THE CAUSES AND CONSEQUENCES OF HYBRIDIZATION IN TWO NORTHERN SWORDTAIL FISHES

A Dissertation

by

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ABSTRACT

Hybridization is a common phenomenon with important evolutionary consequences. It may result in the loss of genetic differentiation between groups, or serve to reinforce reproductive barriers between species. Hybridization may further allow for the introgression of adaptive traits from one species into another, aiding in the exploitation of novel niches. It may even contribute to the creation of new species. Much of the literature focuses on genotypes and phenotypes of individuals that have likely undergone many generations of selection because early generation hybrids are often rare in established hybrid zones. However, many important processes acting on hybrid fitness do so in the early stages of admixture. Thus, a crucial question is how selection acts on the first few generations of hybrids to determine the evolutionary trajectory of future generations. Natural selection on viability during the first generations of hybridization can be critical in shaping patterns of genetic exchange. In contrast, we know less about the evolutionary consequences of sexual selection during the early stages of hybridization, but genetic exchange between divergent populations ultimately depends on the mating decisions of individuals within sympatric populations.

My research addresses the causes and consequences of hybridization between two closely related swordtail fish, *Xiphophorus birchmanni* and *X. malinche,* in its early stages. I explore the fitness of the first two generations of hybrids when compared to parental species in a common-garden rearing experiment. I find little evidence for intrinsic fitness reduction, and hybrids were morphometrically and physiologically intermediate to parentals. Additionally, early generation

female preferences were more permissive than either parental species and hybrid male chemical cues were universally attractive, circumstances that should promote ongoing geneflow between species via rampant back crossing.

For gene flow between species via backcrossing and continued intercrossing to be possible however, there must first be a breakdown in reproductive isolation. I find that a common and deliberately introduced anthropogenic pollutant can not only disrupt chemical communication, but it does so in a way that should promote hybridization, causing female *X. birchmanni* to prefer the chemical cues of the heterospecific *X. malinche* over conspecific ones.

Lastly, the ways in which hybridization can affect trait distributions in hybrid populations depends largely on the genetic architecture underlying those traits. I use controlled intercrosses to perform classical QTL mapping for many of these traits that exhibit correlated phenotypes within the two species. I uncover a single QTL for each of 5 separate traits, none of which colocalize to any one chromosome suggesting independent genetic pathways control these traits. As such, trait combinations might be expected to vary outside the distributions for either parental species in hybrid populations which aligns with patterns observed in the wild.

DEDICATION

For my mother, Theresa LeMaster.

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All work for this dissertation was completed by Daniel Lee Powell, under the advisement of Dr. Gil Rosenthal of the Department of Biology.

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CHAPTER I

INTRODUCTION

It is no longer controversial that hybridization between genetically divergent populations is a common phenomenon with important evolutionary consequences (Abbott et al., 2013; Barton & Hewitt, 1989). Genetic exchange upon secondary contact between diverging species may result in the loss of genetic differentiation between groups if hybrids do not suffer fitness consequences relative to parentals, or it may serve to reinforce reproductive barriers between species if the fitness of hybrids is reduced compared to the offspring of conspecific matings (Coyne & Orr, 2004; Kronforst, Young, & Gilbert, 2007). Hybridization may further allow for the introgression of adaptive traits from one species into the genomic background of the other, aiding in the exploitation of novel niches (Pereira, Barreto, & Burton, 2014; Servedio, 2001). It may even contribute to the creation of new species if hybrids are reproductively isolated from parentals (Abbott et al., 2013; Mallet, 2007; Schumer, Rosenthal, & Andolfatto, 2018).

Early generation hybrids are often rare in established hybrid zones, due in part to the reduced frequency of interactions between parentals in populations consisting primarily of hybrids (Gröning & Hochkirch, 2008), but also because break down in premating barriers may be episodic (Gee, 2004). Accordingly, much of the literature focuses on genotypes and phenotypes of individuals from wild hybrid zones which have likely undergone many generations of selection (Abbott et al., 2013). However, many important processes acting on hybrid fitness do

so in the early stages of admixture. Thus, a crucial question is how selection acts on the first few generations of hybrids to determine the evolutionary trajectory of future generations.

Natural selection on viability during the first generations of hybridization can be critical in shaping patterns of genetic exchange (Abbott et al., 2013; Arnold & Martin, 2010). For example, fitness of reciprocal first generation (F_1) hybrids is also often asymmetric, with a greater reduction of fitness in one F_1 cross than the other (Jiggins, Salazar, Linares, & Mavarez, 2008; Schrader, Fuller, & Travis, 2013). Further, taxa with sex chromosomes are prone to Haldane's rule, wherein F_1s of the heterogametic sex suffer reduced fitness due to deleterious recessive alleles being expressed in the hemizygous state (Good, Dean, & Nachman, 2008; Michael Turelli & Moyle, 2007; Turelli & Orr, 1995). Beyond the first generation of hybrids, novel combinations of alleles can produce extreme traits or combinations of traits outside of typical parental values, which may be deleterious or advantageous with respect to natural or sexual selection (Pereira et al., 2014). Hybrid breakdown, or accelerated inviability of hybrids beyond the first generation, often occurs when closely related species hybridize due to the segregation of species-specific interacting loci as a result of recombination, a phenomenon known as Bateson-Dobzhansky-Muller incompatibility (BDMIs) (Burton, 1990; Matsubara, Ando, Mizubayashi, Ito, & Yano, 2007; Orr & Coyne, 1989; Presgraves, 2003; Turner & Harr, 2014).

We know less about the evolutionary consequences of sexual selection during the early stages of hybridization. The mating signals (McDonald, Clay, Brumfield, & Braun, 2001) and preferences (Rosenthal, 2013; Stein & Uy, 2006) of early generation hybrids can have major effects on fitness and on mating patterns of both hybrids and parental genotypes. The mating biases of parental species and early generation hybrids can act in concert or in opposition to viability

selection. Choosers of one species may be more permissive to heterospecific matings than the other (Ryan & Wagner, 1987). Hybrid courter signals may be attractive or aversive to parentals, either promoting or inhibiting introgression of traits via backcrossing. Likewise, the mating preferences of hybrid females may facilitate ongoing gene flow between species if they are similar to those of parentals or if they are generally permissive, but may limit gene flow if hybrids exhibit preferences for hybrids (von Helversen and von HelversenVon Helversen & Von Helversen, 1975; Ten Cate & Vos, 1999). Thus, the evolutionary dynamics of hybridization depend both on the viability as well as the individual mating decisions of hybrids (Rosenthal, 2013; Rosenthal, 2017), which in turn are shaped by their novel genotypes and phenotypes.

My research addresses the causes and consequences of hybridization between two closely related swordtail fish, *Xiphophorus birchmanni* and *X. malinche,* in its early stages. In chapter II I explore the fitness of the first two generations of hybrids when compared to parental species in a common-garden rearing experiment. By rearing *X. birchmanni* and *X. malinche* alongside F_1 and F_2 intercross hybrids, I compare growth, viability, mophoplogy, and physiology while standardizing environmental variables. There was little evidence for intrinsic fitness reduction, and hybrids were morphometrically and physiologically intermediate compared to parental genotypes.

Mate choice dynamics involving early generation hybrids are of particular interest for exploring the lasting evolutionary consequences of hybridization, as they ultimately determine whether or not gene flow persists when there is weak viability selection (Brelsford & Irwin, 2009). I tested this by assaying mating preference of females from both parental species as well as F_1 and F_2

intercross females. Here I found that early generation female preferences were more permissive than either parental species and that hybrid male chemical cues were universally as attractive as any parental cue across all female genotype classes, a situation that should promote ongoing geneflow between species via rampant back crossing.

For gene flow between species via backcrossing and continued intercrossing to be possible however, there must first be a breakdown in reproductive isolation. Behavioral reproductive isolation between closely related species often occurs long before postmating isolation can evolve via genetic incompatibilities (Grant & Grant, 1997; Servedio & Noor, 2003). Thus, assortative mating may be the strongest barrier to gene flow between recently diverged species in sympatry, so whether or not hybridization occurs depends first on the mating decisions of individuals within those sympatric populations which in turn depends on those individuals' interpretations of intersexual signals.

Reproductive isolation via mate choice based on chemical communication is pervasive across metazoa (Brock & Wagner, 2018; Rosenthal, 2018; Smadja & Butlin, 2008; Wyatt, 2014), including swordtail fishes, where it is known to override preferences in the visual modality for some species (Crapon de Caprona & Ryan, 1990; Hankison & Morris, 2003). Chemical signaling can be extraordinarily fine-tuned compared to other modalities because of the diversity and specificity of odorant receptors with species-specific signals, sometimes varying only stereoisomerically (Leary et al., 2012; Xue, et al., 2007). Because of this specificity, anthropogenic perturbation of the signaling environment can disrupt chemical communication and result in a breakdown in behavioral reproductive isolation.

Chapter III of my dissertation focuses on how, in the face of behavioral reproductive isolation due to conspecific mating preferences in the *X. malinche* - *X. birchmanni* system, hybridization might be possible. Here I find that a common and deliberately introduced anthropogenic pollutant can not only disrupt chemical communication, but it does so in a way that should promote hybridization, causing female *X. birchmanni* to prefer the chemical cues of the heterospecific *X. malinche* over conspecific ones. I further show that this flip in preference valence is the result of a reduced preference for the conspecific cue coupled with a strengthening of preference for the sister species cue.

Lastly, the ways in which hybridization can affect trait distributions in hybrid populations depends largely on the genetic architecture underlying those traits. Mating preferences are often multivariate, but due to depleted genetic variance and mechanistic constraints on trait correlations in courter phenotypes, the trait combinations available in courters of a given species may not align with peak chooser preferences (Rosenthal, 2013; Van Homrigh, et al., 2007). Hybridization has the potential to better address chooser preference by breaking up these trait correlations through recombination and independent assortment of non-linked alleles. If and how this can happen depends on the genetic architecture underlying the multivariate traits under selection. The closely related *X. malinch*e and *X. birchmanni* differ in many male secondary sexual traits including the caudal fin extension for which the group gets the name swordtail. The "sword" is composite trait in and of itself, consisting of both morphological and pigmentation differences fixed between the two species. In chapter IV I use controlled intercrosses to perform classical QTL mapping for many of these traits that exhibit correlated phenotypes within the two

species. Here I uncover a single QTL for each of 5 separate traits, none of which colocalize to any one chromosome suggesting independent genetic pathways control these traits. As such, trait combinations might be expected to vary outside the distributions for either parental species in hybrid populations which aligns with patterns observed in the wild (Rosenthal et al., 2003).

CHAPTER II

FITNESS AND MATING BIASES IN EARLY GENERATION HYBRIDS OF SWORDTAIL FISH

Introduction

A growing body of work has highlighted the evolutionary importance of genetic exchange between divergent populations (Abbott et al., 2013; Barton & Hewitt, 1989): from reinforcement of reproductive barriers (Coyne & Orr, 2004; Kronforst et al., 2007), to the introgression of adaptive traits that allow the exploitation of novel niches (Pereira et al., 2014; Servedio, 2001), and even the creation of new species (Abbott et al., 2013; Mallet, 2007; Schumer, Rosenthal, et al., 2018). Much of this work focuses on the genotypes and phenotypes of individuals from wild hybrid zones which have likely undergone many generations of selection (Abbott et al., 2013). However, a crucial question is how selection acts on the first few generations of hybrids to determine the evolutionary trajectory of future generations. Early generation hybrids are often rare in established hybrid zones due in part to the reduced frequency of interactions between parentals in populations consisting primarily of hybrids (Gröning & Hochkirch, 2008). Accordingly, we often observe only later-generation hybrids that reflect the outcome of multiple generations of selection.

Some basic processes in evolutionary genetics shape and are shaped by the fitness of early generation hybrids. The direction of hybridization is often asymmetric, where traits from one

species introgress into the other with little gene flow in the opposite direction. Fitness of reciprocal F_1 hybrids is also often asymmetric, with a greater reduction of fitness in one F_1 cross than the other (Jiggins et al., 2008; Schrader et al., 2013). For example, cyto-nuclear incompatibilities in cultivated x wild rice hybrids cause F_1s possessing cytoplasm from the cultivated species to be completely pollen sterile (Yamagata et al., 2010). Additionally, taxa with sex chromosomes are prone to Haldane's rule, wherein F_1s of the heterogametic sex suffer reduced fitness due to deleterious recessive alleles being expressed in the hemizygous state (Good et al., 2008; Michael Turelli & Moyle, 2007; Turelli & Orr, 1995). Alternatively, such asymmetric hybridization may be due to premating behavioral mechanisms such as mate choice, where asymmetric mating preferences can lead to unidirectional introgression of display traits (Stein & Uy, 2006; Uyeda, et al., 2009; Wirtz, 1999), as in red-backed fairy wrens where two hybridizing subspecies that differ in a conspicuous male trait, red vs orange back color, show unidirectional introgression of the red back trait into the orange population due to female preference for the red back (Baldassarre, White, Karubian, & Webster, 2014).

Further, selection can act on hybrid generations beyond the F_1 in myriad ways. Novel combinations of alleles can produce extreme traits outside of typical parental values, which may be deleterious or advantageous with respect to selection (Pereira et al., 2014). Hybrid breakdown, or accelerated inviability of hybrids beyond the first generation, often occurs when closely related species hybridize (Burton, 1990; Matsubara et al., 2007; Turner & Harr, 2014). This reduction in fitness is due to the segregation of species-specific interacting loci as a result of recombination. These so-called Bateson-Dobzhansky-Muller incompatibilities (BDMIs) are

common across taxa and represent a strong source of viability selection against hybridization (Gröning & Hochkirch, 2008; Orr & Coyne, 1989; Presgraves, 2003; Turner & Harr, 2014).

Natural selection on viability during the first generations of hybridization can be critical in shaping patterns of genetic exchange (Abbott et al., 2013). By contrast, we know little about the evolutionary consequences of sexual selection during early hybridization. Sexual selection on hybrid mating signals (McDonald et al., 2001) and through hybrid mating preferences (Rosenthal, 2013; Stein & Uy, 2006) can have major effects on fitness and on the distribution of matings among genotypes. The mating biases of parental species and early generation hybrids can act in concert or in opposition to viability selection. For example, females of one species may be more reluctant than the other to mate with heterospecifics. The mating traits of hybrids may be transgressive or intermediate and may be attractive or aversive to parentals, either promoting or inhibiting introgression of traits via backcrossing. Hybrid females may exhibit similar preferences to one parental species or the other, or have intermediate and more permissive preferences facilitating further hybridization, or hybrids may have novel preferences, effectively limiting gene-flow between species. Consequently, the dynamics of genetic exchange between divergent populations depend not only on viability of hybrids but also on individual mating decisions (Rosenthal, 2013; Rosenthal, 2017), which in turn are shaped by the novel genotypes and phenotypes of hybrids.

The morphologically divergent but naturally hybridizing swordtail fish *Xiphophorus birchmanni* and *X. malinche* are an ideal system for exploring the consequences of hybridization in its early stages as interpecific crosses within this genus have been produced for years (Gordon, 1937;

Rosenthal & De León, 2006) and numerous studies have characterized both male secondary sexual traits and female mating preferences for these traits (Basolo & Trainor, 2002; Basolo, 1990; Johnson & Basolo, 2003; Kingston, Rosenthal, & Ryan, 2003; Rosenthal, Evans, & Miller, 1996; Ryan & Wagner, 1987). Hybridization between these two closely related species is likely the result of a breakdown in conspecific mate recognition resulting from anthropogenically induced disruption of olfactory communication (Fisher, Wong, & Rosenthal, 2006). Hybrid zones, which are present in all seven stream reaches where the two species' ranges are known to overlap, conform to a model of bounded hybrid superiority as a function of thermal tolerance (Culumber, et al., 2012; Moore, 1977).

Behavioral studies indicate that some hybrids are more attractive to parental females due to transgressive phenotypes caused by the introgression of secondary traits from one species into the other (Fisher, Mascuch, & Rosenthal, 2009), but hundreds of genetic incompatibilities have been identified (Schumer et al., 2014) suggesting a strong potential for reduced hybrid viability. Here I quantify viability, male sexual signals, and female mating preferences in the early stages of hybridization between *X. birchmanni* and *X. malinche* by rearing F_1 and F_2 intercross hybrids concurrently with both parental species in a common garden.

Methods

Parent collection and offspring production and rearing

Previous attempts to produce reciprocal F_1 crosses between *Xiphophorus birchmanni* and *X. malinche* has been largely unsuccessful. Attempts to cross female *X. birchmanni* to *X. malinche*

males have resulted in severely reduced offspring viability compared to the reciprocal cross. Offspring were born underdeveloped and weak with only two out of 23 pregnancies from 14 different dam-sire combinations producing offspring that survived past the two-week mark. Median brood size was three compared to 20 in the reciprocal cross. There were three additional instances where pregnant females died shortly prior to parturition as indicated by larval development. For this reason, I focused on the *X. malinche* x *X. birchmanni* cross. This cross produces viable broods of similar size to pure parental matings (Kindsvater, et al., 2012). Sperm storage for up to 10 months is a well-documented phenomenon in other poeciliid fishes (Kobayashi & Iwamatsu, 2002; López-Sepulcre, et al., 2013). In *X. malinche,* eggs may be fertilized by sperm stored for more than 12 months (personal observation). Thus, in order to be certain of offspring genotype it was necessary to rear females from juveniles in order to ensure virginity prior to crossing.

In order to make direct comparisons between both parental species and F_1 and F_2 intercross hybrids while controlling for environmental factors outside of epigenetic effects, it was necessary to simultaneously produce offspring from all four of these classes. To accomplish this, I first reared virgin female *X. malinche* derived from pregnant wild females collected in spring of 2014 using baited minnow traps from the Chicayotla locality on the Rio Xontla (20°55'27.24"N 98°34'34.50W). Those females were then crossed to wild collected adult *X. birchmanni* from the Rio Coacuilco at the town of Coacuilco (21°5'50.85 N, 98°35'19.46 W) (Fig. S1). Both parental populations are allopatric to other northern swordtail species. After parturition, all adult fish were removed to avoid potential fertilization from the adult male *X. birchmanni*. These F_1s were allowed to freely interbreed to produce F_2 intercross offspring. Concurrently, a new set of *X.*

malinche offspring were reared to maturity to produce virgin females to serve as dams for the F_1 class for the common garden experiment. Upon maturity this set of virgin *X. malinche* females were crossed to wild collected *X. birchmanni* males producing a cohort of F_1 offspring. In order to produce pure *X. malinche* and *X. birchmanni* offspring of approximately the same age as the two cross generations described above, late term pregnant females of both species were collected from the wild in May of 2016. All crosses and subsequent brood production and rearing was done in 2000 L, semi-natural mesocosms at the CICHAZ field station in Calnali, Hidalgo, Mexico. All offspring of all four classes were born between May 16 and May 24, 2016, at which time offspring from each class were randomly assigned to one of three replicate 2,000 L semi-natural mesocosms for a total of 12 tanks (three per class, n = 34 per tank).

