MATE CHOICE AND MULTIPLE PATERNITY IN THE *XIPHOPHORUS MALINCHE/X. BIRCHMANNI* HYBRID SYSTEM

A Thesis

by

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ABSTRACT

Hybridization represents a collision of genomes that can introduce new genetic and phenotypic variation into a population. Depending on the environment, this may lead to increased individual fitness and allow for integration of novel gene combinations via gene flow between divergent species. Recent work has shown that hybridization is an important evolutionary process in terms of the diversification of species and that it is probably far more common than once thought. To further understand the process of hybridization, studies examining mating decisions can be used to predict not only how hybridization occurs in the first place but also to predict the future evolutionary path of parental and hybrid populations. Here I present two studies on *Xiphophorus malinche*, *X. birchmanni*, and their hybrids. In the first, I examine the chemical and visual preferences of male *X. malinche* with dichotomous choice trials; I found that, unlike females or male *X. birchmanni*, male *X. malinche* show no strong preferences in terms of chemical or visual cues. In my second study, I used microsatellite markers to determine that there is a high degree of polyandry in a subpopulation of an *X. malinche* and *X. birchmanni* hybrid zone after first investigating population structure.

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CHAPTER I

INTRODUCTION TO MATE CHOICE IN HYBRID ZONES AND THE *X. MALINCHE* X *X. BIRCHMANNI* HYBRID ZONE SYSTEM

Introduction

Hybridization represents a coming together of genomes that can introduce new genetic and phenotypic variation into a population. Depending on the environment, this may lead to increased individual fitness and allow for integration of novel gene combinations via gene flow between divergent species (Barton 2008; Abbott et al. 2013; Cui et al. 2013; Paczolt et al. 2014). Recent work also shows that hybridization is an important evolutionary process in terms of the diversification of species and that it is probably far more common than once thought (Rieseberg 2003; Seehausen 2004; Cui et al. 2013).

Hybridization is dynamic depending on the species and ecological circumstances involved, and its consequences can range from production of sterile individuals in the population (Dobzhansky 1935), to gene flow (Cui et al. 2013; Hailer 2015), to hybrid speciation (Mallet 2007). How and if hybridization occurs, and what happens after initial hybridization events, is partially dependent on the mating decisions of the individuals involved (Rosenthal 2013). Mate choice may get more complicated when two recently diverged populations with fragile reproductive barriers meet again in sympatry. New trait phenotypes to choose from—both in terms of the other parental species and in terms of transgressive phenotypes that result from hybridization—and

new preference phenotypes to do the choosing can lead to a wide variety of outcomes within hybrid systems.

Most studies in hybrid zones thus far have examined the mating decisions of the parental species involved in hybrid systems. Here, what happens in hybrid zones can depend on who mates with whom (Are one or both parental species permissive?) and the fitness costs of hybridizing (Are costs low enough that hybrid individuals can stay in the population to mate with each other or backcross with parentals? Or are costs high enough so that hybrid genotypes diminish and reinforcement of parental genotypes/phenotypes occur?). In what appears to be the vast majority of cases, asymmetrical mating decisions and/or fitness consequences drive the patterns we see within hybrid zones. Focusing just on mate choice, asymmetric hybridization (followed closely by asymmetric introgression of certain traits/preferences) occurs when the female of one species mates more often with the male of the other (Wirtz 1999). This may occur because the female prefers the secondary sex characteristics of heterospecifics to conspecifics (Parsons et al. 1993) or because males of one species subvert female choice by indiscriminate coercive matings (Gröning and Hochkirch 2008).

When two closely related species meet in sympatry, females of one species may find certain heterospecific male traits (or lack thereof) more appealing than those of conspecifics. These new males may be appealing because they represent hidden preferences (Arak and Enquist 1993), a better choice given environmental factors (Pearson 2000; Willis et al. 2012), or simply because of their novelty (Hughes et al.

1999). Examples include: female white collared manakins (*Manacus candei*) preferring the bright golden plumes of the golden manakin (*M. vitellinus*) over the white collars of conspecifics (Stein and Uy 2005); and female hermit warblers (*Dendroica occidentalis*) that choose to mate with Townsend warbler (*D. townsendi*) males, as these males are better at holding territories than hermit males (Pearson 2000).

Males can also be the driver of asymmetric matings, but usually because of their indifference to female identity. Theoretically, a male should take advantage of any mating opportunity presented in order to increase his fitness (Bateman 1948), even if that mating occurs with a heterospecific. Appropriately named "the satyr effect" after a mythological half-goat, half-man who mates with anything, in hybrid zones, males of one species may subvert heterospecific females' preferences for conspecifics by coercively mating with said female (Ribeiro and Spielman 1986; Gröning and Hochkirch 2008). This effect has been described in hybridizing damselflies where males may get many mating opportunities, but females may only get one or two (Ribeiro and Spielman 1986) and in some species of leaf-hoppers, where not only does male reproductive interference behavior lead to hybridization, but also to more general competitive exclusion (Hochkirch et al. 2007).

Once hybridization has occurred, transgressive traits and transgressive preference types are found in hybrid zones. Traits used to attract mates may be intermediate between parental forms (Harrison and Bogdanowicz 1997), may resemble one parental form or the other because of asymmetrical mating/introgression (Stein and Uy 2005), or hybrids may display novel trait combinations not found in the parents

(Rosenthal et al. 2003). How fit any of these trait phenotypes are depends on the mating preferences of potential mates. Much less is known about hybrid preferences, but like trait phenotypes, preference phenotypes also probably run the gamut of being more or less like one of the parental species involved, to something in between. There is also the possibility that hybrids may prefer something completely novel.

With the potential of transgressive phenotypes and two parental genotypes complicating mate choice, fitness costs are potentially everywhere: one could mate with the "wrong" type of individual and waste time producing less fit offspring; on the other hand, one could waste time and potential mating opportunities by narrowing the range of acceptable mates too much (Rosenthal 2013). In a hybrid zone, then, there are potential trade-offs between mating more and mating less. Promiscuity is predicted to evolve in populations where genetic incompatibilities may be an issue: for instance, by mating multiply, a female can insure that at least one of her mates will have sperm that are compatible with her eggs (Colegrave et al. 2002). Of course, if there are strong enough reinforcement mechanisms in place (or if they eventually evolve given how much fitness is affected), individuals may mate less.

The extent of multiple mating is important both within and outside of hybrid zones. Multiple mating, where multiple sires (or dams in some cases) contribute to one brood or clutch, has ramifications for numerous evolutionary processes (Andersson 1994). In particular, the degree of multiple mating can affect the strength and direction of sexual selection (Shuster and Wade 2003, Clutton-Brock and Vincent 1991). Generally, the more sires represented in one brood, the weaker sexual selection is

(Kvarnemo and Simmons 2013). This is because more sires usually equals more variation in traits sampled, which means that more trait values will be represented in the brood, and thus no one trait value is "selected" over the others. This can have important implications for how and what traits may introgress through hybrid zones.

To begin understanding how the above processes come together, we need studies detailing mating decisions to better predict their outcomes in hybrid zones. Here, I propose to add to a body of knowledge on the naturally hybridizing swordtails, *Xiphophorus malinche* and *X. birchmanni,* by completing a set of studies on parental species preferences and by investigating multiple paternity in a hybrid zone. These two fish produce viable hybrid offspring and form seven replicated hybrid zones displaying key differences in population structure in the wild (Culumber et al. 2011). That, coupled with their life history characteristics and the growing body of knowledge on preferences and traits important in precopulatory mate choice in these fish, makes this system ideal for studying the roles of mate choice and differential fertilization in hybridizing species.

The *X. birchmanni/X. malinche* System

X. birchmanni and *X. malinche* form at least seven hybrid zones in the Sierra Madre Oriental of eastern Mexico(Rosenthal et al. 2003; Culumber et al. 2011). These hybrid zones likely formed due to anthropogenic disturbance in the form of organic chemical pollution (Fisher et al. 2006; Culumber et al. 2011). Wild adult hybrids of these two species are viable, and there is no evidence of contemporary selection against hybrids (Culumber et al. 2011), although there may be some genetic incompatibilities

between the two (Schumer et al. 2014). *Xiphophorus* are live-bearing fish that, like other poeciliids, may store sperm from multiple males within their reproductive tract for up to 10 months (Potter and Kramer, 2000). Generally, female poeciliids are also highly promiscuous (Simmons et al. 2008; Evans and Magurran 2000) and these two species are probably no exception. Indeed, broods from female *X. birchmanni* are shared by 2.37 sires on average (Paczolt et al. 2014).

There is also a wealth of genetic and molecular tools available for studying *Xiphophorus*. Members of this genus have served as a model system for cancer research for decades (Walter and Kazianis 2001; Fernandez et al. 2012) and this system in particular is becoming a model for responses to hybridization in vertebrate animals (Schumer et al. 2012; Cui et al. 2013). Additionally, Fish and genomic tools are readily available through the *Xiphophorus* Genetic Stock Center in San Marcos, TX, and the Rosenthal and Andolfatto labs have recently generated pseudogenomes for both *X. malinche* and *X. birchmanni* using the fully sequenced *X. maculatus* genome (Schartl et al. 2013) as a reference (Schumer et al. 2014).

