# PHEROMONAL MECHANISMS OF REPRODUCTIVE ISOLATION IN *XIPHOPHORUS* AND THEIR HYBRIDS

A Dissertation

by

CHRISTOPHER HOLLAND

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

| | |
|---|---|
| Chair of Committee, | Gil G. Rosenthal |
| Committee Members, | Michael Smotherman |
| | Kevin W. Conway |
| | Jessica L. Yorzinski |
| Head of Department, | Thomas McKnight |

December 2018

Major Subject: Biology

# ABSTRACT

Pheromones play an important role in conspecific mate preference across taxa. While the mechanisms underlying the pheromonal basis of reproductive isolation are well characterized in insects, we know far less about the mechanisms underlying the production and reception of chemical signals in vertebrates. In the genus *Xiphophorus,* conspecific mate recognition depends on female perception of male urine-borne pheromones. I focused on interspecific differences between the sympatric *X. birchmanni* and *X. malinche,* which form natural hybrid zones as a consequence of changes in water chemistry. First, I identify the organ of pheromone production and compounds comprising chemical signals. I localized pheromone production to the testis; testis extract elicited the same conspecific preference as signals generated by displaying males. I used solid phase extraction (SPE) in combination with high performance liquid chromatography (HPLC)/ mass spectrometry (MS) to characterize pheromone chemical composition. Analyzing HPLC/MS readouts for pure peaks with high relative intensity identified two compounds of interest, which were identified according to their fraction pattern and retention times and then individually assayed for their effect on female behavior. The ability to directly measure the pheromones with paired responses of female conspecific mate recognition gives insight into what specific components are important to female mate choice. Elucidating the chemical composition of *Xiphophorus* signals sheds light into how communication acts as a reproductive barrier between species and how its breakdown facilitates hybridization. Next, I characterize intraspecific variation in pheromone signals. Understanding the relationship between a quantifiable male pheromone profile and measurable female response provides unique insight

into female mate choice. I examined the variation in male morphology in *X. birchmanni*, and used SPE to measure changes in pheromone structure in relation to distinct morphometric traits. Lastly, I evaluate the relationship of male pheromone phenotype to population substructure. If pheromones play a role in reproductive isolation, pheromone profiles should map on to male genotype morphology. Hybrid zones vary from highly structured, with distinct *birchmanni*-like and *malinche*-like subpopulations, to highly admixed hybrid swarms. I measured pheromone profiles for individual males, I show the relationship between male morphology, pheromone profile and population structure.

# DEDICATION

For, Ellen Ford. Love you forever, my mum you will be.

# ACKNOWLEDGEMENTS

I would like to first thank my advisor, Dr. Gil Rosenthal; over the past 4 years he has provided me with support and guidance to navigate my time in graduate school. Sharing his knowledge on mate choice and reproductive isolation with me was pivotal in my development as a young scientist and he has provided constant feedback to all of the proposals, presentations, research and manuscripts I have worked on throughout my graduate career. I would like to thank my committee, Dr. Jessica Yorzinski, Dr. Michael Smotherman, and Dr. Kevin Conway, for their guidance and support throughout the course of this research. I would also like to acknowledge Dr. Adam Jones and Dr. Peter Sorenson for their advice and constructive research contributions. A special thanks to Jennifer Bradford, for all her support and patience with me in my advancement at Texas A&M and for all of her hard work over the years helping me throughout it all.

I would not have grown or enjoyed my graduate career without my lab mates, friends and peers who provided moral and emotional support, thank you so much to: Dan Powell, Gastón Jofre-Rodríguez, Dr. Molly Schumer, Mateo García, Megan Exnicios, Stephen Bovio, Carolyn Henderson, David Saenz, Luke Bower, Chris Figgener, and McKensie Daugherty. I need to thank my squad: Mattie Squire, M.S., Dr. Sarah Flanagan, and Dr. Alejandra Malandando. The three of you have taught me so much and have been there for me through it all. I know I have made lifelong friends and am eternally grateful for you all.

I need to thank my family; my mother and sister who have been my never ending support system. My companion and endless source of love, Renly, who helped get me through my graduate career with early morning walks, belly rubs, puppy kisses, and staying by my side every

late night. Finally, my best friend and life partner, Eric Murray, M.S.. Thank you for understanding me, having my back and making me laugh everyday.

# CONTRIBUTORS AND FUNDING SOURCES

## Contributors

This work was supported by a dissertation committee consisting of my advisor, Professor Gil Rosenthal, and Professor Michael Smotherman of the Department of Biology, and Professors Jessica Yorzinski and Kevin Conway of the Department of Wildlife and Fisheries Sciences.

The HPLC/MS used in Chapter I, II and III were provided by Dr. Bo Wang of the Department of Chemistry at Texas A&M University. The student independently completed all other work conducted for the dissertation.

## Funding sources

Graduate study was supported by a grant from Animal Behavior Society and a fellowship from L.T. Jordan International Travel Foundation at Texas A&M University.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

# CHAPTER I

## INTRODUCTION AND LITERATURE REVIEW

The phenomenon of how a cue (a stimulus whose communicative function is incidental) becomes a signal (a stimulus that has evolved in an explicitly communicative context) remains an integral part of research in communication. This process of exploiting a cue that provides information about an individual's – identity (Brennan, P.A., & Zufall, F. 2006, Buchinger, T. J., *et al*. 2015), reproductive state, (Sorensen, P., *et al*. 1990, Irvine, I. A. S., & Sorensen, P. W. 1993, Gabor, C. R *et al*. 2012), or location (Sorensen, P.W. *et al*., 2005, Niño-Domínguez, A., *et al*. 2015) – may also increase the receiver's overall fitness, creating a selective force for those who are able to exploit it (Burnard, D., *et al*. 2008, Wyatt, T.D. 2010). As a cue becomes more informative to a receiver, the cue transitions to a signal and a form of active communication if the sender benefits as well (Sorensen, P. W., *et al*. 1998, Reding, L., & Cummings, M. E. 2015). As this channel of information transmission is strengthened, the portion of individuals in a population employing it may diverge from those who are not, resulting in a speciation event (Linn, C. E. & W. L. Roelofs. 1995). This phenomenon has been long thought to be one of the major mechanisms for speciation and reproductive isolation (Smadja, C., & Butlin, R. K. 2009). Once this process has been completed, differentiation is maintained due to reduced fitness in hybrids (Abbott, R., *et al*. 2013). This is achieved by constraints on the behaviors and signals one is receptive to (Irvine, I. A. S., & Sorensen, P. W. 1993, Rosenthal, G.G., & Lobel, P. 2006). Multiple sensory modalities are often used to distinguish or attract conspecifics; however most are under constraints by closely related species using similar signals (Crapon de Caprona, M.D., & Ryan, M. J. 1990, Mérot, C., *et al*. 2015). Additionally, many sensory systems are under

selective restriction by their use in other behaviors: hunting/foraging, spatial navigation and orientation, predator avoidance and constrained physiology (Rosenthal, G.G., & Lobel, P. 2006). In addition, to the restraints brought on by closely related sympatric species and selective pressure are the context in which communication is presented: season (Borg, B., 1994), social dynamic (DeColo, S. L., *et al*. 2016, Hesse, S., *et al*. 2016), reproductive state (Schubert, S. N., *et al*. 2006, Stacey, N., & Sorensen, P. W. 2008), and relationship to receiver (Wyatt, T. D. 2014). Therefore, it is not surprising to see the complex behaviors involved in communication in extant animals.

One modality that is used throughout the animal kingdom for communication is olfaction (Sorensen, P. W., *et al*. 1998, Brennan, P.A., & Zufall, F. 2006). The large repertoire of olfactory receptors allows for specialization and diversification of signals, as they are not constrained by the olfactory modality (Linn, C. E., & Roelofs, W. L. 1995, Leary, G.P., *et al.* 2012). The long-term, immediate response and wide array of signals available via chemical signaling is why many animals use it for a multitude of behaviors including species recognition and mate choice (Rosenthal, G.G., *et al*. 2003, Wong, B.B.M., *et al*. 2005). The variable properties of chemical signaling – volatility, weight, classification type, mode of transmission and solubility – play an important role in how signals are used (Stewart, M., *et al*. 2013, Candolin, U. 2014). The type of compound used must be effective at reaching the recipient, efficiently transmitted and last long enough to stimulate receptors (Brennan, P.A., & Zufall, F. 2006, Burnard, D., *et al*. 2008). However, most signals originate as a byproduct or metabolite of some preexisting pathway; they are not commonly novel substances in and of their own (Van Den Hurk, R., & Resink, J. W. 1992, Lienard, M.A., *et al*. 2008, Stacey, N., & Sorensen, P. W. 2008). A common occurrence that compensates for this is the unique biosynthesis of these products that can create isomers, as

well as varying ratios or additional components (Leary, G.P., *et al.* 2012, Mitchell, R. F., *et al*. 2015). When these single or multicomponent chemical signals are unique to a species and elicit an innate behavior or response in conspecifics, it is then considered a pheromone.

Pheromones' uniqueness to species and behavioral specific properties make them an ideal model for understanding reproductive isolation via communication. The species-specific nature of pheromones should allow conspecifics to easily distinguish one another from heterospecifics; however, in some closely related sympatric species we see hybridization (Culumber, Z.W., *et al*. 2011, Rosenthal, G. G. 2013). Several factors may be in effect and result in these events. Constraints imposed on the production of chemical pheromone signals in closely related species that are more likely to hybridize is potentially in part due to the similar pathway from which the pheromone originates (Fujii, T., *et al*. 2015). Another factor is the species' evolutionary history with related species evolving in sympatry or allopatry during reproductive isolation (Willis, P.M. 2013). Specifically, species that overlap in environment tend to have better discrimination between species and/or have very distinct pheromone profiles (Palmer, C. A., & Houck, L. D. 2005, Yang, C.Y., *et al*. 2015). Another possible mechanism resulting in hybridization is interference of pheromone communication. Natural or anthropogenic "noise" in the environment that prevents communication that mediates species recognition can cause a breakdown in reproductive isolation (Fisher, H.S., *et al*. 2006).

Understanding the relationship of how species recognition is lost when chemical signals are altered by chemical interactions with contaminants can explain what components of the signal are important, how noise in an environment directly affects a species, where conservation is needed and how this breakdown may act as a mechanism for speciation and hybridization (Löfstedt, C. 1990). In the case of resulting hybrids, understating and having a means of

measuring the pheromone is crucial to teasing apart the key characteristics of the future interactions between parentals and hybrids in mate choice. In addition, the resulting hybrid pheromone profile and its implication in future mating are a novel model to study and answer questions about the introgression of phenotypes in hybrids and their attractiveness to other hybrids and parentals. If pheromone profile is not under species-specific constraints, hybrids in a population may represent a novel and attractive stimulus to parentals (Xue, B., *et al*. 2007). If the pathway for pheromone production is under the control of multiple genes, the constraint may be relaxed in hybrids of intermediate levels of introgression (Zhang, Y-N., *et al*. 2015). We might expect to see a change in the ratio or change in additional important components both being important to parental species identification (Wang, H. L., *et al*. 2005). In this case, hybrids may have an advantage over pure parentals of the same sex, as hybrid pheromone phenotype is more attractive (Wyman, M., *et al*. 2015). This is likely due to a combination of forces that may not necessarily occur singularly. A novel phenotype may be attractive to parentals by: (1) being a recognizable but less habituated signal, (2) achieving a higher level of stimulation due to relaxation of signal production thereby achieving a wider range in signal amongst hybrids, (3) being an introgressed phenotype that achieves the "best of both", and/or (4) being a novel stimuli that is not recognized as familiar and prevents learned inbreeding avoidance (Rosenthal, G. G., *et al*. 2013). Habituation to common pheromone profile in an individual's population would heighten the sensitivity to a novel stimulus that stimulates the sense at a greater level against the common signature (Fisher, H. S., *et al*. 2009). The hybrid signal does not need to be entirely novel in order to achieve a higher level of stimulation in parental receivers; if the production of pheromone is relaxed in hybrids, the resulting pheromone may be more variable compared to the parental species (Sandkam, B. A., *et al*. 2013, Bailey, R. I., *et al*. 2015). The pheromone is not

novel in structure but the composition is, making it recognizable but novel. Introgression of both parentals pheromones presents a unique opportunity for selection for what both or one species may view as "ideal". This selection may drive the new pheromone to a narrow range under this selective pressure from receiver's preference (Rollmann, S. M. *et al*., 2003). Lastly, if hybrids present a new pheromone profile species that show active inbreeding, avoidance may greatly prefer the novel signal of hybrids as it is unlearned and not associated with kin (Verzijden, M. N., & Rosenthal, G. G. 2011). The above-described mechanisms that can lead to hybridization, if heritable, can also be a driving force to creating homoploid hybrid speciation events (Barton, N. H., & Hewitt, G. M. 1989). This is interesting when considering the natural variation that occurs in wild populations (García-Roa, R., *et al*. 2016, Pascoal, S., *et al*. 2016). If sender signal varies and the receiver preference is fixed in individual receivers, then populations may begin to diverge with respect to the receiver preference for a given sender variation (Culumber, Z. W., *et al*. 2014). This selective pressure may be acting on the novel pheromone profile of hybrids either by parentals preferring hybrid signal to conspecifics or hybrids being isolated from parentals by preferring their own pheromone profile (Morgado-Santos, M., *et al*. 2015). Studying this in a natural system would provide insight into how communication and its breakdown could facilitate hybridization and speciation events, the introgression of hybrid phenotypes and its correlation to genotype and the implications of this relationship to parental backcrossing.

Animals that live in aquatic environments provide a unique opportunity for studying pheromone chemical signaling. The measurable water parameters and quantifiable impacts that perturbation of the environment has on chemical signaling are made possible by studying aquatic animals (Tomkins, P., *et al*. 2015). In swordtail fish (genus *Xiphophorus*), conspecific mate recognition depends on pheromone signals but is abolished by high levels of dissolved organic

compounds. The goals of my project are to identify the location of pheromone production in male *Xiphophorus* and characterize interspecific differences in the chemistry of pheromone signals. Identifying the organ of pheromone production allowed me to concentrate the pheromone and quantifiably measure differences in males and their effects on female mate choice. By analyzing polymorphic differences in males, I directly measured changes in pheromone profile and corresponding female behavior. Quantifying pheromone chemistry enabled me to assess signal variation among species and among populations and to directly test the role of pheromones as mechanisms of reproductive isolation, furthering our understanding of the role communication plays in species maintenance.

