ENVIRONMENTAL AND NEUROGENETIC FRAMEWORK OF MATE-CHOICE RELEVANT BEHAVIORS IN *XIPHOPHORUS* FISHES

A Dissertation

by

PABLO JOSE DELCLOS

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Chair of Committee,	Gil G. Rosenthal
Committee Members,	Adam G. Jones
	Georgianne W. Moore
	Kirk O. Winemiller
Head of Department,	Thomas McKnight

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ABSTRACT

Mate-choice related behaviors are highly variable and sensitive to a wide array of environmental and social factors. Therefore, the stability of a given behavior can largely depend on the level of environmental variability within a population. My research aims to understand the mechanisms whereby behaviors are influenced by social conditions and other environmental factors. I first describe the level of preference variation within a population of swordtail fish across time and small-scale space. Over three years, I found marked, but highly variable differences in female mating preferences between sampling sites. These results highlight the importance of accounting for small-scale heterogeneity when modelling and measuring the evolution of mating preferences and display traits, and may help explain why empirical measures of sexual selection via mate choice are often very weak. Next, I take advantage of the socially-sensitive olfactory mating preferences of female *Xiphophorus birchmanni* to elucidate the neurogenetic mechanisms by which these preferences are learned. I compare whole brain and olfactory epithelial gene expression profiles of females that were socially isolated from adults, or exposed to either adult conspecifics or members of the closely related *X. malinche*. I found that conspecific-exposed females experienced an upregulation of genes with functional roles in immune response and the detection of visual and olfactory cues. Meanwhile, heterospecific-exposed females showed upregulation of genes involved in neurogenesis and synaptic transmission, suggesting a prioritization of processing sensory cues. Lastly, I used this same system to determine the role of cultural transmission - the intergenerational transfer of information - in shaping male and female

personalities. I found that both males and females learn to develop boldness behaviors similar to those of their exposure models. These culturally-sensitive personalities are likely to have important mate choice and evolutionary implications. Together, these studies describe the complex direct and indirect relationships between the environment and female mate choice.

DEDICATION

Per el meu avi, Lluís Delclòs.

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CHAPTER I

INTRODUCTION

A major goal in evolutionary biology is to determine the behavioral and genetic mechanisms that drive reproductive isolation and the maintenance of species. In many closely related species, reproductive isolation occurs due to the evolution of behavioral barriers, which often develop long before postmating isolation can evolve (Grant and Grant 1997). Premating isolation via mate choice depends on divergent behavioral phenotypes between species. Various evolutionary models suggest a set of possible explanations for the formation of premating barriers. For example, ecological heterogeneity could result in divergent mating preferences for ecologically-relevant traits (Schluter 2001, Reynolds and Fitzpatrick 2007), or the development of divergent personalities that are locally adaptive (Ingley and Johnson 2014). Alternatively, a premating barrier could arise from the co-evolution of divergent mating signals and mate preferences from signal-receiving individuals, irrespective of ecological function (Verzijden, Lachlan et al. 2005). Mating preference functions and personalities- consistent differences in behavior over time and across contexts (Ingley and Johnson 2014)- are often environmentally shaped and can be maintained through indirect genetic effects such as learning (Servedio, Sæther et al. 2009). Therefore, the stability of a given behavior can largely depend on the level of environmental variability within a population. My research aims to understand the mechanisms whereby behaviors are coupled to fluctuating environmental factors, or context-dependent.

Context-dependent mating preferences can change an individual's relative fitness by altering mate choice dynamics in response to its environment or physiological state. For example, in the lesser wax moth, *Achroia grisella*, females prefer to mate with males that produce calls with higher pulse rates. However, gene × environment (G × E) interactions result in different pulse rate thresholds females can detect relative to temperature, which could result in differential male reproductive success across environments (Rodríguez and Greenfield 2003). These G × E interactions in response to fluctuating environments can therefore maintain genetic variance in receiver preferences and signaler traits, providing a resolution to the paradox of the lek, the observation that choosers continue to choose courters based on additive genetic benefits for the offspring, despite the fact that directional mate choice depletes additive genetic variation in courter traits, thus preventing female choice from resulting in any genetic benefit (Kirkpatrick and Ryan 1991).

Context-dependent mate preferences have been shown to occur in response to several environmental variables [transmission spectra: (Seehausen and van Alphen 1998, Maan, Seehausen et al. 2010); seasonal effects: (Chaine and Lyon 2008); and social environment: (Alonzo and Sinervo 2001, Verzijden and Rosenthal 2011)], highlighting the ubiquity and advantages of plastic mate preferences in response to social and ecological changes in nature. However, the evolution of secondary sexual traits through female mate choice requires homogeneity in preference functions within populations and consistency in this function throughout time (O'Donald 1983, Mead and Arnold 2004). Therefore, plasticity in mating preferences could affect the strength of sexual selection

and evolution for elaborate traits (Janetos 1980, Gibson and Langen 1996, Cotton, Small et al. 2006, Rodríguez, Boughman et al. 2013).

In order to understand the role of sexual selection via mate choice on a given trait within a population, it is necessary to determine the level of heterogeneity, in space and time, of a given preference for that trait. However, few studies have ever measured the standing temporal and spatial variation in mating preferences within a natural population. In Chapter II, I take advantage of a unique system to decouple temporal, spatial, and genetic effects on preferences in the wild. Hybrid swordtails (*Xiphophorus birchmanni* x *X. malinche*) at the Calnali-mid locality in Calnali, Hidalgo, Mexico fall into two assortatively-mating genetic clusters distributed between two adjacent pools separated by a small natural barrier less than 2 meters high. This distance is small enough that individuals from each pool are genetically indistinguishable (Culumber, Fisher et al. 2011, Schumer, Powell et al. 2017). Over three successive years, I measured pool-level differences in a previously established female olfactory preference for male nutritional condition (Fisher and Rosenthal 2006a, Fisher and Rosenthal 2006b), as well as differences in male phenotypic distributions and female reproductive allotment and condition. The results from this study join previous work suggesting that heterogeneity in expressed preferences could weaken sexual selection. Population structure and environmental heterogeneity are ubiquitous in nature. These results highlight the importance of replicating mate choice studies across both time and even small spatial scales in order to gain a better understanding of the contribution a mating preference for a given trait has on sexual selection.

Despite the large behavioral literature on context-dependent female mate choice, there is relatively little research focusing on the neurogenetic mechanisms that link the environment to a given mating preference. Mating preferences are often developed through learning processes, and can thereby be influenced by an individual's social environment throughout life (ten Cate 1987, ten Cate, Verzijden et al. 2006, Verzijden and Rosenthal 2011). An individual's exposure to different social cues can elicit preferences either for or against a familiar trait, which can have important evolutionary consequences by promoting assortative mating through sexual imprinting or the breakdown of reproductive barriers through developed antipathy. Therefore, determining the proximate mechanisms underlying learned mating preferences is of vital importance to understanding their evolutionary implications.

There are two main neural mechanisms through which the social environment could affect female mate choice. First, a social stimulus could result in increased sensitivity to the signaler cues at the sensory periphery, making the individual more sensitive to the familiar phenotype (Nevitt, Dittman et al. 1994, Harden, Newton et al. 2006). Second, social exposure to a specific stimulus could affect the downstream processing of signaler cues in the brain itself (Corotto, Henegar et al. 1994, Yamaguchi and Mori 2005, Okuyama, Yokoi et al. 2014). In Chapter III, I examine the behavioral and neurogenomic effects of the social environment on *Xiphophorus birchmanni* females. Specifically, I assess how exposure to adult conspecifics, exposure to the sister species *X. malinche*, and social isolation from adults affect female olfactory preferences for conspecific vs. heterospecific males. I then conduct RNA-sequencing analyses on

dissected whole brain and olfactory epithelial tissues to identify groups of genes that are differentially expressed among exposure groups. The results from this study are among the first of its kind, as they describe the transcriptomic imprint that social exposure has on the sensory periphery and brain. This study also identifies avenues for future research aiming to elucidate the neurogenetic framework of learned mating preferences.

Whereas a large portion of studies stressing the importance of behavior in speciation focuses on the importance of mating preferences and their sensitivity to genetic and environmental effects, other behaviors can indirectly have important consequences that influence mate choice dynamics and, ultimately, speciation. Recent studies on individual and population-level personality have highlighted its importance in mate choice dynamics and evolutionary biology (Sih, Bell et al. 2004, Sih, Cote et al. 2012, Wolf and Weissing 2012) and its potential to affect speciation processes. For example, boldness correlates with reproductive success, along with other fitness-related traits (Dingemanse and de Goede 2004, Brown, Jones et al. 2005, Bell and Sih 2007, Ariyomo, Carter et al. 2013, Boulton, Grimmer et al. 2014). Furthermore, bold and aggressive individuals can be more likely to disperse from native habitats and invade novel habitats than shy individuals (Cote, Clobert et al. 2010, Sih, Cote et al. 2012). Repeated bottleneck dispersions from bolder individuals can eventually result in differential selection pressures acting on these populations relative to the source population.

Furthermore, divergent personalities could drive assortative mating within populations, as in the case of the great tit *Parus major*, a bird for which it has been

shown that individuals mate assortatively according to boldness behavior (Both, Dingemanse et al. 2005). Personalities could also covary with courtship or mating preferences and ultimately play a direct role in sexual selection via mate choice. Whereas individual personalities are often assumed to be fixed in time and context (Beekman and Jordan 2017), this is not necessary (Galhardo, Vitorino et al. 2012): plasticity in personalities, like plasticity in mating preferences, can be adaptive in fluctuating environments that alter the reproductive success of specific behavioral phenotypes. In Chapter IV, I examine how social environment affects personality in both *X. birchmanni* males and females. Specifically, I assess the role of cultural transmission - the intergenerational transfer of social information - in shaping boldness behaviors, such as the time spent in open versus sheltered zones, in both *X. birchmanni* males and females. I then test for correlations between these measures of personality and male morphology and female mating preferences, respectively, in order to determine whether personality may play a significant role in shaping mate choice dynamics. The results from this study highlight the sensitivity of developed boldness behaviors to the social environment, providing strong evidence for the context-dependence of personalities. Furthermore, this is among the first studies to show that individuals can learn personality-related traits from heterospecific exposure models. I discuss the potential these plastic personalities have in the maintenance of reproductive isolation between species, as well as fruitful avenues of future research directly testing the role of personality in speciation.

CHAPTER II

HETEROGENEITY IN MATING PREFERENCES ACROSS TIME AND MICROHABITAT: A HARD LIMIT ON MEASURES OF MATE CHOICE?

Introduction

Phenotypic evolution is fastest if selection is consistent and directional over space and time. In particular, sexual display traits should evolve most readily if these are consistently favored by mating preferences (Mead and Arnold 2004). Nevertheless, empirical research amply shows that preferences in natural populations are highly variable over time (Chaine and Lyon 2008, Johnson, Stanis et al. 2013), and that mating preferences can vary according to changes in the environment or individual physiological state (Qvarnström 2001). Such labile preferences can result in dampened selection on a given secondary sexual trait, and may serve as a potential resolution to the paradox of the lek (Kirkpatrick and Ryan 1991). Small-scale habitat heterogeneity could play a particularly powerful role in maintaining behavioral variation within populations, since an individual's environment has been shown to shape individual preferences over very short timescales (Johnson, Stanis et al. 2013). Furthermore, previous studies have linked habitat heterogeneity to variation in morphology (Grether, Millie et al. 2001, Hoffmann and Shirriffs 2002, Gray, Dill et al. 2008) and behaviors relevant to mate choice (Zuk, Rotenberry et al. 2001). However, in many of these cases habitats were still separated by large enough distances that would limit migration, and the degree to which spatial heterogeneity in preference patterns exists in nature has not been examined.

Ultimately, the maintenance of many parapatric or sympatric species can often be attributed to behavioral differences that correlate with microhabitat use (Seehausen, Terai et al. 2008). However, in many of these cases genetic effects cannot be decoupled from environmental ones, as speciation has already occurred. Therefore, it is important to examine the role of microhabitat on behavior within genetic populations in order to understand its potential evolutionary implications.

I studied a population of hybrid swordtails (*X. birchmanni* x *X. malinche*) in two adjacent pools (UP and DOWN) that comprise the Calnali-mid locality in Calnali, Mexico over three successive summers (2013-2015). These pools are adjacent and isolated by < 2 m elevation by a small spillway, allowing for moderate levels of migration between the pools (Culumber, Ochoa et al. 2014). Despite their proximity, differences between the two pools in light availability and substrate drive differences in the availability of periphyton- a direct and indirect food source for swordtails (Arthington 1989, Maddern, Gill et al. 2011)- which could potentially lead to pool-level differences in nutritional condition. Therefore, I used a two-choice assay to measure natural yearly and microspatial variation in a previously established diet-dependent female preference for chemical cues of males differing in nutritional history (Fisher and Rosenthal 2006a). Previous literature suggests that females in better condition show stronger mating preferences (Cotton, Small et al. 2006). However, the opposite result has been found in female swordtails (Fisher and Rosenthal 2006a). This olfactory preference for well-fed males may be driven by differences in protein content of male diet, as seen in other poeciliids (Ward, Herbert-Read et al. 2011). I tested this by measuring female

olfactory preferences for males fed either a high or low protein diet, and I assessed how this preference varies according to sampling site and year.

Local adaptation can result in morphological differences between nearby populations (Houde and Endler 1990, Grether, Millie et al. 2001) that may drive observed differences in mating preference patterns. Behavioral differences at small spatial and temporal scales can be influenced by a myriad of extrinsic and intrinsic factors. Female condition (Fisher and Rosenthal 2006a) and social environment (Verzijden and Rosenthal 2011, Verzijden, Culumber et al. 2012) play particularly important roles in shaping female swordtail mating preferences. Therefore, I collected males and females from both pools between 2013-2015 to assess phenotypic differences between pools and across years to determine whether observed mating preference patterns were correlated with population-level male or female phenotypes.

Although the two pools do not differ in genetic composition (Culumber, Fisher et al. 2011), in 2015, parallel population-genomic analyses revealed that swordtails at both pools in Calnali-mid include distinct, reproductively isolated subpopulations of genetically *birchmanni*-like and *malinche*-like hybrids (Schumer, Powell et al. 2017). Therefore, in 2015 I genotyped females to determine whether preference differences could be attributed to genetic differences among females.

Materials and Methods

Preference trials

Wild-caught hybrid females were collected in May of 2013-2015 from each of

the two pools, UP and DOWN, described above and then acclimated to the lab environment in 40-liter tanks (n=8-11 per tank) for two weeks before being marked with colored elastomer on the dorsal and ventral sides of the caudal peduncle. In 2015, fin clips were used to prepare multiplexed shotgun genotyping (MSG) libraries to assign females to one of two genotype clusters [*malinche*-like or *birchmanni*-like (Schumer, Cui et al. 2014)]. Following tagging and fin clipping, females were allowed to recover for at least two weeks before testing. I tested 51, 45 and 45 UP females in 2013, 2014 and 2015, respectively (38, 32 and 29 responsive), and I tested 17, 18 and 40 DOWN females in 2013, 2014 and 2015, respectively (16, 16 and 26 responsive). Genotypic cluster identities were not known prior to conducting preference trials.

To create diet-dependent cue models, 10 male *X. birchmanni* from the Río Garces locality (Culumber, Fisher et al. 2011) were divided into two 40-liter aquaria and fed a Repashy gel premix diet containing either 55% (HP) or 35% (LP) proteinaceous content for one month. Female swordtails and other poeciliids have been previously shown to differentiate between male olfactory cues according to diet (Fisher and Rosenthal 2006a, Ward, Herbert-Read et al. 2011).

I used a well-established protocol to test female preference for male odors (McLennan and Ryan 1999, Fisher, Wong et al. 2006b, Verzijden and Rosenthal 2011). Association times with a given stimulus have been shown to be predictive of mate choice outcomes in swordtails (Walling, Royle et al. 2010). Briefly, to produce the olfactory cues, groups of 5 males were divided according to stimulus group and separately placed in 20 liters of carbon-filtered water. These males were visually

exposed to 5 females from their own population in adjacent tanks for 4 hours. The water from each of these tanks was then used as a stimulus cue. Female swordtails have previously been shown to respond to male olfactory cues using this design (Crapon de Caprona and Ryan 1990, Fisher and Rosenthal 2006b, Verzijden and Rosenthal 2011).

During preference trials, females were individually placed in a trial lane (75x19x20 cm) and allowed to acclimate for 20 minutes. Stimulus water was then dripped into each of the far ends of the tank until the end of the trial (600 s). Trial tanks were virtually divided into three zones of equal length. Once a female visited all three zones, the time in each zone was recorded for 300 s. Association time in each zone was used as a proxy for female preference, and has been shown to be predictive of female mate choice (Walling, Royle et al. 2010). If a female failed to visit both preference zones within 300 s, she was defined as unresponsive and excluded from analysis, as done in previous studies (Fisher and Rosenthal 2006a, Verzijden, Culumber et al. 2012). To account for potential side bias, each female was tested twice, back-to-back, with the sides from which cues were presented switched. I summed the association time in the two trials for analysis.

Within each given pool/year sample, I used Wilcoxon signed-rank tests to assess significance of mean differences in association time between two stimuli. A two-way ANOVA on net preference was used to test whether year, pool of origin, or their interaction significantly affected female preference. For the 2015 data, I also separately tested whether genotype, pool of origin, or their interaction affected mating preferences. All analyses were conducted in R.

To account for unequal sample sizes between groups, I used a resampling approach to compare net preferences between pools within a given year (Keselman, Wilcox et al. 2002) using the coin package in R. Briefly, 10,000 null datasets were generated to compare to the observed data. For each null dataset, site labels (UP or DOWN) were randomly assigned to the observed net preferences according to the observed sample size. The p-value reported signifies the proportion of scenarios where the difference in mean net preference between UP and DOWN was equal to or greater than the observed difference.

Power to detect a significant effect in behavioral trials

To determine the number of individuals needed to attain 90% power to detect strong mating preferences in Calnali-mid females, I performed power simulations (Schumer, Powell et al. 2017). Since these are the first behavioral data on these hybridizing females, I based my simulations on the results of a previous study quantifying preferences for diet-dependent male chemical cues in well-fed *X. birchmanni* females (Fisher and Rosenthal 2006a). This study found that, during a 300 s trial, well-fed females associated with well-fed male *X. birchmanni* chemical cues for 148 s on average (standard deviation = 62) and hungry male chemical cues for 65 s (standard deviation = 49). Female association times were simulated via random draws from normal distributions with these means and standard deviations truncated at 0 using the truncnorm package in R. Since preference measures are paired and the maximum association time with both stimuli in an individual trial is 300 s, I excluded all draws where the sum exceeded 300. For each replicate simulation, I drew paired simulated

association times until I had the number of measures equal to a given sample size *n*. I then tested whether these distributions were significantly different ($p < 0.05$) using a paired Wilcoxon test, and repeated this simulation for 1,000 replicates. The proportion of replicates with p-values less than 0.05 is the power at a given sample size *n*.

Based on this analysis, I determined that I have greater than 90% power to detect strong preferences for well-fed male chemical cues with a sample size of 14 or more females. I therefore aimed to sample at least this many individuals from each site. However, I recognize that this approach assumes parental levels of mating preferences, and that actual power may be lower if hybridizing females from Calnali-mid have weaker preferences than parentals.

Measurements of male morphology and female physiology

Over three successive years, I measured pool-level means of female and male phenotypes pertaining to 1) female physiological status and 2) the pool-level distribution of sexually selected male morphological traits, respectively, in order to assess whether either trait correlated with the observed preference patterns. In swordtails, the strength of female olfactory preferences for better-fed males is dependent on recent diet (Fisher and Rosenthal 2006a). Furthermore, reproductive status has also been shown to affect mating preferences in poeciliids (Gabor and Page 2003). Female swordtail preferences are also particularly sensitive to social cues, and can change the magnitude and direction of their preferences following changes in the social environment (Verzijden, Culumber et al. 2012). Therefore, for the purpose of this study, I focused on testing whether these two pool-level traits- male morphology and female physiology- were correlated with pool-

level variation in preferences across time. However, preference functions are sensitive to a myriad of other environmental cues beyond the scope of this study, such as light availability (Gamble, Lindholm et al. 2003), water transparency (Maan, Seehausen et al. 2010) and pH (Heuschele and Candolin 2007), and it is possible that these cues interact in a complex manner to shape mating preferences. One goal of this study was to assess the level of temporal and spatial variation in preference patterns within a genetic population and determine whether these patterns followed similar morphological or physiological variation within swordtails.

