choose between quinine-laced (3.2 g/L concentration) and quinine-free food in two treatments. Treatments included plain fly food substrate (consisting of

a standard recipe of agar, malt sugar, inactive dry yeast, and deionized water) laced with quinine, and imitation *M. citrifolia* (noni) food substrate laced with quinine. Imitation noni food substrate was made by adding octanoic and hexanoic acids to the plain food substrate (as in Dworkin and Jones 2008). In the quinine-noni treatment, flies could choose between plain food, and noni containing quinine. In the quinine-plain treatment, flies could choose between noni food, and plain food containing quinine. We also randomized the orientation of the food options. Flies were allowed to explore these options overnight, and their location (on plain food, or on noni food) was recorded the following morning. We tested 4 *D. sechellia* genotypes and 4 *D. simulans* genotypes. For each genotype, 3-20 males were tested, and 4-15 females were tested for a total of 431 individuals. We demonstrate below that *D. simulans* and *D. sechellia* individuals also avoid quinine, as expected.

Learning: overview

To compare learning between *D. sechellia* and *D. simulans*, we investigated change in substrate preference following an aversive conditioning assay using quinine hydrochloride, in either high or low concentrations (6.4 g/L and 3.2 g/L concentrations), as the negative gustatory stimulus. Quinine was paired either with a gustatory stimulus or a visual pattern stimulus. Fruit flies have become a common model system for studying chemosensory learning, as associative conditioning has been demonstrated in flies in many previous studies, and the behavioral methods for measuring learning have been repeatedly validated (Gerber & Stocker 2007; Saltz et al. 2017). In particular, aversive conditioning using gustatory

or olfactory stimuli has become a popular approach for investigating learning in flies (Mery and Kawecki 2002; Saltz et al. 2017). During aversive conditioning, a negative conditioned stimulus is paired with an unconditioned stimulus for a training period; learning is indicated if the animal subsequently avoids the unconditioned stimulus, even in the absence of the negative conditioned stimulus (Ayestaran et al. 2010). Aversive conditioning using a noxious gustatory stimulus has been found to elicit strong learning responses in animals, with many individuals requiring only one training episode or conditioning "experience" for a strong negative association and avoidance of the unconditioned stimulus to occur (Gustavson et al. 1974; Ralphs & Provenza 1999; Yamamoto 1993).

To determine how flies responded to the aversive conditioning, we first measured their initial (naive) substrate preferences to determine which of the two substrates each fly preferred prior to training. Flies were subsequently subjected to an associative conditioning (or training) stage, where quinine was paired with one of the two substrates. Each fly was assigned randomly to a treatment. Following the training stage, substrate preference was measured again (in the absence of quinine), to determine which of the two substrates each fly preferred following training. Learning would be indicated if flies significantly reduced their preference for the food that previously contained quinine (of any concentration).

Learning: stimulus types

For each genotype, species, and sex, we tested learning for two different types of stimuli: gustatory, and visual.

Because these species have been under divergent selection for diet breadth (with one species specializing on a host plant that is toxic to other species of *Drosophila*), they are expected to have also been under divergent selection for gustatory stimuli, whereas this is not expected to be the case for visual stimuli.

For gustatory stimuli, there were two options: plain fly food substrate (consisting of a standard recipe of agar, malt sugar, inactive dry yeast, and deionized water), and imitation *M. citrifolia* (noni) food substrate. Imitation noni food substrate was made by adding octanoic and hexanoic acids to the plain food substrate (as in Dworkin and Jones 2008). Previous work has provided extensive evidence that both the octanoic and hexanoic acids in noni are responsible for the fruit's toxicity to other species of *Drosophila* and are those involved in both attracting *D. sechellia* and repelling *D. simulans* (Jones 2005; Dekker et al. 2006; Ibba et al. 2010; Lavista-Llanos et al. 2014; Prieto-Godino et al. 2017; Auer et al. 2020; Burns et al. 2020). Thus, for the purpose of investigating food preferences, adding octanoic and hexanoic acids to the plain fly substrate provides an adequate, standardized proxy for noni.

For visual stimuli, there were two options: plain food substrate (same as mentioned above) surrounded by a black and white stripes pattern tape, and plain food substrate surrounded by a black and white zigzag pattern tape. Black and white patterned tape was chosen as the visual stimuli to control for possible variation in color perception between species.

Trials

Stage 1: Measuring initial substrate preference

To compare initial substrate preferences between *D. sechellia* and *D. simulans*, we investigated the starting substrate choices for both the gustatory and visual stimuli, prior to associative conditioning. To do this, individual flies were gently aspirated into a short pipette tip and allowed to emerge on their own accord into the initial preference arenas.

Initial preference arenas consisted of one petri dish containing the two substrate options, which were cut into halves, covered by a second petri dish (acting as a lid), and sealed together with tape (Figure 1). The relative location of the substrate options within the arenas was varied (i.e., there was a 1:1 ratio of initial preference arenas with imitation noni on the right-hand side and imitation noni on the left-hand side, and a 1:1 ratio of preference arenas with zigzag pattern tape on the right-hand side and zigzag pattern tape on the left-hand side). Individual flies were assigned randomly to initial preference arenas.

To measure initial substrate preferences, observers first noted the fly's immediate substrate choice (i.e., which part of the arena each fly landed on upon immediate emergence into the arena). Substrate choice was measured every 10 min over the course of a 2 h observation period. In total, 13 substrate choice observations were recorded for each individual fly during the initial preference phase of the experiment.

Stage 2: Associative conditioning with an aversive gustatory stimulus, and controls

Following the initial food preference stage, flies were transferred from the initial food preference arenas into associative conditioning training arenas. To do this, individual flies were gently aspirated into a short pipette tip and carefully moved into the training arenas (Figure 1).

For gustatory stimuli, the associative conditioning training arenas consisted of three treatments: plain food substrate paired with quinine, imitation noni food substrate paired with quinine, or a control treatment containing both plain food substrate and imitation noni food substrate and lacking quinine.

For visual stimuli, the associative conditioning training arenas consisted of three treatments: plain food substrate paired with quinine and surrounded by black and white stripes pattern tape, plain food substrate paired with quinine surrounded by black and white zigzag pattern tape, or a control treatment containing plain food substrate (lacking quinine) surrounded both by stripes pattern tape and zigzag pattern tape.

For all treatments, associative conditioning training arenas consisted of one petri dish containing the food substrate (or substrates), covered by a second petri dish (acting as a lid), and sealed together with tape. For the quinine treatments, one unconditioned stimulus was paired with quinine (i.e., all plain food substrate paired with quinine, or all noni food substrate paired with quinine (Figure 1); all zigzag pattern substrate paired with quinine, or all stripes pattern substrate paired with quinine). For the control treatments, both stimuli were present, but no quinine. The relative location of the substrate options was varied (i.e., there was a 1:1 ratio of control arenas with

imitation noni substrate on the right-hand side and imitation noni substrate on the left-hand side, and a 1:1 ratio of control arenas with zigzag pattern tape on the right-hand side and zigzag pattern tape on the left-hand side), as with initial food preference.

Individual flies were randomly assigned to treatments, and each individual fly was used in only one treatment. Once transferred to the associative conditioning training arenas, flies were left overnight for training prior to measuring change in food preference the following day.

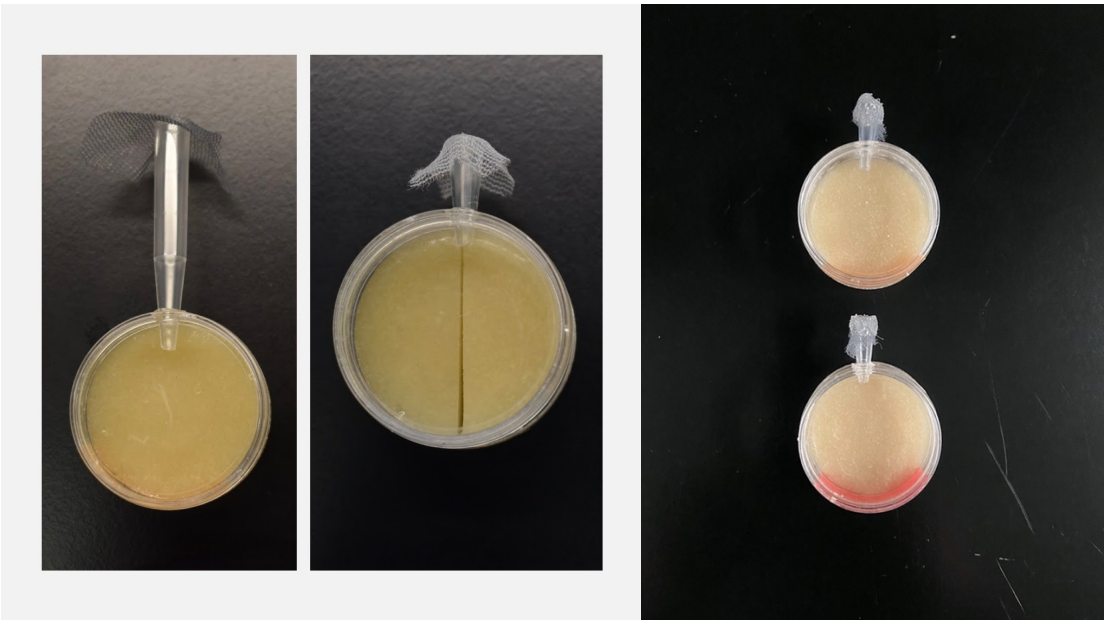


Figure 1. Substrate Preference Arenas and Associative Conditioning Training Arenas: Single flies were gently aspirated into the pipette tips, which were fitted into a small hole in the arena with the two substrate options. A mesh barrier prevented each fly from escaping the pipette tip in the other direction. For the initial preference and preference following training stages (Stage 1 and Stage 3), flies were allowed to emerge into the arena on their own accord and were then scan sampled every 10 minutes over the course of 2 hours, and the substrate choice of each fly was recorded. For the associative conditioning stage (Stage 2), flies were gently transferred into the arenas and left overnight for training to occur.

Stage 3: Measuring learning (change in substrate preference)

Following the associative conditioning training stage, flies were transferred from the training arenas into change-in-preference arenas. To do this, individual flies were gently aspirated into a short pipette tip and allowed to emerge on their own accord into the new change-in-preference arenas (Figure 1).

As in the initial preference stage, the change-in-preference arenas consisted of one petri dish containing the two substrate options, and the relative location of the substrate halves was varied. Individual flies were once again assigned randomly to the change-in-preference arenas. Following each fly's emergence into the new arena, immediate substrate choice was recorded, followed by scan sampling to record the substrate choice every 10 min over the course of a 2 h observation period. In total, 13 change-in-food-preference choice observations were recorded for each individual fly.

Replication

A total sample size of 243 individuals were measured for the gustatory stimuli learning trials. For each of the 8 genotypes, a range of 10 to 17 males and a range of 10 to 24 females were measured. A total sample size of 279 individuals were measured for the visual stimuli learning experiments. For each of the 8 genotypes, a range of 11 to 22 males and a range of 14 to 22 females were measured. The number of replicates varied between genotypes because of variation in the availability of flies on the day of testing (Burns et al. 2020).

Analysis

All analyses were conducted in R version 3.6.1 (Vienna, Austria) (R core team 2019).

Avoidance of the negative gustatory stimulus (quinine avoidance)

We tested whether flies chose the quinine-laced substrate, and whether these choices differed among species, sexes, and genotypes. We also tested whether this decision depended on which substrate the quinine was added to (treatment).

We used a binomial mixed-model with fixed effects of species, sex, treatment, and the location of the quinine-laced substrate (left or right), as well as all 2-way interactions among these. We included random effects of date and genotype.

Initial substrate preference

Our goal was to investigate species differences in initial substrate preference prior to training with an aversive gustatory stimulus. Specifically, we wanted to investigate whether species differed in initial substrate preference when presented with two gustatory substrates (plain substrate or imitation noni substrate), or when presented with two visual substrates (zigzag pattern tape or stripes pattern tape).