# CHAPTER II

# PHEROMONAL MECHANISMS OF REPRODUCTIVE ISOLATION IN TWO HYBRIDIZING SPECIES OF *XIPHOPHORUS*

## *Introduction*

Chemical communication constitutes a widespread barrier to gene flow between species (Burnard, D., *et al*. 2008, Wyatt, TD. 2010, Wyatt, T. D. 2014). This is because the large repertoire of olfactory receptors allows for specialization and diversification of signals (Linn, C. E., & Roelofs, W. L. 1995, Leary GP, *et al.* 2012, Hesse, S., *et al*. 2016). Chemoreception can thus be narrowly tuned to attend to subtle specifics of signal chemistry (Löfstedt, C. 1990, Irvine, I. A. S., & Sorensen, P. W. 1993), like different isomers of the same molecule (Xue, B., *et al*. 2007, Fujii, T., *et al*. 2015, García-Roa, R., *et al*. 2016), to changes in the ratios of different molecules (Wang, H. L., *et al*. 2005, Lienard, M.A., *et al*. 2008, Smadja, C., & Butlin, R. K. 2009), and to the presence or absence of distinct components (Mitchell, R. F., *et al*. 2015, Pascoal, S., *et al*. 2016). These mechanisms of coupling species-typical chemoreception and chemical signal production give chemosignals a key role in speciation. This is because specific odorant receptor proteins can be narrowly tuned to species-typical chemical signals (Barton, N. H., & Hewitt, G. M. 1989, Rollmann, S. M., *et al*. 2003, Palmer, C. A. & Houck, L. D. 2005). However, some sympatric species hybridize despite chemical-based conspecific mate recognition (Culumber, Z.W., *et al*. 2011, Rosenthal, G. G. 2013, Wyman, M. T., *et al*. 2015). This may be because of overlap between conspecific and heterospecific signals (Dekker, T., *et al*. 2015, Yang, C.Y., *et al*. 2015), or through interactions with the chemical environment that cause interference with communication (Fisher, H.S., *et al*. 2006, Candolin, U. 2014, Tomkins, P., *et*

*al*. 2015). Understanding the chemical basis of pheromone signals is therefore critical to understanding their evolutionary role. The chemosignals involved in reproductive isolation have been extensively characterized in a number of systems, primarily in invertebrates. Despite the importance of pheromones to mate choice and reproductive isolation in vertebrates, the mechanisms linking chemical communication to conspecific mating preference remain poorly understood.

Behavioral data indicate that chemical communication is a primary isolating mechanism between two parapatric sister species of swordtail fish (Poeciliidae), *Xiphophorus birchmanni* and *X. malinche* and that conspecific mate preference can be abolished by interference with the chemical environment (Crapon de Caprona, M.D., & Ryan, M. J. 1990, Hankison, S.J. & Morris, M.R. 2002). However, *Xiphophorus birchmanni* and *X. malinche* form at least six natural hybrid zones along elevation gradients in the eastern Sierra Madre Oriental in Hidalgo state, Mexico (Rosenthal, G.G., *et al*. 2011). To date, however, all our insight on chemical communication in *Xiphophorus* comes from the behavioral responses of females; we have had no means to quantify or characterize chemical signals. Here I use analytical chemistry techniques in combination with behavioral assays to characterize species-typical differences in chemical signal composition that are meaningful to conspecific mate preference.

## *Materials and Methods*

### Collection

All subjects were adults collected from allopatric populations using baited minnow traps. *X. birchmanni* (*N*=24) were collected from the Rio Garces (20°57'22 N, 098°16'48 W) and the

Rio Coacuilco (21°5'50.85 N, 98°35'19.46 W). *X. malinche* (*N*=16) were collected from the Rio Xontla (20°55'27.24"N 98°34'34.50W).

**Dissection and identification of organ of pheromone production**

Males were euthanized using buffered MS-222 and kept on ice prior to dissection. In order to identify the organ of pheromone production kidney, liver, testis and muscular tissue were removed from individual males. Organs were stored individually in 1mL of distilled water at -20°C.

**Pheromone SPE and HPLC-MS-MS$^2$**

I purified and concentrated candidate chemical cues using solid phase extraction (SPE). Candidate tissue was suspended in 0.5 mL of distilled (DI) water and stored at -20°C prior to use. Tissue was prepared for extraction by thawing at 23°C and 300 rpm on a mixing tray. Tissue of *X. birchmanni* and *X. malinche* males and 1mL holding water was loaded into a C18 (Bond Elut-C18, 200mg, 3ml, Agilent Technologies, Wilmington, DE, USA) SPE column mounted on a vacuum manifold pressurized at 15 Pa. The C18 column was prepped with 2mL MeOH followed by 2 mL DI water. Tissue was then loaded and washed with 1mL DI water. Tissue elution was performed with 25% MeOH and DI water, 65% MeOH and 100% MeOH. Each eluate was captured in a separate test tube along the manifold then each eluate was split for behavior trials and HPLC. For HPLC, an internal standard of 1μg of the unconjugated bile acid chenodeoxycholic acid (CDCA; Steraloids Inc, Newport, RI, USA) was added to each of the eluates to standardize retention times and injection volumes between samples and to a blank sample of 35% DI water/65% MeOH (control). Eluates were dried under a stream of 99.9% pure nitrogen gas at 1.5 LPM (Cal Gas Inc., Huntington Beach, CA, USA), reconstituted in 2 ml MeOH /water (60/40, v/v) and stored at -20°C till HPLC/MS. The LC column (Nova-Pak

reversed-phase C18, 4μm, Waters Chromatography Division, Milford, MA, USA) was coupled to a mass spectrometer with electrospray ionization (ESI; LCQ-DECA, Thermo Electron Corporation, Houston, TX, USA). The mobile phase was isocratic at 15% MeOH for 4 minutes, increased linearly from 15% to 100% MeOH from 4 to 31 minutes, and allowed to run at 100% MeOH for 5 minutes before increasing back to 15% MeOH for the next sample. Additional run times extended to an hour showed no new additional compounds in HPLC readouts. Peaks were identified if maximum relative intensity was at least 50% above background, not dependent on another compound for presence (including IS), and found in all conspecific samples found in male testis.

I then used a PE SCIEX QSTAR (Applied Biosystems, Foster City, CA, USA) to perform secondary mass spectrometry (MS/MS) using negative and positive-ion high-resolution electrospray ionization to identify compounds. The ion trap was operated in the negative and subsequent positive-ion mode with a spray voltage of 5kV. A stream of 99% pure nitrogen at 60 Pa was used as the sheath gas. Data were collected in the range *m/z* 250–950. The relative peak areas (normalized to the area of the internal standard, CDCA) in the HPLC fractions that elicited female conspecific mate preference in behavior trials were determined with Compass Data Analysis Viewer software (2014, Bruker Daltonik GmbH, Billerica, MA, USA) in conjunction with two open access databases: *m/z* cloud and MassBank, HMDB. I made conservative assumptions about the composition of candidate compounds for identifying peaks in MS: 1) a natural product, 2) synthesis along the urogenital tract, 3) a semi – to highly polar molecule and 4) a stable compound.

**Creating chimeric signals**

Having identified two peaks present in *X. birchmanni* and absent in *X. malinche* (see Results), I fractioned samples based on polarity and molecular weight to create chimeric signals, removing these peaks via SPE from the *X. birchmanni* signal and adding them to *X. malinche* and verified with HPLC. I then used these chimeric signals to evaluate the role of these chemical components in eliciting preferences.

**Female preference trials**

Organs were pooled in groups of 4 of the same tissue type and suspended in 500ml of distilled water 24 hours prior to trials. Tissue is pooled to account for between male variation. Live male cue stimuli were prepared by placing four males into a single 40 L collection aquarium for 6 hours adjacent to a tank containing four conspecific females to provide them visual stimulation. To assess females' responsiveness to SPE pheromone cue, eluates were pooled in groups of 4 males and suspended in 500ml 24 hours prior to trials. Live male cue stimuli, described above, was used for comparison to SPE cue.

Preference trials were carried out following established methods (Fisher, H.S., *et al*. 2006, Rosenthal, G.G., *et al*. 2011). Trials were conducted in an aquarium (75x19x20 cm) divided into 3 equally divided zones defined in the Biobserve Viewer tracking system (Bonn, Germany). Each tank had a stimulus delivery system at each end and was controlled by two peristaltic pumps (VWR Scientific, Sugarland TX, USA) at a rate of 5 ml/minutes. Female *X. birchmanni* were acclimatized 20 minutes prior to trials in their individual test tank lane. To control for side bias, females were tested twice, switching the sides from which cues were presented. Trials ran for 600s each and females who did not respond or visit both sides by 300s were removed from analysis. I summed the association times in the two trials for analysis.

Females were tested on each organ tissue type and then with conspecific (*X. birchmanni*) and heterospecific (*X. malinche*) candidate tissue cue. Trials were carried out on *X. birchmanni* females: control (blank DI water) vs. *X. birchmanni* SPE, *X. birchmanni* vs. live male cue, conspecific (*X. birchmanni*) and heterospecific (*X. malinche*), chimeric interchange of *X. birchmanni* and *X. malinche* species specific peaks, and trials using A and B peaks isolated separately, as identified from HPLC (Figure.1, 2, &3). For each comparison, I used Wilcoxon signed-rank tests to evaluate the null hypothesis of no difference in mean association time between paired stimuli. All analyses were conducted in JMP Pro.

*Results*

**Organ preference trials of pheromone production and pheromone extraction**

Testis (*N*=12, Wilcoxon signed-rank test, Z = -1.83842, p = 0.033), but not non-reproductive tissues (*N*=24, Wilcoxon signed-rank test, Z = -0.6418, p = 0.2605), elicited a female preference over aquarium-water controls. Testis was at least as effective as water collected from live courting males, as used in previous studies (*N*=16, Wilcoxon signed-rank test, Z = -1.50704, p = 0.0659, (Figure 1B), and female *X. birchmanni* strongly preferred testis of conspecifics over *X. malinche* (*N*=20, Wilcoxon signed-rank test, Z = -1.98992, p = 0.0233, Figure 1A).

Eluate following solid phase extraction (SPE) was at least as effective as signals from live males at eliciting conspecific preference (*N*=12, Wilcoxon signed-rank test, Z = -1.38386, p = 0.0832), and *X. birchmanni* females preferred SPE of conspecific over heterospecific males (*N*=14, Wilcoxon signed-rank test, Z = -1.94813, p = 0.0257, Figure 1C).

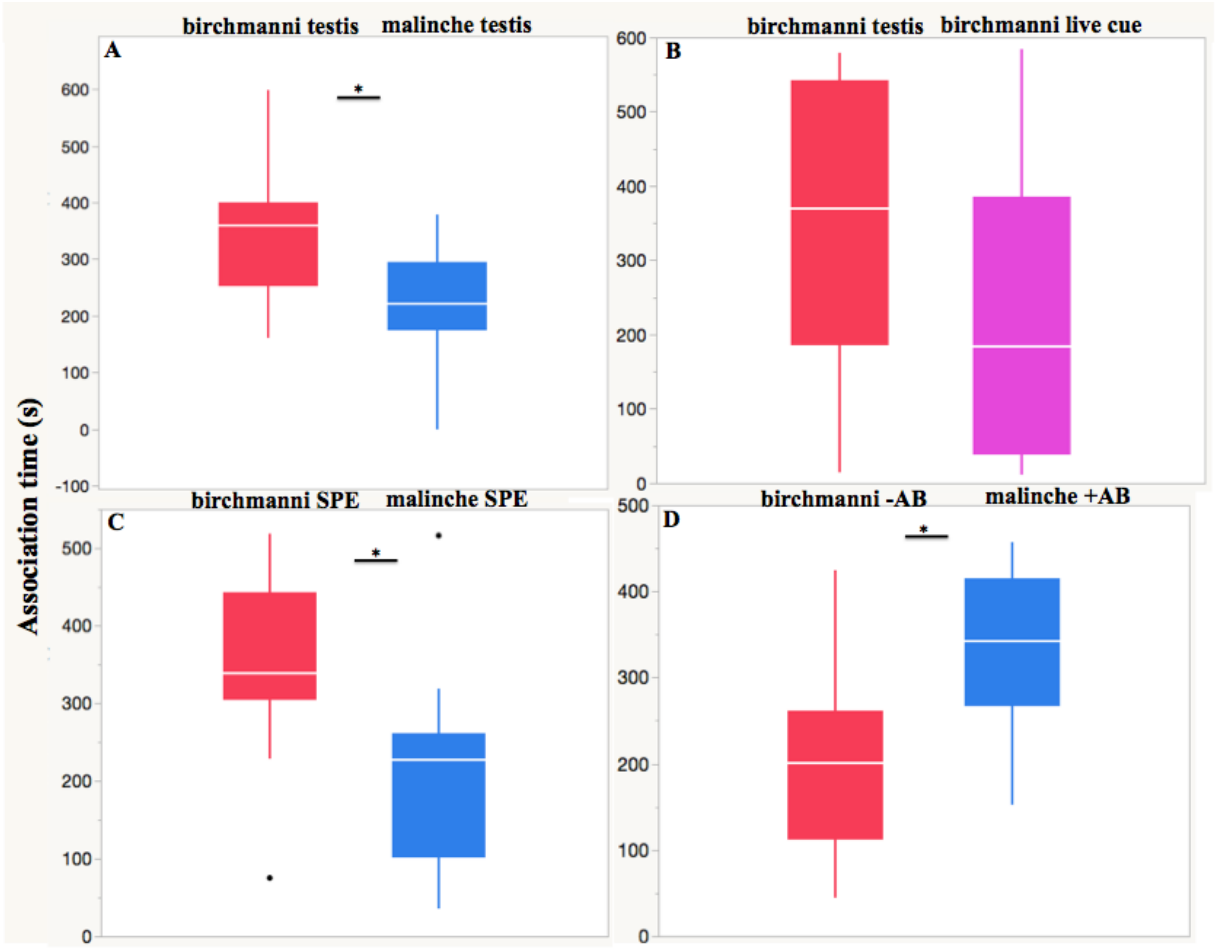


Figure 1. Behavioral data evaluating male chemical signals on female *X. birchmanni.* Association time (mean ± SEM) of female *X. birchmanni* with chemical signals. Testis extract elicits conspecific preferences (A), preferences are maintained after solid-phase extraction (B,C) and chimeric interchanges show that *X. birchmanni* peaks A and B are together necessary and sufficient for conspecific mate preference (D). *p value < 0.05, Wilcoxon Signed-Rank.

**HPLC/MS analysis**

High performance liquid chromatography (HPLC) and mass spectrometry (MS) identified peaks found in male testis eluate (Figure 2A). Peaks 1-4 were conserved between the two species, showing no significant difference in retention time (*N*=24 *X. birchmanni*, *N*=16 *X. malinche*, unpaired *t*-test, p = 0.5, *t* = 2.65, Figure 2B). Two peaks, A and B in figure 2A, were specific to *X. birchmanni*. HPLC readouts of *X. birchmanni* and *X. malinche* show minimal variation among males within a species (Table 1).