Females in these species base mating decisions on both chemical and visual cues. In general, females of both species prefer conspecific chemical cues (Rosenthal and Ryan 2011); however, these preferences may be reversed as a result of learning through ontogeny (Verzijden et al. 2012), Cui 2014), or abolished as a result of environmental or social stressors (Fisher et al. 2006; Willis et al. 2012). When exposed to just visual cues, both *X. malinche* and *X. birchmanni* prefer male *X. birchmanni* cues (Fisher et al. 2009; Cui 2014). The story becomes even more complicated when females

are exposed to computer generated visual signals: both of these species prefer males with larger apparent body size paired with smaller dorsal fins and swords (Wong and Rosenthal 2006). Thus, because fin size increases allometrically with body size, females have strong preferences for males where these traits are decoupled (Fisher et al. 2009). This sort of phenotype is not seen in either parental species, which could lead to certain hybrid males being more globally attractive visually (Culumber et al. 2014).

When it comes to males, we only know part of the picture: *X. birchmanni* prefer the chemical cues of heterospecifics and have no preferences for visual cues from either female(Wong, Fisher, and Rosenthal 2005). *We do not yet know the chemical or visual preferences of* X. malinche. In **Chapter II**, I will test if *X. malinche* males have preferences for, or are indifferent to, the chemical and visual cues of hetero- and conspecific females.

What we do know about precopulatory preferences suggests that *X. birchmanni* males and *X. malinche* females may preferentially mate with one another in areas of species overlap, leading to hybrid swarms. However, this is not what we see in some populations, which instead display a bimodal population structure characterized by hybrids that fall into two genetic clusters: either more *X. birchmanni*-like or more *X. malinche*-like (Culumber et al. 2011, 2014). In **Chapter III**, I will examine multiple paternity, reproductive skew, and confirm population structure in a sample from this particular hybrid population.

CHAPTER II

CHEMICAL AND VISUAL CUE PREFERENCES IN MALE SWORDTAILS, *XIPHOPHORUS MALINCHE*

Introduction

Within the mate choice and sexual selection literature, males are not usually regarded as active choosers when it comes to mating; however their mating preferences, or lack thereof, can be extremely important in the natural history and evolution of a species (Trivers 1972; Emlen and Oring 1977; Edward and Chapman 2011). This may be especially true within hybrid zones, where males may help reinforce mating barriers alongside choosy females or where males might aid in the process of hybridization with their indifference.

Male choice has a wide variety of consequences in instances where recently diverged organisms suddenly find themselves living in sympatry. For example, in the hybridizing leaf beetles, *Chrysochus cobaltinus* and *C. auratus,* sexual isolation has increased because of strong selection against hybrids. Here, female choice is augmented by the chemical preferences of males: in both species, males use cuticular hydrocarbons to preferentially mate with conspecifics (Peterson et al. 2007). Alternatively, in the hybrid zone between white-collared (*Manacus candei*) and golden-collared (*M. vitellinus)* manakins, male indifference coupled with *M. candei* females' preference for the brighter neck plumes of male *M. vitellinus,* has led to asymmetric hybridization. This in turn has lead to introgression of a sexual signal, the golden collar trait, well into

M. candei's range (Parsons, Olson, and Braun 1993). Males that mate indiscriminately can also affect heterospecifics through reproductive interference: by hybridizing and/or limiting heterospecific females' opportunities to mate, these indifferent males can severely affect the fitness of heterospecifics. This phenomenon, termed the "satyr effect", has been found to lead not only to the formation of unfit hybrids, but also to more general competitive exclusion between two species of ground-hopper (*Tetrix* species). Aggressive and indifferent mating by *T. subulata* males leaves *T. ceperoi* females unable to mate with conspecifics and limits *T. ceperoi*'s ability to compete with *T. subulata* in areas of sympatry (Hochkirch et al. 2007). Thus, whether or not males exercise strong preferences can have important implications for hybrid systems and the parental species involved.

In *Xiphophorus*, males are showy, in terms of coloration (Kingston et al. 2003), physical display (Fisher and Rosenthal 2007), and chemical attractants (Rosenthal et al. 2011). This may suggest that they are just courters and may not exercise much choice, as their energies would be better spent trying to court with and mate with as many females as possible (Emlen and Oring 1977). To even have mating opportunities, males may also have to compete with other males, either through direct physical combat or through display (Fisher and Rosenthal 2007). Advertising to females and competition with other males may have quite significant energy costs (Head et al. 2010; Edward and Chapman 2011). Thus, it may behoove males to be choosier and court only certain females based on perceived reproductive benefit (Herdman et al. 2004; Servedio 2007). This may be especially true in hybrid zones where having hybrid offspring could

negatively impact an individual's fitness. Given that there are genetic incompatibilities between *X. birchmanni* and *X. malinche*, this could be a possibility (Schumer et al. 2014). Here then, there is the potential for male species recognition and subsequent mate choice to reinforce female choosiness (Servedio 2007).

In this study, I examine the chemical and visual preferences of *X. malinche* males in particular. Previous studies have shown that *X. birchmanni* males display a complex set of preferences: while they are indifferent to visual cues, *X. birchmanni* males prefer *X. malinche* female chemical cues compared to those of conspecifics (Wong et al. 2005). *X. malinche* may follow in this pattern, prefer conspecifics as females do, or be indifferent.

Methods

Animal Collection

Focal males (N=32, SL = 50.125 mm +/- SD = 3.87 mm) were collected from a pure *X. malinche* site, Chicayotla (Arroyo Xontla, 1000 m; Culumber et al. 2011) in October of 2014. *X. malinche* females used to generate chemical and visual cues were collected from this site (N=8) in December 2013; *X. birchmanni* females (N=8) used for cue generation were collected from the pure *X. birchmanni* site, Garces (Rio Garces, 244 m; Culumber et al. 2011), also in December of 2013.

Chemical Cue Preference Trials

Behavioral trials followed the protocol as in Wong et al. 2005. Briefly, first chemical cue was generated; for each respective cue, two 20 L tanks were placed side by side so fish in either tank could see each other and interact through glass, but not smell each other or interact physically. Four males (non-focal males) were placed in one tank and four conspecific females in the other. The fish were allowed to swim freely and interact for 4 hours. Cue is contained in the urine (Rosenthal et al. 2011), so after this time period, 3-5 L of water (containing the urine/cue) was collected from the female tanks for use in the trials (Figure 2.1).

Trials consisted of dichotomous choice tests (Wong et al. 2005). Trial lanes are divided into three equal sections: two association zones on either end of the trial lane, with a neutral zone in between (Figure 2.1). After a 20-minute acclimation period (acclimation period is extended for *X. malinche* based on preliminary studies, Squire and Rosenthal, unpublished data), chemical cue was dripped into the respective association zones via an automated pump system. Overhead tracking cameras and software allowed us to watch trials remotely and track males' association time with each cue. Each male was tried twice to account for potential side bias. Trials lasted 10 minutes; if the male did not explore both cues or failed to move in the first 5 minutes, he was deemed unresponsive.

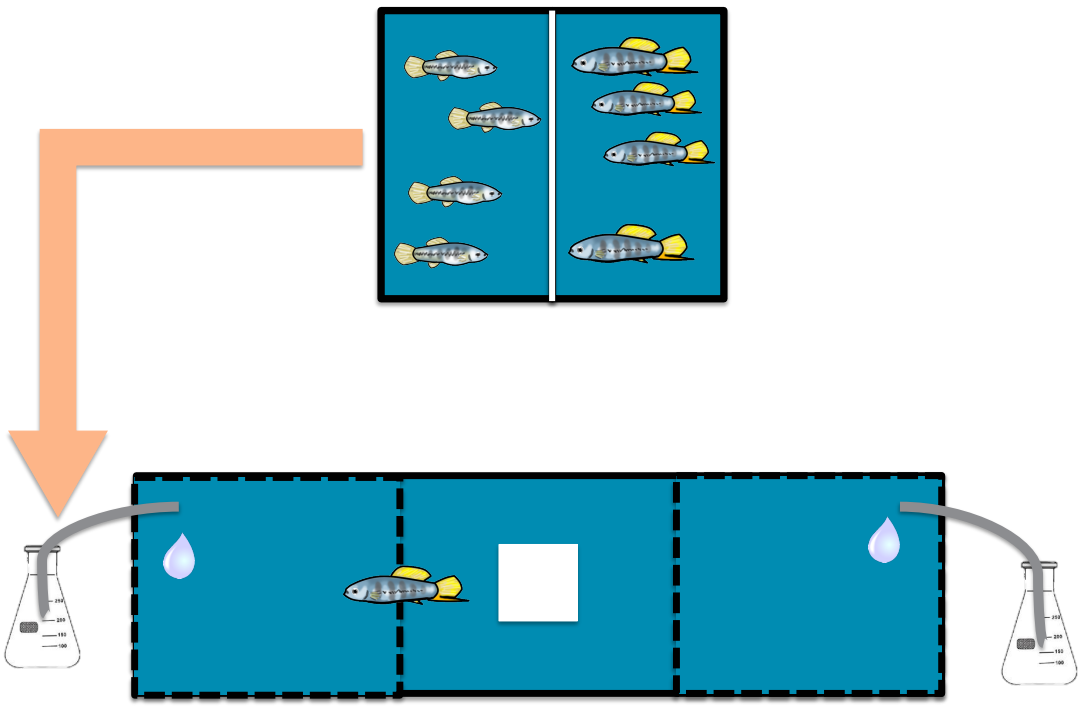

Figure 2.1: Chemical cue collection and dichotomous choice trial set up. Cue is collected from females (see text) and dripped into the association zones (dashed lines) of the trial lane.