Measuring and calculating initial substrate preference

Initial substrate preference was calculated by averaging the proportion of time each fly spent on each of the two substrates. For gustatory stimuli, an observation on the plain substrate was arbitrarily given a value of 0, while an observation on the imitation noni substrate was given a value of 1 (Burns et al. 2020). For visual stimuli, a stripes pattern substrate observation was arbitrarily given a value of 0, while an observation on zigzag pattern substrate was given a value of 1. For observations where the fly failed to make a clear choice between the two substrate options (i.e. were located in the middle of the two substrate options), no decision was indicated, and the observation was not included in the final preference calculation. This situation was rare - out of 5,928 observations, 378 observations were removed because of failure to choose, resulting in a final total of 5,550 observations used for the analysis.

Thus, to calculate initial substrate preference for each individual, the number of observations on either noni substrate or zigzag pattern substrate were added and then divided by the total number of observations (13 total observations, minus any failures to choose). Gustatory stimuli preference values at or closer to 0 indicated higher preference for the plain substrate, while values at or closer to 1 indicated higher preference for the imitation noni substrate. Visual stimuli preference values at or closer to 0 indicated higher preferences for the stripes pattern substrate, while values at or closer to 1 indicated higher preference for the zigzag pattern substrate. Substrate preference values of 0.5 indicated no observable preference for either substrate.

Species differences in initial substrate preference

To test whether species differed significantly in initial substrate preference between gustatory stimuli and visual stimuli, we ran generalized linear mixed models in the lme4 package in R (Bolker et al. 2008), testing the effect of species on initial substrate preference for both gustatory and visual stimuli. Generalized linear mixed models provide a flexible approach to analyzing non-normal data when random effects are of interest (Bolker et al. 2008).

Models were run separately for gustatory and visual stimuli. Species, sex, and arena substrate orientation (whether imitation noni substrate was on the right- or left-hand side; whether zigzag pattern substrate was on the right- or left-hand side) were included as fixed effects. Random effects were included to account for differences among genotypes, as well as the non-independence of the 13 habitat choice observations recorded for each individual fly. A random effect was also included for the experiment date to account for which flies were tested on the same day. We specified a binomial error distribution, since each habitat choice observation was assigned a binomial value of either 0 or 1.

Previous work has demonstrated significant effect of a species by sex interaction, as well as a sex by arena orientation interaction, on substrate preferences for these species (Burns et al. 2020). Thus, initial models included a three-way fixed-effects interaction, and all possible two-way interactions including both Species x Sex and Sex x Arena Orientation fixed-effect interactions. However, models including the three-way fixed effect interaction (Species x Sex x Arena Orientation) failed to converge, and AIC model comparison showed negligible delta AIC values between the first-

and second- best models (with a delta AIC of 0.3 between the first-and second-best models for gustatory stimuli and a delta AIC of 0.9 for visual stimuli). Thus, final models included all possible two-way interactions (Species x Sex + Species x Arena Orientation + Sex x Arena Orientation) for both gustatory and visual stimuli. To calculate p-values for species, sex, and arena orientation, we used Type III Wald Chi-square tests implemented in the car package (Fox and Weisberg 2010). We tested the significance of genotype using likelihood ratio tests.

Learning (change in substrate preference)

Our goal was to investigate species differences in learning (i.e., change in substrate preferences) following training with a negative gustatory stimulus. Specifically, we wanted to investigate whether species differed in learning following associative conditioning using gustatory stimuli (plain substrate or imitation noni substrate) or using visual stimuli (zigzag pattern tape or stripes pattern tape).

Measuring and calculating learning (change in substrate preference)

Substrate preference following aversive conditioning was calculated by averaging the proportion of time each of the flies spent on each of the two substrates, just as in initial preference. Substrate preference following conditioning was calculated by averaging the proportion of time each fly spent on each of the two substrates. For observations where the fly failed to make a clear choice between the two substrate options (i.e., were in the middle of the two substrate options), no decision was indicated, and the

observation was not included in the final preference calculation. This situation was rare - out of 5,928 observations, 347 observations were removed because of failure to choose, resulting in a final total of 5,581 observations used for the analysis. To calculate learning (or change in substrate preference) scores, the food preference score following conditioning was subtracted from the initial food preference score (initial food preference - food preference following conditioning = learning score).

For both the gustatory and visual stimuli, learning scores were calculated based on the specific quinine treatment received, so learning scores would be directly comparable between quinine treatments. For gustatory stimuli, imitation noni substrate was assigned a value of 1 and plain substrate a value of 0 in treatments where quinine was paired with imitation noni; for treatments where quinine was paired with plain, plain substrate was assigned a value of 1 and imitation noni substrate a value of 0. For visual stimuli, zigzag pattern substrate was assigned a value of 1 and stripes pattern substrate a value of 0 in treatments where quinine was paired with the zigzag pattern substrate; for treatments where quinine was paired with the stripes pattern, striped pattern substrate was assigned a value of 1 and zigzag pattern substrate a value of 0. Positive learning scores indicate a decrease in preference for the substrate that was paired with quinine, as expected for aversive associative conditioning. Negative learning scores indicate an unexpected increase in preference for the substrate that was paired with quinine. Learning scores of 0 indicate no change in preference following associative conditioning.

For the control treatments that lacked quinine, imitation noni substrate was arbitrarily given a value of 1 and plain substrate a value of 0 for the gustatory stimuli; zigzag substrate was arbitrarily given a value of 1 and

stripes substrate a value of 0 for the visual stimuli (just as in the initial preference stage). Quinine treatment and control treatment learning scores were analyzed separately, as there was no predicted learning direction for the control treatments.

Species differences in learning

To test whether species differed significantly in learning for gustatory and visual stimuli, we ran linear mixed models in the nlme package in R (Pinheiro et al. 2014), testing the effect of species on learning. The nlme package, an alternative to the lme4 package, provides functions to fit and analyze linear mixed models with structure in the residuals (various forms of heteroscedasticity), incorporating both fixed and random effects (Bates et al. 2015). The nlme package was used in lieu of the lme4 package to account for the heteroscedasticity of variance observed in lmer model residuals.

As in initial preference, models were run both for gustatory and visual stimuli. Models were run and analyzed separately for the quinine treatments and the control treatments. This was because there were no predicted learning score directions for the controls, relative to the quinine treatments (i.e., in the quinine treatments, positive learning scores correspond to learning in the correct direction, relative to the associated treatment, whereas this is not the case for the control treatments). Species, sex, treatment (i.e., which stimulus was paired with quinine), and arena orientation were included as fixed effects in the quinine treatment models. Species, sex, and arena orientation were included as fixed effects in the control treatment models. In preliminary models, quinine concentration was included as a

covariate, but was never found to be significant, so it was removed in subsequent analyses.

Random effects were included to account for the non-independence of genotype, as well as the trial date to account for which flies were tested on the same day. Previous work has demonstrated significant effects of Species by Sex and Sex by Arena Orientation interactions on habitat preference for these species (Burns et al. 2020). Thus, linear mixed models were run for a four-way fixed-effects interaction, all possible three-way fixed-effects interactions, and all possible interaction combinations including Species x Sex x Treatment and Sex x Arena Orientation interactions.

Using AIC model comparison, the best model for gustatory stimuli controls included a Species x Sex interaction and a Sex x Arena orientation interaction (Species x Sex + Sex x Arena Orientation), with a delta AIC of 2.9 between the first- and second-best models. The best model for visual stimuli controls included a Species x Sex interaction and a Sex x Arena orientation interaction (Species x Sex + Sex x Arena Orientation), with a delta AIC of 3.5 between the first- and second-best models. To calculate p-values for species, sex, and arena orientation, we used Type III marginal F tests in the nlme package (Pinheiro et al. 2014). We tested the significance of genotype using likelihood ratio tests.

Using AIC model comparison, the best model for gustatory stimuli quinine treatments included the four-way fixed effects interaction (Species x Sex x Treatment x Arena Orientation), with a delta AIC of 4.9 between the first- and second-best models. The best model for visual stimuli quinine treatments included a Species x Sex x Treatment interaction and a Sex x Arena Orientation interaction (Species x Sex x Treatment + Sex x Arena Orientation), with a delta AIC of 4.8 between the first- and second- best

models. To calculate p-values for species, sex, treatment, and arena orientation, we used Type III marginal F tests in the nlme package (Pinheiro et al. 2014) as an alternative to the Type III Wald Chi-square tests in the car package (Fox and Weisberg 2010). We tested the significance of genotype using likelihood ratio tests.

Results

Avoidance of the negative gustatory stimulus (quinine avoidance)

Across all trials, 80% of the flies (343/431) chose the quinine-free substrate, indicating that flies of these species avoid quinine. This was also represented as a significant intercept in the model ($X^2 = 17.41$, degrees of freedom = 1, $p < 0.0001$). We did not find support for a significant main effect of species ($X^2 = 0.0978$, degrees of freedom = 1, $p = 0.75$), sex ($X^2 = 1.36$, degrees of freedom = 1, $p = 0.244$), treatment ($X^2 = 1.22$, degrees of freedom = 1, $p = 0.267$), or quinine location ($X^2 = 0.83$, degrees of freedom = 1, $p = 0.361$). Most of the interaction terms were likewise not significant (Species x Sex: $X^2 = 0.0004$, degrees of freedom = 1, $p = 0.984$; Species x Quinine Location: $X^2 = 1.35$, degrees of freedom = 1, $p = 0.246$; Sex x Treatment: $X^2 = 1.14$, degrees of freedom = 1, $p = 0.286$; Treatment x Quinine Location: $X^2 = 0.52$, degrees of freedom = 1, $p = 0.469$). However, we did find a significant Species x Treatment interaction ($X^2 = 30$, degrees of freedom = 1, $p < 0.0001$). Inspection of least-squares means indicated that flies of both species strongly avoided the quinine, except for *D. simulans* when the quinine was added to the plain substrate (ls-means, where values indicate the proportion of flies that chose the quinine-laced substrate: *D.*

sechellia when quinine was in plain: 0.12; *D. sechellia* when quinine was in noni: 0.11; *D. simulans* when quinine was in noni: 0.08; *D. simulans* when quinine was in plain: 0.67). This indicates that *D. simulans* flies avoid quinine-laced substrate, unless their other choice is noni. This is consistent with quinine and noni both being toxic to *D. simulans*.

We also found a significant interaction between Sex and Quinine Location ($X^2 = 4.15$, degrees of freedom = 1, $p = 0.042$). Inspection of the least-squares means indicates that females, but not males, particularly avoid quinine-laced substrate when this substrate was on the left (ls-means, where values indicate the proportion of flies that chose the quinine-laced substrate: males with quinine on the left: 0.3324; males with quinine on the right: 0.229; females with quinine on the left: 0.191; females with quinine on the right: 0.235). Despite this variability, flies of both sexes avoided quinine, regardless of which side the quinine was on (as indicated by the least-squares means values below 0.5).

Overall, this experiment demonstrates that flies of these species avoid quinine, similar to what has been found in *D. melanogaster*.

Species differences in initial substrate preference: gustatory stimuli

We did not find support for species differences in initial preference for the gustatory stimuli. The effect of species on initial preference for gustatory stimuli was not significant ($X^2 = 2.39$, degrees of freedom = 1, $p = 0.12$). We also found no effect of sex ($X^2 = 1.18$, degrees of freedom = 1, $p = 0.28$), nor an effect of arena orientation ($X^2 = 0.11$, degrees of freedom = 1, $p = 0.74$). However, we did find evidence that genotypes differed significantly based on the results of our likelihood ratio test, as including

genotype as a random effect significantly improved model fit (likelihood ratio = 29.14, degrees of freedom = 1, $p < 0.005$). These results indicate that genotypes, but not species, differed in initial preference for gustatory stimuli. The presence of genotypic differences in the absence of species differences was likely because while all *D. sechellia* (specialist) genotypes showed a strong preference for the noni substrate, only two of the four *D. simulans* (generalist) genotypes showed stronger preference for the plain substrate. The other two *D. simulans* genotypes showed a strong preference for the noni substrate, in contrast to previous findings (Burns et al 2020). Additionally, when genotype was removed as a random effect from the model, we observed a significant effect of species ($X^2 = 6.95$, degrees of freedom = 1, $p = 0.008$). This supports our hypothesis that the gustatory stimuli should be associated with variation in initial preference but does not support our hypothesis that species should differ significantly in their initial preference for the gustatory stimuli.