MS/MS data of 4 peaks gave insight into candidate compounds. Ideal matches were chosen based on lowest tolerance across databases and adherence to assumptions as described above. Additional matches were excluded, as they did not meet assumptions or tolerance. *m/z* peak 3 showed a dominant negative ion of 512.49 and MS/MS showed a similar dominant negative ion of 512.41, tolerance 0.02. Database searches noted a similar pattern to L-tyrosine 4-hydroxyphenylalanine. *m/z* peak A showed two low intensity negative ions at 269.10 and 287.20 with a dominant negative ion 367.15, tolerance 0.02. Data base searches suggest a testosterone sulfate compound. *m/z* peak 4 showed a negative dominant ion at 514.47, and MS/MS showed two negative 255.21 and 273.22, tolerance 0.01. Database hits showed a testosterone glucuronic acid. *m/z* peak B showed a dominant negative ion at 407.48 and a smaller ion at 815.78, tolerance 0.03. This pattern gave a database hit of a small urinary conjugated bile acid, cholanic acid. The internal standard *m/z* was verified by a dominant negative ion 391.28, tolerance 0.001, matching the chemical in the database.

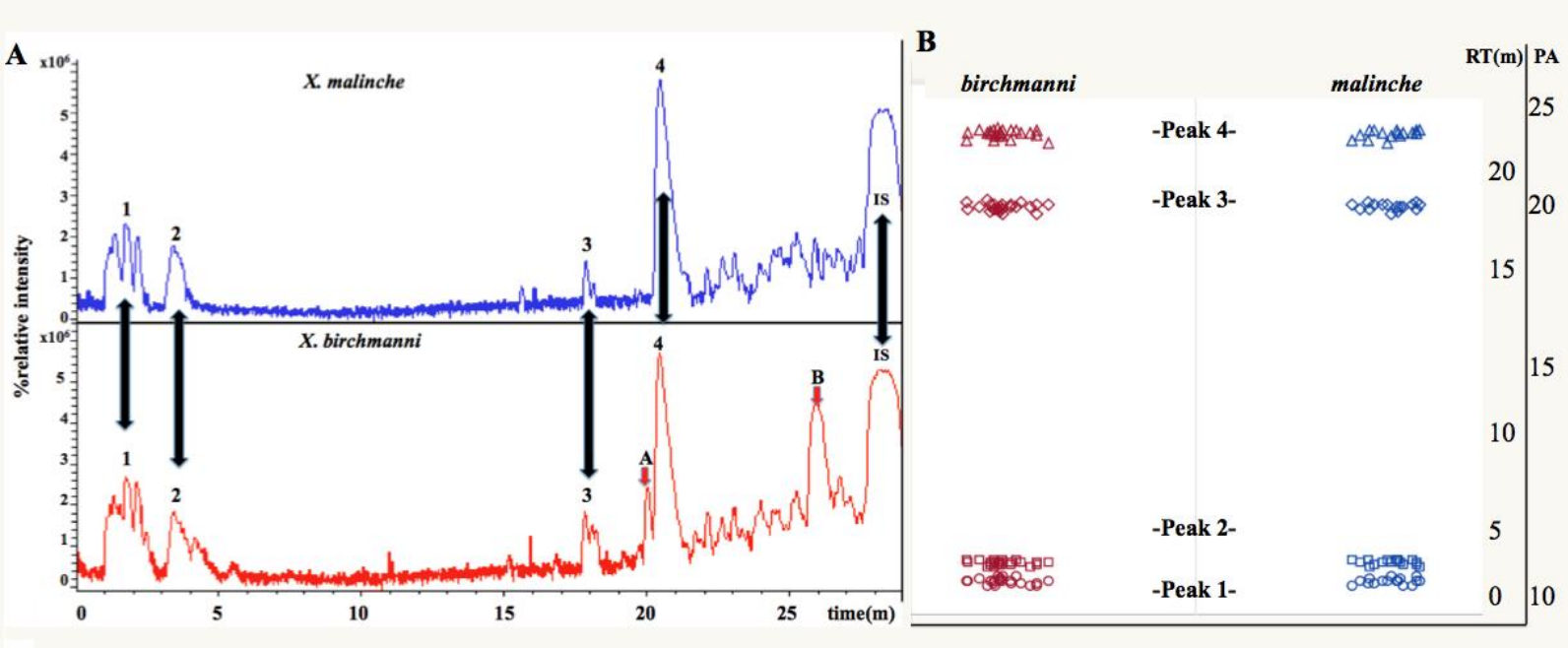


Figure 2. HPLC readouts of *X. malinche* and *X. birchmanni* SPE from testis (A) scatterplot of *birchmanni* and *malinche* peaks showing retention time and peak area( B). A.) HPLC readouts of *X. malinche* (top) and *X. birchmanni* (bottom) SPE from testis. Peaks 1-4 are shared while A&B are found only in *birchmanni*. IS, internal standard. B.) Scatterplot of *birchmanni* (red) and *malinche* (blue) peaks 1-4 showing retention time (RT) and peak area (PA) are not significantly different. (unpaired *t*-test, p= 0.5)

**Chemical composition and mating preference**

Female *X. birchmanni* preferred the *X. malinche* signal with *X. birchmanni* peaks over the *X. birchmanni* signal with peaks removed (*N*=14, Wilcoxon signed-rank test, Z = -1.80933, p = 0.0352, Figure 3D). These components are therefore together both necessary to elicit preference for conspecifics. Females did not show a preference between signals containing peak A alone versus peak B alone (*N*=22, Wilcoxon signed-rank test, Z = -0.4383, p = 0.3299, Figure 3B&C). Females preferred the *X. malinche* signal with peak B added (A-B+) over *X. malinche* signal (*N*=22, Wilcoxon signed-rank test, Z = -1.769, p = 0.0383) and showed the same trend for *X. malinche* signal with peak A added (A+B-; *N*=19, Wilcoxon signed-rank test, Z = -1.4085, p = 0.0792.)

SPE is an important step in analyzing the chemical signal but may also introduce noise into the system by removing important components from the resulting eluates. However, the C18 column should have retained the non-polar molecules and allowed the passage of more polar molecules. Due to the limited gradient of the mobile phase used, some important compounds may have been left in the column and not been detected as a peak in HPLC. My experimental results (Figure 1B &3A) suggest that any compounds removed during SPE would be neither necessary nor sufficient to elicit a preference for conspecific signal.

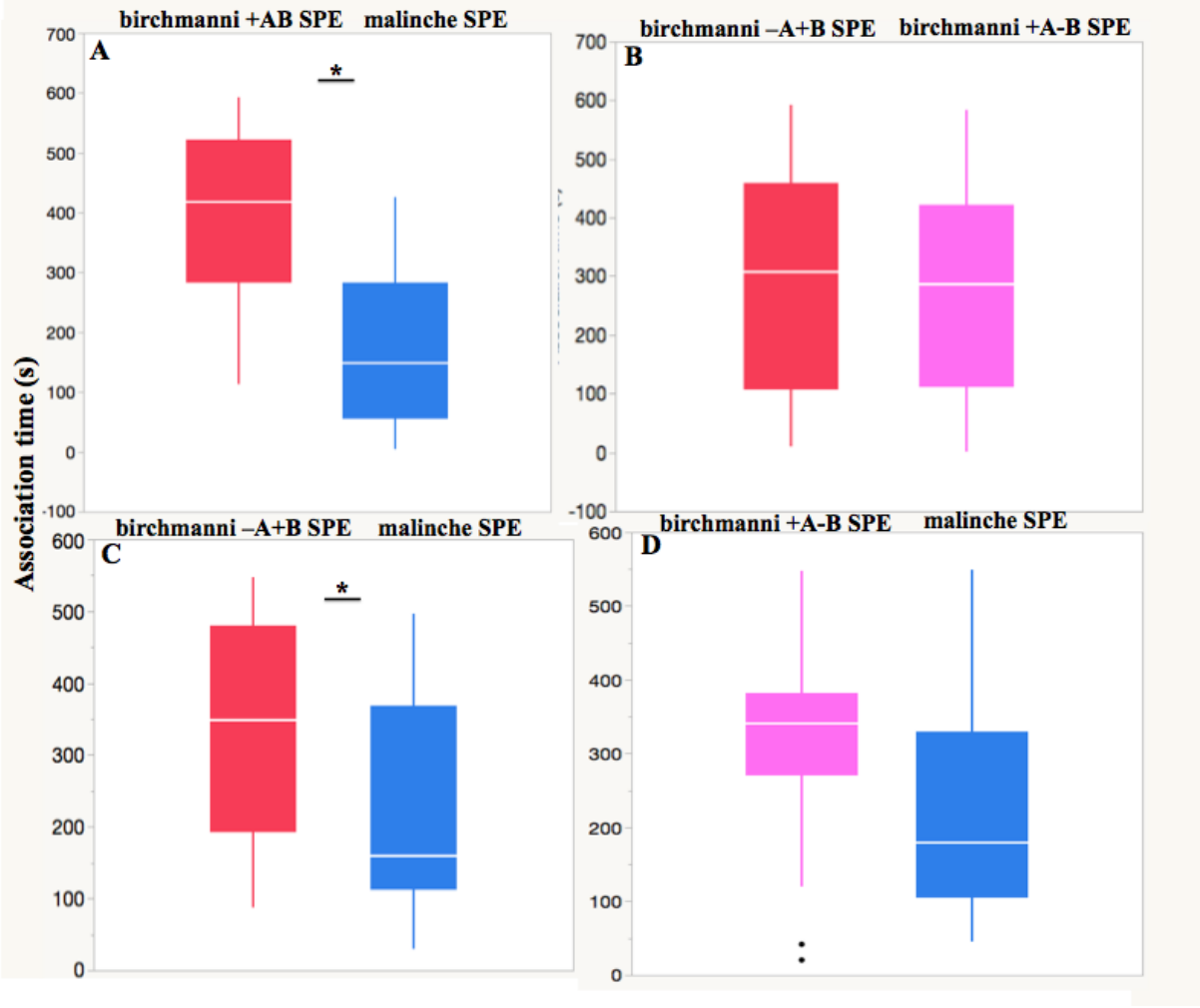


Figure 3. Behavioral data elucidating the interaction of species-specific peaks using female *X. birchmanni* with chemical signals. Association time (mean ± SEM) of female *X. birchmanni* with chemical signals. Testis SPE elicits conspecific preferences (A), preferences are lost when peaks A & B are tested against each other (B), and interchanges show that *X. birchmanni* peak B regains conspecific mate preference (C) while peak A does not (D). *p value < 0.05, Wilcoxon Signed-Rank.

*Discussion*

I localized pheromone production to the testis (Figure 1A), which allowed us to harvest and purify concentrated samples for solid phase extraction (SPE). Importantly, reconstituted SPE extracts were as effective as unmanipulated cues at eliciting conspecific mate preference in a simultaneous-choice assay (Figure 1B). Our experimental results suggest that any compounds removed during SPE would be neither necessary nor sufficient to elicit a preference for conspecific signal. Analysis of reconstituted eluates with HPLC showed four distinct peaks (Figure 2A) shared between *X. birchmanni* and *X. malinche,* and two peaks unique to *X. birchmanni* (Figure 2A). Peaks 1-4 showed the same retention times and *m/z* patterns between species (Figure 2B) between samples and replicates of both species. By contrast, fractions A and B were found only in *X. birchmanni.* Isolation of fractions A & B was achieved from elution (SPE) and verified with HPLC. I created chimeric signals by isolating these fractions, allowing me to add them to *X. malinche* and remove them from *X. birchmanni.* When fractions A&B were removed from *X. birchmanni* signal and added to *X. malinche* signal, females reversed their preference (Figure 3D). This result shows that the species-typical compounds present in these fractions are sufficient to elicit a behavioral preference. I tested combinations of A and B to dissect the functional importance of each component. There was no difference in response to signals of *X. birchmanni* with only fraction A present versus only fraction B (Figure 3B). When females were tested with *X. birchmanni* signal containing only one of the two species-typical peaks, they showed a significant preference for *X. birchmanni* with fraction B over *X. malinche,* and a similar, non-significant trend for fraction A (Figure 3C&D). This suggests that these components may combine additively to elicit conspecific mating preference (Partan, S., & Marler, P. 1999).

Using MS, MS/MS data and the available databases I identified confident candidate compounds found in the *Xiphophorus* pheromone. The compounds identified were: *m/z* peak 3, L-tyrosine 4-hydroxyphenylalanine, *m/z* peak A, testosterone sulfate compound, *m/z* peak, testosterone glucuronic acid, *m/z* peak B, and a small urinary conjugated bile acid, cholanic acid.

*Conclusions*

This is one of the first studies to characterize the chemical signals involved in reproductive isolation among vertebrate species. The candidate compounds for the components involved in conspecific mate recognition include a conjugated sex steroid and a bile acid, both of which play a role in sex communication in other fishes (Sorensen, P. W., *et al*., 1998). In several fishes, including another poeciliid, urine-borne sex-steroid metabolites have a stimulatory effect on sexual behavior (Burnard, D., *et al*. 2008). Communication between senders and receivers constitutes a key mechanism of reproductive isolation (Brennan, P.A., & Zufall, F. 2006). Pheromones play an important role in conspecific mate preference across taxa (Rosenthal, G.G., & Lobel, P. 2006). Among insects, mutations to pheromone-production pathways and to pheromone receptor proteins are sufficient to generate behavioral isolation between species (Sorensen, P. W., *et al*. 1998). In vertebrates, chemical communication is just as important to reproductive isolation, but we know far less about the mechanisms underlying signal production and reception. Understanding the chemical basis of premating isolation is particularly important in aquatic species, where both signals and receivers are sensitive to water chemistry. Perturbation of the chemical environment can interfere with species recognition and relax mating preferences, resulting in hybridization between sympatric species. This study presents a novel step towards understanding the complexity of chemical communication in a model aquatic vertebrate. Here I show that pheromones are produced in the testis; testis extract elicited the same conspecific

preference as signals generated by displaying males. I used solid phase extraction (SPE) preparation in combination with high performance liquid chromatography / mass spectrometry (HPLC/MS) to characterize pheromone chemical composition. Analyzing HPLC/MS readouts for pure peaks with high relative intensity identified two discrete chemical components present in *X. birchmanni* but absent in *X. malinche.* Experimental manipulation of signal composition showed that the presence of these components is critical to conspecific mate preference by *X. birchmanni*. Characterization of chemical signals allows for powerful tests of how they interact with the environment and receiver perception, thereby contributing to both the maintenance and breakdown of reproductive isolation.