Offspring were then reared to maturity in the common garden. Mesocosms were fed by a constant flow of dechlorinated municipal tap water which is captured from a small tributary of the Rio Calnali upstream of the town of Calnali. CICHAZ is situated at 900m above sea level, a typical elevation for hybrid zones between *X. birchmanni* and *X. malinche,* one of which occurs in the Rio Calnali less than 300 meters from the mesocosm site (Culumber et al., 2011). In addition to naturally occurring food items such as periphyton and macroinvertebrates, fish were fed twice daily with a combination of high-quality granules (Ken's fish) and freeze dried newly hatched brine shrimp (Brine shrimp direct).

Thermal tolerance (critical thermal maxima)

Due to experimental power considerations and the limited number of common garden individuals available I focused on critical thermal maxima which were more informative than minima in a

previous study that tested both common garden reared and wild fish caught in this system (Culumber et al., 2012). Accordingly, critical thermal maxima were measured in February 2018 using trials closely following Culumber et al. (2012). Briefly, the test fish, a standard glass thermometer and a HOBO temperature logger (Onset) were placed in an enamel container holding 4 L of water at ambient mesocosm temperature (16.1 ± 0.2°C) was nested in a larger container of water which was suspended above a gas burner. Water was heated at a rate of 0.3 °C/min until the fish lost equilibrium. Temperature at initial loss of equilibrium was recorded. Because the data departed from the assumption of normality, a Kruskal-Wallis rank sum test to test the effect of genotype class was performed in R.

Counts and measures

All fish were lightly anesthetized with tricaine methanesulfonate and digitally photographed for morphometric analysis in July, September and November of 2016, and January and March of 2017 at which time number surviving as well a sex and maturity status of developing males was recorded. Tissue samples were taken from the upper third of the caudal fin in January 2017 after which each fish was tagged by elastomer injection (Northwest Marine Technologies) with a unique color code for tracking. Sex was recorded upon the onset of differentiation as determined by the presence of a gravid spot in females and initial development of the gonopodium, the sexual intromittent organ formed from by modification of the anal fin, in males. Upon sexual maturity, indicated by complete gonopodial development, traditional morphometric measurements for standard length, body depth, dorsal fin width, dorsal fin height, gonopodium length, and sword extension length in mm (Fig. S2) were made for males using ImageJ (Abràmoff, Magalhães, & Ram, 2004). Principal components analysis (PCA) was conducted on

measurements standardized by standard length using the rda function in the vegan package of R. During a period of extended elevated temperature in late summer 2017 most of the *X. malinche* including all but one sexually mature male died. Because of this I further analyzed wild adult male *X. birchmanni* and *X. malinche* from the same populations as the common garden specimens for additional morphometric comparison. For both sets of analyses the first principal component (PC1) was largely influenced by sword extension, dorsal fin height, and dorsal fin width, with PC1 explaining approximately 33.5% of the phenotypic variation in the initial data set and 53.1% in the data set including wild fish. PC1 was used in all subsequent analyses of male phenotype. Mean PC1 scores across genotype class were compared using one-way ANOVAs and Tukey HSD post hoc tests for significant differences in R.

Xiphophorus males are known to have almost determinate growth in that growth rate slows substantially at the onset of sexual development (Evans, Pilastro, & Schlupp, 2011). Because of this feature I compared standard length of all males showing the initial stages of gonopodial development or later across all genotype classes following previous studies (Boulton, Rosenthal, Grimmer, Walling, & Wilson, 2016; Meffe & Snelson, 1989).

Mate choice assays

In order to assay the relative attractiveness of hybrid male chemical cues and mating preferences of hybrid females in the olfactory modality, a battery of simultaneous mate choice trials were conducted using *X. birchmanni* (n = 39), *X. malinche* (n = 43), F_1 (n = 27), and F_2 (n = 33) focal females from the common garden described above. Assays performed are summarized in Table 1. Briefly, parental females were tested for both heterospecific vs conspecific (Fisher et al., 2009;

Verzijden, Culumber, & Rosenthal, 2012), and conspecific vs F_1 cue combinations. F_1 females were tested for *X. birchmanni* vs *X. malinche* as well as *X. birchmanni* vs F_1, and *X. malinche* vs F_1 cue combinations. F_2 females were tested for *X. birchmanni* vs *X. malinche* cues. Cue combinations were randomized and all trials were run over the course of two weeks with a minimum of two days' rest between different cue combinations trials for any given focal female. Mate preference was tested following a well-established protocol in which two males stimuli are presented at either end of a trial lane (McLennan & Ryan, 1999; McLennan & Ryan, 1997). Male chemical stimuli for both conspecific (*X. birchmanni*) and heterospecifics (*X. malinche*) were prepared by placing five male fish in a 22 L tank filled with 20 liters of previously aerated and carbon filtered tap water for four hours. In order to elicit release of urine-born pheromones, visual stimulus was provided by an adjacent tank containing five conspecific females. Cues were prepared no more than 24 hrs in advance of testing and new cue was prepared for each day of tests.

Trials were conducted by placing a female *X. birchmanni* in a 75x19x20 cm trial lane and allowing her to acclimate for ten minutes. Lanes were divided into three sections of equal size (one association zone on either side where stimuli were presented and a central neutral zone containing a small acrylic shelter). Each lane was fitted with a stimulus delivery system at the end of each association zone run by peristaltic pumps. After the ten-minute acclimation period stimulus flow was initiated and delivered for 600s. To control for side bias each female was tested a second time with the cue presentation reversed. Water was changed and lanes were cleaned between trials.

Once a female visited both association zones, I recorded time spent in each stimulus association zone for 300s. Association time with a given stimulus has been shown to be a robust proxy for realized mate choices in swordtail fishes (Walling, Royle, Lindström, & Metcalfe, 2010). Association times were summed across both trials for each female. Females that failed to visit both association zones within 300s were scored as unresponsive and excluded from analysis. Association times across genotype classes were compared using ANOVA Tukey HSD post hoc tests for significant differences. One sample T tests were used to determine significant net association times within classes and two sample T tests for between cue combination comparisons for any given genotype class. All statistical analyses were performed in R.

Results

Survivorship, sex ratio, male maturity proportion

There was no difference in survivorship at the 10-month time point across groups (two-sided Fisher's exact multinomial test: $p = 0.7575$). Within-group sex ratios did not differ from 1:1 for any group (Binomial test, two-sided: *X. birchmanni*, $N = 91$, $p = 0.1418$; *X. malinche*, $N = 90$, $p = 0.9161$; F_1, $N = 80$, $p = 0.1456$; F_2, $N = 81$, $p = 0.2664$). However, proportion of fully mature males at the 10-month time point varied significantly across genotype groups (two-sided Fisher's exact multinomial test: $p = 5.237e\text{-}07$) (Fig. 1). A larger proportion of surviving F_2 ($N = 21/40$) males were fully mature than *X. birchmanni* ($N = 15/53$: two-sided pairwise binomial test: $p = 0.0241$) or F_1 males ($N = 11/45$: two-sided pairwise binomial test: $p = 0.0196$) which did not differ from one another (two-sided pairwise binomial test: $p = 0.819$) but had larger maturity

proportions than *X. malinche* (N = 1/46: two-sided pairwise binomial test: p = 0.00338 and p = 0.000876 for X. birchmanni and F_1s respectively).

Table 1. Cue combinations tested for each genotype class. Order of cue was combination presentation randomized across individuals.

Female focal group	**Cue combination**
X. birchmanni	*birchmanni* vs *malinche,* *birchmanni* vs F_1
X. malinche	*birchmanni* vs *malinche,* *malinche* vs F_1
F_1	*birchmanni* vs *malinche,* *birchmanni* vs F_1, *malinche* vs F_1
F_2	*birchmanni* vs *malinche*

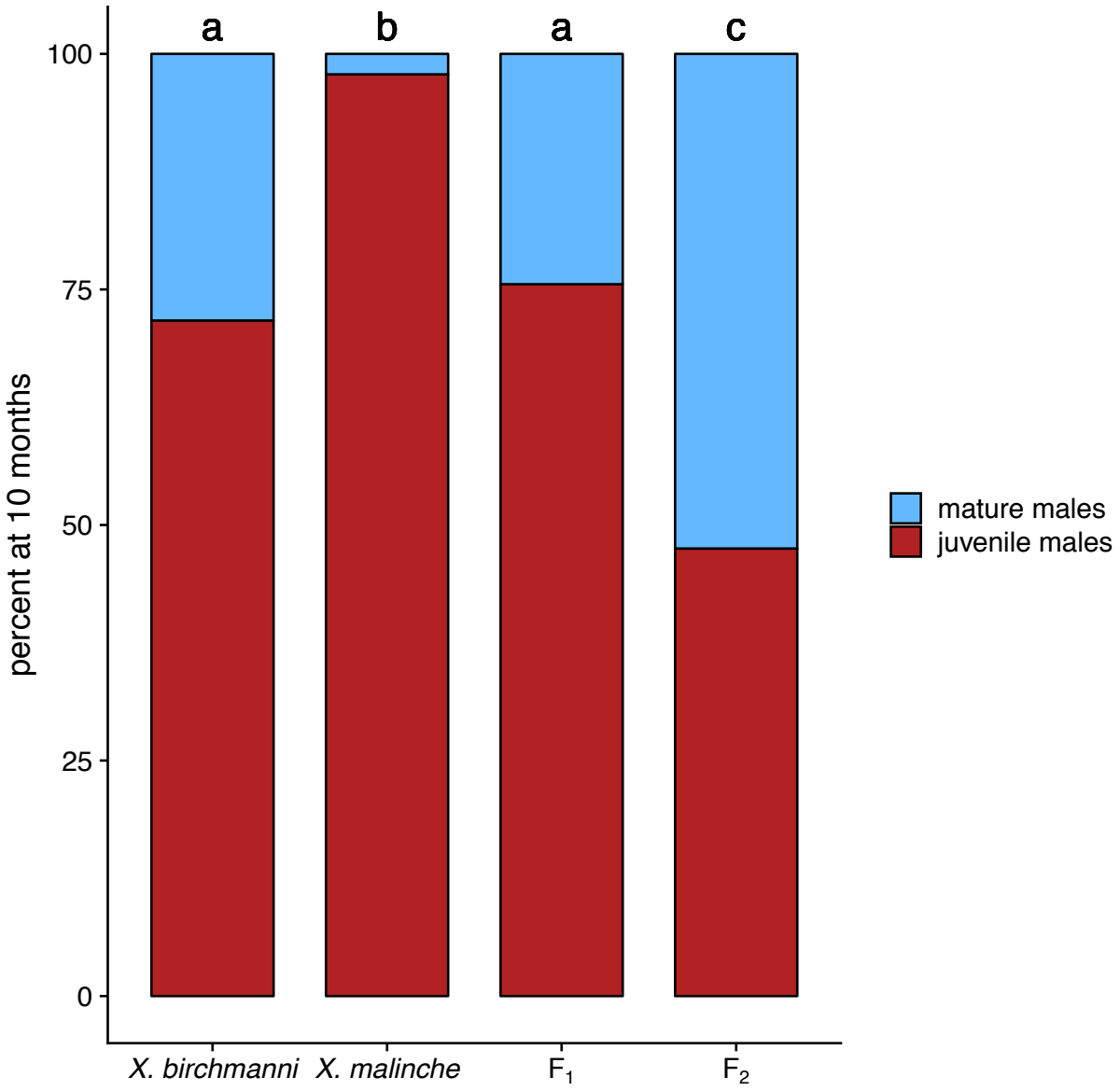


Figure 1. Proportion of mature males (blue) vs immature males (red) at 10 months of age. Different letters represent significantly different proportions.

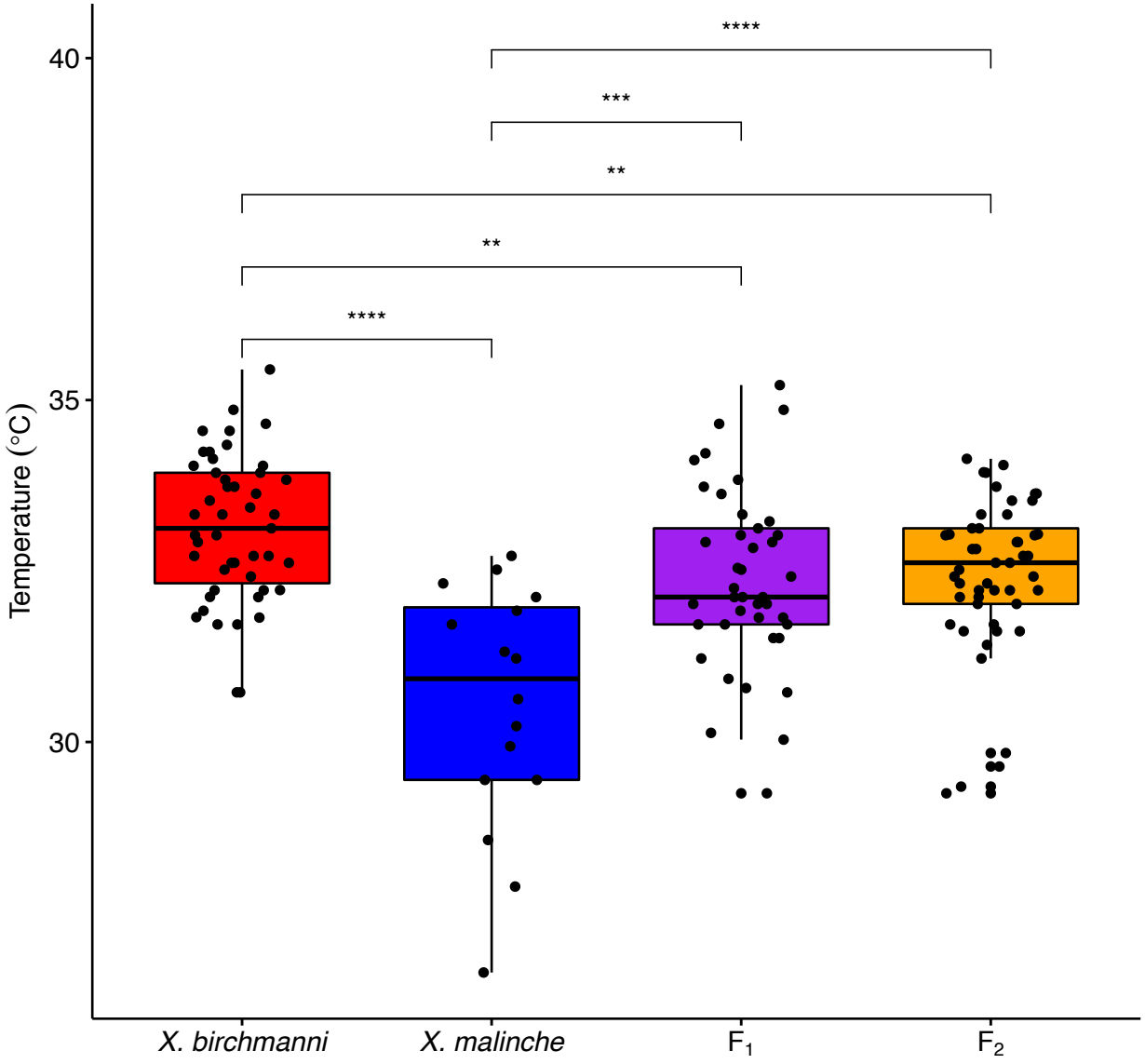


Figure 2. Critical thermal maxima for X. *birchmanni* (red), *X. malinche* (blue), F_1 (purple), and F_2 (orange) common garden fish. Asterisks indicate significant differences between groups.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

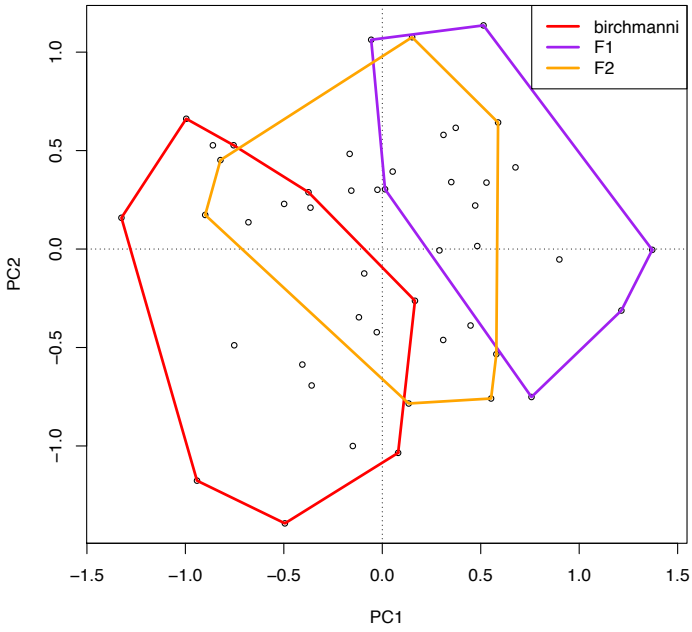
Thermal critical maxima

Genotype significantly affected critical thermal maxima across genotype groups (Kruskal-Wallis chi-squared = 30.783, df = 3, p = 9.442e-07) with *X. birchmanni* exhibiting a higher mean maximum than all other groups and *X. malinche* a lower mean (Dunn test, Benjamani-Hochberg adjusted p-values: p = 0.016 - 3.46e-07 and p = 0.0029 - 3.46e-07 respectively) (Fig 2). F_1 and F_2 males were intermediate to both parental groups but did not differ significantly from one another (Dunn test, Benjamani-Hochberg adjusted p-values: p = 0.54).