Male morphometric data

Wild-caught males (N=10-40 per pool per season) were lightly anesthetized with tricaine methanesulfonate and photographed for traditional morphometrics measurements using the ImageJ program (Abràmoff, Magalhães et al. 2004). Average measurements were taken for standard length, dorsal fin length, gonopodium length and sword extension length in mm. In addition, males were weighed to the nearest 0.01 g. Principal components analysis (PCA) was conducted on log + 1 transformed measurements using the rda function in the vegan package of R (center and scale = TRUE). Approximately 64.6% of total variance was explained in the first principal component, corresponding largely to male size, and this was used as an estimate of male phenotype in subsequent analyses (see Table A-1 for loading scores).

Female reproductive allotment and fat content analysis

Females (N=10 females per pool, UP or DOWN, per season) were collected in June 2013-2014 and October 2015, and preserved in 10% formalin. Reproductive tissue

was removed and weighed to determine reproductive allotment (RA), and a previously established petroleum-ether based method was used to determine fat content of the rest of the body as a proxy for body condition (Reznick and Braun 1987, Tobler 2008). Briefly, reproductive tissue was removed from females, and whole carcasses and reproductive tissue were placed in a drying oven at 65 °C for 5 days and separately weighed. The ratio of reproductive tissue mass to total mass was used to assess RA. Fish carcasses were then rinsed 4 times over 24 hrs with petroleum ether, and dried again at 65 °C for 24 hours and weighed. The difference in dry mass between the first and second measurements was divided by the mass of the first measurement to determine fat content. PCA was conducted on log + 1 transformed measurements as previously mentioned. Approximately 81% of total variance was explained in the first principal component, and this was used as an estimate of female phenotype in subsequent analyses (see Table A-2 for loading scores).

Assessing yearly and microspatial differences in male and female morphology

To determine the effects of year and pool on male morphology and female physiological state, I conducted Type III sum of squares ANOVA's on PC1 of each sex phenotype in R. Year, pool and their interaction were included as fixed effects. I then calculated Tukey's Honest Significant Difference (HSD) to report p-values for between-group comparisons.

Testing for phenotypic effects on female preference

To determine whether pool-level means of male morphology or female physiology predict observed preference patterns observed, I tested whether female

preference was correlated with either mean female physiological state or the mean male phenotype at each site within a given year. Specifically, I conducted a two-way ANOVA on net preference, with mean PC1 values of female and male phenotypes at each site/year combination included as fixed effects.

Results

Yearly and microspatial mating preference patterns

I found a significant interaction effect between pool and year on net preference ($F(2,157) = 4.56$, $p = 0.012$, Figure 1). While UP females failed to show a significant preference in any year (all $p > 0.05$), DOWN females first showed a net preference for males fed a high-protein diet in 2013 (Wilcoxon signed-rank, $Z = -2.54$, $p = 0.015$), followed by a strong change in the magnitude and direction of preference in 2014, with DOWN females preferring males fed a low-protein diet ($Z = -3.21$, $p = 0.0013$). Finally, in 2015, DOWN females showed, on average, no significant preference for either male cue ($p > 0.05$). DOWN females had significantly stronger preferences for males fed a high-protein diet than UP females in 2013, and stronger preferences for males fed a low-protein diet than UP females in 2014 (2013: $Z = 2.1032$, $p = 0.032$; 2014: $Z = -3.05$, $p = 0.0022$). In 2015, UP and DOWN female preferences did not significantly differ ($Z = -0.298$, $p = 0.771$, Figure 2).

Phenotypic comparisons

Approximately 64.6% of the total variance in male morphology was modeled by principal component 1 (PC1, Figure 3a). This component was largely influenced by

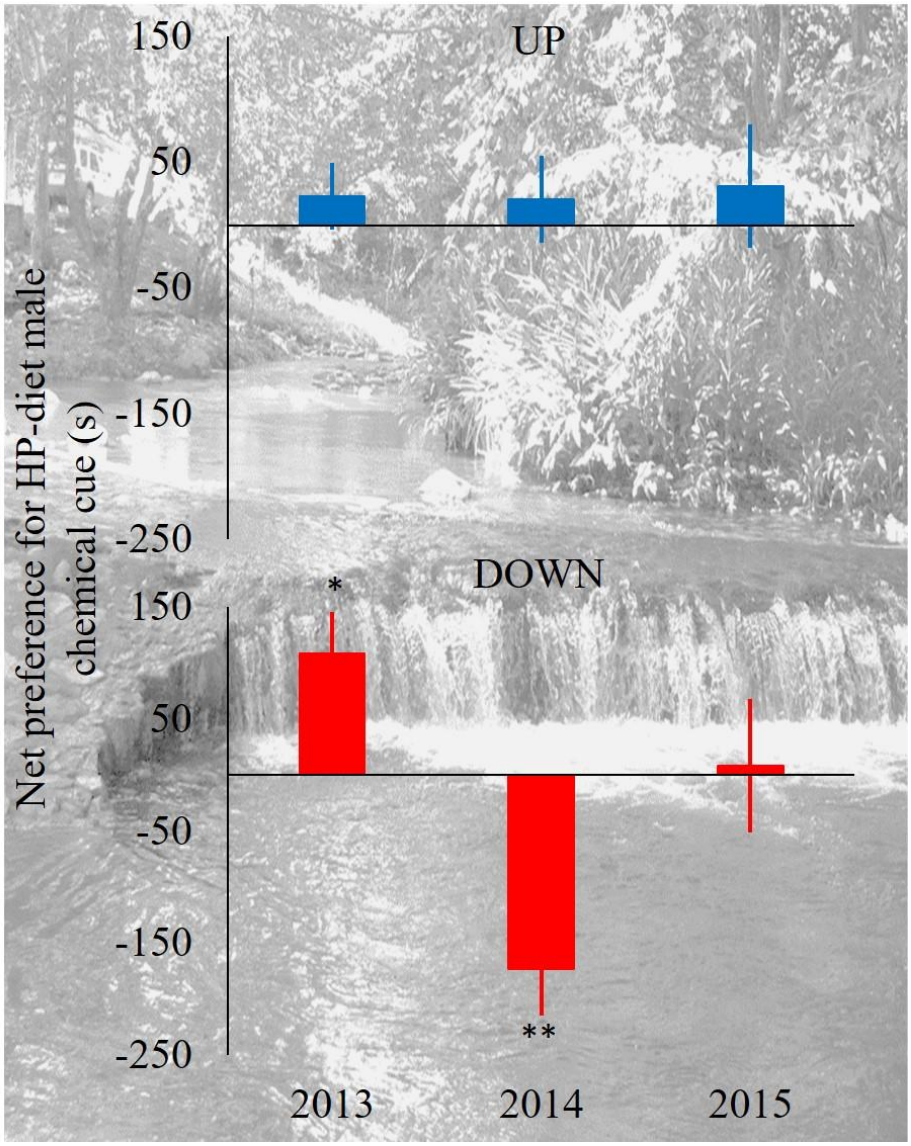

Figure 1 - Net female olfactory preference for males fed a high-protein diet, 2013-2015. UP: Calnali-mid upstream pool, DOWN: Calnali-mid downstream pool. Bar height represents mean association time with water containing pheromones of *X. birchmanni* males fed a high-protein diet ± S.E.M. * $p < 0.05$, ** $p < 0.01$ (Wilcoxon signed-rank test). Background image shows the adjacent pools on the Río Calnali, separated by less than 20 meters by a small spillway.

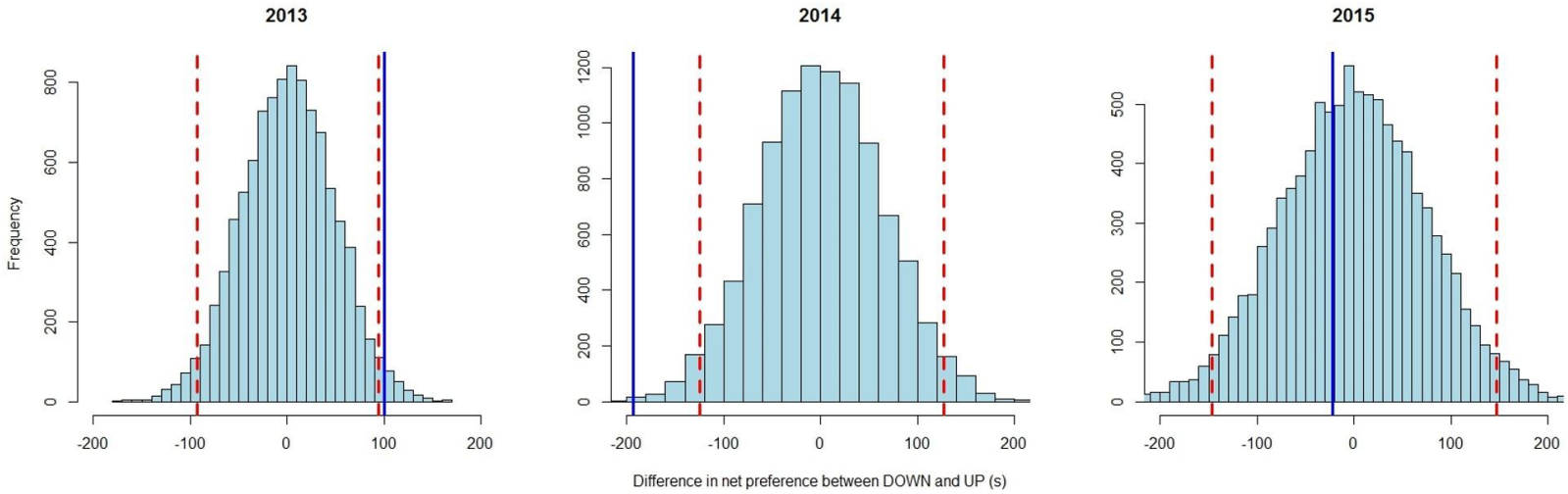

Figure 2 – Distribution of differences in net olfactory preference for males fed a high-protein diet between DOWN and UP pools. Positive values indicate relatively stronger preferences for males fed a high-protein diet in DOWN compared to UP. Dashed red lines indicate 95% confidence intervals of 10,000 resampled differences in net preference between pools (blue bars), and blue lines indicate the observed differences in net preference between DOWN and UP pools.

standard length, gonopodium and dorsal fin length, and body mass. I found a strong interaction effect of pool and year on this component (ANOVA: $F(2,154) = 6.27$, $p = 0.0024$). This effect was largely explained by significantly larger UP males in 2015 relative to DOWN males in the same year (Tukey HSD: $p < 0.001$), as well as larger UP males in 2015 relative to UP males from previous years (2013-2015: $p = 0.016$, 2014-2015: $p = 0.033$, Figure 3b).

Approximately 81.4% of the total variance in female reproductive allotment and fat content was modeled by principal component 1 (PC1, Figure 4a). I found a strong main effect of year on this component ($F(2,54) = 40.1$, $p < 0.0001$), and no significant effect of pool ($F(1,54) = 0.017$, $p = 0.897$) or their interaction ($F(2,54) = 0.091$, $p = 0.913$). Specifically, females had the highest reproductive allotment and fat content in 2014 (Tukey HSD: 2013-2014: $p < 0.0001$, 2014-2015: $p < 0.0001$), and the lowest levels in 2015 (2013-2015: $p < 0.0001$, Figure 4b).

Testing for phenotypic and genetic effects on behavior

Neither female PC1 nor male PC1 explained much of the variation in preference observed between pools and across years (ANOVA: female PC1: $F(1,157) = 1.55$, $p = 0.22$, male PC1: $F(1,157) = 0.93$, $p = 0.34$). Furthermore, in the 2015 dataset, I found no effect of female genotypic cluster identity on female preference for male diet-dependent cues (two-way ANOVA $p > 0.3$, Figure 5a). As part of a previous study conducted on the same females collected in 2015, it was shown that site, but not female cluster identity, had an effect on female preference for male cluster identity, as female swordtails from the UP pool showed preferences for *birchmanni*-like male chemical

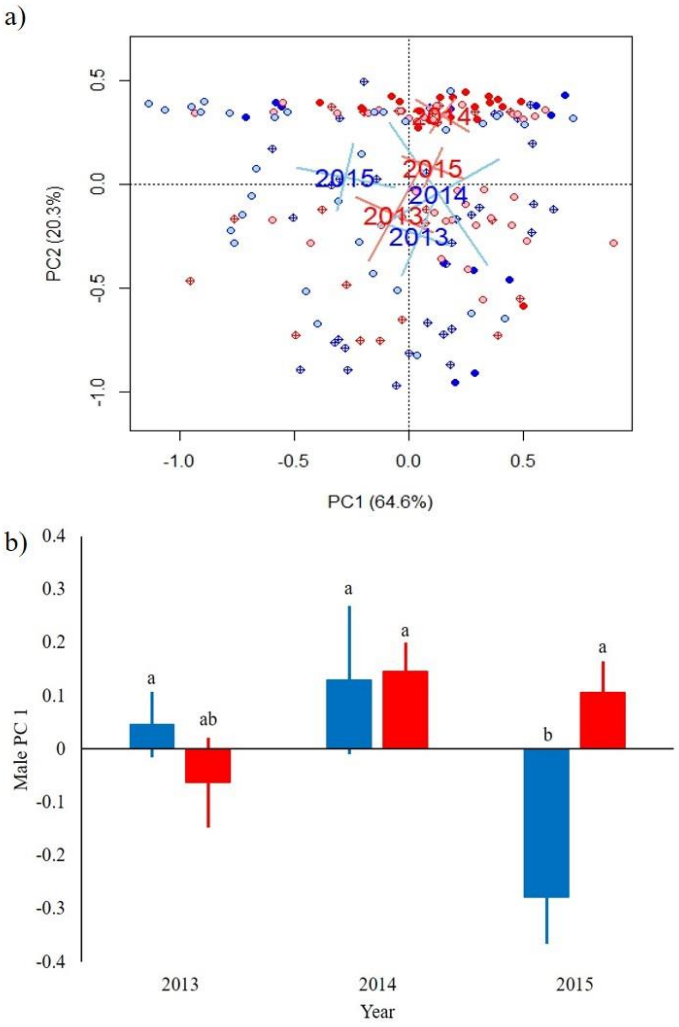

Figure 3 – Principal components analysis of male phenotypic distributions in UP and DOWN pools. a) Male phenotypic distribution in UP (blue) and DOWN (red) pools from 2013 (crosshair), 2014 (dark fill), and 2015 (light fill). Principal component 1 (PC1) is most influenced by standard and dorsal fin lengths and mass (larger individuals on the left), and PC2 is most influenced by sword extension length (larger swords at the bottom). b) Mean PC1 value according to year and pool. Groups with non-matching letters have significantly different values of PC1 ($p < 0.05$). Error bars in both figures represent S.E.M.

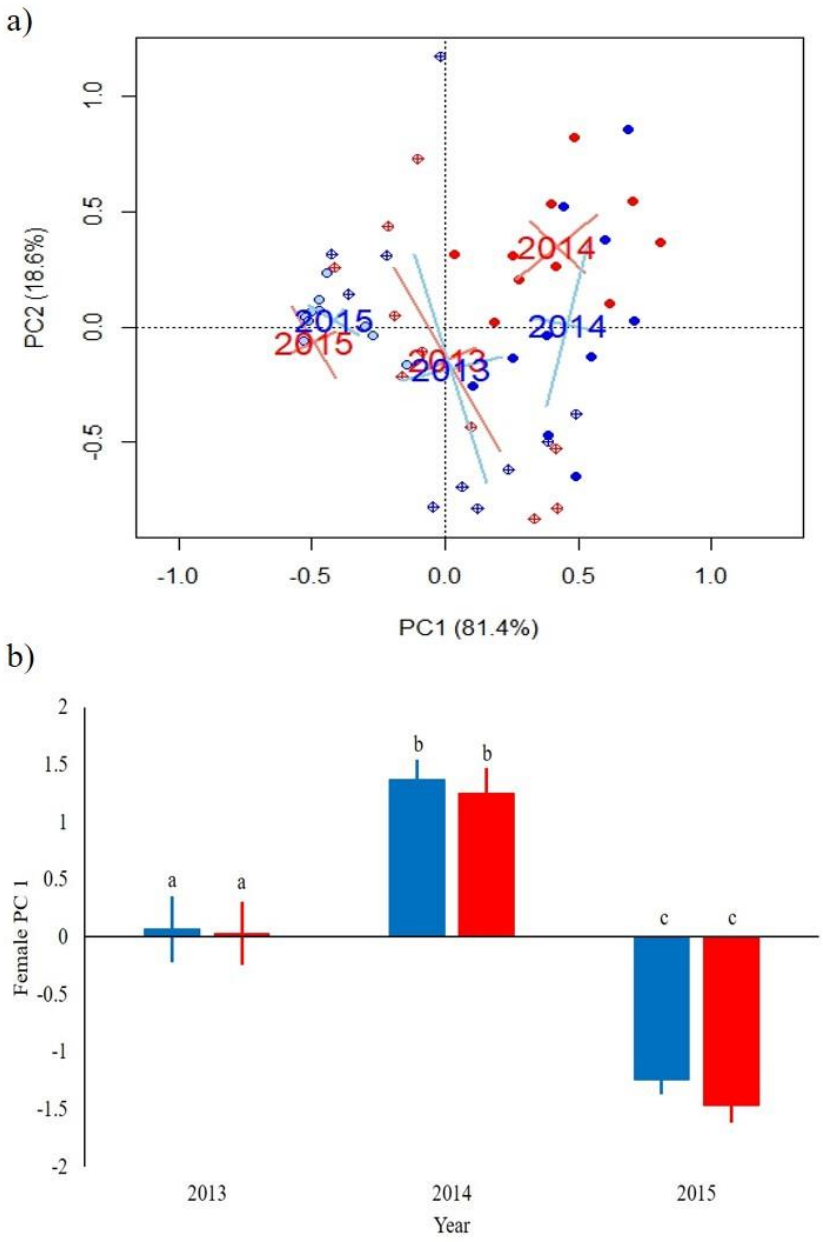

Figure 4 - Principal components analysis of female physiological state in UP and DOWN pools. a) Female physiological state in UP (blue) and DOWN (red) pools from 2013 (crosshair), 2014 (dark fill), and 2015 (light fill). b) Mean PC1 value according to year and pool. Groups with non-matching letters have significantly different values of PC1 ($p < 0.05$). Error bars in both figures represent S.E.M.

cues, while females from DOWN showed preferences for *malinche*-like males (Schumer, Powell et al. 2017)(Figure 5b). Female cluster identity did affect sexual responsiveness, as a greater proportion of females with a more *X. malinche* genetic background were responsive during both sets of preference trials (40 of 52 genotyped) than those from the *birchmanni*-like cluster (11 of 22 genotyped) (preference for genetic cluster: X^2 (1, N = 74) = 17.9, $p < 0.0001$, preference for diet-dependent cue: X^2 (1, N = 74) = 4.05, $p = 0.044$).