**Table 1.** HPLC analysis of *X. birchmanni (N=22)* and *X. malinche (N=16)* testis SPE.

| | *X. birchmanni* | | *X. malinche* | |
|---|---|---|---|---|
| Peak | RT (m) | Peak Area | RT (m) | Peak Area |
| 1 | 1.3±0.11 | 10.877±0.242 | 1.5±0.31 | 11.867±0.312 |
| 2 | 4.1±0.18 | 12.995±0.113 | 2.3±0.19 | 13.845±0.143 |
| 3 | 19.1±0.43 | 17.076±0.194 | 17.5±0.53 | 16.076±0.124 |
| A | 20.1±0.32 | 19.336±0.261 | — | — |
| 4 | 21.5±0.18 | 24.263±0.344 | 20.3±0.22 | 24.563±0.334 |
| B | 25.9±0.34 | 17.521±0.217 | — | — |
| IS | 28.4 ±0.082 | 45.122±0.322 | 28.2 ±0.082 | 45.132±0.212 |

# CHAPTER III

# MALE PHEROMONES CO-VARY WITH ALTERNATIVE REPRODUCTIVE STRATEGIES IN A TELEOST FISH

## *Introduction*

Across taxa, males employ alternative reproductive tactics (Gross, M. R. 1996, Sinervo, B., & Lively, C. M. 1996). These males typically mature faster and express few secondary sexual characteristics, while investing more in primary reproductive structures (Rasotto, M. B., & Mazzoldi, C. 2002). While often at a disadvantage with respect to mate choice and intrasexual competition, these males are more likely to survive to sexual maturity and avoid direct competition with larger courting males (Reichard, M. 2016). Numerous studies to date have shown differences between strategies not only in behavioral tactics, but also in the repertoires of visual and acoustic signals used to attract mates (Aubin-Horth, N., & Dodson, J. J. 2004, Morris, M. R., *et al*. 2016, Partridge, C. G., *et al*. 2016). Despite the ubiquity of chemical communication in mate choice, few if any studies have addressed differences in olfactory cues as a function of mating strategy. Chemical signaling has been highlighted in the invertebrate literature for the vital role it plays in mate choice and species recognition; in vertebrates we know far less about this interaction (Brennan, P.A., & Zufall, F. 2006).

Here I identify striking quantitative differences in chemosignal composition between alternative male morphs of the poeciliid *Xiphophorus birchmanni*, where females show well-characterized premating preferences for sexually-dimorphic visual cues and for urine-borne pheromones (Wong, B.B.M., *et al*. 2005, Rosenthal, G.G., & Lobel, P. 2006, Fisher, H. S., *et al*. 2009, Kindsvater, H. K., *et al*. 2013, Culumber, Z. W., *et al*. 2014). Male *X. birchmanni* show

two distinct phenotypic clusters: large and ornamented (M+) and small and drab (M-) (Figure 4B). Additionally I test natural variation I found in a pheromone component within M- males, high (M-H) and low (H-L). Here I tie together the relationship between male reproductive tactic and pheromone cue by identifying the organ of pheromone production, quantify both chemical signal and male visual traits and measure female preference for male pheromone cue.

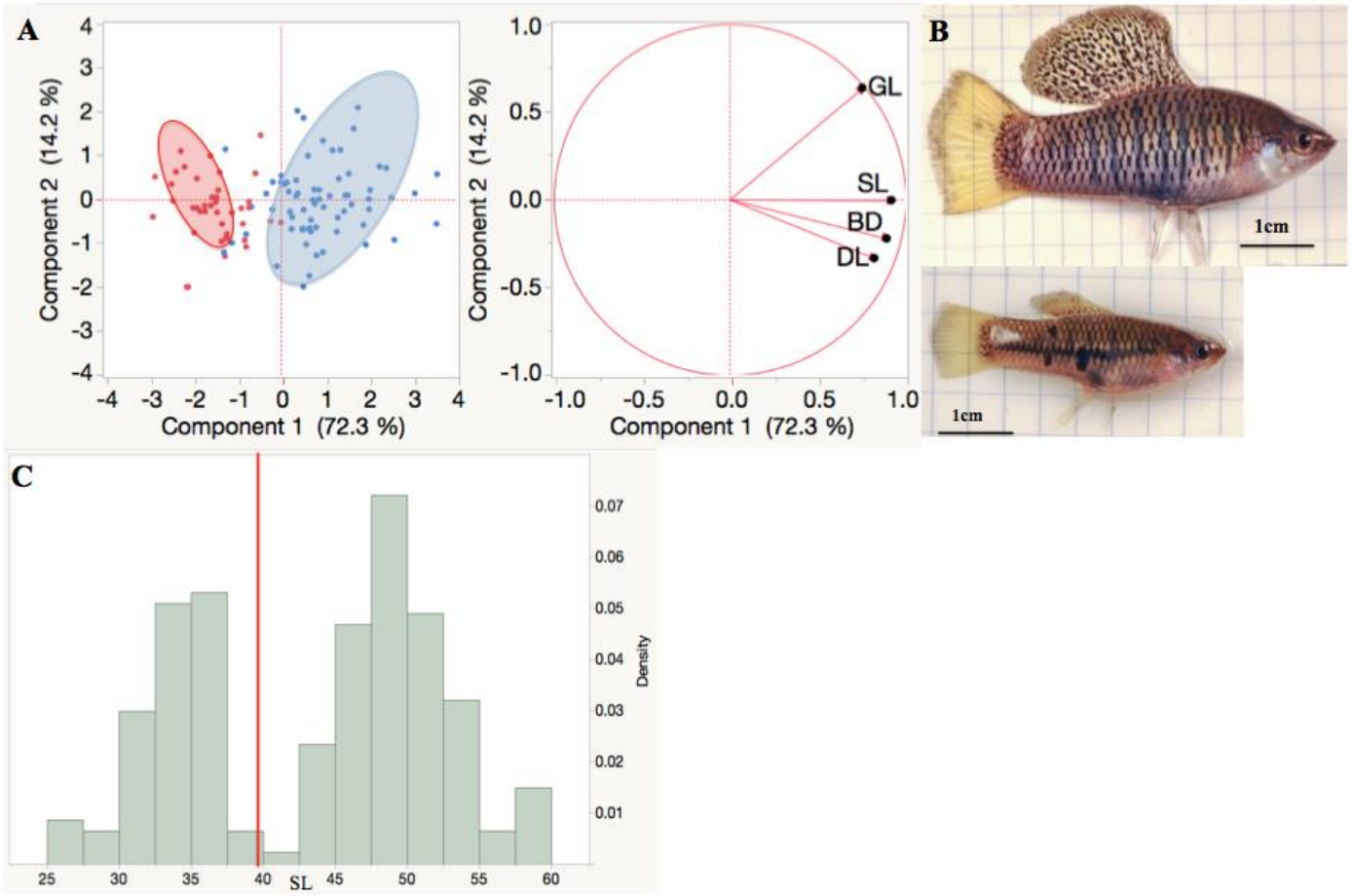


Figure 4. Data showing male polymorphism persists in *X. birchmanni.* A.) PCA of male morphometric traits, M+ and M- cluster separately. B.) Images of *X. birchmanni* males, M+ phenotype (top) and M- male (bottom). C.) Histogram of male standard length distribution with

*Materials and Methods*

**Collection**

All subjects were adult *X. birchmanni* collected using baited minnow traps from the Rio Garces (20°57'22 N, 098°16'48 W) and Rio Coacuilco (21°5'50.85 N, 98°35'19.46 W).

**Dissection of testis**

Males were euthanized using buffered MS-222 and kept on ice prior to dissection. Testis and duct were stored individually in 1ml of distilled water at -20°C.

**Male morphometric data**

Wild-caught males (*N*=189), were lightly anesthetized with MS-222 and photographed for traditional morphometric measurements using the ImageJ program. Measurements were taken for standard length (SL), dorsal fin length (DL), gonopodium length (GL), and body depth (BD) in mm. To standardize for male body length, the ratio of GL to SL was taken by dividing GL by SL. PCA was conducted on log + 1 transformed measurements in JMP. Fully mature males less than 39cm in standard length (as shown by wild population's natural distribution, (Figure 4C) that exhibited – no vertical bars, lacked a nuchal hump, reduced dorsal fin, and either or both of the two female traits, a false gravid spot or horizontal bar – were classified as M-.

**Male GSI analysis**

For male gonadosomatic index (GSI) analyses, a subset of males (*N*=20) males were preserved in 95% EtOH after being photographed for morphometrics and dissecting the testis. Testis tissue was used for solid phase extraction and then stored in 95% EtOH. 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 GSI. PCA was conducted on log + 1 transformed measurements in JMP.

**Pheromone SPE and HPLC-MS**

Purification and concentration of the male *Xiphophorus* pheromone was achieved using solid phase extraction (SPE). Testis tissue was suspended in 0.5 mL of distilled water (DI) and stored at -20°C prior to use. For extraction, tissue was thawed at 23°C and 300 rpm on a mixing tray. Tissue and holding water were loaded into a C18 (Bond Elut-C18, 200mg, 3ml, Agilent Technologies, Wilmington, DE, USA) SPE column mounted on a vacuum manifold pressurized at 15 psi. The C18 column was prepped with 2mL MeOH followed by 2 mL DI water. Tissue was then loaded and washed with 1mL DI water. Tissue elution was performed with 25% MeOH and DI water, 65 MeOH and 100% MeOH. Each elute was captured in a separate test tube along the manifold then split for behavior trials and high performance liquid chromatography (HPLC). For HPLC, an internal standard of 1μg of the unconjugated bile acid chenodeoxycholic acid (CDCA; Steraloids Inc, Newport, RI, USA) was added to each of the eluates to standardize retention times and injection volumes between samples and to a blank sample of 35% DI water/65% MeOH (control). Eluates were dried under a stream of 99.9% pure nitrogen gas (Cal Gas Inc., Huntington Beach, CA, USA) reconstituted in 2 ml MeOH /water (60/40, v/v) and stored at -20°C till HPLC/MS. The LC column (Nova-Pak reversed-phase C18, 4 μm, Waters Chromatography Division, Milford, MA, USA) was coupled to a mass spectrometer with electrospray ionization (ESI; LCQ-DECA, Thermo Electron Corporation, Houston, TX, USA). The mobile phase was isocratic at 15% MeOH for 4 minutes, increased linearly from 15% to 100% MeOH from 4 to 31 minutes, and allowed to run at 100% MeOH for 5 minutes before increasing back to 15% MeOH for the next sample. Preliminary analysis of HPLC showed no

new compounds after 30 minutes when run for a full hour. I then used a PE SCIEX QSTAR (Applied Biosystems, Foster City, CA, USA) to perform MS/MS using negative and positive-ion high-resolution electrospray ionization to identify peaks found in male testis. The ion trap was operated in the negative and subsequent positive ion mode with a spray voltage of 5 kV. A stream of 99% pure nitrogen at 60 psi was used as the sheath gas. Data were collected in the range m/z 250–950. The relative peak areas (normalized to the area of the internal standard, CDCA) in the HPLC fractions were determined with Compass Data Analysis Viewer software (2014, Bruker Daltonik GmbH, Billerica, MA, USA).

**Female preference trials**

To assess females' responsiveness to pheromone eluate following SPE, eluates were pooled in groups of 4 males and suspended in 500ml of DI water 24 hours prior to trials. Preference trials were carried out following established methods (Fisher, H.S., *et al*., 2006, Rosenthal, G.G., *et al*., 2011). Trials were conducted in an aquarium (75x19x20 cm) divided into 3 equally divided zones defined in the Biobserve Viewer tracking system (Bonn, Germany). Each tank had a stimulus delivery system at each end and was controlled by two peristaltic pumps (VWR Scientific, Sugarland TX, USA) at a rate of 5 ml/minutes. Female *X. birchmanni* were acclimated for 20 minutes prior to trials in their individual test tank lane. To control for side bias, females were tested twice, switching the sides from which cues were presented. Trials ran for 600s each and females who did not respond or visit both sides by 300s were removed from analysis. I summed the association times in the two trials for analysis. Females ($N$=24) were carried out on *X. birchmanni* females, M+ vs. M- male and M-H vs. M-L. I used Wilcoxon signed-rank test to assess significance of differences in mean association time between two stimuli per group and all analysis was conducted in JMP Pro.

*Results*

**Pheromone SPE and HPLC-MS**

HPLC analysis of both male phenotypes showed peak 3 (ANOVA F (1,18) 414.6467, p = 0.0001) and peak A (ANOVA F (1,18) 905.1234, p = 0.0001) were significantly different between the two male types (Figure 5&6A)). While peaks 4 (ANOVA F (1,18) 0.5063, p = 0.4859) and B (ANOVA F (1,18) 0.2608, p = 0.6157 (Figure 5&6A)) were not. Notably the StDev in peaks 3 (±0.9856), A (±0.945), and B (±1.215) in M- males were all low, while peak 4 showed high variation (±8.83, Brown-Forsythe F ratio 7.8258, p = 0.0019 (Figure 6A)).

**Male morphometric and GSI/GPI data**

Morphometric data showed that the SL (ANOVA F (1,18) = 74.5174, p = 0.0001), BD (ANOVA F (1,18) = 83.1323, p = 0.0001), and DL (ANOVA F (1,18) = 78.6521, p = 0.0001) in the M+ male phenotype were all significantly different than M- males. While GL was not significantly different between the two types (ANOVA F (1,18) = 1.8661, p = 0.1887). The mean trait GPI (the ratio of SL to GL) was significantly different with M- males having larger GPI (ANOVA F (1,18) = 1.1472, p = 0. 0.0029 (Figure 6B)). Male BM (ANOVA F(1,18) = 54.0133, p = 0.0001 was significantly different with M+ males weighing more while TM (ANOVA F (1,18) = 0.2967, p = 0.5926) was not significantly different. GSI (the ratio of TM to BM) was significantly different between male types (ANOVA F (1,18) = 53.2311, p = 0.0001) with M- males having larger GSI (Figure 6B).

