Influences of environmental variation on the bold-shy continuum

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Abstract

Boldness is an axis of personality defined by the propensity to take risks. As a personality, behaviors associated with boldness are highly consistent within individuals across different time scales. Because there is considerable variation within and between populations, behavioral ecologists have long been interested in boldness behaviors and their relationship with fitness. One of the most noticeable examples of boldness variation between populations is between wild and captive-bred stocks. Captive or domesticated individuals tend to show reduced fear and take more risks than wild individuals, and this pattern has been observed repeatedly across a variety of animal species.

My work in this dissertation is focused around the impact that environmental variables have on the evolution of boldness behaviors. Some variables, such as predation pressure, affect the fitness landscape and evolutionary trajectory of behaviors along the bold-shy continuum. Using an agent-based simulation, I show that differences in predation pressure is a likely cause of of the patterns we observe between wild and domestic populations.

Other variables, such as complexity in the environment, can have a plastic effect on the development and expression of boldness behaviors. I test this hypothesis with a zebrafish model and show that early experience to complexity can affect boldness behaviors later in life, but the effect is most pronounced in males over females.

In addition, boldness behaviors display a high degree of sexual dimorphism in zebrafish. I test whether this might affect pharmaceutical responses to the anxiolytic substances nicotine and fluoxetine. While I find no sex-specific effects of each drug, I attribute this to observing no sex differences in the control, suggesting that sex differences in our sample might not have been present.

Because boldness is associated with domestication and adaptation to captivity, these behaviors are of interest to conservation and wildlife management efforts. With captive breeding and rearing programs used to mitigate population loss, care must be taken to ensure that we do not produce individuals that are maladapted for reintroduction to the natural environment.

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CHAPTER 1

Introduction

Behavioral plasticity, the ability to change one's actions in response to a variety of stimuli, is thought to be advantageous. The ability to alter one's behavioral responses should maximize survival in a changing environment. But despite this prediction, behavioral traits appear that are constrained, causing consistent individual variation across a variety of contexts (Wilson, 1998). In humans, these traits are often called personalities. For example, some people are more likely to engage in risky activity than others (Zuckerman and Kuhlman, 2000). Similar axes of personality have also been observed in animal populations (Sih *et al.*, 2004a,b; Wilson, 1998), and evidence suggests these axes have an impact on fitness (Dingemanse, 2005; Smith and Blumstein, 2008).

This dissertation focuses on behaviors associated with the bold-shy continuum, also referred to as boldness. Behaviors along the bold-shy continuum are associated with risk-taking (Wilson *et al.*, 1993), and the associated costs and benefits often impact fitness. Some of the measures and tests used to quantify boldness include the responses to a novel object, a predator, a human observer, exploratory response in a novel environment, place preference, and feeding latency (Toms *et al.*, 2010). All of these measures assume that boldness is related to anxiety and stress where shy individuals show a stronger anxiety response to a stimulus and are less likely to engage in risky behavior than bold individuals. Evidence that boldness is correlated with stress response has been seen in salmonids (Overli *et al.*, 2005) and zebrafish (Oswald *et al.*, 2013a), but the evidence also highlights the complex nature of the relationship between physiology and behavior. It is not necessarily the case that all measures of boldness are in fact measuring the same anxiety or stress response. One study using pumpkinseed sunfish (*Lepomis gibbosus*) found that bold-shy behaviors associated with a novel object did not correlate with bold-shy behaviors associated with a novel food source suggesting that boldness may be context-specific (Coleman and Wilson, 1998). However within a population, individuals may show consistent variation across

a variety of contexts (Dall *et al.*, 2004). In zebrafish, consistent responses across contexts have been observed. Oswald (2010) found that bold individuals retained their relative positions on the bold-shy continuum regardless of social pairings and exposure to a threatening stimulus. In stickleback, bold-shy behaviors correlated across contexts of feeding latency after a simulated aerial predator, shoaling tendency, resource competition and shoal position preference where bold individuals resumed feeding quickly, preferred not to shoal with other individuals, out-competed shy individuals for food, and preferred front positions when they did join a shoal (Ward *et al.*, 2004).

Boldness behaviors are observed to evolve during domestication or adaptation to captivity with captive populations becoming more bold than their wild ancestors. Vincent (1960) noticed that hatchery strains of trout spend more time in the upper water column and show a reduced fear of humans than individuals from a wild population, even when hatched and reared in the same hatchery environment. This trend toward more more bold and tame behavioral patterns has been observed consistently in different species including salmon (Fleming *et al.*, 1994), catfish (Simmons *et al.*, 2006), zebrafish (Drew *et al.*, 2012), and foxes (Kenttamies, 2002; Trut *et al.*, 2004).

Interest in understanding the evolutionary effects of domestication events has been sparked by conservation concerns with releasing captive-bred individuals into a wild environment with native populations. The behavioral characteristics of a domesticated species make it maladapted for survival in the wild. This is because the offspring have been observed to exhibit similar behavior patterns of the parents regardless of rearing environment (Vincent, 1960; Fleming *et al.*, 2000). This suggests that behaviors associated with domestication have a heritable genetic component. Selection experiments provide more evidence that tameness is heritable. Selection experiments in rats (Albert *et al.*, 2009) and foxes (Kenttamies, 2002; Trut *et al.*, 2004) have produced populations that diverge in their affinity and aggressiveness toward humans. Many of the behaviors measured in the studies above are often associated with the bold-shy continuum.

The mechanism by which boldness behaviors change during the process of domestication remains up for debate. The repeated changes observed could simply be

a result of artificial selection in which humans either intentionally or unintentionally select for boldness because it is desirable or correlated with other desirable traits. This is certainly true of agricultural stock and animals bred for the pet industry. But changes in boldness may also result from a change in the selective landscape simply due to a change in environment. For example, captive environments tend remove any selective pressure from predation, thus eliminating much of the cost to being bold while the benefits remain (Price, 1999). In addition to changes in selective pressures across environments, changes in environmental variables can also induce plastic effects. For example, embryos exposed to maternal stress (cortisol) develop to be more shy (Eriksen *et al.*, 2011). Adding sources of enrichment to a captive environment tends to reduce stress, leading to more bold-like behaviors(Benaroya-Milshtein *et al.*, 2004; Näslund *et al.*, 2013).

This dissertation contributes work that explores the relationship of environmental variation on the plastic and evolutionary effects of behaviors on the bold-shy continuum. With domestication as a primary context of this work, I focus on three sources of environmental variation between wild and domestic environments: predators, resource abundance, and structural complexity.

Chapter 2 uses a simulation approach to test predictions around the role of predators and resource density on the fitness landscape and evolution of risk-taking behaviors. The simulation is influenced by our work with zebrafish in which bold individuals spend more time near the surface of the water than shy individuals. Using an artificial neural network, an agent moves about a bounded environment collecting resources that translate into gametes. In this study, I predict that spatially heterogeneous risk drives animals to spend less time near the source of risk. I also predict that reducing resources might alter the way in which predators select against risk-taking. Finally, I look at the mechanics of risk-taking and ask whether selection on place preference is acting on choice to target specific resource pieces or an inherent bias in movement patterns.

Chapter 3 tests the hypothesis that structural complexity in the rearing environment alters boldness in adults. Using zebrafish (*Danio rerio* as a study system, I reared fry in tanks containing plastic aquarium plants or no plants and collect behavior on

adults in an open field drop test as well as a home tank scan-sampling assay (Robison *et al*., 2012). Complexity tends to reduce anxiety and neurotic behavior in captive animals, and I predict that early exposure to complexity will produce individuals that are more bold throughout life.

Chapter 4 tests the hypothesis that sex differences in boldness behavior influence plastic responses to anxiolytic substances. Again, I use the zebrafish as a study system, dosing them with fluoxetine, nicotine, or a water control before collecting behavior data with a novel tank drop test. Previous studies in the Robison lab have identified sex differences in boldness in zebrafish where females are more bold than males (Oswald *et al*., 2013a). Therefore I predict that the behavioral effect of exposure to anxiolytic substances might be stronger in males than females given that females are inherently showing a reduced stress response.

CHAPTER 2

Predation, but not resource abundance, affects evolution of place preference behavior *in silico*

2.1 SUMMARY

Boldness is an axis of personality associated with risk-taking that shows consistent evolutionary shifts during adaptation to captivity in which wild populations are more risk-averse compared with captive populations. One explanation for this phenomenon is that there is an underlying fitness trade-off in which bold individuals experience increased mortality from predators, but have a reproductive advantage from collecting more resources if they survive their risky behavior. However in some species, the relationship between boldness and predation risk runs counter to our expectations. In this paper, we develop an agent based simulation to run *in silico* experiments to test three main hypotheses: (1) that predators drive the evolution of risk-averse behavior in wild populations, (2) that resource abundance alters the underlying fitness trade-off leading to the evolution risk-taking with predation, and (3) that when predation is removed, bold behavior is selectively favored. Predation pressure is spatially heterogeneous along the y axis such that predation is stronger at the top and non-existent at the bottom. A simple neural network controls movement as well as choice of resources to target. We find that predation selects for place preference away from the risky locations, and that resource availability does not alter this pattern. When predation is removed, populations evolve behavior that is indistinguishable from a population that never experienced predation risk. Our results suggest that predation is a major source of evolutionary pressure, and that behavioral adaptation to captivity could result simply from the removal of predators in a captive environment.

2.2 INTRODUCTION

Boldness is an axis of animal personality that encompasses behaviors associated with risk-taking and risk-aversion. These behaviors are often measured using location preferences (ie, top vs bottom of water column with some fish), tendencies to explore a novel environment, reactions to predators, and reactions to humans. Individuals are considered bold if they spend more time in open areas, explore new environments, inspect predators, or associate with humans, and these behaviors may be measured either as a latency to engage in a particular activity, or a proportion of observed time engaged in such activities (Toms *et al.*, 2010).

Risk-taking behaviors evolve during domestication or adaptation to captivity. Many behavioral changes between wild and domestic populations have been repeatedly observed in a variety of taxa. Vincent (1960) noticed that hatchery strains of trout spend more time in the upper water column and show a reduced fear of humans than individuals from a wild population, even when hatched and reared in the same hatchery environment. This trend toward more tame behavioral patterns has been consistently documented in different species including salmon (Fleming *et al.*, 1994), catfish (Simmons *et al.*, 2006), zebrafish (Drew *et al.*, 2012), and foxes (Kenttamies, 2002; Trut *et al.*, 2004). These trends lead to the hypothesis that pressures in nature, such as predation, are selecting against the more risk-taking bold phenotypes (Bell and Sih, 2007)

In captivity, any selective pressures due to predators are removed. Therefore, there are three scenarios that might explain the rise of boldness in captivity: (1) artificial selection, where boldness is directly or indirectly selected for by humans, (2) natural selection, where boldness is naturally the more fit phenotype in the absence of human intervention, and (3) the relaxation of selection, where neither bold nor shy phenotypes have any selective advantage and drift and random mating allow the bold phenotype to increase in frequency in the population (Price, 1999). In many situations, it is likely that bold behavior types are artificially selected. For example, artificial selection has produced new foxes that are tame to humans (Kenttamies, 2002; Trut *et al.*, 2004). In aquaculture, high growth rates are desirable for producing food

stocks, and if boldness is correlated with growth rates (Stamps, 2007; Ward *et al.*, 2004), then culture practices inadvertently select for bold individuals.

Defining boldness in the context of risk-taking assumes that there are fitness trade-offs associated with these behaviors. In a natural system, bold individuals might benefit by collecting more resources to put toward growth and reproduction (Stamps, 2007), or perhaps these behaviors simply afford more mating opportunities (Ballew *et al.*, 2017), while risking mortality due to predation. Shy individuals might enjoy higher survival but at a cost of reproductive potential. Selection should favor behaviors that maximize fitness by balancing predation risk with resource consumption. For boldness to be advantageous in the face of predation, we should expect that other environmental variables alter the risks or benefits associated with fitness.

In the absence of any cost to boldness (ie, in the absence of predators), we should predict that bold behaviors are selectively favored. In the presence of predation cost, then we should predict that a less bold behavior type is favored. This pattern is supported by observations comparing wild and captive populations. But among wild populations, the patterns aren't as clear. Populations of the Trinidad guppy (*Poecilia reticulata*) vary in predation pressure depending on their locations in streams (Reznick *et al.*, 2001). Populations from high predator environments are more bold than populations from low predator environments (Harris *et al.*, 2010). The same trend has been observed in *Brachyraphis episcopi* populations in Panama (Brown *et al.*, 2005), as well as stickleback from ponds, but not rivers (Brydges *et al.*, 2008). These examples contradict the initial predictions and suggest that predation may not be the only factor influencing selection pressures on boldness behaviors.

There are many hypotheses as to what other factors may interact with predators to influence selection along the bold-shy continuum. For example, the amount of resources available may influence the evolutionary trajectory of boldness. In environments where resources are abundant, individuals can make fewer risky actions in order to collect benefits. On the other hand, where resources are scarce, individuals may need to make more risky actions just to keep up with the needs for maintaining growth and survival.

In this paper, we investigate the nature of this risk-reward trade-off regarding the evolution of boldness. We test whether predators should select for a less bold behavioral strategy and whether resource density might alter this pattern. Second, we ask whether the mechanism underlying boldness is one of choice between target destinations or an inherent bias in movement patterns. Third, we test whether boldness is favored when predators are removed, possibly explaining rapid changes in behavior during domestication. We utilize an agent-based simulation with an artificial neural network to model spatial use across a bounded habitat. The simulation is inspired by recent work in the Robison lab analyzing zebrafish swimming and feeding behaviors that diverge among wild and laboratory strains, most notably in depth preference. While zebrafish have a relatively short generation time for a vertebrate model organism, they are still a poor choice for experimental evolution within a short time frame. Simulations let us speed up this process while also giving us the ability to manipulate environmental conditions that would otherwise be unfeasible or unethical with laboratory experiments.

2.3 METHODS

2.3.1 *The simulation*

Our simulation is inspired by the work we have completed utilizing zebrafish swimming behavior in an open field. When stressed, zebrafish tend to retreat to the bottom of their tanks, and shy individuals spend more time near the bottom than bold individuals (Oswald *et al.*, 2013b; Singer *et al.*, 2016). In this program, we interpret the y-axis as the vertical "depth" of the arena, with zero representing the "top" and 500 representing the "bottom." Agents move about the defined two-dimensional area collecting resource particles that we call "food". The movements are defined by a simple neural network in which the inputs are the x-distance and y-distance to the nearest piece of food, a random component to movement used to encourage exploration, and a bias node with a constant input of 1. The inputs are passed through a series of weights which act as the genes that vary between individuals and evolve

in the population over time (Figure 2.1). Inputs are multiplied by their weights and then summed to produce the output values move x distance and move y distance.

The agents are diploid sexually reproducing organisms, and the gene weights are comprised of two "alleles" each on their own "chromosomes." Thus for this simulation, all genes assort randomly and independently when forming haploid "gametes." Each gene weight is simply the sum of the value of the alleles. For example, if an individual at has alleles valuing 2 and 3 at gene 0, the gene value of that individual at that locus would be 5. The food that the agents collect translate into fitness. For each food particle, the agent produces two gametes that are added to the gene pool. After all individuals have run gametes are randomly selected and combined to produce individuals in the next generation.

To make the simulation more realistic, limits are placed on the distance an agent can move in a single time step. For these experiments, agents cannot move more than 8 in the x and y directions. The agents are also given a sensing radius of 125, unable to detect any objects outside of the sensing field. When agents consume all food in a particular area of the environment, they will move with a correlated random walk to explore until more food particles appear within their sensing range.

2.3.2 *Experiment 1: Effects of predation and resource abundance on the evolution of risk-taking.*

Populations are kept at a size of 1000 individuals, each of which are run for a maximum of 3000 time steps. Each population then evolves independently for 1000 generations. Initial gene weights at each locus are drawn randomly from a standard normal distribution with a mean of zero and standard deviation of one. Genes are also allowed to mutate with a probability of 1/(2*genes), or the expectation of 1 mutation per individual. There are 18 genes total, ten of which do not influence the outputs and are therefore considered neutral for the expected patterns of genetic drift.

Individuals start at the center of an arena measuring 1000 units on the x-axis and 500 units on the y-axis. Food is populated according to a random normal distribution with either 100 pieces for the high density treatment or 20 pieces for the low density treatment.

We add predation by varying the probability of survival with respect to position along the y-axis. This probability was defined with a logistic curve

$$p(d) = \frac{I}{1 + e^{r(y-c)}} + a \tag{2.1}$$

where $p(d)$ is the probability of dying before the last time step if the agent were to spend every time step at a given y position, I represents the intensity of predation, r represents the rate of transition, c is the center of the logistic curve, and a is an additive value used to shift the function up or down as needed. The probability of death at each time step is then calculated using the function

$$p(d_t) = 1 - (1 - p(d))^{(1/T)} \tag{2.2}$$

where T is the maximum number of time steps that each individual will be observed. We chose this predation function due to its versatility for creating different shapes of predation curves. We varied predation pressure by setting values of I to 1.0, 0.5, and 0.0 for high, low, and no predation pressure. Ten replicate populations are run at every combination of predation level and resource density and predation curve. Since it is unclear what an ecologically relevant predation curve might look like, we replicated this experiment under a variety of curve shapes as seen in figure 2.2. Because risk was only introduced along the y-axis, we defined boldness as an individual's average position along the y-axis, with bolder behavior having an average y position closer to zero.

2.3.3 *Experiment 2. Testing the mechanistic basis of location preference*

We wanted to find out whether adding an element of choice to the model would change behavior patterns as well as the genes targeted for selection. Therefore we added the ability for the agent to sense two pieces of food and added a neural network preceding the movement network in order to define choice. In the previous model, agents detected the nearest piece of food and moved toward it. In this model, the agent can choose between the two nearest pieces of food and decide which is the more

optimal to move toward based on attributes associated with each food piece. The new network consists of two weights, one evaluating the x-distance with directionality (+ or -) to each food, and one evaluating the y-distance with directionality. The network then produces choice values, with either the maximum or minimum value being set to the most desirable. We expect that that both choice methods should produce the same results with one evolving positive weights and the other evolving negative weights to make the actual optimal food piece match the choice criteria.