Discussion

Mating preferences are extraordinarily labile, and sensitive to a wide range of extrinsic and intrinsic factors (Houde and Endler 1990, Godin and Dugatkin 1995, Wagner Jr, Murray et al. 1995). In this study, I found that, depending on the year, females from one pool exhibited drastically different preferences from those in an adjacent one, and these differences could not be explained by yearly or spatial differences in female physiological state, genotype or the male social environment. Specifically, females from the DOWN pool showed strong preferences for, then against, chemical cues of males fed a high-protein diet in 2013 and 2014, respectively, whereas females from the UP pool showed no significant preference, on average, for either male cue from 2013-2015. This result highlights the exceptionally fine-scale level at which behavior can covary with microhabitat. These behavioral differences were detected between two pools separated by only a slight barrier that has previously been shown to allow for a moderate level of downstream migration (Culumber, Ochoa et al. 2014),

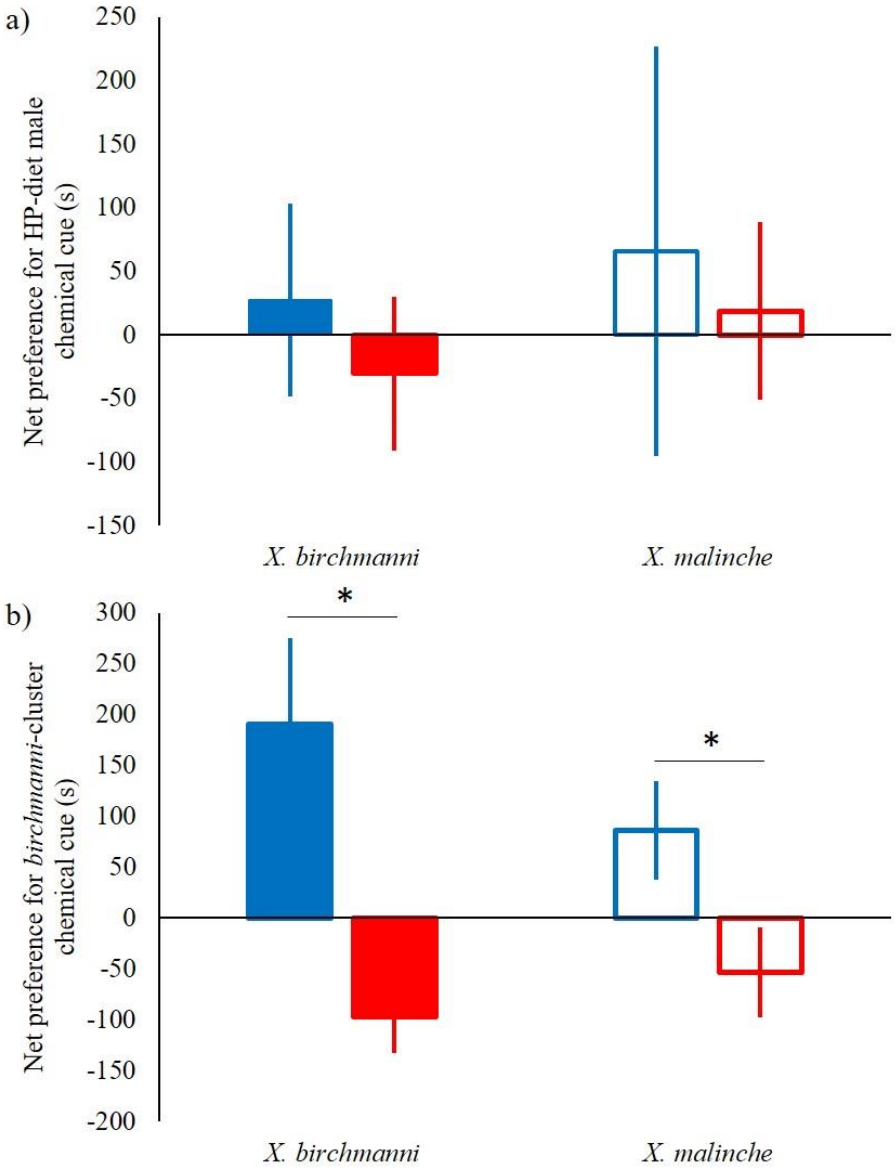

Figure 5 - Net female olfactory preferences in UP and DOWN pools, according to female genotypic cluster identity. Preferences for a) males fed a high-protein diet, and b) *birchmanni*-like males in Calnali-mid in the summer of 2015. Blue and red bars indicate UP and DOWN pools, respectively. Filled and unfilled bars indicate *birchmanni*-like and *malinche*-like females based on genetic ancestry determined by MSG. Bar height represents mean association time ± S.E.M. * $p < 0.05$.

thereby suggesting that females may be adjusting certain preferences at a surprisingly high rate. These fluctuations in preferences at small spatial and temporal scales could act to weaken sexual selection within populations, thereby explaining why strengths of sexual selection through mate choice are often very weak (Jennions and Petrie 1997, Qvarnstrom, Brommer et al. 2006).

In many studies, sample sizes are obtained through collecting at various nearby locations, and then pooled together to measure a given behavior. However, this method could potentially be hiding fine-scale, or alpha-level (Whittaker 1972), heterogeneity in preference patterns that could play an important role in maintaining phenotypic and genetic diversity within a population. Sampling exclusively at one pool versus another, or in one year versus another, would have yielded markedly different behavioral results that could have subsequently led to misleading generalizations about species- or population-level preferences. Likewise, pooling the samples would have hidden this alpha-level of preference variation within the population, which could reinforce microhabitat-driven assortative mating. Therefore, it is important for future studies to clearly define the temporal and spatial resolution at which a given population-level relationship between the environment and a behavior is being described.

One possible explanation for the preference differences observed in this study is that they were an artifact of sampling or methodological errors. Power simulations performed on parental species suggest that I had sufficient statistical power to detect moderate differences in preference between groups with the sample sizes obtained in this study. While low sample sizes are often assumed to result in reduced chances of

detecting true effects, they can also increase false positive rates (Button, Ioannidis et al. 2013). This is an issue that is pervasive throughout mate choice and all of behavioral research, and the sample sizes used in the smallest groups (DOWN 2013 and DOWN 2014: N = 16 responsive females) in this study are well within the range of many past behavioral studies (Pfennig and Tinsley 2002, Witte and Sawka 2003, Cummings, Larkins-Ford et al. 2008, Farris and Ryan 2011, Rosenthal and Ryan 2011, Willis, Rosenthal et al. 2012, Root, Denny et al. 2014, Kozak and Boughman 2015, Zhuang, Sun et al. 2016). Therefore, behavioral experiments should be repeated across space and time in order to assess the reproducibility of previously published results, as well as to gain an understanding of the alpha-level diversity in behaviors within genetic populations. In this system, just a few meters separating the ranges of local populations drastically affected population-level estimates of mating preference. Estimates of the strength of sexual selection and reproductive isolation at the population level may therefore often be artifacts of limited sampling in space and time.

Conclusions

Population-level estimates of mating preferences are a cornerstone of sexual selection research. Investigators often use behavioral measures to characterize how choosers in a natural population express preferences for one trait over another. Sample sizes are often obtained by pooling across microhabitats and over time, thereby potentially introducing unaccounted variance due to small-scale heterogeneity in preferences. I examined this in a natural population of hybrid swordtails (*Xiphophorus*

birchmanni x *X. malinche*) distributed between two adjacent pools separated by a small spillway. Over three successive years, I found marked, but highly variable, differences between pools in female preference for olfactory cues associated with male nutritional condition. While females from the upstream pool never showed preferences, females from immediately downstream showed strong preferences that reversed across years. These differences in preference could not be attributed to population-level differences in phenotypes previously shown to be mate-choice relevant. The heterogeneity in observed mating preferences and the intrinsic error generated by behavior trials call attention to the challenges inherent in estimating population-level distributions of complex phenotypes, and to the importance of accounting for small-scale heterogeneity when modelling and measuring the evolution of mating preferences and display traits.

CHAPTER III

NEUROGENETIC FRAMEWORK OF LEARNED FEMALE MATING PREFERENCES IN THE SWORDTAIL FISH *XIPHOPHORUS BIRCHMANNI*

Introduction

A major goal in evolutionary biology is to determine the mechanisms behind the maintenance of reproductive isolation between species. Mate choice can play an important role in reproductive isolation between lineages long before the evolution of postmating barriers (Grant and Grant 1997). Mating preferences are often learned rather than genetically specified**,** and can thereby be influenced by an individual's social environment throughout life (Grant and Grant 1997, ten Cate, Verzijden et al. 2006, Verzijden, Ten Cate et al. 2012).

Experience can elicit preferences either for or against a familiar trait, which can have important ecological and evolutionary consequences by promoting assortative mating through imprinting on familiar phenotypes (Verzijden, Lachlan et al. 2005, Servedio, Sæther et al. 2009) or outcrossing through learned "antipathy" (Hughes, Houde et al. 2013). Individuals can be genetically predisposed to favor early learning of familiar stimuli, thereby strengthening reproductive isolation between species (Marler 1991). For example, in stickleback fish learned preferences for familiar stimuli act to minimize hybridization between ecotypes (Kozak and Boughman 2009, Kozak, Head et al. 2011). However, changes in the social environment can also facilitate hybridization (Irwin and Price 1999). Zebra finches can develop preferences for hybrid males through

early exposure to a mixed social environment (ten Cate 1987). In Darwin's finches, mis-imprinting on heterospecific songs promotes hybridization (Grant and Grant 2008). These studies show that learned mating preferences can be a major driver in maintaining or eroding reproductive isolation between species. It is therefore crucial to determine the mechanisms underlying conspecific mating preferences in order to gain a better understanding of their behavioral and evolutionary consequences.

As individuals are developing, this behavioral tuning of the social environment on mating preferences is likely to leave a neurogenomic imprint on the brain. Despite the large behavioral literature on learned mating preferences and their ubiquity across systems (ten Cate 1987, Bischof and Clayton 1991, Payne, Payne et al. 2000, ten Cate, Verzijden et al. 2006, Kozak, Head et al. 2011, Verzijden, Ten Cate et al. 2012), the neurogenomic mechanisms driving this behavior are relatively unknown. The majority of sexual imprinting studies have focused on a small set of genes, usually non-specific, immediate-early genes that serve as general markers of neural activity, in a subset of functionally relevant brain regions (Changeux and Mikoshiba 1978, Bolhuis, Zijlstra et al. 2000, Bolhuis and Gahr 2006, Sadananda and Bischof 2006, Maekawa, Nakamori et al. 2007, Meparishvili, Nozadze et al. 2015). While these studies are of vital importance for understanding the roles of specific genes within a given brain region in the formation of learned mating preferences, they do not allow for the detection of more general gene expression patterns throughout the whole brain. Recent advances and reduced costs in next-generation sequencing have made individual-level, transcriptome-wide analyses more practical. Examining the neurogenomic effects of learning through this wider lens

will allow for the detection of new genes previously unknown to have roles in the development of learned preferences. Furthermore, these studies can address how networks of co-varying genes respond to changes in an individual's social environment.

Across the animal kingdom, and notably in fishes, olfactory cues play an important role in conspecific mate preference. As with other cues, response to these cues can depend on previous experience. Exposure to artificially-supplied chemicals during development alters sensitivity to these odorants later in life (Nevitt, Dittman et al. 1994, Harden, Newton et al. 2006), and sensitivity to new odors is accompanied by increased expression of genes involved in odorant receptor cell neurogenesis at the sensory periphery (Harden, Newton et al. 2006). Differences in social exposure can result in differential regulation of certain odorant receptor genes in the olfactory epithelium (Cui, Delclos et al. 2017). Odorant receptor neurons project to the olfactory bulb, which further project throughout the forebrain and other brain regions. Furthermore, individuals have been shown to have a neuronally plastic response in the forebrain to varying levels of social exposure (Corotto, Henegar et al. 1994, Yamaguchi and Mori 2005, Makinodan, Rosen et al. 2012, Okuyama, Yokoi et al. 2014). Therefore, social exposure is likely to have transcriptomic implications throughout the entire brain as well as the sensory periphery. Specifically, I expect exposure to different species to result in the relative upregulation of species-typical odorant receptor genes, which will have differential consequences on downstream processing in the whole brain. Applying RNA-sequencing methods on whole brain and sensory tissue can serve as a powerful means of identifying species-typical odorant receptor genes (Cui, Delclos et al. 2017) as well as

networks of genes that are differentially regulated at the sensory periphery and brain across exposure treatments.

Swordtail fish *Xiphophorus birchmanni* serve as an ideal system for studying mechanisms of learned mating preferences. Many swordtail species rely on olfactory signaling for conspecific mate preference (Crapon de Caprona and Ryan 1990, McLennan and Ryan 1999, Fisher, Wong et al. 2006b, Verzijden and Rosenthal 2011). Wild-caught female *X. birchmanni* generally prefer the chemical cues of conspecific males over males of a closely related congener, *X. malinche*. However, this preference is sensitive to both early (Verzijden and Rosenthal 2011) and recent (Verzijden, Culumber et al. 2012) experiences; female *X. birchmanni* prefer familiar olfactory cues regardless of whether they are conspecific or heterospecific. This study aims to describe the transcriptomic differences between brains and sensory tissue of sexually mature female *X. birchmanni* raised under different social exposure treatments in order to elucidate the general transcriptomic effect of the social environment on the whole brain and sensory periphery.

Materials and Methods

Fish collection and exposure treatments

Fifteen *X. birchmanni* females were collected from the Río Coacuilco at Coacuilco, Hidalgo, Mexico (Culumber, Fisher et al. 2011) in March 2014 and transported to Texas A&M University facilities where they gave birth. When offspring reached approximately 3 weeks of age, broods were evenly pooled, separated into

groups of 30 juveniles, and assigned to one of 3 treatment groups (3 replicates each): 1) B-EXP: exposed to 2 males and 2 females of adult conspecifics (*X. birchmanni* from the Río Garces locality); 2) M-EXP: exposed to 2 males and 2 females of adult heterospecifics [*X. malinche* from the Chicayotla locality (Culumber, Fisher et al. 2011)] and 3) NO-EXP: controls which did not receive adult stimulus exposure. Exposure treatments were performed at 23 °C, 12:12 light:dark cycle in adjacent, though visually isolated, 120-liter aquaria where adults and juveniles were divided by a transparent, perforated Plexiglas board which allowed for transmission of both visual and olfactory cues (Verzijden and Rosenthal 2011, Cui, Delclos et al. 2017). Sufficient shelter was provided to both adults and juveniles, and water was continually refreshed via a flow-through system. Juvenile males were removed from aquaria upon the first sign of maturation (thickening of the anal fin to form the gonopodium). At an average of 11 months of age, I tested female preference for olfactory cues of *X. birchmanni* and *X. malinche* as described below. After all behavioral trials, females were rinsed in aquarium water and returned to their respective treatment for an additional week before sample collection to minimize possible short-term effects from behavioral trials. Visual and olfactory exposure thus continued until the time of tissue collection.

Olfactory preference trials

I tested female preference for conspecific vs. heterospecific male odors following a well-established protocol (McLennan and Ryan 1999, Verzijden and Rosental 2011, Fisher et al. 2006). Briefly, to produce the olfactory cues, 20 L aquaria were thoroughly rinsed with Alconox and a 1:1 mixture of hydrogen peroxide, followed by rinsing with

carbon-filtered water 4 times. Groups of 5 males of *X. birchmanni* and 5 *X. malinche* were separately placed in 20 L of carbon-filtered water and visually exposed to 5 females from their own population in adjacent tanks for 4 hours. The water from the male tanks was used as the olfactory stimulus in preference trials. None of the males or females used to produce olfactory cues were used as exposure models in the rearing tanks. All preference trials were conducted with the same stimulus water.

The preference trial tanks (75 x 19 x 20 cm filled to a depth of 15 cm) were opaque on all sides. A small shelter was provided in the middle of each tank. Female position during the trial was recorded using an overhead camera connected to the Viewer (Biobserve GmbH, Bonn, Germany) recording software. The tank was equally divided along its length into three virtual zones: two preference zones on either end of the tank, with the middle defined as a neutral zone. Twenty minutes before each trial, the focal female was introduced to the testing tank for acclimation. Immediately afterward, stimulus water started dripping on both ends of the tank via computer-controlled peristaltic pumps (VWR) until the end of the trial at a rate of approximately 5 ml/min. Upon initiation of the cue pumps, I allowed 5 minutes for the focal female to visit both preference zones. If the subject failed to do so, she was defined as unresponsive. Starting at the moment the subject visited both preference zones, the time in each zone was recorded for a total of 5 minutes. Each female was tested a second time after another 20 minutes of acclimation and switching the sides from which each cue was dripped to account for potential positional biases. The association times from both trials were summed for data analysis. I tested a total of 30 B-EXP (22 responsive), 30 M-EXP (21

responsive), and 32 control females (27 responsive).

Statistical analysis of olfactory preferences

I tested the normality of association time datasets using Shapiro-Wilk tests. For datasets fitting the normality assumption, I used paired Student's *t*-tests to detect differences in mean association time between the two stimuli for each group. Unpaired *t*-tests assuming equal variance were then used to test for differences in net preference between groups. A one-way mixed effects ANOVA on net preference (time with *X. birchmanni* – time with *X. malinche* in seconds) was used to test whether exposure experience significantly affected mate preference. Replicate was included as a random effect, and exposure treatment was the fixed effect. I used non-parametric tests (Wilcoxon signed-rank tests and Kruskal-Wallis rank-sum tests) for datasets violating the normality assumption. All analyses were conducted in R. ANOVA was conducted using the nlme package in R.

Tissue sample collection for RNAseq

I randomly selected 5 females from each replicate of each treatment group (45 total samples). Females were euthanized with an overdose of MS-222, then decapitated. For RNAseq analyses, whole heads were preserved in either RNALater solution, placed at 4 °C overnight, then whole brains, optic nerves and both nares, including olfactory epithelia were dissected and stored at -80 °C until use (Figure B-1).

RNA extraction and library preparation

RNA was extracted from the above tissue using a standard Trizol reagent (Life Technologies) protocol following manufacturer's instructions and quantified and

assessed for quality on a Bioanalyzer 2100 (Agilent Technologies). Briefly, tissue was removed from the RNALater solution and dried on a sterile, RNAse-free wipe, then completely homogenized in Trizol with a hand-held TissueRuptor (Qiagen). RNA was extracted with 100 µL of bromochloropropane, followed by overnight precipitation with isopropanol in -20 °C and two washes of 75% ethanol. RNA quality (RIN) indices from the Bioanalyzer ranged from 6.9-8.5. Only those samples with RIN > 7 were used for further analyses (36 samples total). 500 ng of total RNA was used to prepare libraries following Illumina's TruSeq RNA Library Preparation Kit v2 (Set A) with minor modifications. Briefly, mRNA was purified from total RNA using manufacturer provided beads. Following cDNA synthesis, mRNA was chemically fragmented and following end repair and A-tailing, unique index adapters were ligated to each sample. Libraries were PCR-amplified for 15 cycles and library size distribution and quality was verified on an Agilent 2200 TapeStation using the D1000 ScreenTape Assay. Libraries were quantified on a Qubit fluorimeter, pooled in equal quantities, and sequenced on three Illumina HiSeq 2500 lanes (125x125 paired-end reads). Adaptor and PCR primer sequences and low quality bases in the raw reads were removed and trimmed by cutadapt (leading, trailing and sliding window quality >= 20 PHRED scale)(Martin 2011). Only reads > 30 bp after filtering were kept for the downstream analyses.

Read mapping

I use the previously described de-novo genome assembly for *X. maculatus* (Schartl, Walter et al. 2013) as the reference sequence for read mapping. First, I mapped pooled reads from all individuals using TopHat 2.0.10 to obtain a comprehensive

alternative junction list. I then mapped reads for each individual sample separately guided by this junction list. I allowed 5 mismatches to the reference per read (5/125 bp) and used default settings for all other parameters (--read-gap-length 1 –read-mismatches 5 –read-edit-dist 5 –b2- very-sensitive)(Cui, Delclos et al. 2017). Allowing for this number of mismatches did not substantially change the percentage of multiple-aligned reads, which was < 1% for all samples.

Differential expression analysis

Gene models (v. 82) for *X. maculatus* were downloaded from Ensembl. I counted the number of reads mapping to each gene using the python package htseq-count (strand specific: no, mode: union, counted feature: exon), requiring a mapping quality of 20. These raw counts were imported into the DESeq2 package in R (Love, Huber et al. 2014) for differential expression (DE) analysis. I visualized gene expression profiles of individuals by conducting a multidimensional scaling analysis on normalized gene counts.

Using DESeq2, I performed a transcriptome-wide analysis, following the DESeq2 default parameters. To control for differences among tanks, replicate was included in the design formula. I defined significance as genes differentially expressed between treatment groups at $p < 0.05$ after Benjamini-Hochberg adjustments.

Weighted gene co-expression network analysis

Systems genetics uses the connectivity of genes to describe the relationship between the transcriptome and a trait of interest. To identify modules of interconnected genes that correlate with social treatments, I used weighted gene co-expression network

analysis [WGCNA (Langfelder and Horvath 2008)] on log-transformed count data for genes that passed an initial 0.5 CPM threshold filter. Two outliers (one B-EXP and one M-EXP sample) were found and excluded from subsequent WGCNA analyses, based on a standardized connectivity threshold of 2.0 standard deviations from mean connectivity. Therefore, final sample sizes for WGCNA analyses were 10 B-EXP, 11 M-EXP and 13 NO-EXP females. Sample network connectivity was then reanalyzed among the remaining samples. For all possible pairs of variable genes, Pearson correlation coefficients were calculated across all samples. An unsigned matrix was created, and I adjusted the soft-threshold value to ensure a scale-free topology ($\beta = 12$), thereby creating a weighted network. Within this topological overlapping network (Yip and Horvath 2007), genes were hierarchically clustered, and modules were identified based on the degree of similarity among genes (Langfelder and Horvath 2008). A merging threshold of 0.2 was used (mergeCutHeight=0.2), with a minimum module size of 30 genes (minModuleSize=30) and a mean connectivity threshold of greater than or equal to 0.7 (minkMEtoStay=0.7). Default parameters were used for the rest of the analyses.