Our chemical cue trials consisted of both a positive control (*X. malinche* female chemical cue vs. water) and experimental (*X. malinche* vs. *X. birchmanni* female cue) trials. After trials were completed, a *t*-test was conducted in R (R Core Team 2013) to determine if males associated more with either conspecifics or heterospecifics.

Visual Cue Preference Trials

The protocol for visual trials is identical to that of chemical trials, except for the nature of cue presentation. Here, males were presented with either one tank containing two live *X. malinche* females and an empty tank, or two tanks containing two conspecific or heterospecific females, respectively, placed at either end of the trial lanes.

In this way, males could see and interact with females through the glass, but could not smell the females (Figure 2.2). During the 20-minute acclimation period, an opaque divider was placed between the visual cue tank and the male's trial tank. At the start of the 10-minute trial, this divider was removed. Again, if the male failed to interact with both cues or did not move in the first 5 minutes of the trial, the trial was terminated. Association time with each cue was recorded by the overhead tracking system and these data were analyzed using a *t*-test in R (R Core Team 2013).

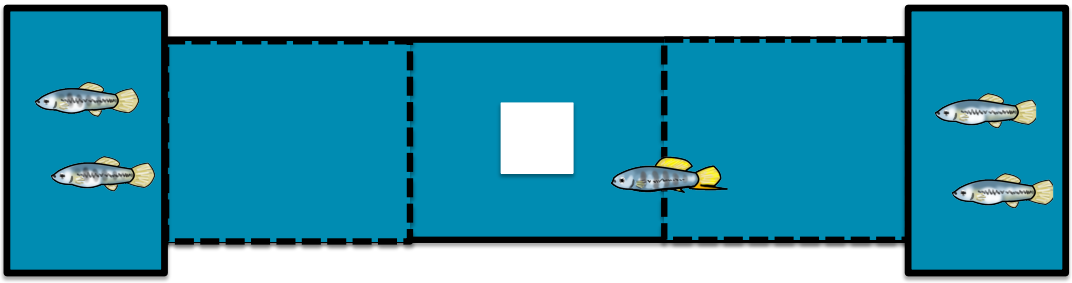

Figure 2.2: Visual dichotomous choice trial set up. Females are visible to males; males can court and associate with females from the association zones (dashed lines) of the trial lane.

Results

Chemical Cue Preference Trials

Male *X. malinche,* when they chose to respond, did have a preference for *X. malinche* cue over water (Welch's 2 sample t-test, n=10, $t = 4.8449$, $p < 0.01$; Figure 2.3a). However, they showed no significant preference for either conspecific or

heterospecific female chemical cues (Welch's 2 sample t-test, n=32, t = 0.1159, p-value = 0.9081; Figure 2.3b).

a) b)

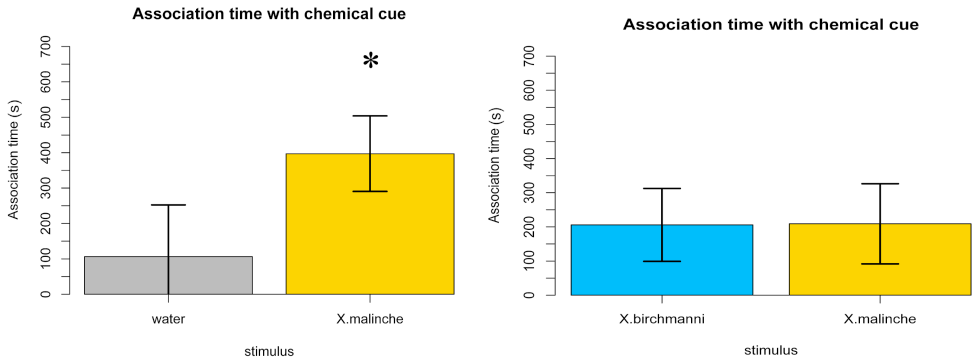

Figure 2.3: Association time (mean +/- SD) of male *X. malinche* with either a) water versus female *X. malinche* chemical cue or b) *X. birchmanni* versus *X. malinche* female chemical cue.

Visual Cue Preference Trials

Just as with the chemical cue trials, male *X. malinche* displayed no significant preference when choosing between conspecific and heterospecific female visual cues (Welch's 2 sample t-test, n=15, t = -0.5456, p-value = 0.594; Figure 2.4). We found that half our males did not respond to this test at all, hence the lower n, even when tried a second time. During the control trial, males were largely unresponsive, and those few that did respond had no significant preferences for water or visual cue (Welch's t-test, n= 5, t = -0.3627, p = 0.7223).

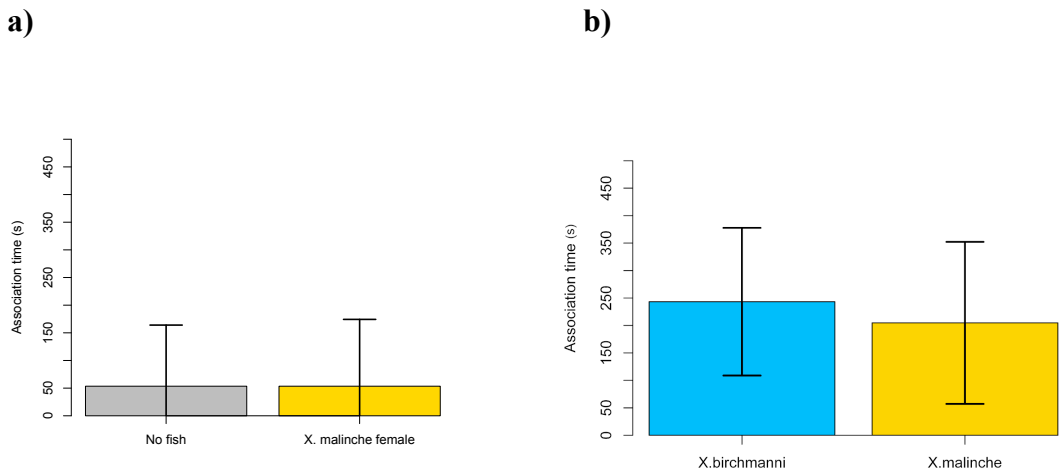

Figure 2.4: Association time (s) male *X. malinche* spent associating with either a) No fish or *X. malinche* female visual cue, or, b) *X. birchmanni* or *X. malinche* female visual cue.

Discussion

In this study, we found that, male *X. malinche* were found to have no statistically significant preferences for conspecific or heterospecifics in either chemical or visual preference trials. This adds to a rich body of evidence suggesting that males in many systems are not overly choosey when it comes to mating decisions. We also can now complete the "preference picture" in that we now have basic preferences information for the wild-caught parental species involved in this *Xiphophorus* hybrid system (See Figure 2.5a and 2.5b).

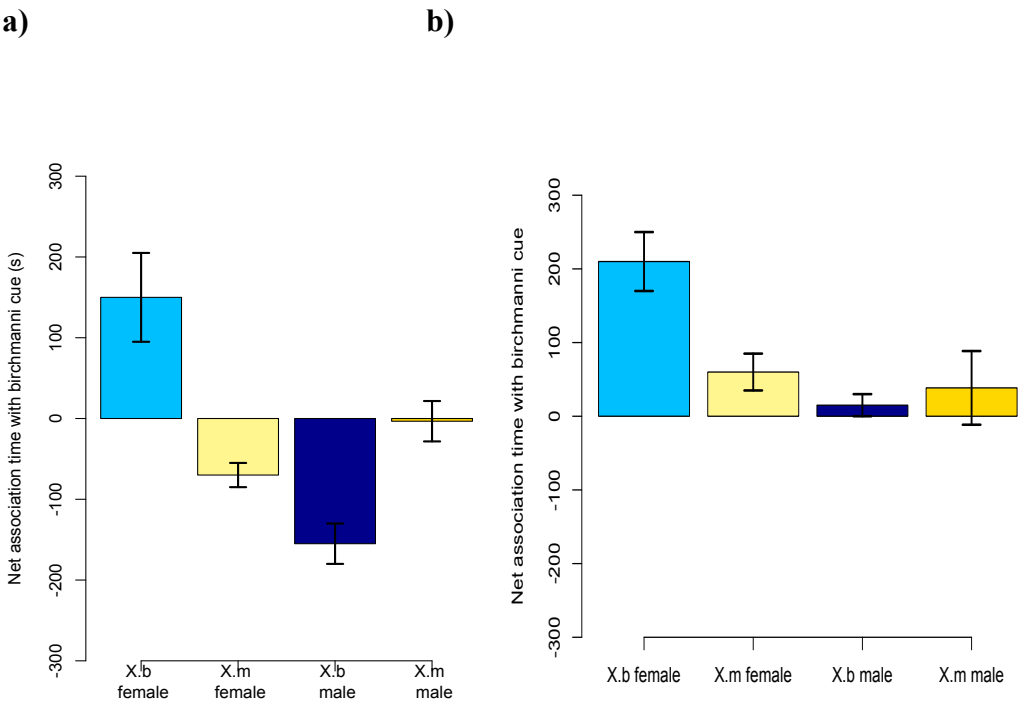

Figure 2.5: a) Net association time with *X. birchmanni* chemical cue; data from Fisher et al. 2006, Wong et al. 2005, and current study; b) Net association time with *X. birchmanni* visual cue; data from Fisher et al. 2006, Wong et al. 2005, and current study.