Using this framework, we predicted that in the predation environments, evolution of place preference would be driven by selection on the choice genes rather than the movement bias. Since the shape of the predation curve did not affect behavioral outcome, this experiment is performed with a basic logistic curve with values of $r = 0.02, c = 250, a = 0$, and i is set to either $1.0, 0.5$, or 0.0 to represent predation levels that are high, low, and absent.

2.3.4 *Experiment 3. Testing for place preference selection in the absence of predators*

In the first two experiments, naive populations are allowed to evolve under high, low, and no predation pressures. But domestication often involves moving wild populations that into an environment where predation threat is reduced or eliminated altogether. For this experiment, we evolve 10 populations under high predation and no predation each for 1000 generations. Then the predation is removed and all populations experience another 1000 generations. If there is selection for place preference in the no predation environment, we expect to see a rapid shift in both behavior and gene weight values to converge with those populations that originally evolved without predation. On the other hand, If there is no selection, then behavioral patterns and gene weights will shift more slowly as within-population variation increases due to mutation and genetic drift. Since the choice methods tested in experiment 2 produced identical results, this experiment uses only choice based on the maximum choice value.

2.3.5 *Analysis*

Data from each individual is recorded and summarized to means whenever possible. This data includes the averages for x-position, y-position, speed, as well as the number of food items collected (fitness) and the number of time steps each individual survived. The genome weight values for each individual are also recorded. The individual summaries are then summarized to produce population level means at each generation for each variable. For the behavior data, these population level summaries from the last generation are then used to evaluate divergence across treatment groups. This method is rationalized in that many empirical studies only have access to data in the current generation, and that we must use contemporary data to make inferences about past processes that lead to the current patterns. Means and standard errors for each treatment group are generated from a two way ANOVA. For the gene data, we visually analyze the population mean values across time as the data are not always normally distributed. A multiple linear regression is used to to associate changes in y-axis behavior (boldness) with the genes.

2.4 RESULTS

2.4.1 *Experiment 1*

ANALYSIS OF BEHAVIORAL TRAITS — Similar patterns of y-axis use are observed across the five different predation curves (Figure 2.2). When predation is absent, agents utilize the entire y-axis space leading to a mean y-axis position of 250, or right in the middle. As predation pressure increases toward $y = 0$, space use along the y-axis gets biased away from the predation shifting the means away from the center and closer to 500. The shifts match the predation pressure, with high predation pressure ($I = 1$) displacing the mean y position the furthest, and low predation pressure ($I = 0.5$) displacing the mean an intermediate amount. Both resource density treatments follow the same pattern, though predator selection on y-axis behavior is slightly stronger when resource density is low.

Position along the x-axis has no fitness consequences with respect to the predation model and therefore acts as a check that the patterns observed with y-axis behaviors are due to selection pressures introduced by the predation treatments. Figure 2.3 shows that behaviors along the x-axis are not affected by any of the treatment groups, with all treatments averaging in the middle. Figure 2.4 shows that average speeds are also not affected with all groups evolving to move about at near the maximum speed. Figure 2.5 shows that life spans are reduced as predation increases, and the amount by which this occurs does vary with the shape of the predation curve. Predation has the strongest effect on lifespan when the curve approximates a discrete threshold model as depicted by predation curve 2. This model also produces the most pronounced interaction with resource density with high predation environments having a greater effect on lifespan in high density environments than in low density environments.

Analysis of the gene weights — There are eight gene weights in the model for this experiment, all of which are in the movement network depicted in Figure 2.1. All of the genes appear to experience selection as shown by the consistent changes depicted in Figure 2.6. Genes 0 and 5, which represent the influence of the inputs of x and y distance to food on the x and y movement respectively, both experience strong shifts in the positive direction. Genes 1 and 4 are the diagonal genes, representing the influence of the x and y distance to food on the movement along the opposite axis. These genes experience disruptive selection where values farther away from zero appear to be more beneficial, but this can occur in both the positive and negative directions. At the population level, these genes evolve together with a tight negative correlation (Figure 2.7). Genes 2 and 6 represent the effects of randomness on movement the x and y directions respectively. These genes also suggest that non-zero values are more desirable. The random movements keep the agent moving and exploring when no food is present within the sensing range. Genes 3 and 7 are associated with the bias node and movement in the x and y directions respectively. Gene3 remains at an average of zero with populations diverging randomly in a pattern consistent stabilizing selection and drift. Gene 7 shows selection in the positive direction in the environments experiencing predation

while remaining around a value of zero in the no predation environments. Of all of the genes, gene 7 is the only one to associate with evolution of behavior along the y-axis (Table reftab:generegression and Figure 2.8).

2.4.2 *Experiment 2*

Because experiment 1 shows no major deviations in behavioral and genetic patterns between different shapes of predation curves, we used the parameters of predation curve 1 for the remaining experiments in this paper. In this experiment, an additional network layer was added to allow the agent to choose among two pieces of food. When the agent is programmed to select the food with the highest choice output value, the gene weights on x and y direction of the food (genes 16 and 17 respectively) evolve in the negative direction (Figure 2.9). When the choice is changed to the minimum value, the weights of the choice genes evolve in the positive direction (Figure 2.10). Behavior patterns are similar to those observed in the first experiment (Figure 2.11) and do not appear to differ among the two choice methods. The patterns of evolution in the gene weights of the movement network are also similar, with one notable exception (Figures 2.9 and 2.10). The addition of choice appears to alleviate selection pressures on the diagonal genes, genes 1 and 4. Instead of a pattern of disruptive selection away from a value of zero, these genes now display patterns more in line with genetic drift with an overall mean around zero.

2.4.3 *Experiment 3*

In this experiment, populations evolved under the same conditions as in experiment 2, and then evolved for an additional 1000 generations with all predation removed. Figure 2.12 shows the results of the evolution of behavior on the y-axis. When predation is removed, all treatments evolve toward a mean y position of 250, which is in the middle of the environment. While the ANOVA on the last generation returns a significant predation by food interaction ($F_{2,54} = 3.659, p = 0.0323$), pairwise t-tests do not distinguish among the treatment groups. The values of gene 7 also evolve

back to a mean value of zero (figure 2.13) suggesting that in the absence of predation, selection favors equal use of the top and bottom.

2.5 DISCUSSION

In nature, populations experiencing different environments evolve and develop different levels of boldness. We predict that environments with higher predation pressure select for less risk-taking in order to maximize survival probabilities, leading to an overall increase in lifetime fitness. Our simulation, though simple, confirms this prediction when predation risk is spatially heterogeneous. A higher risk of death near the "top" of our environment leads agents spending more time away from that source of risk.

However, natural populations aren't quite so simple, and sometimes we see patterns that contradict our expectations. Therefore we introduced resource abundance as a possible explanation that might alter the selective landscapes and favor bolder behavior in the presence of higher predation pressure. We are unable to support this hypothesis with this simulation model. While there is some discrepancy in the y axis behavior between high and low food environments, these differences are small when compared with the effect of the predator. We were unable to produce any scenario where high predator populations evolved average positioning that are equal to or closer to the "top" than low predation populations.

In the guppy system, per-capita resources have been determined to be more abundant in the high predation environment than in the low predation environment (Reznick *et al.*, 2001). This is because juveniles tend to grow faster in high predation environments than in low predation environments, though they also mature at a smaller body size. The pace of life syndrome hypothesis equates fast growth with more bold behavior (Biro and Stamps, 2008; White *et al.*, 2016), and fast growth rates are indicative of high resource abundance citepReznick2001. Our model does not yet include such life history traits, which could make a suitable addition for testing this hypothesis *in silico*. Whether the same explanations apply to the the stickleback system is unknown. Brydges *et al.* (2008) does not include any ecological descriptions

of the pond and river habitats with regard to resource abundance and productivity, nor are any life history traits such as growth rates discussed.

While predators tend to select for a shy behavior type, our results suggest that a more bold behavior type is advantageous in the absence of predation. The trend for the no-predator environments was to remain, on average, in the middle of the tank. What this really means is that the agents were utilizing the top half and bottom half evenly. The results of the third experiment suggest that this is an evolutionarily advantageous solution rather than a symptom of agents starting in the middle with mean location preferences diverging due to random drift. Not only did the mean y position return to the middle when the predation threat was removed, but it did so relatively quickly. These results aren't entirely unsurprising. Since we defined fitness as a linear relationship to the amount of food collected, agents that collect more food will have a better probability of contributing alleles to the next generation, and the best way to collect more food is to utilize as much of the habitat as possible. Thus while we didn't program a fitness trade-off directly, we do see that a trade-off exists resulting from the uniform distribution of food and the non-uniform distribution of predation risk. A recent study by Ballew *et al*. (2017) confirms that a trade-off may exist in natural populations of largemouth bass. They find a strong negative association of boldness with juvenile survival. They also present that bolder males had higher reproductive success.

The speed at which evolution occurs differs between the first 1000 generations and the second 1000. Initially, all populations are naive of all environments. Not only are they terrible at responding to the environmental conditions presented, but they are terribly inefficient at navigating to food. Thus in addition to evolving to the predator, the populations are also evolving to efficiently move about their environment and collect resources, evidenced by the evolution of genes 0 and 5. Additionally, it takes time for genetic variation from mutation to build. By the 1000th generation, the organisms are not only good at collecting food, but there is more variation in the genes for selection to act upon.

In the context of domestication, the results of the third experiment suggest that the lack of predation in the captive environment can, on its own, begin the evolution

toward a bold behavior type. Thus, the evolution of boldness in captive-bred animals isn't solely due to artificial selection. For example, based on the results of Ballew *et al*. (2017), we should predict that boldness would be selectively favored for largemouth bass in captivity as the mortality on bold juveniles is reduced. In reality, the effects of artificial and natural selection are not necessarily independent. Bold individuals, being less afraid and less reactive to stimuli, are the easiest individuals to catch in a net or a trap (Biro and Adriaenssens, 2013). The result is that biases for boldness are introduced even when sampling individuals from the wild, and then continued when sampling individuals as breeders for subsequent generations. Thus it's likely that in most captive breeding programs, artificial selection and natural selection work complimentary to one another to evolve bolder individuals. This is worth noting as captive breeding and rearing programs are also used to mitigate population losses in critically endangered species. Breeding behaviorally and genetically maladapted individuals for return to the wild could actually hamper conservation efforts (Araki *et al*., 2007).

Finally, we investigate what boldness is from a mechanistic perspective. When we first started programming the simulation, we approached the problem with the understanding that boldness represents a series of decisions that an animal makes to move about its environment. However the first versions of the program did not really incorporate any sort of decision making. Instead, agents moved about the environment deterministically according to the layout of the food and the values of the gene weights in the movement network. The agent only detects one piece of food at a time and moves toward it. As a result, it is unsurprising that in the first experiment, changes in mean position along the y axis is determined solely by the weight on the bias node. In the second experiment, we set out to add decision making by letting the agent choose between two pieces of food, using attributes of the food be the criteria for decision making. We predicted that selection pressure induced by the predation would shift from the bias gene to the genes in the decision making network. Instead we observe that neither predation nor resource ability affect the choice criteria, and evolution remains on the bias. The implications here are that position along the bold-shy continuum might represent an inherent bias in movement toward a particular area

in the habitat that might be deemed as safe. It's not uncommon for animals to seek out edges (thigmotaxis), for which the bottom of an aquarium tank has an analogous effect in fish (Levin *et al.*, 2007). We wonder whether this association is inherent as an unconscious and instinctual movement, or whether it represents a conscious choice as animals evaluate their surroundings. While our results suggest the former, we must interpret them with caution. Our simulation is incredibly simple and does not represent the complexity and nuances of the brain's neural circuitry. Even a relatively simple organism such as *Caenorhabditis elegans* has a more complex relationship with its environment than our simulation is able to account for at this time. It's likely that for choice to be a meaningful addition, we will have to include more attributes to weigh in on the choice, both extrinsically from the environment, and intrinsically about the state of the agent.

Boldness is an incredibly complex behavioral trait that interacts with many facets of the environment. Here we present a set of *in silico* experiments to try and understand how two environmental variables, predation and resource abundance, might interact to affect behavioral evolution along the bold-shy continuum. Simulation experiments such as these can be useful as they allow us to manipulate subjects without ethical considerations. They also let us work with larger populations and more generations than a laboratory or field study might permit. Still, the nature of simulations means that we must be careful when interpreting the results. The goal of a simulation is to determine the simplest set of parameters that might describe patterns observed in empirical studies. In this paper, we can conclude that predators should have an effect on selecting for risk-averse behavior. However we define boldness in the context of location preference. Boldness can also be defined with behaviors associated with latency, such as latency to move after a stressful event (freezing behavior), latency to emerge from a shelter, or latency to approach a novel object (Toms *et al.*, 2010). Our simulation currently won't produce these responses without a much more complex neural network and a stimulus that can be sensed by the agent. Therefore we are missing a lot of valuable insight into the multivariate nature of boldness and how each facet impacts the total selective landscape. Sociality may also affect risk. When a group, individuals reduce their probability of being preyed upon, while

employing behavioral tactics that further reduce risk. However more individuals in an environment will also increase competition for resources. We hope to add more layers to the simulation in the future in order to test hypotheses associated with such environmental conditions that are also known to influence boldness and might impact the selective landscape.

2.6 TABLES

TABLE 2.1: Results of the multiple linear regression to determine gene association with average y axis position.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	15.6613	0.2008	77.99	0.0000
Gene0	-0.0030	0.0073	-0.40	0.6880
Gene1	0.0076	0.0109	0.70	0.4892
Gene2	-0.0010	0.0014	-0.74	0.4637
Gene3	-0.0120	0.0194	-0.62	0.5401
Gene4	0.0112	0.0084	1.33	0.1894
Gene5	0.0067	0.0065	1.02	0.3102
Gene6	0.0017	0.0020	0.83	0.4124
Gene7	0.3017	0.0088	34.26	**0.0000**

2.7 FIGURES

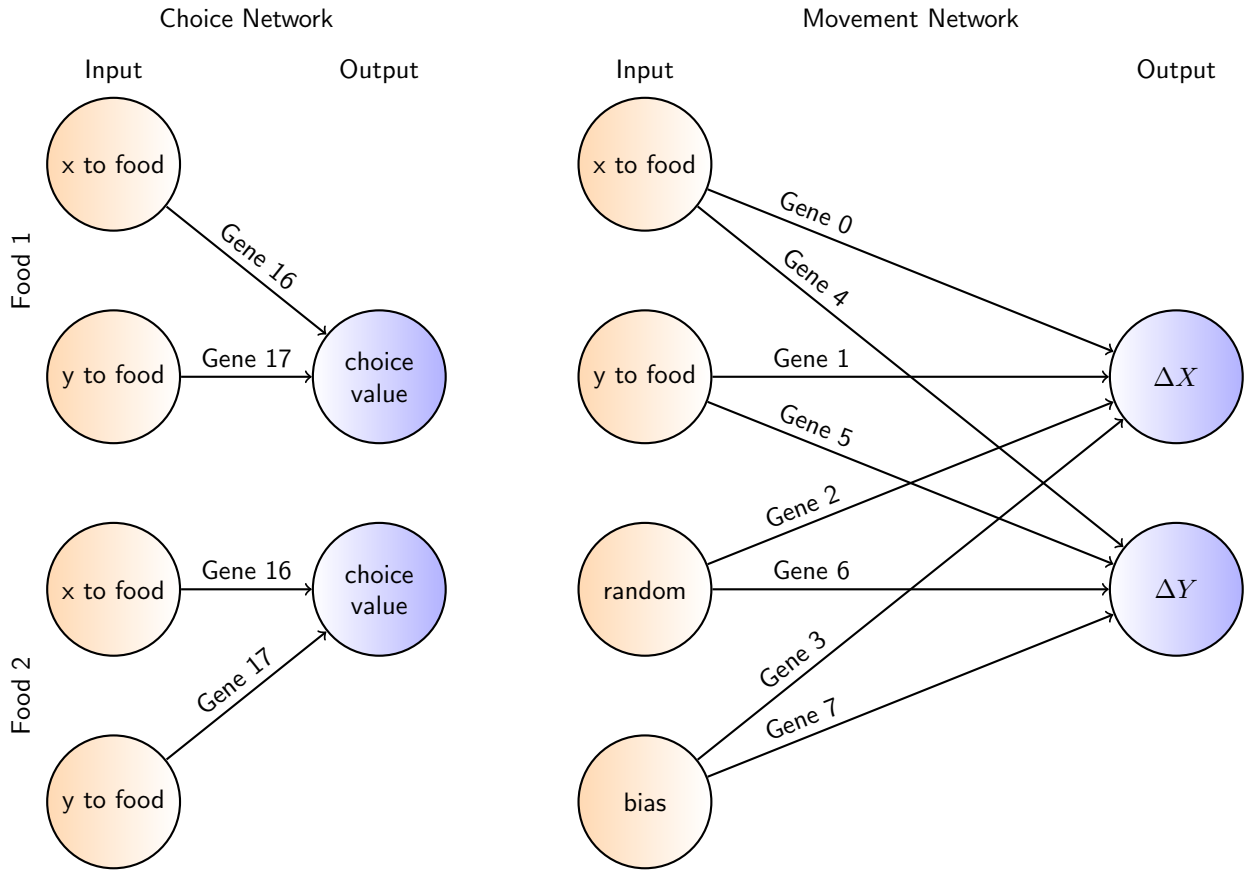

FIGURE 2.1: A diagram of the neural networks that evolve during the simulation. The network on the right controls the agent's movement around the environment. The first two inputs are the x and y distance (with positive and negative direction) to a food piece. The random input adds a bit of random movement, drawn from a standard normal distribution, and is activated when no food is detected in the sensing range to keep the agent moving and exploring. The bias is a constant input of 1 and is a standard component of artificial neural networks. These output to control the direction and distance of movement along the x and y axes. The diagram on the left controls choice when the agent can detect more than one piece of food at a time. The inputs include the x and y distance to a food piece that output to a choice value. This diagram shows the choice network being acted upon two pieces of food. Attributes of the food with the highest or lowest choice value are then used as inputs of the movement network.