GO enrichment and PANTHER pathway analysis

To determine whether particular functional categories and pathways might be implicated in developing socially sensitive learned mating preferences, I performed gene ontology (GO) enrichment analysis on: 1) the three lists of differentially expressed genes between pairwise comparisons of the three exposure groups (B-EXP/M-EXP, B-EXP/control and M-EXP/control), and 2) WGCNA modules that were significantly differentially regulated ($p < 0.05$) between exposure treatments (B-EXP/M-EXP). I used

the annotated *X. maculatus* genome to assign Human Genome Organization (HUGO) gene symbols to each gene. All genes that passed coverage filtering in DESeq2 were included as part of the gene universe, and I tested for significant enrichment (FDR < 0.05) of different biological processes and pathways by comparing the gene universe to gene lists using the PANTHER Classification System [release 20160321 (Mi, Muruganujan et al. 2013)]. Briefly, genes are organized into families and subfamilies according to sequence homology and functional similarity. They are then assigned GO terms ("GO biological processes complete") and placed within one of 177 "PANTHER Pathways". Gene lists are compared to the gene universe to find GO terms or biological pathways that are statistically over- or underrepresented using a binomial test. I used Revigo (Supek, Bošnjak et al. 2011) to visualize GO categories clustered by semantic similarities (SimRel).

Results

Female preference behavior

Early social experience had a significant effect on olfactory preference ($F(2, 65) = 5.17$, $p = 0.0083$, Figure 6). Females exposed to adult *X. birchmanni* showed a significant olfactory preference for conspecific (*X. birchmanni*) male water (Wilcoxon signed-rank, $Z = 2.65$, $p = 0.0067$), and females exposed to heterospecific adult *X. malinche* showed a trend towards an olfactory preference for heterospecific male water (Wilcoxon signed-rank, $Z = -1.58$, $p = 0.06$). Socially isolated females did not show significant olfactory preferences ($Z = 0.120$, $p = 0.9153$). Conspecific-exposed females had significantly greater preferences for *X. birchmanni* cues than both heterospecific-

exposed ($p = 0.0024$) and females isolated from adults ($p = 0.038$).

Transcriptome-wide differential expression

Social exposure had a large effect on female gene expression profiles (Figure 7). After applying a 0.5 CPM coverage filter with DESeq2, 19,126 genes were retained, of which 17,715 had annotated gene symbols. B-EXP and M-EXP females showed the greatest level of transcriptomic separation, while socially isolated females showed a large variance in gene expression profile resulting in relatively little separation from either exposure treatment (Figure 8). After false discovery correction at FDR = 0.05, 919 genes were significantly differentially expressed between B-EXP and M-EXP females, and only 6 (two unique) and 2 (one unique) genes were significantly differentially expressed in B-EXP and M-EXP groups compared to the socially isolated group respectively (Table S1).

Gene ontology and PANTHER pathway analysis

To determine the functional roles of differentially expressed genes between group comparisons, I performed gene ontology (GO) enrichment analysis. No significant terms were statistically overrepresented in the list of 6 and 2 differentially expressed genes between B-EXP/NO-EXP and M-EXP/NO-EXP comparisons. Of the genes differentially expressed between B-EXP and M-EXP groups, a term related to mRNA nuclear export, “SRP-dependent cotranslational protein targeting to membrane” (GO:0006614) was the most significantly enriched process. In total, 220 GO categories were statistically enriched or underrepresented at $p < 0.05$ after FDR correction between the two exposure treatments (Table S2).

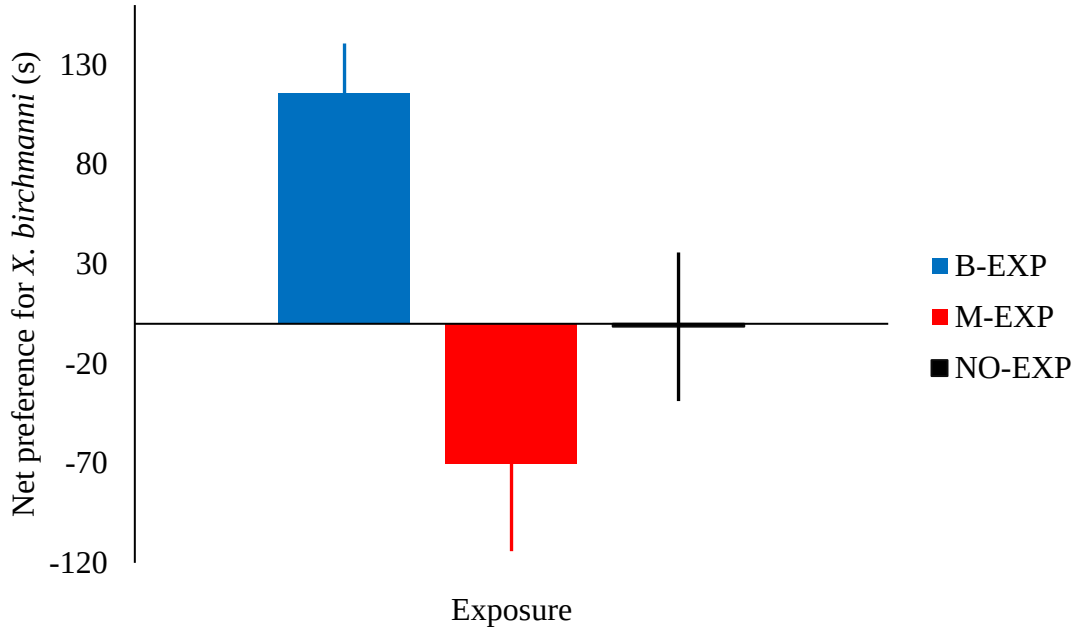

Figure 6 – Mean net association time of *Xiphophorus birchmanni* females for conspecific versus heterospecific (*Xiphophorus malinche*) male chemical cues according to social exposure treatment. Blue bar: exposed to conspecific adults, red bar: exposed to heterospecific adults, and black bar: isolated from adults throughout development. Error bars represent SEM.

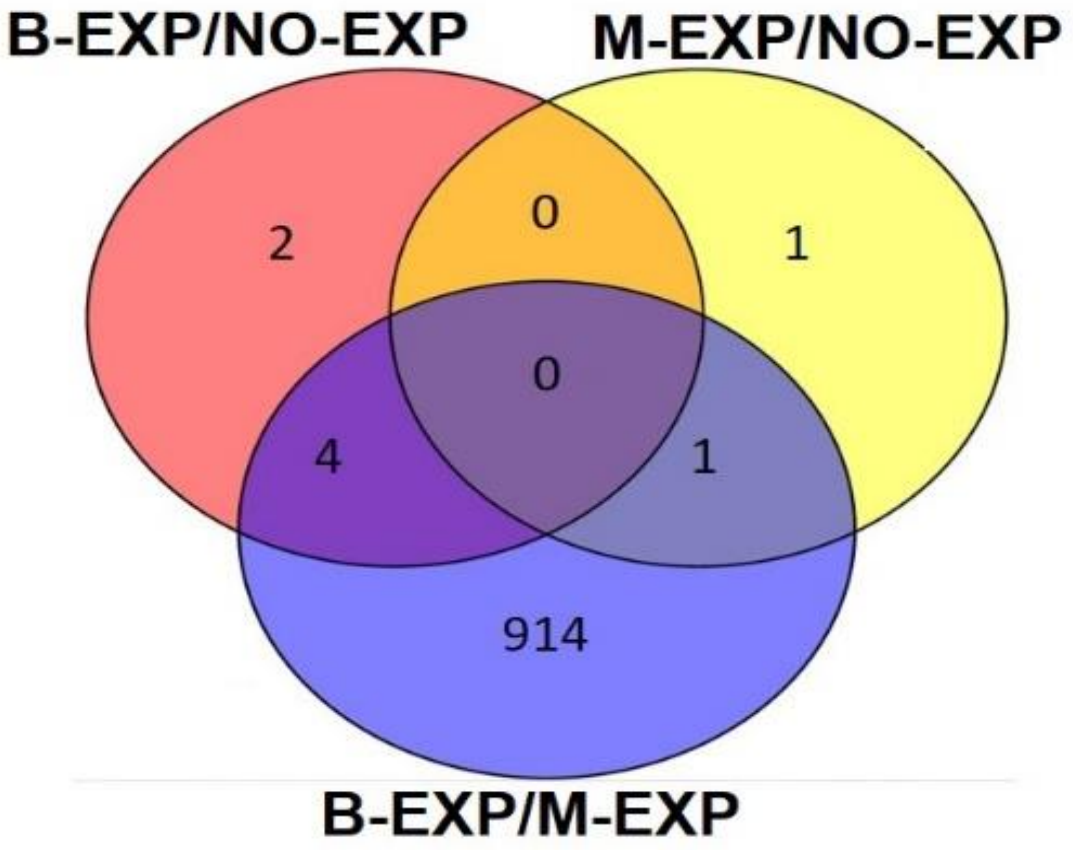

Figure 7 – Venn diagram showing overlaps of differentially expressed genes between social exposure treatments. Numbers indicate the number of differentially expressed genes in a given pairwise comparison of social exposure treatments. B-EXP/NO-EXP: conspecific-exposed vs. socially isolated *X. birchmanni* females, M-EXP/NO-EXP: heterospecific (*X. malinche*)-exposed vs. socially isolated females, and B-EXP/M-EXP: conspecific-exposed vs. heterospecific-exposed females.

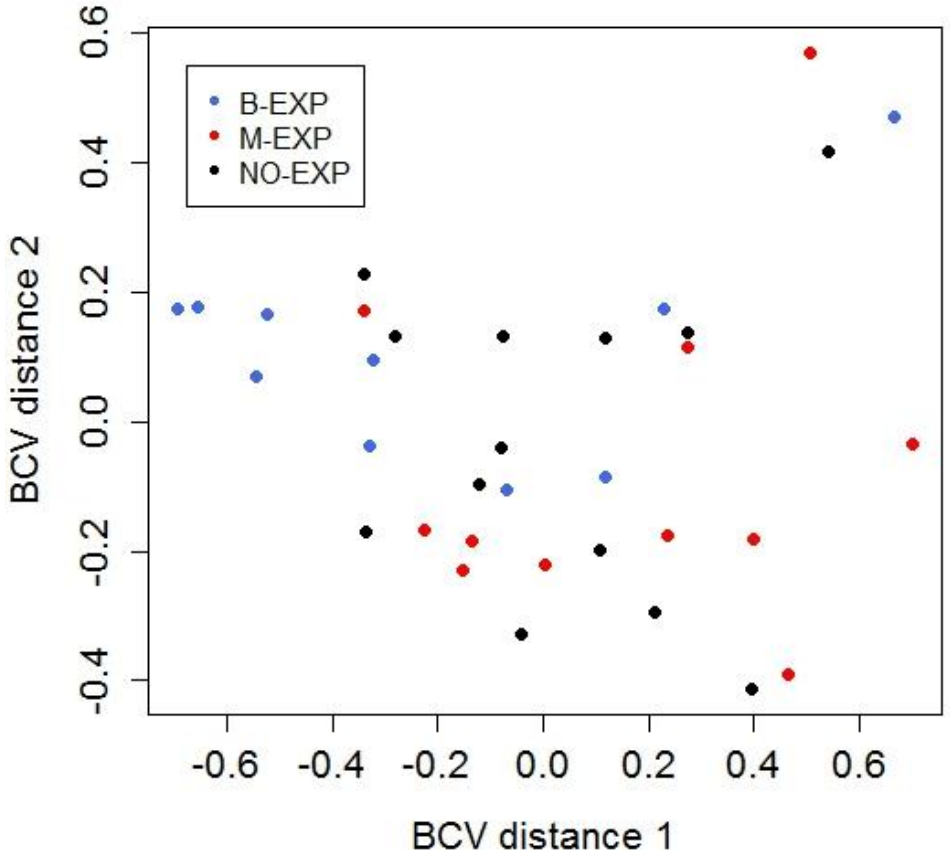

Figure 8 – Multidimensional scaling plot depicting within- and among-treatment variation in female neural gene expression profiles according to social exposure treatment. blue dots: conspecific-exposed *X. birchmanni*, red dots: heterospecific (*X. malinche*)-exposed females, and black dots: socially isolated females. Data points correspond to individual gene expression profiles.

To gain a better understanding of the functional relationships of differentially expressed genes between the two exposure groups, I divided the gene list according to direction of gene regulation (Hong, Zhang et al. 2014). A total of 468 and 451 genes were significantly upregulated and downregulated, respectively, in the B-EXP group compared to the M-EXP group. In the list of upregulated genes, "SRP-dependent cotranslational protein targeting to membrane" (GO:0006614) was the most significantly enriched process out of a total 374 significant GO terms, while "generation of neurons" (GO:0048699) was the most enriched process in the list of relatively downregulated genes out of a total 34 significant GO terms (Table S3). Using Revigo to visualize the list of enriched GO terms revealed that a large proportion of relatively upregulated genes pertain to biological processes related to the immune system, behavioral responses to stimuli and ribosome assembly (Figure 9a). Meanwhile, genes that were significantly upregulated in the M-EXP group were largely related to synaptic plasticity and neurogenesis (Figure 9b).

PANTHER pathway analyses revealed a total of seven significantly enriched biological pathways in the lists of differentially expressed genes. Specifically, four pathways mostly pertaining to immune system processes were significantly upregulated in B-EXP females, with "B cell activation" (P00010) being the most enriched. Three pathways pertaining to glutamate receptor and G-protein signaling pathways were upregulated in M-EXP females, with "metabotropic glutamate receptor group 1 pathway" (P00041) being the most enriched (Table 1).

WGCNA results

WGCNA analysis revealed no significantly differentially regulated gene modules between either M-EXP and NO-EXP or B-EXP and NO-EXP after corrections for multiple comparisons (see figure B-2). When comparing B-EXP and M-EXP gene expression profiles, WGCNA analysis revealed that the brain transcriptome of female *X. birchmanni* can be grouped into 12 modules of similarly coregulated genes (Figures 10-12). Three of these modules were significantly differentially expressed, on average, between B-EXP and M-EXP females, and I refer to these modules according to their functional roles (synapse module, $p = 0.02$; vision module, $p = 0.02$; olfaction module, $p = 0.01$, Figure 12b). The synapse module consists of 2,768 genes (Table S4), and gene ontology enrichment analysis revealed that this module is largely comprised of genes with similar GO terms to those found in the traditional differential expression analysis, such as “synaptic transmission” and “immune response” (Figure 13, Table S5). On average, this module was significantly upregulated in M-EXP females. The genes with highest module membership- a measure of intramodular connectivity and relative importance within a module- were *mmp16b*, *cntn2*, and *rc3*. These genes have roles in tissue remodeling (Hotary, Allen et al. 2000), neuronal migration and adhesion (Tsiotra, Karagogeos et al. 1993, Yoshihara, Kawasaki et al. 1995), and synaptic vesicle scaffolding (Nagano, Kawabe et al. 2002), respectively.

The vision module consists of 86 genes (Table S6), is upregulated in B-EXP females, and consists of genes with roles in visual detection (Figure 14a, Table S7). Four of the five genes most central to the vision module (i.e., highest module membership

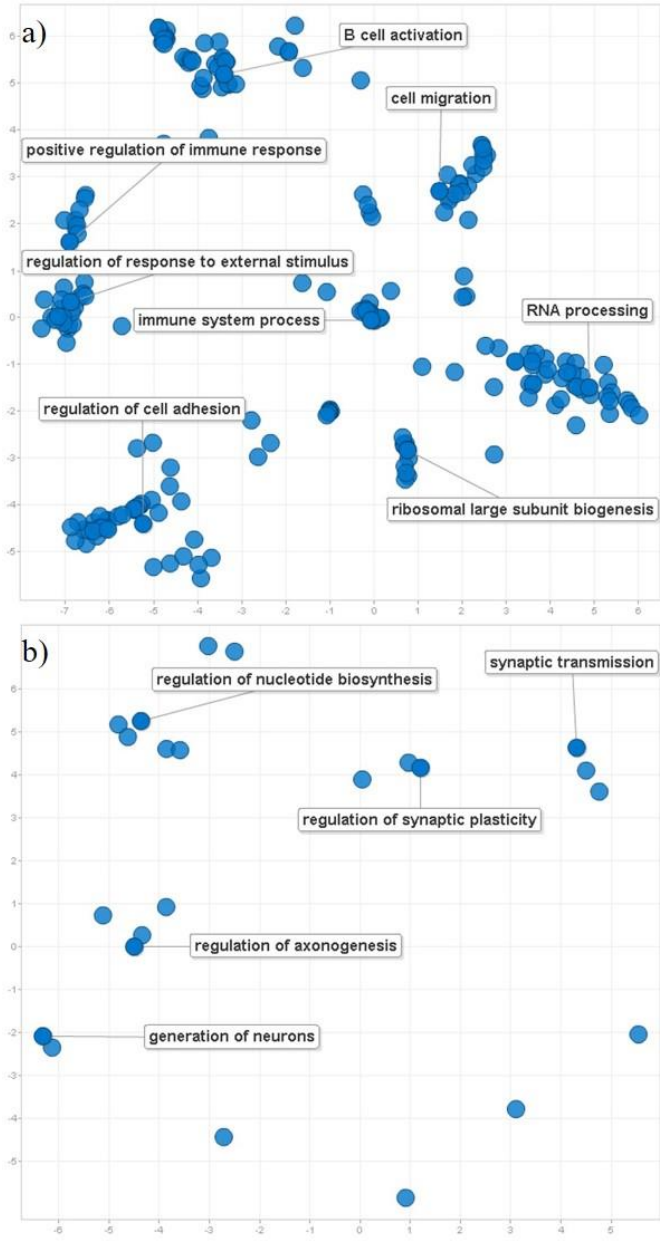

Figure 9 – Significantly enriched gene ontology terms of genes significantly (FDR < 0.05) a) upregulated and b) downregulated in conspecific vs. heterospecific-exposed female *X. birchmanni*. Visualized with Revigo using the SimRel similarity index (Schlicker, Domingues et al. 2006). Positioning in semantic space indicates functional similarity of GO terms, although the semantic space units have no intrinsic meaning.

Table 1 – List of significantly enriched PANTHER biological pathways in list of genes differentially up- or down-regulated between B-EXP and M-EXP females. P-value shown is FDR adjusted.

