NEUROGENETICS OF LEARNED MATING PREFERENCES

An Undergraduate Research Scholars Thesis

by

SANTIAGO A. FORERO

Submitted to the Undergraduate Research Scholars program at
Texas A&M University
in partial fulfillment of the requirements for the designation as an

UNDERGRADUATE RESEARCH SCHOLAR

Approved by Research Advisor: Dr. Gil G. Rosenthal

May 2017

Major: Biomedical Sciences

TABLE OF CONTENTS

Page

ABSTRACT 1

DEDICATION 2

ACKNOWLEDGMENTS 3

CHAPTER

I. INTRODUCTION 4

Swordtail fish as models 4

II. METHODS 8

Primers, PCR and *in situ* hybridization 8

III. RESULTS 10

IV. DISCUSSIONS 11

LITERATURE CITED 12

ABSTRACT

Neurogenetics of Learned Mating Preferences

Santiago A. Forero
Department of Veterinary Medicine & Biomedical Sciences
Texas A&M University

Research Advisor: Dr. Gil G. Rosenthal
Department of Biology
Texas A&M University

Mate choice plays an important role in reproductive isolation and hybridization between species. Mating preferences are often learned in early social development; therefore an individual's social environment can have important evolutionary consequences by shaping individual preference functions. While decades of research on mate choice have studied the behavioral implications of social exposure, the neural network and specific genes active in developing a learned mating preference remain unidentified. The purpose of this experiment is to shed light on some of the neural mechanisms involved in learned mating preferences of a swordtail fish *(Xiphophorus birchmanni*) for chemical cues of either conspecific adults or those of a closely related sister species (*Xiphophorus malinche*) based on social exposure (exposed to adult conspecifics vs. heterospecifics). Studying previously gathered RNAseq data, we identify possible target genes which have been shown to be differentially expressed between exposure treatments. Creating *in situ* hybridization probes for the candidate genes, we then aimed to identify brain regions where differential expression is occurring in order to gain a better overall understanding of the neural framework of learned mating preferences.

DEDICATION

I would like to dedicate this work to my family. I would particularly like to dedicate it to my parents Helena Lozano and Alvaro Forero who have been my support group my whole life. I owe this all to them.

ACKNOWLEDGEMENTS

I would like to thank my faculty advisor Dr. Gil Rosenthal and PhD candidate Pablo Delclos. They have given me such a special opportunity to work with them, I am so grateful and honored to have worked with them and their research. They have been a such a positive influence in my undergraduate career and I hope they know how much I appreciate their support.

CHAPTER I

INTRODUCTION

An expanding area of evolutionary biology has focused on how individual mating preferences can result in species isolation or hybridization[1]. Servedio argues that persistent hybridization and gene flow in nature can eventually result in adaptive speciation [1]. Mating preferences are often learned early in life and influenced through social exposure [2]. A study performed on zebra finches looking at sexual imprinting on a novel trait (a red feather on the forehead) showed that only females sexually imprinted on the novel trait if there were exposed to it (both parents adorned, or father adorned) in early social development; this supports the evolutionary importance for male traits and how social exposures influences mate preference later in life. Mechanisms such as social and sexual imprinting explain the behavioral consequences of varying social exposures on an individual [2]. In 1935, Konrad Lorenz showed how birds raised by a heterospecific foster species choose to mate with other birds of the foster species instead of their own [3]. Similarly, in an experiment performed on closely related species *Pundamilia pundamilia* and *Pundamilia nyererei,* it was found that females of three treatment groups (interspecific treatment with *P. pundamilia* & *P. nyererei,* and an intraspecific treatment with *P. nyererei*) demonstrated a significant preference for males of the foster mother's species [4]. Although empirical examples of such sexual imprinting have been documented for decades, few researchers have began to investigate the neural mechanisms that lead to these biological preferences.

Past research performed on zebra finches has focused on a few key brain regions involved in sexual imprinting [5]. Bischof discusses how the hippocampus may be playing a role

in sexual imprinting of birds [5]. This research however, is too narrow in its localization and only investigates auditory and visual modalities. In this study, we aim to take a more generalized approach by selecting candidate genes from an RNA-seq study examining whole brain gene expression profiles of females exposed to varying social environment. Furthermore, we examine the olfactory modality in this study, as olfactory cues are important to conspecific mating preferences. In insects, odorant receptors at the periphery play a determining role in mate choice. The vertebrate olfactory periphery has the potential to play a similar role: it includes a relatively large repertoire of olfactory receptors, allowing for the evolution of specialized receptor families, such as olfactory receptors (OR) and vomeronasal (V1R and V2R). This receptor diversity at the olfactory periphery allows for the evolution of specific behavioural responses to unique cues and therefore deserves further examinaton.