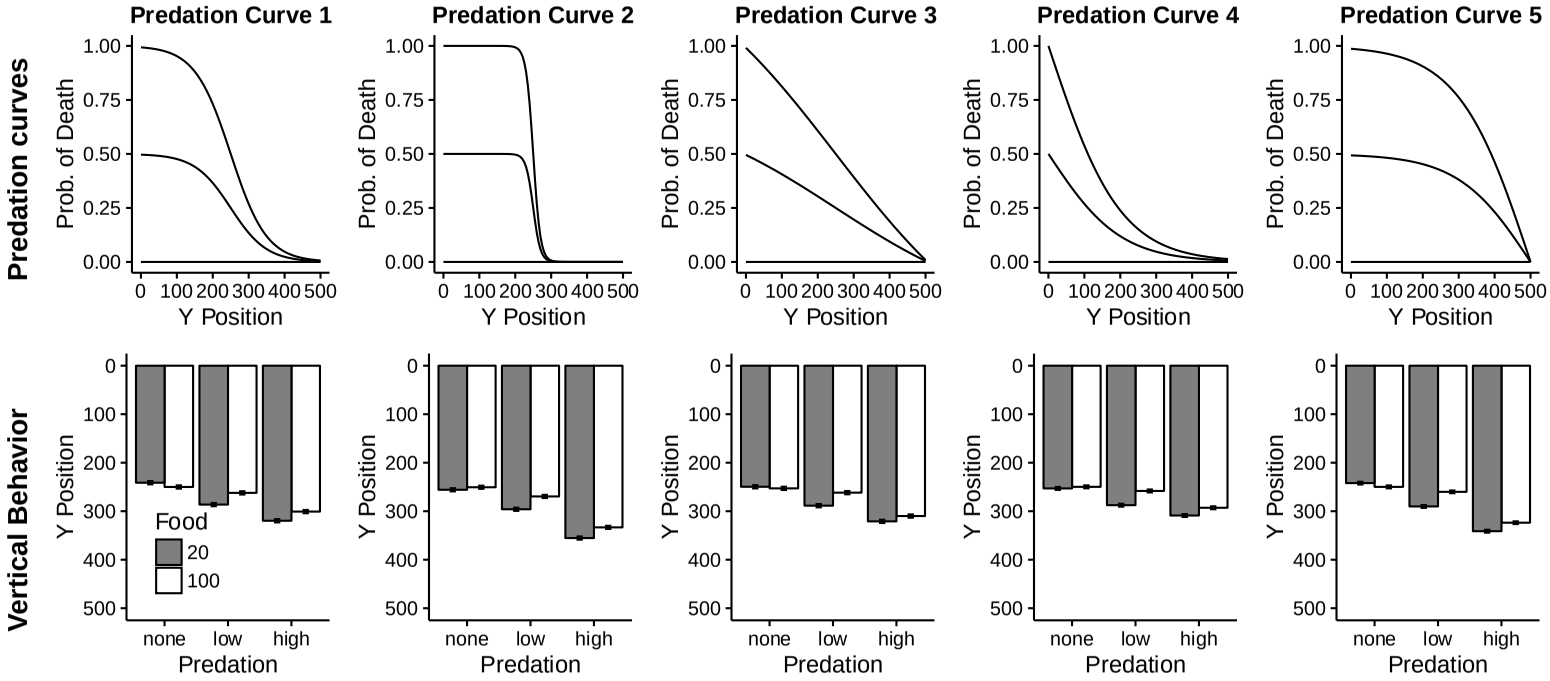

FIGURE 2.2: Five different shapes of the predation curve along the y-axis and their effects on average position along the y-axis. The curves approximate a sigmoid, threshold, linear, and two variations on an exponential decay, and will be referenced as predation curves 1-5 respectively. A y-value of 0 represents the "top" of the simulation environment while a value of 500 represents the "bottom". The behavior plots show the overall means and standard errors of each treatment group within each predation curve type in the last generation.

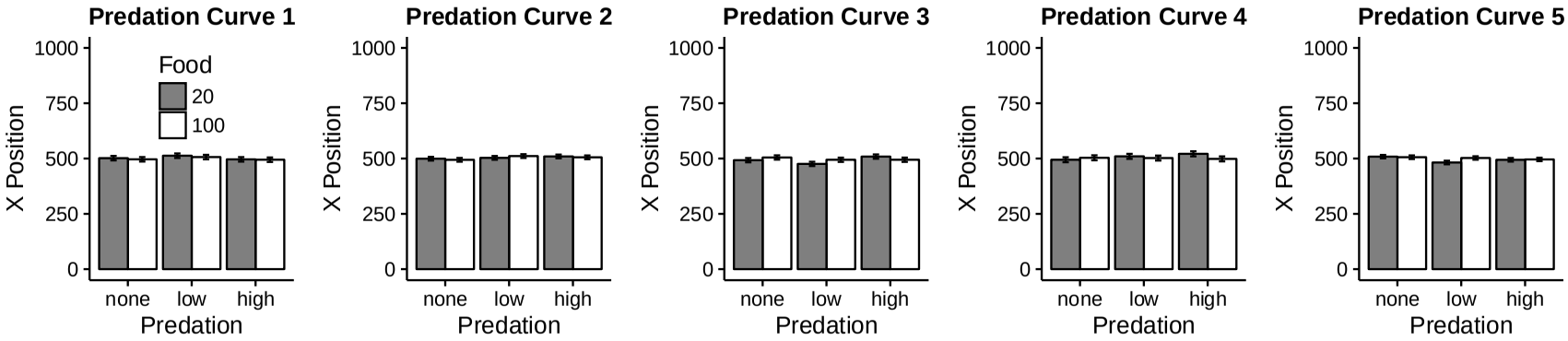

FIGURE 2.3: Mean x position and standard errors for all treatment groups and predation curve types. All groups stay in the middle on average since there is no selection built in the model to shift place preference to the right or left. These figures act as a check to verify that the predation curves along the y-axis are behaving as expected.

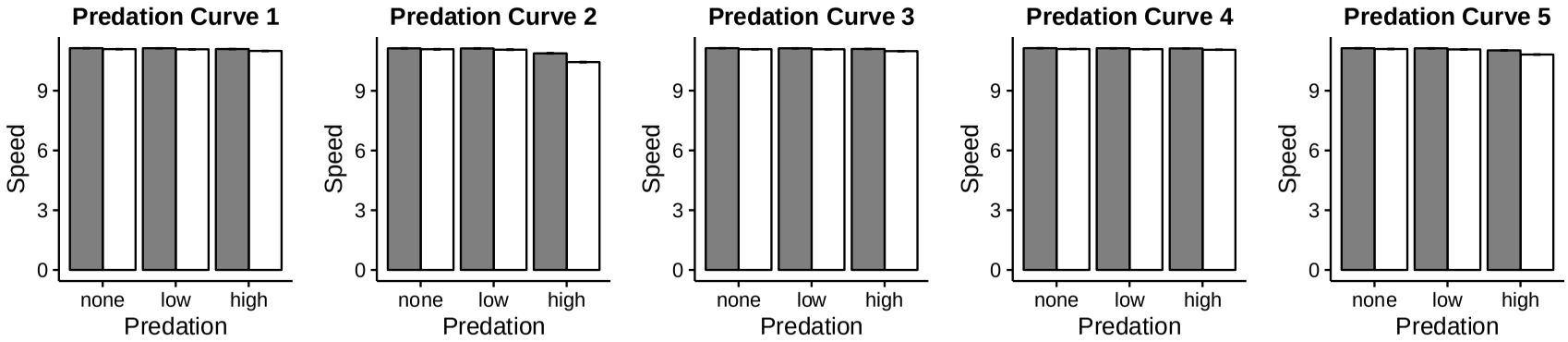

FIGURE 2.4: Mean speeds are unaffected by predation, food density, or predation curve type, except in predation curve 2 where the high predation high food evolve a slightly slower speed on average. Food density treatments are denoted by the shade of the bars with dark bars having a low density and light bars having a high density.

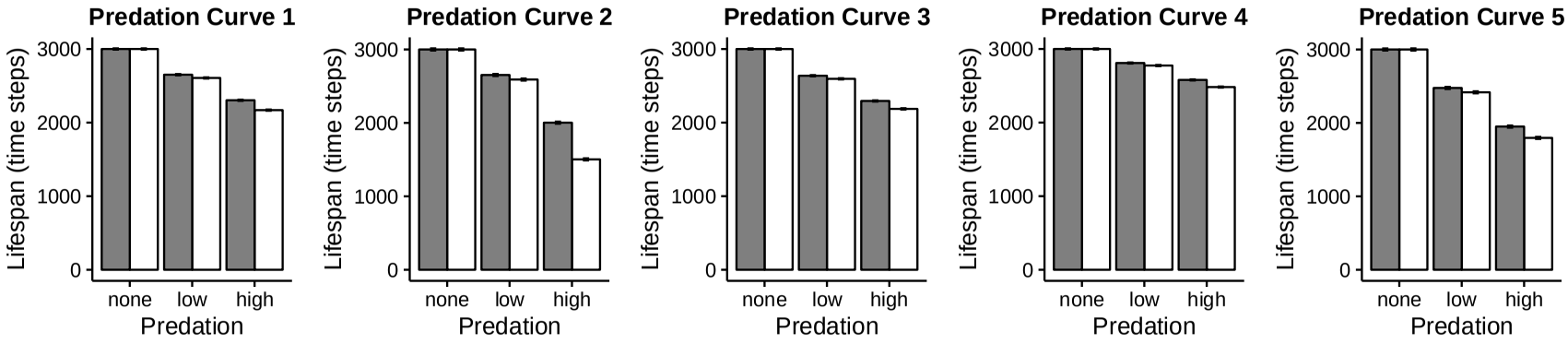

FIGURE 2.5: Mean lifespan and standard errors in each treatment and predation curve type. Food density treatments are denoted by the shade of the bars with dark bars having a low density and light bars having a high density. Predation levels affect life spans with high predation environments having the shortest average lifespans. Predators have a greater effect on lifespan in the high food environments, though this difference is not likely to be biologically relevant except with predation curve 2.

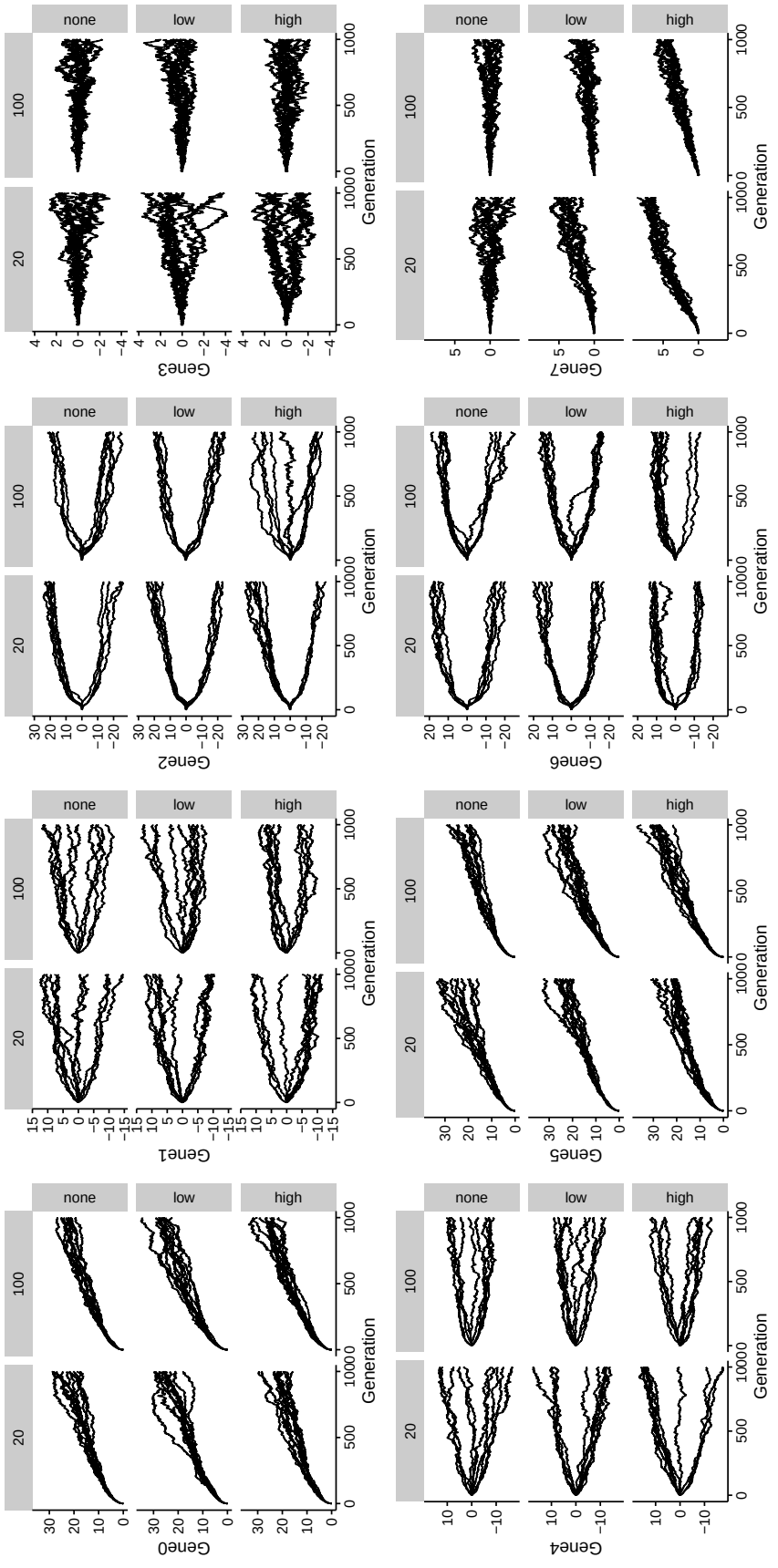

FIGURE 2.6: Gene weights across time. Weights for predation curve 1 are shown, but the same patterns occur in predation curves 2-5. Refer to Figure 1 for a description of each gene. Only Gene 7, the bias on the y movement evolves according to the y position.

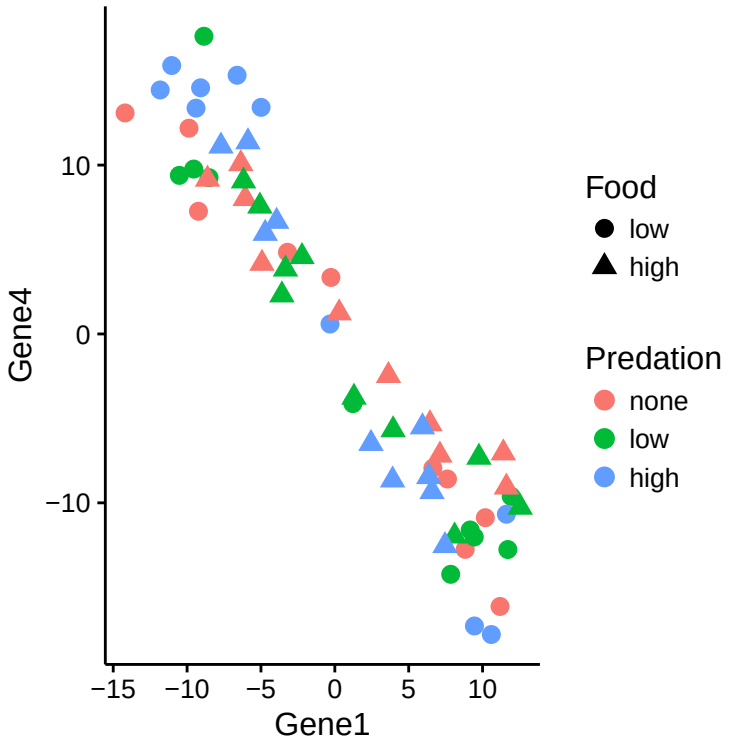

FIGURE 2.7: The diagonal genes, Gene 1 (y distance to food on x movement) and Gene 4 (x distance to food on y movement) diverge across populations with a strong negative correlation ($r = -0.95, t_{58} = -23.461, p < 0.001$). Data and statistics are shown for predation curve number 1, but the other predation curves produce similar results.

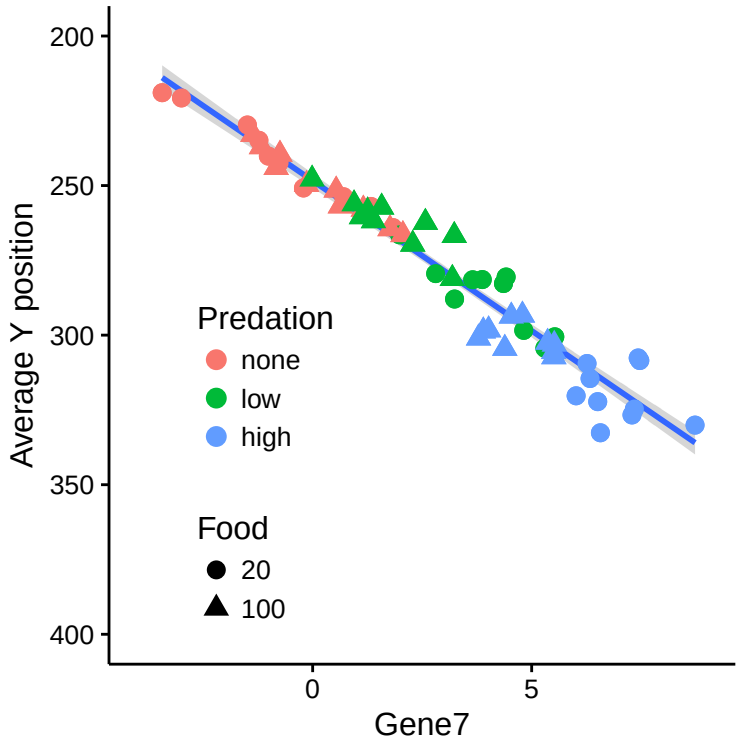

FIGURE 2.8: The relationship between gene 7 and average y position. This relationship extends both within and between the predation and food treatment groups. Each point is a single population with the mean value of gene 7 and mean value of the y position. A linear regression line with the standard error is also presented. The data in this figure come from predation curve 1, but the other curves produce similar results.