ID	Description	Relative B-EXP expression	p-value
P00010	B cell activation	↑	< 0.0001
P00031	Inflammation mediated by chemokine and cytokine signaling pathway	↑	< 0.0001
P00053	T cell activation	↑	< 0.0001
P00009	Axon guidance mediated by netrin	↑	0.0147
P00041	Metabotropic glutamate receptor group I pathway	↓	0.0441
P00026	Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway	↓	0.0458
P00027	Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway	↓	0.0480

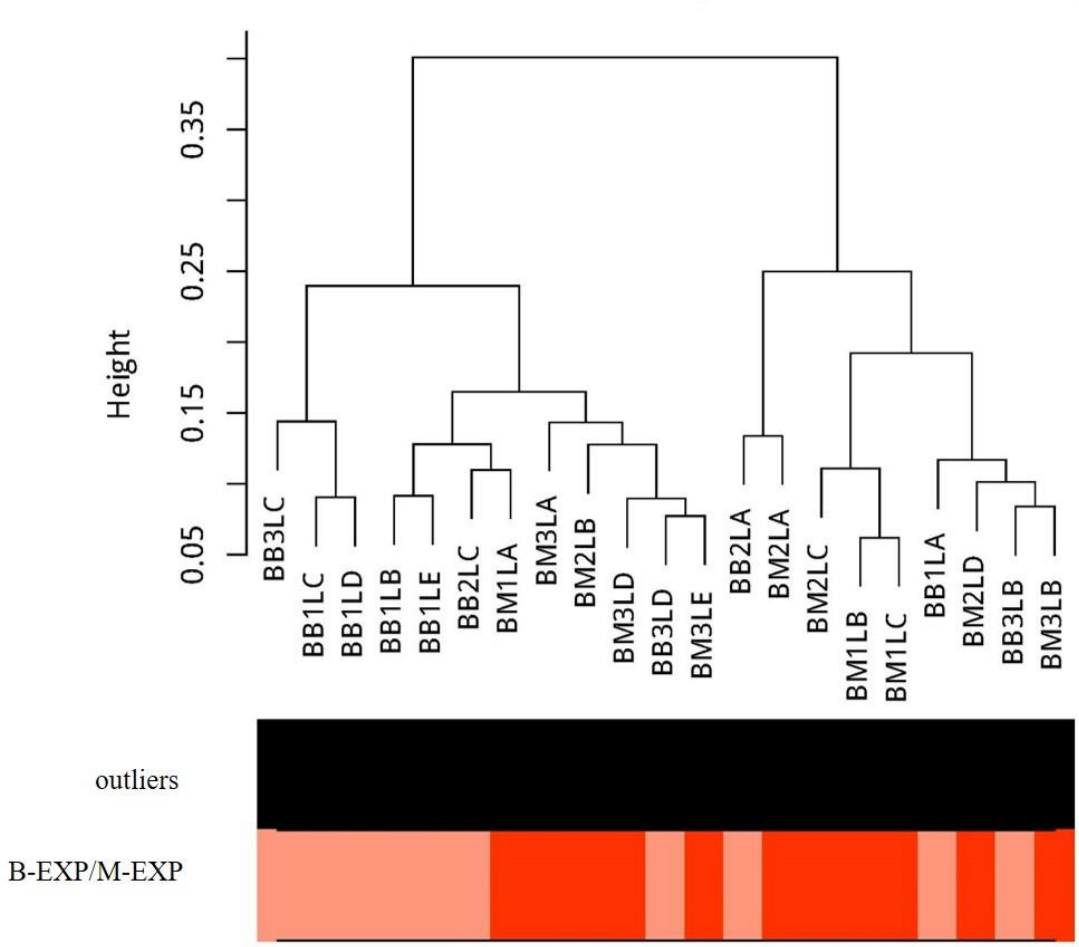

Figure 10 – Cluster dendrogram measuring similarity among conspecific- and heterospecific-exposed female *X. birchmanni* gene expression profiles. Pink bars represent conspecific-exposed females (B-EXP), and red bars represent heterospecific-exposed females (M-EXP). Lower height values indicate greater similarity between samples. Similarity was assessed via a Euclidean distance based network in the WGCNA package in R

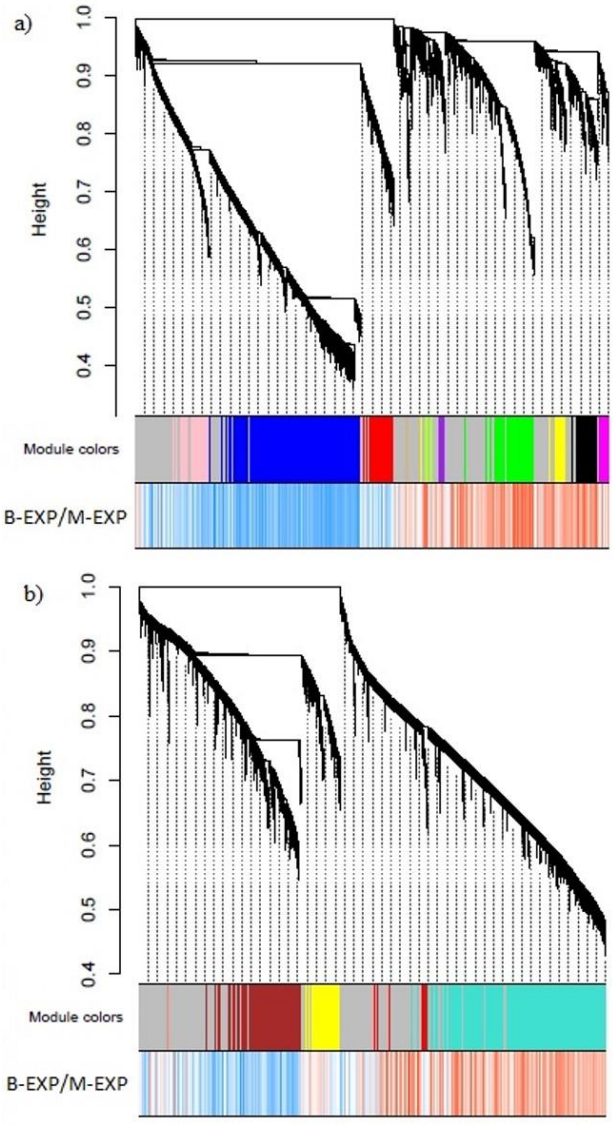

Figure 11 – Hierarchical cluster tree of all genes passing coverage filter based on similarities in expression. Module color bands represent module identities of individual genes (individual tree leaves) using the blockwise automatic module detection method in the WGCNA package in R (a = block 1, b = block 2). The bottom row of colors denotes the association of a gene with a given exposure treatment (blue: relatively upregulated in M-EXP, red: upregulated in B-EXP).

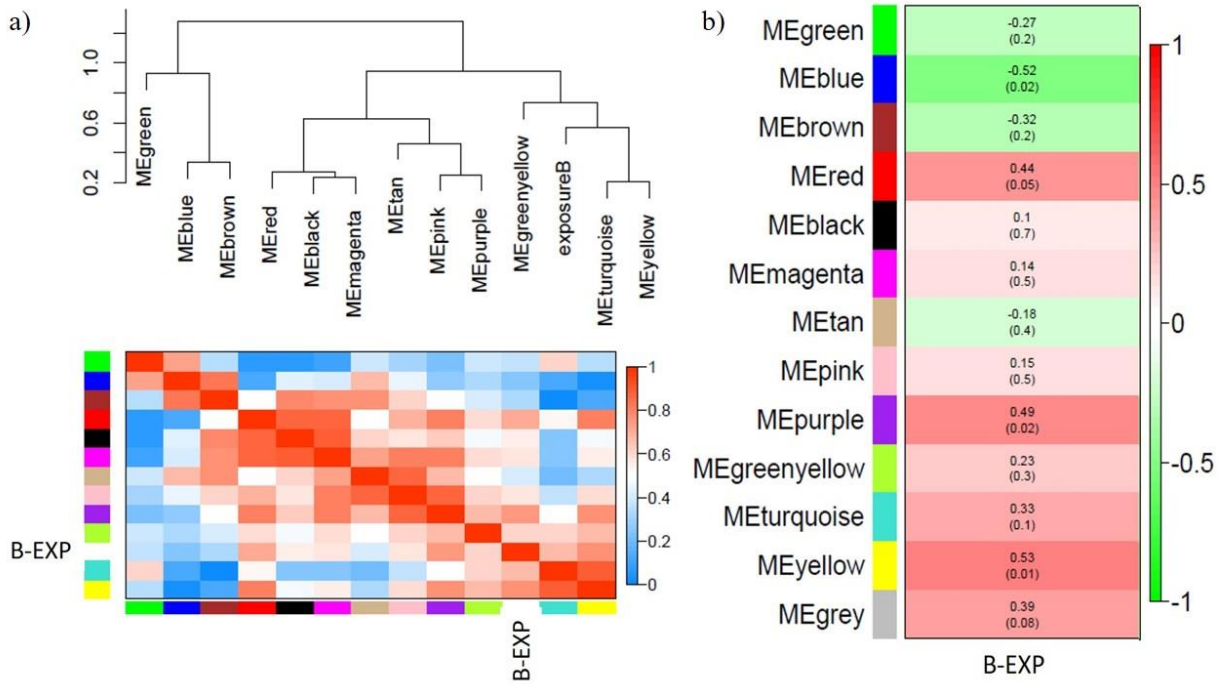

Figure 12 – Heatmap of the eigengene network representing relationships among modules and between modules and exposure to conspecifics. a) The top panel shows a hierarchical clustering dendrogram of the modules according to similarity, while the bottom panel shows the module adjacency values as calculated in the WGCNA package in R. b) Table of module-trait correlations and p-values for comparison of B-EXP and M-EXP female gene expression profiles. The table is color-coded by relative upregulation in B-EXP females according to the legend on the right. MEblue: “synapse” module, MEpurple: “vision” module, MEyellow: “olfaction” module.

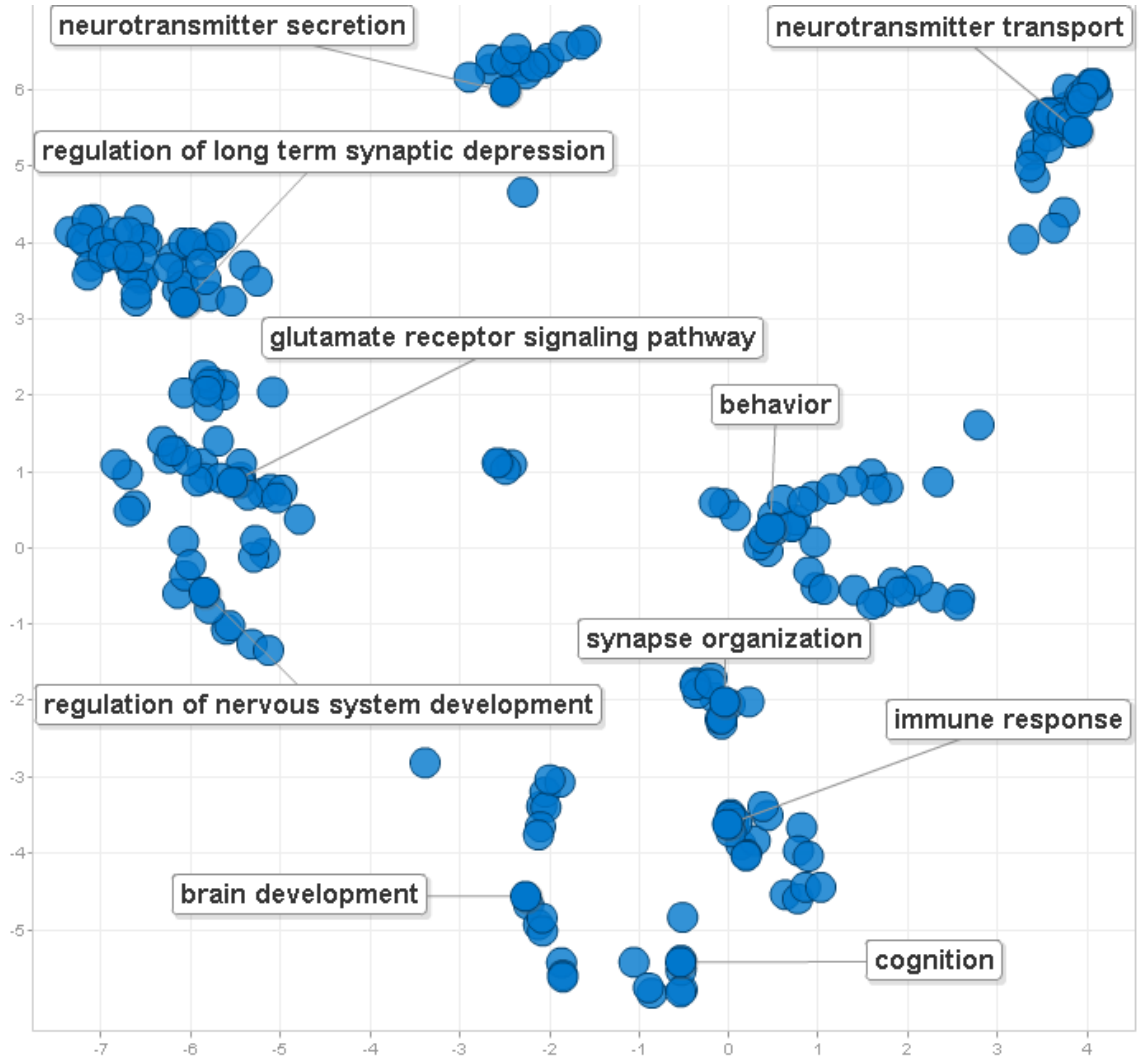

Figure 13 - Significantly enriched gene ontology terms of the list of genes within the synapse module. These genes were, on average, upregulated in heterospecific-exposed female *X. birchmanni*. Visualized with Revigo using the SimRel (Schlicker, Domingues et al. 2006) term similarity index. Positioning in semantic space indicates functional similarity of GO terms, although the semantic space units have no intrinsic meaning.

values, *pde6g, gnat1, aipl1,* and *rlbp1a*) have direct roles in visual detection (Pittler, Baehr et al. 1990, Jacobson, Cideciyan et al. 2011, Naeem, Chavali et al. 2012, Nagashima, Barthel et al. 2013).

The olfaction module consists of 951 genes (Table S8), is upregulated in B-EXP females, and consists of genes with roles in ribosomal activity and a wide variety of GO terms (Figure 14b, Table S9). In this module, the genes with the highest modular membership, *rpl35*, *rpl7*, *btf3*, *nsa2* and *ddx21*, all have known roles in the assembly or maintenance of ribosomes. This module also contains a high proportion of odorant receptors. Interestingly, all but two of the annotated genes with roles in sensory perception of smell are found in this one module. Furthermore, all show the same trend of relative upregulation in B-EXP females relative to M-EXP females (Figure 15).

Discussion

Social experience is necessary for the development of olfactory conspecific recognition

Female *X. birchmanni* showed positive experience-dependent preferences for olfactory cues, in contrast to females of the sister species *X. malinche* which develop relative antipathy for familiar olfactory cues (Cui, Delclos et al. 2017). These results confirm previous research showing that *X. birchmanni* female preferences for the familiar can occur after both short-term and long-term exposure to different social cues (Verzijden and Rosenthal 2011, Verzijden, Culumber et al. 2012). Furthermore, this is the first study to show that *X. birchmanni* do not develop an innate olfactory preference

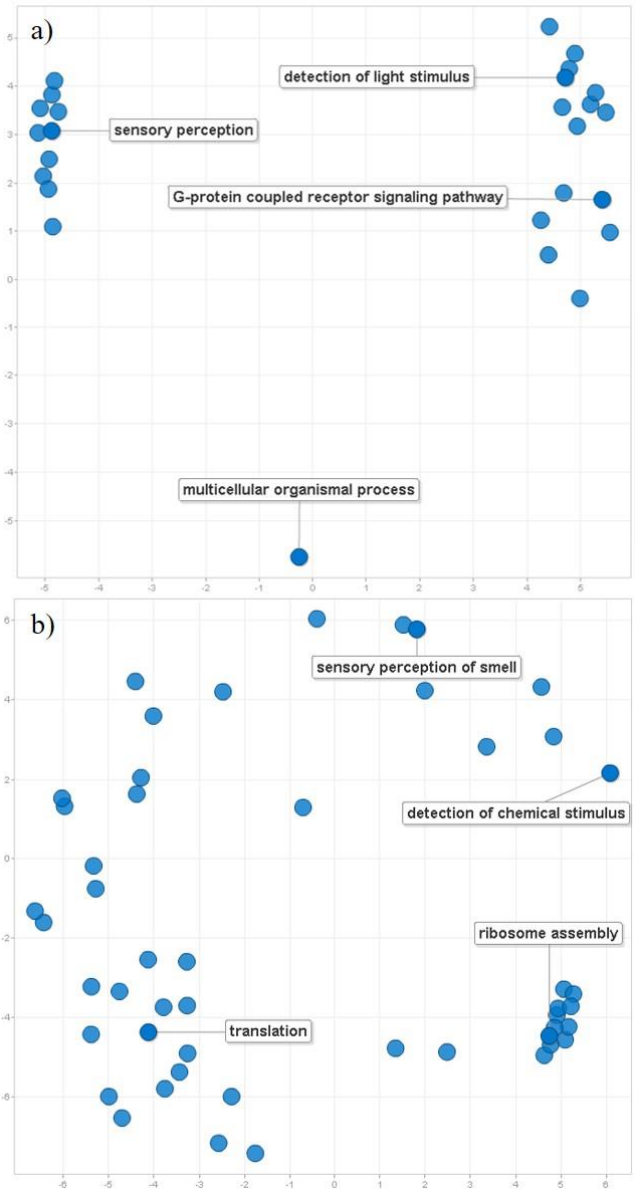

Figure 14 - Significantly enriched gene ontology terms of the lists of genes within the vision and olfaction modules. The genes within the (a) vision module and (b) olfaction module were, on average, upregulated in conspecific-exposed female *X. birchmanni*. Visualized with Revigo using SimRel index (Schlicker, Domingues et al. 2006). Positioning in semantic space indicates functional similarity of GO terms, but the axis units have no intrinsic meaning.

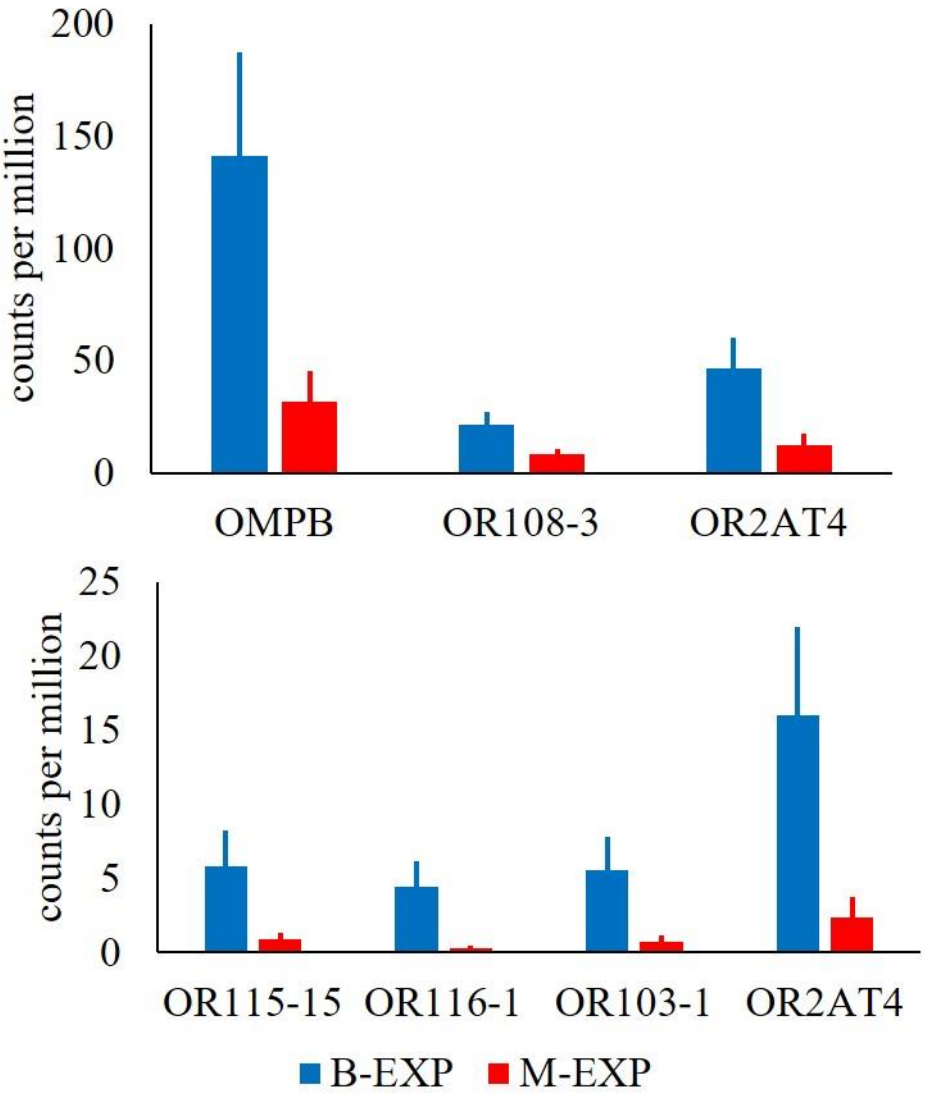

Figure 15 – Representative expression levels for genes within the gene ontology term "sensory perception of smell". Y-axis represents total reads in counts per million. All genes shown are significantly upregulated in conspecific-exposed (blue) relative to heterospecific-exposed (red) females after FDR correction.

for conspecifics, as females that were socially isolated from adults throughout development formed no olfactory preference for conspecific cues; rather, learning from the social environment may be necessary for development of these preferences. This crucial role of social experience in the development of mating preferences has been shown in other systems as well (Bischof and Clayton 1991), and may differ significantly from learning mechanisms in *X. malinche*, where exposure to heterospecifics, but not conspecifics, was required to elicit conspecific mating preferences. Because of these stark differences in learning between such closely related species, *X. birchmanni* and *X. malinche* could serve as a model system for understanding the behavioral and neural mechanisms of sexual imprinting, and its evolutionary implications.

Exposure type affects neural development more than exposure *per se* in maturing adults

Our results revealed greater differentiation between neural gene expression profiles of conspecific- and heterospecific-exposed females than between either of these exposure groups and socially isolated females (Figure 7-8). Mirroring the behavioral differences between *X. birchmanni* and *X. malinche* females (Figure 6), this is strikingly different from patterns of gene expression change in *X. malinche* in response to social exposure. In *X. malinche*, the differences in gene expression profiles between socially exposed and isolated females were greater than between females exposed to conspecifics versus heterospecifics (Cui, Delclos et al. 2017). This large degree of separation between M-EXP and B-EXP transcriptomes in *X. birchmanni* further suggests that learning mechanisms are not only necessary for the development of certain mating preferences,

but that a female's social environment plays a crucial role in neural development.

The observed differences in gene expression profiles may be driven by morphological differences caused by exposure to conspecifics versus heterospecifics. It is possible that females from one exposure group were, on average, larger than those from another group, as seen in the male swordtails (see Chapter IV). While the females selected for RNAseq analyses were chosen based on size similarities across treatments, it is possible that the observed transcriptomic differences are influenced by treatment-level differences in population structure. Future studies focusing on the role of population-level phenotypic distribution on an individual's neural development will provide a clearer interpretation of the results obtained in this study.