Morphometric analysis

The first principal component (PC1) explained approximately 33.5% of the phenotypic variance in the traits measured among common-garden reared males, with sword extension, dorsal fin height, and dorsal fin width loading most heavily on this axis (Fig. 3a). PC2 which was most influenced by body depth, peduncle depth, and sword extension explained an additional 27.0% of phenotypic variance (Tables S1-2). Genotype class had a significant effect on PC1 scores (ANOVA: F(2,44) = 19.14, p = 1.05e-06) with F_2 male having larger mean PC1 scores than *X. birchmanni* and F_1 males having larger scores than F_2 and *X. birchmanni* males (Tukey HSD: p = 0.017 - 0.0000007)(Fig 3b). For the extended male morphology data set including wild male *X. birchmanni* and *X. malinche,* PC1 which was most influenced by sword extension explained approximately 53.1% of phenotypic variance (Fig. 4a). PC2 which was most influenced by dorsal fin width explained approximately 40.9% of variance (Tables S3-4). Genotype class had a highly significant effect on PC1 scores (ANOVA: F(4,97) = 169.7, p < 2e-16) with wild caught *X. malinche* having the highest scores and tank reared *X. birchmanni* the lowest. All groups

a)



b)

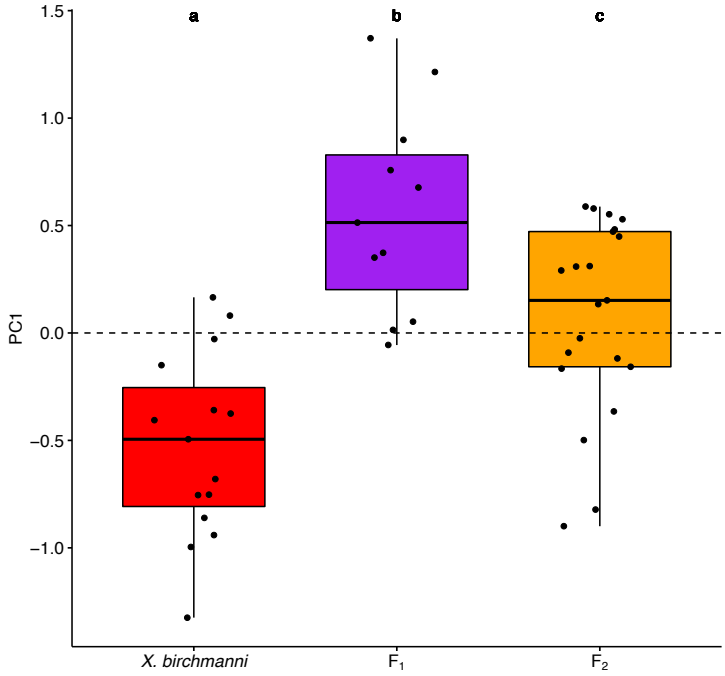


Figure 3. Male phenotypic distribution of mesocosm reared fish and boxplot of PC1 scores. a) Male phenotypic distribution of mesocosm reared fish, red = *X. birchmanni*, purple = F_1, orange = F_2 males. PC1 is positively associated with sword extension and negatively associated with dorsal fin height, and width. PC2 is positively associated with peduncle depth, body depth and sword extension. b) Boxplot of PC1 scores. Groups with nonmatching letters have significantly different means, $p < 0.05$.

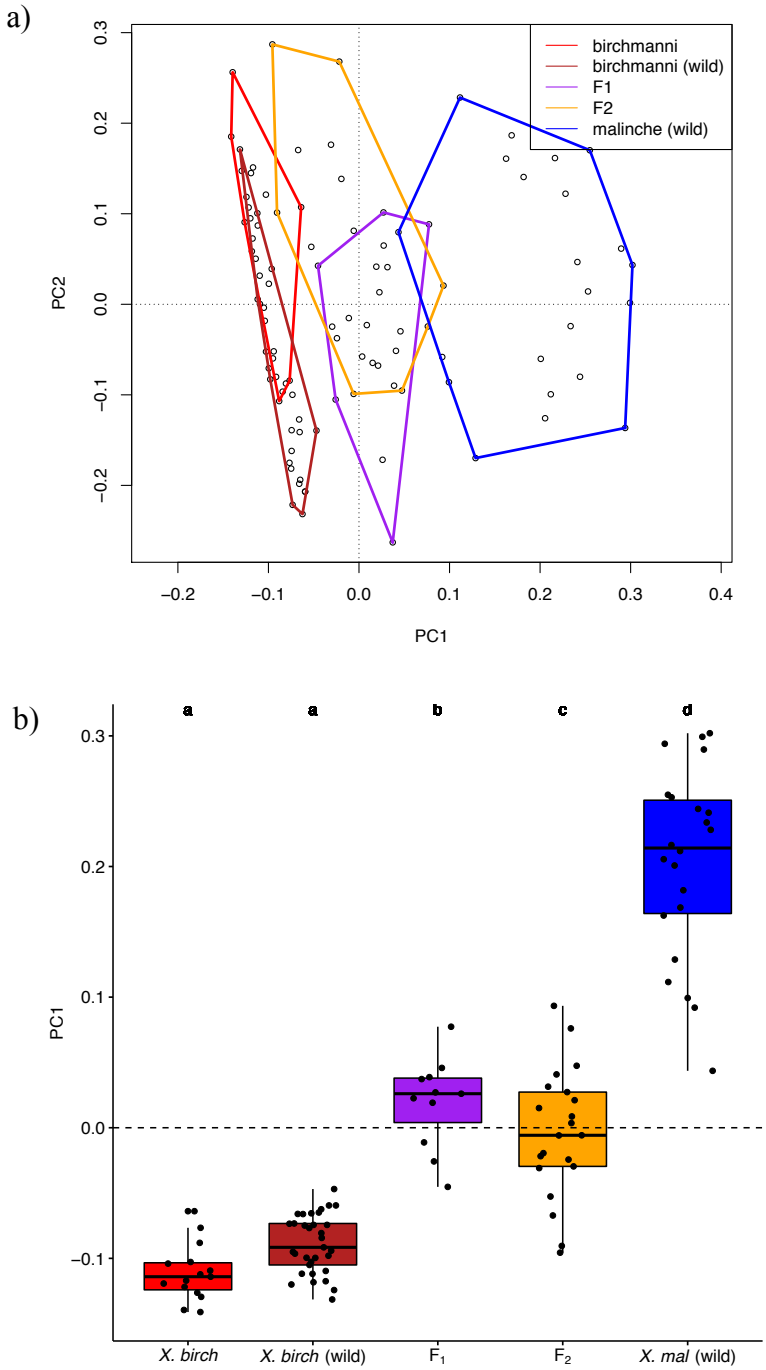


Figure 4. Male phenotypic distribution of mesocosm reared fish and wild caught parentals and boxplot of PC1 scores. a) Male phenotypic distribution of mesocosm reared fish and wild caught parentals, red = *X. birchmanni*, brick red = wild *X. birchmanni*, purple = F_1, orange = F_2 males, blue = wild *X. malinche*. PC1 is positively associated with sword extension. PC2 is positively associated with dorsal height. b) Boxplot of PC1 scores. Groups with nonmatching letters have significantly different means, $p < 0.05$.

differed except for tank reared *X. birchmanni* and wild caught *X. birchmanni*, and F_1 and F_2 tank reared males (Tukey HSD: p = 0.000) (Fig 4b). Mean male standard length, including all males with at least partially developed gonopodia, did not vary across genotype classes (one way ANOVA: F(3,177) = 1.41, p = 0.241).

Chemical cue mate choice assays

Responsiveness did not vary across genotype class (*X. birchmanni* N = 27/39*, X. malinche* N = 22/43, F_1 N = 15/27, F2 N = 20/33; two-sided Fisher's exact multinomial test: p = 0.887). Association time with pooled male *X. birchmanni* chemical cue differed significantly according to genotype group when presented opposite the pooled *X. malinche* cue (one way ANOVA: F(23,76) = 3.263, p = 0.0259) with net association time of *X. birchmanni* females with the *X. birchmanni* cue being significantly greater than that of *X. malinche* females (TukeyHSD: p = 0.0365647) (Fig. 5). *X. birchmanni* females spent significantly more time in association with the *X. birchmanni* chemical cue over *X. malinche* (one-tailed, one sample T test: t = 2.0535, df = 20, p = 0.02667) while *X. malinche* females associated significantly with *X. malinche* male chemical cue over *X. birchmanni* cue (two-tailed, one sample T test: t = 2.3692, df = 21, p = 0.02749). Neither F_1 (two-tailed, one sample T test: t = 0.025177, df = 16, p = 0.9802) nor F_2 (two-tailed, one sample T test: t = 1.1316, df = 19, p = 0.2719) females differed from zero in their mean net preference for *X. birchmanni* versus *X. malinche* cues (Fig. 5). Further variance in net association time did not differ across genotype (Levene's test for homogeneity of variance: F(3,76) = 1.4185, p = 0.2439).

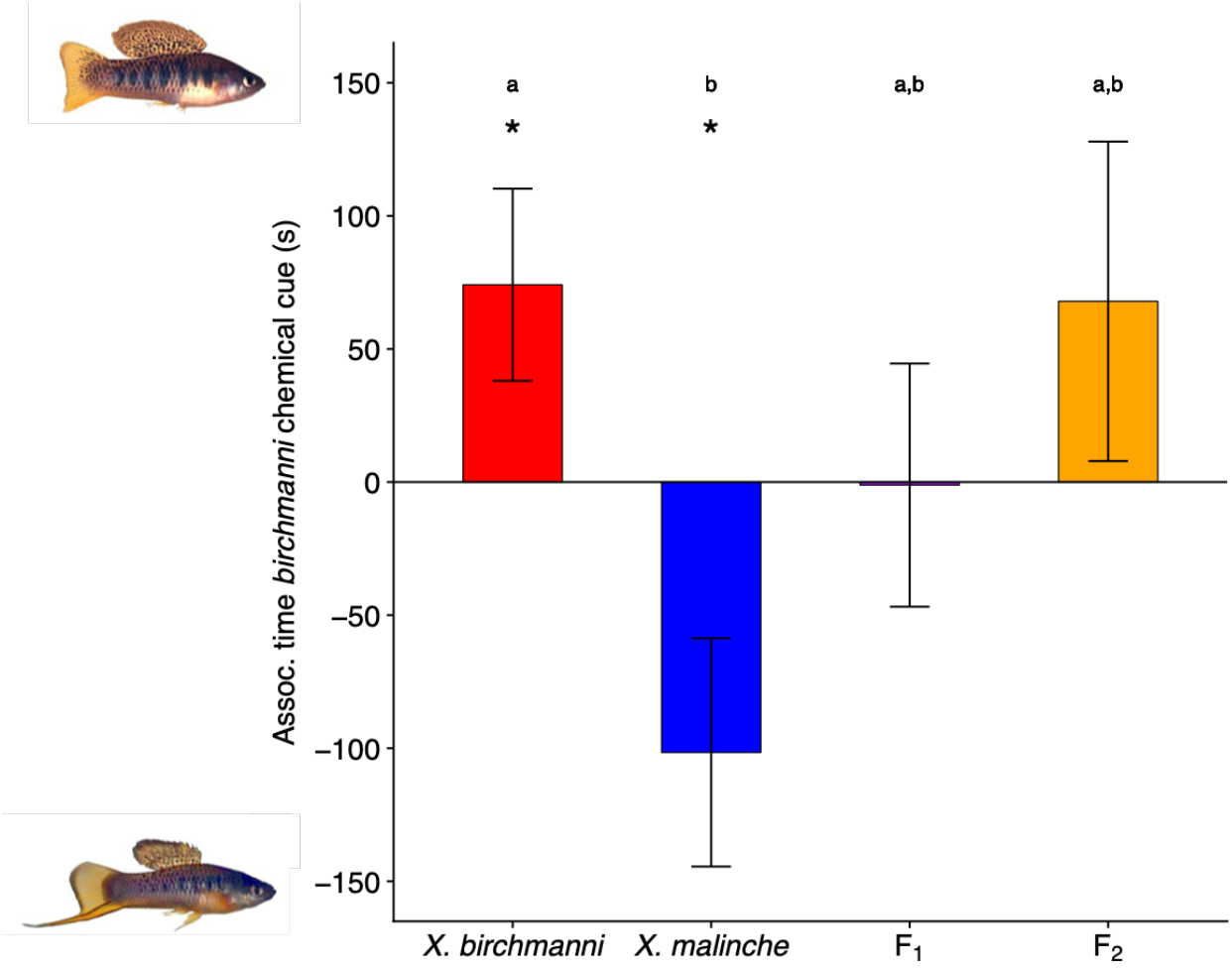


Figure 5. Female mate preference: *X. birchmanni* vs *X. malinche* chemical cues. Bar height represents mean net association time ± SE with *X. birchmanni* over *X. malinche* pooled male chemical cues for female *X. birchmanni* (red), X. malinche (blue), F_1 (purple), and F_2 (orange). Groups with nonmatching letters have significantly different means, $p < 0.05$. Asterisks indicate mean net association times significantly different from 0. * $p < 0.05$ (two-tailed one sample T tests).

F1s showed intermediate chemical signals and preferences. Neither *X. birchmanni* (two-tailed, one-sample test: t = -1.872, df = 20, p = 0.0759) nor *X. malinche* (two-tailed, one-sample test: t = -0.98972, df = 26, p = 0.3314) females showed a significant net association time when tested for pooled conspecific male chemical cues versus F_1 male cues. Likewise, F_1 females did not show a significant preference when either *X. birchmanni* (two-tailed, one-sample test: t = 1.2134, df = 23, p = 0.2373) cues or *X. malinche* (two-tailed, one-sample test: t = -0.23576, df = 23, p = 0.8157) cues were tested against F_1 male cues. F_1 cues were significantly more attractive than heterospecific cues for both female *X. birchmanni* (two-tailed, two-sample T test: t = 2.746, df = 38, p = 0.009142) (Fig. 6a) and *X. malinche* (Fig. 6b) (two-tailed, two-sample T test: t = 2.2635, df = 42, p = 0.02872). F_1 females showed no differences in association times across cue combinations (two-tailed, two-sample T tests: *birch* v *mal* / *birch* v F_1, t = -0.84641, df = 36, p = 0.4028; *birch* v *mal* / *mal* v F_1, t = 0.18703, df = 38, p = 0.8526) (Fig, 6c-d).

Discussion

Early-generation hybrids of *Xiphophorus malinche* and *X. birchmanni* are viable, attractive and have permissive mating preferences. Despite hundreds of identified genetic incompatibilities (Schumer et al., 2014) and inviability in the reciprocal cross, I found no evidence for reduced viability relative to the parentals for the female *X. malinche* x male *X. birchmanni* cross. Though I did not assay fertility in males (the heterogametic sex in swordtail fish) I take the recovery of even sex ratios across all groups including early generation hybrids as evidence that Haldane's

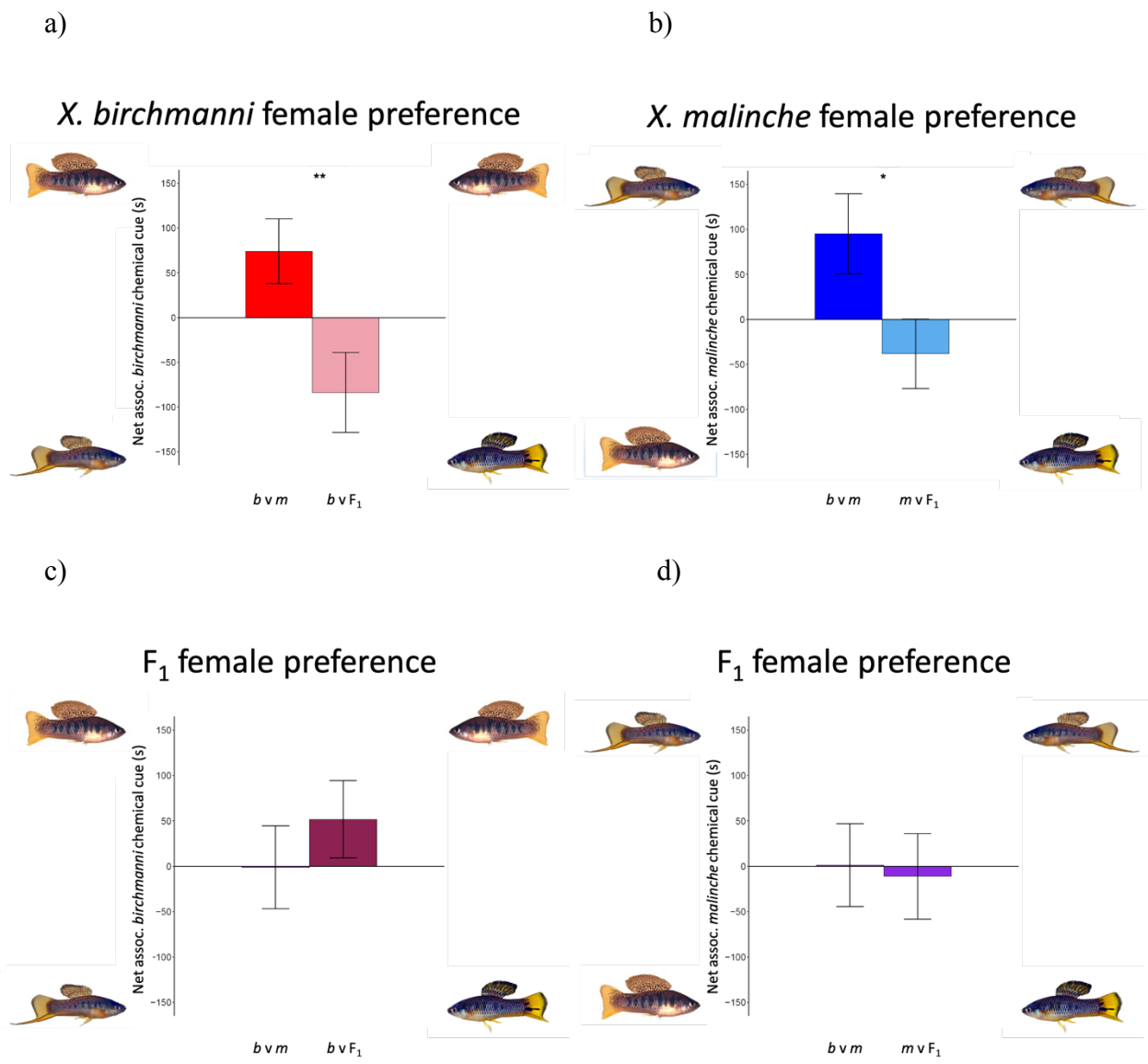


Figure 6. Female mate preference comparisons. a) Female *X. birchmanni* mean net association time with *X. birchmanni* male chemical cues when tested against *X. malinche* on the left (b v m, red) and F_1cues on the right (b v F_1, pink). b) Female X. malinche mean net association time with *X. malinche* male chemical cues when tested against *X. birchmanni* on the left (b v m, blue) and F_1 cues on the right (m v F_1, light blue). c) Female F_1 mean net association time with *X. birchmanni* male chemical cues when tested against *X. malinche* on the left (b v m, purple) and F_1 cues on the right (b v F_1, mauve). d) Female F_1 mean net association time with *X. birchmanni* male chemical cues when tested against *X. malinche* on the left (b v m, purple) and F_1 cues on the right (b v F_1, orchid). Error bars represent SE. Asterisks indicate significantly different mean net association times across cue combinations. * $p < 0.05$, ** $p < 0.01$ (two-tailed one sample T tests).

rule is not a strong source of reproductive isolation in these fish. This finding is potentially explained by the relatively young age and small non-recombining region of the Y chromosome in this system (Volff & Schartl, 2002). Hybrids did not exhibit reduced survivorship but male time to full sexual maturity differed, with F_1s developing at a similar rate to one partental species and F_2s developing faster than all other classes. There is often a trade-off between maturation rate and final adult size in organisms with determinate growth representing alternative reproductive tactics, as has been shown for other members of the genus *Xiphophorus* (Boulton et al., 2016; Lampert et al., 2010; Ryan, Pease, & Morris, 1992). However, I did not find evidence for that phenomenon here. Despite the different male maturation rates, standard length did not differ across genotype class at the onset of gonopodial development the time when growth rate drastically slows in these fish (Evans et al., 2011).