Swordtail fish as models

Swordtail fish are an ideal study system for examining the neural mechanism of mate choice [6,7]. Studies have found that the expression of the neuroligins may play an important role in making mating decisions in swordtail fish *Xiphophorus nigensis* [7,8,9,10]. Neuroligin-3 is a mitogen, playing an important role in cell replication (cancer growth)[11] and the formation of synapses in the central nervous system. Specifically, nlgn-3 was found to be associated within glutamatergic and GABAergic synapses [12]. Neuroligins were found to be expressed strongly in the social decision-making network (SDMN) a cluster of brain regions associated with assessing sexual and social stimuli [7]. However, this study focused on assessing brain gene expression profiles immediately after preference trials and therefore depict its role during the act of mate choice (short term potentiation). Long-term neurogenetic effects of the social environment throughout development remain largely unknown. In a previous study, we addressed this by

conducting olfactory preference trials on female swordtail fish *Xiphophorus birchmanni* that, were either socially isolated from adults throughout development, raised with adults of their respective species, or those of a closely related sister species (*Xiphophorus malinche)*. Olfactory cues play a primary role in conspecific recognition in swordtails[13, 14, 15, 16]. Research has shown that wild fish from both species prefer a conspecific male [17]. However, manipulating the social environment over both short and long periods of time in *X. birchmani* has revealed a preference for the species they were exposed to [18]. This behavior differs from X. *malinche* who have been shown to have relative disdain for familiar phenotypes [19]. In this study, we take advantage of the RNAseq data previously collected on conspecific- and heterospecific- exposed female *X. birchmanni* whole brains and olfactory tissue, and have chosen to localize the expression of the following genes: *btk* (bruton tyrosine kinase), *ngln-2b* (neurolign 2b), *cxcr4a* (Chemokine (C-X-C Motif) Receptor 4) and a novel gene (Ensembl ID ENSXMAG00000003934) which was the most differentially expressed gene between conspecific and heterospecific exposed subjects (Figure 1).

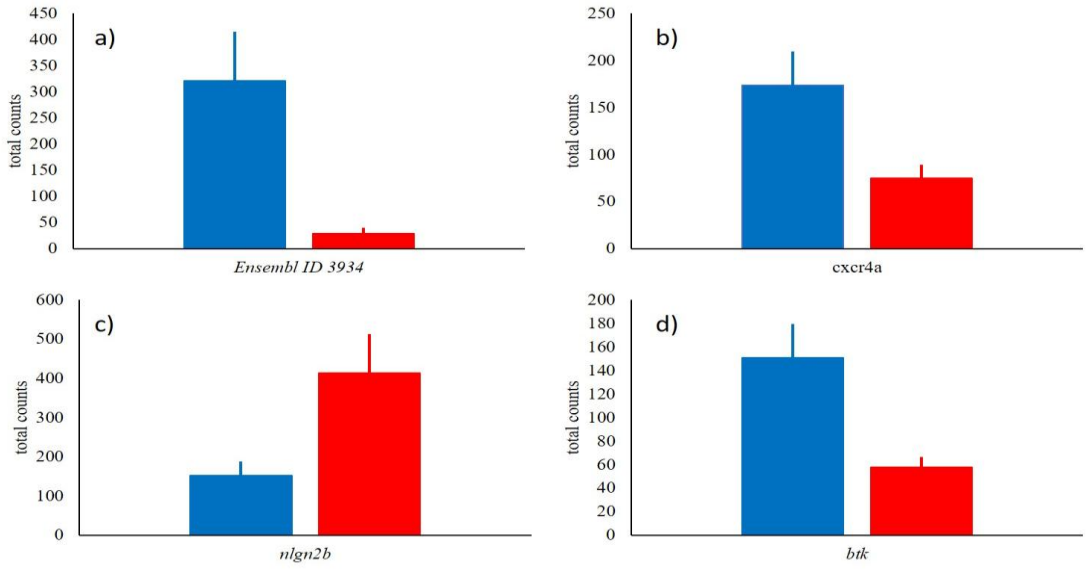

Figure 1: Expression of candidate genes between sister species *X. birchmanni* (blue) and *X.malinche* (red)