Although I found a high level of separation between conspecific- and heterospecific-exposed female gene expression profiles, I found little to no significant difference between either exposure group and socially isolated females. One possible explanation for this is that a lack of social experience results in greater variation in transcriptomic profiles due to a relatively greater contribution from unmeasured environmental variables as well as stochastic influences on development. Another possibility is that social exposure results in a sort of canalization process of neural development. In zebra finches, social exposure during critical time periods results in a neuronal "shedding" process in key brain regions where only those neurons that are activated in response to a given social stimulus are retained, and inactive neurons are lost (Changeux and Mikoshiba 1978, Bischof 2003). In socially isolated juveniles, a lack of exposure significantly slows down this process (Bischof, Geißler et al. 2002). If this

mechanism is similar in *X. birchmanni*, then this process could potentially cause the observed variation in the transcriptomes of socially-isolated individuals.

Lastly, this relatively greater separation in M-EXP and B-EXP transcriptomes may be a result of the time at which brains were examined. While anatomical research has shown that swordtails' olfactory epithelia undergo a rapid maturation process shortly before sexual maturation (Schreibman, Margolis-Kazan et al. 1984), behavioral studies show that juveniles as young as seven days old can attend to olfactory cues of adult conspecifics and exhibit social preferences (Coleman and Rosenthal 2006). Social exposure during the first ten weeks of life are sufficient for developing olfactory preferences for familiar phenotypes upon maturation (Verzijden and Rosenthal 2011). In other systems, learned social preferences in juveniles during critical periods of development often translate to later sexual preferences in adults (Bischof 1979, Bischof 1994). Therefore, social environment likely affects neural development in discrete stages throughout early life, and that the largest transcriptomic separation between socially isolated and exposed individual brains may occur much earlier in life. Meanwhile, separation in neural gene expression profiles may be expected to become more prominent between social exposures shortly after maturation, at a time when these developed mating preferences are becoming more relevant to the individual in preparation for future mate choice. Future studies should focus on addressing the temporal nature of sexual imprinting in order to identify 1) the trajectory of neural development as it relates to the social environment and 2) the transcriptomic imprint of critical periods in development where sexual preferences become consolidated.

Exposure type alters regulation of immune processes and neurogenesis

A traditional analysis of differential expression between conspecific and heterospecific-exposed female neural tissues revealed 919 differentially regulated genes. Of these genes, 468 were upregulated in conspecific-exposed females, and GO enrichment analysis revealed that these genes largely have functional roles in immune response (Figure 9a). Exposure to adult male conspecifics may induce a female defense response, as seen in other species (Lawniczak and Begun 2004, Bailey, Gray et al. 2011, Immonen and Ritchie 2012). Previous studies have described a typical upregulation of immune-related genes when comparing virgin to mated females (McGraw, Gibson et al. 2004). Furthermore, in fruit flies, exposure to conspecific courtship displays also resulted in a similar upregulation of immune-related and odorant-binding genes in conspecific-exposed relative to heterospecific-exposed females (Immonen and Ritchie 2012). This response by females could be in anticipation of future sexual conflict caused by internal fertilization and sperm competition (Neville and Goodwin 2012), a typical mating tactic in poeciliid fish (Pilastro, Benetton et al. 2003, Paczolt, Passow et al. 2014). However, the results from this study suggest that this transcriptomic response may not require mating to be induced; rather, exposure throughout development, specifically to adult conspecifics, may be sufficient to activate the observed immune-related processes.

The remaining 451 genes that were upregulated in heterospecific-exposed females had roles largely pertaining to neurogenesis and synaptic plasticity (Figure 9b). This is the first study, to the best of my knowledge, to find these classes of genes to be

upregulated in heterospecific-exposed individuals relative to conspecific-exposed ones. In many songbirds, juveniles have auditory biases that lead to a development of species-specific song predispositions (Marler and Peters 1977, Konishi 1985, Marler 1997, Wheatcroft and Qvarnström 2015). These predispositions can also lead to biases in neural development. For example, embryonic transplants of brain regions with functional roles in auditory learning have been shown to result in the development of donor mating preferences (Long, Kennedy et al. 2001). While the behavioral results from my study suggest that females do not have an innate *behavioral* preference for conspecific chemical cues, it is possible that there is still a *neural* predisposition towards developing a brain that is tuned towards learning to prefer conspecific cues, a so-called "instinct to learn" (Marler 1991). A deviation from this predisposition via exposure to a novel phenotype, such as adult heterospecifics, could potentially result in a neuronal "rewiring" process that results in a relative upregulation of genes related to neurogenesis and synaptic plasticity. However, future experiments are required to validate this hypothesis. Further studies examining the differential neural mechanisms between exposure types will provide a much-needed understanding of the neural and behavioral consequences of exposure to novel experiences, such as a heterospecific environment.

Conspecific exposure fine-tunes the sensory periphery

WGCNA revealed three gene modules that are differentially regulated between conspecific and heterospecific-exposed females (Figures 7-9). The largest of these modules (synapse: 2,768 genes), which was relatively upregulated in M-EXP females, contained 380 of the genes found to be differentially expressed between conspecific and

heterospecific-exposed females in the traditional differential expression analysis. Due to the large overlap between datasets, many similar gene ontology terms were significantly enriched in the synapse module. However, the synapse module was more heavily comprised of genes with functional roles in synaptic transmission, neurogenesis and brain development relative to immune response genes. Furthermore, WGCNA revealed that these genes strongly covary with genes related to cognition and behavior. Therefore, this result provides strong evidence that heterospecific-exposed females exhibit relative upregulation of neuroplasticity-related genes that are either directly or indirectly tied to cognition and behavior.

The other two significant modules (vision: 86 genes, olfaction: 951 genes) were relatively upregulated in conspecific-exposed females. The vision module contained one gene that was significantly differentially expressed between the two exposure treatments. However, on average, genes in this module tended to be more highly expressed in B-EXP females. Gene ontology enrichment analyses on the vision module revealed that it is largely comprised of genes pertaining to visual detection. Specifically, many of these genes have important roles in the retina, which was not included in the tissue sample. However, most of these genes also have moderate expression levels within the brain, though their functions in these regions are not well known. The results from this study suggest an alternative role of these genes in downstream visual detection and processing. Future studies should focus on localizing where these genes are expressed in the brain in order to better determine their functional relevance to learned mating preferences.

The olfaction module contains 146 genes found to be differentially expressed

between conspecific and heterospecific-exposed females. I identified a large group of genes within this module directly involved in the sensory perception of smell (GO: 0007608). All but two of the annotated genes within this biological process were found within the olfaction module. Furthermore, all of these genes showed relatively greater expression in conspecific-exposed versus heterospecific-exposed females (Figure 15). This suggests that conspecific recognition may not rely on the activation of a single or few types of odorant receptors, but rather an entire suite of species-specific receptors. The results from the vision and olfaction modules support the hypothesis that exposure to conspecifics upregulates sensory *detection* genes through a potential "fine-tuning" mechanism. This follows similar previous studies which suggest that exposure to a given stimulus increases the expression of odorant receptor genes for that stimulus, and thereby improves detection (Bazáes, Olivares et al. 2013, Saraiva, Ahuja et al. 2015).

While I found several odorant receptor genes that are upregulated with conspecific exposure, no annotated odorant receptor genes were upregulated in heterospecific-exposed females. Male *X. birchmanni* chemical cues might elicit upregulation of cue-specific odorant receptors beyond a baseline level of expression, whereas *X. malinche* males may lack this chemical structure. For conspecific-exposed females, detection of these chemical cues may be sufficient for triggering a mating preference for them. However, heterospecific-exposed females show a relative preference for *X. malinche* olfactory cues despite a lack of upregulation of any annotated odorant receptor genes. Instead of preferring the cue that elicits the strongest activation of the sensory periphery, females in this scenario might be relying more on *processing*

the cues they are detecting. This mechanism would explain the observed relative upregulation of synaptic-plasticity related genes in heterospecific-exposed females. Future studies should follow up on this experiment by directly testing the hypothesis that conspecific exposures result in the prioritization of sensory *detection* while heterospecific or novel exposures prioritize sensory *processing*.

PANTHER analysis reveals candidate molecular pathways implicated in learned mating preferences

To gain a better understanding of the specific biological pathways that might be functionally implicated in the development of learned mating preferences, I conducted a PANTHER molecular pathway enrichment analysis on the list of differentially expressed genes between conspecific and heterospecific-exposed female *X. birchmanni*. The analysis revealed seven molecular pathways that may directly or indirectly explain the behavioral differences between B-EXP and M-EXP females (Table 1). Three immune-related pathways were identified, all of which were upregulated in conspecific-exposed females.

I also identified one pathway related to axon guidance by netrin that was upregulated in conspecific-exposed females. This result suggests that, while on average heterospecific-exposed females exhibit a relative upregulation in genes related to the generation and development of neurons, conspecific-exposed females are also experiencing a localized upregulation of specific neural processes. Netrins can minimize spatial memory impairment (Bayat, Baluchnejadmojarad et al. 2012) and have previously been found to be upregulated in key imprinting brain regions in juveniles that

successfully imprinted (Yamaguchi, Fujii-Taira et al. 2008). This result is in accord with the hypothesis that different exposure types elicit the activation of different learning mechanisms. Future studies on the functional relevance of netrin-mediated axon guidance with regards to imprinting will help explain why its regulation may be sensitive to different types of exposure.

PANTHER pathway analysis also revealed three pathways upregulated in heterospecific-exposed females pertaining to glutamate receptor pathways and pathways involved in the signaling of G-protein alpha subunits s, i, q, and o. Group 1 metabotropic glutamate receptors have been shown to be required for the formation of memories (Rodrigues, Bauer et al. 2002, Homayoun, Stefani et al. 2004), and G-protein alpha subunits o, s, and i are expressed at the sensory periphery and implicated in the olfactory signal transduction needed to process odors (Jones and Reed 1987, Jia and Halpern 1996, Leinders-Zufall, Brennan et al. 2004). Furthermore, *rgs2*, which actively inhibits G-protein alpha subunit s signaling in the olfactory epithelium (Sinnarajah, Dessauer et al. 2001, Kehrl and Sinnarajah 2002), exhibits upregulation in conspecific-exposed females (FDR < 0.05). Interestingly, this same upregulation is seen in conspecific-exposed female *X. malinche*, who learn to disdain the familiar (Cui, Delclos et al. 2017), suggesting that conspecific exposure may have similar effects on the sensory periphery of both species, while downstream changes in the processing and valuation of these detected cues result in the observed behavioral dichotomy. The molecular pathways identified in the PANTHER analysis will serve as good starting points for future studies assessing their functional roles in the development of learned mating preferences.

Conclusions

Female mate choice can play a pivotal role in the nature and extent of reproductive isolation between species. Mating preferences are often learned from an individual's social experience with adult phenotypes throughout development. The results from this study revealed that social exposure is necessary for the development of learned olfactory preferences in *X. birchmanni*. This exposure evokes strong gene expression responses in both the sensory periphery and the brain. Specifically, in maturing females, the type of social exposure (conspecific vs. heterospecific) can result in greater neural differentiation than exposure itself relative to isolation from adults. Furthermore, conspecific exposure appears to result in a relative upregulation of genes pertaining to the immune system and visual and olfactory detection, while heterospecific exposure is characterized by an increased expression of neurogenesis and synaptic-plasticity related genes. These results suggest that the neural mechanisms for developing and expressing a learned preference may be dependent on the type of social exposure a female experienced. Specifically, females exposed to adult conspecifics may rely more heavily on the detection of chemical cues, while those exposed to heterospecifics may rely more on the downstream processing of these cues in the brain. Lastly, this study identified molecular pathways that will serve as the foundations for future studies assessing their causal implications in the development of learned mating preferences.

CHAPTER IV

CULTURAL TRANSMISSION OF A HETEROSPECIFIC PERSONALITY TRAIT IN A VERTEBRATE WITHOUT PARENTAL CARE

Introduction

Personality - consistent behavioral variation among individuals across time and contexts - interacts in important ways with individual fitness (Ariyomo and Watt 2012, Ariyomo and Watt 2013) and social structure (Wilson, Grimmer et al. 2013, Briffa, Sneddon et al. 2015). Personality-related traits are often under strong ecological selection (Reale, Dingemanse et al. 2010, Sih, Cote et al. 2012, Wolf and Weissing 2012) and may play a key role in speciation through its effects on mate choice (Ingley and Johnson 2014). In particular, assortative mating according to personality is widespread and could result in reproductive isolation over time (Schuett, Tregenza et al. 2010, Nosil 2012). Alternatively, differences in personality could impede reproductive isolation by affecting individual preference functions (Coleman, Patricelli et al. 2004, David and Cézilly 2011, Sommer-Trembo, Bierbach et al. 2016). Personality could thus constitute a 'magic trait' (Servedio, Van Doorn et al. 2011), simultaneously under divergent ecological selection and coupled to divergence in mate-choice mechanisms (Boughman and Svanbäck 2017, Rosenthal 2017).

Personality traits are often highly heritable (Schuett, Tregenza et al. 2010, Thomson, Watts et al. 2011, Wisenden, Sailer et al. 2011, Ariyomo, Carter et al. 2013) and subject to contextual cues and recent experience (Suboski, Bain et al. 1990, Mathis,

Chivers et al. 1996). Few studies, however, have addressed the intergenerational transfer - or cultural transmission - of personality traits [but see (Schuett, Dall et al. 2013)]. Cultural transmission can increase individual fitness by allowing for the development of relatively rapid behavioral adaptations to fluctuating environments (Danchin 2011). Cultural transmission through social learning is sufficient for the development of mating preferences that maintain reproductive isolation between closely related species (Verzijden and ten Cate 2007, Verzijden, Ten Cate et al. 2012). As personality and mate choice dynamics are intricately coupled (David and Cézilly 2011, Bierbach, Sommer-Trembo et al. 2015, Sommer-Trembo, Bierbach et al. 2016), it is likely that personality-related traits have culturally inherited components as well.

I studied the effects of social exposure to adults during ontogeny on personality-related traits in the swordtail fish *Xiphophorus birchmanni*. Boldness, the measure of how likely an individual is to explore and take risks in a relatively novel environment (Wilson, Clark et al. 1994, Sih, Bell et al. 2004), is the most well-studied axis of personality in fish (Toms, Echevarria et al. 2010) and has been shown to be positively correlated with certain fitness-related traits, such as reproductive success, social dominance and anti-predator behaviors (Dingemanse and de Goede 2004, Bell and Sih 2007, Ariyomo and Watt 2012), although bolder individuals also experience a greater risk of mortality (Dugatkin 1992, Stamps 2007). *X. birchmanni* show repeatable within-individual correlations in traits related to boldness (Boulton, Grimmer et al. 2014). *X. birchmanni* are relatively bold compared to the closely related sister species *X. malinche* (Johnson, Culumber et al. 2015).

Hybridization between the two species occurs in areas of coexistence, and hybrid individuals display intermediate boldness behaviors (Johnson, Culumber et al. 2015). However, it remains unknown whether this observed difference is genetically-based or may be influenced through the social environment. Studies of cultural transmission largely focus on social interactions within species. However, interspecific encounters can often affect behavioral development among individuals (Mathis, Chivers et al. 1996, Verzijden, Korthof et al. 2008, Verzijden and Rosenthal 2011). In this study, I assess whether boldness can be culturally inherited across species by male and female *X. birchmanni* through observational learning. If the development of boldness behaviors has a socially sensitive component, then I expect individual *X. birchmanni* boldness traits to mimic the species to which an individual was exposed throughout development.

Differences in personality-related traits have been shown to affect growth in *X. birchmanni* males (Wilson, Grimmer et al. 2013). Over longer timescales, the behavioral consequences of differing social environments during ontogeny may shape adult morphology (Stamps 2007), further affecting mate choice dynamics, as female swordtails rely on both visual and chemical cues to choose mates (Fisher, Mascuch et al. 2009). To test whether social exposure affects the development of secondary sexual traits, I assessed the effects of social environment on male morphology, and correlated this to observed boldness measures.

Lastly, personality could potentially play an important role in predicting individual preference functions, particularly those preferences that are shaped via social cues, as shyer individuals across systems have been shown to typically prioritize social

over private information (Kurvers, Van Oers et al. 2010, Trompf and Brown 2014). To determine whether differences in personality and mating preferences are linked, I evaluated the association between female boldness measures and observed mating preferences for conspecific vs. heterospecific male odorant cues.

Materials and Methods

Fish collection and exposure treatments

Swordtails for this experiment were collected from the rearing experiment described in Chapter III. To summarize briefly, fifteen *X. birchmanni* females were collected from the Río Coacuilco in Coacuilco, Hidalgo, Mexico (Culumber, Fisher et al. 2011) in March 2014 and transported to Texas A&M University facilities where they gave birth. When offspring reached approximately 3 weeks of age, broods were evenly pooled, separated into groups of 30 juveniles, and assigned to one of 3 treatment groups (3 replicates each): 1) B-EXP: exposed to 2 males and 2 females of adult *X. birchmanni* from the Río Garces locality; 2) M-EXP: exposed to 2 males and 2 females of adult *X. malinche* from the Chicayotla locality (Culumber, Fisher et al. 2011) and 3) NO-EXP: controls which did not receive adult stimulus exposure. Exposure treatments were performed at 23 °C, 12:12 light:dark cycle in adjacent, though visually obstructed, 120-liter aquaria where adults and juveniles were divided by a transparent, perforated Plexiglas board which allowed for transmission of both visual and olfactory cues (Verzijden and Rosenthal 2011). Sufficient shelter was provided to both adults and juveniles, and water was continually changed via a flow-through system.

Juvenile males were removed from aquaria upon the first sign of maturation (hardening of the anal fin to form the gonopodium). The age at which the male was removed was noted, and the male was then placed into an individual tank, isolated from all individuals for 90 days to allow for complete development of secondary sexual traits. Male *X. birchmanni* are slightly aggressive and exhibit dominance in social environments (Wilson, Grimmer et al. 2013). Therefore, I isolated all males to account for any potential effects of the peer social environment on behavior. After 90 days in isolation, I performed shy-bold trials, and then photographed the males for morphological analysis.

Male shy-bold open field trials

Boldness behavior is commonly assessed across animal systems using an open-field trial (OFT) paradigm (Warren and Callaghan 1975, Walsh and Cummins 1976, Budaev 1997, Burns 2008, Boulton, Grimmer et al. 2014, Johnson, Culumber et al. 2015), where an individual is placed in an empty, open arena, and its behavior is observed for a predetermined amount of time. Therefore, to assess boldness, I conducted OFTs on male *X. birchmanni* swordtails as in previous experiments (Boulton, Grimmer et al. 2014) with minor modifications. I used a 75x19x20-cm tank filled to a depth of 15 cm with room temperature water (22 °C). Male swordtails were caught individually from their respective tanks with a dip net and immediately placed into the OFT tank, and behavior was immediately filmed with a video camera suspended above the tank. Water was changed between individual trials to prevent chemical cues from affecting subsequent behavior.

To gain a more complete estimate of boldness, we measured three behaviors in males: latency to enter an open area (L_o) and amount of time spent in an open area (T_o) as measures of risk-taking behavior, and total distance travelled (T_d) as a measure of exploratory behavior. I measured multiple behaviors likely to reflect variation along a shy-bold axis, as is frequently done in studies of boldness (Huntingford 1976, Moretz 2003, Boulton, Grimmer et al. 2014), in accordance with previous studies that warn against using any one measure of boldness on a given species which can be easily misinterpreted (Boulton, Grimmer et al. 2014). Furthermore, these three measures were chosen as they have each previously been suggested to be reliable indicators of boldness (Dingemanse, Wright et al. 2007, Boulton, Grimmer et al. 2014). Using a custom video-scoring script, the tank was divided into two equal zones, a perimeter and middle or "open" zone. With this script, I first noted L_o in seconds. Upon first entering the open zone, I then measured the amount of time spent in the open zone over the following 600 seconds (T_o).

In addition, I also measured T_d during the 600-second trial using the software program Tracker (Brown 2012). Briefly, every 20 frames (2 seconds) an individual's location in xy-space (origin placed at the bottom left of the arena) was measured. T_d was then calculated as the sum of all linear distances between successive data points.

Measuring female boldness behavior

Estimates of female shy-bold behavior were extracted from video footage of previously conducted mating preference trials. Briefly, female olfactory preferences were assessed in successive, 600-second trials (see Chapter III for method details). Due

to differences in experimental setup (i.e. the presence of a small shelter in the center of the trial arena), I was not able to obtain an accurate measure of L_o and did not directly compare differences in T_d behavior between the sexes. T_d was assessed for female swordtails during the entire 600-s trial as previously described. To gain a better understanding of shy-bold behavior in female swordtails, we also measured the amount of time females spent within the shelter provided during preference trials (T_s), a measure frequently used to estimate boldness behaviors (Boulton, Grimmer et al. 2014).