Hybrid males' visual phenotypes generally appeared intermediate when compared to wild parentals. Groups primarily separate across the PC1 axis which is largely explained by sword extension, a trait that is generally absent in *X. birchmanni*. X. malinche have longer swords than hybrids which have longer swords than *X. birchmanni* (Fig. 3). When only the common garden *X. birchmanni*, F_1s and F_2s were considered, dorsal fin measurements along with sword extension differentiated the groups (Fig. 2).

As expected from previous work on wild hybrids in this system (Culumber et al., 2012), thermal critical maxima were intermediate to the parentals (Fig. 3). Though not addressed by this study, intermediate thermal tolerance may represent a fitness benefit or hybrids at the intermediate

elevations where hybrids zones occur if maximum summer temperatures exceed tolerances for *X. malinche* and winter minima are lower than tolerances for *X. birchmanni* helping to explain the abundance of later generation hybrid individuals at intermediate elevation which better fits a model of bounded hybrid superiority than the classical tension zone (Barton & Hewitt, 1989; Culumber et al., 2011; Culumber et al., 2012; Hewitt, 1988; Moore, 1977).

Though I found no evidence for natural selection against hybrids in our crosses, sexual selection via mate choice potentially represents a strong source of behavioral reproductive isolation. For natural selection to act on hybrid phenotypes, individuals must first make heterospecific mating decisions. Further the attractiveness of and preferences of the resulting hybrid individuals will determine whether those initial choices have lasting evolutionary consequences within sympatric populations. Here I show that pooled F_1 hybrid male chemical cues are at least as attractive as conspecific cues are to females of both parental species. Both *X. birchmanni* and *X. malinche* females significantly preferred conspecific chemical cues over heterospecific cues (Fig. 5), but when conspecific cues were tested against first generation hybrid cues, mean net association time with the conspecific cue was not significant. In fact, the trend for both parental female groups was a mean net F_1 preference, and the differences in mean net association with the conspecific cue between the two choice trials (conspecific vs heterospecific and conspecific vs F_1) was significant for both species (Fig. 6a,b). In addition, despite responsiveness to cues similar to parental levels, early generation hybrid females are less choosy. F_2 females showed no preference for either *X. birchmanni* or *X. malinche* male chemical cues, and F_1 females

attended to both cues in all cue combinations equally displaying no significant mean net preference. One possible explanation for this reduced choosiness in hybrid females is mis-expression of receptors within olfactory receptor neurons in the sensory periphery as seen in hybrid *Rhagoletis* flies (Olsson et al., 2006).

Hybridization has the potential to produce universally more attractive courters because multivariate trait preferences of choosers are often mismatched with realized trait distributions (Rosenthal, 2013). Phenotypic correlations due to mechanistic constraint and depleted genetic variance can be broken in recombinant genotypes to produce courter traits more closely matched to chooser preferences (Fisher et al., 2009; Van Homrigh et al., 2007). This may be the case for the *X. birchmanni* - *X. malinche* hybrid chemical cue. The pheromone profiles of these two species are complex but differ in only two chemical compounds, a testosterone sulfate compound and a small urinary conjugated bile acid, cholanic acid, always found in male *X. birchmanni* and never in male *X. malinche* (Holland, 2018). Here there is potential for recombinant pheromone profiles to be more attractive to females of one or both parental species as seen in this study.

Conclusions

Even if relatively strong behavioral barriers to gene flow are present in the form of conspecific mate preferences, the rare hybridization event may lead to continued genetic exchange if

hybrid courter signals are universally attractive and hybrid choosers are permissive. Hybrids from one cross direction did not show reduced viability despite many known genetic incompatibilities, though it is clear the these incompatibilities shape the evolutionary trajectories of hybrid populations (Schumer et al., 2014). This finding is complemented by no evidence of reproductive isolation between hybrids and parentals, so even if the reciprocal cross never produce viable offspring, gene flow may still be bi-directional due to backcrossing of female *X. birchmanni* to hybrid males. As such the results of this study support a scenario of ongoing genetic exchange between these two species which reflects patterns observed in the wild.

CHAPTER III

A WIDELY-USED POLLUTANT CAUSES REVERSAL OF CONSPECIFIC MATE PREFERENCE IN A FRESHWATER FISH

Introduction

It is increasingly apparent that hybridization is common and can have important evolutionary consequences, ranging from the reinforcement of reproductive barriers, to the loss of genetic differentiation between divergent populations (Abbott et al., 2013; Barton & Hewitt, 1989; Coyne & Orr, 2004). It also allows for the introgression of adaptive traits from one species into another (Kronforst et al., 2007; Ru et al., 2018) and may rarely be a direct cause of speciation (Buerkle, Morris, Asmussen, & Rieseberg, 2000; Mallet, 2007; Schumer, et al., 2018). However, gene flow between divergent populations is ultimately mediated by the mating decisions of individuals within those populations (Ritchie, 2007). Thus, mate choice dynamics can play a critical role in assortative mating with respect to genotype allowing it to contribute to intrapopulational divergence (Butlin et al., 1994). Conversely, it can also promote gene flow between lineages if it induces individuals to mate with heterospecifics (Rosenthal, 2013; Servedio, 2001). Heterospecific mating decisions often incur fitness costs for individuals, and can lead to biodiversity loss through hybridization (Seehausen, et al., 2008).

Behavioral reproductive isolation based on chemical communication is pervasive across metazoans (Brock & Wagner, 2018; Rosenthal, 2018; Smadja & Butlin, 2008; Wyatt, 2014).

Such chemical signaling can be extraordinarily fine-tuned compared to other modalities because of the diversity and specificity of odorant receptors with species-specific signals sometimes varying only stereoisomerically. (Leary et al., 2012; Xue et al., 2007). Because of this specificity and the reliance of some species on chemical communication, environmental disturbances that either change chemical signals or their receptors in some way has the potential to disrupt olfactory signaling and break down pre-mating reproductive isolation between species (van der Sluijs et al., 2011; Wyatt, 2014).

Hybridization between two northern swordtail fish, *Xiphophorus birchmanni* and *X. malinche* is one such example, as it is likely mediated by a breakdown in behavioral isolation due to anthropogenically induced chemical signal disruption (Fisher, Wong, & Rosenthal, 2006; Rosenthal et al., 2003). In these fish, mate choice decisions are based on both visual and olfactory signaling, but as is the case in closely related congeners, responses to olfactory cues in these species likely override visual preferences (Crapon de Caprona & Ryan, 1990; Hankison & Morris, 2003). Further, Fisher and colleagues convincingly showed that intersexual communication in the olfactory modality can be disrupted by anthropogenic inputs into the river water (Fisher, Wong, & Rosenthal, 2006). Specifically, humic acid, a common byproduct of agricultural run-off and untreated sewage was sufficient to abolish otherwise strong conspecific preferences for male pheromones in female *X. birchmanni*. Likewise, conspecific preferences in clean river water were abolished when females were tested in polluted water from river stretches where *X. birchmanni* and *X. malinche* currently hybridize.

Many other compounds that find their way into stream water are known to affect chemical communication in fishes (reviewed in: Klaprat, Evans, & Hara, 1992; Lürling & Scheffer, 2007). Studies have largely focused on heavy metals and pesticides known to be endocrine disruptors (e.g. Honda, Fernandes-de-Castilho, & Val, 2008; Lee, et al., 2006; Sárria et al., 2011; Tomkins, et al., 2016; van der Sluijs et al., 2011; Ward, et al., 2007), however, other chemical compounds thought to be environmentally safe based on mortality assays have not been tested for their effects on chemical communication in aquatic organisms. One such inorganic compound that is often deliberately introduced into aquatic environments is calcium hydroxide ($Ca(OH)_2$), otherwise known as slaked lime, and as "cal" in the native range of *X. malinche* and *X. birchmanni*. Beyond its many industrial applications, $Ca(OH)_2$ is used for water treatment in several different capacities. It is often used to treat wastewater treatment plant effluents to reduce pathogen concentrations (Gerba, 1981; Grabow, Middendorff, & Basson, 1978b; Reinthaler et al., 2010) and as a flocculant for particulate matter and to precipitate out phosphate and heavy metal ions in an effort to prevent sedimentation and phosphate associated eutrophication (del Bubba, et al., 2004; Semerjian & Ayoub, 2003). Similarly, it is used to help combat eutrophication in lacustrine environments that are afflicted by agricultural run-off (Murphy, Hall, & Northcote, 1988). An example of such guidelines can be found in Murphy, et al. (1990) . Though "liming" of acidified lakes and streams affected by acid rain and mining activity to recover historical pH levels using calcium hydroxide was common prior to the 1990s this practice has been largely discontinued and $Ca(OH)_2$ has been replaced by the less caustic calcium carbonate ($CaCO_3$) (Clair & Hindar, 2005; Fraser & Britt, 1982; Johnson & Hallberg, 2005). Several assessments of the effects of "liming" in lakes on invertebrate fauna assemblages, which are generally detrimental on an immediate time scale but beneficial in the long term, have

been made but there is little mention in the literature of effects, adverse or otherwise, on vertebrates (Ghadouani, et al., 1998; Leoni et al., 2007; Miskimmin, Donahue, & Watson, 1995).

Over the course of 16 years of research in the *X. birchmanni – X. malinche* range researchers have observed many instances of the aftermath of calcium hydroxide being deliberately introduced into streams as an inexpensive method of disease prevention (Rosenthal, personal communication). Large amounts of powdered calcium hydroxide are introduced into riffles up-stream of pools frequented by humans and cattle as a method to reduce bacterial load via pH shock and flocculation (Grabow, Middendorff, & Basson, 1978a; Lucena et al., 2004; Reinthaler et al., 2010). Of course, it also creates a hostile environment for other stream organisms. In fact, because it often kills fishes at high enough doses it is employed, illegally (as indicated by local prohibition signage), as a fishing technique to catch food fish like mojarra (Cichlidae) and solemiche (Ictaluridae). Any organism, including swordtail fish, caught in the main flow of the poisoning suffers high mortality. However, many vagile organisms are able to avoid experiencing the most concentrated areas of the plumes and thus experience only nonlethal concentration of the chemical. Field observations indicate that exposure to unknown but sublethal concentrations of calcium hydroxide can affect some fishes' ability to perceive olfactory food cues and may disrupt chemical communication (personal observation).

There are three likely pathways by which altering the chemical environment can affect olfactory communication. First, it can affect the molecular structure of the signal itself by changing the composition or conformation of the signal molecules (Hubbard, Barata, & Canario, 2002). Second, it can affect the response sensitivity of olfactory receptor neurons (ORNs) to signal

molecules (Burnard, Gozlan, & Griffiths, 2008; Dew & Pyle, 2014; Lazzari, et al., 2017; Reisert & Matthews, 2001). Third, it may affect signal processing down-stream of the sensory periphery (Rosenthal, 2018).

Due to the intromittent pulsed nature of this point source pollution, increased calcium hydroxide concentration is likely to effect signal perception due to interaction with ORNs or down-stream neural processing rather than signal molecules themselves. Here I test the effects of short-term exposure of a sublethal concentration of calcium hydroxide on mate preference in the olfactory modality for female *X. birchmanni*. Based on reduced response to baited minnow traps after exposure observed in the wild, I predicted that chemical communication would be disrupted and the species typical strong conspecific preference would be abolished similar to the effect of humic acid shown by Fisher et al (Fisher, Wong, & Rosenthal, 2006). I then tested mate choice in the visual modality to control for the effect of calcium hydroxide on overall motivation to mate.

Though the water chemistry can be complex given variation in specific local conditions, the addition of calcium hydroxide to stream water has two main hydrochemical effects. First, it increases the Ca^{2+} ion concentration which may alter the efficacy of neuronal signaling as calcium plays an important role in ORN function (Nicholls, Martin, Wallace, & Fuchs, 2001). Calcium has been shown to be an odorant in *Pimephales* minnows which show neuronal excitation during electro-olfactography targeting the nasal epithelia as well as an avoidance behavioral response (Dew & Pyle, 2014). Second, it increases the pH of the solution due to the influx of OH^- ions. The pH of a saturated calcium hydroxide solution at 24 C is 12.2. Changes in

pH are known to affect chemical communication in many aquatic organisms including teleost fishes. For example, increasing the pH of male chemical cues in three-spined stickleback cause an increased response from gravid females over non-manipulated cues (Heuschele & Candolin, 2007). In order to dissect apart the effects of the two ions formed from the dissolution of calcium hydroxide in water we ran further mate preference trials after exposing female *X. birchmanni* to either calcium chloride ($CaCl_2$) which does not affect the pH or sodium hydroxide (NaOH) which raises the pH but does not affect Ca^{2+} ion concentrations.

Methods

Fish collection and housing

All *X. birchmanni* (focal females and males chemical cue production) were collected between March 2018 and January 2019 from the Rio Coacuilco near the town of Coacuilco (21°5'50.85 N, 98°35'19.46 W). *X. malinche* males for chemical cue production were collected from the Chicayotla location on Rio Xontla (20°55'27.24"N 98°34'34.50W) (Fig. S1). All fish were housed at Texas A&M University in single sex 120 L tanks maintained at 22 °C and fed twice daily a combination of Repashy brand gel foods formulated for, algae-based flake foods, and live food items for at least two weeks prior to testing.

Simultaneous olfactory signal presentation preference trials

For all tests of the female olfactory modality, mate preference was tested following a well-established protocol in which two males stimuli are presented simultaneously at either end of a trial lane (McLennan & Ryan, 1999; McLennan & Ryan, 1997). Male chemical stimuli for both

conspecific (*X. birchmanni*) and heterospecifics (*X. malinche*) were prepared by placing five male fish in a 22 L tank filled with 20 liters of previously aerated and carbon filtered tap water for four hours. In order to elicit release of urine-born pheromones, visual cues of females were provided by an adjacent tank containing five conspecific females. Cues were prepared no more than 24 hrs in advance of testing and new cue was prepared for each day of tests.

Trials were conducted by placing a female *X. birchmanni* in a 75x19x20 cm trial lane and allowing her to acclimate for ten minutes. Lanes were divided into three sections of equal size (one association zone on either side where stimuli were presented and a central neutral zone containing a small acrylic shelter). Each lane was fitted with a stimulus delivery system at the end of each association zone run by peristaltic pumps. After the ten-minute acclimatization period, stimulus flow was initiated and delivered for 600s. To control for side-bias each female was tested a second time with the cue presentation reversed. Water was changed and lanes were cleaned between trials.

Once a female visited all three zones following the initiation of stimuli flow, time spent in each stimulus association zone was recorded for 300s. Association time with a given stimulus has been shown to be a robust proxy for realized mate choices in swordtail fishes (Walling et al., 2010). Association times were summed across both trials for each female. Females that failed to visit both association zones within 300s were scored as unresponsive and excluded from analysis.

For assays testing the effects of calcium hydroxide on odor preference between conspecific and heterospecific stimuli females were randomly assigned to the control (n = 14) or exposure group (n = 14) and tested on four separate days. On day zero both groups were given a sham exposure in which they were held in 4 L tanks (two per container) containing carbon filtered tap water for ten minutes prior to being placed into the trial lanes for acclimation. On day 2 the control group was given the same sham exposure while the exposure group was placed into identical 4 L tanks containing carbon filtered tap water and 24 mg/Lcalcium hydroxide for ten minutes prior to testing. On day 4, two days after exposure, and again on day 12, ten days post-exposure, both groups were once again treated with a sham exposure containing only carbon filtered water prior to testing. A 24 mg/L concentration is similar to some published recommended concentrations for eutrophication remediation and 10x less than the 24hr LC50 for *Gambusia affinis*, a closely related poeciliid (Wallen, et al., 1957). The 10-minute duration represents a typical breeding season flow time through pools in the Rio Calnali containing hybrid populations in the wild (mean surface flow rate = 0.0013 m/s ± 0.0003).

A similar set of trials using new females was performed for fish exposed to an equimolar concentration of $CaCl_2$ (35 mg/l) (control n = 16, exposure n = 16). A further set of trials was run using a NaOH exposure (control n = 16, exposure n = 16) titrated to a pH of 9.21 (15.4 mg/l, equal to the calcium hydroxide exposure). For both of these trial sets, day twelve was omitted as females had recovered species typical responses by day four.

Olfactory signal versus water control preference trials

In order, to further explore how calcium hydroxide exposure affects mate preference in *X. birchmanni* females, we tested a new set of females (control n = 16, exposure = 16) for conspecific chemical (*X. birchmanni*) signal against a water blank and heterospecific signal (*X. malinche*) against water. Each female was tested twice for each combination to control for side bias (four tests total per fish). The order and side of cue presentation was randomized. Trials were performed as described above for $Ca(OH)_2$ exposure trials.