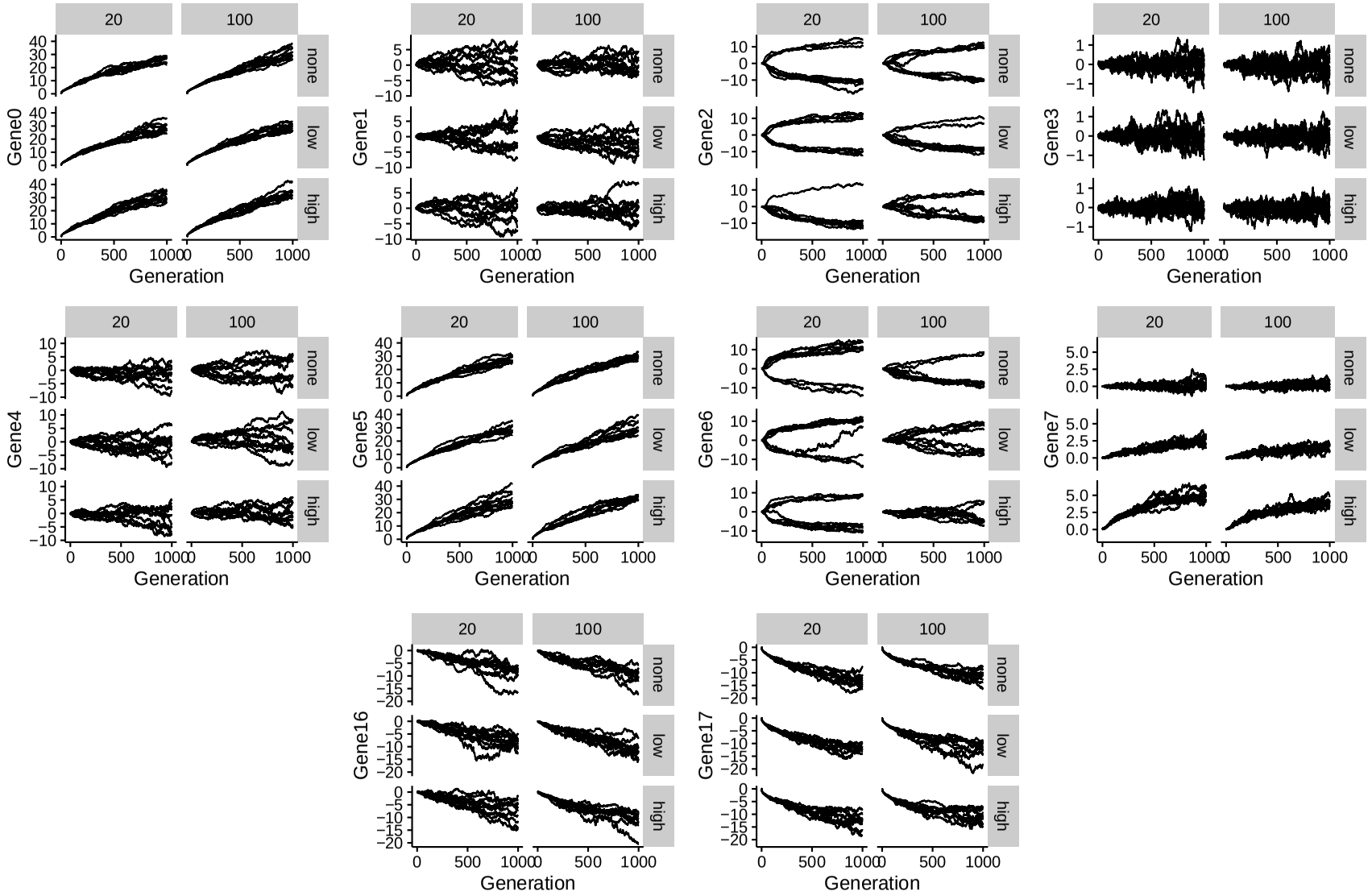

FIGURE 2.9: Average gene weights across time for each population when choice is set to the maximum choice value. In each panel, columns represent the food density while rows represent the predation level.

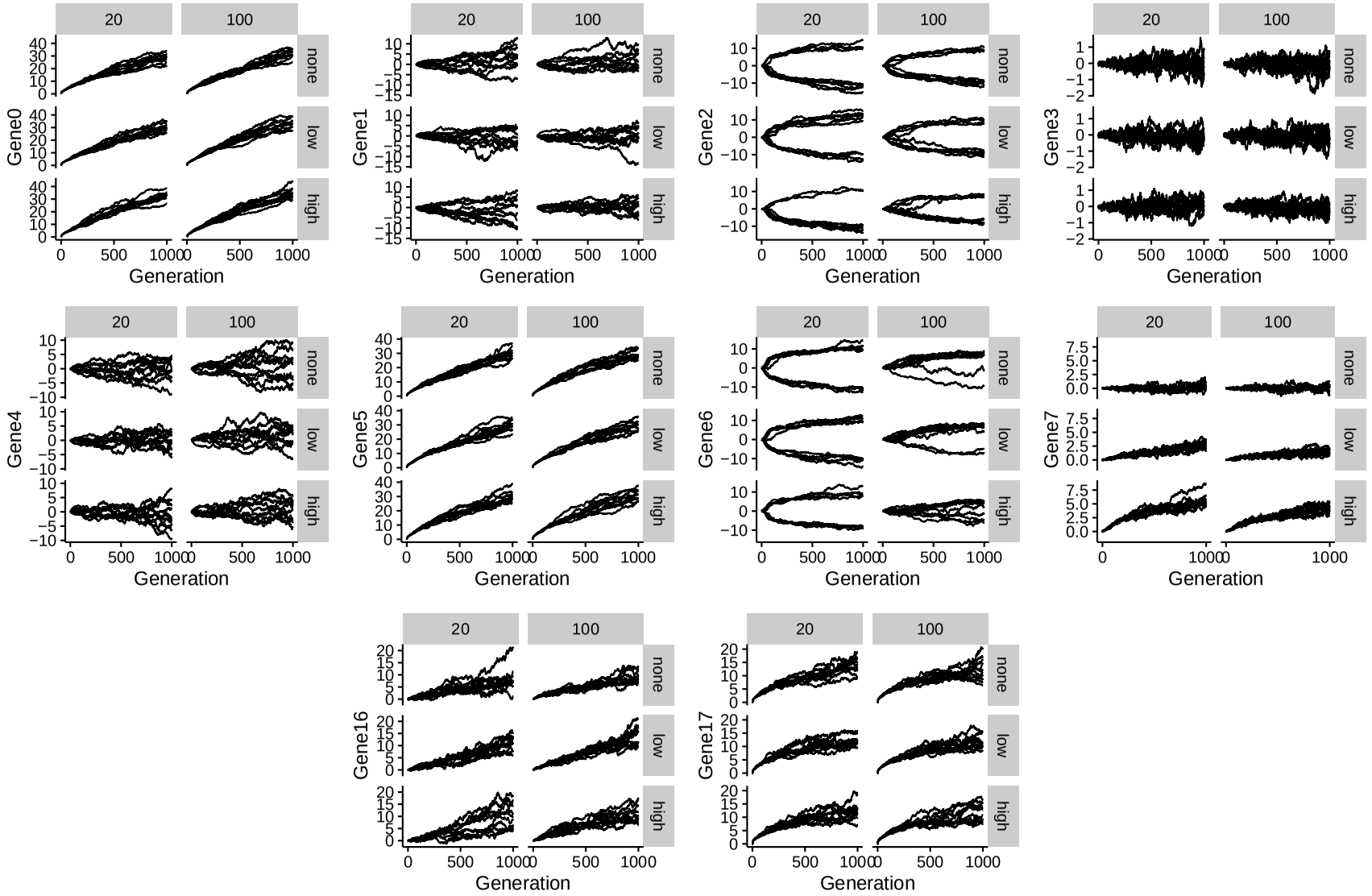

FIGURE 2.10: Average gene weights across time for each population when choice is set to the minimum choice value. In each panel, columns represent the food density while rows represent the predation level.

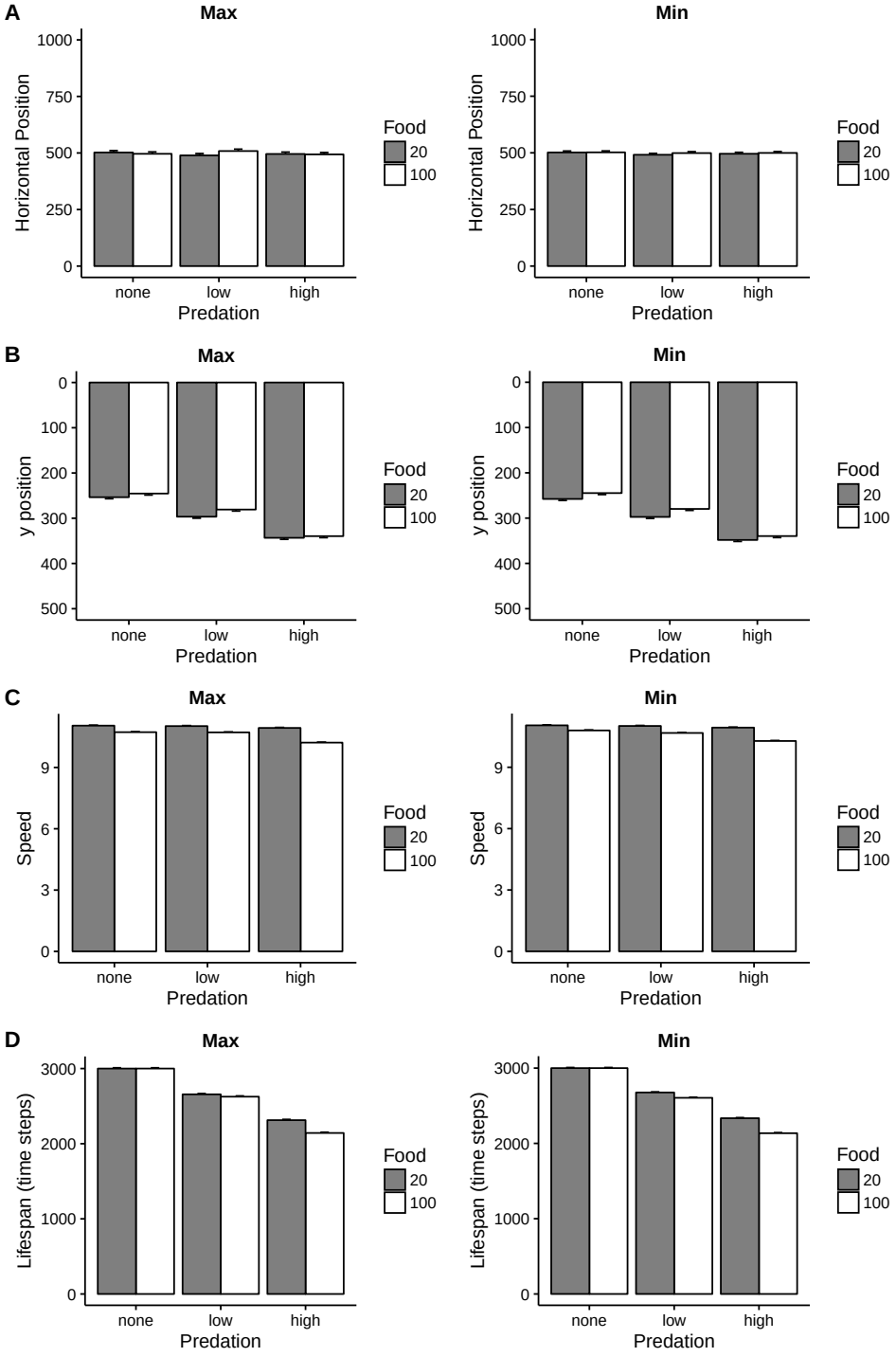

FIGURE 2.11: Behavioral outcomes in the last generation when choice is applied. General patterns regarding average x position (A), average y position (B), speed (C), and lifespan (D) are not different than without the choice (see figures 2.2-2.5).

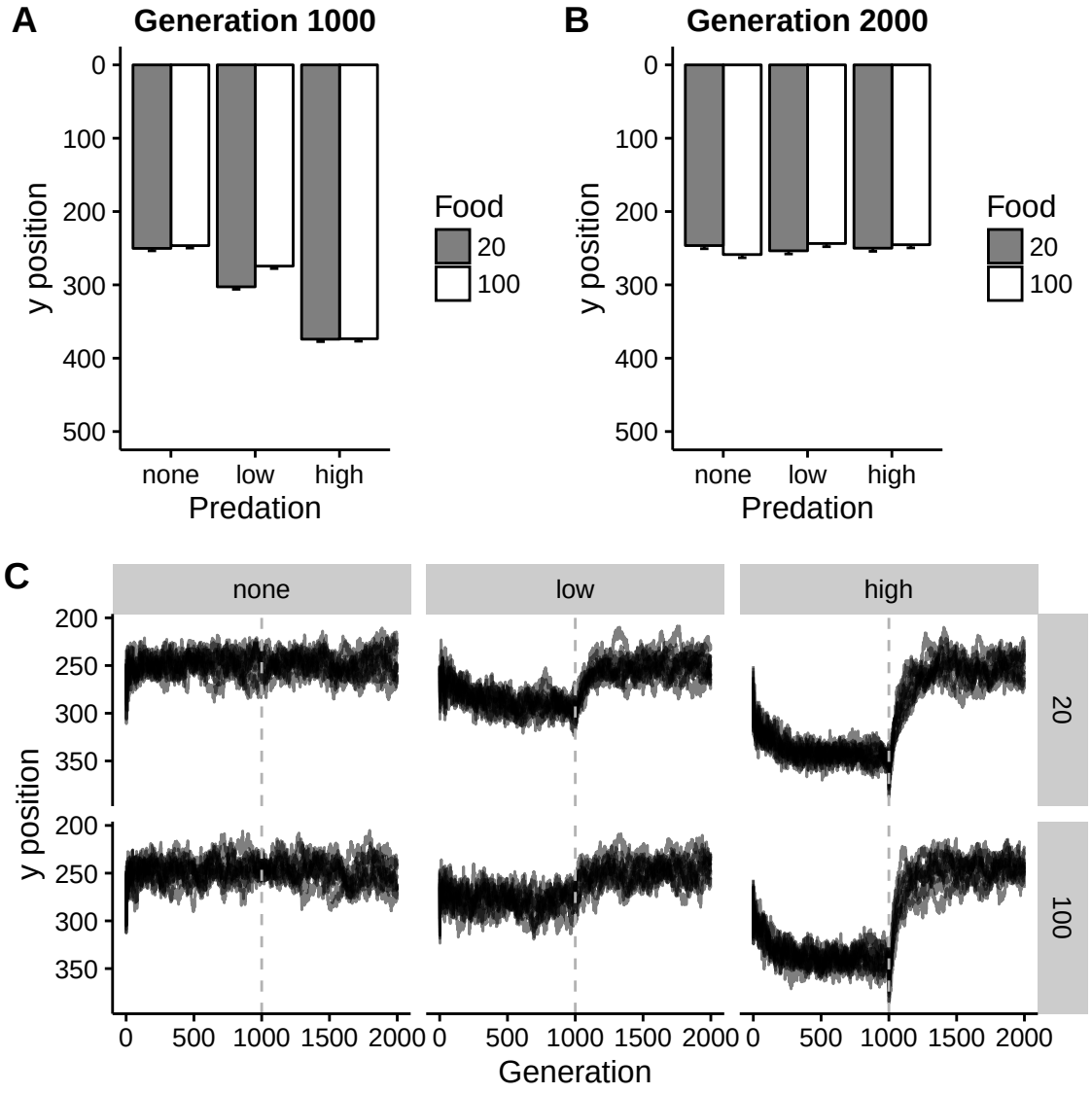

FIGURE 2.12: Results of y-axis behavior with an additional 1000 generations after the predation is removed. A and B show the means and standard errors at generation 1000 and 2000 respectively. C shows the population means at each generation with predation treatments of the first 1000 generations shown by column and food density shown by rows.

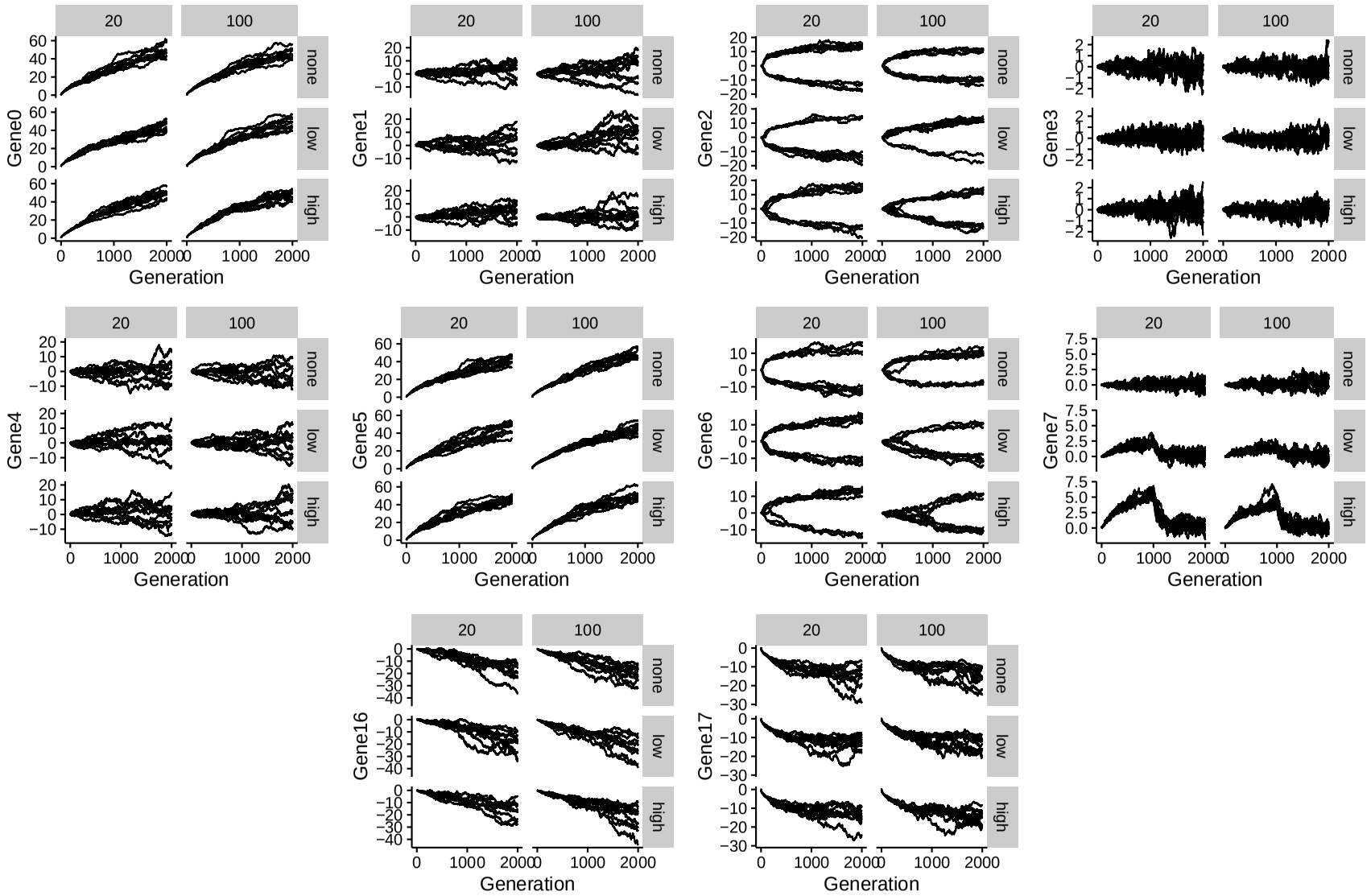

FIGURE 2.13: Average gene weights across time for each population when an additional 1000 generations is included with the predation turned off. In each panel, columns represent the food density while rows represent the predation level experienced during the first 1000 generations.