Measuring male morphology

To assess the effects of social environment on male morphology, males were weighed to the nearest 0.01 g and photographed on both sides after behavior trials. The images were then loaded on the ImageJ program (Abràmoff, Magalhães et al. 2004), and I measured males' body depth, dorsal fin, gonopodium and standard lengths to the nearest 0.1 mm. Measurements were averaged from both sides. I also recorded the number of vertical bars on each side of the male, as well as the presence or absence of a false gravid spot, a dark pigment near the base of the gonopodium.

I conducted a principal components analysis (PCA) on log + 1 transformed measurements using the rda function in the vegan package of R (center and scale = TRUE). Approximately 40.4% of total variance was explained in the first principal component (PC1), and 27.8% was explained by the second (PC2). Loading scores indicated that high PC1 scores correspond to longer, heavier males with large gonopodia and dorsal fins, and high PC2 scores correspond to early-maturing males with more vertical bars.

Measuring effect of social exposure on behavior and morphology

L_o, T_o, T_s and T_d were all square-root transformed to reduce positive skew (Boulton, Grimmer et al. 2014). To relate male morphology and behavior to exposure treatment, I used linear mixed-effects ANOVAs on each behavior and PC1 and PC2 measures of male morphology. Replicate was included as a random effect, and exposure treatment as a fixed effect. Boldness measures, PC1 and PC2 of male morphology were included as covariates to assess how personality-related traits and morphological measures covaried with one another. I then ran Tukey's Honest Significant Difference contrasts to compare between-group differences in all behaviors and morphology using the multcomp package in R. ANOVAs were conducted using the nlme package in R.

To compare individual female boldness behaviors with observed mating preferences, I conducted a similar analysis, including replicate as a random effect, exposure as a fixed effect, and boldness measures and net mating preference as covariates.

Results

Exposure effects on boldness

Early social experience had a significant effect on both the amount of time taken for a male to enter the open zone ($F(2, 62) = 20.7$, $p < 0.0001$, Figure 16a), as well as the amount of time spent in the open zone during a 10-minute period ($F(2, 62) = 4.07$, $p = 0.022$, Figure 16b). Specifically, male *X. birchmanni* that had been exposed to heterospecific *X. malinche* adults took significantly longer to enter the open area of the

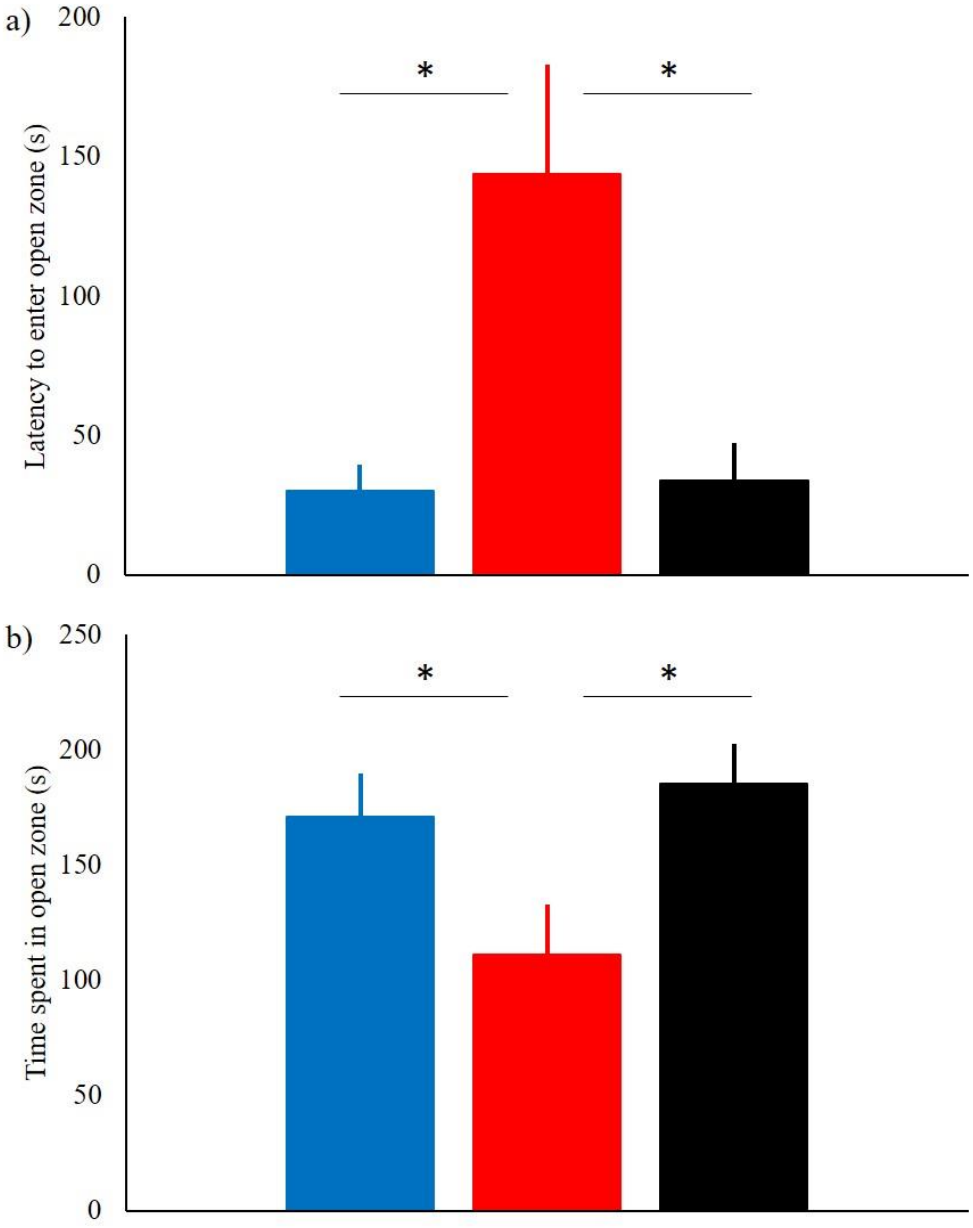

Figure 16 – Mean times a) taken to enter open zone and b) spent in open zone by male *X. birchmanni* during open-field shy-bold behavior trials according to social exposure treatment. Blue bars: exposed to conspecific adults throughout development, red bars: exposed to heterospecific *X. malinche* adults, and black bars: isolated from adults. Error bars represent SEM. Asterisks denote significant differences between groups ($p < 0.05$).

arena (143.8 ± 39.0 s) than those exposed to adult conspecifics (30.1 ± 9.4 s, $p < 0.0001$) or those isolated from adults altogether (34 ± 13.1 s, $p < 0.0001$). Meanwhile, males exposed to heterospecific adults throughout development spent the least amount of time within the open zone of the arena (111.3 ± 21.7 s), and significantly less time in this area than males exposed to adult conspecifics (171.1 ± 18.7 s, $p = 0.042$) and males isolated from adult swordtails (185.7 ± 16.8 s, $p = 0.011$). Males exposed to conspecific adults did not significantly differ from socially isolated males in either latency to enter or time spent within the open area ($p = 0.76$ and $p = 0.40$, respectively).

Social exposure had no significant effect on the total distance traveled during shy-bold trials ($F(2,62) = 1.74$, $p = 0.18$, Figure 17). NO-EXP males (1459.0 ± 113.7 cm) traveled relatively further than M-EXP (1275.3 ± 136.5 cm) and B-EXP males (1223.9 ± 78.2 cm), though neither difference was significant (NO-EXP/M-EXP: $p = 0.363$, NO-EXP/B-EXP: $p = 0.107$, B-EXP/M-EXP: $p = 0.818$).

Exposure type had a considerable role in shaping female shy-bold behaviors, as heterospecific-exposed female *X. birchmanni* spent significantly more time within the provided shelter than conspecific-exposed females (M-EXP: 41.6 ± 11.7 s, B-EXP: 15.9 ± 4.3 s, $p = 0.042$, Figure 18a). Neither exposure treatment differed significantly in T_s from females isolated from adult swordtails (NO-EXP: 28.9 ± 9.7 s, both $p > 0.05$). Furthermore, heterospecific-exposed female *X. birchmanni* traveled significantly less distance (M-EXP: 489.9 ± 55.6 cm) than both conspecific-exposed (B-EXP: 685.3 ± 79.7 cm, $p = 0.047$) and isolated females (NO-EXP: 690.0 ± 64.4 cm, $p = 0.020$, Figure 18b).

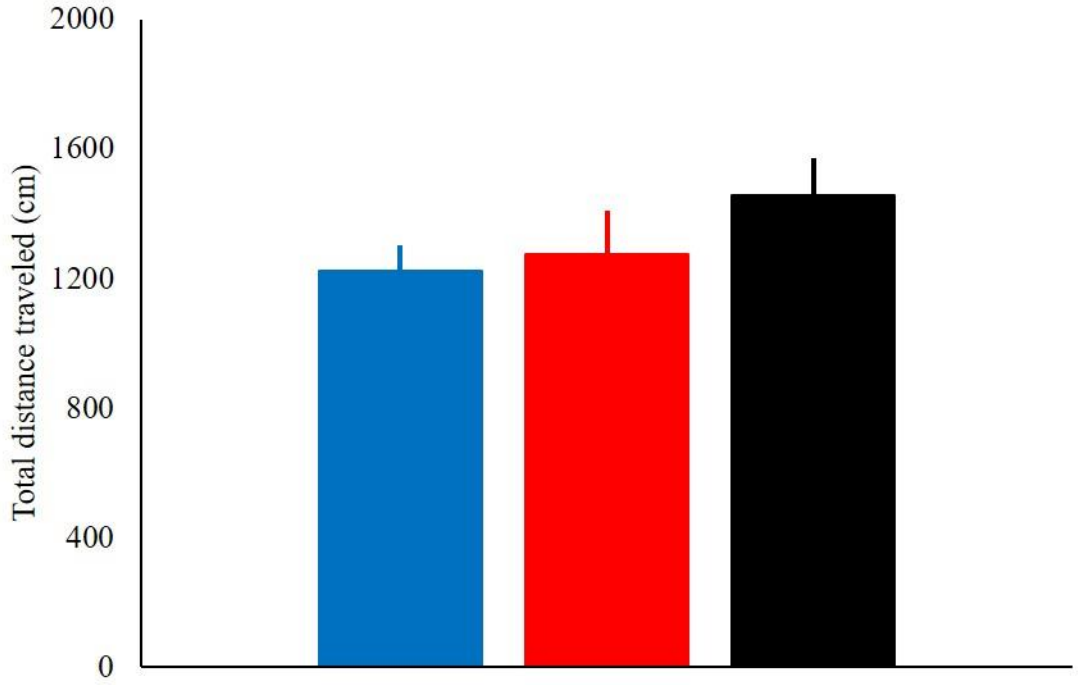

Figure 17 - Mean distance *Xiphophorus birchmanni* males traveled during open-field shy-bold behavior trials according to social exposure treatment. Blue bar: exposed to conspecific adults throughout development, red bar: exposed to heterospecific *X. malinche* adults, and black bar: isolated from adults). Error bars represent SEM.

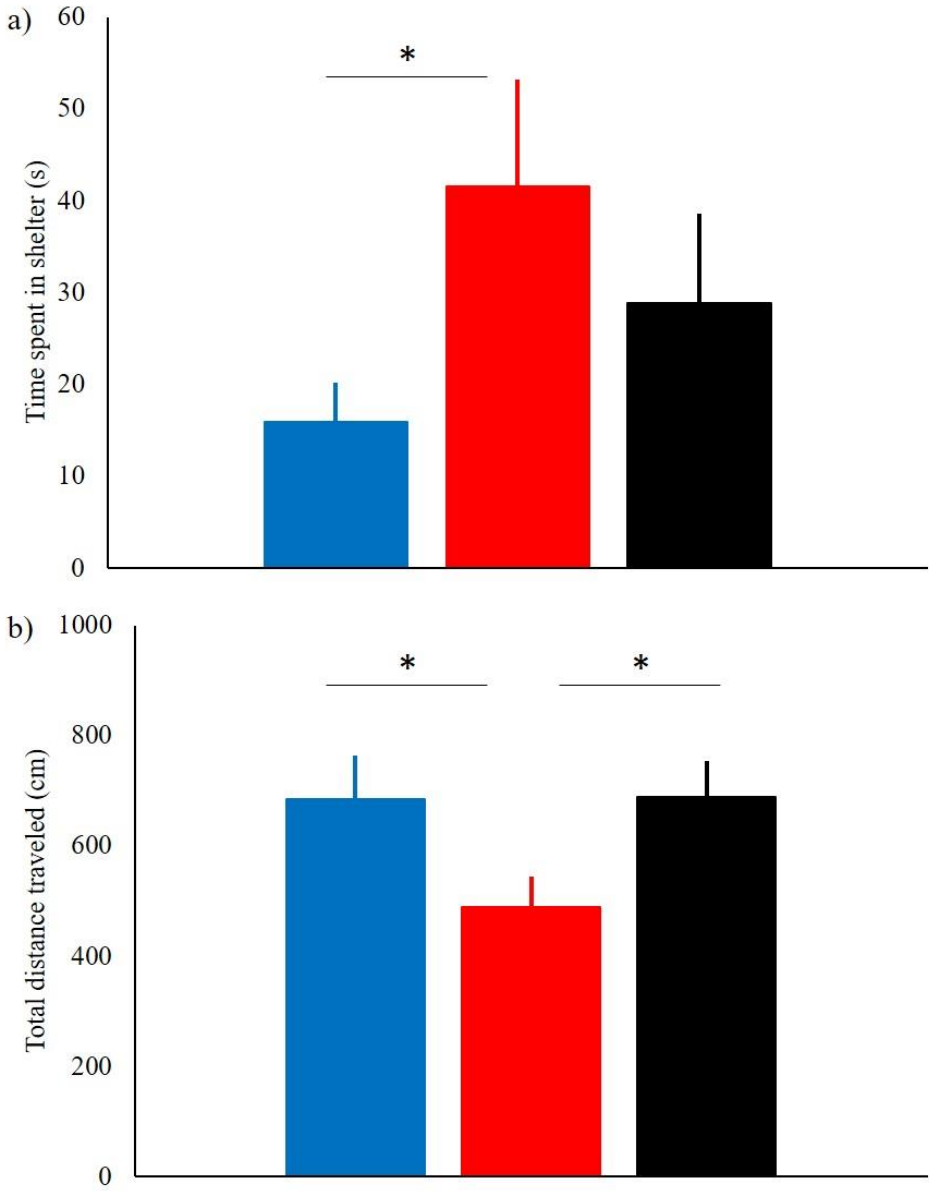

Figure 18 – a) Mean time spent in sheltered area and b) mean distance traveled by *Xiphophorus birchmanni* females during mating preference trials according to social exposure treatment. Blue bars: exposed to conspecific adults throughout development, red bars: exposed to *X. malinche* adults, and black bars: isolated from adults. Error bars represent SEM. Asterisks denote significant differences between groups ($p < 0.05$).

Exposure effects on male morphology

Early social experience had a significant effect on male morphology. Specifically, exposure significantly affected PC1 ($F(2,62) = 6.47$, $p = 0.003$). Male *X. birchmanni* exposed to adult conspecifics were, on average, larger than males exposed to adult *X. malinche* (B-EXP: PC1 = 0.067 ± 0.081, M-EXP: PC1 = -0.216 ± 0.096), $p = 0.0049$). Socially isolated males were intermediate in size, though not significantly different than either exposure group (NO-EXP: PC1 = 0.033, 0.092, both $p > 0.05$, Figure C-1). No significant effect of exposure on PC2 was detected ($F(2,62) = 1.36$, $p = 0.27$).

Correlations between boldness and sexual traits

Male boldness behaviors significantly covaried with male morphology. Specifically, PC2 was significantly correlated with the total distance traveled during shy-bold trials ($F(1,62) = 5.82$, $p = 0.019$). However, this effect was relatively weak ($r^2 = 0.059$). No significant covariance between PC1 and observed boldness measures were observed (all $p > 0.5$).

The total distance a male traveled during shy-bold trials significantly covaried with the latency to enter the open zone ($F(1,62) = 4.67$, $p = 0.035$) and the amount of time spent in the open ($F(1,62) = 12.05$, $p = 0.001$), with males that traveled greater distances spending more time in, and less time to enter, the open zone. Latency and time spent in the open zone did not significantly covary with one another ($F(1,62) = 0.62$, $p = 0.43$).

Lastly, female boldness behaviors did not significantly covary with one another

($F(1,84) = 0.09$, $p = 0.77$) nor with observed female mating preferences (time in shelter: $F(1,84) = 0.36$, $p = 0.55$; distance traveled: $F(1,84) = 3.60$, $p = 0.06$).

Discussion

Boldness behaviors are culturally transmitted between swordtail species

In this study, I found a significant effect of social exposure on boldness-related behaviors. Whereas previous studies have highlighted the influence of the social environment on learned female mating preferences [see Chapter III and (Verzijden and Rosenthal 2011)], the results from this study show that cultural transmission plays an important role in shaping personality-related traits, and that the development of these traits are not bounded by species.

Previous studies examining the relationship between the social environment and boldness have focused on the short-term effects of direct encounters or observed contests on the focal individual (Frost, Winrow-Giffen et al. 2007, Nomakuchi, Park et al. 2009, Harcourt, Biau et al. 2010). However, this study is among the first to directly test the role of learning through social communication between generations in the development of boldness behaviors. Furthermore, the results from this study show that these social effects on boldness have long-term implications on personality later in life, as males were isolated from all social cues for 90 days prior to testing.

I previously found that the social environment plays a direct role in shaping female mating preferences (see Chapter III), and the results from this study suggest that social upbringing may also indirectly affect mate choice dynamics through changes to

personality-related traits in both males and females. Previous studies in other fish systems have found that personalities play an important role in mate choice by affecting both female mating preferences (Sommer-Trembo, Bierbach et al. 2016) as well as male mating preferences (Bierbach, Sommer-Trembo et al. 2015) and attractiveness (Godin and Dugatkin 1996). Swordtail females have previously been shown to prefer more active male courtship behaviors (Wong, So et al. 2011). Furthermore, the combination of personalities between males and females could have post-mating implications, as pairings of dissimilar personality types can result in lower reproductive success (Ariyomo and Watt 2013). I found no significant correlation between individual female boldness measures and mating preferences for conspecific versus heterospecific male chemical cues, although it is possible that preferences for other traits may be affected by differences in personality. The results from these studies suggest that the social environment can have a complex effect on mate choice dynamics via both direct effects on mating preferences as well as indirect effects through shaping the personalities of both sexes.

Social environment affects male morphology

In this study, I found a significant effect of social environment on male morphological development. Specifically, males raised with conspecifics tended to grow larger than those raised with heterospecific adults. Although the mechanisms behind this result are unclear, these effects might result from long-term physiological effects caused by differences in the social environment. Boldness measures are often correlated with testosterone levels (Chang, Li et al. 2012, Raynaud and Schradin 2014), which in turn

drive the development of secondary sexual traits in swordtails (Sangster 1948, Offen, Blum et al. 2008). *X. birchmanni* males have been shown to grow differentially according to personality, with more dominant and aggressive males growing at a faster rate (Wilson, Grimmer et al. 2013). This result is in accordance with my finding that heterospecific-exposed males, on average, grew less and were shyer than conspecific-exposed males. This morphological effect can be expected to have important mate choice implications, as female swordtails typically prefer larger and bolder males (Cummings, Larkins-Ford et al. 2008, Wong, So et al. 2011), which may allow for the cultural evolution of certain personalities. Furthermore, future studies should aim to address whether the observed morphological response to social exposure in males is driven by behavioral and physiological mechanisms, or if the reverse is true, and the behavioral responses to social exposure observed are driven by differences in morphology. Regardless of the result, this study has shown that *X. birchmanni* swordtails' social experiences throughout development can have important long-term consequences on both morphology and personality.

Future studies

Female *X. birchmanni* learn to prefer familiar visual and chemical cues [(Verzijden and Rosenthal 2011) and see Chapter III]. However, it remains unknown whether this learning process also translates to preferences for familiar personalities. Future rearing experiments should test whether learned male personalities confer an advantage in female mate choice. Developing a familiar personality would therefore benefit males by behaviorally matching the species preferred by female *X. birchmanni*.

Results from such a study would shed light on the potential selective implications of these learned personalities.