Visual preference tests

Because we hypothesized that calcium hydroxide exposure would reduce or abolish conspecific preference mediated by chemical communication it was necessary for us to show that exposed fish were still motivated to mate despite an inability to perceive olfactory signals. To accomplish this, we tested mate preference of calcium hydroxide exposed females using *X. birchmanni* and *X. malinche* animated stimuli created in anyfish 2.0 (Ingley et al., 2015). Body shapes of animations were based on population means calculated from 42 morphometric landmarks according to the anyfish 2.0 user manual. A digital photograph of a representative male from each species was then used to generate body texture. The animations follow the same simple conserved courtship display typical for both species in which a male swim on screen raises his dorsal fin and tilts toward the direction of the test lane and shimmies briefly then swims off screen. Female *X. birchmann*i are known to prefer male conspecifics over *X. malinche* in the visual modality and are responsive to animation of males (Wong & Rosenthal, 2006).

Visual tests were run for three sets of previously untested *X. birchmanni* females (control n = 14, calcium hydroxide exposed n = 14, calcium chloride exposed n = 20). Visual trials for NaOH exposed fish were not conducted as their response in the olfactory trials was similar to the calcium hydroxide exposed fish with both groups responding strongly to sexual stimuli on the day of exposure.

Visual assays were conducted in the same test lanes as the olfactory trials. Animations were simultaneously displayed on either end of the test lanes using Samsung SyncMaster E1920X display monitors connected to a macbook pro laptop computer. Presentation closely followed Wong and Rosenthal (2006). Briefly, after chemical exposures as outlined above, females were acclimated for 10 minutes during which only the background image of the animations was displayed followed by a 5-minute simultaneous presentation of the test stimuli. The focal female was once again presented only the background image for 10 minutes, followed by another 5-minute presentation of the test stimuli presented on opposite sides as the first trial in order to correct for side bias. As in the olfactory trials the test lanes were divided into three equal zones and time spent in each association zone was recorded. Any fish that spent more than 90% of the time in any one zone across both trials was scored as nonresponsive and excluded from analysis.

Statistical analysis

To assess female preference in all chemical trials within a given group (control or exposure) for a given timepoint we used one sample t-tests for net association time with the conspecific cue. Where distributions did not meet the assumption of normality a Wilcoxon Signed-rank tests were substituted. To compare chemical trials within treatments (control, and exposure) across all time

points we used one way repeated measures ANOVA s for each chemical exposure ($Ca(OH)_2$, $CaCl_2$, and NaOH). Where response distributions or variances did not meet the assumption of normality or equality a Kruskal-Wallis rank-sum tests was substituted. Female preference in visual trials was assessed using a Kruskal-Wallis rank-sum test. All analyses were done in the R computing environment.

Results

Simultaneous olfactory signal presentation preference trials

Net association time with the conspecific olfactory cue did not differ significantly for control females across the $Ca(OH)_2$ trials (repeated measures ANOVA, $F(3, 39) = 0.226$, $p = 0.878$) (Fig. 7a). However, net association time for the $Ca(OH)_2$ exposed fish did differ significantly across time points (repeated measures ANOVA $F(3,39) = 6.17$, $p = 0.00132$) with net association time with the conspecific cue being lower on the day of exposure (day 2) than two days prior to exposure (day 0) and ten days after exposure (day 12) (TukeyHSD, $p < 0.05$) (Fig. 7b). Net association time two days after exposure (day 4) was not significantly different from any other time point. Unexpectedly, on the day of exposure ($N = 14$, two tailed one sample *t*-test, $t = -2.18$, $p = 0.048$) and two days after exposure ($N = 14$, two tailed one sample *t*-test, $t = -2.66$, $p = 0.019$) the mean net association times were significantly negative representing a switch in preference valence from conspecific to heterospecific male chemical cues (Fig. 7b). By 10 days post exposure (day 12) the species typical conspecific preference was recovered ($N = 14$, two tailed Wilcoxon signed-rank test, $p = 0.019$).

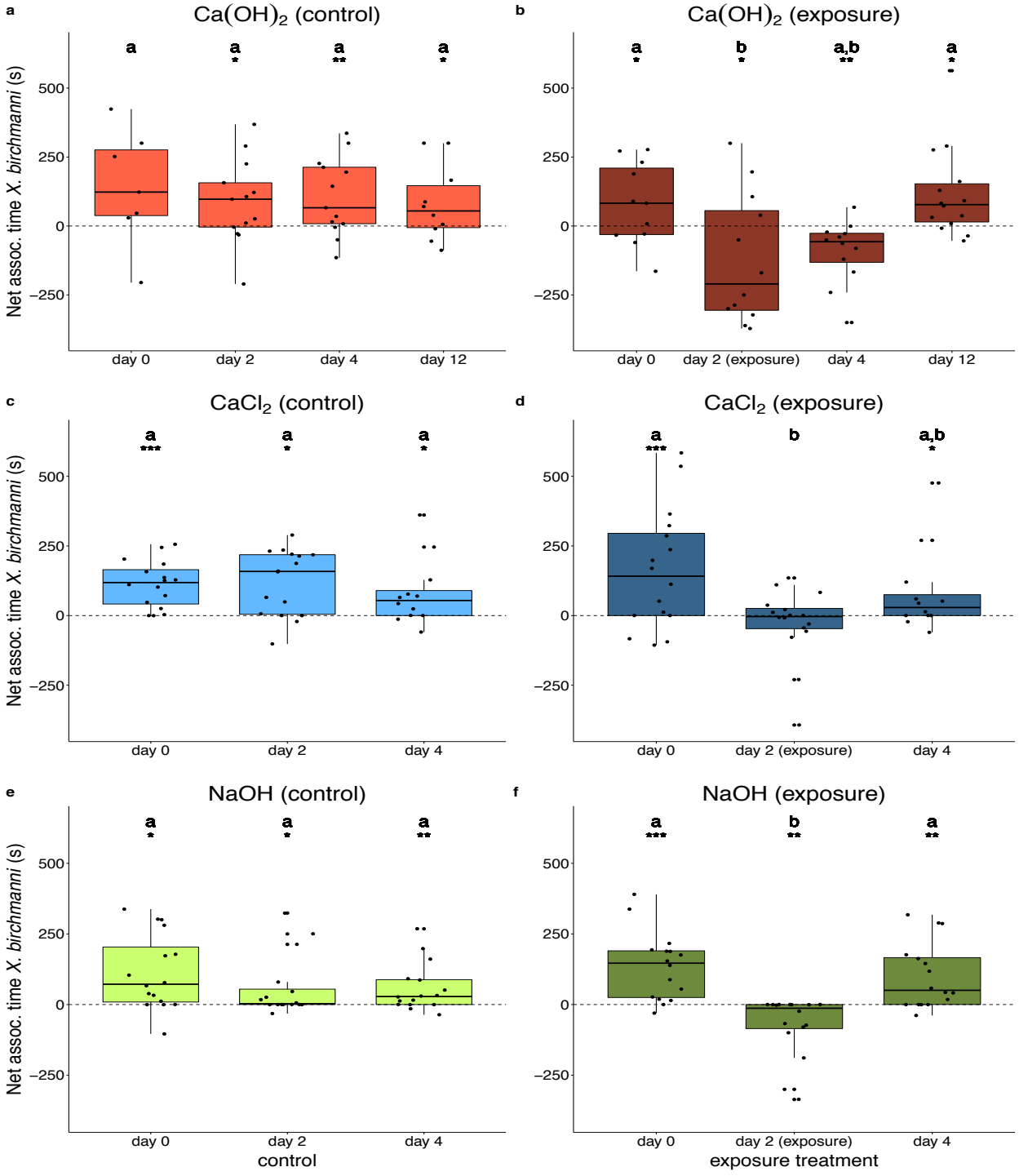


Figure 7. Effect of chemical exposure on female *X. birchmanni* preference for male chemical stimuli. Net association time of female *X. birchmanni* for conspecific (*X. birchmanni*) heterospecific (*X. malinche*) male chemical stimuli for $Ca(OH)_2$ exposure trials, a) control treatment and b) exposure treatment; $CaCl_2$ exposure trials, c) control treatment and d) exposure treatment; and NaOH exposure trials, e) control treatment and, f) exposure treatment. Letters represent significantly different means across treatment time points (ANOVA, $p < 0.05$). Astrisks represent mean association times different from 0. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Net association time with the conspecific olfactory cue did not differ significantly for control females across the $CaCl_2$ trials (repeated measures ANOVA, $F(1, 42) = 0.601$, $p = 0.442$) (Fig. 7c). However, net association time for the $Ca(OH)_2$ exposed fish did differ significantly across time points (Kruskal-Wallis rank sum, chi-squared = 6.81 $p = 0.033$, d.f. = 2) with net association time with the conspecific cue being less on the day of exposure (day 2) than two days prior to exposure (day 0) (TukeyHSD, $p < 0.05$). In this case, on the day of exposure there was no significant preference ($N = 16$, Wilcoxon signed-rank test, $p = 0.712$) (Fig. 7d).

As with the previous two sets of trials, net association time with the conspecific olfactory cue did not differ significantly for control females across the NaOH trials (Kruskal-Wallis rank sum, chi-squared = 3.00 $p = 0.273$, d.f. = 2) (Fig 7e). The results for the NaOH exposed females were qualitatively similar to the $Ca(OH)_2$ exposed fish with net conspecific preference being significantly less on the day of exposure (day 2) than two day prior to (day 0) and two days post exposure (day 4) (repeated measures ANOVA, $F(2, 45) = 14.9$, $p = 0.000011$) (TukeyHSD, $p < 0.001$). On the day of exposure (day 2) females subjected to NaOH significantly preferred the conspecific cue ($N = 16$, Wilcoxon signed-rank test, $p = 0.0091$). Species typical conspecific cue preference was recovered by day 4 ($N = 16$, Wilcoxon signed-rank test, $p = 0.0042$) (Fig 7f).

Olfactory signal versus water control preference trials

Control females showed significant mean net association time for conspecific chemical cues over a water blank ($N = 16$, two tailed one sample *t*-test, $t = 3.097$, $p = 0.0074$), but no preference for heterospecific cues over water ($N = 16$, two tailed one sample *t*-test, $t = -1.39$, $p = 0.185$) (Fig. 8). Whereas, $Ca(OH)_2$ exposed females showed no preference for conspecific cues over water

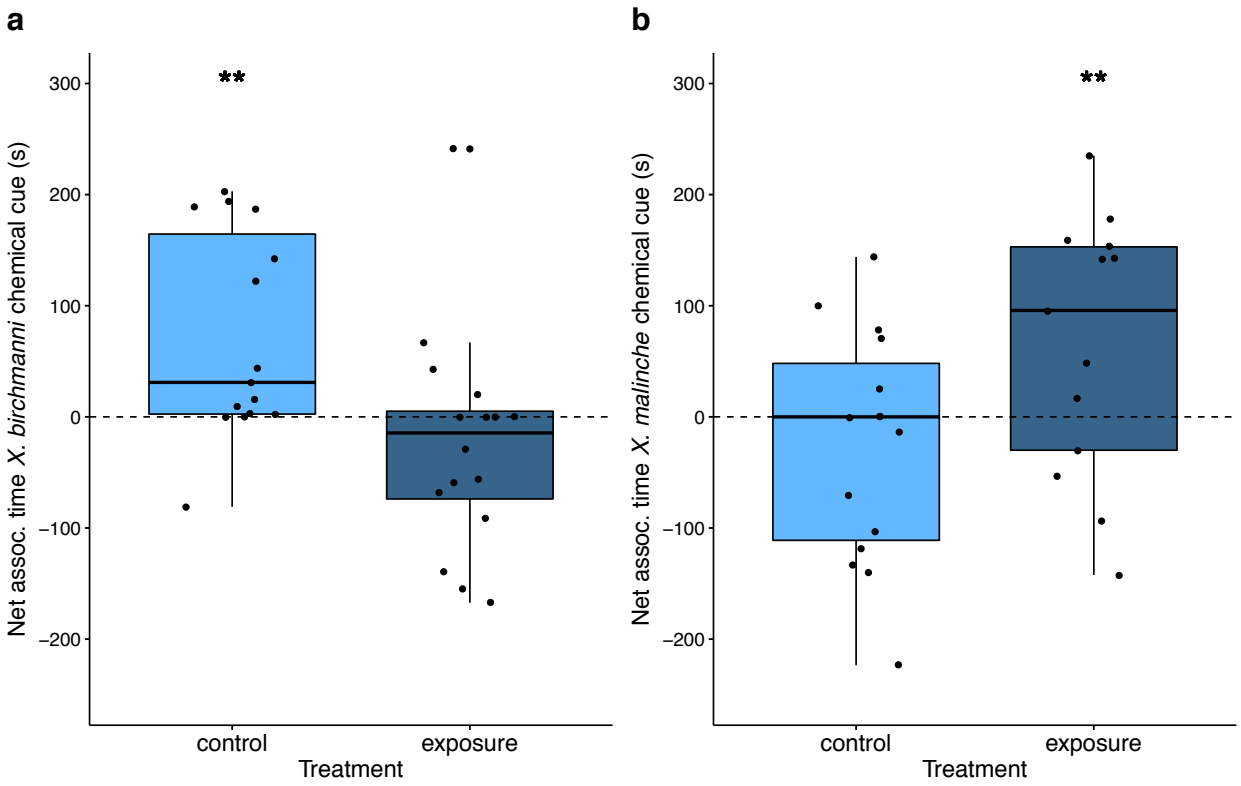


Figure 8. Net association time of control and $Ca(OH)_2$ exposed female *X. birchmanni.* a) For conspecific (*X. birchmanni*) male chemical cue over a water blank and b) heterospecific (*X. malinche*) male chemical cue over a water blank . Asterisks represent mean association times different from 0. ** $p < 0.01$.

($N = 16$, two tailed one sample t-test, $t = -0.994$, $p = 0.336$) but a strong preference for heterospecific cues over water ($N = 16$, two tailed one sample t-test, $t = 2.99$, $p = 0.0094$) (Fig. 8).

Visual preference tests

There was no significant difference across treatments for net association time when control, $Ca(OH)_2$ exposed and $CaCl_2$ exposed females were conspecific vs heterospecific animated visual stimuli (Kruskal-Wallis rank sum, chi-squared = 0.834 $p = 0659$, d.f. = 2) (Fig. 9). Both control ($N = 14$, Wilcoxon signed-rank test, $p = 0.024$) females and $CaCl_2$ exposed ($N = 20$, Wilcoxon signed-rank test, $p = 0.0025$) females significantly preferred the conspecific over the heterospecifc stimulus. $Ca(OH)_2$ exposed females showed a nonsignificant trend toward conspecific preference ($N = 14$, Wilcoxon signed-rank test, $p = 0.066$) (Fig 9).

Discussion

In this study, female preference assays show that not only can a common, deliberately introduced anthropogenic pollutant disrupt chemical communication in *X. birchmanni,* but it does so in a way that should promote hybridization. When exposed to low concentrations of calcium hydroxide, strong conspecific preference typically shown by *X. birchmanni* females is not just abolished as predicted, but rather a strong preference for the sympatric sister species *X. malinche* emerges, an effect which lasts for at least two days but less than ten, since by day twelve the expected conspecific preference is recovered (Fig. 7b). This unexpected switch in valence to heterospecific preference could lead directly to interspecific matings in the days following a

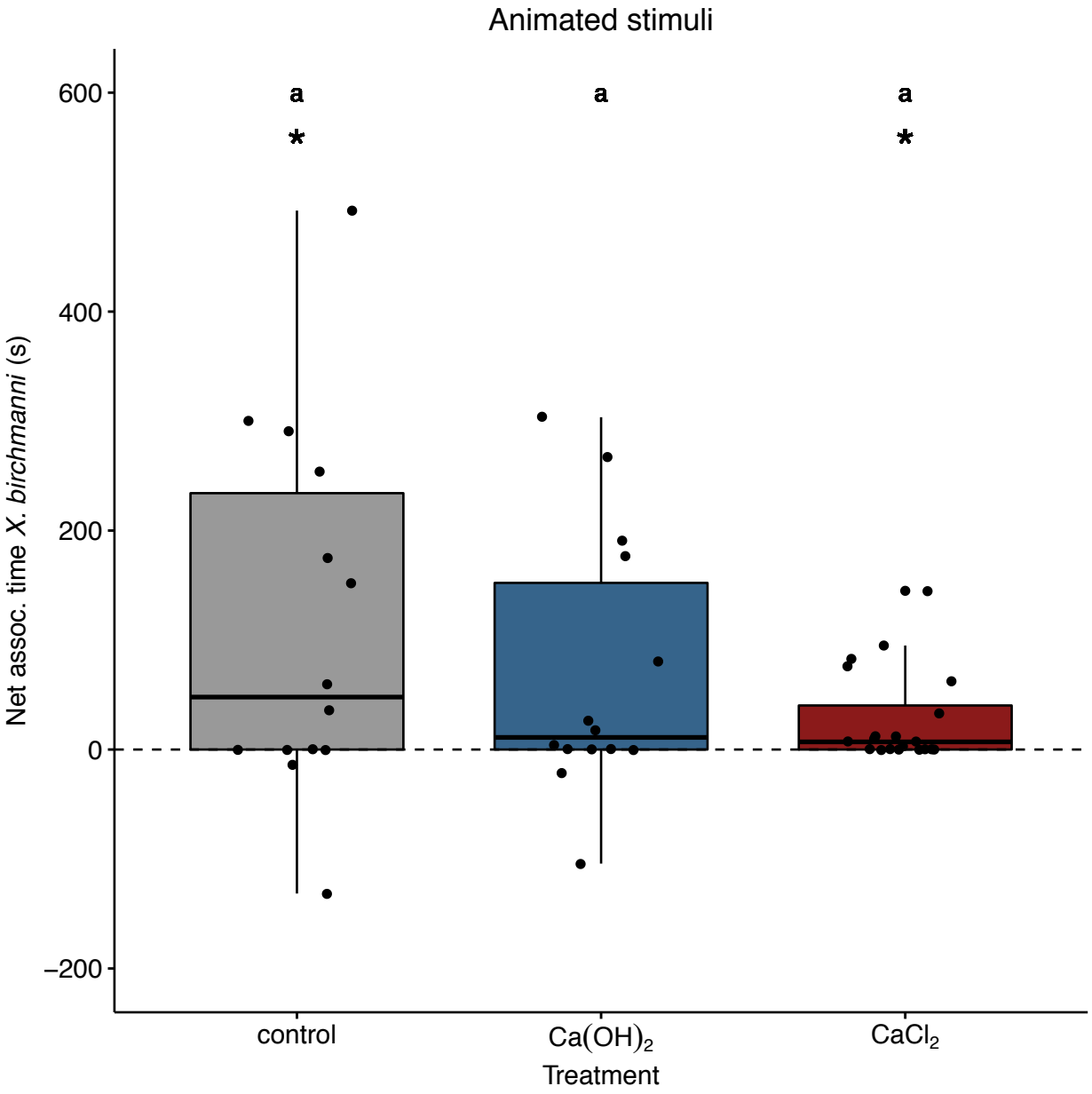


Figure 9. Net association time of female *X. birchmanni* for conspecific (*X. birchmanni*) heterospecific (*X. malinche*) male animated visual stimuli for control, $Ca(OH)_2$, and $CaCl_2$ exposed females. Letters represent significantly different means across treatment time points (ANOVA, $p < 0.05$). Asterisks represent mean association times different from 0. * $p < 0.05$.

pulse disturbance since females are more likely to exert behavioral reproductive isolation than are males in species with no post mating paternal care (Rosenthal, 2013). Calcium ions on their own were not sufficient to cause the switch in valance seen in the calcium hydroxide exposed fish, but an increase concentration seems to interact with the olfactory periphery given that exposure abolished preference in the olfactory modality while not affecting motivation to mate, as exposed fish showed a preference for conspecific visual stimuli (Fig. 7d). Raising the pH had a qualitatively similar effect to calcium hydroxide exposure though recovery of conspecific preference for chemical cues was quicker, suggesting that many other compounds that affect pH (e.g. soaps) may have similar effects on chemical signaling (Fig. 7f).