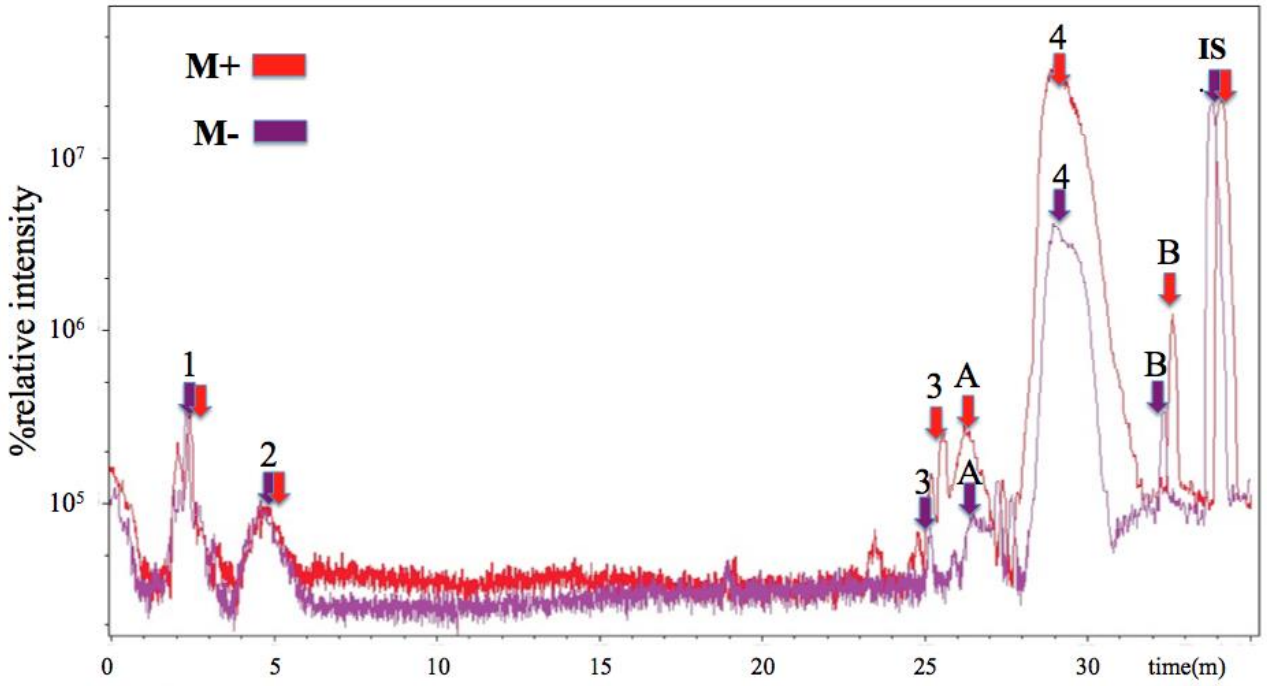


Figure 5. HPLC readouts of both male polymorphisms. Individual *X. birchmanni* M+ male (red line and arrows) and *X. birchmanni* M- (purple line and arrows) SPE from testis. Peaks 1,2 and B are not different, while 3, A and 4 show variation between the two *birchmanni* male types. IS, internal standard.

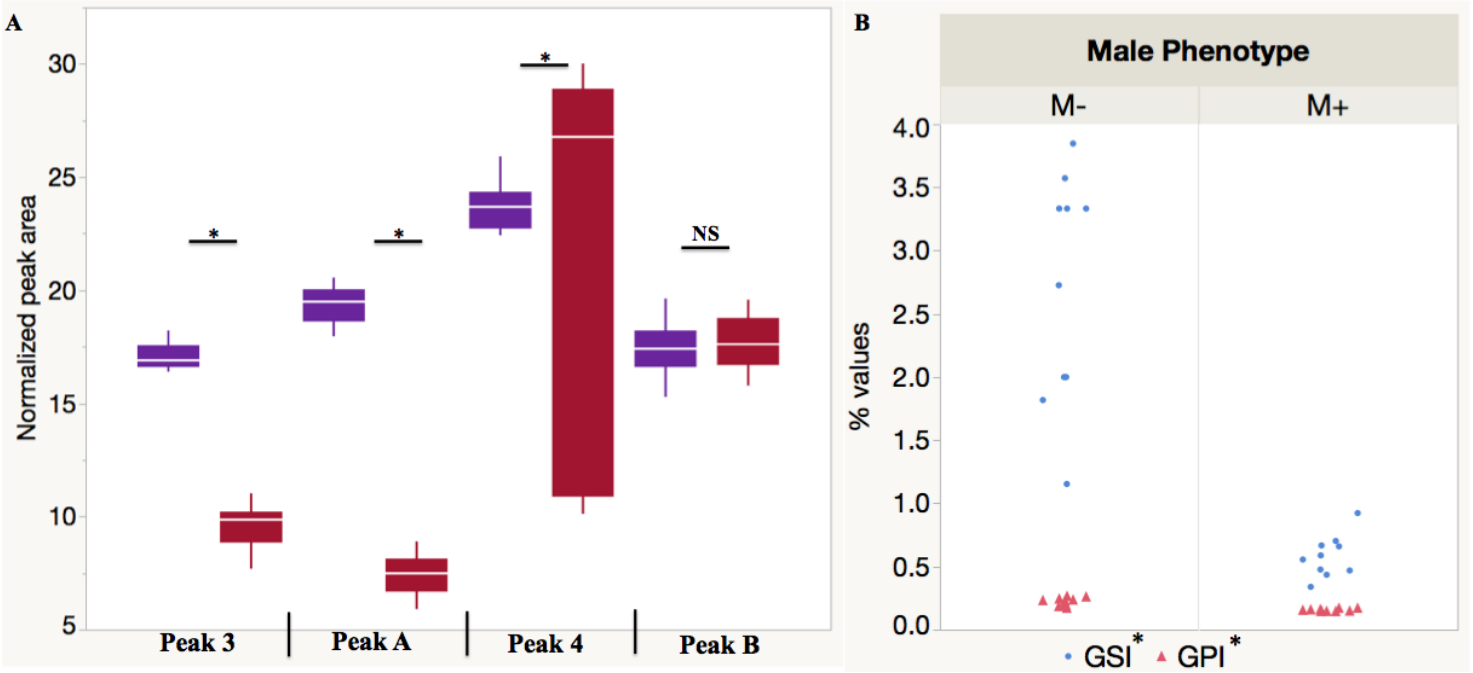


Figure 6. Comparison of traits (± SD) by male phenotype. A.) HPLC peaks 3, A, and 4 were all significantly higher in M+ males however, there was no difference between males at peak B (M+ in purple, M- in red). B.) Gonadosomatic index (GSI) and Gonopodium length (GPI) were significantly higher in M-males. (ANOVA, *p value < 0.05)

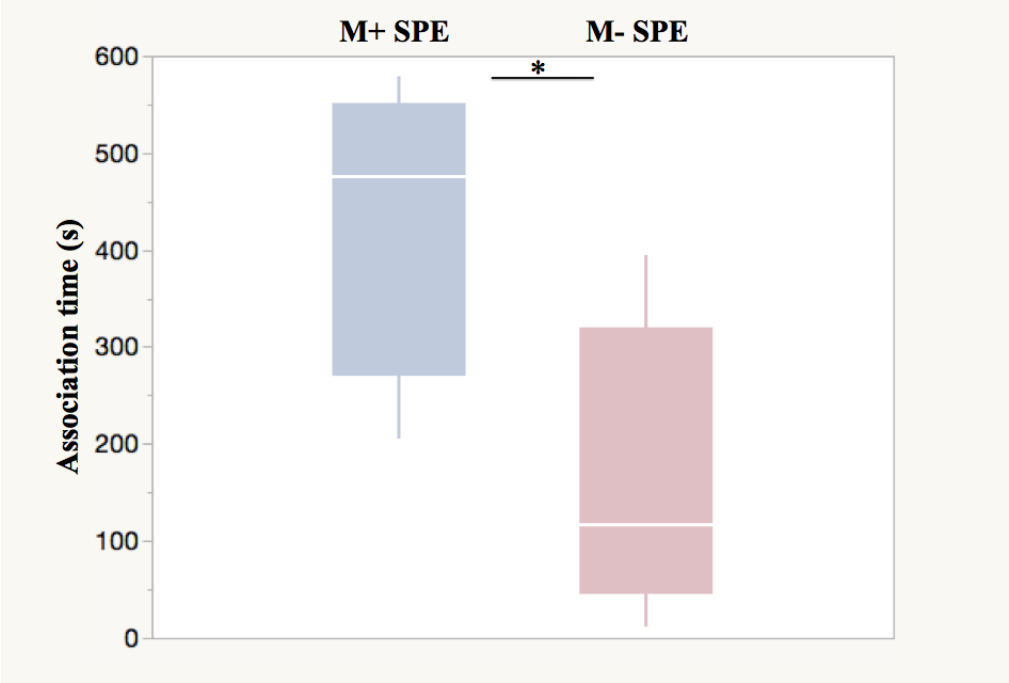


Figure 7. Preference trials of female *X. birchmanni* with chemical signals of M+ and M-males. Association time (mean ± SEM) of female *X. birchmanni* with chemical signals. Females prefer testis solid-phase extraction of M+ *X. birchmanni* males over M- males. *p value < 0.05, Wilcoxon Signed-Rank

**Pheromone extraction preference trials**

Female *X. birchmanni* preferred the M+ male signal over M- male (*N*=24, Wilcoxon signed-rank test, Z = -2.7262, p = 0.00317, (Figure 7)) but showed no preference for M-H or M-L males (*N*=16, Wilcoxon signed-rank test, Z = -0.6525, p = 0.61700).

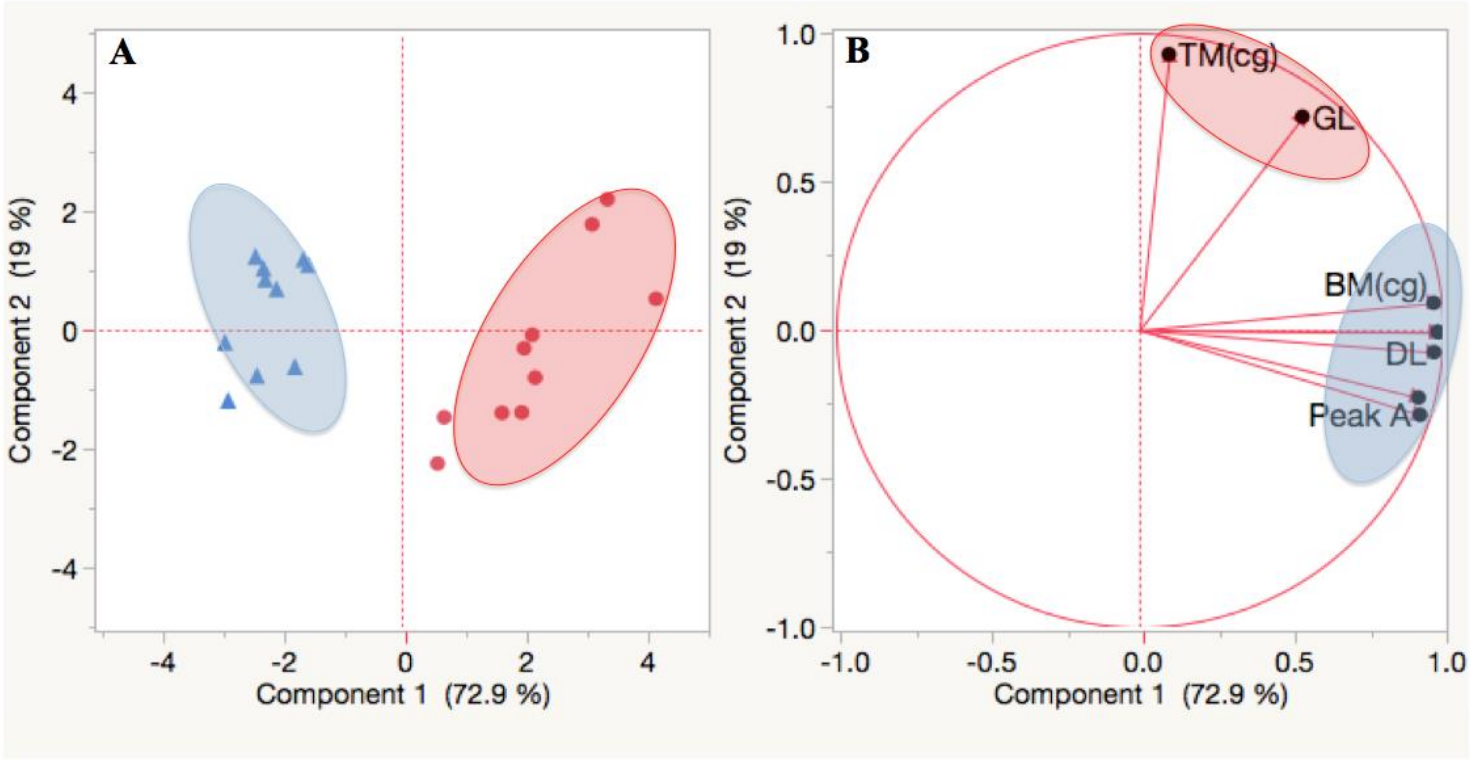


Figure 8. Principal components analysis of male traits. A.) M+ males (blue) and M- males (red) cluster separately. B.) Testis mass (TM) and gonopodium length (GL) explain the biggest difference between male phenotype in the M- phenotype. Body mass (BM), body length (BL), peak 3, and peak A explain the difference for M+ males.

**PCA analysis**

Approximately 72.9% of the total variance in male phenotype was predicted by principal component 1 with M+ males clustering (PC1, Figure 8A). This component was largely influenced by SL, BD and DL, and BM and all peaks in HPLC (PC1, Figure 8B) While M- males clustered separately (PC2, Figure 8A), principal component two was largely influenced by GL and TM (Figure 8B).

*Discussion*

HPLC analysis of testis SPE showed significant differences between the M+ males and M- males (Figure 5&6A). All compounds were shared between the two males (Figure 5); however, peaks 3, A and B were at a significantly greater intensity and showed less variation in M+ males (Figure 6A). Female *X. birchmanni* significantly preferred SPE of M+ males over M- males (Figure 7). However, females showed no preference between the variations within M- (M-H and M-L) males that differentially expressed amounts of peak 4. These results show even though pheromone profile is conserved across males in this species, intraspecific variation between male types is important in female mate choice. This variation in male pheromone relating to phenotype may be influenced by relaxation of selective pressure on males not expressing male traits used for courting. Alternatively, females may be able to distinguish males between phenotypes using chemical signals. Morphometric data from both male types showed that M+ males have significantly larger SL, BD, DL and BM, while M- males have significantly larger GSI and GPI (Figure 6B). Respectively, these traits are also tightly correlated to each male type in a PCA (Figure 8B) causing males to cluster separately (Figure 8A). These morphometric data are in agreement with a large expanse of research on alternative reproductive tactics. Mature smaller males express traits typically important for high reproductive output: large testis, longer

intermittent organs and higher sperm counts (Gross, M. R. 1984, Ruchon, F., *et al*. 1995). Alternatively, large courting males typically express traits that improve mating via female preferences and direct male competition: highly colored, large body size, and increased size of secondary sexual characteristics (Taborsky, M. 1998, Norman, M. D., *et al*. 1999, Oliveira, R. F., *et al*. 2001).

### *Conclusions*

Sexual selection can favor the evolution of alternative mating strategies. A well-documented strategy is for smaller males to employ a "sneaker" strategy in an attempt to bypass direct competition with larger and/or more ornate males. In swordtail fish (genus *Xiphophorus*), males express a wide range of secondary sexual characteristics as ornate morphological traits. Males I call M+ males, deploy these traits during courtship in concert with a stereotyped motor pattern directed towards females. "Sneaker" males, or M- males, by contrast, are small and unornamented, and can resemble females. In *X. birchmanni,* I found two distinct male types consistent with these two strategies. As in other species, M+ males were larger and more ornamented, while small males had longer intromittent organs and a higher gonadosomatic index. Further, the two types differed significantly in the abundance of discrete pheromone components likely to be androgen conjugates. Females significantly preferred the pheromone cue of M+ males over sneaker males. Chemosignals may therefore represent an underappreciated component of variation in male sexual strategies.