Furthermore, this gene exhibits opposite signs of gene regulation between the two sister species, as it is relatively upregulated in conspecific-exposed *X. birchmanni* but downregulated in conspecific-exposed *X. malinche* females. *Btk* is a gene that plays a major role in innate and adaptive immunity by activating B cells [20]. Previous data show that *nlgn-2b* is downregulated in *X. birchmanni* females exposed to conspecific mates while being upregulated in *X. malinche* females exposed to conspecific mates (Cui et al. in review). While *nlgn-3* was found to be directly correlated with immediate mate choice decisions [7], it is possible that *nlgn-2b* may play a prominent role in the long-term associations with a social sitmulus. Meanwhile, *cxcr-4a* is upregulated in both species exposed to conspecific mates. This gene is also known to play a role in axon guidance and neuronal wiring in the hippocampus [21]. Furthermore, *cxcr-4a* has been suggested to have a direct, causal role in learning [22] and chemokine receptors have previously been implicated in social recognition [23] and could therefore have important mate choice implications. The goal of this study is to determine the brain regions where these candidate genes are expressed in order to determine their functional relevance in shaping learned mating preferences**.**

CHAPTER II

METHODS

Xiphophorus birchmanni juveniles were raised under controlled; light, food, temperature, and density conditions. Shortly after birth, they were exposed to either 1) conspecific adults 2) adult *X. malinche*, or 3) socially isolated from adults (n=30 juveniles per tank, 3 replicates per treatment. Upon maturation, we tested female olfactory preference for conspecific vs. heterospecific males. A subset of fish were later sacrificed and their brains dissected, frozen, and sliced for later *in situ* hybridization analyses using a well-established protocol [19]. RNA sequence data was collected from the whole brains of the remainder of females.

Primers, PCR and *in situ* hybridization

From this RNAseq data, the four previously described candidate genes were selected based on the level of significant differential expression between the conspecific and heterospecific exposure treatments, as well as functional relevance based on previous mate choice and learning literature (see Introduction). Candidate gene primers were made using transcripts and scaffolds for each gene identified in the *X. birchmanni* genome. Sequences were retrieved from the annotated *X. maculatus* genome in Ensembl.[24] Primers were designed using primer3 [25] as indicated in by the geneius program [26]. Primers were ordered online from NCBI and re-suspended in nuclease free water. PCR was performed on candidate genes following the New England Biolab PCR protocol for Phusion High-Fidelity DNA Polymerase. PCR products were quantified using Qubit 3.0 then quality-checked on gel electrophoresis to ensure the specificity and base-pair size of the product. A 500 base pair *nlgn-2b* digoxigenin-labeled (DIG) probe template was subcloned by using primer pair 5'-

TTACCCCCCAACCTTCTTTGCC-3’ and 5’-TGTATGGGGTGCCAAATCCC-3’. A 500 base pair *cxcr-4a* DIG probe template was subcloned by using primer pair 5’-TTGGCCTACTTCCACTGCTG-3’ and 5'-TGCGGCACACACACAAAAAT-3'. A 485 base pair DIG probe template for *ENSXMAG00000003934* was subcloned using primer pair 5’-AGAGGGCAGAATGCTG-3’ and 5’-CATTTATGGATCTGGTTCTGC-3’. A 514 base pair *btk* DIG probe template was subcloned by using primer pair 5’-ACAGCTGGCCAATGAGAAGA-3’ and 5’-TGCAAGCCCTTCTTACTTGGT-3’.

Following PCR, products were then cleaned and concentrated following the ZYMO Research: DNA Clean & Concentrator kit with a minor modification after step 4 of centrifuging the column for two and half minutes at max speed to get rid of residual wash buffer. PCR products were then A-tailed using Taq DNA Polymerase to create overhangs for subsequent ligations into pGEM-T Easy vectors (Promega). We then performed a standard heat-shock transformation using JM109 high efficiency competent cells (Promega) as a vector for DIG-labeled RNA probe synthesis. Uncut plasmid were used as positive controls for transformations.

CHAPTER III

RESULTS

We received a high enough concentration from each of our PCR products (all >40 ng/uL) and deducted from gel electrophoresis that our PCR products were in fact expanding our gene of interest (at around 500 base pairs). We then successfully cleaned up and concentrated our PCR products following A-tailing (Figure 2). We successfully performed ligations on our concentrated PCR products. We received negative results from our transformations (only 3-4 white colonies were seen on the *3934* and *btk* plates) telling us that our candidate genes were not inserted into the plasmid and replicated as expected except for our few white colonies. We expect this to be a problem with the transformation step because our positive control group also gave negative results, suggesting that our competent cells were no longer viable.