Calcium hydroxide-exposed *X. birchmanni* females showed no preference for conspecific cues when tested against a water blank but strongly preferred *X. malinche* (Fig. 8b), whereas control fish preferred conspecific cues over water and were indifferent toward heterospecific cues (Fig. 8a). These results indicate that the preference reversal after exposure seen in the simultaneous choice trials is explained by the interaction of flips in hedonic value assigned to both species-specific cues (i.e. from positive to neutral for the *X. birchmanni* cue and neutral to positive for the *X. malinche* cue). It has been shown that the urine-born pheromones of these fish are identical in chemical profile with the exception of two compounds, a testosterone sulfate compound and a small urinary conjugated bile acid, cholanic acid, present in *X. birchmanni* and absent in *X. malinche* (Holland, 2018). These two compounds are necessary and sufficient to elicit strong conspecific preference in female *X. birchmanni*. Chemoreceptors in *Xiphophorus* have not been characterized, but given the strong olfactory based species recognition exhibited *X. birchmanni* (Fisher & Rosenthal, 2006a, 2006b; Fisher, Wong, & Rosenthal., 2006; Verzijden

et al., 2012; Wong, Fisher, & Rosenthal, 2005) under natural conditions it seems reasonable that the hedonic flips shown here are related to the interaction of $Ca(OH)_2$ exposed chemoreceptors and one or both of these *X. birchmanni*-specific compounds. Additionally, it is possible that downstream neural processing of inputs from the periphery are responsible for the observed flip (Rosenthal, 2018). Further, given the inexact nature of pooled cue collection used in this study, it is also possible that the treated fish are more responsive to other cues present in the stimulus water that are not associated with mate preference. For example, heightened sensitivity to multiple signals including aggression signals might present as indifference toward that stimulus. Using electro-olfactography (Evans & Hara, 1985) on the sensory periphery using testis extracted cue to dissect apart these scenarios is an intriguing path for future studies.

Though not explicitly tested, the two-day post exposure recovery is faster than reported ORN regeneration in other vertebrates including teleost fishes, suggesting that either exposure at these concentrations does not cause neuronal degeneration (Cancalon, 1982; Graziadei & Graziadei, 1979), or that full regeneration of chemoreceptors may not be necessary for expressing species typical responses as is the case for some discriminatory tasks in goldfish (Zippel, 2000). That strong preferences were still exhibited after exposure to two of the three chemicals tested, albeit with inverted hedonic values, is taken as further evidence that the ORNs were not ablated.

Conclusions

Hybridization ultimately requires the breakdown of reproductive barriers, an important first step of which is individuals choosing heterospecific mates. Here we show that an inorganic

compound generally thought to be safe for aquatic environments and used actively and heavily in remediation can not only disrupt chemical communication in these closely related sympatric swordtails, but actually causes a switch in preference from the conspecific to heterospecific, a scenario that promotes ongoing gene flow. This study adds to the growing body of evidence that sublethal concentrations of pollutants can have unforeseen evolutionary consequences (Boettcher & McManus, 2015; Crispo, et al., 2011; Dudgeon et al., 2006; Fisher, Wong, & Rosenthal, 2006; Hayes et al., 2002; Seehausen, Van Alphen, & Witte, 1997).

CHAPTER IV

GENETIC ARCHITECTURE OF SECONDARY SEXUAL TRAITS IN NATURALLY-HYBRIDIZING SWORDTAIL FISHES

Introduction

Genetic architecture shapes the evolutionary trajectory of hybridizing species (Abbott et al., 2013). In particular, genetic correlations and physical linkage among secondary sexual display traits and traits under viability selection can facilitate speciation (Servedio & Noor, 2003) and can impact preference evolution (Kirkpatrick & Hall, 2004).

Mating preferences are often multivariate (Brooks et al., 2005; Chenoweth & Blows, 2006; Gerhardt & Brooks, 2009; Hohenlohe & Arnold, 2010) and due to mechanistic constraints as well as depleted variation because of ongoing selection, the suite of traits courters of a given species can display may not match up with the peak preference combinations of choosers (Rosenthal, 2013; Van Homrigh et al., 2007). This is especially the case if pleiotropic effects generate phenotypic correlations among multiple traits of interest to choosers (Lipson, Pollack, & Suh, 2002; Wagner, Pavlicev, & Cheverud, 2007; Wagner, 1996). Hybridization has the potential to break up trait correlations and better address chooser preference through recombination and independent assortment, but if and how this happens depends on the genetic architecture controlling these traits (Barton & Hewitt, 1989; Chenoweth & Blows, 2006; Seehausen, 2004).

Thus, when exploring the genetic architecture of multivariate traits, an important question is: does the correlated evolution of such trait components result in governance by pleotropic and/or linked loci or allelic variation in independent genetic pathways? The genetic architecture of such correlated traits can influence the way in which individual components can respond to selection. For example, in the Hawaiian cricket radiation females show repeatable preferences for at least three components of male song when decoupled, with the strongest difference in preference across closely related species being for pulse duration. Yet interspecific divergence in male song is greatest for the other axes of variation likely because of trait correlation and differential heritable variability between trait components (Oh & Shaw, 2013).

Likewise, in the poeciliid fish genus *Xiphophorus*, females attend to multiple aspects of male phenotypes. Males in some species have a prominent sword extension, a composite trait consisting of both an elongation of the ventral caudal fin rays as well as several pigmentation patterns. In *X. hellerii,* decoupling the components of this composite trait result in reduced response from females (Basolo & Trainor, 2002; Rosenthal et al., 1996). In this case, sexual selection favors co-expression of elongation of the fin rays along with pigmentation whether or not these traits are governed by the same allele or by a suite of unlinked genes. In contrast, *X. birchmanni* females prefer males with small dorsal fins and large body sizes despite a strong positive allometry for this trait combination (Fisher et al., 2009). Thus, intersexual selection should favor a decoupling of these traits if they are regulated by separate genetic mechanisms.

Studies of *Xiphophorus* genetics have identified polygenic control over some traits (Kallman, 1989; Zander & Dzwillo, 1969) and pleiotropy for others (Kallman, 1989) as well other architectures. For example, the yellow-blue polymorphism in male *Xiphophorus pygmaeus* is controlled by a single y-linked locus (Kingston, Rosenthal, & Ryan, 2003) and from a genetics stand point perhaps the most well understood sexually dimorphic phenotype, body size, is controlled by copy number variation for the melanocortin receptor mc4r (Smith et al., 2015).

Despite being only 0.5 % divergent genome wide (Culumber et al., 2011; Schumer et al., 2014) the naturally hybridizing and sexually dimorphic *X. malinche* and *X. birchmanni* differ in a suite of male secondary sexual traits (Rauchenberger, Kallman, & Morizot, 1990; Rosenthal et al., 2003). The sword extension components described above are present in *X. malinche* but absent in *X. birchmanni. X. birchmanni* is further characterized by a hypertrophied dorsal fin, a polymorphism for the false brood patch pigment pattern which has been shown to be important in male-male aggression in a closely related congener (Morris, Darrah, & Rios-Cardenas, 2010), as well as the nuchal hump, a fatty cephalic deposit that functions as a sexual signal in other fish groups (Barlow & Siri, 1997). Here I use traditional QTL mapping based on over 12,000 genome-wide markers to search for genomic regions associated with trait variation between these two species. Of particular interest are visual traits likely to be under sexual selection.

Methods

Collection crossing, rearing and housing

I used the *X. malinche* (female) x *X. birchmanni* (male) cross to produce an F_1 generation of sires and dams for the intercross mapping population because previous attempts to produce viable offspring from the reciprocal cross were largely unsuccessful (see chapter II). Sperm storage is a phenomenon common in poeciliid fishes including *Xiphophorus* (López-Sepulcre et al., 2013). Accordingly, I reared virgin *X. malinche* (n = 24) born to dams collected at the Chicayotla (n = 5) locality on the Rio Xontla (20°55'27.24"N 98°34'34.50W) using baited minnow traps. Wild *X. birchmanni* sires (n = 10) were similarly collected from the Rio Coacuilco at Coacuilco (21°5'50.85 N, 98°35'19.46 W) (Fig. S1). The resulting F_1 offspring from this cross were reared to maturity and allowed to interbreed to produce a F_2 mapping generation.

In June of 2016, as part of a long-term selection study, an array of eight 2000 L seminatural mesocosms at three separate outdoor locations in the Calnali region of Hidalgo Mexico (Ahuacatlan, Calnali, and Achiquihuixla) were stocked with a combination of new F_1 (n = 21 per tank) offspring produced as described above and F_2 (n = 6 per tank) generation juvenile swordtails all crossed from the same original wild populations (Coacuilco and Chicayotla) (24 tanks total). An additional 3 mesocosms at the Calnali location were stocked with only F_2 (n= 34 per mesocosm) offspring for the common garden experiment outlined in chapter II. Original stocks consisted of mixed brood cohorts with individual offspring assigned randomly to each mesocosm. In addition to naturally occurring periphyton and macroinvertebrates, fish were fed a high-quality algae-based granular feed (Ken's fish) once daily.

I sampled the long term mesocosms in January and May of both 2017 and 2018, at which time all adult males were lightly anesthetized with tricaine methanesulfonate, given individually color-coded elastomer tags for future identification (Northwest Marine Technologies), digitally photographed for phenotyping, and nonlethally fin-clipped for genotyping before being returned to the mesocosms. I similarly sampled the common garden mesocosms at CICHAZ in Calnali in January, 2017.

Morphometrics

I measured standard length (distance from the proximal tip of the mandible to the vertical midpoint of the distal edge caudal peduncle), body depth (distance from the insertion of the first dorsal ray to the vent), sword extension (distance from the , dorsal fin width (distance between the insertion of the first ray to the last), dorsal fin height (length of the second to last branched ray), the length of the gonopodium (modified anal fin and intromittent sexual organ), and caudal peduncle depth (distance from the dorsal insertion to the ventral insertion of the caudal fin) from digital images of all adult males using the ImageJ software package (Abràmoff et al., 2004). All measures except for standard length were then standardized by dividing by standard length. Hereafter all continuous measures referred to are standardized by standard length. I also scored the presence or absence of the nuchal hump, and the melanin patterns, false brood patch (Rauchenberger et al., 1990), dorsal sword pigmentation (upper sword edge) and ventral sword pigmentation, (lower sword edge) (Fig. S3).

DNA extraction and library preparation

DNA was extracted using either a DNeasy blood and tissue extraction kit (Qiagen) or an Agencourt bead-based purification kit (Beckman Coulter) following the manufacturer's instructions. We prepared multiplexed shotgun genotyping (MSG) libraries (Andolfatto et al., 2011) using a new, transposase-based shearing approach (Schumer et al., 2017; Stern, 2017). Ten nanograms of DNA was digested using Tn5 enzyme produced by the Andolfatto lab (Princeton University) charged with adapter sequence in 5X TAPS buffer at 55 C. The reaction was killed with 0.2% SDS. One µl of each digested sample was amplified with primers containing custom index sequences for 12 cycles. This approach generates low-coverage data throughout the genome, and simulations with simMSG suggest it has even higher accuracy than the MseI MSG pipeline previously used to assign ancestry in this system. (Schumer, et al., 2015; Schumer et al., 2017).

Due to multiplexing with other samples, libraries were sequenced on a total of four Illumina HiSeq 4000 lanes. Raw paired end reads were parsed by index and barcode and trimmed for quality, then run through the MSG ancestry calling pipeline (Andolfatto et al., 2011). The MSG pipeline uses a hidden Markov model to assign ancestry probabilities at marker loci based on polymorphism masked parental reference genomes (Andolfatto et al., 2011) and has been extensively validated for *X. malinche X. birchmanni* hybrids (Andolfatto et al., 2011; Schumer et al., 2014; Schumer et al., 2017; Schumer, Xu, et al., 2018). Soft ancestry calls were then converted to hard calls using a custom perl script. Marker density was then thinned by physical distance of 50 kb resulting in 12,794 markers spread evenly across the genome (Fig. 10). The resulting genotype file was used for all downstream QTL analyses.

QTL mapping

I used the scanone function of r/QTL to perform single QTL model standard interval mapping using the EM algorithm on the thinned data set for each trait of interest with mesocosm array as a covariate (Broman & Sen, 2009). Nuchal hump, false brood patch, upper sword edge and lower sword edge were run as binary traits using the scanone(model= "binary") option. Since genome-wide hybrid index was significantly correlated with measures of standardized sword extension length ($r = 0.20373$, $p = 2.067e-06$), standardized dorsal fin height ($r = -0.1195987$, $p = 0.005564$), and nuchal hump presence ($r = -0.09583706$, $p = 0.02665$), I included it as a covariate for these phenotypes. Genome-wide likelihood of odds (lod) significance thresholds ($p = 0.05$) were determined based on 1,000 permutations of genotype scores for each phenotype. 1.5 lod intervals around each QTL peak marker were considered for downstream analyses.

QTL overlay with complementary data sets

I then overlaid the 1.5 lod QTL intervals which were generally large (4.1 – 11.5 Mb) with several other data sets to identify an initial list of narrower candidate regions associated with the QTL. I used bedtools (Quinlan & Hall, 2010) to intersect each QTL lod interval with time series selection analyses for structured populations performed following Mathieson and McVean (2013) comparing samples spanning 2006 to 2018 from two independent wild *X. malinche*-*X. birchmanni* hybrid zones, Acuapa and Tlatemaco (Schumer et al., 2017; Schumer, et al., 2018; Schumer et al., unpublished data). I report the closest annotated gene from the *X. birchmanni* transcriptome within 10 kb of overlapping markers under selection ($p < 1e-4$) (Schumer et al., 2017; Schumer, et al., 2018; Schumer et al., unpublished data).

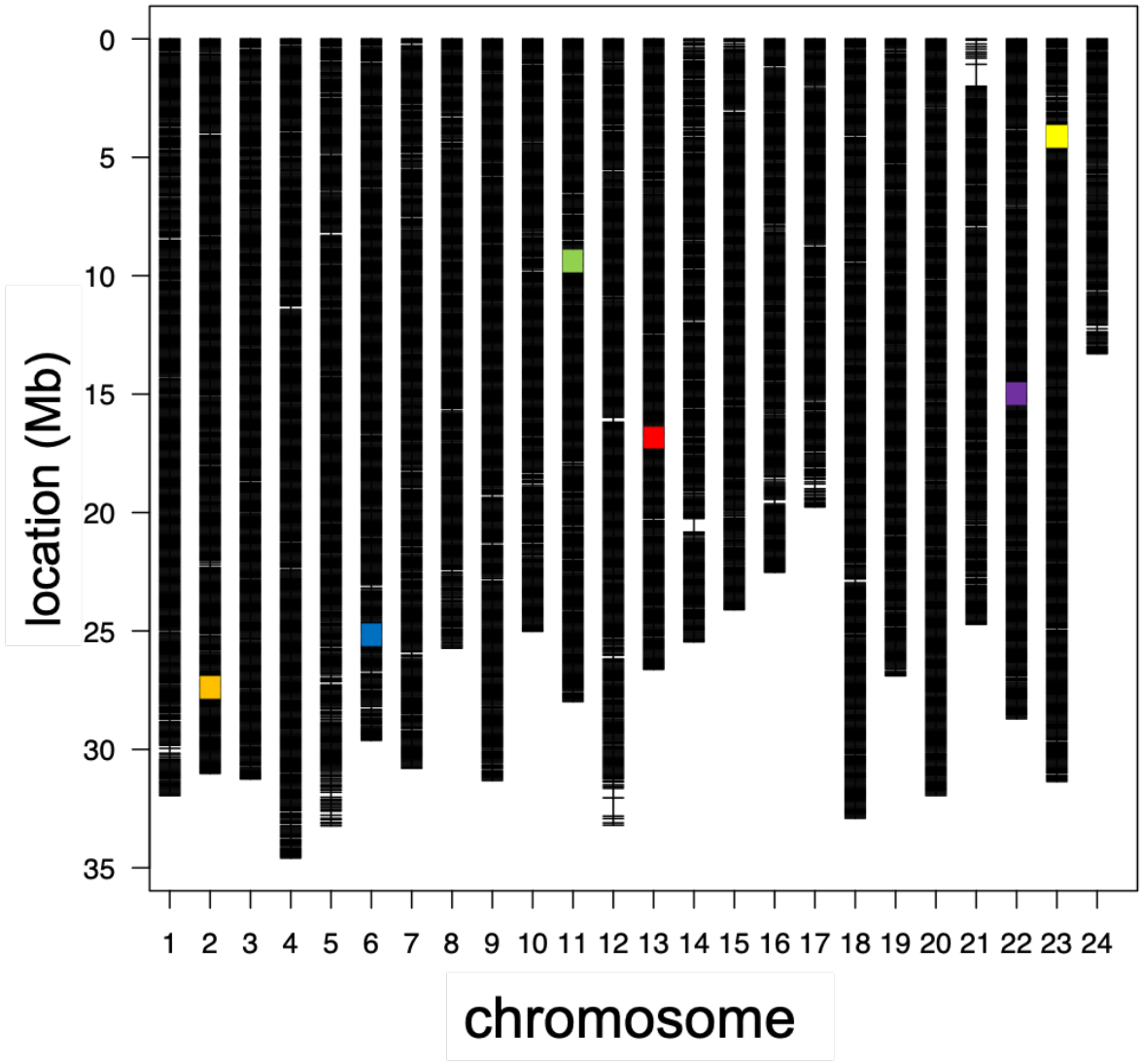


Figure 10. Genetic marker distribution across the genome with QTL locations. Black bars indicate marker locations. Colored bars indicate the positions of QTL peaks above the p =0.05 genome wide lod threshold for each phenotype. Red = sword extension, blue = upper sword edge,
orange = dorsal fin height, purple = caudal peduncle depth, green = false brood patch, and yellow = nuchal hump.