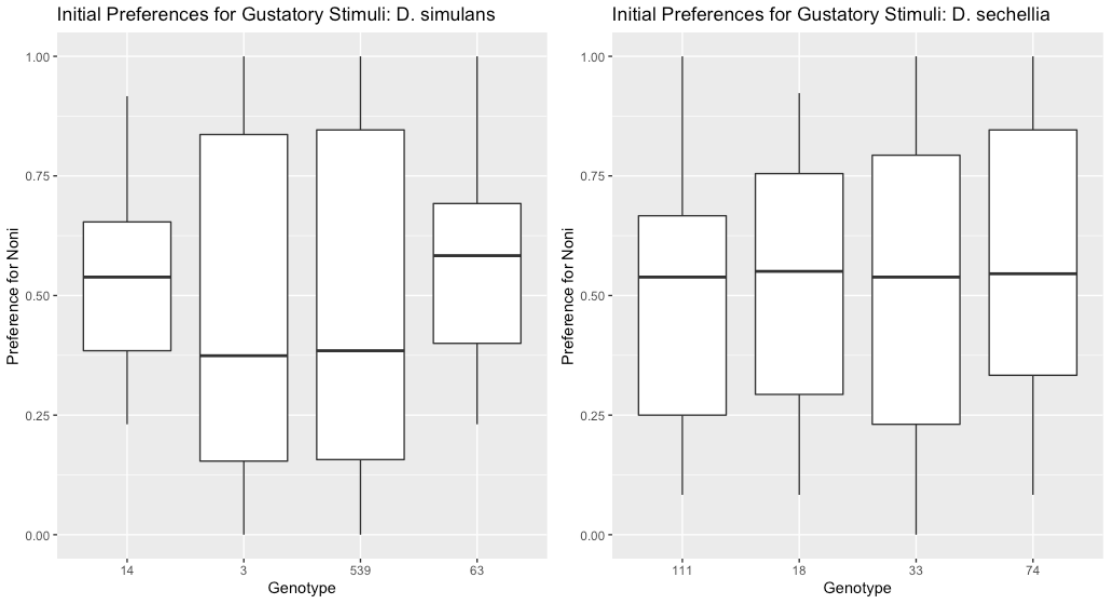


Figure 2. Species and Genotype Differences in Initial Preference for Gustatory Stimuli: These plots represent genotypic differences in initial preference for gustatory stimuli, as noted in the main text. The y-axis represents the proportion of time spent on the imitation noni substrate. Lower values closer to 0 indicate higher preference for the plain substrate, while higher values closer to 1 indicate higher preference for the imitation noni substrate. Values at or closer to 0.5 indicate no demonstrable preference for either the plain or imitation noni substrates. Our findings indicate that genotypes, but not species, differ in initial preference for the gustatory stimuli.

Species differences in learning: gustatory stimuli - control treatments (no quinine)

As expected, we found no evidence that learning occurred in the gustatory stimuli control treatments (Intercept F-value = 0.006, denominator degrees of freedom = 41, p = 0.94). We did not find support for species differences in learning for the gustatory stimuli control treatments. We found no effect of species on learning scores (F-value = 0.0002, denominator degrees of freedom = 6, p = 0.99). We also found no effect of sex on

learning scores (F-value = 0.02, denominator degrees of freedom = 26, p = 0.89), nor an effect of arena orientation (F-value = 0.22, denominator degrees of freedom = 26, p = 0.64), nor an effect of any two-way interactions. We did not find evidence that genotypes differed significantly in learning (likelihood ratio = 1.28, degrees of freedom = 1, p = 0.26). These results indicate that learning did not occur in the control treatments, and that there were no significant differences in learning between species or genotypes. This was consistent with our prediction that no change in preference should be observed for the control treatments, as no quinine associative conditioning training took place for individuals assigned to this treatment.

Species differences in learning: gustatory stimuli - quinine treatments

We found support for both species and sex differences in learning for the gustatory quinine treatments. We found a significant Species x Sex interaction on learning scores (F-value = 5.33, denominator degrees of freedom = 82, p = 0.02). We also found a significant Sex x Treatment interaction (F-value = 7.09, denominator degrees of freedom = 82, p = 0.01), a Sex x Arena Orientation interaction (F-value = 10.01, denominator degrees of freedom = 82, p < 0.005), a Species x Sex x Treatment interaction (F-value = 6.83, denominator degrees of freedom = 82, p = 0.01), a Species x Sex x Arena Orientation interaction (F-value = 11.75, denominator degrees of freedom = 82, p < 0.005), a Sex x Treatment x Arena Orientation interaction (F-value = 9.97, denominator degrees of freedom = 82, p < 0.005), and a Species x Sex x Treatment x Arena Orientation interaction (F-value = 10.97, denominator degrees of freedom = 82, p < 0.005) on learning

scores. We did not find evidence that genotypes differed significantly in learning (likelihood ratio = 0.31, degrees of freedom = 1, p = 0.58). These results indicate that both species and sexes differ significantly in learning for the gustatory stimuli quinine treatments, and that learning performance varies between treatments. Additionally, these results indicate that orientation of the substrate within the preference arenas (i.e., whether imitation noni substrate was located on the right or left-hand side) significantly influences learning scores, contributing to the differences in learning performance observed between species and sexes.

While both species averaged positive learning scores, indicating that both were learning to decrease preference for whichever substrate was previously paired with quinine, inspection of the raw data means indicated that the specialist species showed higher positive learning score averages than the generalist (Figure 3). This does not support our hypothesis that the generalist species should outperform the specialist in associative conditioning learning trials. Additionally, inspection of the raw data means indicated that males generally averaged higher positive learning scores than females (Figure 3). Between treatments, the specialist averaged higher learning scores in the treatment where quinine was paired with noni substrate, indicating that the specialist was better at learning to avoid the noni substrate following a negative experience than with the plain substrate (Figure 4). The generalist showed no substantial differences in learning between quinine treatments (Figure 4). Interestingly, the orientation of the substrate options within the preference arenas significantly influenced learning scores. Specialist females showed a left side bias, demonstrating higher learning performance in arenas where the correct choice, relative to the quinine treatment received, was located on the left-hand side. Specialist

males showed a right-side bias, demonstrating higher learning performance in arenas where the correct choice, relative to the quinine treatment received, was located on the right-hand side - so much so that negative learning scores were observed in the treatment where quinine was paired with noni substrate. Even more interestingly, generalist males and females both demonstrated higher learning performance in preference arenas where plain substrate was located on the left-hand side, regardless of the treatment received.

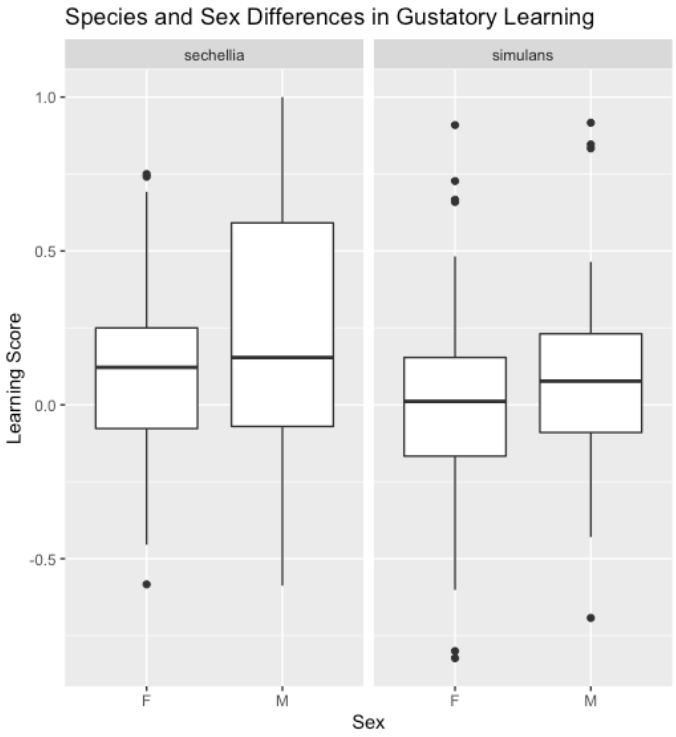


Figure 3. Species and Sex Differences in Learning for Gustatory Stimuli (Quinine Treatments): This plot represents species and sex differences in learning for the gustatory stimuli quinine treatments, as noted in the main text. The y-axis represents the learning score or change in preference following associative conditioning using a negative gustatory stimulus. Positive learning score values indicate a decrease in preference for the substrate that was paired with quinine (i.e., learning in the "correct" direction), while negative learning score values indicate an increase in

preference for the substrate that was paired with quinine (i.e., learning in the "incorrect" direction). Learning scores of 0 indicate no change in substrate preference following associative conditioning. Our findings indicate that both species and sexes differ in learning for the gustatory stimuli quinine treatments.

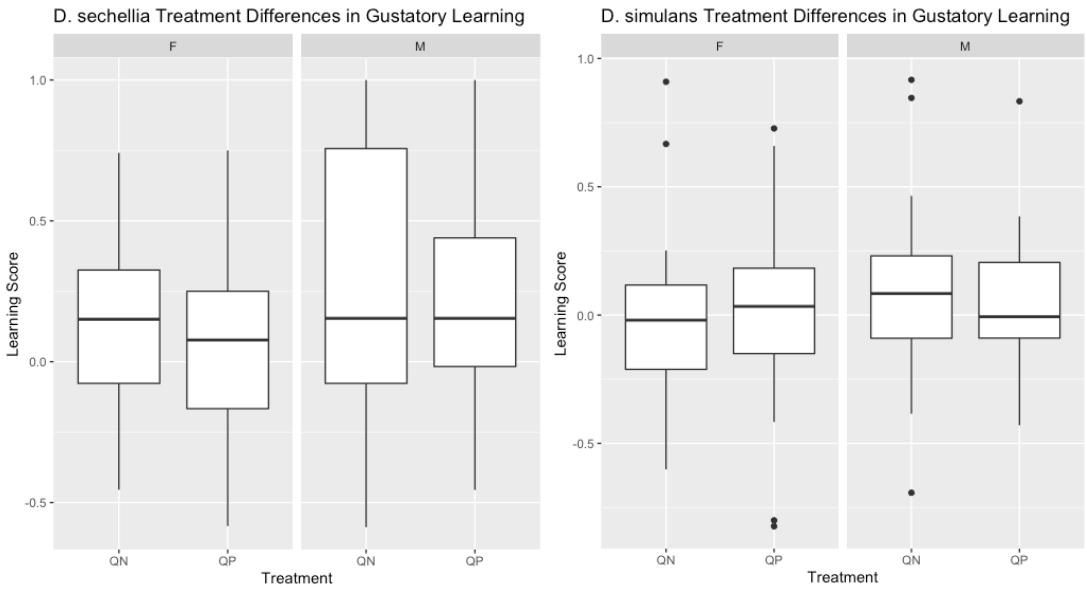


Figure 4. Species, Sex, and Treatment Differences in Learning for Gustatory Stimuli (Quinine Treatments): These plots represent the species, sex, and treatment differences in learning for the gustatory stimuli quinine treatments, as noted in the main text. The y-axis represents the learning score or change in preference following associative conditioning using a negative gustatory stimulus. Positive learning score values indicate a decrease in preference for the substrate that was paired with quinine (i.e., learning in the "correct" direction), while negative learning score values indicate an increase in preference for the substrate that was paired with quinine (i.e., learning in the "incorrect" direction). Learning scores of 0 indicate no change in substrate preference following associative conditioning. Our findings indicate that both species and sexes differ between quinine treatments in learning for the gustatory stimuli.