CHAPTER 3

Complexity in early rearing environment affects adult boldness behavior.

3.1 SUMMARY

Boldness is a set of behaviours associated with risk-taking and is known to influence an individual's fitness. Boldness behaviours can be heritable, yet most of the variation between individuals cannot be explained by genetic variation alone. Environmental differences experienced early in life contribute to an animal's behaviour. Here we test the hypothesis that structural complexity in the early rearing environment will alter boldness as an adult. We reared zebrafish (*Danio rerio*) fry in tanks that were either barren or contained plants and a marble substrate until they were adults. We then tested behaviour in an open-field dive test that was also either barren or contained a plant, followed by an individual home tank scan sample test. We found that complexity in the rearing environment produced more bold individuals, but this effect was only observed in males. Additionally, we found complex-reared fish are more bold if assayed in a barren tank, but not when assayed in a complex environment. We found no evidence for the effects of rearing environment on the scan sample behaviours. We concluded that while complexity of the rearing environment affects the development of boldness personality as an adult, the effect depends on both sex and the context in which behaviour is assayed.

3.2 INTRODUCTION

Boldness is a set of behavioural traits that define an individual's propensity to take risks (Réale *et al.*, 2007). Boldness behaviours are important in an ecological context because the risks associated with them can be associated with a reduction in fitness (Smith and Blumstein, 2008). Boldness has a heritable genetic component which allows animals to respond to evolutionary forces (Dochtermann *et al.*, 2015; Oswald

et al., 2013b). Therefore, adaptive hypotheses have been proposed to explain why populations in different environments display different levels of boldness. For example, stickleback from lakes with predators tend to be more shy than populations from lakes without predators (Brydges *et al*., 2009). Differences in boldness are also observed between captive and wild populations across many species. Animals that have been raised in captivity for several generations tend to be tamer and more bold while their wild counterparts tend to be more shy and fearful. These patterns have been well documented in fish where captive raised populations tend to spend more time at the top of the water (Vincent, 1960; Robison and Rowland, 2005), take more risks (Einum and Fleming, 1997; Fleming and Einum, 1997), and are more likely to associate with humans (Drew *et al*., 2012). In these examples, behavioural differences across populations could be due to differential selective pressures in each environment. But heritability estimates of boldness and other related behaviours are typically around 0.3 to 0.5 (Dochtermann *et al*., 2015), leaving much of the variation in these behaviours to non-additive and non-genetic effects including variation in the environment.

Behavioural plasticity can be defined as the contribution of environmental variables to behavioural traits being observed. Plasticity can come in two categories. Developmental plasticity describes the effects of previous exposure on a trait later in life. Contextual plasticity describes the effects of the immediate environment on a trait. The environment experienced during critical periods of development is known to play a role in the formation of many traits. DiRienzo *et al*. (2012) show that the presence of conspecific acoustic signals during juvenile stages reduces aggressiveness in adults of the field cricket *Gryllus integer*. Garduño-Paz *et al*. (2010) showed that the structural complexity of the juvenile rearing environment can affect the expression of morphological traits.

In the context of domestication, wild and captive environments tend to differ in their structural complexity. Wild fish populations experience rich and structurally complex environments while captive environments such as a hatchery or lab setting are often more uniform and homogeneous (Johnsson *et al*., 2014). Adding structure as a form of enrichment is known to reduce stress and anxiety and alter behaviours in captive animals. Mice housed with objects to interact with (tunnels, ladders, running

wheels) showed reduced anxiety behaviours in the form of increased exploratory activity when compared with mice housed in an empty plastic housing (Benaroya-Milshtein *et al.*, 2004). In fish, the addition of plants and gravel substrates have been shown to reduce startle responses, aggression (reviewed in Näslund and Johnsson (2014)), and basal cortisol levels (Näslund *et al.*, 2013). These studies show a contextual effect associated with structural complexity in the environment. They do not address whether early exposure to complexity has any long-term influence on the development of an animal's behaviour. Less is known about the permanent effects of structural complexity on the formation of boldness personalities during critical stages of development.

The primary aim of this experiment is to investigate the effects of the complexity of early rearing environment on boldness as an adult. We use zebrafish (*Danio rerio*) as a model system to study individual differences in bold-shy behaviours because they can be reared in the absence of parental care. We reared fry from 2 days to adult in experimental treatments that were either simple or complex, and then assayed behaviour using the novel open-field dive test and home tank scan sampling techniques.

3.3 METHODS

Wild-type zebrafish of the Scientific Hatcheries strain (Huntingdon, CA) were bred in August 2014 from 10 of our breeding stock tanks, each containing between 5 and 10 fish of mixed sex. Embryos were collected, pooled, and distributed into 200mL beakers containing system water in an initial density of 40 per beaker and kept until two days post hatch, about five days after initial collection, at which point the fry were transferred into their experimental rearing environments. The simple rearing environment consisted of a blank 9L Aquaneering tank (approximately 20 cm wide, 30 cm long, 18 cm deep) filled only with water, while the complex rearing environment consisted of a layer of blue and clear glass marbles for a substrate and five plastic aquarium plants. There were three replicate tanks of each environment, and each tank received 100 fry of which we expected an average survival rate between 10 and

20 percent. The tanks were then placed on our main system and separated with opaque dividers so that individuals could not see the neighbouring tank. The fry were raised for three months in their experimental environment before being sampled for behavioural assays.

3.3.1 *Novel tank dive test assay*

We measured behaviour using the novel tank diving test (Egan *et al.*, 2009). Ten fish from each of the six tanks were randomly selected (except for tank 1 which only had 8 fish remaining), with five from each tank being placed in one of two testing environments. The fish were placed one at a time into a rectangular tank measuring 25 cm wide, 12 cm high (from water level to bottom), and 6 cm thick (front to back). The volume of water in the tank was approximately 2 L. In the simple assay environment, the tank contained only water while the complex assay environment contained a 2 cm layer of marbles and a single plastic aquarium plant off-set to one side. The side that the plant was placed was randomised for each complex trial to distinguish a left-right side bias from plant preference behaviour. The test arena was top-lit using a standard aquarium light and the sides and back were covered using a black background. This allowed for maximum contrast for automated video tracking. Behavioural assays occurred over four consecutive days with 15 fish randomly sampled each day, and each being randomly assigned to an assay environment. Videos were recorded using a Canon Rebel T3 and Ef-s 18-200mm lens at 720p and 25 frames per second. Videos were imported into Pro Analyst (Xcitex) for automated tracking.

3.3.2 *Scan sample assay*

After each fish was recorded in the tank diving test, it was placed into its own individual 1.5L tank and housed on the top rack of the main system. After two weeks of acclimation, we recorded behaviours associated with place preference and feeding using a high-throughput assay we call scan sampling (Oswald *et al.*, 2013b; Robison *et al.*, 2012). In this assay, the fish were visually isolated from one another using opaque dividers between the tanks. An observer stood in front of a tank and

recorded the location of the fish. The depth was recorded by dividing the tank into six vertical zones approximately 2 cm deep. The horizontal position, also called observer orientation, was recorded as either being within one body length of the front or not. These observations were scored three times for each fish and repeated twice a day (morning and afternoon) for five consecutive days.

3.3.3 *Analysis of swim tracks*

The tracks output from ProAnalyst contain a list of x, y, and time coordinates for each frame of the video. Tracking began with the frame that the fish entered the surface of the water and continued to the end of the video. We cleaned and processed the data in R. During the first five seconds, the fish sank to the bottom but otherwise remained motionless. This portion of the track was removed and the tracks were then standardised to a length of 4 minutes, or 6000 frames. We estimated the velocity in each frame using the data from two frames prior and two frames ahead of the focal frame. This method sufficiently smoothed noise due to positional changes of the tracking point on the fish from frame to frame while retaining changes in velocity at small time intervals.

Freezing was defined as time spent motionless (velocity $<$ 0.01 cm/frame) on the bottom of the tank for more than 20 consecutive frames. Short bursts of motion less than 40 frames within a longer freezing period were considered to be artifacts of the tracking software and were converted to be part of the freezing period. Freezing time was then calculated by counting the total number of frames classified as frozen.

The remaining frames not classified as frozen were considered to be active movement. During this time, the fish was swimming about and making decisions about how and where to move about the tank. We were interested in differences in location preferences during activity. Using the active time points, we calculated the individual average and variance of speed, average and variance of depth, proportion of time spent in the upper half (0-6 cm below the surface), and number of times an individual entered the upper half. We also calculated the latency to enter the the upper half, though this time is calculated from the beginning of the trial and includes freezing.

For subjects assayed in the complex environment, we also measured an association with the plant using the individual average x position and the percentage of time spent in the third of the tank containing the plant. Since the plant appeared on either the right or left hand side of the tank, we redefined the x-axis so that the edge nearest the plant was defined as 0 and the opposite side as 25 cm for the purpose of analysing these variables.

3.3.4 *Statistical Analysis*

For the dive test, we analysed the effects of sex, rearing environment, testing environment, and all interactions on the average speed, speed variance, distance travelled, average depth, depth variance, latency to enter the top half, proportion of time in the top half, and proportion of time in the horizontal middle third of the test arena using a type II linear mixed model (LMM) with rearing tank included as a random effect. We tested the results of all models for the assumptions of normality using a Shapiro-Wilk test and equality of variance using Levene's test. Variables that deviated from these assumptions were transformed according to a Box-Cox analysis. We applied a logit transformation to all proportional variables prior to analysis. Freezing behaviour deviated from the LMM assumptions due to an over-abundance of zeros in the data. Therefore freezing was defined in two variables. Freezing occurrence was defined as a yes or no response and analysed using a generalized linear mixed model (GLMM) with a binary distribution. Freezing duration, or the time spent motionless, was then analysed using an LMM for all individuals who did display freezing behaviour ($N = 46$). The number of crosses into the top half was analysed using a GLMM with the assumption of a poisson distribution.

Since tests for plant association were performed only on individuals assayed in the complex environment, we only included the effects of sex, rearing environment, and the interaction in our type II LMM. Similarly, the home tank environment used for the scan sampling assay does not vary in complexity, therefore the type II LMM on these data only included the fixed effects of sex, rearing environment, and the interaction.

3.4 RESULTS

3.4.1 *Novel tank dive test*

Statistical results of the fixed effects in the linear mixed models and generalized linear mixed models are presented in table 3.1.

EFFECTS OF REARING ENVIRONMENT — We observed no main effects of rearing environment on behaviour in either of the assays performed. However in three instances, we observed sex differences that were mediated by the rearing environment (Figure 3.1). Males from a simple environment were more variable in their swimming velocity than females from a simple environment (Tukey post-hoc: $T_{45} = -3.11, P = 0.0165$), yet no differences were detected between males and females from a complex environment.

Males reared in a complex environment travelled a further total distance and visited the top half of the assay tank than females reared in a complex environment as well as all fish raised in a simple environment.

We observed a significant interaction between the effects of the rearing environment and the testing environment with the latency to enter the top half (LMM: $F_{1,45} = 8.60, P = 0.01$) . Of the individuals reared in a simple environment, those tested in a simple environment waited on average 215 +/- 20 seconds to enter the top half of the test environment while those tested with complexity waited only 89 +/- 22 seconds on average (Tukey post-hoc: $T_{45} = -4.187, P = 0.0007$). Individuals reared in a complex environment waited an intermediate time of around 150 +/- 22 and 156 +/- 22 seconds and for the complex and simple assay environments respectively (Figure 3.2A).

EFFECTS OF TESTING ENVIRONMENT — Structural complexity in the testing arena also affected three behavioural measures and interacted with sex in two others in the dive test (Figures 3.2 and 3.3). Individuals moved with more variable speed when assayed in complex environment than in a simple environment (LMM: $F_{1,45} = 7.96, P = 0.01$), after accounting for the effects of sex and rearing environment. Fish assayed in a simple environment also swam approximately twice the distance as those

tested in a complex environment (LMM: $F_{1,45} = 5.12, p = 0.03$). Individuals assayed in a complex environment spent more time in the top half of the tank compared with individuals assayed in a simple environment (LMM: $F_{1,45} = 9.48, P = 0.004$).

Interactions between sex and testing environment were detected regarding latency to enter the top half (LMM: $F_{1,45} = 5.98, P = 0.02$) and the number of crosses into the top half (GLMM: $\chi^2_1 = 13.24, P < 0.001$). Males assayed in a complex environment were quicker to enter the top half than females assayed in the same environment, while no such sex differences occurred when fish were assayed in a simple environment (Figure 3.2B). In a simple environment, males visited the upper half of the tank fewer times than females, but no sex differences were observed when fish were assayed in a complex environment (Figure 3.2C).

Effects of Sex — Sex differences in freezing time were observed (LMM: $F_{1,33} = 5.97, P = 0.02$) and were not associated with variation in rearing or testing environments. When fish did display freezing behavior, females froze on average a minute longer than males (Figure 3.3A).

3.4.2 *Scan sampling assay*

In the scan sampling assay, we observed clear sex effects with regards to observer preference, depth preference and feeding latency. Females spent more observations near the observer, higher in the water column, and were quicker to feed than males. However, we observed no effects of rearing environment on these behaviours (Figure 3.4 and Table 3.2).

3.5 DISCUSSION

Behavioural plasticity is variation either due to previous experience (developmental) or more immediate effects of the current environment (Dingemanse and Wolf, 2013) (behavioral flexibility, or contextual plasticity). Much of the literature assessing the effects of structural complexity on behaviour focus on the contextual effects. We tested the hypothesis that early exposure to complexity in the rearing environment affects

the developmental plasticity of boldness behaviours. This experiment detected limited support for this hypothesis, with the caveat that effects of the rearing environment appear to be mostly sex-specific. We included sex effects in our experiment because we have previously observed strong sexual dimorphism in boldness in laboratory strains of zebrafish (Oswald *et al*., 2013a). Typically, we have observed patterns where females are more bold than males (Oswald *et al*., 2013a), and our observations in the present scan sampling data continue to support this pattern. However the role of sex in determining behavioural variation in the open field is less clear. In this assay, males tend to exhibit behaviours that we would describe as being more bold. Males froze for a shorter duration, travelled longer distances, made more visits the top half, and entered the top half sooner than females. However many of these differences only occurred in males raised in the complex environment leading us to hypothesize that complexity in the rearing environment can generate or enhance sex differences in boldness exploratory behaviour. Similar sex-specific plasticity has been observed in brain size where enrichment caused a larger brain volume in male stickleback reared in a complex enriched environment (Herczeg *et al*., 2015). Larger brains are associated with a more bold behaviour type in guppies (Kotrschal *et al*., 2014), though we can't say for certain from our data whether brain size is the underlying mechanism influencing boldness in zebrafish.

We varied complexity in the testing environment as a way to account for potential effects of environment induced neophobia into the data. For example, if we had only gathered behaviour in a simple tank environment as the dive test is typically performed, we might have observed anxious behaviours from the complex-reared treatment group because the simple environment was more unfamiliar to them. We found no patterns to suggest neophobia, but in one instance, we did observe a significant interaction effect between the rearing and assay environments, and the patterns we observed raise the argument that context of the behavioural test matters. For example, had we only assayed dive test behaviour in the traditional barren environment, we would have concluded that the complex rearing environment reduces latency to enter the top half of the water after a stressful event. Instead we find the opposite effect when fish are placed in a assay environment containing complex structures. This

observation suggests that the complexity in the early rearing environment could alter the plasticity of a behavioural response. We might conclude that early exposure to complexity stabilizes an animal's behaviour type across different environments while a simple rearing environment facilitates a more plastic response.

There is some evidence that behavioural flexibility is related to boldness. Shy behaviour types seem to be more flexible in changing environments than bold types (Herborn *et al*., 2014). Our data seem to support this idea when we look at the interaction effects with the testing environment. Individuals reared in a simple environment develop a shy personality, but appear to be somewhat flexible between environments compared with individuals reared with complexity. Similarly, males in our population tend to be shy, and also show the most variability across testing environments, at least when it comes to latency to visit the top half of the tank.