For the caudal fin phenotypes, sword extension and upper sword edge, I compared 1.5 lod QTL intervals to allele specific expression (ASE) data for tissue including caudal fin tissue from F_1 *X. malinche* x *X. birchmanni* hybrids collected as part of another ongoing study (Powell and Schumer, unpublished data). Briefly, for each QTL region I used fastahack to acquire the DNA sequence encompassed the 1.5 lod interval from a de novo 10X *X. birchmanni* genome assembly and NCBI Blast-n (e-value < 1e-50) to blast to the annotated *X. maculatus* genome (Schartl et al., 2013). I then used bedtools to pull out the gene names that overlapped those in the ASE data with an FDR < 0.1. For these matches, I looked for nonsynonymous substitutions between the *X. birchmanni* and *X. malinche* alleles for this subset of genes using ExPASy (Gasteiger et al., 2003) for sequence translation and CLUSTAL omega to align amino acid sequence (Madeira et al., 2019). Lastly, I compared the sword extension QTL to a differential expression study examining caudal fin tissue regeneration between *X. birchmanni* and *X. malinche* as described for the ASE comparison above (Schumer et al., unpublished data).

Results

Of the 751 adult males phenotyped and genotyped, 215 F_1 males (heterozygosity > 0.9) were removed from analysis leaving 536 males in the mapping population. Thinning genetic markers by 50 kb physical distance intervals resulted in 12,794 markers spread evenly across the genome (95% quantile, 60,010.15 bp) with 98.2% of loci genotyped across all individuals (Fig. 10).

Table 2. Summary of significant QTL. Mean standardized phenotypes are shown for each genotype: MM = homozygous *X. malinche* MB = heterozygous, BB = homozygous *X. birchmanni*

trait	chrom	peak position (MB)	1.5 lod interval (Mb)	peak lod score	% variation explained	mean phenotype		± se
sword extension	13	16.761606	13.23201 - 17.33781	5.539369	5.112849	MM	0.0962973	0.00393724
						MB	0.0863749	0.0029692
						BB	0.0615336	0.00514853
dorsal fin height	11	9.308359	5.268017 - 15.543669	5.508586	4.589777	MM	0.2247186	0.00287717
						MB	0.239453	0.00193301
						BB	0.2436685	0.0027051
caudal peduncle depth	23	4.244965	3.201736 - 9.707757	4.141691	3.061675	MM	0.2070244	0.00129463
						MB	0.2038561	0.00099908
						BB	0.1993036	0.00134671
upper sword edge pigment	6	25.129565	18.10039 - 29.61709	4.358197	3.777529	MM	0.3409565	0.03523407
						MB	0.4139607	0.02855434
						BB	0.6815032	0.0654885
false brood patch	1	27.396161	26.58771 - 31.01519	5.997539	4.278533	MM	0.2279117	0.03652964
						MB	0.3751081	0.02617834
						BB	0.5146761	0.03988471
nuchal hump	22	14.862147	11.74453 - 16.48131	5.072957	6.926118	MM	0.1563501	0.03498465
						MB	0.1568883	0.02388618
						BB	0.3627293	0.03452655

QTL mapping

Table 2 summarizes genome-wide significance thresholds, lod scores and 1.5 lod intervals. I recovered one significant QTL each for sword extension, dorsal fin height, caudal peduncle depth, upper sword edge pigment, false brood patch, and nuchal hump. No significant QTL were recovered for dorsal fin width, standard length or lower sword edge. Significant QTL explained between 3.1-6.7% of phenotypic variation (Table 2) over LOD intervals ranging from 4.1-11.5 Mb. All identified QTL effects display patterns of incomplete dominance (Figs. 11-13), except nuchal hump which is primarily associated with the *X. birchmanni* homozygote genotype (Fig. 13c). Further, all patterns of genotype-phenotype effects reflect trait patterns in the parental lineages, except upper sword edge which was associated with the *X. birchmanni* allele despite it being a trait only occurring in *X. malinche* (Fig. 11c) (Cui et al., 2013; Rauchenberger et al., 1990).

QTL overlay with complementary data sets

Table 2 summarizes significant QTL regions that intersect with loci found to be under selection through analysis of time series data of allele frequency shifts (2006 - 2018) from two independent wild hybrid zones. that intersect with significant QTL regions identified in this study are summarized in (Table 3). Briefly, when I compared all QTL regions from this study to loci under selection in the Acuapa hybrid population, I identified one overlapping locus for sword extension, three loci for dorsal fin height, and one for false brood patch were identified. All five loci showed significant ($s > 0.43$ for all, $p < 0.05$) positive selection coefficients for the *X. birchmanni* allele. When compared to the Tlatemaco data, one locus each for upper sword edge and nuchal hump overlapped the QTLs. Both of these showed strong negative selection

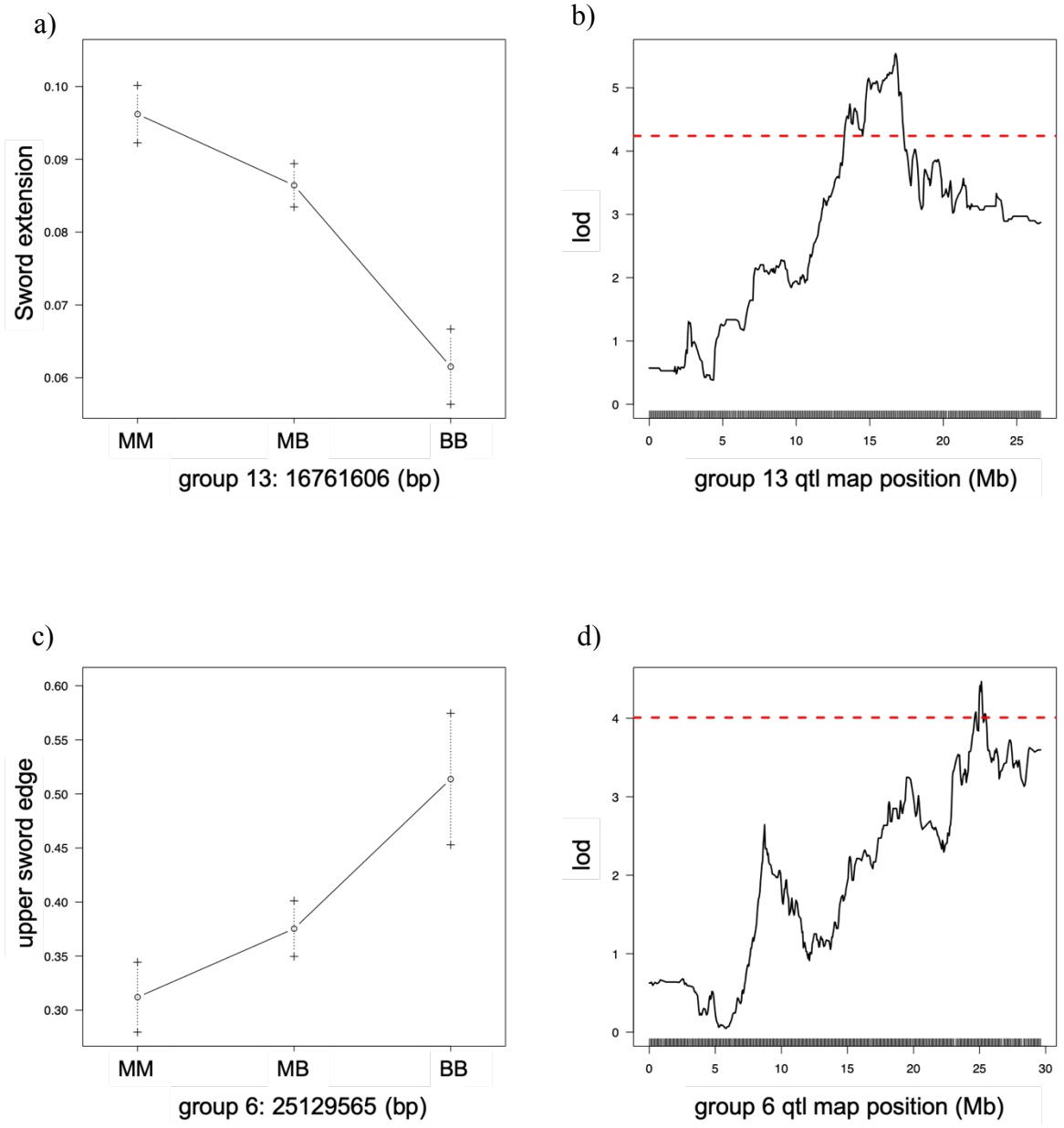


Figure 11. Effect size and genomic position for sword extension and upper sword edge. Mean effect sizes ± SE by genotype for a) sword extension and c) upper sword edge pigmentation. Lod peak for b) sword extension and d) upper sword edge pigmentation.
MM = homozygous *X. malinche*, MB = heterozygous, BB =homozygous *X. birchmanni*. Red dashed lines are genome-wide lod significance thresholds (sword extension = 4.10, upper sword edge = 4.00, p =0.05).

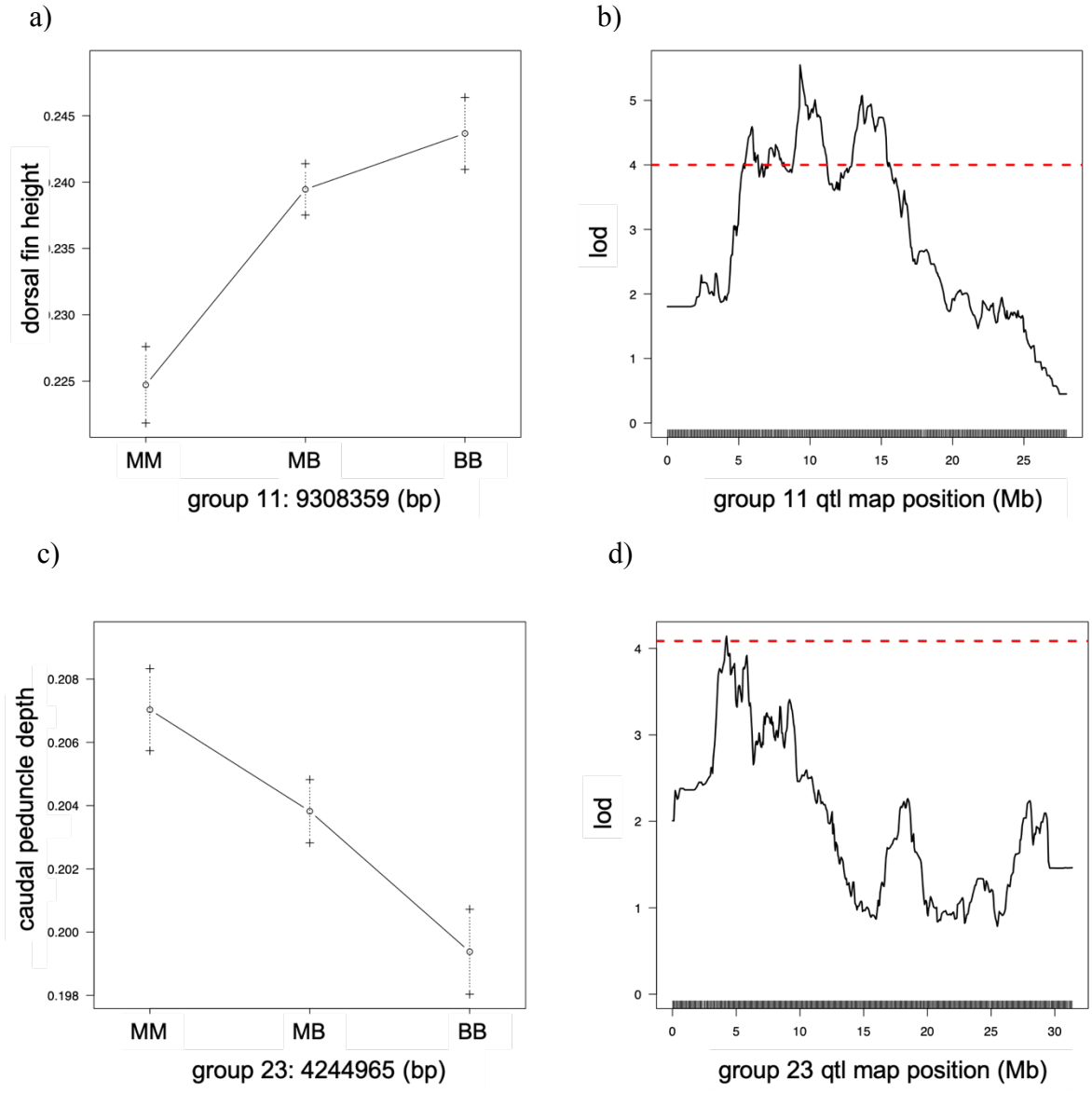


Figure 12. Effect size and genomic position for dorsal fin height and caudal peduncle depth. Mean effect sizes ± SE by genotype for a) dorsal fin height and c) caudal peduncle depth. Lod peak for b) dorsal fin height and d) caudal peduncle depth.
MM = homozygous *X. malinche*, MB = heterozygous, BB =homozygous *X. birchmanni*. Red dashed lines are genome-wide lod significance thresholds (dorsal fin height = 4.00, caudal peduncle depth = 4.13, p =0.05).

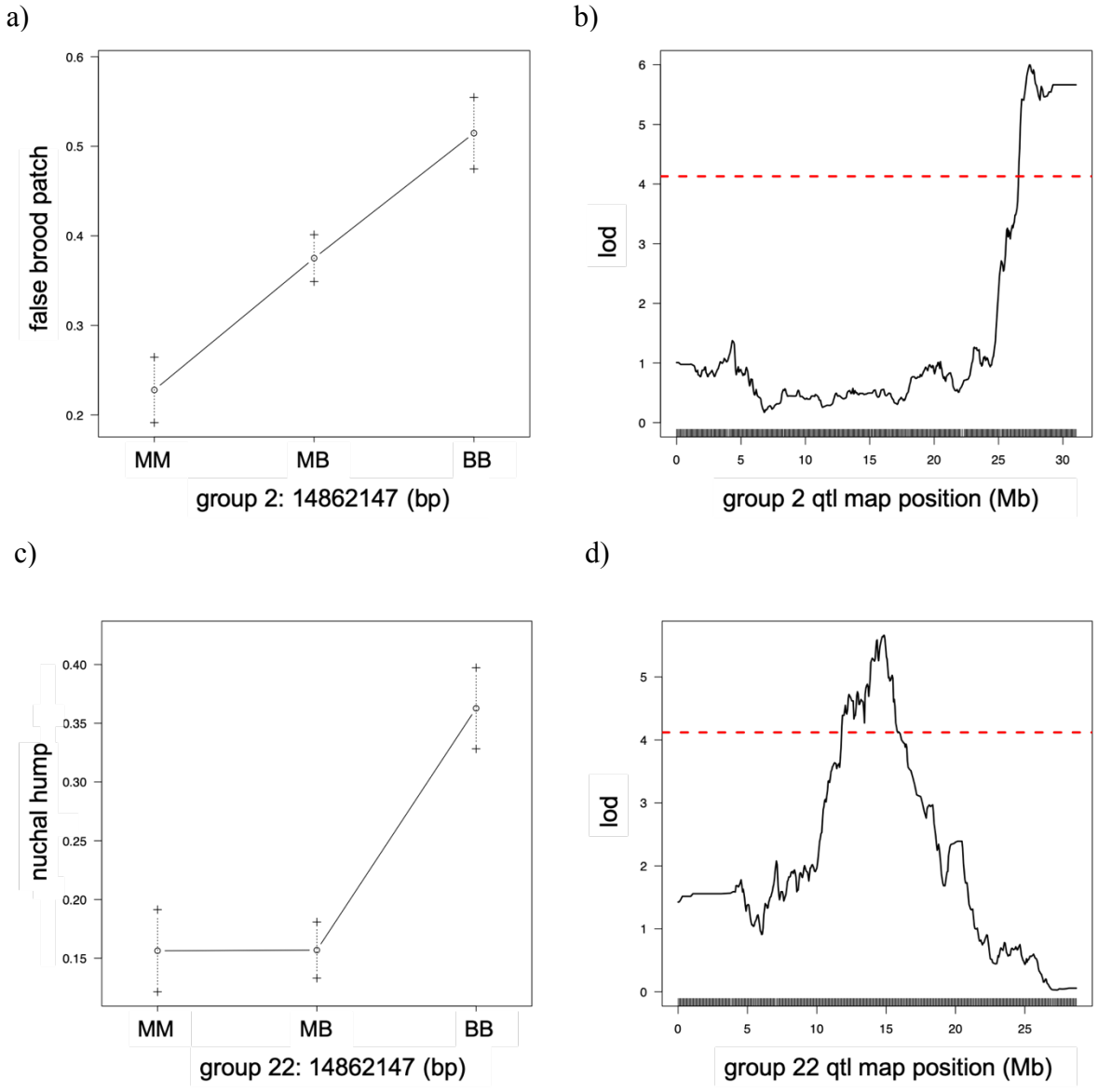


Figure 13. Effect size and genomic position for false brood patch and nuchal hump. Mean effect sizes ± SE by genotype for a) false brood patch and c) nuchal hump. Lod peak for b) false brood patch and d) caudal nuchal hump. MM = homozygous *X. malinche*, MB = heterozygous, BB =homozygous *X. birchmanni*. Red dashed lines are genome-wide lod significance thresholds (dorsal fin height = 4.13, caudal peduncle depth = 4.12, p =0.05).

coefficients for the *X. birchmanni* allele ($s < -0.43$ for both, $p < 0.05$). Loci showing allelic imbalance in the F_1 ASE study that overlapped QTL regions (FDR < 0.1) for the caudal fin phenotypes sword extension (5 genes) and upper sword edge (one gene) are summarized in (Table 4). No genes in this overlay contained any non-synonymous substitutions. Additionally, one gene, *Hoxa3a,* found to be upregulated in *X. malinche* compared to *X. birchmanni* in an ongoing caudal fin regeneration gene expression study, overlapped the QTL region for sword extension. *Hoxa3a*, which is located approximately 360 kb from the estimated sword extension QTL peak, the 14th most differentially regulated (3.66 fold) gene in the data set. In total these comparisons have highlighted 15 candidate loci for further analysis.