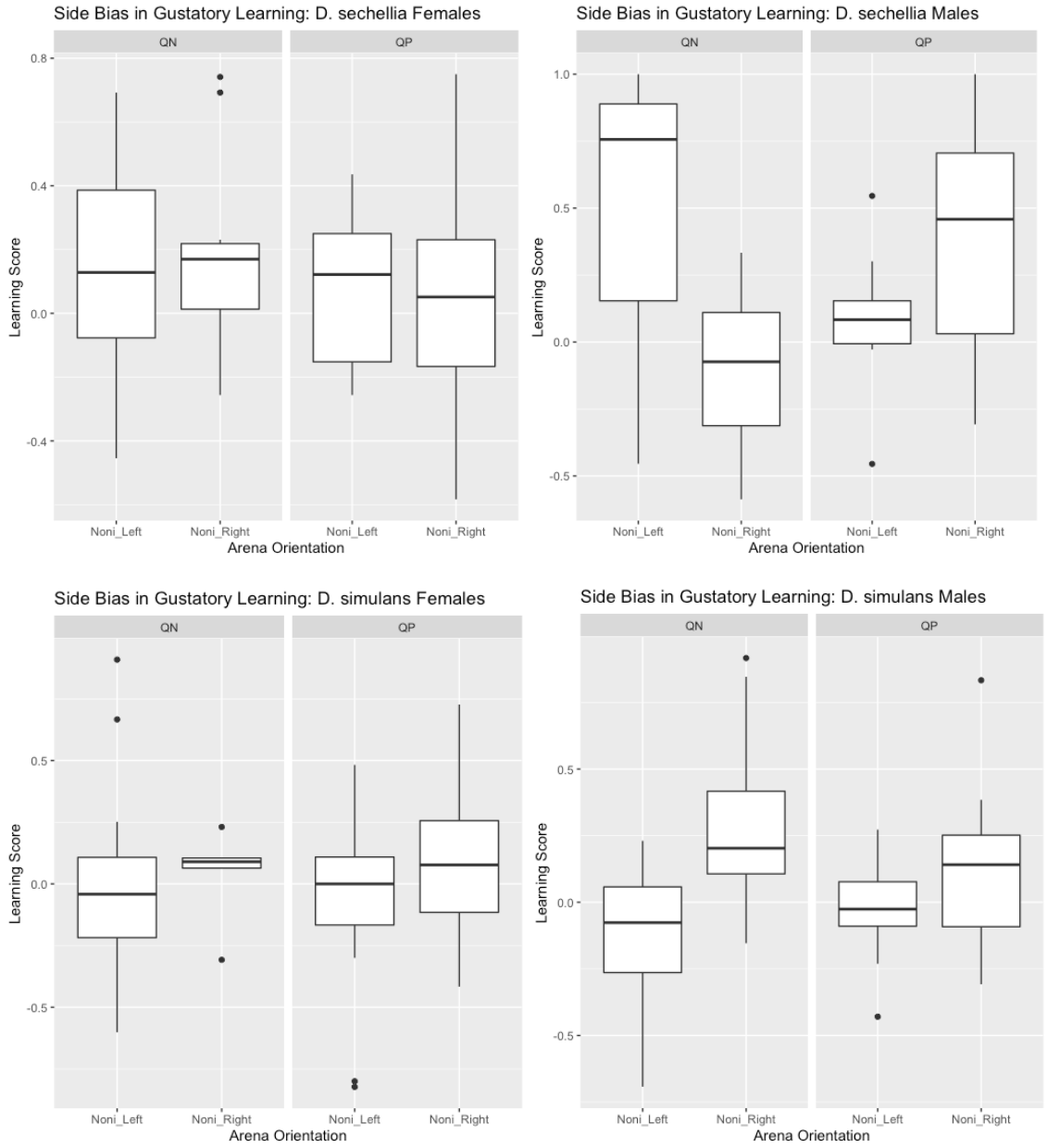


Figure 5. The Effect of Side Bias on Species, Sex, and Treatment Differences in Learning for Gustatory Stimuli (Quinine Treatments): These plots represent the effect of side bias on species, sex, and treatment differences in learning for the gustatory stimuli quinine treatments, as noted in the main text. The y-axis represents the learning score or change in preference following associative conditioning using a negative gustatory stimulus. Positive learning score values indicate a decrease in preference for the substrate that was paired with quinine (i.e., learning in the "correct" direction), while negative learning score values indicate an increase in preference for the substrate that was paired with quinine (i.e., learning in the

"incorrect" direction). Learning scores of 0 indicate no change in substrate preference following associative conditioning. Our findings indicate that learning scores are influenced by the presence of a side bias for both species and sexes.

Species differences in initial substrate preference: visual stimuli

We found evidence of overall initial preference differences between the zigzag and stripes pattern tape ($X^2 = 7.03$, degrees of freedom = 1, $p = 0.008$). This finding indicates that the flies can differentiate between these visual stimuli.

We did not find support for species differences in initial preference for the visual stimuli. We found no effect of species on initial preference for visual stimuli ($X^2 = 2.39$, degrees of freedom = 1, $p = 0.12$). We also found no effect of sex on initial preference ($X^2 = 1.39$, degrees of freedom = 1, $p = 0.24$), nor an effect of arena orientation ($X^2 = 3.46$, degrees of freedom = 1, $p = 0.06$). We did not find evidence that genotypes differed significantly in initial preference (likelihood ratio = 1.25, degrees of freedom = 1, $p = 0.26$). This result supports our hypothesis that species should not differ in initial preference for the visual stimuli.

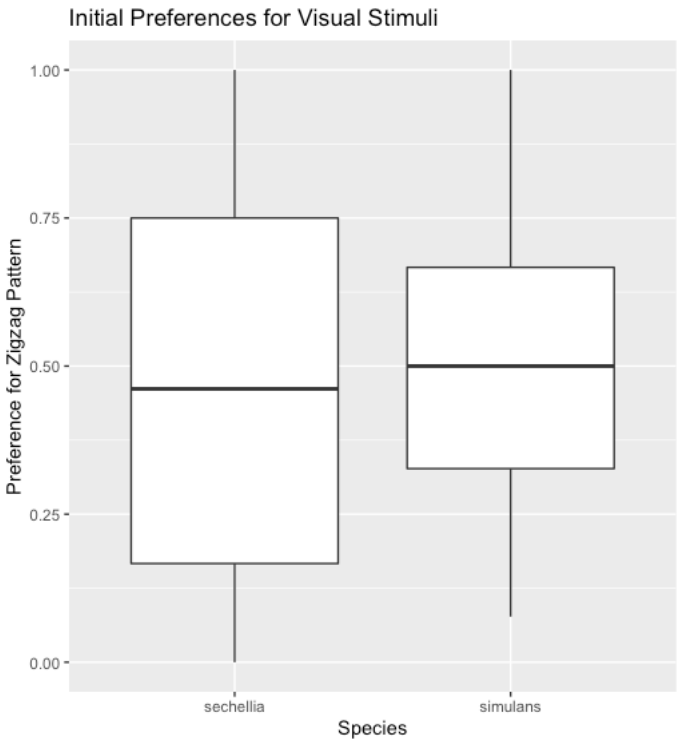


Figure 6. Species Differences in Initial Preference for Visual Stimuli: This plot represents species differences in initial preference for visual stimuli, as noted in the main text. The y-axis represents the proportion of time spent on the zigzag pattern substrate. Lower values closer to 0 indicate higher preference for the stripes pattern substrate, while higher values closer to 1 indicate higher preference for the zigzag pattern substrate. Values at or closer to 0.5 indicate no demonstrable preference for either the stripes pattern or zigzag substrates. Our findings indicate that species do not differ in initial preference for the visual stimuli.

Species differences in learning: visual stimuli - control treatments (no quinine)

We found no evidence that learning occurred overall in the visual stimuli control treatments (Intercept F-value = 0.38, denominator degrees of freedom = 44, p = 0.54).

We did not find support for species differences in learning for the visual stimuli control treatments. We found no effect of species on learning

scores (F-value = 0.02, denominator degrees of freedom = 6, p = 0.9), nor an effect of sex (F-value = 0.07, denominator degrees of freedom = 44, p = 0.8). However, we did find a significant effect of arena orientation on learning scores (F-value = 8.96, denominator degrees of freedom = 44, p = 0.005). We did not find evidence that genotypes differed significantly in learning (likelihood ratio = 2.32, degrees of freedom = 1, p = 0.13). This was mostly consistent with our prediction that no change in preference should be observed for the control treatments, as no quinine associative conditioning training took place for individuals assigned to this treatment. However, the significant effect of arena orientation on learning scores was unexpected, as we observed an increase in preference over time for whichever pattern is oriented on the left-hand side (Figure 7).

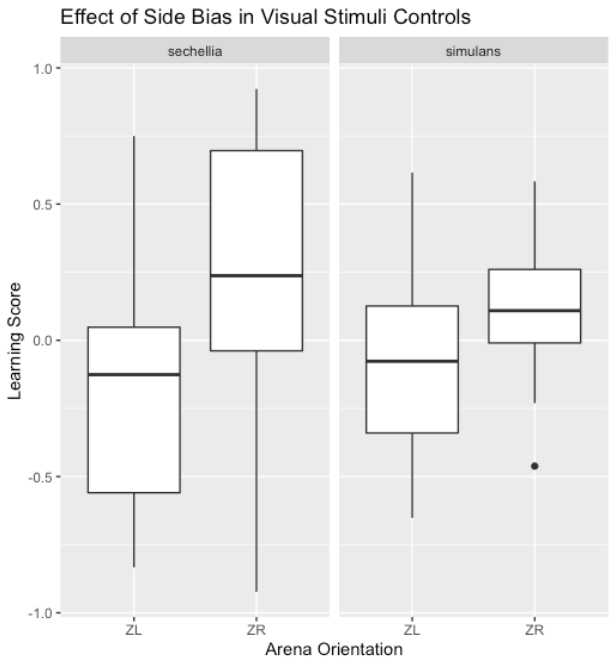


Figure 7. The Effect of Side Bias in Visual Stimuli Control Treatments: This plot represents the effect of side bias on learning in the visual stimuli control treatments, as noted in the main text. The y-axis represents the learning score or change in preference following additional exposure to the

two substrate options in the control treatments during the "training" stage of the experiment. Positive learning score values indicate an increase in preference for the stripes pattern substrate, while negative learning score values indicate an increase in preference for the zigzag pattern substrate. Learning scores of 0 indicate no change in substrate preference following the training stage of the experiment. Our findings indicate an effect of a left-side bias, with both species and sexes increasing preference for whichever substrate pattern was located on the left-hand side.

Species differences in learning: visual stimuli - quinine treatments

Overall, we found evidence that average learning scores in the visual stimuli - quinine treatments significantly differed from zero (Intercept F-value = 11.0, denominator degrees of freedom = 119, $p < 0.005$). However, we found that both species were learning in the incorrect direction, relative to the quinine treatment received (i.e., both species increased preference for whichever visual stimulus was previously paired with quinine).

We did not find support for species differences in learning for the visual stimuli quinine treatments. We found no effect of species on learning scores (F-value = 2.86, denominator degrees of freedom = 6, $p = 0.14$), nor an effect of sex (F-value = 0.06, denominator degrees of freedom = 119, $p = 0.8$), nor an effect of treatment (F-value = 0.53, denominator degrees of freedom = 119, $p = 0.47$) nor arena orientation (F-value = 2.72, denominator degrees of freedom = 119, $p = 0.1$). We did not find evidence that genotypes differed significantly in learning (likelihood ratio = 2.9, degrees of freedom = 1, $p = 0.09$). These results indicate that species and sexes do not differ in learning for visual stimuli quinine treatments.