Taken in context with previous data (Figure 2.5), this current data allows us to make more robust predictions about the history and future of the *Xiphophorus* hybrid system. In the absence of chemical cues, *X. malinche* females prefer the visual signals of *X. birchmanni* males (Fisher et al. 2009); in areas with organic pollution that may abolish females' ability to detect chemical cues, female *X. malinche* are then more prone to mating with heterospecifics (Fisher et al. 2006). Even in areas that have not been disturbed chemically, *X. birchmanni* males may attempt mating with *X. malinche*

females where they co-occur, given *X. birchmanni* male chemical preferences (Wong et al. 2005). In contrast, *X. birchmanni* females strongly prefer conspecifics (Fisher et al. 2006), and, as this current study shows, male *X. malinche* are indifferent.

Because of this, one could predict that asymmetric hybridization between *X. malinche* females and *X. birchmanni* males was key for the inception of these hybrid zones. As mentioned previously, geographic cline data on males traits across hybrid zones suggest that more *X. birchmanni*-like male sexual signals may be introgressing into hybrid zones and potentially may even introgress into "pure" *X. malinche* populations (Rosenthal and Garcia de León 2011). However, there are fluctuations in this pattern, and clines between these two species need to be further resolved (Jofre, unpublished data). The reciprocal cross (*X. birchmanni* female X *X. malinche* male) has also proven difficult to produce in the lab (Powell, unpublished data).

Here, though, we have completed the "preference picture" and now know where *X. malinche* males fit in with the other players in this hybrid system. Now that we have a complete idea about what each parental species might be doing in hybrid zones, we can better predict how these hybrid zones began, as well as make more informed hypotheses about their future.

CHAPTER III

POPULATION STRUCTURE AND MULTIPLE PATERNITY WITHIN A HYBRID ZONE

Introduction

While hybrid zones have proven valuable in the study of various aspects evolution, from speciation (Abbott et al. 2013) to reticulate evolution (Arnold 1992; Seehausen 2004), in the past decades certain areas within hybrid zone research remain understudied. Specifically, this includes studies that examine population structure of hybrid populations and mating decisions within hybrid zones. How parental and hybrid individuals interact and mate have myriad effects on hybrid systems and thus, the future of the species involved.

Mating patterns within hybrid zones can contribute to overall population structure. Several different mechanisms are predicted to generate structure within hybrid zones: selection against hybrids, continuous migration of parentals into the hybrid zones, and/or assortative mating. When hybrids have very low fitness, hybrid genotypes are disproportionately removed from the population, generating a multimodal population structure consisting of parental individuals and early generation hybrids (Harrison and Bogdanowicz 1997; Lindtke et al. 2014). Similar patterns are seen in populations where parentals continuously migrate into the hybrid population; if migration levels are high enough, parental genotypes will increase in the population even in the absence of selection against hybrid individuals (e.g. Charpentier et al. 2012).

Specifically, assortative mating—either between parentals and hybrids or different groups of hybrids—tends to lead to complex population structure versus hybrid swarms (Bailey et al. 2004). While it is expected that parentals will discriminate against hybrids when it comes to mating decisions (Naisbit et al. 2001; Servedio and Noor 2003; Latour et al. 2014), how hybrids will choose to mate is less predictable. Hybrids may have preferences that are intermediates of their parental species and thus may exhibit weak or distinct mate preferences, they may be sensitive to cues from both parental species (Rosenthal 2013), or they may have distinct sensory experiences and thus perhaps novel preferences (Sandkam et al. 2013).

In addition to deciding with whom they should mate, hybrids are also faced with how often they should mate. There may be trade-offs between mating more and mating less (Jennions and Petrie 2000). However, the potential benefits that come with mating multiply may outweigh costs for hybrid individuals. Promiscuity is predicted to evolve in populations where genetic incompatibilities may be an issue: by mating multiply, a hybrid female may be more likely to find a male with sperm that are compatible with her eggs (Jennions and Petrie 2000; Colegrave et al. 2002).

Multiple mating and its extent has ramifications for numerous evolutionary processes (Andersson 1994). In particular, the degree of multiple mating can affect the strength and direction of sexual selection (Shuster and Wade 2003; Clutton-Brock and Vincent 1991). Generally, the more sires represented in one brood, the weaker sexual selection is. This is because more sires usually equals more variation in traits sampled, which means that more trait values will be represented in the brood(s), and thus no one

trait value is “selected” over the others(Tatarenkov et al. 2008; Shuster et al. 2013; Kvarnemo and Simmons 2013). This can have important implications for how and what traits may introgress through hybrid zones.

In a hybrid zone between the swordtails *X. malinche* and *X. birchmanni*, we have found a population with complex structure that may be due to assortative mating within genetic clusters(Culumber et al. 2014). These two fish, sister species that diverged 2-3 mya, are found in the Sierra Madre Oriental of Central Mexico (Cui et al. 2014; Rosenthal et al. 2003). Hybrid zones have formed at least seven separate times between these species, and research suggests that past hybridization events were likely due to anthropogenic disturbance (Culumber et al. 2011; Fisher et al. 2006). As stated, in one of these hybrid zones, the Rio Calnali, hybrid populations seem to break down into several defined clusters versus hybrid swarms: in one study, individuals sampled were deemed to be either pure parentals or backcrossed hybrid individuals. No F1s were found (Culumber et al. 2014). Here, our aim is to add to this study by, in addition to investigating structure with microsatellite markers, examining the level of multiple paternity in this population.

In general, female pregnant fish tend to be polyandrous (Coleman and Jones 2011) and *Xiphophorus* are no exception: in fact, on average, *Xiphophorus* females tend to mate slightly more often than other livebearers (Luo et al. 2005; Simmons et al. 2008; Tatarenkov et al. 2008; Paczolt et al. 2014). In pure *X. birchmanni* populations, females were found to have a mean of 2.48 sires represented in their broods (Paczolt et al. 2014).

It is hypothesized that females in hybrid zones may mate more often, especially

if genetic incompatibilities are present and if there is the opportunity for post-copulatory sexual selection (Colegrave et al. 2002). *X. malinche* and *X. birchmanni* are known to have potentially hundreds of genetic incompatibilities (Schumer et al 2014); *Xiphophorus* in general are also internal fertilizers, and females can store sperm for up to 10 months at a time, making post-copulatory sexual selection a real possibility (Potter and Kramer 2000). We thus predicted that females will indeed mate multiply in this population and that they will probably mate more often than other poeciliids or parental populations.

Methods

Sample Collection and DNA Extraction

32 females and 22 males were collected from a *X. malinche/X. birchmanni* hybrid zone in one small pool ("Sycamore") behind the CICHAZ field station on the Rio Calnali (981 m) in 2012. Females were photographed, fin-clipped, and measured, then, if gravid (N= 27), dissected to remove embryos for DNA extraction. Males were also fin-clipped for DNA extraction.

DNA from adults and embryos was extracted from fin-clips and whole embryos using proteinase-K digestion followed by an isopropyl alcohol clean up step. Briefly, tissue (fin-clip or embryo) was digested overnight in a solution of proteinase K (Promega), dithiothreitol (DTT, Promega) and cell lysis solution (0.1 M Tris HCl, .1 M EDTA, 1% SDS); digested samples were then treated with a protein precipitation

solution (Promega), and then cleaned with 95% isopropanol followed by 70% ethyl alcohol before being resuspended in TE (pH 8.0).

Of the collected families, twenty-two were used for subsequent multiple paternity analyses, as multiple paternity could not be confidently assigned to n=5 of 27 families (see below).