Here I show the first correlation, to my knowledge, between male alternative reproductive strategy, olfactory signals and female preference. Females across many taxa rely on olfactory cues for mate recognition and preference (Wyatt, TD. 2010, Wyatt, T. D. 2014). However, in vertebrates we know very little about how these intersect and few studies can

measure this in a quantitative evaluation. This work highlights the important role pheromones play in intraspecific mate choice and their relationship with other traits in male reproductive strategy.

# CHAPTER IV

# PHEROMONE SIGNALS, MORPHOLOGY, AND POPULATION STRUCTURE IN NATURALLY HYBRIDIZING SWORDTAIL FISH

## *Introduction*

Chemoreception is a ubiquitous communication mechanism among organisms and constitutes a barrier to gene flow between species (Rosenthal, G.G. & Lobel, P. 2006, Wyatt, T. D. 2014). This is because the large repertoire of olfactory receptors allows for specialization and diversification of signals (Leary GP*, et al.* 2012, Hesse, S., *et al.* 2016) due to the ability of odorant receptor proteins to be narrowly tuned to species-typical chemical signals (Barton, N. H., & Hewitt, G. M. 1989, Rollmann, S. M., *et al.* 2003, Palmer, C. A., & Houck, L. D. 2005). However, some sympatric species hybridize despite chemical-based conspecific mate recognition (Culumber, Z.W., *et al*. 2011, Rosenthal, G. G. 2013, Wyman, M. T., *et al*. 2015). This may be because of overlap between conspecific and heterospecific signals (Yang, C.Y. 2015) or through interactions with the chemical environment that cause interference with communication (Fisher, H.S *et al*. 2006). Environmental effects are particularly acute for animals that live in aquatic environments, since water chemistry can interact directly with the olfactory periphery and/or with chemical signaling (Candolin, U. 2014). Alteration of the chemical environment can abolish mating preferences for conspecific signals, thereby facilitating hybridization (Rosenthal, G.G., *et al*. 2003). Hybrids, in turn, may produce novel combinations of odorant signals (Morgado-Santos, M., *et al*. 2015) and odorant preferences (Sandkam, B. A., *et al*. 2013, Reding, L., & Cummings, M. E. 2015), which can lead to gene flow between parent species or to the formation of novel hybrid species. Studying how natural hybridization affects chemosignals provides

insight into how communication and its breakdown could facilitate the formation and breakdown of reproductive barriers. Chemical communication likely played an important role in hybridization between two sister species of poeciliid fish in the genus *Xiphophorus.* The swordtails *X. birchmanni* and *X. malinche* form at least six natural hybrid zones along elevation gradients in the eastern Sierra Madre Oriental in Hidalgo state, Mexico (Culumber, Z.W., *et al*. 2011, Culumber, Z. W., *et al*. 2014). Previous work has shown that pheromones are the primary mechanism for conspecific mate preference in *Xiphophorus* (Wong, B.B.M., *et al*. 2005) and that conspecific mate preference can be abolished by interference with the chemical environment (Fisher, H.S. *et al*. 2006).

Having identified pheromone signal components used in conspecific mate recognition, I will test the hypothesis that pheromone signals act as mechanisms of reproductive isolation in hybrid populations. We do this by taking advantage of the wide variation in structure among hybrid populations. Specifically, some populations are hybrid swarms with random mating with respect to genotype (Tlatemaco, TLMC), some are random-mating with migration from parental species (Acuapa, ACUA), and some are highly structured with two reproductively isolated parental-like forms (Aguazarca, AGZC) (Schumer, M., *et al*. 2017). I predict that pheromone signals will correspond tightly with genotype cluster morphology in these latter populations, but not in populations that mate randomly with respect to species ancestry. Further, I examine whether random mating in hybrid swarms is correlated between admixture and pheromone phenotypes by the expression of transgressive pheromone phenotypes in hybrids. By collecting samples of testis from each distinctive population, I can use a SPE/HPLC protocol to examine the signal structure in comparison to the genomic structure represented in each population. In populations that are now reproductively isolated in which hybrids have formed distinctive

genetic clusters, we would expect to see retention of majority-species pheromone blends. Hybrid swarms, by contrast, should exhibit either novel compounds, a mixture of the parental pheromones within individuals, or just one parental pheromone blend. Correlating hybrid population to pheromone phenotype sets the stage for mapping studies of the genetic basis of species-typical signal components and our understanding of how human pollutants affect the chemistry in pheromone signaling in *Xiphophorus*.

## *Materials and Methods*

### Collection

All subjects were adult *X. birchmanni* X *X. malinche* hybrid males collected using baited minnow traps from three independently formed hybrid zones in different river systems in Hidalgo, Mexico. In the Río Calnali, hybrid-cluster individuals (*N*=30, 15 *malinche*-cluster, 15-*birchmanni*-cluster) were collected from the Aguazarca stream reach. Admixed hybrid samples came from the Río Huazalingo (Acuapa) locality; *N*=13) and the Río Claro (Tlatemaco) locality; *N*=14).

### Dissection of testis

Males were euthanized using buffered MS-222 and kept on ice prior to dissection. Testis and duct were stored individually in 1ml of distilled water at -20°C.

### Male morphometric data

Wild-caught males (*N*=57), were lightly anesthetized with MS-222 and photographed for traditional morphometric measurements using the ImageJ program. Measurements were taken for standard length (SL), dorsal fin length (DL), gonopodium length (GL), body depth (BD), and sword extension length (SEL) in mm (Rosenthal, G.G., *et al*. 2003).

**Analysis**

We divided males into *birchmanni*-cluster and *malinche*-cluster by sword extension length; males with SEL over 0.1 mm were classified as having the *X. malinche* specific trait of significant sword development. A previous study by Schumer (2017) showed that SEL predicted male genetic cluster assignment with 95% accuracy.

**Pheromone SPE and HPLC-MS**

Purification and concentration of the male *Xiphophorus* pheromone was achieved using solid phase extraction (SPE). Testis tissue was suspended in 0.5 mL of distilled water (DI) and stored at -20°C prior to use. For extraction tissue was thawed at 23°C and 300 rpm on a mixing tray. Tissue and holding water was loaded into a C18 (Bond Elut-C18, 200mg, 3ml, Agilent Technologies, Wilmington, DE, USA) SPE column mounted on a vacuum manifold pressurized at 15 psi. The C18 column was prepped with 2mL MeOH followed by 2 mL DI water. Tissue was then loaded and washed with 1mL DI water. Tissue elution was performed with 25% MeOH and DI water, 65 MeOH and 100% MeOH. Each elute was captured in a separate test tube along the manifold. An internal standard of 1μg of the unconjugated bile acid chenodeoxycholic acid (CDCA; Steraloids Inc, Newport, RI, USA) was added to each of the eluates to standardize retention times and injection volumes between samples and to a blank sample of 35% DI water/65% MeOH (control). Eluates were dried under a stream of 99.9% pure nitrogen gas (Cal Gas Inc., Huntington Beach, CA, USA) reconstituted in 2 ml MeOH /water (60/40, v/v) and stored at -20°C till HPLC/MS. The LC column (Nova-Pak reversed-phase C18, 4 μm, Waters Chromatography Division, Milford, MA, USA) was coupled to a mass spectrometer with electrospray ionization (ESI; LCQ-DECA, Thermo Electron Corporation, Houston, TX, USA). The mobile phase was isocratic at 15% MeOH for 4 minutes, increased linearly from 15% to

100% MeOH from 4 to 31 minutes, and allowed to run at 100% MeOH for 5 minutes before increasing back to 15% MeOH for the next sample. Preliminary analysis of HPLC showed no new compounds after 30 minutes when run for a full hour. I then used a PE SCIEX QSTAR (Applied Biosystems, Foster City, CA, USA) to perform MS/MS using negative and positive-ion high-resolution electrospray ionization to identify peaks found in male testis. The ion trap was operated in the negative and subsequent positive ion mode with a spray voltage of 5 kV. A stream of 99% pure nitrogen at 60 psi was used as the sheath gas. Data were collected in the range m/z 250–950. The relative peak areas (normalized to the area of the internal standard, CDCA) in the HPLC fractions and were determined with Compass Data Analysis Viewer software (2014, Bruker Daltonik GmbH, Billerica, MA, USA).

## *Results*

### Pheromone SPE and HPLC-MS

HPLC analysis of male hybrid pheromones showed TLMC and ACUA had no relationship between male morphology and pheromone profile (Pearson Chi Square test, $p = 0.6780$). Alternatively, AGZC hybrids pheromone matched tightly with respect to morphology (Pearson Chi Square test, $p = 0.001$). However, pheromone structure correlated with population structure. AGZC *malinche*-cluster and TLMC showed greater *X. malinche* pheromone structure with AB being absent in the profile (ANOVA $F(3,57) = 7.5092$, $p = 0.0029$, Figure 9). The population at ACUA and the AGZC *birchmanni*-cluster had greater presence of *X. birchmanni* pheromone with AB and/or B only (ANOVA $F(3,57) = 5.7081$, $p = 0.0018$).

### PCA analysis and Male morphometric data

Approximately 51.3% of the total variance in population was predicted by principal component 1 with ACUA and the AGZC *birchmanni*-cluster clustering (PC1, Figure 10). This

component was largely influenced by SL, BD and DL, and peak B presence in pheromone structure. While TLMC and AGZC *malinche*-cluster males clustered separately (PC2, Figure 10), principal component 2 was largely influenced by SEL and absence of peak A or B. Interestingly, pheromone structure A+B- did not occupy similar principal component space with any population. Discriminant function analysis was used on pheromone components to differentiate between these populations and morphometric traits were overlaid to measure any covariance with the pheromone components. I found that the absence of B in pheromone structure distinguished the AGZC *malinche*-cluster from the *birchmanni*-cluster that could be explained by the presence of B (assignment accuracy of the discriminant function: 93%).

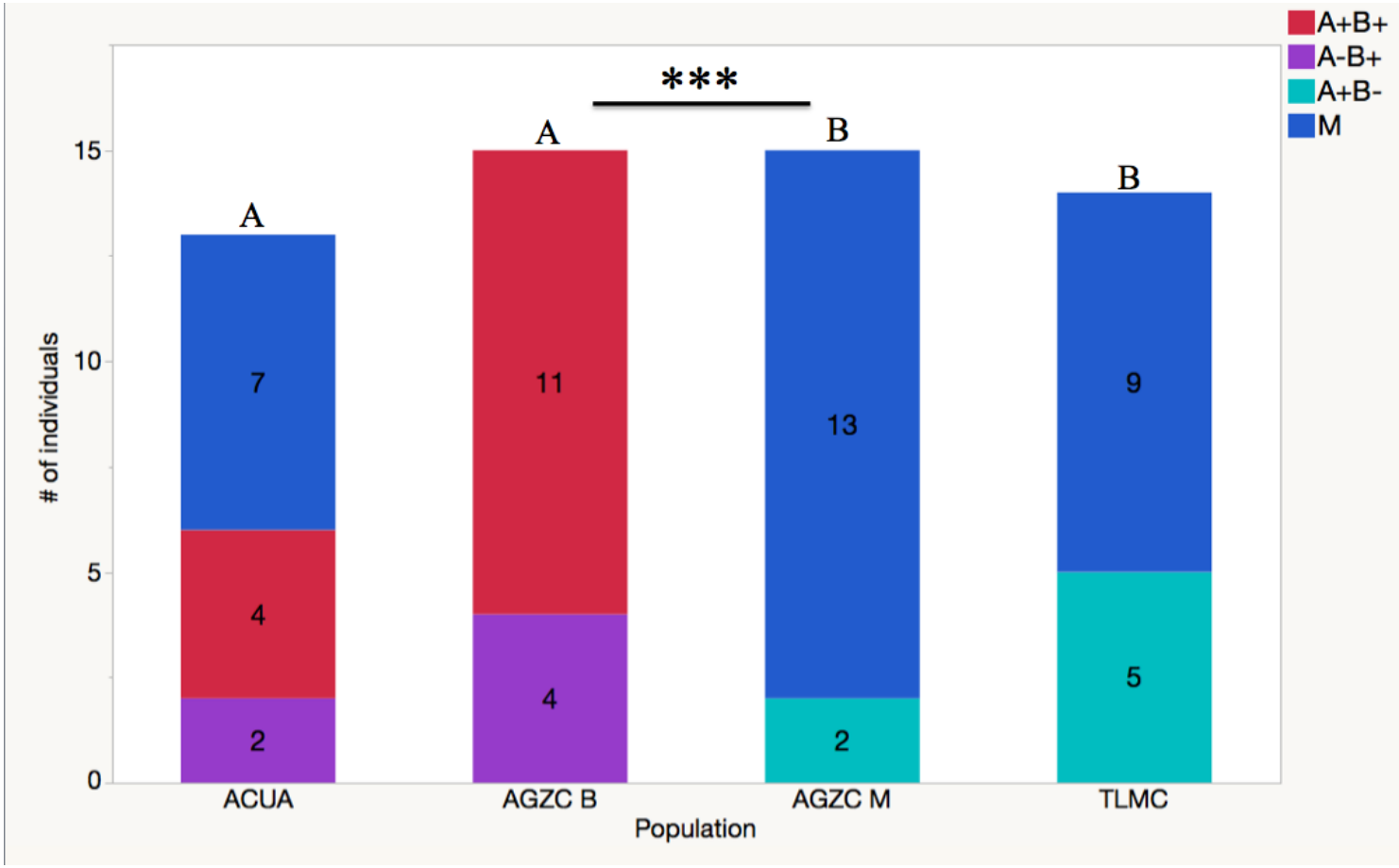


Figure 9. Pheromone profile distribution by population. No A+B+ males were present in the malinche skewed population, TLMC, and no A-B- males were found in the birchmanni skewed population, ACUA of the birchmanni morphology. AGZC malinche-cluster and TLMC showed greater malinche pheromone structure, AB being absent or A only in the profile (ANOVA $F_{(3,57)} = 7.5092$, $p = 0.0029$). ACUA and the AGZC birchmanni-cluster had greater presence of birchmanni pheromone with AB and/or B only (ANOVA $F_{(3,57)} = 5.7081$, $p = 0.0018$).