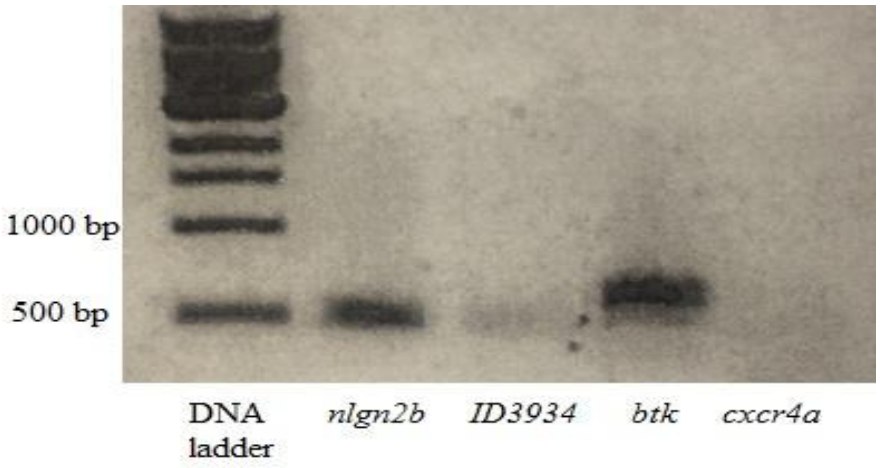

Figure 2: Gel electrophoresis of candidate genes after clean up and A-tailing

CHAPTER IV

DISCUSSIONS

After successfully obtaining the gene primers of our genes of interest, running PCRs, A-tailing the products, cleaning up the products and performing ligations; we could not successfully perform a transformation to insert our candidate genes into a vector for *in situ* hybridization. We had initially chosen this route because it was efficient at creating probes out of low-expression genes, but now after obtaining a high enough concentrations of PCR product after A tailing and cleaning (>40 ng/ul) we concluded to use an alternative method for probe synthesis that does not require a ligation or transformation step.

Next, we will conduct PCR on new modified primers that will include a T7 RNA Polymerase promoter sequence on the 5' end of the reverse primer. This product will then be able to go directly into RNA probe synthesis following Roche DIG-RNA Labeling Kit protocol. Allowing us to conduct *in situ* hybridization.

Fluorescent *in situ* hybridization (FISH) protocol will be performed on the previously obtained brain slices in order to discover where candidate genes are being differentially expressed in subject brains. We will follow the methods of a previous *in situ* hybridization study [27] with minor modifications. To identify brain regions, we will rely on a previously published brain atlas of the closely related *Xiphophorus hellerii* [28].

LITERATURE CITED

1) Servedio M R, J Hermisson & GS van Doorn. 2013. Hybridization may rarely promote speciation. *J. Evol. Biol*. 26: 282–285. doi:10.1111/jeb.12038

2) Witte K & N Sawka. 2003. Sexual imprinting on a novel trait in the dimorphic zebra finch: sexes differ. *Animal Behaviour.* 65(1): 195-203

3) Lorenz KJ. 1937. The Companion in the Bird's World. *The Auk.* 54(3): 245-273. doi: 10.2307/4078077

4) Verzijden MN &CT Cate. 2007. Early learning influences species assortative mating preferences in Lake Victoria cichlid fish. *Biol Lett.* 3(2): 134-136. doi: 10.1098/rsbl.2006.0601

5) Bischof HJ. 2003. Neural mechanism of sexual imprinting. *Animal Biology.* 53(2): 89-112

6) Cummings ME. 2012. Looking for sexual selection in the female brain. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 367: 2348–2356.

7) Wong RY & ME Cummings. 2014. Expression patterns of neuroligin-3 and tyrosine hydroxylase across the brain in mate choice contexts in female swordtails. *Brain, Behavior and Evol*. 83(3): 231-243

8) Cummings ME, J Larkins-Ford, CRL Reilly, RY Wong, M Ramsey & HA Hofmann.2008. Sexual and social stimuli elicit rapid and contrasting genomic responses. *Proc. R. Soc. B.* 275: 393-402; DOI: 10.1098/rspb.2007.1454.