Microsatellite Genotyping

Four highly polymorphic tetranucleotide microsatellite markers were chosen for paternity analyses based on their success in previous studies (Paczolt et al. 2015). Microsatellites sequences were modified from *X. maculatus* sequences (Walter 2004) to incorporate *X. birchmanni-X. malinche* specific mutations. Microsatellite amplification followed PCR protocols described in Paczolt et al. 2015 (Table 3.1). PCR product labeled with fluorescent markers (6-FAM, NED, VIC, PET) from the four microsatellites was multiplexed and sent to the Yale DNA Analysis Facility on Science Hill for fragment analysis on a 3730x1 DNA analyzer. Individuals were genotyped via manual scoring in PeakScanner v1.0 (Life Technologies). Microsatellites optimized for this study were highly variable in the study population (Table 3.1) with 7-20 alleles per loci. Some families were excluded from final analysis due to failed microsatellite amplification, that is, if only 3 or fewer microsatellites amplified (N=5).

Table 3.1: Microsatellite details, including annealing temperature (Temp), size range in base pairs (bp), number of alleles at each locus in this subpopulation (A), and p-value from Fisher's Exact Test, and F_{IS} value.

Locus	Temp	Range (bp)	Alleles	p_{HW}	F_{IS}
Msd029	58	166-268	20	< 0.000	0.2103
Msd036	58	157-295	15	0.215	-0.0061
Msd049	60	135-229	7	< 0.000	0.2584
Msd072	60	206-231	13	0.0012	0.1294

Fisher's Exact Test for violation of Hardy-Weinberg Equilibrium (HWE) and F_{IS} scores were calculated for each microsatellite with Genepop 4.2 (Raymond and Rousset 1995; Rousset 2008). We found statistically significant deviations from HWE in three of the four microsatellites when all of our individuals were considered together (Table 3.1). This is perhaps to be expected, given evidence of bimodal structure/assortative mating in this population (Culumber et al. 2014) We also found that all microsatellites displayed high F_{IS} scores, suggesting a Wahlund effect. To investigate if these patterns were due to structure within our microsatellite data, multilocus genotypes from adults in the population were further analyzed (see next section).

Tests for Population Subdivision

The program STRUCTURE v2.3 (Falush et al 2003) was used to determine possible number of distinct genetic clusters within the sample. While previous research shows that there are likely two separate clusters in the population sampled (Culumber et al. 2014), we ran K = 1-6 for exploratory purposes. We simulated each K value five times (50,000 burnin, 100,000 replications) using correlated allele frequencies and admixture models. STRUCTURE does run under assumptions of HWE and linkage equilibrium, which could be problematic. To cross-check our STRUCTURE results we analyzed our data with a Principle Coordinates Analysis (PCoA), a multivariate statistical method that does not rely on the aforementioned assumptions, with our data using GenAlEx v6.5 (Peakall and Smouse 2006). Additionally, using ancestry data obtained through Multiplex Shotgun Genotyping (MSG, see methods below), we tagged the microsatellite genotypes of individuals with known ancestry information to see if those groups also lined up with our STRUCTURE and GenAlEx results.

Using the clusters determined with MSG, we returned to Genepop 4.2 to test our microsatellites against HWE for each cluster. We also estimated F_{IS} and F_{ST} values (Cockerham and Weir 1984) between our two clusters; these were calculated using F_{STAT} v.2.9.3 (Goudet 1995). Additionally, we explored whether there were differences in multiple paternity between our genetic clusters by running a generalized linear model to see if cluster could predict number of sires, given female size and number of offspring.

Multiplex Shotgun Genotyping

A parallel study not presented here on this same sample makes use of Muliplex Shotgun Genotyping (MSG; Andolfatto et al. 2011) to further investigate population structure and genetic ancestry of individuals in this population. Though we will not get into the full details of that other study, this method allows us to differentiate our sample in to two genetic clusters with thousands of markers versus our four microsatellite markers. We compared the genetic clusters found in that data set with the structure we see given the analyses described above to determine if they were concordant.

The library preparation and data analysis methods we used for MSG follow Schumer et al. 2014. Briefly, samples were digested with MseI (NEB, Ipswich, MA) and custom barcode adapters were ligated to each sample. Following this step, the ligation reaction was stopped with 5 ul of sodium acetate and 50 ul of isopropanol. Samples with unique barcodes were pooled (in groups of 48) and precipitated overnight at -20 C. Pools were resuspended in TE (pH 8.0) and were bead purified using the Agencourt AMPure PCR purification kit (Beckman Coulter Inc., Brea, CA). Libraries were then size selected for fragments between 250-500 bp on a 2% agarose gel, and 2 ng of the purified product was amplified with Illumina indexed primers for 14-16 cycles using the Phusion PCR kit (NEB, Ipswich, MA). After bead purification, samples were assessed for quality on a Bioanalyzer 2100 (Agilent, Santa Clara, CA) and sequenced on an Illumina HiSeq 2000 or 2500 (100 bp or 140 bp reads respectively). Up to 150 individuals were pooled on a single lane.

After libraries were prepared, we parsed raw reads by index and barcode and trimmed to remove low quality base pairs (Phred quality score <20); reads with fewer than 30 bp of high quality contiguous sequence were discarded. The number of reads per individual ranged from 0.3-7.9 million, but reads in excess of 2 million were excluded to improve pipeline speed.

Following quality trimming, data was processed with the MSG pipeline (https://github.com/JaneliaSciComp/msg). The following parameters were specified for the analysis: recRate = 420, rfac=0.0001, *X. birchmanni* error (deltapar1) = 0.05, *X. malinche* error (deltapar2) = 0.05. The recombination rate was set based an expectation of 0.0018 cM/Mb in *Xiphophorus* and of at least 35 generations of recombination (Amores *et al.* 2014; Schumer et al. 2014) and the error rate was set based on observed error rates in parental individuals (Schumer et al. 2014). Each population was analyzed separately. Individuals were initially run with naïve MSG priors (expectation par1, par1par2, par2 = 0.33,0.33,0.33), and then were re-analyzed with informed priors based on genome-wide ancestry in the initial run.

MSG reports genotypes in the form of posterior probabilities. To calculate hybrid index, we treated posterior probabilities >0.95 as support for a particular genotype, and for each individual divided the total number of *X. malinche* genotype calls by the total number of genotype calls with >0.95 posterior probability support. For calculations of hybrid index, we used only markers that were sampled in >70% of individuals (720,040 ancestry informative markers). Of the adults in this sample, we

were only able to achieve high enough coverage to confidently ascertain hybrid index in 33 of our 53 individuals thus far.

Parentage Analysis

GERUD 2.0 (Jones 2001, 2005) was used to reconstruct paternal genotypes and determine the minimum number of sires per brood. GERUD reconstructs parental genotypes from full- and half-sibling genotype arrays using an algorithm that tests all possible paternal genotypes against offspring arrays to determine the minimum number of sire genotypes (Jones et al. 2010) and uses Mendelian probabilities (Jones 2005) to calculate the most likely groups of sires (within a 95% confidence interval); these are ranked by likelihood and displayed to the user, and we are then able to chose the most likely set of potential sire genotypes.

To determine our power to detect multiple paternity, we used PrDM (Neff and Pitcher 2002) to measure the probability that we could detect multiple mating given the allele frequencies in our population. We used average sire numbers and skew data from our GERUD analyses as a basis for our simulations.

Results

Population Subdivision

STRUCTURE results showed us that the optimal K for this population was 2. The mean ln P(D) reached it's largest values at K = 2 (mean = -864.96) followed by K = 3 (mean = -889.46) before declining further. At K = 3, however, variance among the

runs in ln P(D) was much larger. Additionally, at $K \geq 3$, Q-values of individuals dropped precipitously, and are thus implausible at $K \geq 3$. Individuals were thus assigned to one of two sub-populations if their Q-value for that group was ≥ 0.7. Of our 53 individual multilocus genotypes, all had a Q-value of ≥ 0.7. Of those, three had a Q-value between 0.7 and 0.8 (5.6%), six had a Q-value between 0.81 and .90 (11.3%), and 44 had a Q-value > 0.9 (83.1%). Additionally we plotted our individuals' multilocus genotypes with a PCoA using GenAlEx, with the populations found in STRUCTURE highlighted in different colors (Figure 3.1). The PCoA also revealed that our individuals fall out into two groups, though there is some slight overlap.