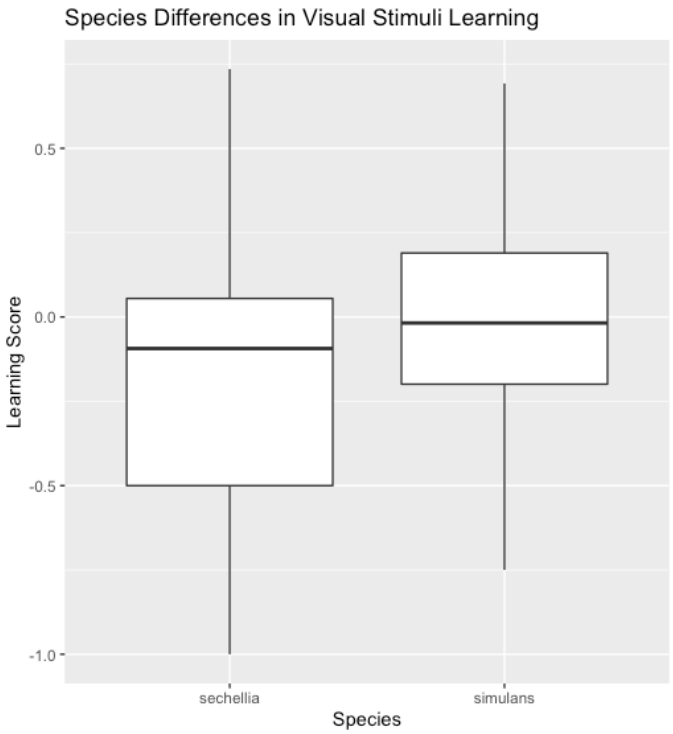


Figure 8. Species Differences in Learning for Visual Stimuli (Quinine Treatments): This plot represents species differences in learning for the visual stimuli quinine treatments, as noted in the main text. The y-axis represents the learning score or change in preference following associative conditioning. Positive learning score values indicate a decrease in preference for the substrate that was paired with quinine (i.e., learning in the "correct" direction), while negative learning score values indicate an increase in preference for the substrate that was paired with quinine (i.e., learning in the "incorrect" direction). Learning scores of 0 indicate no change in substrate preference following associative conditioning. Our findings indicate that species do not differ in learning for the visual stimuli quinine treatments.

Discussion

Identifying the various selection pressures that demand higher learning abilities for animals to survive and succeed in an environment, as well as how the fitness benefits of learning compare to the associated energy costs, is important for understanding how individual differences in learning

arise and evolve (Buchanan et al. 2013). However, we currently lack a full understanding of the types of environments that favor learning, and whether selection for improved learning in one context carries over to generate improvements in general cognitive ability. In this study, we investigated whether an evolutionary decrease in the degree of environmental variability experienced in nature is associated with divergence in learning. Additionally, we aimed to investigate how learning evolves - more specifically, if divergence in learning impacts general learning ability, with responses correlated across a wide range of stimuli and contexts, or if divergence in learning is limited to specific stimuli and contexts.

In our species comparison of *D. sechellia* and *D. simulans*, we did not find evidence that higher degree of environmental variability was associated with higher learning performance. In fact, we found the opposite: the species that has evolved under low environmental variability (the specialist, *D. sechellia*) outperformed the species that has evolved under high environmental variability (the generalist, *D. simulans*) in associative conditioning learning trials. These species differences in learning were observed for the gustatory stimuli quinine treatments (with the specialist outperforming the generalist), but not for the visual stimuli quinine treatments. These findings provide support for the evolutionary based theory, indicating that divergence in learning for these species is limited to more specific stimuli and contexts (i.e., the stimuli that were predicted to be the most relevant in terms of fitness consequences). No species differences in learning were observed in the control treatments for either the gustatory or visual stimuli, which is consistent with what was expected (since no quinine treatment was received). Additionally, while we found species differences in learning for the gustatory stimuli - quinine treatments, we did not find

evidence for genotypic differences in learning for either the gustatory or visual stimuli quinine treatments.

In addition to the observed species differences in learning, we also observed sex differences, with males generally outperforming females in learning performance. However, as with species differences, this effect was only observed in the gustatory quinine treatments. This was a particularly surprising result for the gustatory stimuli, considering that in addition to foraging, female *Drosophila* must also lay eggs on substrates. Thus, females would be expected to be under strong selection for being able to learn about these important gustatory cues. Additionally, female *D. sechellia* glean substantial fertility benefits from being on their noni host (Lavista-Llanos et al. 2014; Dworkin & Jones 2015; Jones 2005). However, a similar result was also observed in a previous study, with *D. sechellia* and *D. simulans* males generally outperforming females in assays testing decision-making accuracy (Burns et al. 2020). One possible explanation is that virgin females were used for this experiment, and thus may have been less motivated to learn about the gustatory cues than males. In *D. melanogaster* males are known to locate and defend substrate patches for mating purposes (Burns et al. 2020; Saltz & Foley 2011; Saltz 2013). Future experiments measuring both virgin and mated flies could help parse out if the unexpected sex differences in learning performance are due to differences in motivation, based on mated status.

We found a significant effect of arena orientation on learning in the gustatory stimuli quinine treatments and the visual stimuli control treatments, implying the influence of a side bias on both familiarity (observed in the visual stimuli controls) and learning (observed in the gustatory stimuli quinine treatments). Side bias, also commonly referred to

as “handedness”, has been observed in a number of animals (Jackson 1998; Alves et al. 2007; Kight et al. 2008; Collins 1975; Andrade et al. 2001; Castellano et al. 1987; Glick & Ross 1981; Sherman et al. 1980), and previous work has demonstrated that it can contribute to variation in learning performance and outcomes (Alves et al. 2007; Anfora et al. 2009; Letzkus et al. 2006; Vallortigara & Andrew 1994). For the visual stimuli controls, we observed a significant left-side bias, with both species and sexes showing an increase in preference for whichever pattern was located on the left-hand side. However, the influence of side bias on the gustatory stimuli learning scores was more complicated. Female specialists (*D. sechellia*) showed an influence of left-side bias, demonstrating higher learning performance in arenas where the correct choice (relative to the quinine treatment received) was located on the left-hand side, whereas the male specialists showed an influence of right-side bias. In addition, both male and female generalists (*D. simulans*) showed higher learning performance in substrate preference arenas where plain substrate was located on the left-hand side, regardless of the quinine treatment received. These findings indicate the potential of certain cognitive biases, such as side bias, to influence learning outcomes, and illustrate that their effects are not entirely straightforward or consistent across stimuli and contexts.

This study, while powerful, was limited in several ways. First, our species comparison was limited to two species - one specialist and one generalist. While *D. sechellia* and *D. simulans* provide a good model system for investigating how divergent selection for diet breadth and environmental variability contribute to variation in learning, there have also been a range of physiological adaptations that have accompanied *D. sechellia*’s specialization on the toxic noni fruit. This includes resistance to the octanoic

and hexanoic acids in noni, which are responsible for the toxicity of noni to other species of *Drosophila*, changes to olfaction, reduced ovariole and egg production in females, and changes in egg and larval morphology (Jones 2005; Dekker et al. 2006; Ibba et al. 2010; Lavista-Llanos et al. 2014; Dworkin & Jones 2015; Prieto-Godino et al. 2017; Auer et al. 2020; Burns et al. 2020). Additionally, species differences in starting gustatory stimuli preferences and the possible associated fitness consequences may have contributed to the observed species differences in learning. Because *D. sechellia* are preferential specialists that can survive either on noni or plain substrates (while noni is toxic to *D. simulans*) (Dworkin & Jones 2015), it is possible that the observed species differences in learning for gustatory stimuli are caused by something other than divergence in habitat breadth. This possibility is further corroborated by the species differences in quinine avoidance, where *D. simulans* was far less likely to avoid quinine-laced plain substrate if the only other option was noni substrate. However, the quinine avoidance experiment included a much larger sample range of genotypes (13 *D. sechellia* and 11 *D. simulans*) than the learning experiment (4 *D. sechellia* and 4 *D. simulans*). Additionally, 2/4 *D. simulans* genotypes measured for species differences in learning started with higher initial preferences for the noni substrate, similar to the initial preferences of *D. sechellia*. Thus, it is unlikely that the observed species differences in gustatory stimuli learning are entirely due to differences in starting gustatory stimuli preferences. Future work incorporating additional specialist/generalist species comparisons within the *D. melanogaster* subgroup would help to elucidate whether these findings are unique to the *D. sechellia*/*D. simulans* species pair or are indicative of a larger pattern.

In regard to our comparative study with *D. sechellia* and *D. simulans*, these findings do not support our hypothesis that species evolving in highly variable environments also evolve higher learning performance. Instead, we saw the opposite pattern, suggesting that the existing theory on the evolution of learning may not be relevant to all species pairs. However, these findings provide support for the evolutionary based theory of divergence in learning, indicating that divergence in learning for these species is limited to more specific stimuli and contexts. Additionally, these findings demonstrate the potential of cognitive biases, such as side bias, to influence learning outcomes, and that the effects of these biases are not always consistent across individuals, stimuli, and contexts.

Chapter 3

Do Dietary Sources of Neurotransmitters Contribute to the Plasticity and Evolution of Learning? A Comparative Study Between *Drosophila sechellia* and *Drosophila simulans*

Introduction

Learning, defined as the modification of behavior following an informative event or experience (Thompson 1986), is an important cognitive ability that has the potential to substantially impact fitness outcomes (Shettleworth 2001). As such, many previous studies have aimed to elucidate why we observe variation in learning, primarily focusing on the various selection pressures that may demand higher cognitive abilities for individuals to survive and succeed in a given environment, as well as how the fitness benefits of learning compare to the associated energy costs (Buchanan et al. 2013). Previous studies have identified several associations between learning and various environmental pressures and stressors, contributing to our understanding of the types of environments that favor higher cognitive abilities. Examples include the degree of environmental variability (Burns et al.2020; Kotrschal & Taborsky 2010; Roth et al. 2010; Roth et at. 2012), predation pressures, and complexity of the social environment (Cummings & Ramsey 2015; Dunbar 1998). However, understanding individual differences in learning, and how those differences evolve, remains a challenge.

Previous work has demonstrated that one of the most consistent factors influencing variation in learning, both for individuals and over evolutionary timescales, is diet. For example, a large body of literature has

demonstrated an association between dietary macronutrient ratios and learning performance in humans, rodents, and honeybees (Cordner & Tamashiro 2015; Messier et al. 2007; Molteni et al. 2002; Arien et al. 2018), and that diet influences the levels of certain neurotransmitters in the brain, namely dopamine, serotonin, and acetylcholine (Reichelt 2016; Reichelt et al. 2017; Nguyen et al. 2017; Growdon & Wurtman 1981). In addition to the diet-induced phenotypic plasticity that may be occurring within an individual's lifetime, previous work has also demonstrated that diet can be an important driving force in evolutionary divergence in learning. For example, one study found that dietary nutrient content predicted brain size across 42 species of butterflies, and that the variation in brain size was due to genetically based differences across species, rather than developmental plasticity (Snell-Rood et al. 2020). A previous study by Snell-Rood et al. confirmed that higher brain volume was associated with higher learning performance in cabbage white butterflies, *Pieris rapae*, (Snell-Rood et al. 2009). This finding was consistent with many others that have found positive associations between brain size/volume and cognitive performance (Cummings & Ramsey 2015; Dunbar 1998).

While many aspects of diet have been previously considered when investigating individual differences in learning, one remains largely overlooked: dietary sources of neurotransmitters (Briguglio et al. 2018). Various foods including animal products, fruits, edible plants, roots, and botanicals have been reported to contain neurotransmitters (Briguglio et al. 2018). The lack of studies investigating the role of dietary neurotransmitters play in individual differences in learning is surprising, given that a large body of literature has robustly demonstrated the strong role neurotransmitters play in learning and cognition, at least when produced

endogenously (Barron et al. 2011; Riemensperger 2011; Yamamoto & Seto 2014). Some previous work has demonstrated that neurochemicals in the environment have the potential to influence brain and behavior, but most work has focused primarily on investigating the effects of neurotoxins, rather than neurotransmitters, on cognitive performance and brain morphology (Rice 2000; Nicolescu 2010). While some limited studies have found that intake of dietary neurotransmitter precursor supplements, namely 5-HTP (the serotonin precursor) and l-DOPA (the dopamine precursor), can influence cognition (Turner et al. 2006; Jenkins & Groh 1970), the significance of dietary neurotransmitters on cognitive performance and the levels of neurotransmitters in the brain remains largely unknown (Briguglio et al. 2018).