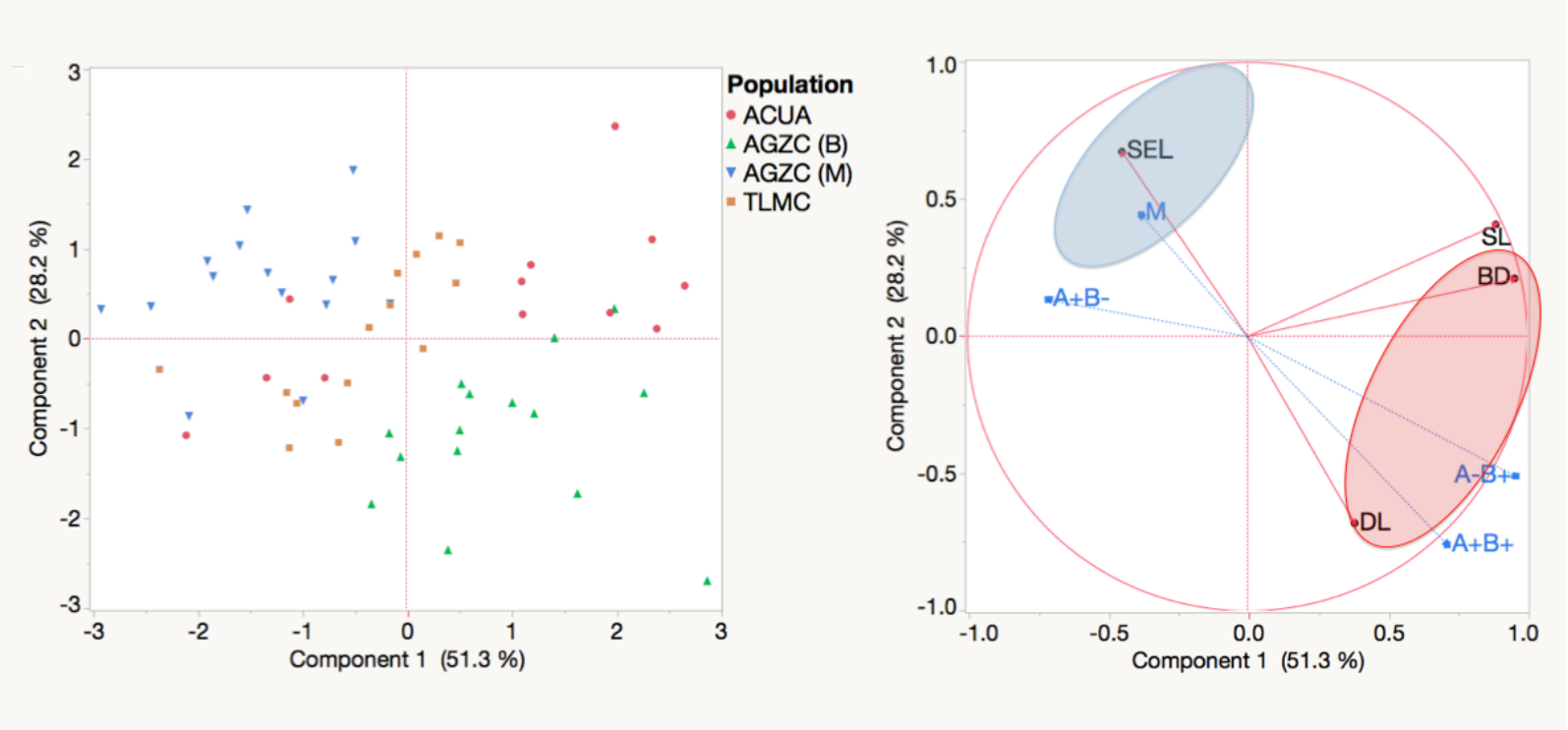


Figure 10**.** Principal components analysis of morphometric traits and pheromone components in hybrid males. ACUA and the AGZC *birchmanni*-cluster males cluster together (PC1, red). This component was largely influenced by SL, BD and DL, and peak B presence in pheromone structure. TLMC and AGZC *malinche*-cluster males clustered together (PC2, blue), principal component 2 was largely influenced by SEL and absence of peak A or B. Pheromone structure A+B- did not occupy similar principal component space with any population.

*Discussion*

I found that within the random mating populations of TLMC and ACUA, male hybrids showed a weak relationship with respect to morphology and pheromone structure. In the AGZC population, males of both clusters had a pheromone profile tightly correlated to their respective morphology. These different population structures are not explained by selection (Schumer, M., *et al*., 2017) and these differences found in pheromone structure may be indicative of hybrids in AGZC re-stabilizing after pulses of interruptions in intraspecific communication. Humic acid, a product of organic decomposition, has been shown to abolish reproductive isolation in the *Xiphophorus* system when human impact increases it beyond naturally occurring levels (Fisher, H.S *et al*. 2006). The effects of humic acid have been shown to have a 7-12 day period before reproductive isolation is recovered. If random mating persisted longer in TLMC and ACUA, then the admixing of male traits between hybrids could have led to the loss of species-specific pheromone structure seen in AGZC. This may be suggested by the high conservation of *X. birchmanni* pheromone components AB found in the AGZC *birchmanni*-cluster. The finding that the ACUA hybrid *birchmanni* males show a decreased level of AB in pheromone structure when compared with AGZC further highlights the unusual strength of pheromone/morphology relationship found at AGZC. Interestingly, the relationship of pheromone components with population structure was found in all three populations (Figure 9). The population at TLMC showed low variation in absence of either component, while ACUA showed a greater presence of *X. birchmanni* component B at the population level with a large percentage of individuals having either component, A or B. This absence of either peak in some individuals at ACUA may be a reflection of parental *X. malinche* migration previously shown in population genotyping. This is not expected based on the lack of a relationship within individual males. This finding

highlights the complex nature of hybrid zones and suggests multiple interactions are contributing to the different mating patterns seen at each population.

It had previously been shown that there is a strong relationship of genotype with male secondary traits in male *Xiphophorus* hybrids (Schumer, M., *et al*., 2017). Males with a large ancestry block of *X. malinche* express the SEL and reduced DL found in parental *X. malinche* males. Conversely, *X. birchmanni* dominate males show an increased DB and DL associated with *X. birchmanni* parental males. However, this is the first time pheromone structure was compared to male morphology. I found that SEL was correlated to the absence of AB in pheromone structure, both associated with parental *X. malinche* male traits. Additionally, DL was correlated with the presence of peak B associated with *X. birchmanni* parental males. In PCA analysis, ACUA and AGZC *birchmanni*-cluster were strongly correlated with the presence of B while TLMC and AGZC malinche-cluster grouped tightly with the absence of B peaks (Figure 10). Strikingly, peak A was not found to have a relationship with any population. This is consistent with the findings within individual males; peak A could be found in *malinche*-like males in random mating populations. This relationship between male traits and population may give insight into what could be a driving force in the AGZC populations. In a discriminant function analysis, peak B differed significantly between the two sub-populations at AGZC. Collectively these findings begin to scratch the surface of complicated interactions in hybrid zones. Specifically, I found that assortative mating at AGZC may be maintained by conservation of parental species pheromone structure and male traits are closely related to pheromone structure.

## *Conclusions*

Correlating hybrid morphology to pheromone phenotype sets the stage for genome associations leading to studies like QTL mapping of species-typical signal components, and furthering our understanding of how human pollutants affect the chemistry in pheromone signaling in *Xiphophorus*. I focused on interspecific differences between the sympatric *X. birchmanni* and *X. malinche,* which form natural hybrid zones as a consequence of changes in water chemistry. If pheromones play a role in reproductive isolation, pheromone profiles should map on to male genotype morphology. Natural hybrid zones vary from highly structured, with distinct, assortatively mating *birchmanni*-like and *malinche*-like subpopulations, to highly admixed hybrid swarms. Analysis of individual male pheromone profiles allows me to test the prediction that pheromones mediate assortative mating in structured hybrid populations. I found that pheromone profile is tightly related to morphology in assortative mating populations, while population structure is correlated with pheromone profile abundance at each distinct hybrid zone.

# CHAPTER V

# CONCLUSIONS

In vertebrates, chemical communication is important to reproductive isolation, but we know very little about the mechanisms underlying signal production and reception (Wyatt, T. D. 2014). In aquatic species, both signals and receivers are sensitive to water chemistry. This is important because perturbation of the chemical environment can interfere with species recognition and relax mating preferences, resulting in hybridization between sympatric species (Tomkins, P., *et al*. 2015). In the swordtail fish (genus *Xiphophorus*), conspecific mate recognition depends on pheromone signals (Wong, B.B.M., *et al*. 2005) but is abolished by high levels of dissolved organic compounds (Fisher, H.S., *et al*., 2006). The major goal of my project was to identify the location of pheromone production in male *Xiphophorus* and characterize interspecific differences in the chemistry of pheromone signals. Identifying the organ of pheromone production allowed me to concentrate the pheromone and quantifiably measure differences in males and their effects on female mate choice. By analyzing natural variation in male morphology, I directly measured changes in pheromone profile and corresponding female behavior. The ability to directly measure the pheromone product with paired responses of female conspecific mate recognition and mate preference related to male phenotype gave insight into what specific components are important to female mate choice. Quantifying pheromone chemistry enabled me to assess signal variation among species and among populations and to directly test the role of pheromones as mechanisms of reproductive isolation, furthering our understanding of the role communication plays in species maintenance in a vertebrate.

The main focus of my work in chapter II, elucidating the chemical composition of *Xiphophorus* signals, sheds light into how communication acts as a reproductive barrier between species and how its breakdown facilitates hybridization. Characterizing the chemical components of pheromone identity at the species level was a necessary first step in understanding the complexity of the signal, introgression of genes associated with pheromone production and how natural and anthropogenic variation in water chemistry interacts with receptors and pheromones. In chapter II, I identified the organ of pheromone production in *Xiphophorus* males, and provided insight into the possible chemical composition of pheromones. This enables future work to directly collect samples from males in the field. The ability to directly collect samples is necessary for concentration and purification steps needed for quantifiable chemical analysis.

Chapter III sought to answer how proximate forces influence mate choice in pheromone signaling. Male reproductive strategy is a major influence in female mate choice both in the *Xiphophorus* system and other well studied taxa, understanding how male phenotype co-varies with differences in chemosignals showed what role it has in olfactory signaling. Measuring the differences in pheromone profile of males and observing the changes in profile (e.g. change in signals associated with morphometric interactions) with male reproductive strategy type identified what in the profile females find attractive or aversive.

Lastly, chapter IV evaluated the relationship between pheromone profile and population structure. The variation in population structure of fully admixed hybrid zones with random mating as well as structured hybrid zones characterized by assortative mating provided me with a unique opportunity to examine how pheromone profile varies across and within populations. Using relative intensity and peak area in HPLC readouts I quantified relative abundance of pheromone components identified in Chapter II. I evaluated the correlation between pheromone

profile and genotype morphometrics across multiple populations and found consistent measurable introgression in hybrid males. This resulting relationship gave us insight into what possible mechanism caused the reproductive isolation to breakdown between the two species, how is it maintained or lost in different population structures and what the control of pheromone production between the two species may be affecting pheromone structure in individuals. These results further our understanding of how communication can breakdown or stabilize reproductive isolation and provided detailed insight into how communication mechanisms can cause hybridization.

# REFERENCES


Abbott, R., Albach, D., Ansell, S., Arntzen, J.W., Baird, S.J., Bierne, N., Boughman, J., Brelsford, A., Buerkle, C.A., Buggs, R., Butlin, R.K. (2013). Hybridization and speciation. *Journal of evolutionary biology*, 26(2), 229-246.

Andolfatto, P., Davison, D., Erezyilmaz, D., Hu, T. T., Mast, J., Sunayama-Morita, T., & Stern, D. L. (2011). Multiplexed shotgun genotyping for rapid and efficient genetic mapping. *Genome research*, gr-115402.

Aubin-Horth, N., & Dodson, J. J. (2004). Influence of individual body size and variable thresholds on the incidence of a sneaker male reproductive tactic in Atlantic salmon. *Evolution*, *58*(1), 136-144.

Barata, E. N., Fine, J. M., Hubbard, P. C., Almeida, O. G., Frade, P., Sorensen, P. W., & Canário, A. V. (2008). A sterol-like odorant in the urine of Mozambique tilapia males likely signals social dominance to females. *Journal of chemical ecology*, 34(4), 438-449.

Barton, N. H., & Hewitt, G. M. (1989). Adaptation, speciation and hybrid zones. *Nature*, 341(6242), 497–503.

Basolo, A. L. (1990). Female preference for male sword length in the green swordtail, *Xiphophorus helleri* (Pisces: Poeciliidae). *Animal Behaviour*, *40*(2), 332-338.

Borg, B. (1994). Androgens in teleost fishes. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology*, *109*(3), 219-245.

Borowsky, R., & Kallman, K. D. (1976). Patterns of mating in natural populations of *Xiphophorus* (Pisces: Poeciliidae). I: *X. maculatus* from Belize and Mexico. *Evolution*, 693-706.

Brennan, P.A., & Zufall, F. (2006). Pheromonal communication in vertebrates. *Nature*, 444(7117):308-315.

Burnard, D., Gozlan, R.E., & Griffiths, S.W. (2008). The role of pheromones in freshwater fishes. *Journal of Fish Biology* 73(1):1-16.

Candolin, U. (2014). The complexity of fish communication in human-disturbed environments: comment on Radford, *et al*. *Behavioral Ecology*, aru075.

Crapon de Caprona, M.D., & Ryan, M. J. (1990). Conspecific mate recognition in swordtails, *Xiphophorus nigrensis* and *X. pygmaeus* (Poeciliidae): olfactory and visual cues. *Animal Behaviour*, *39*(2), 290–296.

Culumber, Z. W., Fisher, H. S., Tobler, M., Mateos, M., Barber, P. H., Sorenson, M. D., & Rosenthal, G. G. (2011). Replicated hybrid zones of *Xiphophorus* swordtails along an elevational gradient. *Molecular ecology*, *20*(2), 342-356.

Culumber, Z. W., Ochoa, O. M., & Rosenthal, G. G. (2014). Assortative Mating and the Maintenance of Population Structure in a Natural Hybrid Zone. *The American Naturalist*, *184*(2), 225–232.

DeColo, S. L., Aspbury, A. S., Ostrand, K. G., & Gabor, C. R. (2016). Male-male interactions and their influence on the mating behavior and success in the fountain darter, *Etheostoma fonticola*. *Acta Ethologica*, 19(1), 15-20.

Dekker, T., Revadi, S., Mansourian, S., Ramasamy, S., Lebreton, S., Becher, P.G., Angeli, S., Rota-Stabelli, O., Anfora, G. (2015). Loss of *Drosophila* pheromone reverses its role in sexual communication in *Drosophila suzukii*. *Proceedings of the Royal Society of London B: Biological Sciences*, *282*(1804), 20143018.

Dobzhansky, T., & Dobzhansky, T. G. (1982). *Genetics and the Origin of Species* (Vol. 11). Columbia university press.

Evans, J. P., Pilastro, A., & Schlupp, I. (Eds.). (2011). *Ecology and evolution of poeciliid fishes*. University of Chicago Press.