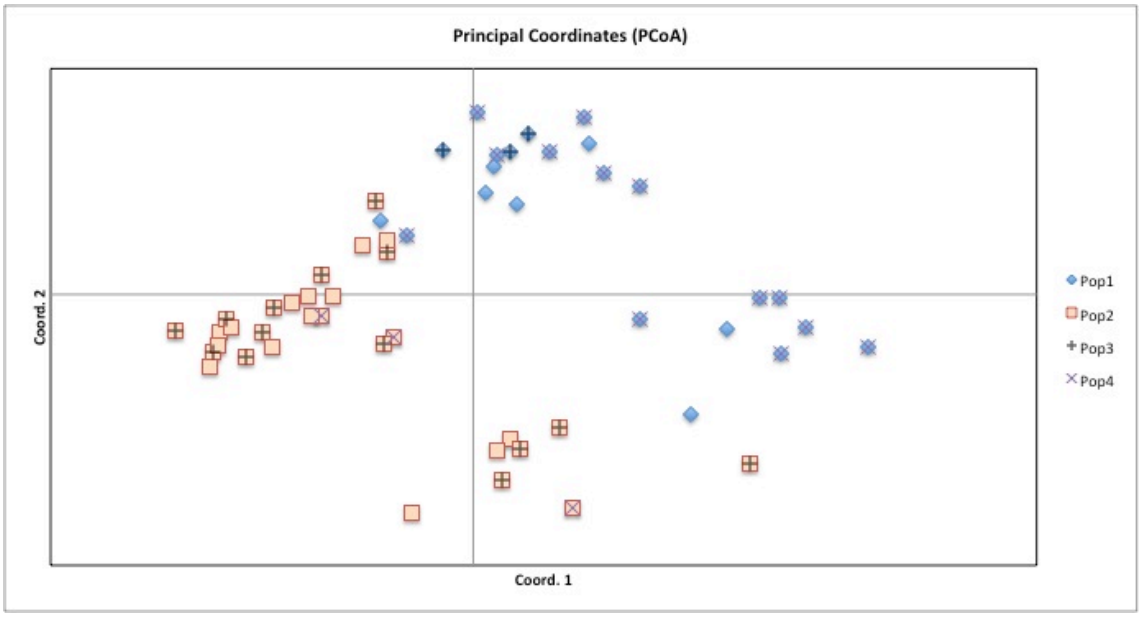

Figure 3.1: PCoA generated in GenAlEx 6.5. Subpopulations predicted by STRUCTURE ("Pop 1" and "Pop 2") are marked by blue diamonds and orange squares, respectively. The microsatellite genotypes of individuals with known ancestry (determined by MSG) overlay this data: "Pop 3" (black plus-signs) are *X. malinche*-like individuals, and "Pop 4" (purple X's) are *X. birchmanni*-like individuals.

From the MSG data, we found that adult individuals sampled from this hybrid zone fall into one of two clusters—an *X. birchmanni*-biased genotype cluster with 73±2% of the genome derived from *X. birchmanni* and an *X. malinche* genotype cluster with 95±2% of the genome derived from *X. malinche* (Figure 3.2). When MSG data is laid over the "sub-populations" found in STRUCTURE and GenAlEx, we see that, in general, *X. birchmanni*-like individuals line up with "Pop 1" and *X. malinche*-like individuals line up with "Pop 2" (Figure 3. 1). Given that the PCoA was made using microsatellite genotypes, and the fact that past hybridization events/introgression has occurred in this population as evidenced by MSG genotypes, some overlap is to be expected.

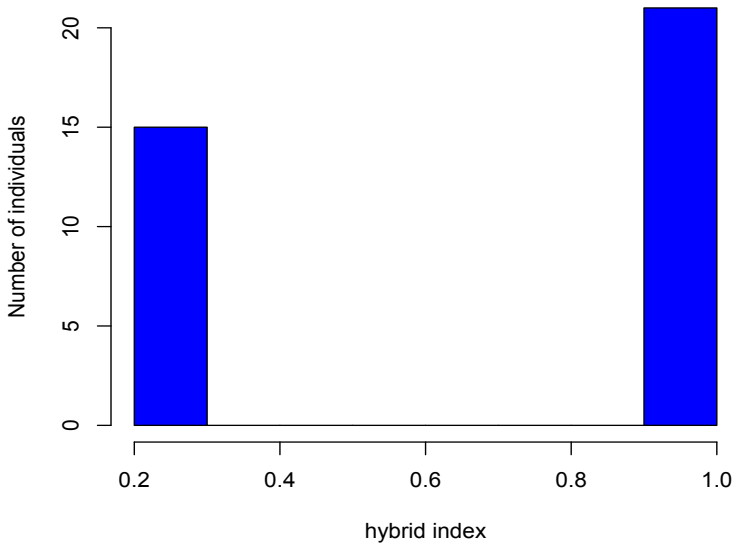

Figure 3.2: Hybrid indexes of adult individuals from MSG analysis, where 0 = pure *X. birchmanni* and 1 = pure *X. malinche.* Individuals in the *X. birchmanni*-biased genotype cluster have 73±2% of their genome derived from *X. birchmanni,* and those in the *X. malinche* genotype cluster have 95±2% of the genome derived from *X. malinche*

We used GenAlEx to further visualize allele frequencies between the two groups (Figure 3.3). Overall, there were no significant differences in number of alleles, number of private alleles between clusters, or expected levels of hetrozygosity. The *X. malinche*-like cluster did have slightly more private alleles, but this was mainly driven by one microsatellite marker (Msd029, where *X. malinche*-like had seven private alleles over the four of the *X. birchmanni*-like group). This was slightly unexpected given the MSG data that shows heavy *X. malinche* introgression the *X. birchmanni*-like cluster.

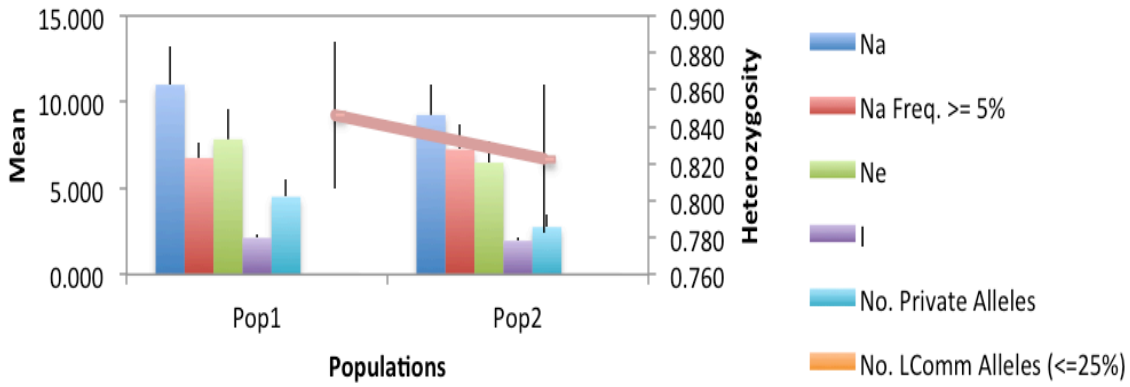

Figure 3.3: Allelic patterns across clusters, where "Pop1" are *X. malinche*-like individuals and "Pop2" are *X. birchmanni*-like individuals. Na is the number of alleles represented; Na Freq. >= 5% are the number of different alleles with a frequency ≥ 5%; Ne is the number of effective alleles; I represents the Shannon's information index; No. Private Alleles are the number of unique alleles represented in each cluster; and No. LComm Alleles (<=25) is the number of locally common alleles (freq. >= 5%) found in 25% or fewer populations. The line represents H_e, or expected hetrozygosity within each cluster.

With this new population structure knowledge, we re-ran our microsatellite data in Genepop 4.2 (ref) and calculated F-statistics using F_{STAT}, following the procedures

described in the Methods section. These results are presented in Table 3.2. When considered separately, microsatellite markers within each genetic cluster are in Hardy-Weinberg equilibrium. F_{IS} values also vary when the two groups are considered separately: F_{IS} seems to be, in general, reduced. However, in the *X. malinche*-like cluster F_{IS} increased for some of the microsatellites, suggesting a higher level of inbreeding. The small sample size may be a factor here. Despite the structure between the two groups, overall differentiation is somewhat low, with total F_{ST} between clusters = 0.053.

Table 3.2: Microsatellite loci assayed from adult male and female individuals. Name of locus and pairwise F_{ST} values are reported for each locus. Number of individuals with successfully amplified markers out of 16 (N), number of alleles (A), P value of Hardy-Weinburg exact test (p_{HW}), and F_{IS} scores are reported for each population at each locus.

	X. malinche-like cluster				***X. birchmanni-like cluster***				
Locus	**N**	**A**	**p_{HW}**	**F_{IS}**	**N**	**A**	**p_{HW}**	**F_{IS}**	**F_{ST}**
Msd029	16	16	0.2783	-0.0269	16	9	0.5062	0.1880	0.033
Msd036	13	13	0.0260	0.2018	9	11	0.2473	0.1176	0.025
Msd049	15	6	0.6997	0.0270	14	5	0.4018	0.0335	0.094
Msd072	16	9	0.1377	0.2246	13	8	0.6880	0.0827	0.061

Parentage Analysis and Minimum Number of Sires

Females from our sample varied in standard length from 37.11 mm to 60.66 mm (mean = 50.36mm, SD = 0.64mm). Brood sizes for females used in the final analysis

were between 8 and 61 offspring (mean= 32.31, SD = 13.82). All females in the final analysis were found to have two or more sires represented in their broods (mean= 3.09 +/- 0.99 SE Figure 3.4).