The results of our dive test and scan sample paradigms are somewhat contradictory. For example, we observe males to be the more bold sex in the dive test, but females to be more bold in the scan sample data. Additionally, The rearing environment does not have any effect on the scan sample behaviours, but does affect behaviours in the open field. This could be explained by two non-mutually exclusive hypotheses. The first is that the behaviours measured in the open field test and the scan sample test are not related and that we may be measuring two independent axes of boldness. A number of recent studies have highlighted the importance in the context which behavioural data is collected. For example, novel environment and novel object tests do not necessarily measure the same aspects of boldness (White *et al*., 2013) and measuring latency behaviours in a novel and familiar environment can yield uncorrelated results (Beckmann and Biro, 2013). There are a number of notable differences between the two paradigms including the stimulus (dive test: novel environment and stress of being handled; scan sample: presence of a human observer in front of the tank), and the familiarity of the tank (dive test: novel; scan sample: familiar with two weeks of acclimation). We can interpret the scan sample paradigm as a test for tameness, or a lack of fear of humans, which are often associated with domestication (Oswald *et al*., 2013b).

A second explanation for the difference between the dive test and scan sample results could stem from the timing of these tests. The fish were subjected to the dive test directly after being removed from the experimental rearing environments. They were then subjected to an individually separated, but common, environment on the main system before being subjected to the scan sampling. In hindsight, we are unable to properly distinguish between the short-term effects of having been immediately exposed to complexity before data collection and the long-term effects of having been exposed to complexity during early development. As a result, we cannot say for certain whether the absence of effect of rearing environment on the scan sample behaviours is due to having been exposed to a common environment prior to data collection, or if it is due to the unrelatedness of the tests.

The impacts of habitat complexity are often discussed in the context of animal welfare where adding some kind of complexity to the captive environment is thought to reduce stress and anxiety levels and thus increase the quality of life in captivity (Benaroya-Milshtein *et al*., 2004; Giacomini *et al*., 2016). However these same behaviours used to indicate stress and anxiety in an individual may be under selection during adaptation to captivity or adaptation to a changing environment. This is important because a number of studies have shown that in just a few generations, captive populations become genetically distinct from wild conspecifics (Christie *et al*., 2012). These behavioural shifts may be maladaptive if the animals are slated to be returned to the wild as in the case of salmon and other fish hatcheries. Assuming that in captive environments the selective optimum favours a more bold behaviour type, the shyness effects of being raised in a simple environment may initially shift a wild population away from this optimum when first brought into captivity. While this may have negative fitness consequences on the population at first, selection for bolder behaviour types ought to be stronger, leading to a faster adaptive process. On the other hand, the large behavioural differences seen between wild and captive populations might also be artificially enhanced by collecting data in a simple captive environment. In other words, the behavioral difference between captive and wild stocks might not be as large if we could accurately measure the behaviour of the populations in a wild habitat.

In conclusion, our study has shown that structural complexity in the early rearing environment has the potential to impact the development of personality traits. However these effects may depend both on the sex of the organism as well as the context in which adult behaviour is assayed. While further investigation is necessary, our data support a link between boldness and behavioural flexibility that is also influenced by complexity in the early rearing environment.

3.6 ACKNOWLEDGEMENTS

We thank Dr. Craig McGowan for the use of the ProAnalyst software to track the fish.

3.7 TABLES

TABLE 3.1: Results of the novel tank dive test. P-values from the fixed effect F-tests from the linear mixed models (* and χ^2 tests where generalized linear mixed models were appropriate) are presented. RE = rearing environment. TE = testing environment.

	Sex	RE[§]	TE	Sex:RE	Sex:TE	RE:TE	Sex:RE:TE
Freezing Occurrence[*]	1.0000	0.2875	0.0936	0.9982	0.9991	0.9992	0.9995
Freezing Time[†]	**0.0201**	0.1237	0.2870	0.1383	0.8974	0.2788	0.4483
Speed	0.2408	0.9197	0.2400	0.2072	0.7772	0.8206	0.2996
Variance Speed	0.0587	0.7612	**0.0071**	**0.0224**	0.3617	0.4282	0.0821
Distance	**0.0032**	0.2269	**0.0286**	**0.0348**	0.7100	0.1379	0.4666
Average Depth	0.5370	0.4791	**0.0000**	0.1311	0.2760	0.3592	0.2055
Variance Depth	0.9058	0.9702	0.1198	0.5784	0.0577	0.3916	0.5751
Latency to top	**0.0009**	0.9680	**0.0039**	0.8492	**0.0184**	**0.0085**	0.1911
% Time in top	0.8826	0.7678	**0.0035**	0.3027	**0.0619**	0.2157	0.4904
# Crosses to top[*]	**0.0042**	**0.0197**	**0.0000**	**0.0000**	**0.0003**	**0.0496**	0.0941
% Time in mid	0.5221	0.7648	0.5010	0.8302	0.6243	0.8321	0.1248
% Time near plant[‡]	0.3998	0.5080	—	0.3494	—	—	—

Degrees of freedom for F test are 1,45 except: * χ^2 df = 1; [†] 1,33; [‡] 1,20; [§] 1,4 (tank nested within RE)

TABLE 3.2: Results of the scan sample assay. P-values from the fixed effect F-tests from the linear mixed models (* and χ^2 tests from generalized linear models) are presented. RE = rearing environment.

	Sex	RE[§]	Sex:RE
Observer Preference[*]	0.0000	0.7238	0.2629
Depth Preference	0.0017	0.4617	0.3433
Feeding Latency	0.0045	0.2101	0.2102

Degrees of freedom for F test are 1,49 except: * χ^2 df = 1;[§] 1,4 (tank nested within RE)

3.8 FIGURES

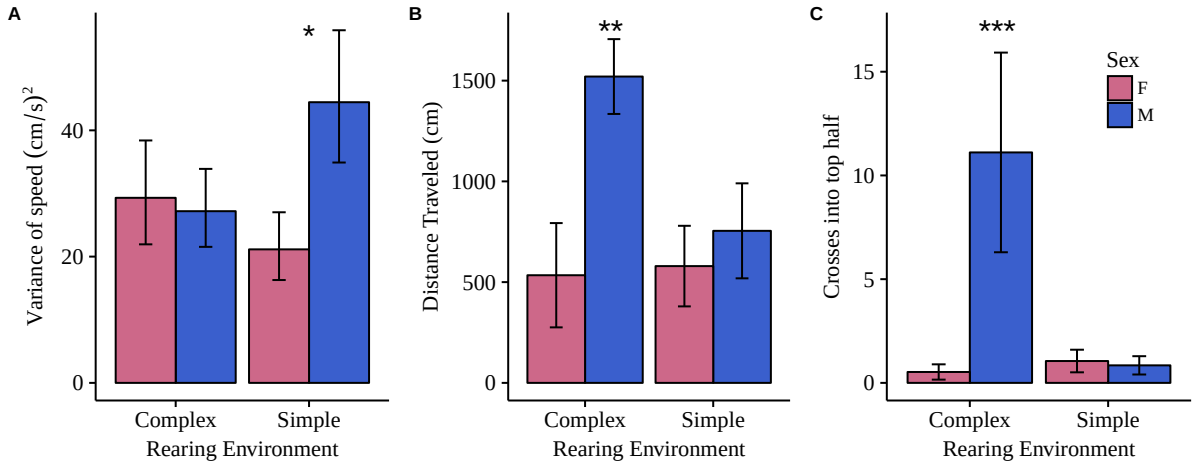

FIGURE 3.1: Interactions between sex and rearing environment were detected in three behavioural measures: (A) variance or consistency of swimming speed, (B) total distance traveled, and (C) number of visits into the top half of the tank. Means and standard error bars are shown with asterisks denoting significance level of the Tukey-adjusted post-hoc tests. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.01$

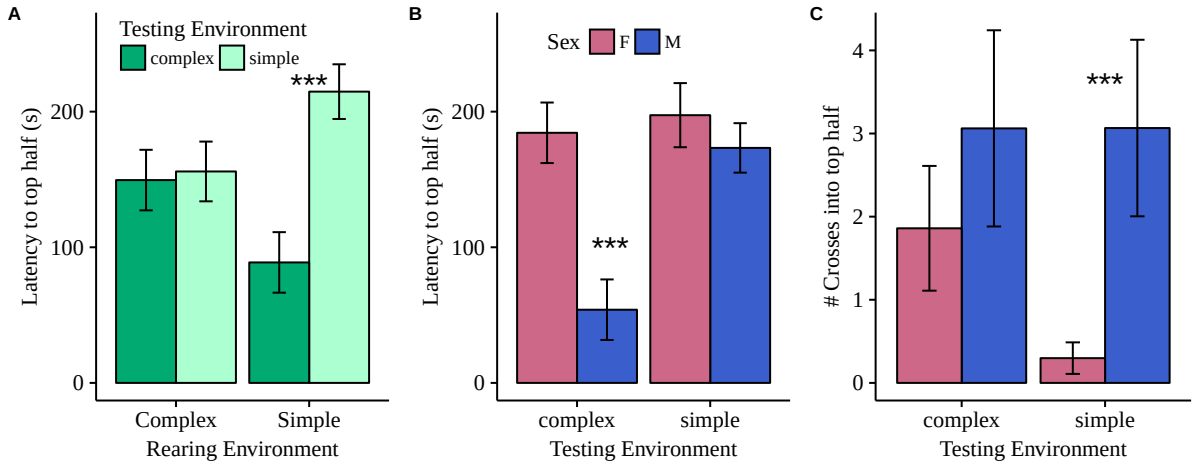

FIGURE 3.2: Interactions between the rearing environment and testing environment (A) and between sex and the testing environment (B) were observed in the latency to enter the top half of the tank. Interactions between sex and the testing environment were also observed with regard to the number of visits to the top half (C). Means and standard error bars are shown with asterisks denoting significance level of the Tukey-adjusted post-hoc tests. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.01$

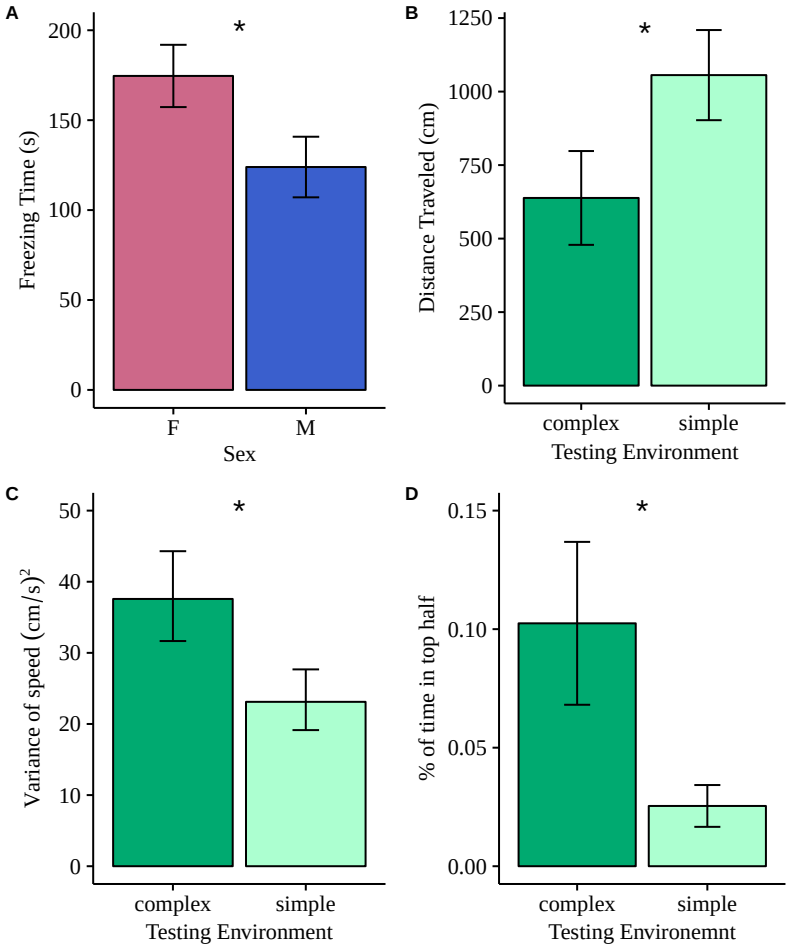

FIGURE 3.3: Effects of sex (A) and testing environment (B-D) were observed with no interactions regarding (A) the time frozen (excluding animals that did not freeze), (B) the total distance traveled in four minutes, (C) variation, or consistency, of swimming speed, and (D) the proportion of active swimming time spent in the top half of the tank. Means and standard error bars are shown with asterisks denoting significance level of the Tukey-adjusted post-hoc tests. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.01$

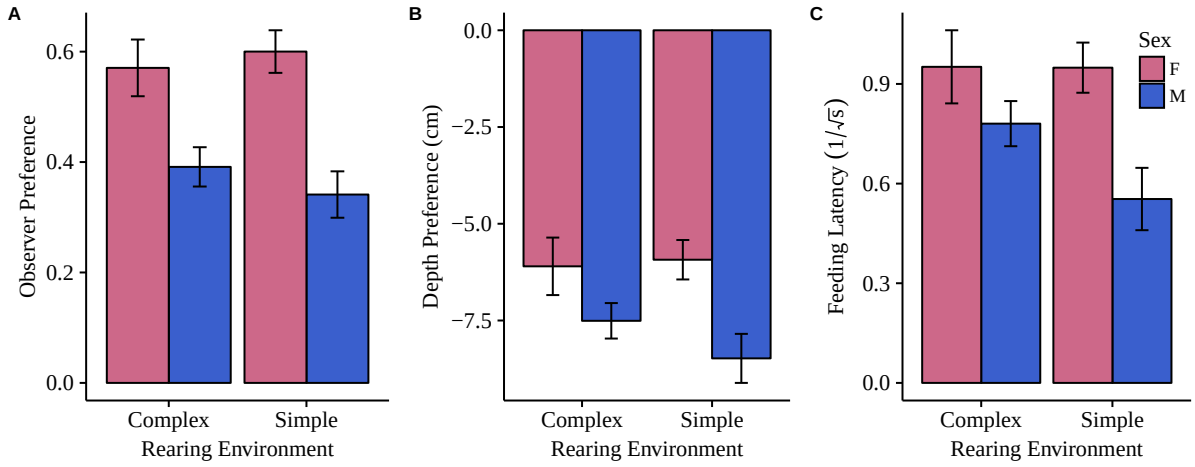

FIGURE 3.4: Results of the scan sampling assay show no effects of rearing environment on observer preference (A), depth (B), and feeding latency (C), but do show a sex effect in all three behaviours in which females are the more bold sex. Means and standard error bars are shown.

CHAPTER 4

Anxiolytic effects of Fluoxetine and Nicotine exposure on exploratory behavior in Zebrafish [1]

4.1 SUMMARY

Zebrafish (*Danio rerio*) have emerged as a popular model for studying pharmacological effects on behavior and anxiety. While there have been numerous studies documenting the anxiolytic and anxiogenic effects of common drugs in zebrafish, many do not report or test for behavioral differences between the sexes. Previous studies of zebrafish have indicated that males and females differ in their behavioral responses to anxiety. In this study, we test for sex-dependent effects of fluoxetine and nicotine. We exposed fish to system water (control), 10 mg/L fluoxetine, or 1 mg/L nicotine for three minutes prior to being subjected to four minutes in an open-field drop test. Video recordings were tracked using ProAnalyst. Fish from both drug treatments reduced swimming speed, increased vertical position, and increased use of the top half of the open field when compared with the control, though fluoxetine had a larger effect on depth related behaviors while nicotine mostly affected swimming speed. A significant sex effect was observed where females swam at a slower and more constant speed than males in all treatments. No interactions between sex and the drugs were observed across the entire study.

4.2 INTRODUCTION

The zebrafish (*Danio rerio*) is a popular research model for studying pharmacology (summarized in Barros *et al.*, 2008; Langheinrich, 2003) and behavior (Gerlai, 2015), particularly with regard to stress and anxiety. The zebrafish provides a vertebrate

[1] Published as: Singer, M. L., Oreschak, K., Rhinehart, Z., & Robison, B. D. (2016). Anxiolytic effects of fluoxetine and nicotine exposure on exploratory behavior in zebrafish Anxiolytic effects of Fluoxetine and Nicotine exposure on exploratory behavior in Zebrafish. PeerJ, 4, e2352.

model that breeds rapidly, is easy to maintain in large numbers, and can be administered drugs through immersion. Zebrafish also share many of the same neurotransmitters (Shin and Fishman, 2002) and stress pathways as humans, utilizing cortisol rather than corticosteroids as used by rats and mice (Barcellos *et al.*, 2007). These features have facilitated zebrafish studies on addiction (Mathur and Guo, 2010), learning (Sison and Gerlai, 2010), social behavior (Buske and Gerlai, 2014; **?**) and anxiety behaviors (Mathur and Guo, 2010; Maximino *et al.*, 2010).

Anxiety-related behaviors are known to vary by sex in zebrafish and other model organisms, and these differences may be explained by gonadal hormones(Zimmerberg and Farley, 1993; **?**). Male and female rats differ in their time spent in the center of an open field and a plus maze, though the nature of these differences are also dependent on the strain observed (Mehta *et al.*, 2013). In zebrafish, females tend to be less anxious, or more bold, than males when measuring location preferences in the presence of a human observer (Benner *et al.*, 2010; Oswald *et al.*, 2013a).

Drugs are used to manipulate anxiety and related disorders in humans and are also a utilized as tool for understanding behavior. Fluoxetine, for example, is a drug used to treat depression and anxiety. It works by blocking the reuptake of serotonin in the brain (Beasley *et al.*, 1992). Serotonin and its transporters have been associated with anxiety (Graeff *et al.*, 1997; Lesch *et al.*, 1996). Nicotine is naturally found in tobacco products and binds to nicotinic cholinergic receptors (nAChRs) to release dopamine (Benowitz *et al.*, 2009). The result is an anxiolytic response (Picciotto *et al.*, 2002).