Fisher, H. S., & Rosenthal, G. G. (2007). Male swordtails court with an audience in mind. *Biology Letters*, 3(1), 5-7.

Fisher, H. S., Mascuch, S. J., & Rosenthal, G. G. (2009). Multivariate male traits misalign with multivariate female preferences in the swordtail fish, *Xiphophorus birchmanni*. *Animal Behaviour*, 78(2), 265–269.

Fisher, H.S., B.B.M. Wong, and G.G. Rosenthal,.( 2006). Alteration of the chemical environment disrupts communication in a freshwater fish. *Proceedings of the Royal Society B-Biological Sciences*, 273(1591): p. 1187-1193.

Fujii, T., Yasukochi, Y., Rong, Y., Matsuo, T., & Ishikawa, Y. (2015). Multiple Δ11-desaturase genes selectively used for sex pheromone biosynthesis are conserved in *Ostrinia* moth genomes. *Insect biochemistry and molecular biology*, 61, 62-68.

García-Roa, R., Cabido, C., López, P., & Martín, J. (2016). Interspecific differences in chemical composition of femoral gland secretions between two closely related wall lizard species, *Podarcis bocagei* and *Podarcis carbonelli*. *Biochemical Systematics and Ecology*, 64, 105-110.

Gordon, M. (1953). The ecological niche of the pygmy swordtail, *Xiphophorus pygmaeus*, in the Rio Axtla, Mexico. *Copeia*, 148-150.

Gross, M. R. (1984). Sunfish, salmon, and the evolution of alternative reproductive strategies and tactics in fishes. *Fish reproduction: Strategies and tactics*, 55-75.

Hankison, S. J., & Morris, M. R. (2002). Sexual selection and species recognition in the pygmy swordtail, *Xiphophorus pygmaeus*: conflicting preferences. *Behavioral Ecology and Sociobiology*, *51*(2), 140-145.

Hesse, S., Bakker, T. C., Baldauf, S. A., & Thünken, T. (2016). Impact of social environment on inter-and intrasexual selection in a cichlid fish with mutual mate choice. *Animal Behaviour*, 111, 85-92.

Irvine, I. A. S., & Sorensen, P. W. (1993). Acute olfactory sensitivity of wild common carp, *Cyprinus carpio*, to goldfish hormonal sex pheromones is influenced by gonadal maturity. *Canadian Journal of Zoology*, 71(11), 2199-2210.

Jones, G. R., & Oldham, N. J. (1999). Pheromone analysis using capillary gas chromatographic techniques. *Journal of Chromatography A*, *843*(1-2), 199-236.

Kindsvater, H. K., Simpson, S. E., Rosenthal, G. G., & Alonzo, S. H. (2013). Male diet, female experience, and female size influence maternal investment in swordtails. *Behavioral Ecology*, *24*(3), 691–697.

Kobelkowsky, A., & Armando,. A. G. (2000). Anatomía sexual de *Gambusia regani* (Pisces: Poeciliidae). *Anales del Instituto de Biología serie Zoología*, 71(002).

Leary, G. P., Allen, J. E., Bunger, P. L., Luginbill, J. B., Linn, C. E., Macallister, I. E., Kavanaugh, M.P., & Wanner, K. W. (2012). Single mutation to a sex pheromone receptor provides adaptive specificity between closely related moth species. *Proceedings of the National Academy of Sciences*, *109*(35), 14081-14086.

Lebreton, S., Trona, F., Borrero-Echeverry, F., Bilz, F., Grabe, V., Becher, P. G., & Witzgall, P. (2015). Feeding regulates sex pheromone attraction and courtship in *Drosophila* females. *Scientific reports*, *5*, 13132.

Liénard, M. A., Strandh, M., Hedenström, E., Johansson, T., & Löfstedt, C. (2008). Key biosynthetic gene subfamily recruited for pheromone production prior to the extensive radiation of *Lepidoptera*. *BMC evolutionary biology*, *8*(1), 270.

Linn, C. E. & Roelofs., W. L. (1995). Pheromone communication in moths and its role in the speciation process. *In Speciation and the Recognition Concept: Theory and Application,* 263-300.

Löfstedt, C. (1990). Population variation and genetic control of pheromone communication systems in moths. *Entomologia Experimentalis et Applicata*, 54(3), 199-218.

Mitchell, R. F., Reagel, P. F., Wong, J. C., Meier, L. R., Silva, W. D., Mongold-Diers, J., & Hanks, L. M. (2015). *Cerambycid* beetle species with similar pheromones are segregated by phenology and minor pheromone components. *Journal of chemical ecology*, 41(5), 431-440.

Morgado-Santos, M., Pereira, H. M., Vicente, L., & Collares-Pereira, M. J. (2015). Mate choice drives evolutionary stability in a hybrid complex. *PloS one*, *10*(7), e0132760.

Morris, M. R., Batra, P., & Ryan, M. J. (1992). Male-male competition and access to females in the swordtail *Xiphophorus nigrensis*. *Copeia*, 980-986.

Morris, M. R., Friebertshauser, R. J., Rios-Cardenas, O., Liotta, M. N., & Abbott, J. K. (2016). The potential for disruptive selection on growth rates across genetically influenced alternative reproductive tactics. *Evolutionary Ecology*, *30*(3), 519-533.

Norman, M. D., Finn, J., & Tregenza, T. (1999). Female impersonation as an alternative reproductive strategy in giant cuttlefish. *Proceedings of the Royal Society of London B: Biological Sciences*, *266*(1426), 1347-1349.

Oliveira, R. F., Canario, A. V., & Grober, M. S. (2001). Male sexual polymorphism, alternative reproductive tactics, and androgens in combtooth blennies (Pisces: Blenniidae). *Hormones and behavior*, *40*(2), 266-275.

Palmer, C. A., & Houck, L. D. (2005). Responses to sex-and species-specific chemical signals in allopatric and sympatric salamander species. In *Chemical signals in vertebrates 10*(pp. 32-41). Springer, Boston, MA.

Partan, S., & Marler, P. (1999). Communication goes multimodal. *Science*, *283*(5406), 1272-1273.

Partridge, C. G., MacManes, M. D., Knapp, R., & Neff, B. D. (2016). Brain Transcriptional Profiles of Male Alternative Reproductive Tactics and Females in Bluegill Sunfish. *PloS one*, *11*(12), e0167509.

Pascoal, S., Mendrok, M., Mitchell, C., Wilson, A. J., Hunt, J., & Bailey, N. W. (2016). Sexual selection and population divergence I: The influence of socially flexible cuticular hydrocarbon expression in male field crickets (*Teleogryllus oceanicus*). *Evolution*, *70*(1), 82-97.

Rasotto, M. B., & Mazzoldi, C. (2002). Male traits associated with alternative reproductive tactics in *Gobius niger*. *Journal of fish biology*, *61*(1), 173-184.

Reding, L., & Cummings, M. E. (2015). Does sensory expansion benefit asexual species? An olfactory discrimination test in Amazon mollies. *Behavioral Ecology*, *27*(2), 411-418.

Reichard, M. (2016). Male–Male Strategies. *Encyclopedia of Evolutionary Psychological Science*, 1-7.

Rolen, S. H., Sorensen, P. W., Mattson, D., & Caprio, J. (2003). Polyamines as olfactory stimuli in the goldfish *Carassius auratus*. *Journal of Experimental Biology*, 206(10), 1683-1696.

Rollmann, S. M., Houck, L. D., & Feldhoff, R. C. (2003). Conspecific and heterospecific pheromone effects on female receptivity. *Animal Behaviour*, 66(5), 857-861.

Rosen, D. E. (1960). Middle-American poeciliid fishes of the genus *Xiphophorus* (Vol. 5). University of Florida.

Rosenthal, G. G. (2013). Individual mating decisions and hybridization. *Journal of Evolutionary Biology*, *26*(2), 252–255.

Rosenthal, G. G., & De León, F. J. G. (2006). Sexual behavior, genes, and evolution in *Xiphophorus*. *Zebrafish*, *3*(1), 85-90.

Rosenthal, G. G., & De León, F. J. G. (2011). Speciation and hybridization. Ecology and evolution of poeciliid fishes., Evans, J.P., Pilastro, A., & Schlupp, I., ed. University of Chicago Press. 109-119.

Rosenthal, G. G., de la Rosa Reyna, X. F., Kazianis, S., Stephens, M. J., Morizot, D. C., Ryan, M. J., & García de León, F. J. (2003). Dissolution of sexual signal complexes in a hybrid zone between the swordtails *Xiphophorus birchmanni* and *Xiphophorus malinche* (Poeciliidae). *Copeia*, *2003*(2), 299-307.

Rosenthal, G. G., Evans, C. S., & Miller, W. L. (1996). Female preference for dynamic traits in the green swordtail, *Xiphophorus helleri*. *Animal Behaviour*, *51*(4), 811-820.

Rosenthal, G.G., & Lobel, P. (2006). Communication. In Behaviour and Physiology of Fish (vol. 24), Sloman, K., Balshine, S., Wilson R., eds. (Academic Press), 39-78.

Rosenthal, G.G., Fitzsimmons, J.N., Woods. K.U., Gerlach. G., Fisher H.S. (2011). Tactical Release of a Sexually-Selected Pheromone in a Swordtail Fish. PLoS ONE 6(2): e16994. doi:10.1371/journal.pone.0016994

Ruchon, F., Laugier, T., & Quignard, J. P. (1995). Alternative male reproductive strategies in the peacock blenny. *Journal of Fish Biology*, *47*(5), 826-840.

Ryan, M. J., Pease, C. M., & Morris, M. R. (1992). A genetic polymorphism in the swordtail *Xiphophorus nigrensis*: testing the prediction of equal fitnesses. *The American Naturalist*, *139*(1), 21-31.

Sandkam, B. A., Joy, J. B., Watson, C. T., Gonzalez-Bendiksen, P., Gabor, C. R., & Breden, F. (2013). Hybridization leads to sensory repertoire expansion in a gynogenetic fish, the Amazon molly (*Poecilia formosa*): a test of the hybrid-sensory expansion hypothesis. *Evolution*, 67(1), 120-130.

Schartl, A., Schartl, M., & Anders, F. (1982). Promotion and regression of neoplasia by testosterone-promoted cell differentiation in *Xiphophorus* and *Girardinus*. *Carcinogenesis; a comprehensive survey*, *7*, 427.

Schreibman, M. P., Berkowitz, E. J., van den Hurk, R. (1982). Histology and histochemistry of the testis and ovary of the platyfish, *Xiphophorus maculatus*, from birth to sexual maturity. *Cell and Tissue Research*, 224(1), 781-787.

Schumer, M., Powell, D. L., Delclós, P. J., Squire, M., Cui, R., Andolfatto, P., & Rosenthal, G. G. (2017). Assortative mating and persistent reproductive isolation inhybrids. *Proceedings of the National Academy of Sciences*, 201711238.

Serrano, R. M., Lopes, O., Hubbard, P. C., Araújo, J., Canário, A. V., & Barata, E. N. (2008). 11-Ketotestosterone stimulates putative sex pheromone production in the male peacock blenny, *Salaria pavo* (Risso 1810). *Biology of reproduction*, *79*(5), 861-868.

Sinervo, B., & Lively, C. M. (1996). The rock-paper-scissors game and the evolution of alternative male strategies. *Nature*, *380*(6571), 240.

Smadja, C., & Butlin, R. K. (2009). On the scent of speciation: the chemosensory system and its role in premating isolation. *Heredity*, 102(1), 77-97.

Sorensen, P. W., & Stacey, N. E. (2008). Hormonally Derived Sex Pheromones in Fish. In *Fish Reproduction* (pp. 215-258). CRC Press.

Sorensen, P. W., Christensen, T. A., & Stacey, N. E. (1998). Discrimination of pheromonal cues in fish: emerging parallels with insects. *Current Opinion in Neurobiology*, 8(4), 458-467.

Sorensen, P. W., Fine, J. M., Dvornikovs, V., Jeffrey, C. S., Shao, F., Wang, J., Vrieze, L.A., Anderson, K.R., & Hoye, T. R. (2005). Mixture of new sulfated steroids functions as a migratory pheromone in the sea lamprey. *Nature Chemical Biology*, *1*(6), nchembio739.

Sorensen, P. W., Hara, T. J., Stacey, N. E., & Dulka, J. G. (1990). Extreme olfactory specificity of male goldfish to the preovulatory steroidal pheromone 17α, 20β-dihydroxy-4-pregnen-3-one. *Journal of Comparative Physiology A*, *166*(3), 373-383.

Stewart, M., Baker, C. F., & Sorensen, P. W. (2013). Chemical analysis of aquatic pheromones in fish. In *Pheromone Signaling* (pp. 55-69). Humana Press, Totowa, NJ.

Taborsky, M. (1998). Sperm competition in fish: bourgeois' males and parasitic spawning. *Trends in Ecology & Evolution*, *13*(6), 222-227.

Tomkins, P., Saaristo, M., Allinson, M., & Wong, B. B. (2016). Exposure to an agricultural contaminant, 17β-trenbolone, impairs female mate choice in a freshwater fish. *Aquatic Toxicology*, *170*, 365-370.

Van den Hurk, R., & Resink, J. W. (1992). Male reproductive system as sex pheromone producer in teleost fish. *Journal of Experimental Zoology*, *261*(2), 204-213.

Wang, H. L., Zhao, C. H., & Wang, C. Z. (2005). Comparative study of sex pheromone composition and biosynthesis in *Helicoverpa armigera*, *H. assulta* and their hybrid. *Insect*

*biochemistry and molecular biology*, 35(6), 575-583.

Wong, B.B.M., Fisher H.S. & Rosenthal, G.G. (2005). Species recognition by male swordtails via chemical cues. *Behavioral Ecology 16(4), 818-822.*

Wyatt, T. D. (2010). Pheromones and signature mixtures: defining species-wide signals and variable cues for identity in both invertebrates and vertebrates. *Journal of Comparative Physiology A*, *196*(10), 685-700.

Wyatt, T. D. (2014). *Pheromones and animal behavior: chemical signals and signatures*. Cambridge University Press.

Wyman, M. T., Locatelli, Y., Charlton, B. D., & Reby, D. (2016). Female sexual preferences toward conspecific and hybrid male mating calls in two species of polygynous deer, *Cervus elaphus* and *C. nippon*. *Evolutionary biology*, *43*(2), 227-241.

Xue, B., Rooney, A. P., Kajikawa, M., Okada, N., & Roelofs, W. L. (2007). Novel sex pheromone desaturases in the genomes of corn borers generated through gene duplication and retroposon fusion. *Proceedings of the National Academy of Sciences*, 104(11), 4467-4472.

Yang, C. Y., Kim, S. J., Kim, J., Kang, T. J., & Ahn, S. J. (2015). Sex pheromones and reproductive isolation in five mirid species. *PloS one*, *10*(5), e0127051.