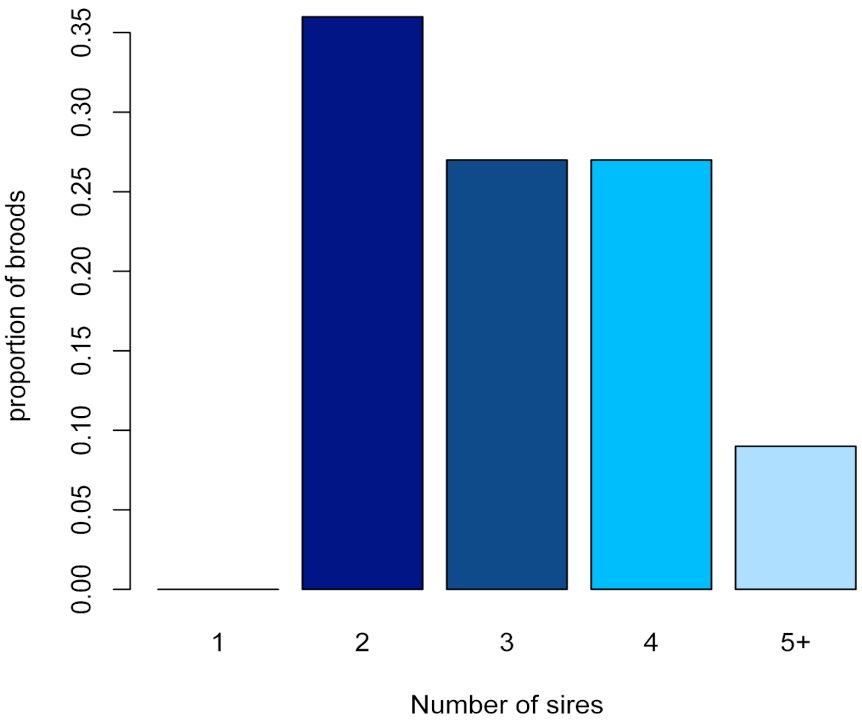

Figure 3.4: Distribution of broods with a given number of sires; mean= 3.09 +/- 0.99

As with a past study, (Paczolt et al. 2014) simulations done in PrDM suggest a high probability of detecting multiple mating in our sample. Simulations with broods of less than 8 individuals showed a detection probability of < 0.95, but in simulations that

reflected similar brood sizes and reproductive skew as our sample showed a probability of detection >0.98.

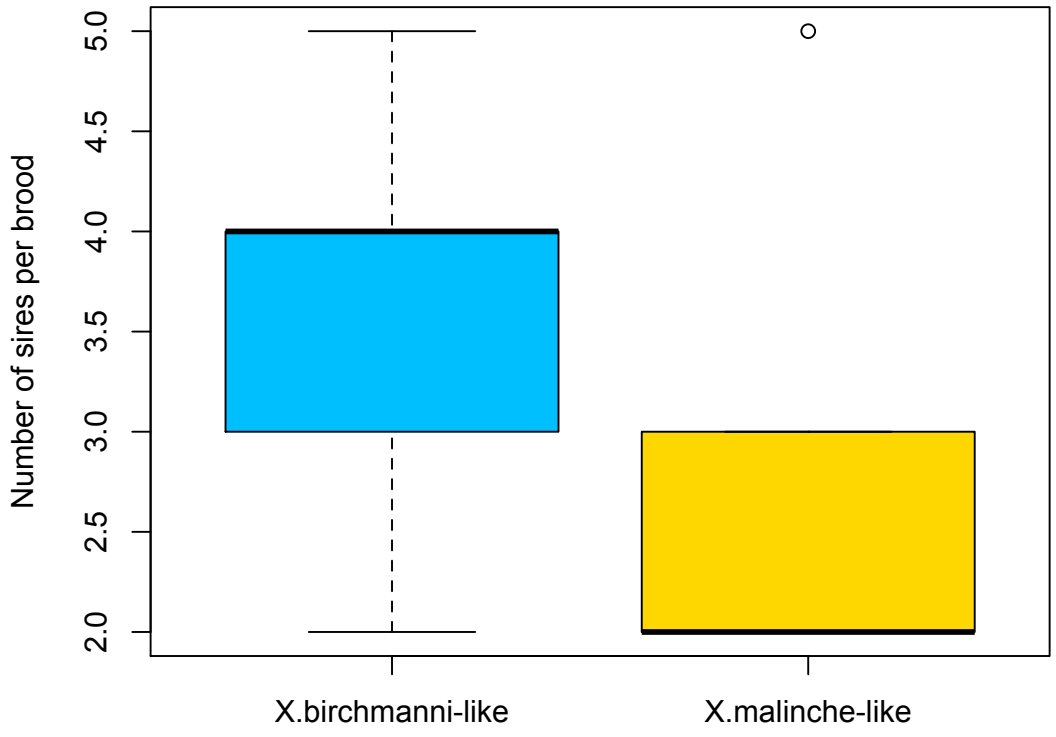

Figure 3.5: Number of sires per brood in hybrid cluster types, before being corrected for offspring number and female body size

X. birchmanni-like hybrids had more sires per brood than *X. malinche*-like hybrids (Welch's 2 sample t-test; t = 2.2942, df = 19.998, p-value = 0.03274; Figure 3.5). To account for female size and brood size, we ran a generalized linear model with number of sires as the dependent variable and genetic cluster and the residuals of brood

size given female size (which were highly correlated: Pearson's product-moment correlation: $r(20) = 0.759$, $p < 0.001$) as predictor variables. When we did this, we found that *X. birchmanni*-like individuals were slightly more likely to have more sires (0.8971 more sires versus a *X. malinche*-like individual of the same size) represented in their broods (linear model; overall model: adjusted $R^2 = 0.1284$, $F_{2,19} = 2.546$, $p = 0.1048$; genetic cluster: $F_{2,19} = 5.0193$, b'=-0.8971, $p = 0.0405$; residuals of brood size given female size: $F_{2,19} = 0.0732$, b'=0.0061, $p = 0.7896$).

Discussion

Factors such as whom individuals mate with and how often individuals mate have implications for how populations are structured and are important in evolutionary processes such as sexual selection. Though studies on multiple paternity in wild populations are quite common, few studies have investigated multiple paternity in populations where hybridization has taken place specifically (although see Anderson, et al. 2008). Given the acceptance of the significant role hybridization may play in the evolution of species (Arnold 1992; Abbott et al. 2013), studies detailing hybrid mating decisions and structure within hybrid zones are relevant and necessary.

In our current study, we first investigated population structure; at first, our microsatellite markers appeared to be out of HWE, suggesting structure. Analyses in STRUCTURE, GenAlEx, and additional information provided by MSG allowed us to show that the population under study could be broken into two distinct clusters. F_{ST} values were somewhat low, as one might predict from two subpopulations (of sister

species) that recently hybridized. F_{IS} values within our two groups were for the most part high, suggesting some degree of inbreeding within genetic clusters. This may be a product of small sample size and the use of only four markers, but freshwater fish are also known to have a lower degree of genetic diversity in general (DeWoody and Avise 2000). This study supports past work suggesting that there is distinct population structure in hybrid zones of *X. malinche* and *X. birchmanni* in this region of the Rio Calnali (Culumber 2011, 2014). The F_{IS} values, MSG data, and population structure results again suggest that this structure is due to assortative mating.

Additionally, as predicted, we determined that females within this hybrid population successfully mate with multiple sires and that the level of multiple mating is slightly higher than in populations of one of the parentals (*X. birchmanni*, Paczolt et al. 2014). While at first it appeared that the level of multiple mating may also differ between hybrid genetic clusters, once we factored in female size and brood size, these differences were found to be non-significant. On average, females in this population had 3.09 sires (+/- 0.99 SE) represented in each brood; this is high, considering studies done in other *Xiphophorus* fishes (Luo et al. 2005; Simmons et al. 2008; Tatarenkov et al. 2008) and studies on poeciliids and female pregnant fish, more generally (Avise and Liu 2010; Coleman and Jones 2011). However, this level of multiple paternity is only slightly higher than what is seen in pure and introgressed *X. birchmanni* populations (Paczolt et al. 2014, Figure 3.6).

Theoretically, this higher level of multiple paternity is to be expected in hybrid zones. Polyandry may provide non-additive genetic benefits to females such as

incompatibility avoidance or allow females to "hedge their bets" (Colegrave et al. 2002; Zeh and Zeh 2003). Schumer et al. (2014) showed that there are hundreds of potential genetic incompatibilities between *X. birchmanni* and *X. malinche*; even a low amount of incompatibilities may be enough for polyandry to evolve (Colegrave et al. 2002).