Observations of male and female differences in anxiety-related behavior have led us to ask whether the effects of anxiolytic substances also differ by sex. There is evidence that the effectiveness of anxiolytic drugs may vary with sex in mammals. Differential responses have been observed in humans utilizing Sertraline, a selective serotonin reuptake inhibitor (SSRI) where females showed an enhanced response compared to males. (Kornstein *et al.*, 2000). Sex-specific differences were observed in the effectiveness of the SSRI Fluoxetine in humans (Marténgyi *et al.*, 2001), and studies utilizing rats (Mitic *et al.*, 2013; Leuner *et al.*, 2004; Lifschytz *et al.*, 2006) and mice (Monleón *et al.*, 2002; Hodes *et al.*, 2010) have shown a discrepancy between the

sexes in both the physiological and behavioral responses to this drug where efficacy tends to be greater in females than in males. Evidence in rats also suggest that nicotine's effects on stress and anxiety may also differ between the sexes with males exhibiting a greater anxiolytic effect (Faraday *et al.*, 1999). This is important from a pharmacological standpoint in that effective doses may differ between males and females. On a broader level, studies utilizing a single sex, or ignoring the effect of sex altogether ought not to be used to draw broad conclusions about the effects of that drug.

While zebrafish are becoming a model for pharmacological research, literature describing sex-dependent effects of anxiolytic drugs in this system are lacking. In this experiment, we test the hypothesis that zebrafish exhibit sex-dependent responses to fluoxetine and nicotine. These substances were chosen because they have known anxiolytic effects across a wide variety of model systems including humans (Gilbert, 1979; Griffin and Mellon, 1999), rats (Cohen *et al.*, 2009; Zhang *et al.*, 2000) and zebrafish (Bencan and Levin, 2008; Bencan *et al.*, 2009; Cachat *et al.*, 2010; Levin *et al.*, 2007), and while sex-specific effects have been observed in mammals, studies in zebrafish utilizing these substances largely ignore the effects of sex.

4.3 METHODS

4.3.1 *Subjects*

Experimental fish were bred from adult Scientific Hatcheries strain (Huntingdon, CA) that has been maintained in our facility. Water in our Aquaneering Inc. (San Diego, CA) system was constantly circulating and kept at a temperature of 28.5 ºC on a 14 hour light:10 hour dark cycle. The fish were fed a diet of brine shrimp twice and flake food (Tetramin) once for a total of three daily feedings. At the time of data collection, the fish were four months old and housed in three-liter tanks in groups of five to achieve maximal growth rates. Though zebrafish stocked at this density are known to develop social hierarchies that can influence stress and behavior (Pavlidis *et al.*, 2013), we randomly assigned individuals to a drug treatment group such that these effects should be equally distributed across treatments. All aspects of this study

were approved by the University of Idaho's Animal Care and Use Committee under protocol 2014-14.

4.3.2 *Dosing*

Fluoxetine (generic (Teva Pharmaceuticals) from Wal Mart) and nicotine (Sigma Aldrich) treatments were administered at concentrations of 10 mg/L for the fluoxetine and 1 mg/L for the nicotine. These doses vary from standard doses in the zebrafish literature. Fluoxetine is often given at concentrations up to 100 μg/L, but administered chronically over a two-week period (Egan *et al.*, 2009). We used a higher dose than the chronic concentrations reported in the literature, however it is important to note that this choice could yield non-target effects due to higher concentrations. Nicotine is often administered as a ditartrate salt at concentrations up to 100 mg/L (Levin *et al.*, 2007). We used pure nicotine and were unsure at the time of the experiment how the two forms compared with each other. We chose our dose based on the LD50 concentration (4 mg/L) to avoid lethal effects on our subjects. Each drug was dissolved in system water to make a working solution each morning of administration. A third treatment of only system water served as a control. Fish were netted from their home tank and immediately placed into a beaker containing 100mL of one of the three treatments. After three minutes of exposure to the drug dose, the fish were transferred to an open field test tank filled with untreated system water for behavioral recording. Dosing and behavioral observations were made on one fish at a time and the treatment type and order were randomized across individuals.

4.3.3 *Behavior Assay & Video tracking*

The fish were placed in a rectangular tank with interior dimensions measuring 25cm wide, 12 cm high (from water level to bottom), and 6 cm thick (front to back). The volume of water in the tank was approximately 2 L. Each fish was filmed for four minutes (240 seconds) at 25 frames/second beginning from the time that the subject entered the water. The camera and operator were hidden behind a blind during the recorded observation time. The tank was backlit with an opaque diffuser for the

purposes of creating a silhouetted object for motion tracking. After the four-minute period, the fish was netted out of the test tank, placed into its own individual 1.5 L housing and returned to the main system. Observations were recorded over three days between the hours of 10:00 am and 2:00 pm. After all subjects had been recorded, weight and standard length measurements were obtained by first anesthetizing the individual in MS-222 solution and blotting excess water with a paper towel. At this time, we also recorded the sex of the individual using visual cues: larger, rounded abdomen and dull fins for females, smaller and leaner abdomen and bright yellow fins for males.

Videos were digitally tracked using ProAnalyst® (Xcitex, Cambridge, MA). Tracking began with the frame in which the fish hit the surface of the water, and proceeded to the end of the video. The tracking data were imported into R for cleaning and processing. Each track was truncated to exclude the first five seconds during which the fish would sink, but remain otherwise motionless, as it recovered from the initial shock of being released from the net. Tracks were then standardized to 4 minutes, or 6000 frames. We computed velocity from the x-y data points. Since the tracking software did not always track the exact same position on the fish, velocity was estimated using the change in coordinates between two frames before and two frames after the focal frame. This algorithm sufficiently smoothed the speed data while retaining detail at small time intervals.

4.3.4 *Analysis*

Freezing — Freezing time was defined as the time a subject spent motionless on the bottom of the tank. We defined motionless as maintaining a velocity of less than .01 cm/frame for more than 20 consecutive frames. Any short bursts of motion flanked by considerable freezing times were verified in the video to be true motion. If a time period of activity was less than 40 frames, it was re-categorized as part of the freezing time as this motion is likely an artifact of the automated tracking. The freezing time was then calculated by counting the total number of frames marked as frozen. We also characterized freezing behavior as a binary 'yes' or 'no' response

as the propensity to show any freezing behavior can be considered an independent response from duration of freezing.

Speed — We computed the average speed for each individual using only the active (non-frozen) data points from the swim tracks. Freezing behaviors can cause a high degree of correlation with average swimming behaviors such as speed and depth use. Since we analyzed freezing behavior separately, we chose to analyze the effects of anxiolytic drugs on velocity during active swimming only. We predicted that anxious individuals would swim slower on average than less anxious individuals (Gerlai *et al.*, 2009). In addition, we computed the variance in velocity for the active data points. The variance represents the consistency in swim speed within an individual. Less anxious individuals should display more consistency in velocity than more anxious individuals due to erratic behavior (Gerlai *et al.*, 2009).

Depth — Depth was measured by the y-coordinate position in the swim track. We aligned the y origin with the water's surface, and measured depth as increasing negatively toward the bottom of the tank. As with velocity, depth variables were calculated using only the active points in the tracks. We analyzed both the mean and variance (consistency) of depth. We predicted that anxious individuals should spend more time near the bottom of the tank and should have a lower variance in depth (Levin *et al.*, 2007; Oswald *et al.*, 2013b). Conversely, we predicted that less anxious individuals will position themselves higher in the water on average and spend more time exploring the entire tank, resulting in a larger variance in depth usage. We also quantified at the number of times an individual entered the top half of the tank from the bottom half. Such behavior may be indicative of anxiety, as anxious individuals tend to enter the top half less often than less anxious individuals (Egan *et al.*, 2009). We also expected that anxious individuals would spend a smaller proportion of active swimming time in the top half, and that they would exhibit a longer latency to enter the top from the beginning of the trial (Egan *et al.*, 2009). The threshold between the top and bottom halves was defined at -6 cm.

Horizontal Place Preference — The width of the tank was divided into three equal sections and the proportion of time in the middle section calculated to differentiate preference to be located in the center versus the edge of the test environment. While we had clear expectations for location preference with respect to depth, it was unclear at the time of analysis whether the middle or the edges represent a "safe" zone with respect to horizontal preference. Experiments with rodents have found that stressed individuals prefer the edges of their arenas (thigmotaxis), but that this behavior is analogous to stressed fish preferring the bottom (Levin *et al.*, 2007).

Statistical Analysis — We began with a MANOVA on all continuous variables where all individuals could be included. We applied transformations where they were required to conform to the assumptions of normality in the residuals (see Results for transformations). The initial model included the effects of weight as a covariate, sex, drug treatment, and the sex by drug interaction. No significant effect of weight was observed, and there was no improvement to the model by keeping the term, so we excluded weight from all subsequent analyses. We performed individual ANOVAs on each of the continuous variables. Since freezing occurrence is a binary response, it was analyzed using a logistic GLM to estimate and compare the probability that an individual will freeze based on a given treatment group. In order to accurately assess freezing time, only individuals that froze were used (N=52). All tests were performed with a significance threshold of $\alpha = 0.05$. When a significant effect of drug treatment was detected, we performed pairwise T-tests among the three treatments with a Tukey correction.

4.4 RESULTS

We recorded observations from 90 individuals divided equally and randomly among the 3 treatments (n=30 per treatment). Due to complications with the filming, observations on three of the individuals had to be removed leaving us with final sample size of 87 individuals broken down by treatment and sex as follows: 29 in the control

treatment (17 females and 12 males), 30 in the fluoxetine treatment (16 females and 14 males), and 28 in the nicotine treatment (14 females and 14 males).

4.4.1 *Multivariate*

The full model Type-II MANOVA included the effects of weight, sex, drug treatment, and the sex by drug interaction on average depth, variance of depth, average speed, variance of speed, percent of time spent in the top half, number of crosses into the top half, latency to enter the top half, and proportion of time spent in the middle third horizontally (ie, away from the edges). There was a non-significant effect of sex ($\Lambda = 0.17896$, $F_{8,73} = 1.9889, p = 0.05974$) and a significant effect of drug treatment ($\Lambda = 0.56646, F_{16,148} = 3.6551, p = 0.00001305$) on behavior, but no significant interaction. There was no significant effect of weight as a co-variate, and including weight in the model showed no improvement over removing it ($\Lambda = 0.95793, F_{5,76} = 0.66755, p = 0.6492$). With the reduced model, we observed a significant effect of sex ($\Lambda = 0.22404, F_{8,74=2.6707}, p = 0.01237$) and drug treatment ($\Lambda = 0.56659, F_{16,150} = 3.7057, p = 0.00001014$). Therefore, for all subsequent analyses we considered only the effects of sex, drug treatment, and the interaction term.

4.4.2 *Individual components of behavior*

We observed no significant interactions between sex and drug treatment in any of the individual behavior components (see Table 4.1), consistent with the results of the MANOVA above. All components indicated a significant effect of drug treatment ($p < 0.05$) except for freezing occurrence and freezing duration. The subsequent descriptions describe the results of the post-hoc pairwise comparisons of the drug treatments using the least-squared means and Tukey adjusted p-values based on 3 tests. We also observed a significant effect of sex with regard to average swimming speed ($F_{1,81} = 10.7178, p = 0.001562$) and consistency (variance) of swimming speed ($F_{1,81} = 13.9196, p = 0.0003528$). Males were on average faster than females, but also exhibited less consistency in their swimming speeds. These were the only instances in which the sexes differed in their behavior.

Freezing behavior — Freezing behavior is a commonly observed anxiety related behavior in zebrafish (Egan *et al.*, 2009). Of the 87 individuals observed, 52 exhibited freezing behavior. Though males tend to be more likely to freeze than females on average, this difference was not statistically significant ($\chi^2 = 3.7866, p = 0.05167$). We also failed to observe a significant effect of drug treatment on freezing occurrence ($\chi^2 = 3.7964, p = 0.14983$) as well as a sex by drug interaction ($\chi^2 = 0.3949, p = 0.82083$). For freezing duration, or latency to explore, we only included the 52 individuals that exhibited freezing behavior (control: F=11, M=10; fluoxetine: F=7, M=8; nicotine: F=6, M=10). This improved the assumptions of normality required for the ANOVA. Results of the type II ANOVA suggest that neither sex nor drug treatment have any significant effect on freezing duration (Sex: $F_{1,46} = 1.9604, p = 0.1682$; Drug: $F_{2,46} = 1.3707, p = 0.2641$). Figure 4.1 shows the results of freezing behaviors.

Speed — When analyzing only the active swimming data from the trials, fish from both drug treatments appear to reduce their average swimming speed compared with the control, however this pattern is only significant in the nicotine treatment ($t = 3.373, p = 0.0032$, see figure 4.2). Drugged fish also swam at a more consistent speed than the undrugged control fish ($F_{2,81} = 4.0654, p = 0.0207731$), but again this trend was only significant in the nicotine treatment ($t = 2.818, p = 0.0166$).

Depth — Both the subjects dosed with nicotine and fluoxetine positioned themselves higher in the water column than the control fish (nicotine: $t = -2.462, p = 0.0417$; fluoxetine: $t = -4.711, p < .0001$). Fish dosed with fluoxetine explored more of the water column than control subjects ($t = -3.172, p = 0.0060$). Subjects dosed with nicotine also exhibited more variation in depth use on average than the control subjects, but this difference was not significant (see figure 4.3).

We also divided the tank into two discrete and equal vertical zones and compared the proportion of time spent in the upper half (figure 4.4). Subjects dosed with fluoxetine tended to spend more than twice as much time in the upper half as control subjects and this difference is significant ($t = -3.883, p = 0.0006$). Subjects in both

the nicotine and fluoxetine treatments exhibited a reduced latency time to first enter the top half than control subjects (nicotine: $t = 3.333, p = 0.0037$; fluoxetine: $t = 2.652, p = 0.0258$). When comparing the total number of visits to the top half, only the fluoxetine group showed a significant increase over the control ($t = -3.801, p = 0.0008$).

Horizontal Place Preference — All subjects spent most of their time near the edges avoiding the center (figure 4.4), consistent with the concept of thigmotaxis. However, subjects dosed with fluoxetine spent less time in the center and more time near the edges than subjects in the control and nicotine treatments ($t = 3.257, p = 0.0046$) which is inconsistent with a reduction in thigmotaxis resulting from a reduction in stress. At this time we are unsure how these results relate to anxiolytic properties of the drug.

4.5 DISCUSSION

4.5.1 *Differences in fluoxetine and nicotine behavioral responses*

Small prey fish such as zebrafish tend to behave in such a way as to reduce risk of predation. When placed in a novel open field, such behavioral strategies include diving to the bottom and remaining motionless (Egan *et al.*, 2009), and avoiding potentially risky locations such as the surface of the water (Wilson and Godin, 2009; Oswald *et al.*, 2013b). Exposure to anxiolytic drugs alters these behaviors in ways that may indicate an association between anxiety related behaviors and risk management. We observed a decrease in bottom dwelling and an increase in time spent in the top half of the tank in fish exposed to fluoxetine (figures 4.3 and 4.4). This is consistent with patterns observed by Egan *et al.* (2009) who also report an increased use of the top of the water column by zebrafish exposed to fluoxetine. However, the study by Egan *et al.* (2009) also reports a reduction in freezing bouts and freezing time, a pattern we failed to observe. One explanation for this discrepancy could be differing effects of chronic and acute dosing. Fluoxetine is metabolized into norfluoxetine, its active metabolite, in the liver by cytochrome P450 enzymes (Rasmussen *et al.*, 1995).

It then travels through the bloodstream to the brain where it blocks the reuptake of serotonin (Beasley *et al*., 1992). Metabolism of the drug could delay its effect until after the animal had already recovered from freezing behavior.While most fluoxetine studies utilize chronic exposure, we have shown that similar behavioral changes can occur with just a single acute dose. Acute exposure to fluoxetine has also been shown to reduce cortisol levels of zebrafish exposed to a stressful environment (de Abreu *et al*., 2014). We speculate that the behaviors we observed may be due to a reduction in physiological stress response resulting from exposure to the drugs, though more experiments are needed to confirm this.

We observed changes in swimming speed, average depth, and latency to enter the top in fish exposed to nicotine. Fish exposed to nicotine were quicker to enter the top and swam higher in the water column on average compared to control fish. This is consistent with a reduction in anxiety related behaviors as seen in the fluoxetine treatment group. Exposure to nicotine and fluoxetine appeared to decrease swimming speed while increasing the consistency at which the fish swam. The increased consistency (reduction of individual variance) might be explained by a reduction in anxiety, where individuals that are calm should move at a fairly normal and constant pace, while anxious individuals may constantly alter their swimming speeds in an erratic fashion Gerlai *et al*. (2009). Egan *et al*. (2009) reported an increase in average swim speed with exposure to fluoxetine, which contrasts with our observations of slower average swim speeds with exposure to either fluoxetine or nicotine. Sackerman *et al*. (2010) suggests that nicotine may have sedating effects which could account for the slower swim speeds. However, we also observe slower average swim speeds in the fluoxetine treatment, and though the difference is not statistically different from the control, it is also not different from the nicotine effect. We observed a similar pattern in the nicotine treatment with respect to the time spent at the top and the variation in depth use, where the nicotine treatment was statistically indistinguishable from both the control and the fluoxetine treatments. In these two instances, it is likely that the nicotine is having an anxiolytic effect, but that we used too low of a dose to observe an effect that is different from the control. Sackerman *et al*. (2010) also failed to observe an effect of nicotine on swim depth using a low dose of 25 mg/L, but noted that

higher doses such as 50 mg/L and 100 mg/L do produce a significant effect (Levin *et al.*, 2007). Our dose of 1 mg/L is noticeably lower than other studies of nicotine in adult Zebrafish, accounting for the our use of pure nicotine liquid while the other studies used a nicotine tartrate salt (Levin *et al.*, 2007; Sackerman *et al.*, 2010). It should be noted that the relationship between the tartrate salt and pure form is about 0.325, such that a concentration of 100mg/l of the tartrate equates to a concentration of 32.5mg/l of pure nicotine (Matta *et al.*, 2007).

Both nicotine and fluoxetine affected behavior in ways indicative of a reduction of anxiety. However, the two drugs also appear to affect different components of behavior. Nicotine had its highest effect on swimming speed, while fluoxetine mostly affected behaviors related to vertical positioning. This suggests that anxiety is not a simple condition, but rather a complex idea encompassing a number of components that are sometimes correlated, but not always connected. These behavioral components may be separated by different physiological pathways which could explain why different classes of drugs affect specific behaviors.