Investigating how dietary sources of neurotransmitters influence learning performance has important implications for our understanding of how individual differences in learning arise, and how those differences evolve. For example, if a population were to shift to foods containing dietary sources of neurotransmitters, immediate changes in learning and cognition may occur. This could result in the Baldwin effect, where physiological or behavioral modifications occurring within an individual's lifetime provide certain fitness benefits, and then heritable genetic factors producing similar phenotypes (and thus similar fitness benefits) are favored by natural selection and spread throughout the population over generations (Simpson 1952). Another possibility is that as populations shift to foods containing dietary sources of neurotransmitters, the environmental perturbation could result in new genotype-by-environment interactions that "reveal" cryptic genetic variation for selection to subsequently act upon (Gibson & Dworkin 2004). Additionally, as populations evolve in the presence of abundant

dietary neurotransmitters, they might experience reduced endogenous production of those neurotransmitters over time. Failure to consider these possibilities could result in misleading or incomplete information about how learning evolves, particularly when investigating individual differences in learning under laboratory rearing conditions.

Do dietary sources of neurotransmitters contribute to individual differences in learning performance? Do dietary sources of neurotransmitters contribute to the evolution of learning? To investigate, we compared two closely related species of *Drosophila*: *D. sechellia* and *D. simulans*. *D. simulans* is a dietary generalist that feeds on a broad range of fruit, while *D. sechellia* is a dietary specialist that has evolved to feed and breed preferentially on the toxic *Morinda citrifolia*, or noni, fruit (Jones 2005). Noni is toxic to other species of Drosophila, including *D. simulans*, and contains relatively high levels of the dopamine precursor, l-DOPA (Schrider et al. 2018; Jones 2005; R'Kha et al. 1990; Lavista-Llanos et al. 2014; Dworkin & Jones 2015). *D. sechellia*, while specialized on noni, are not obligate specialists (i.e., they are able to survive and reproduce on other fruits and standard fly medium in laboratory settings) (Dworkin & Jones 2015). Previous work has shown that *D. sechellia* are dopamine deficient in regard to fecundity when not raised on noni in the presence of dietary sources of l-DOPA, and have evolved a nutritional requirement for high levels of exogenous l-DOPA (Lavista-Llanos et al. 2014; Dworkin & Jones 2015). However, we currently do not know if *D. sechellia* are also dopamine deficient in the brain when not raised on noni. In particular, the neurotransmitter dopamine has been identified as having a significant impact on learning, both in *Drosophila*, as well as other species (Barron et al. 2011; Riemensperger 2011; Yamamoto & Seto 2014).

To investigate how being reared in the presence of dietary l-DOPA influences learning performance, we reared the flies in two different rearing environments: plain fly medium and plain fly medium supplemented with l-DOPA. We then conducted associative conditioning assays to measure individual learning performance. Comparing species that have diverged in diet breadth and have simultaneously been exposed to different levels of dietary l-DOPA over evolutionary time, allowed us to investigate how dietary sources of l-DOPA contribute to evolutionary divergence in learning. We hypothesize that evolving in the presence of dietary l-DOPA will be associated with decreased learning performance when not reared in the presence of dietary l-DOPA. Comparing learning performance across two rearing environments allowed us to investigate how dietary sources of l-DOPA contribute to the short-term plasticity of learning. We hypothesize that being reared in the presence of dietary l-DOPA will be associated with higher learning performance.

Materials and Methods

Study System: A comparative approach using D. sechellia and D. simulans

We compared the two closely related species of *Drosophila* recently diverged in dietary breadth: *D. sechellia* and *D. simulans*. Previous work has estimated very recent divergence times for these species, ranging from 250,000 to 413,000 years ago (Garrigan et al. 2012; Kliman et al. 2000; Schrider et al. 2018). *Drosophila* habitats consist of ephemeral rotting fruit patches where flies eat, mate, lay eggs, and spend the majority of their time (Powell 1997). As a dietary generalist, *D. simulans* has a broad range of

dietary options that, depending on the region and time of year, can vary substantially across time and space. This is in direct contrast to *D. sechellia*, a dietary specialist that has evolved to feed and breed preferentially on *M. citrifolia* fruit, which is ubiquitous in the Seychelles, present year-round, and toxic to other species of *Drosophila* (including *D. simulans*) (Schrider et al. 2018; Jones 2005; R'Kha et al. 1990). As *D. sechellia* is an island specialist recently diverged from ancient *D. simulans*, within-species genotypic variation is expected to be higher in *D. simulans* than in *D. sechellia*.

Genotypes

In addition to species differences, we also investigated genotypic variation. Isofemale lines, hereafter "genotypes", were selected randomly from a range of samples generously provided by D. Matute in 2016 (Burns et al. 2020). *D. sechellia* genotypes (specifically NF 33, NF 52, NF 103, and NF 127) were collected from various locations across the Seychelles, while the *D. simulans* genotypes were collected across various locations in central and southern Africa (specifically NMB-024 collected in Namibia, NS-39 collected in Nairobi) and Madagascar (specifically MD 223) (Matute et al. 2014; Schrider et al. 2018; pers.comm.). Each genotype was established by inbreeding a single wild female; therefore, individuals of the same genotype are more genetically similar to one another than they are to individuals of other genotypes. Thus, these genotypes represent a sample of natural genetic diversity of these species.

Rearing

To rear flies for the trials, 10 virgin females were placed with 10 males of the same species and genotype and housed in vials containing either standard fly medium, which consisted of corn meal, corn syrup, malt sugar, dead yeast, soy flour, tegosept (methyl paraben), propionic acid, and phosphoric acid, or standard fly medium supplemented with l-DOPA (0.4mM; 0.08 g/L). The parents were allowed to mate and lay eggs for 14 days and newly eclosed virgin male and female offspring were collected under light CO_2 anesthesia on day 15. Collected flies were housed individually in vials containing standard fly medium and allowed to recover from the CO_2 anesthesia for 3 days prior to beginning trials. Flies were not starved prior to trials due to concerns that food deprivation could influence energy levels or habitat choice, as previous work has demonstrated that flies subjected to starvation are more likely to settle for less preferred habitats (Davis 2007).

Learning: overview

Fruit flies have become a common model system for studying chemosensory learning, as associative conditioning has been demonstrated in flies in many previous studies, and the behavioral methods for measuring learning have been repeatedly validated (Gerber & Stocker 2007; Saltz et al. 2017). In particular, aversive conditioning using gustatory or olfactory stimuli has become a popular approach for investigating learning in flies (Mery and Kawecki 2002; Saltz et al. 2017). During aversive conditioning, a negative conditioned stimulus is paired with an unconditioned stimulus for a

training period, after which the valence of the unconditioned stimulus is altered and subsequently avoided, even in the absence of the negative conditioned stimulus (Ayestaran et al. 2010). Aversive conditioning using a noxious gustatory stimulus has been found to elicit strong learning responses in animals, with many individuals requiring only one training episode or conditioning "experience" for a strong negative association and avoidance of the unconditioned stimulus to occur (Gustavson et al. 1974; Ralphs & Provenza 1999; Yamamoto 1993).

To investigate variation in learning between our specialist and generalist species, we employed an aversive conditioning assay using quinine hydrochloride (6.4 g/L), as the negative gustatory stimulus. Previous work has demonstrated that quinine, a bitter tasting substance, is noxious to flies, with flies showing strong avoidance of quinine that does not lessen over time (i.e., flies do not habituate to quinine) (Mery and Kawecki 2002). During the aversive conditioning assays, flies were provided with a choice between two distinct types of food medium (standard, or "plain", fly medium, and imitation noni medium) and trained to associate one of the food mediums with the presence of quinine. To determine how flies responded to the aversive conditioning, we first measured initial food medium preferences to determine which of the two food mediums (plain or noni) each fly preferred prior to training. Flies were then subjected to an associative conditioning (or training) stage, where quinine was paired with one of the two food medium types. Following the training stage, food medium preference was measured again (in the absence of quinine) to determine which of the two mediums each fly preferred following aversive conditioning, relative to the treatment that was received. Avoidance conditioning arenas consisted of one of three treatments: plain fly medium

paired with quinine, imitation noni medium paired with quinine, or a control treatment, containing both plain fly medium and imitation noni medium and lacking quinine.

Trials

Stage 1: Measuring initial food preference

We began by measuring the initial food medium preferences of *D. sechellia* and *D. simulans* prior to the associative conditioning training. To do this, individual flies were placed into habitat choice arenas, which consisted of two medium options: plain fly medium (consisting of a standard recipe of agar, malt sugar, inactive dry yeast, and deionized water), and imitation *M. citrifolia* (noni) fruit medium. Imitation noni medium was made by adding octanoic and hexanoic acids to the plain fruit fly medium, as in Dworkin and Jones 2008. Previous work has provided extensive evidence that both the octanoic and hexanoic acids in noni are responsible for the fruit's toxicity to other species of *Drosophila* and are those involved in both attracting *D. sechellia* and repelling *D. simulans* (Jones 2005; Dekker et al. 2006; Ibba et al. 2010; Lavista-Llanos et al. 2014; Dworkin & Jones 2015; Prieto-Godino et al. 2017; Auer et al. 2020; Burns et al. 2020). Thus, for the purpose of investigating habitat preferences, adding octanoic and hexanoic acids to the plain fly medium provides an adequate proxy for noni. Because previous work has demonstrated that the levels of octanoic and hexanoic acids in noni can vary substantially based on fruit ripeness (Jones 2005; Dekker et al. 2006), imitation food substrates were used in lieu of real fruit patches. This allowed for reproduction of foods with a molecularly defined

composition and ensured that flies were presented with identical choices across trials.

Initial preference arenas consisted of one petri dish containing the two medium options, which were cut into halves, covered by a second petri dish (acting as a lid), and sealed together with tape (Figure 1). Relative location of the substrate halves within the initial preference arenas was varied (i.e., there was a 1:1 ratio of initial preference arenas with imitation noni on the right-hand side and plain on the left, and imitation noni on the left-hand side and plain on the right). Individual flies were assigned randomly to initial preference arenas.

To measure initial preference, individual flies were gently aspirated into a short pipette tip and allowed to emerge on their own accord into the initial preference arenas. Observers noted the fly's immediate substrate choice (i.e., which part of the arena each fly landed on upon immediate emergence into the arena). Substrate choice was measured every 10 min over the course of a 2 h observation period. In total, 13 substrate choice observations were recorded for each individual fly during the initial preference phase of the experiment.

Stage 2: Associative conditioning with an aversive gustatory stimulus

The aversive conditioning training arenas consisted of one petri dish containing the food medium (or mediums) covered by a second petri dish (acting as a lid) and sealed together with tape (Figure 1). For the quinine treatments, only the food medium paired with quinine was present in the training arenas (i.e., all plain medium paired with quinine, or all noni medium paired with quinine). This was done to ensure that each fly was

subjected to the same training experience for each treatment, and that flies were not able to simply avoid the substrate paired with quinine by moving to the other substrate option. For the control treatments, both the plain and noni mediums were present (as in the initial preference arenas) and the relative location of the substrate halves was varied (i.e. there was a 1:1 ratio of change-in-preference arenas with imitation noni on the right-hand side and imitation noni on the left-hand side). Individual flies were randomly assigned to treatments. Once transferred to the aversive conditioning training arenas, flies were left in the arenas overnight for associative conditioning prior to measuring change in preference the next day.