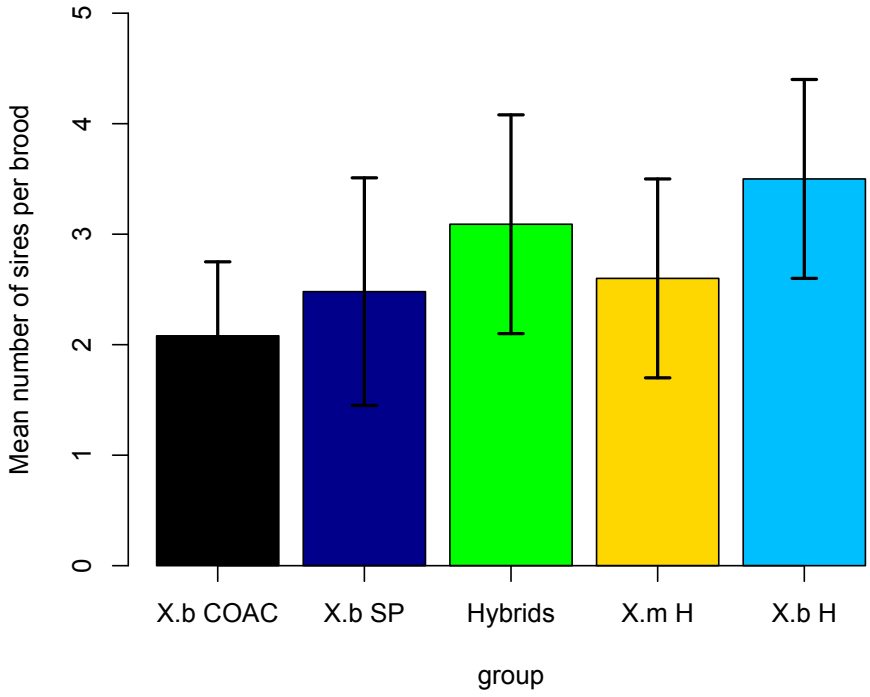

Figure 3.6: Comparison of multiple paternity across *Xiphophorus birchmanni* and *X. malinche* populations. Black and deep blue are *X. birchmanni* from Coacuilco (X.b COAC) and San Pedro (X.b SP) localities, respectively (Paczolt *et al.* 2014). Green is hybrids from the current study; yellow and bright blue represent *X. malinche*-like hybrids (X.m H) and *X. birchmanni*-like hybrids, respectively.

While this study sheds light on important information regarding this hybrid zone, further work is necessary. The results we obtained here will go on to inform an additional study that further investigates assortative mating as a cause of structure in this population. So far MSG results from offspring (of the females sampled here) sequenced for that study reveal that their genotype distribution is almost identical to the adult population and, using offspring genotypes to infer sire ancestry, we can see that there appear to be no cross-cluster matings in this subpopulation (Schumer et al, unpublished data). This means that females are only successfully mating with males within their genotype cluster and that there is probably no post-fertilization selection against these hybrid offspring. However, because of the nature of our study, we cannot with complete confidence report that no cross-cluster matings occurred.

We know that, in the parental species, females generally show strong preferences for conspecific males (Fisher et al. 2006; Fisher et al. 2009; Cui 2014), but rearing environment also plays an important role in the development of female preference (Verzijden et al. 2012). Females from this hybrid zone do prefer to associate with males of similar genotype in mesocosm social assays (Culumber et al. 2014), suggesting that they would also choose to mate with individuals with similar hybrid indices. Also, we can predict that forced copulations (either within or between ancestry clusters) may not be overly important in this system, given the showiness, courtship displays, and relatively short gonopodia of males (Rosen and Tucker 1961).

Given the high degree of structure seen in this population, mate choice and possible reinforcement mechanisms may be very important in this system. If high levels

of polyandry are an adaptation to avoid incompatibilities/allow females to hedge their bets, it may be a relic from when *X. malinche* and *X. birchmanni* first began hybridizing in this area. However, studies into multiple paternity and interactions with individual genotype need to be done in other hybrid zones between these species before we can speculate further.

CHAPTER IV

SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS

Hybridization is an important evolutionary process (Arnold 1992; Abbott et al. 2013; Cui et al. 2013); understanding the consequences of hybridization requires that we investigate the mating decisions of individuals within hybrid zones. Mate choice and other mating decisions, such as mating frequency, are crucial to how genes are spread through hybrid zones; thus, the study of these elements is vital to predicting the evolutionary future of species involved in hybrid systems. Here, I conducted two studies: the first investigated the chemical and visual preferences of male *X. malinche*, one of two swordtail fish species that hybridize in rivers of central Mexico. My second study detailed the beginning of a larger analysis; I start a more refined investigation into assortative mating in a hybrid zone by first examining multiple paternity in a hybrid population.

In my first study, I found that male *X. malinche* have no significant preferences for conspecific or heterospecifics in either chemical or visual preference trials. This study, along with findings from previous studies (Wong et al. 2005, Cui 2014), allows us to make more accurate predictions about the history and future of the *Xiphophorus* hybrid system. To summarize, although females of both species have strong preferences for conspecific chemical cues (Fisher et al. 2006) in the absence of chemical cues, *X. malinche* females prefer the visual signals of *X. birchmanni* males (Fisher et al. 2009). This means that in areas contaminated by anthropogenically derived organic

pollution, where females' ability to detect chemical cues is diminished, female *X. malinche* may be more prone to mating with heterospecifics (Fisher et al 2006). Even in areas that have not been disturbed chemically, *X. birchmanni* males may attempt mating with *X. malinche* females where they co-occur, as *X. birchmanni* males somewhat prefer heterospecific chemical cues (Wong et al. 2005). As this current study shows, male *X. malinche* are indifferent, meaning they will not reinforce female *X. malinche* preferences and that they may attempt courting and mating with *X. birchmanni* females. Whether or not the potential amorous attentions of *X. malinche* males could lead to reproductive interference and fitness costs (Gröning and Hochkirch 2008) for *X. birchmanni* females remains to be tested.

Given the preferences and behavioral studies done on the parental species in this system, one could predict that asymmetric hybridization between *X. malinche* females and *X. birchmanni* males was key for the inception of these hybrid zones. Geographic cline data on male traits across hybrid zones seems to suggest that more *X. birchmanni*-like male sexual signals may be introgressing into hybrid zones and potentially into *X. malinche* populations. This pattern has been seen to fluctuate, and clines between these two species need to be more fully resolved (Jofre, unpublished data). Further study of trait introgression/gene flow and of understanding how preferences lead to mating decisions in the hybrid zones are needed.

In addition to elucidating how mating preferences operate for parental species that hybridize, studies that examine the mating decisions of hybrids within hybrid zones are important. To start, I investigated population structure and multiple paternity in a

sample consisting of *X. malinche* and *X. birchmanni* hybrids. Though studies on multiple paternity in wild populations are quite common, few studies have investigated multiple paternity in populations where hybridization has occured specifically. In this study, we determined that, as predicted, females within this hybrid population successfully mate with multiple sires and that the level of multiple mating is slightly higher than in populations of one of the parentals (*X. birchmanni*, Paczolt et al. 2014). On average, females in this population had 3.09 sires (+/- 0.99 SE) represented in each brood; this is somewhat high, considering studies done in other *Xiphophorus* fishes (Luo et al. 2005; Simmons et al. 2008; Tatarenkov et al. 2008) and, more generally, studies on poeciliids and female pregnant fish (Avise and Liu 2010; Coleman and Jones 2011). However, this level of multiple paternity is only slightly higher than what is seen in pure and introgressed *X. birchmanni* populations (Paczolt et al. 2014, Figure 3.4).

In hybrid zones, females mating with multiple sires is expected: polyandry may provide non-additive genetic benefits to females such as incompatibility avoidance (Colegrave et al. 2002; Zeh and Zeh 2003). Even a low amount of incompatibilities may be enough for polyandry to evolve (Colegrave et al. 2002) and here, we know that that there are hundreds of potential genetic incompatibilities between *X. birchmanni* and *X. malinche* (Schumer et al. 2014). However, given the high degree of structure seen in this population it would seem that mate choice and possible reinforcement mechanisms may also have evolved here. If high levels of polyandry are an adaptation to avoid incompatibilities/allow females to hedge their bets, it may be a relic from when *X. malinche* and *X. birchmanni* first began hybridizing in this area. Thus more research on

premating barriers is needed in this hybrid zone and investigations into paternity patterns in other *X. malinche/X. birchmanni* hybrid zones are needed.

As stated, parental females generally show strong preferences for conspecific males (Fisher et al. 2006; Fisher et al. 2009; Cui 2014), but rearing environment also plays an important role in the development of female preference (Verzijden et al. 2012). Females from this hybrid zone do prefer to associate with males of similar genotype in mesocosm social assays (Culumber et al. 2014), suggesting that they would also choose to mate with individuals with similar hybrid indices. We can also predict that forced copulations (either within or between ancestry clusters) may not be overly important in this system, given the showiness, courtship displays, and relatively short gonopodiums of males (Rosen and Tucker 1961). However, additional observational and behavioral studies are needed to determine if the patterns we see are strictly because of strong precopulatory mate choice and lack of coerced matings, or if postcopulatory mechanisms, such as conspecific sperm precedence, occur as well. Additionally, reproductive interference—where males of one species/genetic cluster waste the time, energy, and mating opportunities of heterospecific females by coercive matings or other sexual harassment—may occur, given the indifference of males. How this affects females in hybrid zones is worthy of investigation.

Historically, hybridization was thought of as nothing more than a fitness-reducing mistake on the part of the individuals involved. Through studying the consequences of the mating decisions of individuals within hybrid systems, we have come to find that in many cases, hybridization represents another of those powers that

allows for the evolution of, as Darwin (1859) wrote, “endless forms most beautiful and most wonderful.”

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