4.5.2 *The effect of sex on behavior and drug efficacy*

Sex differences in anxiety behaviors have been described in a number of species including rats (Mehta *et al.*, 2013), stickleback (King *et al.*, 2013), and guppies (Harris *et al.*, 2010). While most of these studies find that males are typically more bold (less anxious) than females, our lab has previously observed the opposite trend in the Scientific Hatcheries strain of zebrafish with regard to association with humans, vertical position, and feeding latency in individual home-tank observations (Oswald *et al.*, 2013a,b; Benner *et al.*, 2010). These differences are the basis for our inquiry as to whether substances known to alter these behaviors might work at different efficacy in males and females. In the present study, we only observe significant behavioral differences between the sexes with respect to swimming speed. While males swim slightly faster than females, it's the females that swim at a more constant rate. In addition, males seem to show a higher probability to exhibit freezing behavior across all three treatments, and even though this trend isn't statistically significant, it still

leads us to suggest that males **could be** behaving with higher anxiety levels than females.

With the active swimming behaviors, we fail to observe differences between the sexes, and across all of the behaviors, the data do not suggest any indications of sex-specific effects of either drug. There is plenty of literature in mammalian models that contradict these findings (Mitic *et al*., 2013; Leuner *et al*., 2004; Lifschytz *et al*., 2006; Monleón *et al*., 2002; Hodes *et al*., 2010). One possible explanation for our lack of sex-specific effects stems from our general lack of sex differences in the behaviors analyzed, and perhaps a baseline difference in behavior is necessary to elicit a sex-specific effect. The results of Mitic *et al*. (2013); Leuner *et al*. (2004) and Lifschytz *et al*. (2006) in rats all observe sex-specific responses to fluoxetine only when the sexes differed in behaviors without the drug. We do not have adequate data to confirm this explanation and more experimentation along with physiological data would be necessary.

Another possible explanation for our lack of sex-specific drug effects could be our choice of dose. Our choice of 1mg/L of nicotine is quite low compared with other studies in zebrafish (Levin *et al*., 2007; Sackerman *et al*., 2010), and while our dosage of fluoxetine was much higher than is typically reported (Egan *et al*., 2009; Wong *et al*., 2013), it is typically administered chronically. We would also like to note that the sex-specific results of Faraday *et al*. (1999) utilizing nicotine in rats was only observed in one of the two strains used. Zebrafish are highly genetically diverse (Parichy, 2015) and strain differences in behavior (Benner *et al*., 2010; Egan *et al*., 2009) and drug efficacy (Sackerman *et al*., 2010) have been reported. Therefore the possibility exists for sex-dependent drug effects to be observed in another strain.

Finally, we cannot dismiss the possibility that zebrafish simply don't exhibit sex-specific effects with fluoxetine or nicotine. While there is no literature in this species to compare our results with, a recently published study utilizing medaka (*Oryzias latipes*), another small teleost fish from southeast Asia, fails to find sex-specific effects of chronic fluoxetine on many of the same behaviors described in the present study (Ansai *et al*., 2016). More research is necessary to confirm any of the explanations given for our lack of observed sex-drug interactions. The absence of studies consider-

ing sex-specific effects of drugs is problematic if zebrafish are to remain a relevant model of pharmacology research. The topic has become a concern in all animal models that NIH is going to start requiring all animal research to include sex as part of the study unless deemed unnecessary (Clayton and Collins, 2014). If it turns out that strain is a major factor influencing our results, then the abundance of genetically diverse populations could make zebrafish an exciting tool to aid in the growing field of pharmakogenetics and personalized medicine in which genetic background, among other traits, will be important for determining what drugs will be most effective for treating disorders.

4.6 TABLES

TABLE 4.1: Table of P-values summarizing results. **Bold** items are considered to show significant differences among treatment groups ($\alpha = 0.05$). P-values for the Fluoxetine and Nicotine columns represent pairwise comparisons with the control and are adjusted using the Tukey method for 3 comparisons.

	Sex	Drug	Interaction	Fluoxetine	Nicotine
Freezing Time	0.17	0.26	0.76	0.99	0.33
Average Speed	**0.00**	**0.01**	0.63	0.13	**0.00**
Variance Speed	**0.00**	**0.02**	0.47	0.57	**0.02**
Average Depth	0.98	**0.00**	0.94	**0.00**	**0.04**
Variance Depth	0.62	**0.01**	0.91	**0.01**	0.19
Proportion in Top	0.86	**0.00**	0.72	**0.00**	0.22
Crosses to Top	0.57	**0.00**	0.89	**0.00**	0.45
Latency to Top	0.64	**0.00**	0.89	**0.03**	**0.00**
Proportion in Center	0.19	**0.00**	0.36	**0.00**	0.99

4.7 FIGURES

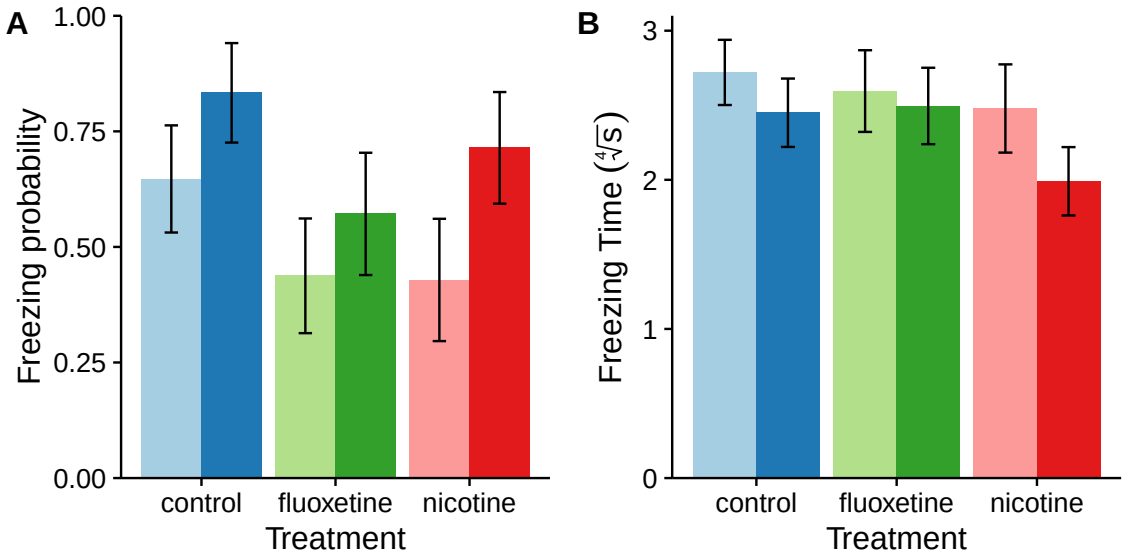

FIGURE 4.1: Freezing behaviors (motionless at the bottom of the tank) appear not to be affected by exposure to fluoxetine or nicotine. These graphs show the probability of freezing ± SE. (A) and the mean time spent frozen ± SE (B) for both sexes in each drug treatment group. Females are represented as light bars and males as dark bars. The freezing probability was calculated from a logistic GLM and transformed back into probabilities for this figure using the 'lsmeans' package in R. Freezing time was transformed using a fourth root in order to meet the assumptions of normality in the ANOVA.

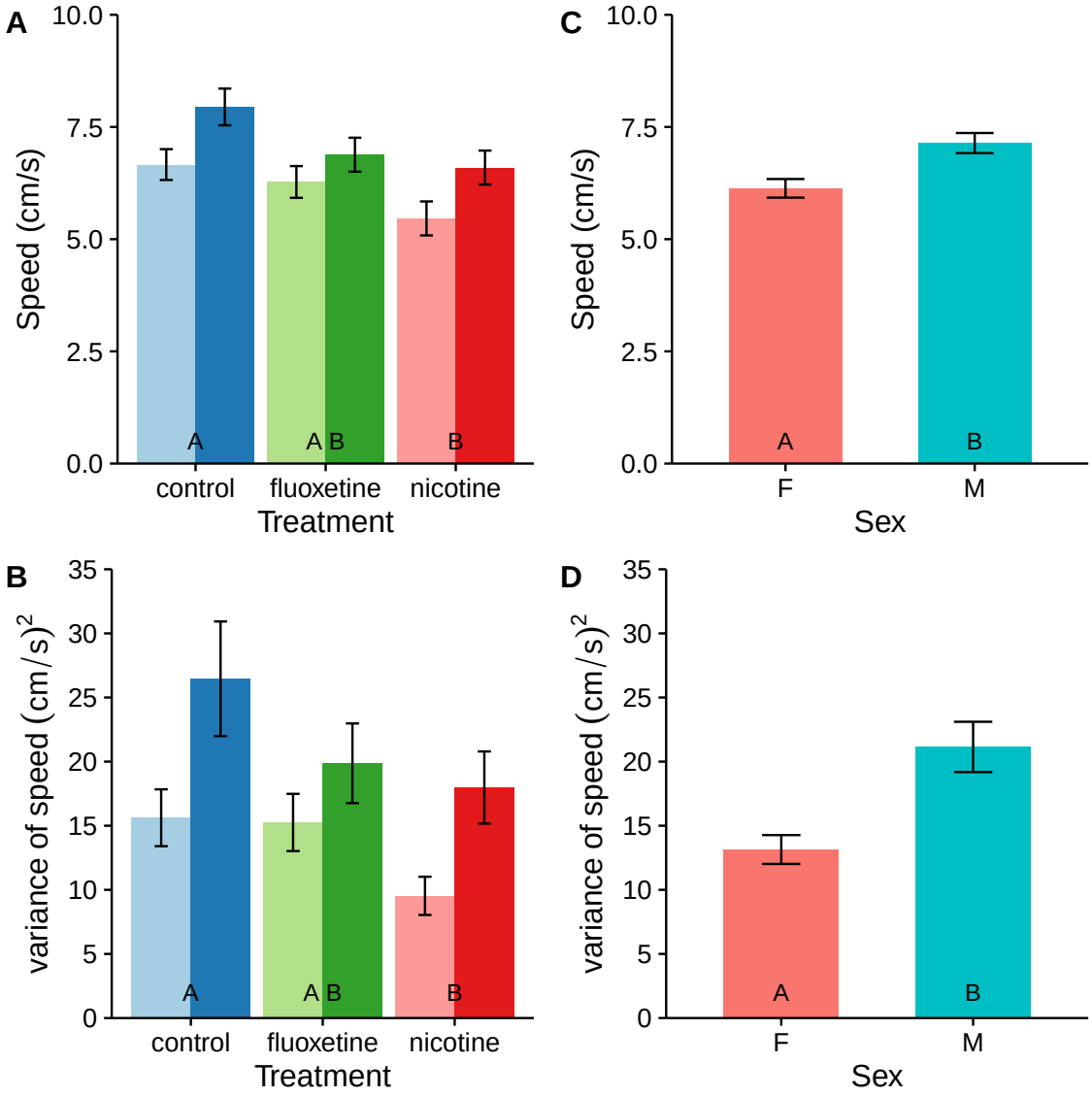

FIGURE 4.2: Average swimming speed (top) and consistency (individual variance) of swimming speed (bottom) are affected by fluoxetine and nicotine (A & B) as well as by sex (C & D). The fluoxetine treatment is not statistically different from the control, but is also not different from the nicotine treatment. Means $\pm$ SE are reported. Results of the Tukey pairwise comparisons of drug treatment groups are delineated with letter groupings where similar letters represent a non-significant difference between treatments ($p > 0.05$). In panels A & B, females are represented with light bars and males with dark bars.

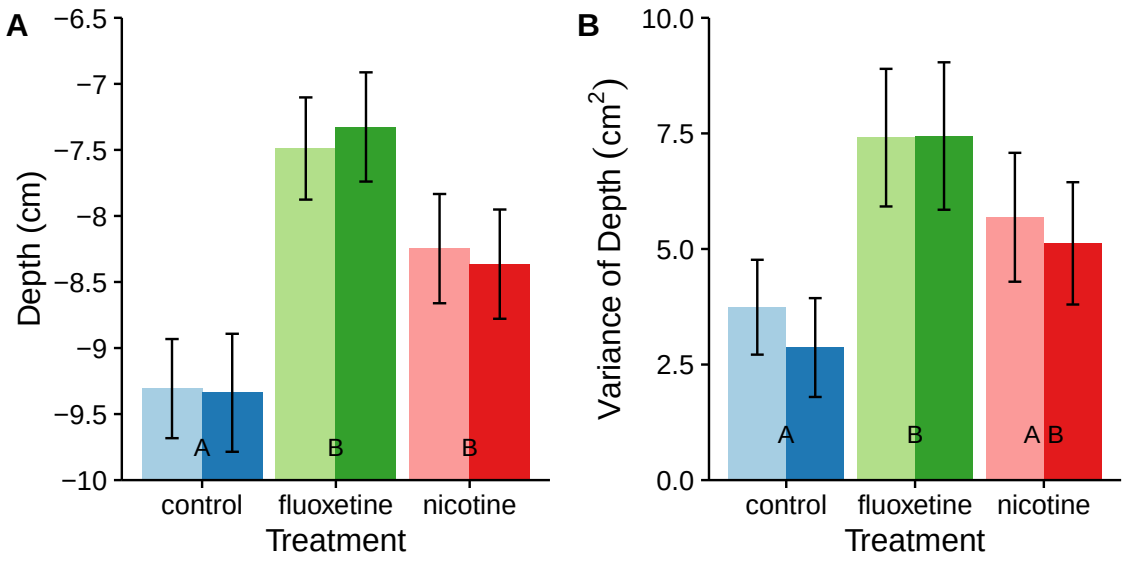

FIGURE 4.3: Average swimming depth (A) and average consistency (individual variance) of vertical usage (B) are affected by fluoxetine and nicotine. The nicotine treatment was not significantly different than the control with depth variance, but was also not different from the fluoxetine treatment. Means $\pm$ SE are reported and the results of the Tukey pairwise comparisons of drug treatment groups are delineated with letter groupings where similar letters represent a non-significant difference between treatments ($p > 0.05$). Sex is distinguished by females with light bars and males with dark bars.

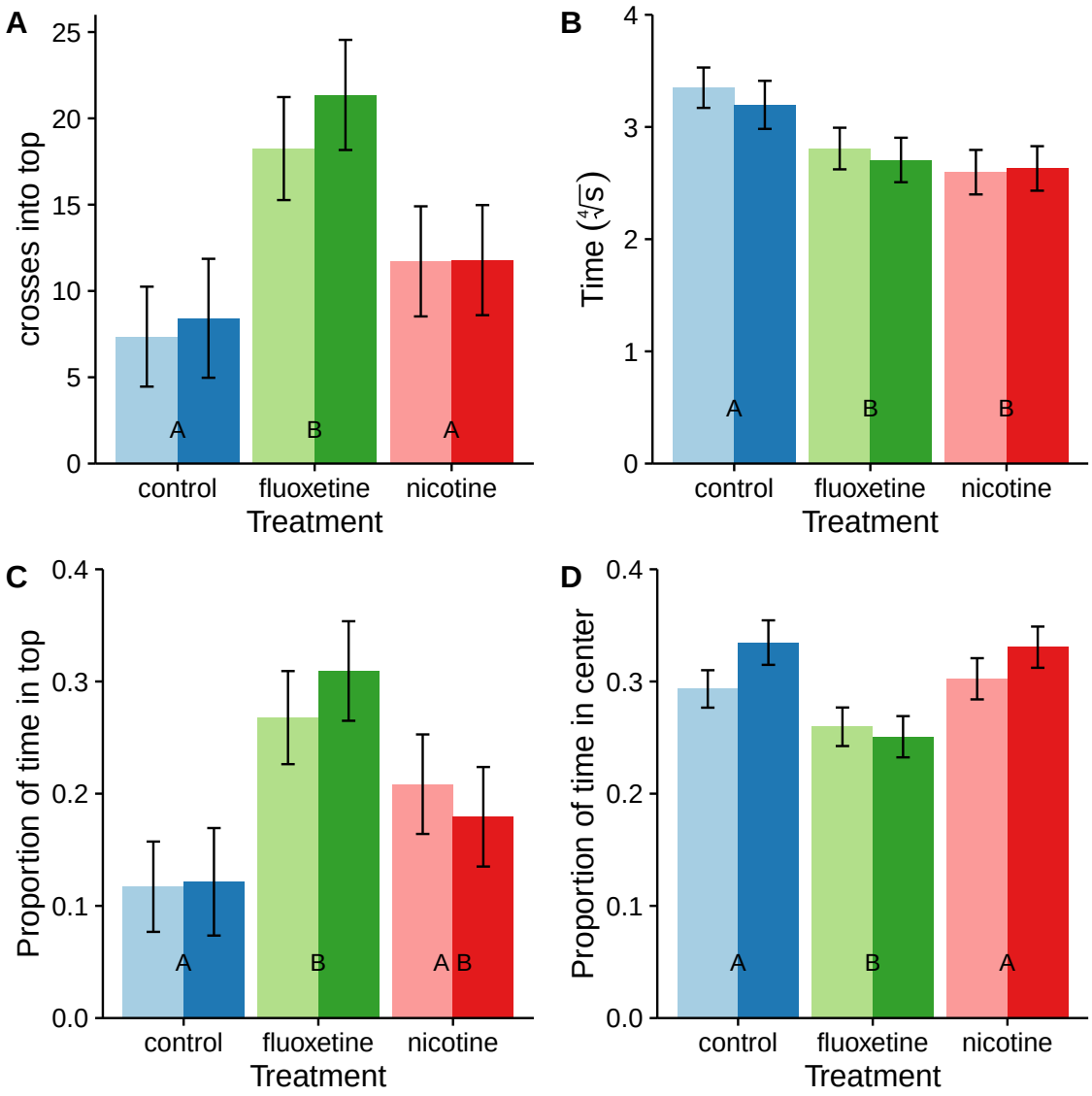

FIGURE 4.4: Average number of entries into the top half (A), latency to enter the top half (B), proportion of time spent in the top half (C), and proportion of time spent in center (D). Means ± SE are reported and the results of the Tukey pairwise comparisons of drug treatment groups are delineated with letter groupings where similar letters represent a non-significant difference between treatments ($p > 0.05$). Sex is distinguished by females with light bars and males with dark bars. Latency to enter the top half is transformed using a fourth root transformation in order to meet the assumption of normality in the ANOVA.

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APPENDIX A

Supplementary Information to Chapter 4

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