9) Lynch KS, ME Ramsey & ME Cummings.2012. The mate choice brain: comparing gene profiles between female choice and male coercive poeciliids. *Genes, Brain and Behavior*. 11: 222–229. doi:10.1111/j.1601-183X.2011.00742.x

10) Ramsey ME, TL Maginnis, RY Wong, C Brock & ME Cummings. 2012. Identifying context-specific gene profiles of social, reproductive, and mate preference behavior in a

fish species with female mate choice. *Front. Neurosci.* 6: 62. doi: 10.3389/fnins.2012.00062

11) Venkatesh HS, TB Johung, V Caretti , A Noll, Y Tang,S Nagaraja &M Monje. 2015. Neuronal activity promotes glioma growth through Neuroligin-3 secretion. *Cell*, 161(4): 803–816. http://doi.org/10.1016/j.cell.2015.04.012

12) Budreck EC & P Scheiffele. 2007. Neuroligin-3 is a neuronal adhesion protein at GABAergic and glutamatergic synapses. *European Journal of Neuroscience*. 26: 1738–1748. doi:10.1111/j.1460-9568.2007.05842.x

13) McLennan DA & MJ Ryan. 1999. Interspecific recognition and discrimination based upon olfactory cues in northern swordtails. *Evolution* 53(3): 880-888

14) Crapon MD & MJ Ryan. 1990. Conspecific mate recognition in swordtails, *Xiphophorus nigrensis* and *X. pygmaeus* (Poeciliidae): olfactory and visual cues. *Anim. Behav*. 290-296

15) Fisher H S & GG Rosenthal. 2006. Female swordtail fish use chemical cues to select well-fed mates. *Animal Behaviour*. 72: 721-725. doi: 10. 1016/j. anbehav. 2006.02.009

16) Verzijden MN & GG Rosenthal. 2011. Effects of sensory modality on learned mate preferences in female swordtails. *Animal Behaviour*. 82: 557-562

17) Rosenthal GG, JN Fitzsimmons, KU Woods, G Gerlach & HS Fisher. 2011 Tactical release of a sexually-selected pheromone in a swordtail fish. *PLoS ONE* 6(2), e16994. (doi:10.1371/journal.pone.0016994).

18) Culumber ZW, HS Fisher, M Tobler, M Mateos, PH Barber, MD Sorenson & GG Rosenthal. 2011 Replicated hybrid zones of *Xiphophorus* swordtails along an elevational gradient. *Mol. Ecol.* 20(2): 342-356. (doi:10.1111/j.1365-294X.2010.04949.x).

19) Verzijden MN, ZW Culumber & GG Rosenthal. 2012. Opposite effects of learning cause asymmetric mate preferences in hybridizing species. *Behavioral Ecology* 23(5): 1133-1139. (doi:10.1093/beheco/ars086).

20) Khan WN, FW Alt, RM Gerstein, BA Malynn, I Larsson, G Rathbun, L Davidson, S Muller, AB Kantor, LA Herzenberg, FS Rosen & P Sideras. 1995. Defective B cell development and function in Btk- defecient mice. *Immunity.* 3(3): 283-299

21) Chong SW, A Emelyanov, Z Gong, V Korzh. 2001. Expression pattern of two zebrafish genes, cxcr4a and cxcr4b. *Mechanisms of Development.* 100(2): 347-354

22) Parachikova A & CW Cotman. 2007. Reduced CXCL12/CXCL4 results in impaired learning and is downregulated in a mouse model of Alzheimer disease. *Neurobiology of Disease.* 28(2): 143-153

23) Kalkonde YV, R Shelton, M Villarreal, J Sigala, PK Mishra, SS Ahuja, I Barea-Rodriguez, P Moretti, & SK Ahuja. 2011. The CC chemokine receptor 5 regulates olfactory and social recognition in mice. *Neuroscience.* 197: 153-161

24) Bronwen L. Aken, S Ayling, D Barrell1, L Clarke, V Curwen, S Fairley, J Fernandez Banet, K Billis, C Garcín Girón, T Hourlier, K Howe, A Kähäri, F Kokocinski, FJ Martin, DN Murphy, R Nag, M Ruffier, M Schuster, YA Tang, J Vogel, S White, A Zadissa, P Flicek & SM J Searle The Ensembl gene annotation system. *Database* 2016, baw093. doi: 10.1093/database/baw093

25) Rozen S & HJ Skaletsky. 2000. Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, NJ, 365-386. Source code available at http://sourceforge.net/projects/primer3/

26) Kearse M, R Moir, A Wilson, S Stones-Havas, M Cheung, S Sturrock, S Buxton, A Cooper, S Markowitz, C Duran, T Thiere, B Ashton, P Mentjies, & A Drummond. 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data.. *Bioinformatics* 28(12): 1647-1649

27) Wong RY, ME Ramsey & ME Cummings. 2012. Localizing brain regions associated with female mate preference behavior in a swordtail. *PLoS ONE.* 7(11), e50355. doi:10.1371/journal.pone.0050355

28) Anken RH & H Rahmann. 1994. Brain atlas of the adult swordtail fish, *Xiphophorus helleri,* and of certain developmental stages. G. Fischer.