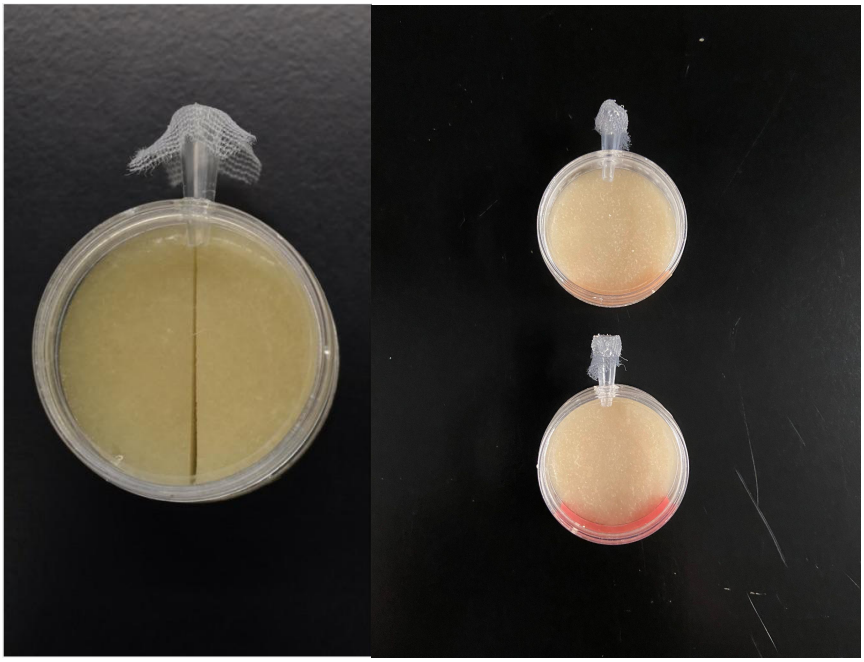


Figure 1. Food Substrate Preference Arenas and Associative Conditioning Training Arenas: Single flies were gently aspirated into the pipette tips, which were fitted into a small hole in the arena with the two substrate options. A mesh barrier prevented each fly from escaping the pipette tip in the other direction. For the initial preference and preference following training stages (Stage 1 and Stage 3), flies were allowed to emerge into the

arena on their own accord and were then scan sampled every 10 minutes over the course of 2 hours, and the food substrate choice of each fly was recorded. For the associative conditioning stage (Stage 2), flies were gently transferred into the arenas and left overnight for training to occur.

Stage 3: Measuring learning (change in food preference)

To compare learning between *D. sechellia* and *D. simulans*, we investigated change in medium preference following aversive conditioning training with a negative gustatory stimulus. To do this, individual flies were gently aspirated into a short pipette tip for transfer to the new change-in-preference arenas and allowed to emerge on their own accord (Figure 1). The arenas consisted of the same food substrate options provided prior to avoidance training: plain fly medium and imitation *M. citrifolia* (noni) medium.

Once again, the change-in-preference arenas consisted of one petri dish containing the two food substrates, cut in halves to be flush, covered by a second petri dish (acting as a lid), and sealed together with tape. As with initial preference, the relative location of the substrate halves was varied (i.e., there was a 1:1 ratio of change-in-preference arenas with imitation noni on the right-hand side and imitation noni on the left-hand side). Individual flies were once again randomly assigned to the change-in-preference arenas. Following each fly's emergence into the new arena, immediate substrate choice was recorded, followed by scan sampling to record the substrate choice every 10 min over the course of a 2 h observation period. In total, 13 change-in-preference substrate choice observations were recorded for each individual fly.

Stage 4: Measuring dopamine levels in the brain

This portion of the experiment is in collaboration with the Yamamoto Lab at Baylor College of Medicine, Houston, TX. We are currently working with the Yamamoto Lab, providing them with brain samples for each of the genotypes reared in both the plain substrate rearing environment and the l-DOPA-supplemented substrate rearing environment. The Yamamoto Lab is using high performance liquid chromatography to measure the amount of dopamine in the brain so we can compare species and genotype differences across the two rearing environments. We expect to have this data shortly.

Analysis

All analyses were conducted in R version 3.6.1 (Vienna, Austria) (R core team 2019)

Learning (change in food preference)

Our goal was to investigate if evolving in the presence of dietary l-DOPA was associated with decreased learning performance (i.e., change in food preferences following associative conditioning with a negative gustatory stimulus) when not reared in the presence of dietary l-DOPA. Additionally, our goal was to investigate if being reared in the presence of dietary l-DOPA was associated with higher learning. Specifically, we wanted to investigate whether species differed in learning when not reared in the presence of dietary l-DOPA, and whether species differed in learning when reared in the presence of dietary l-DOPA.

Measuring and calculating learning (change in food substrate preference)

Food substrate preference before and after aversive conditioning was calculated by averaging the proportion of time each of the flies spent on each of the two substrates. For observations where the fly failed to make a clear choice between the two substrate options (i.e., were in the middle of the two substrate options), no decision was indicated, and the observation was not included in the final habitat choice calculation. To calculate learning (or change in food substrate preference) scores, the food preference score following conditioning was subtracted from the initial food preference score (initial food preference - food preference following conditioning = learning score).

Learning scores were calculated based on the specific quinine treatment received, so learning scores would be directly comparable between quinine treatments. For the treatment where quinine was paired with imitation noni food substrate, imitation noni was assigned a value of 1 and plain substrate a value of 0. For the treatment where quinine was paired with plain food substrate, plain was assigned a value of 1 and imitation noni substrate a value of 0. Positive learning scores indicate a decrease in preference for the food substrate that was paired with quinine, while negative learning scores indicate an increase in preference for the food substrate that was paired with quinine. Learning scores of 0 indicate no change in food preference following associative conditioning. For the control treatments that lacked quinine, imitation noni substrate was arbitrarily given a value of 1 and plain substrate a value of 0.

Species differences in learning

To test whether species differed significantly in learning when reared in the presence of dietary l-DOPA and when not raised in the presence of dietary l-DOPA, we ran linear mixed models in the nlme package in R (Pinheiro et al. 2014), testing the effect of species on learning. The nlme package, an alternative to the lme4 package, provides functions to fit and analyze linear mixed models with structure in the residuals (various forms of heteroscedasticity), incorporating both fixed and random effects (Bates et al. 2015). The nlme package was used in lieu of the lme4 package to account for the heteroscedasticity of variance observed in lmer model residuals.

Models were run and analyzed separately for the plain food substrate rearing environment (in the absence of dietary l-DOPA) and the plain substrate supplemented with dietary l-DOPA. Species, sex, treatment (whether quinine was paired with plain or imitation noni substrate), and arena orientation were included as fixed effects. Random effects were included to account for the non-independence of genotype, as well as the trial date to account for which flies were tested on the same day. Linear mixed models were run for a four-way fixed-effects interaction, all possible three-way fixed-effects interactions, all possible two-way fixed-effects interactions, and no interactions.

Using AIC model comparison, the best model for the plain rearing environment treatments included all two-way interactions (Species + Sex + Treatment + Arena Orientation)^2, with a delta AIC of 12.1 between the first- and second-best models. The best model for the l-DOPA-supplemented rearing environment included two-way interactions between Species and Treatment, and Sex and Arena Orientation (Species x Treatment + Sex x

Arena Orientation). To calculate p-values for species, sex, and arena orientation, we used Type III marginal F tests in the nlme package (Pinheiro et al. 2014). We tested the significance of genotype using likelihood ratio tests.

Results

Species differences in learning: plain substrate rearing environment (no l-DOPA)

We found support for species differences in learning for individuals raised on the plain food substrate in the absence of dietary l-DOPA. We found no effect of sex on learning scores. However, we did observe a significant Species x Treatment interaction ($X^2 = 8.14$, degrees of freedom = 2, $p = 0.02$), as well as a Treatment x Arena Orientation interaction ($X^2 = 7.73$, degrees of freedom = 2, $p = 0.02$). We did not find evidence that genotypes differed significantly in learning (likelihood ratio = 0, degrees of freedom = 1, $p = 1.0$). These results indicate that species, but not genotypes, differed significantly in learning across quinine treatments when reared in the absence of dietary l-DOPA. Additionally, these results indicate that the orientation of the food substrate within the food preference arenas (i.e., whether the imitation noni substrate was located on the right or left-hand side) significantly influenced learning scores, contributing to the differences in learning performance observed between treatments.

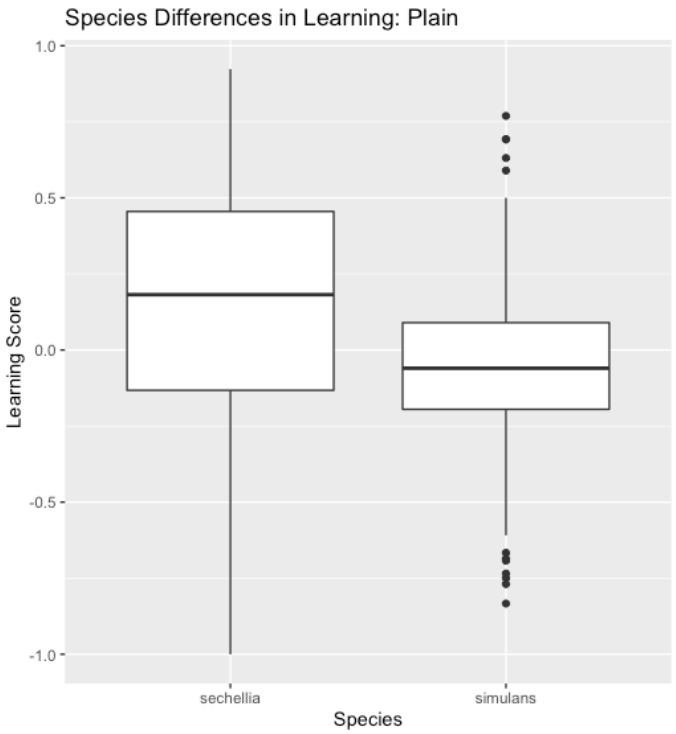


Figure 2. Species Differences in Learning for Plain Substrate Rearing Environment: This plot represents species differences in learning for individuals reared in the plain substrate environment (lacking dietary sources of l-DOPA), as noted in the main text. The y-axis represents the learning score or change in preference following associative conditioning using a negative gustatory stimulus. Positive learning score values indicate a decrease in preference for the food substrate that was paired with quinine (i.e., learning in the "correct" direction), while negative learning score values indicate an increase in preference for the food substrate that was paired with quinine (i.e., learning in the "incorrect" direction). Learning scores of 0 indicate no change in food preference following associative conditioning. Our findings indicate that both species differ in learning when raised in the absence of dietary sources of l-DOPA.

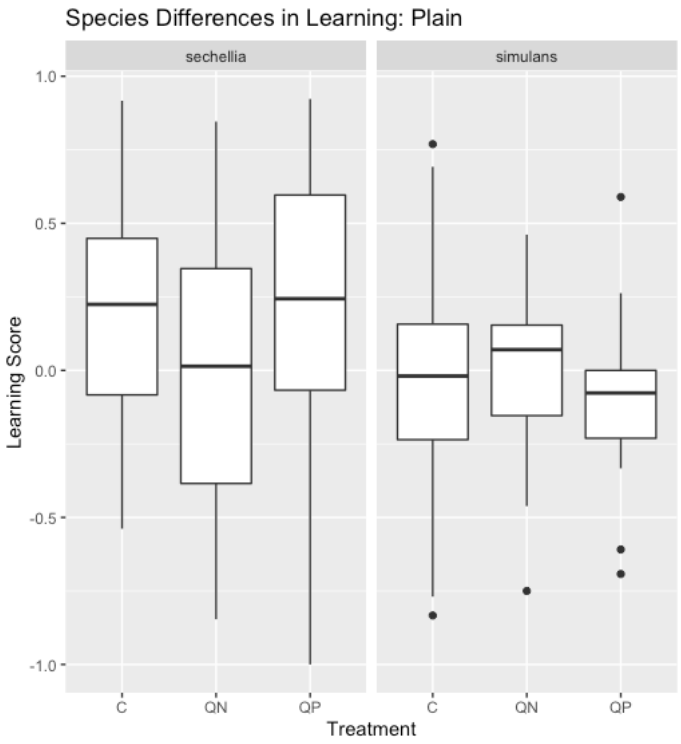


Figure 3. Species and Treatment Differences for the Plain Substrate Rearing Environment: These plots represent the species and treatment differences in learning for individuals reared in the plain substrate environment (lacking dietary sources of l-DOPA), as noted in the main text. The y-axis represents the learning score or change in preference following associative conditioning using a negative gustatory stimulus. Positive learning score values indicate a decrease in preference for the food substrate that was paired with quinine (i.e., learning in the "correct" direction), while negative learning score values indicate an increase in preference for the food substrate that was paired with quinine (i.e., learning in the "incorrect" direction). Learning scores of 0 indicate no change in food preference following associative conditioning. Our findings indicate that both species differ in learning across treatments when raised in the absence of dietary sources of l-DOPA.