Whereas female *X. birchmanni* prefer familiar phenotypes, females of the sister species *X. malinche* learn to relatively disdain familiar chemical cues (Verzijden, Culumber et al. 2012). However, visual preferences in this system appear to be insensitive to learning (Cui, Delclos et al. 2017). Therefore, further research is required in the *X. malinche* system to determine whether male and female personalities are sensitive to the social environment, and whether females exhibit preferences for particular personalities. Regardless of the results, these studies would provide a foundation towards making the *X. birchmanni-X. malinche* system a powerful one for studying the evolution of personality.

The finding that personalities in *X. birchmanni* are sensitive to the social environment also makes this system highly suitable for future research on the neural mechanisms of learning-dependent personalities. Outside of studies involving human subjects, relatively little research has been conducted on the neural correlates of boldness (Beaton, Schmidt et al. 2008). The swordtail system could provide a basis for examining the neurogenetic framework of personality development in novel ways by being amenable to next-generation sequencing methods.

Conclusions

Recent theoretical and empirical research has highlighted the importance of considering individual personality in a variety of fields. However, the mechanisms by

which personality is developed in an individual remains largely unknown. While mating preferences have been shown to be largely influenced by the social environment through learning processes, the potential for personality-related traits to be culturally transmitted across species has yet to be examined. In this study, I tested whether male and female *Xiphophorus birchmanni* boldness behaviors varied according to exposure to conspecific adults, to adults of the sister species *Xiphophorus malinche*, and social isolation from adult swordtails. I found that both male and female *X. birchmanni* learned to develop boldness behaviors similar to their exposure models, mirroring the previous finding that female *X. birchmanni* learn to prefer familiar cues. Their ability to learn was not limited by species, as individuals raised with adult *X. malinche* developed personalities similar to their interspecific models. Furthermore, male morphology was significantly affected by social exposure, as conspecific-exposed males developed relatively larger than heterospecific-exposed males. The results from this study highlight the complexity with which cultural transmission can shape mate choice dynamics by affecting the development of mate-choice related morphology and behavior.

CHAPTER V

CONCLUSIONS

A major goal in evolutionary biology is to determine the behavioral and neural mechanisms by which reproductive isolation is maintained between populations. Mating preferences are extraordinarily sensitive to environmental and social factors. In my dissertation, I took advantage of the *Xiphophorus birchmanni – Xiphophorus malinche* swordtail system to determine how environmental and social cues affect the expression of behaviors that are implicated in female mate choice.

In Chapter II of my dissertation, I used behavioral, morphological and genetic data to describe standing variation in swordtail mate-choice related phenotypes across time and small-scale space. The results from this study highlight the level of variation in preference patterns across year and small-scale space, with female preferences for a condition-dependent chemical cue differing according to pools separated by only a few meters. Furthermore, these preferences switched according to year, highlighting the importance of replicating across time and space in order to more confidently determine the potential evolutionary implications of an observed mating preference. Measures of selection strengths through mate choice are often very weak (Qvarnstrom, Brommer et al. 2006), and the results from this study provide a clear example of how fluctuations in mating preferences at microspatial and temporal scales could weaken sexual selection within populations. Whether these differences in association times are related to real mating preferences or methodological error, the results from this study provide a strong

caution for past and future behavioral studies that extrapolate observed preference patterns to the population or species level.

In Chapter III, I heeded the cautions discussed in the previous chapter and tested the reproducibility of a previous finding that female *Xiphophorus birchmanni* learn to prefer the olfactory cues of familiar males when raised with conspecifics or heterospecifics. Furthermore, I tested a control group where juvenile swordtails were socially isolated from all adults and showed that preference for conspecifics may not be completely innate, but that social exposure may be *necessary* for the development of these preferences. I then used RNA-sequencing methods on sensory and brain tissue to identify differentially expressed genes and significantly enriched molecular pathways that correlate with this observed preference for a familiar phenotype. I found greater differentiation between gene expression profiles of conspecific- and heterospecific-exposed females than the differentiation observed between socially isolated females and either of these exposure groups. Specifically, conspecific-exposed female *X. birchmanni* experienced net upregulation of genes pertaining to immune response and visual and olfactory detection. Meanwhile, heterospecific-exposed females exhibited net upregulation of genes related to neurogenesis and synaptic plasticity. These results suggest that the type of social exposure elicits downstream transcriptional regulation of different biological processes that ultimately lead to learned preferences. Specifically, conspecific-exposed female *X. birchmanni* may rely more on sensory *detection* in order to realize a preference for conspecific males, whereas heterospecific-exposed females may rely on greater sensory *processing* to prefer heterospecific males. Previous theory

(Marler 1991, Marler 1997) has suggested the presence of "innate releasing mechanisms", where individuals may be neurally primed to learn from conspecific stimuli in social interactions. Consistent exposure to this stimulus subsequently improves the ability to detect the cue, as seen in other species (Corotto, Henegar et al. 1994, Bazáes, Olivares et al. 2013). However, exposure to a heterospecific cue may require a sort of neuronal rewiring of this primed architecture in order to create a modified template that allows the individual to learn to prefer the familiar heterospecifics. The findings from this study have opened several promising avenues of research that will elucidate the neurogenetic framework of learned mating preferences.

While I and other authors have shown that female mating preferences can be learned from their social environment via cultural transmission (Verzijden and Rosenthal 2011, Verzijden, Culumber et al. 2012), it was unknown whether other mate-choice relevant behaviors, such as personality-related traits, are culturally learned. In Chapter IV, I tested behavioral indicators of boldness in male and female *X. birchmanni* according to the social exposures described in Chapter III. Furthermore, I tested whether social exposure affected male morphology, which would have drastic mate choice consequences as females also rely on the visual modality when selecting mates (Fisher, Mascuch et al. 2009). Lastly, I correlated behavioral measures of female boldness to observed mating preferences to determine whether preference and personality behaviors are linked to one another. I found that, much like how female *X. birchmanni* develop mating preferences typical of the species to which they are exposed, both males and females learn to mirror some of the personalities of their exposure model as well.

Specifically, males and females exposed to adults of the relatively shy sister species *X. malinche* (Johnson, Culumber et al. 2015) became relatively shy themselves. This is the first study showing that male behaviors in this system can be learned from their developmental social environment, and is the first, to my knowledge, showing that individuals can learn personality-related traits from heterospecific models of a previous generation. Furthermore, heterospecific-exposed males developed relatively smaller than conspecific-exposed males. These results are likely to have important mate choice consequences, as female swordtails typically prefer larger and more active males (Cummings, Larkins-Ford et al. 2008, Wong, So et al. 2011). The results from this study highlight the complexity with which the social environment can influence female mate choice, not only by directly shaping learned female mating preferences, but also by affecting mate-choice related behavior and morphology.

The results from the studies described in this dissertation highlight the environmental sensitivities of mate-choice related traits, and bring to light some of the complex mechanisms through which these traits can fluctuate within and between populations. In Chapter II, I concluded that interannual and small-scale spatial differences can be associated with behavioral differences and may contribute to a resolution to the paradox of the lek. In Chapter III, I focused on the role of the social environment in directly shaping learned mating preferences, and provided the necessary foundation for future studies examining the neural mechanisms of these learned behaviors. Finally, in Chapter IV, I revealed how the social environment can also have indirect implications on mate choice by affecting personality-related traits and

morphology that, in turn, are expected to have important consequences on mate choice dynamics. Together, these studies describe the complex direct and indirect relationships between the environment and female mate choice.

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APPENDIX A

SUPPORTING INFORMATION FOR

HETEROGENEITY IN MATING PREFERENCES ACROSS TIME AND MICROHABITAT: A HARD LIMIT ON MEASURES OF MATE CHOICE?

Table A-1 - Principal components analysis loading scores and summary statistics for measurements of male swordtails from the UP and DOWN pools of the Calnali-Mid locality taken from 2013-2015.

		PC1	**PC2**	**PC3**	**PC4**	**PC5**
Measurements	*standard length*	2.31	-0.08	0.276	-0.29	0.336
	dorsal fin length	2.16	-0.27	0.658	0.694	-0.05
	sword length	-0.01	-2.36	-0.24	-0.08	-0.02
	gonopodium length	1.73	0.256	-1.59	0.155	-0.01
	mass	2.29	0.129	0.309	-0.47	-0.28
Importance of components	*eigenvalue*	3.23	1.02	0.571	0.146	0.035
	% explained	64.6%	20.3%	11.4%	2.9%	0.7%

Table A-2 - Principal components analysis loading scores and summary statistics for measurements of female swordtails from the UP and DOWN pools of the Calnali-Mid locality taken from 2013-2015.

		PC1	**PC2**
Measurements	*reproductive allotment*	2.1	-1.0
	fat content	2.1	-1.0
Importance of components	*eigenvalue*	1.63	0.37
	% explained	81.4%	18.6%

APPENDIX B

SUPPORTING INFORMATION FOR

NEUROGENETIC FRAMEWORK OF LEARNED FEMALE MATING PREFERENCES IN THE SWORDTAIL FISH *XIPHOPHORUS BIRCHMANNI*

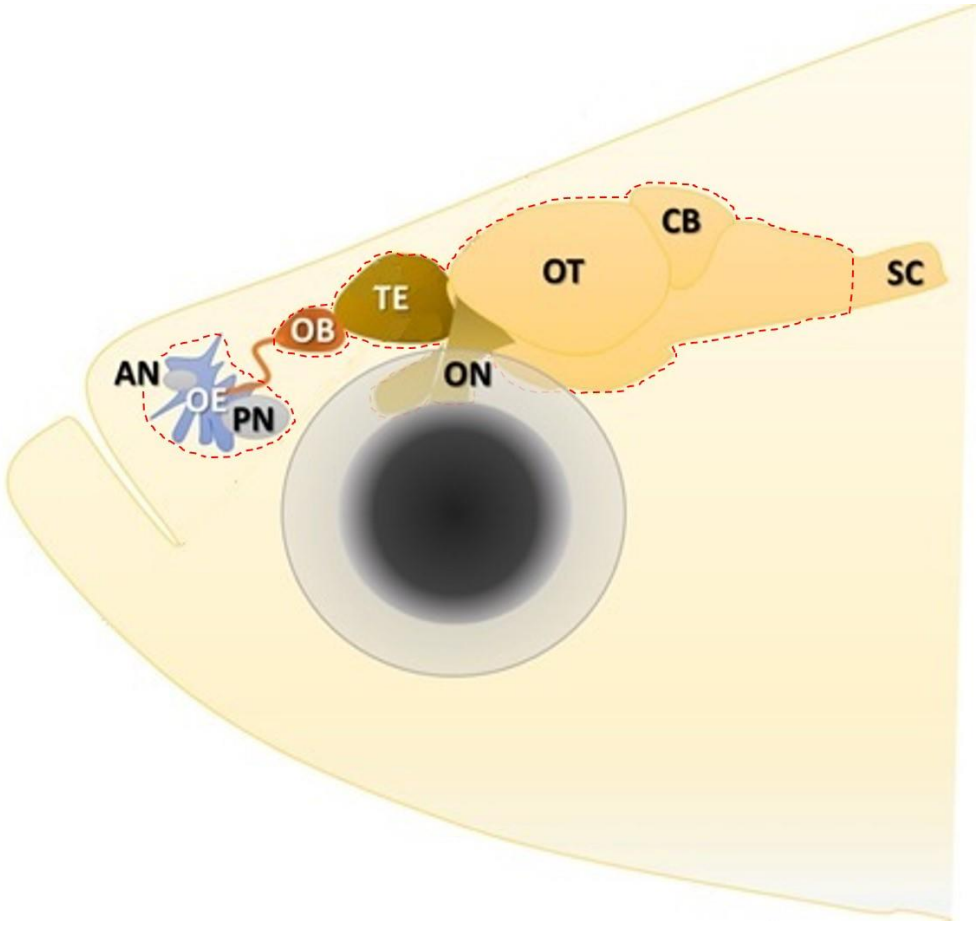

Figure B-1 - Sampling method of pooled sensory and brain tissue. Whole brain was dissected and cut at the base of the hindbrain, and olfactory epithelia were removed from cuts around fish nares (dashed red lines). Abbreviations: AN- anterior nare, PN- posterior nare, OE- olfactory epithelium, OB- olfactory bulb, TE- telencephalon, ON- optic nerve, OT- optic tectum, CB- cerebellum, SC- spinal cord.

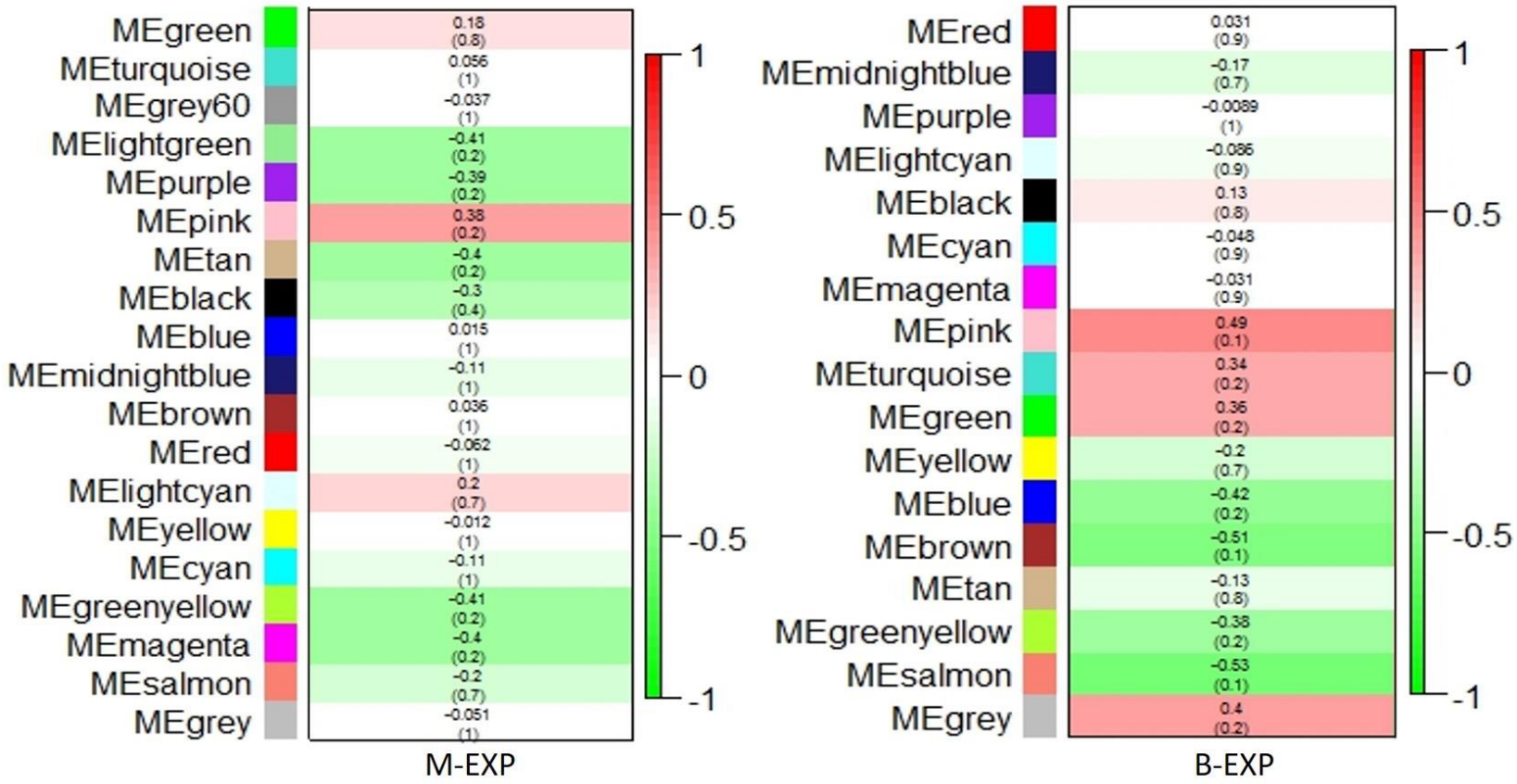

Figure B-2 - Table of module-trait correlations and adjusted p-values for comparisons of a) M-EXP and NO-EXP, and b) B-EXP and NO-EXP female *X. birchmanni* gene expression profiles. The table is color-coded by correlation according to the legend on the right. Negative correlations denote relative upregulation of module genes in NO-EXP samples.

Supplementary Table Titles

Table S1- List of significant differentially expressed genes for all pairwise comparisons between conspecific-exposed, heterospecific-exposed, and socially isolated female *X. birchmanni*.

Table S2- Biological process gene ontology results for conspecific-exposed versus heterospecific-exposed differentially expressed genes at FDR < 0.05. Table restricted to gene ontology terms significantly enriched at FDR < 0.05.

Table S3- Biological process gene ontology results for conspecific-exposed versus heterospecific-exposed differentially expressed genes at FDR < 0.05, with gene lists divided according to relative up- and down-regulation according to treatment. Table restricted to gene ontology terms significantly enriched at FDR < 0.05.

Table S4- Full list of genes assigned to the "synapse" module comparing conspecific-exposed and heterospecific-exposed female *X. birchmanni* gene expression profiles.

Table S5- Biological process gene ontology results for list of genes found within the "synapse" module. Table restricted to gene ontology terms significantly enriched at FDR < 0.05.

Table S6- Full list of genes assigned to the "vision" module comparing conspecific-exposed and heterospecific-exposed female *X. birchmanni* gene expression profiles.

Table S7- Biological process gene ontology results for list of genes found within the "vision" module. Table restricted to gene ontology terms significantly enriched at FDR < 0.05.

Table S8- Full list of genes assigned to the "olfaction" module comparing conspecific-

exposed and heterospecific-exposed female *X. birchmanni* gene expression profiles.

Table S9- Biological process gene ontology results for list of genes found within the "olfaction" module. Table restricted to gene ontology terms significantly enriched at FDR < 0.05.

APPENDIX C

SUPPORTING INFORMATION FOR

CULTURAL TRANSMISSION OF A HETEROSPECIFIC PERSONALITY TRAIT IN A VERTEBRATE WITHOUT PARENTAL CARE

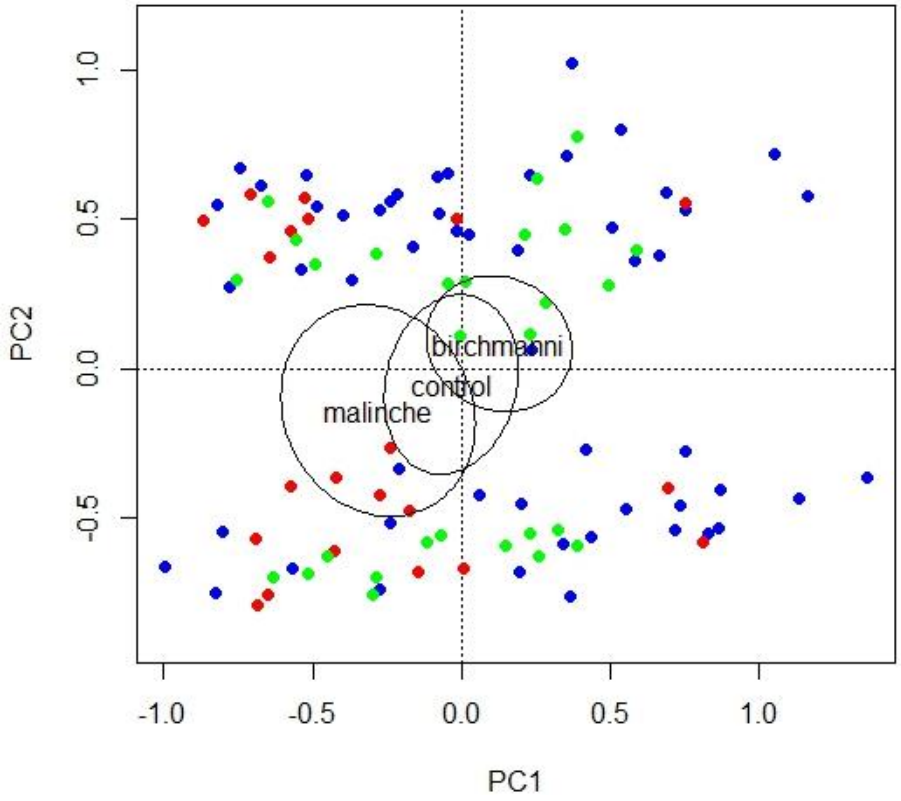

Figure C-1 – Principal components analysis of male *X. birchmanni* phenotypic distribution according to social exposure treatment. Blue dots: exposed to conspecific adults, red dots: exposed to *X. malinche* adults, and green dots: socially isolated from adults. PC1 is most influenced by general size of the male (larger individuals on the right), and PC2 is most influenced by maturation age and vertical bar number (early-maturing individuals with more vertical bars towards the top). Circles represent 95% confidence intervals of the standard error of the mean centroid value of a given group.