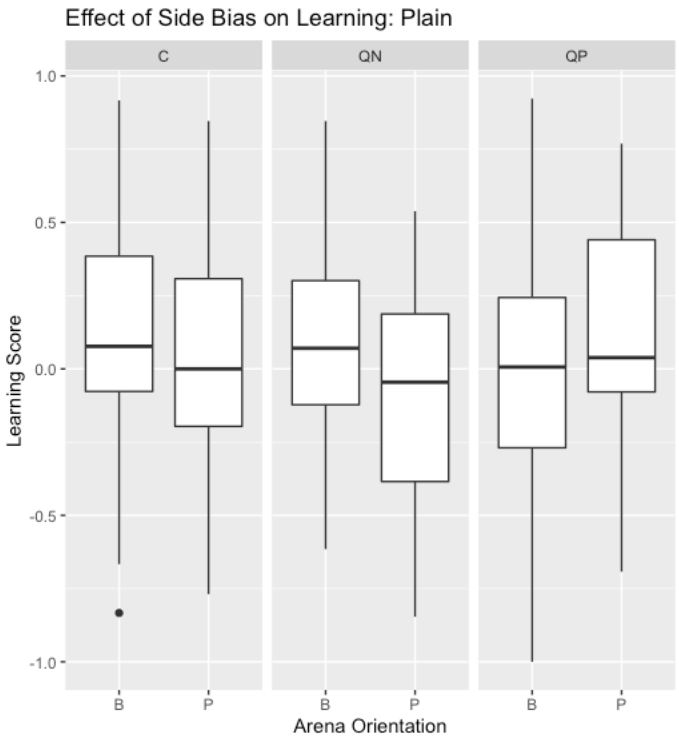


Figure 4. The Effect of Side Bias in Learning for the Plain Substrate Rearing Environment: This plot represents the effect of side bias on learning for individuals raised in the absence of dietary l-DOPA, as noted in the main text. The y-axis represents the learning score or change in preference following additional exposure to the two substrate options in the control treatments during the "training" stage of the experiment. Positive learning score values indicate an increase in preference for the stripes pattern substrate, while negative learning score values indicate an increase in preference for the zigzag pattern substrate. Learning scores of 0 indicate no change in food preference following the training stage of the experiment. Our findings indicate an effect of a left-side bias, with all individuals demonstrating higher learning performance when the correct choice, relative to the quinine treatment received, was located on the left-hand side.

Species differences in learning: l-DOPA supplemented rearing environment

We did not find evidence for species differences in learning for individuals raised on the plain substrate supplemented with l-DOPA (X^2 = 0.92, degrees of freedom = 1, p = 0.34). We also found no effect of sex (X^2

= 1.51, degrees of freedom = 1, p = 0.22) or treatment (X^2 = 5.05, degrees of freedom = 2, p = 0.08) on learning scores. However, we did observe a significant effect of arena orientation on learning scores (X^2 = 7.37, degrees of freedom = 2, p = 0.03). We did not find evidence that genotypes differed significantly in learning (likelihood ratio = 0.79, degrees of freedom = 1, p = 0.37). These results indicate that neither species, sexes, nor genotypes differ significantly in learning when reared in the presence of dietary l-DOPA. Additionally, these results indicate that the orientation of the food substrate within the food preference arenas (i.e., whether the imitation noni substrate was located on the right or left-hand side) significantly influenced learning scores for both species.

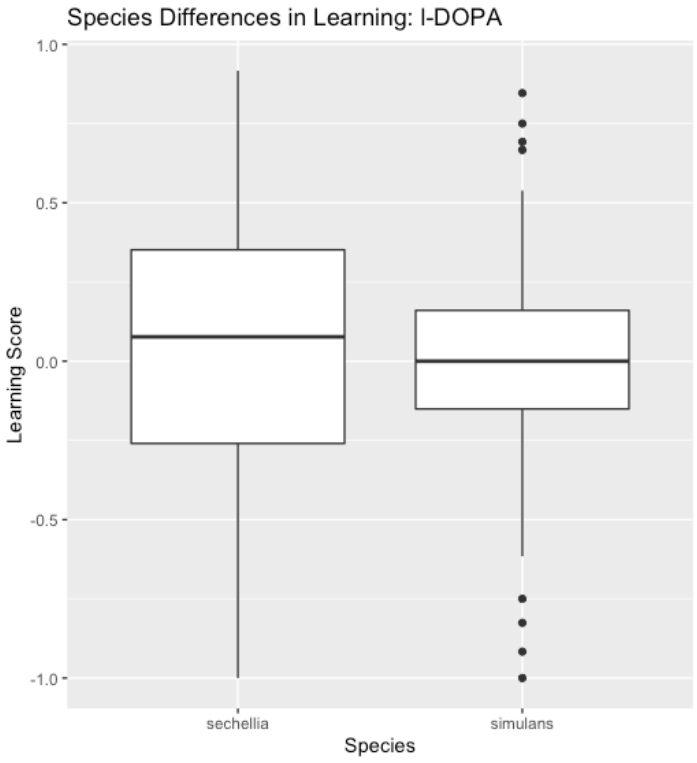


Figure 5. Species Differences in Learning for the l-DOPA Supplemented Substrate Rearing Environment: This plot represents species differences in learning for individuals reared in the l-DOPA supplemented substrate

environment, as noted in the main text. The y-axis represents the learning score or change in preference following associative conditioning using a negative gustatory stimulus. Positive learning score values indicate a decrease in preference for the food substrate that was paired with quinine (i.e., learning in the "correct" direction), while negative learning score values indicate an increase in preference for the food substrate that was paired with quinine (i.e., learning in the "incorrect" direction). Learning scores of 0 indicate no change in food preference following associative conditioning. Our findings indicate that species do not differ in learning when raised in the presence of dietary sources of l-DOPA.

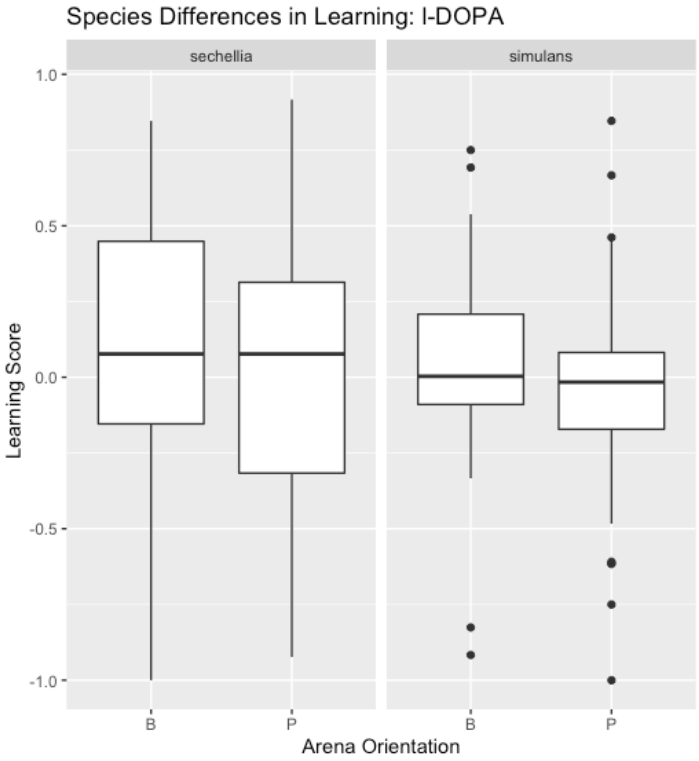


Figure 6. The Effect of Side Bias on Learning for the l-DOPA Supplemented Substrate Rearing Environment: These plots represent the effect of arena orientation/side bias on species differences in learning for individuals reared in the l-DOPA supplemented substrate environment, as noted in the main text. The y-axis represents the learning score or change in preference following associative conditioning using a negative gustatory stimulus. Positive learning score values indicate a decrease in preference for the food substrate that was paired with quinine (i.e., learning in the "correct" direction), while negative learning score values indicate an increase in preference for the food substrate that was paired with quinine (i.e., learning

in the "incorrect" direction). Learning scores of 0 indicate no change in food preference following associative conditioning. Our findings indicate an effect of side bias on learning scores for individuals raised in the presence of dietary sources of l-DOPA.

Discussion

Identifying the various selection pressures that demand higher cognitive abilities for animals to survive and succeed in an environment, as well as how the fitness benefits of learning compare to the associated energy costs, is important for understanding how individual differences in learning arise and evolve (Buchanan et al. 2013). However, we currently lack a full understanding of the types of environments that favor learning, and whether selection for improved learning in one context carries over to generate improvements in general cognitive ability. In this study, we investigated how dietary sources of l-DOPA contribute to evolutionary divergence in learning. Specifically, we investigated if evolving in the presence of dietary l-DOPA was associated with decreased learning performance when not reared in the presence of dietary l-DOPA. Additionally, we investigated if being reared in the presence of dietary l-DOPA was associated with higher learning performance.

In our species comparison of *D. sechellia* and *D. simulans*, we did not find evidence that evolving in the presence of dietary l-DOPA was associated with decreased learning performance when not reared in the presence of dietary l-DOPA. In fact, we found the opposite: with the species that has been evolving in the presence of dietary l-DOPA (*D. sechellia*) outperforming the species that has not been evolving in the presence of dietary sources of l-DOPA (*D. simulans*) in associative learning trials. These

species differences were observed for individuals that were reared on the plain food substrate (lacking l-DOPA supplementation), but not for individuals that were reared on the plain food substrate supplemented with l-DOPA. These findings partially support our hypothesis that being reared in the presence of dietary l-DOPA should be associated with higher learning performance - as being reared on food substrate supplemented with l-DOPA was associated with higher learning scores in *D. simulans*, but not in *D. sechellia*. No sex or genotype differences were observed in learning performance for individuals raised on either rearing environment.

We found a significant effect of arena orientation on learning for individuals reared both in the presence and absence of dietary l-DOPA, implying the influence of a side bias on learning performance. For individuals reared in the absence of dietary l-DOPA, both species showed higher learning performance when the correct choice, relative to the quinine treatment that was received, was located on the left-hand side. For individuals raised in the presence of dietary l-DOPA, both species showed higher learning performance in food preference arenas where the plain food substrate was located on the left-hand side, regardless of the quinine treatment received. These findings indicate the potential of certain cognitive biases, such as side bias, to influence learning outcomes, and illustrate that their effects are not entirely consistent.

In particular, the neurotransmitter dopamine has been identified as having a significant impact on learning. In previous studies, *D. sechellia* outperformed *D. simulans* in negative association conditioning trials. This is surprising, given that *D. sechellia* are supposed to be dopamine deficient, and that dopamine plays a big role in learning, particularly in forming negative associations. Previous studies have suggested that noni has

neurotoxic effects (due to the presence of octanoic and hexanoic acids), and that *D. sechellia* may have higher levels of dopamine to counteract the neurotoxic effects of noni (Lavista-Llanos et al. 2014). If this is the case, perhaps *D. sechellia* need more dopamine in the brain to survive on noni - and the observed higher learning ability is just a by-product of specialization on noni specifically, and not indicative of the fitness benefits of being able to learn in their environment.

In regard to our comparative study with *D. sechellia* and *D. simulans*, these findings do not support our hypothesis that species evolving in the presence of dietary l-DOPA would exhibit decreased learning performance when not reared with dietary sources of l-DOPA. Instead, we found the opposite: with the species that has been evolving in the presence of dietary l-DOPA (*D. sechellia*) outperforming the species that has not been evolving in the presence of dietary sources of l-DOPA (*D. simulans*) in associative learning trials. However, these findings do partially support our hypothesis that individuals reared in the presence of dietary l-DOPA would exhibit higher learning performance, though this was only the case for the generalist (*D. simulans*). Additionally, these findings demonstrate the potential of cognitive biases, such as side bias, to influence learning outcomes.

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