Discussion

In this study I identify single QTL of low to moderate effect for several male traits that are likely under sexual selection in the *Xiphophorus birchmanni - X. malinche* species pair. One QTL each for sword extension, dorsal fin height, caudal peduncle width, nuchal hump, false brood patch, and upper sword edge pigmentation are recovered (Figs. 11-13, Table 2). These QTL explain between 3.1 and 6.9% of the variation in these traits, suggesting a highly polygenic architecture for all, though phenotypic plasticity may also reduce power to detect loci (Walling et al., 2010). This is especially true for the continuous measures of sword extension and dorsal fin height, and the binary trait nuchal hump, all three of which are significantly correlated with genome wide hybrid index in the mapping population. Even in the absence of other mechanisms such as

Table 3. QTL-time series overlay. Intersection of QTL and loci identified to be under selection from allele frequency time series data spanning 2006-2018 for two natural *X. malinche – X. birchmanni* hybrid zones Acuapa and Tlatemaco. s = selection coefficient for the *X. birchmanni* allele. Identified closest genes are within 10kb of the marker locus.

trait	time series population	chr	position (Mb)	s B allele	95% CI upper	95% CI lower	p value	closest gene
sword extension	Acuapa	13	15397312	**0.4332992**	-0.5896746	-0.2190061	7.60E-05	NDRG1 isoform X1
dorsal fin height	Acuapa	11	8143521	**0.5631508**	-0.7177638	-0.3551243	1.25E-07	PKNOX2 isoform X3
		11	11580924	**0.5483741**	-0.7387397	-0.3359828	4.57E-06	wdr44
		11	12054778	**0.5254899**	-0.6844934	-0.3062223	3.92E-06	stk36
upper sword edge pigment	Tlatemaco	6	21503972	**-0.5675278**	0.3738772	0.7830978	9.29E-06	VAV3-like isoform X1
false brood patch	Acuapa	2	26887045	**0.4312934**	-0.5820834	-0.2136167	9.77E-05	poc1b
nuchal hump	Tlatemaco	22	14980207	**-0.4312639**	0.2194994	0.5782469	3.07E-05	pyroxd2

Table 4. Sword component QTL - F_1 Allele Specific Expression (ASE) overlay. Intersection of QTL and genes exhibiting allelic imbalance in caudal tissue of *X. malinche – X. birchmanni* F_1 hybrids. Upturned arrows indicate up-regulation of the *birchmanni* allele relative to the *malinche* allele. Down turned arrows indicate down-regulation.

trait	Relative *X. birchmanni* allele expression	FDR	ensembl gene ID	gene name	dN
sword extension	↑	0.031406	ENSXMAT00000003439	brd2a	0
	↑	0.049153	ENSXMAT00000014489	HERPUD2	0
	↑	0.054171	ENSXMAT00000001052	hoxa13a	0
	↓	0.0552	ENSXMAT00000014569	hnrnpr	0
	↑	0.061783	ENSXMAT00000014186	prrc2a	0
	↑	0.076419	ENSXMAT00000003330	trib1	0
upper sword edge	↓	0.031406	ENSXMAT00000016394	usp33	0

linkage between loci, such variation in overall individual ancestry proportion can be expected by the chance pairings of gametes with complementary sets of ancestry blocks. The phenotype genotype correlations make sense in light of the parental phenotypic distributions for these traits. Increased *X. malinche* ancestry genome-wide is correlated with increased sword length, a *X. malinche* trait, and likewise, increased dorsal fin height and nuchal hump presence, both *X. birchmanni* associated traits, are correlated with increased *X. birchmanni* ancestry. This is an important consideration that if overlooked may result in spuriously significant QTL (Geldermann et al., 2003; Slate, 2013). For example, when I run a single QTL model for sword extension without adding genome-wide hybrid index as a covariate, I recover two additional QTL on chromosome 1 and 20. These loci may still warrant further investigation by building a multiple QTL model using the makeqtl and fitqtl functions in r/QTL but the current analysis does not have the power to confidently detect them (Broman & Sen, 2009).

Genotypic effects aligned with the phenotypes of the parental species, with the notable exception of upper sword edge pigmentation, where *X. birchmanni* alleles predicted a phenotype only found in *X. malinche.* This suggests epistatic interactions between a *X. birchmanni* allele at this locus and a *X. malinche* allele elsewhere. A two dimensional scan for interacting loci employing the scantwo function of r/QTL is an important next step in analyzing these data (Broman & Sen, 2009).

Because of the wide 1.5 lod intervals surrounding the QTL peaks (from 4.1 for sword extension to 11.4 for upper sword edge), there are potentially hundreds of genes to explore for each of these regions. In the future I will combine the statistical power of this classical QTL approach with the precision of admixture mapping results for the same suite of traits in natural hybrid population (Schumer, unpublished data) in order to narrow the genomic regions and help guide fine scale mapping to identify genes responsible for variation in these (Payseur & Place, 2007). Currently, in order to identify a first list of potential genes in the QTL regions I compared these genomic regions to times series data sets of two distinct wild *X. birchmanni* - *X. malinche* hybrid zones which vary in estimated time since initial hybridization and admixture proportions (Schumer et al., 2014). By overlapping loci identified as under selection from these data sets with the QTL regions I was able to recover seven potential genes of interest.

I also used differential expression data within and among individuals to further interrogate QTL for candidate loci. First, I used allele-specific expression data that leveraged F_1 males to identify alleles that exhibited intra-individual upregulation of one parental allele over the other. Here I

uncovered another seven genes that overlapped with the QTL region for sword extension and one for upper sword edge pigmentation. Add to these, *Hoxa3a*, which is located near the sword extension QTL peak was the 14th most differentially expressed gene in a *X. birchmanni -X. malinche* RNA data set, for a total of fifteen candidate genes for future study (Schumer et al., unpublished data).

Importantly, no QTLs for any trait co-localize to the same chromosome. Indeed, all QTLs are located on separate chromosomes and even under relaxed conditions where genome wide hybrid index is not considered for sword extension and three QTLs are recovered, none of them colocalize to the same chromosome as any other trait QTL (Fig. 10). This lack of co-localization, even among parts of a composite phenotype like the sword, indicates that these traits, are under separate genic control (Wagner et al., 2007; Wagner, 1996) and supports a scenario in which recombinant hybrid genotypes may result in trait combinations outside the typical ranges of the parental species and may address female preferences that do not align with available male parental phenotypes (Rosenthal, 2013).

CHAPTER V

CONCLUSIONS

Uncovering the dynamics of hybridization in its early stages is integral to understanding the evolutionary consequences of genetic exchange between divergent populations. These dynamics include not only how gene flow is initiated and its consequences on the fitness of those early hybrids, but also how sexual selection via mate choice acts on those hybrid phenotypes, the combinations and variation of which are ultimately determined by the genetic architecture underlying those traits. In my dissertations I explore these processes using the naturally hybridizing *Xiphophorus malinche – X. birchmanni* system.

In chapter II I show that in the common garden, F_1 and F_2 hybrids from the *X. malinche* x *X. birchmanni* (female x male) cross do not differ in survivorship, sex ratio, or male size at the onset of sexual maturity from either parental species, though time to sexual maturity does vary significantly with male F_2s maturing the fastest, *X. malinche* the slowest, and F_1s and birchmanni being intermediate. For both morphology and the physiological measure, critical thermal maximum, early generation hybrids were intermediate to parentals. Thus, I recovered no evidence for reduced viability in at the first stages of hybridization for these two species for one cross direction. Further, intermediate thermal tolerances could provide a selective advantage over parentals at the intermediate elevations where hybrid zones prevail (Culumber et al., 2012).

Behavioral assays of female mating preferences showed F_1 hybrid male chemical cues to be at least as attractive as conspecific cues for both parentals and that F_1 and F_2 females have more permissive preferences, not showing a preference for any one cue class over another while still attending to stimuli. Preferences in the olfactory modality are known to override visual preferences in *Xiphophorus* (Crapon de Caprona & Ryan, 1990; Hankison & Morris, 2003). Consequently, if premating behavioral isolation breaks down, the universal attractiveness of F_1 male chemical signals and the general permissiveness of early generation hybrid females coupled with no apparent viability reduction in one of the crosses should promote ongoing hybridization between *X. malinche* and *X. birchmanni* via backcrossing, even if the reciprocal cross rarely produces viable offspring. These conditions are consistent with the patterns of complete admixture observed in some natural hybrid populations (Schumer et al., 2014; Schumer et al., 2017). In chapter III I explore one way in which such behavioral isolation might initially dissolve. Calcium hydroxide, $Ca(OH)_2$, is used for disease prevention via bacterial load reduction in natural waterways throughout the range of *X. malinche* and *X. birchmanni*, as well as for wastewater treatment and lake eutrophication remediation elsewhere. Here I find that this anthropogenic pollutant, generally thought to be environmentally safe, not only disrupts chemical communication in *X. birchmanni*, but does so in a way that should promote ongoing genetic exchange with its sister taxon. When exposed to non-lethal concentrations of calcium hydroxide, female *X. birchmanni* switch from expressing the species typical conspecific olfactory preference to a preference for the heterospecifc *X. malinche*. This effect lasts for at least two days during which females are still motivated to mate and thus are likely to mate heterospecifically when given the opportunity.

The intermittent addition of calcium hydroxide to rural streams where regulations barring its use are less often enforced may explain the puzzling pattern observed across hybrid zones for these two fish, where less anthropogenically impacted sites which should be less polluted exhibit a breakdown of population structure while other sites where the human population is comparatively large but regulations are likely better enforced have highly structured hybrid populations where assortative mating is near complete (Schumer et al., 2017). Whether or not this is actualy the case, point source pulses of calcium hydroxide for any number of purposes has the potential to promote ongoing hybridization in populations where migration of non-hybrid parentals might otherwise re-establish assortative mating since exposed fish prefer the odor of heterospecfics. This, coupled with the universal attractiveness of early generation hybrid male cues presented in chapter II, outlines a scenario for ongoing interspecific geneflow even in the face of hundreds of identified genetic incompatibilities (Schumer et al., 2014). Taken together, the behavioral results from chapters I and II suggest a need for future studies examining female olfactory receptor neuronal response, before and after $Ca(OH)_2$ exposure to whole chemical signal profiles as well as the components that differ between species. *X. malinche* and *X. birchmanni* male pheromone profiles differ only in two *X. birchmanni* specific components (Holland, 2018) so it seems reasonable to first examine how exposure to calcium hydroxide alters perception of one or both of them at the periphery before considering alterations in down-stream signal processing.

The ways in which multivariate female preferences can affect and be affected by multivariate trait combinations in hybridizing populations depends in large part on the genetic architecture underlying these suites of traits (Iwasa & Pomiankowski, 1994; Rosenthal et al., 2003).

In chapter IV I produced an intercross mapping population in order to perform classical QTL mapping for a suite of secondary sexual traits that differ between *X. malinche* and *X. birchmanni*, most notably the composite trait for which swordtails earn their name the sword-like ventral caudal fin extension found in many *Xiphophorus* species males. Here I recovered one QTL each for five different correlated traits explaining between 3 and 6% of the variation and all of which were located on separate chromosomes. This was true even for the two components of the sword, relative length and a pigmentation character, upper sword pigment which always co-occur in *X. malinche*. Though all of these traits are likely highly polygenic given the low proportion of variation explained by the QTL identified, the lack of a colocalization pattern may indicate that these traits are control by separate genetic pathways, a situation that would allow hybridization to more easily produce trait combinations that better align with female multivariate preferences (Fisher, 2009), which could in turn promote ongoing gene between species and the introgression of some male secondary sexual traits across species boundaries.

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APPENDIX

SUPPORTING INFORMATION

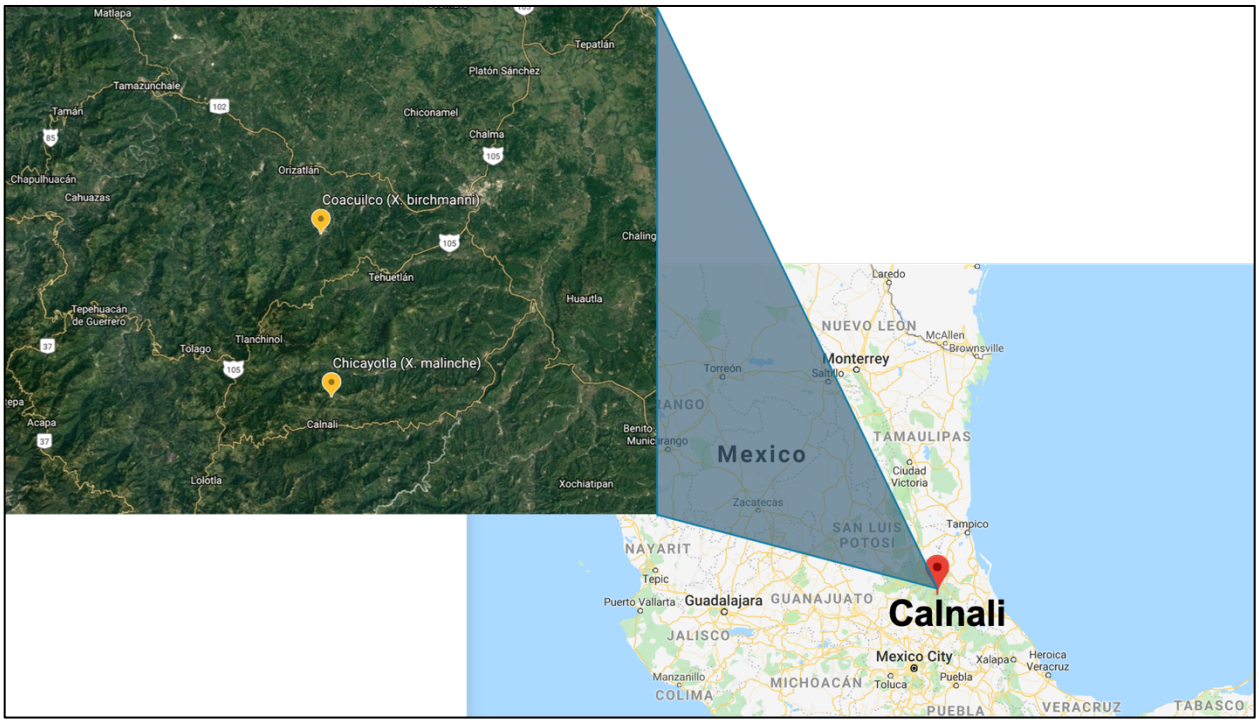

Figure S1. Collection sites for allopatric populations of *X. birchmanni* and *X. malinche*. *X. birchmanni* were collected from the Rio Coacuilco at the town of Coacuilco (21°5'50.85 N, 98°35'19.46 W). *X. malinche* were collected from the Chicayotla locality on the Rio Xontla (20°55'27.24"N 98°34'34.50W).

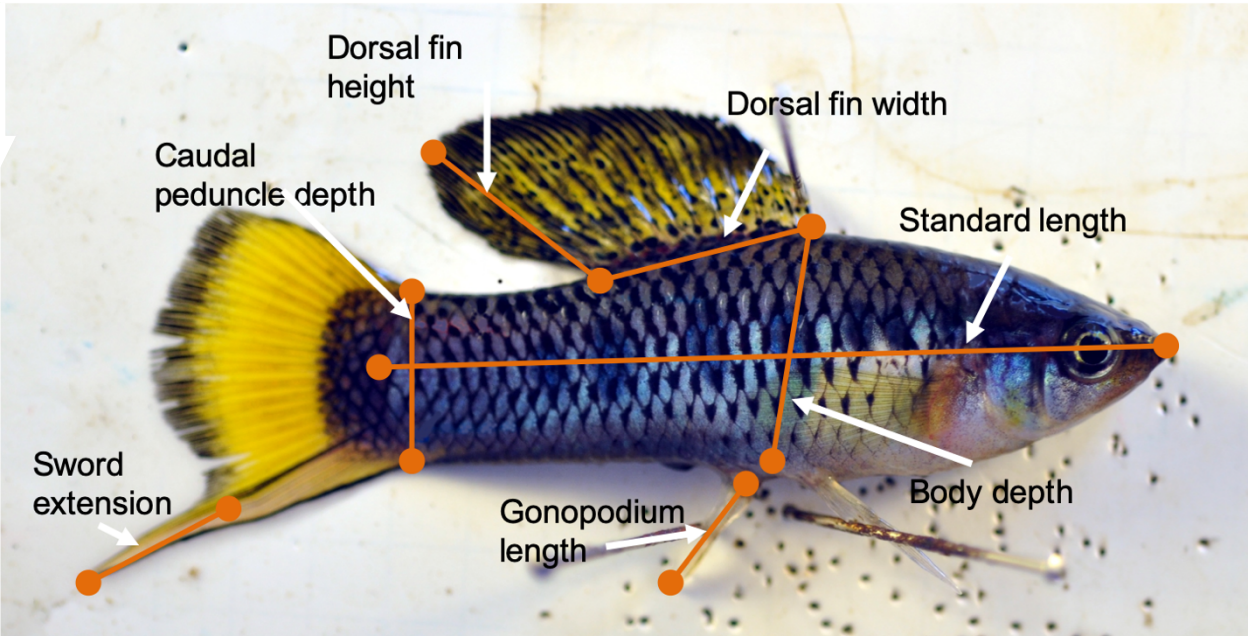

Figure S2. Schematic of morphometrics used for phenotypic comparisons in chapters II and IV. Measurements include: standard length (distance from the proximal tip of the mandible to the vertical midpoint of the distal edge caudal peduncle), body depth (distance from the insertion of the first dorsal ray to the vent), sword extension (distance from the , dorsal fin width (distance between the insertion of the first ray to the last), dorsal fin height (length of the second to last branched ray), the length of the gonopodium (modified anal fin and intromittent sexual organ), and caudal peduncle depth (distance from the dorsal insertion to the ventral insertion of the caudal fin.

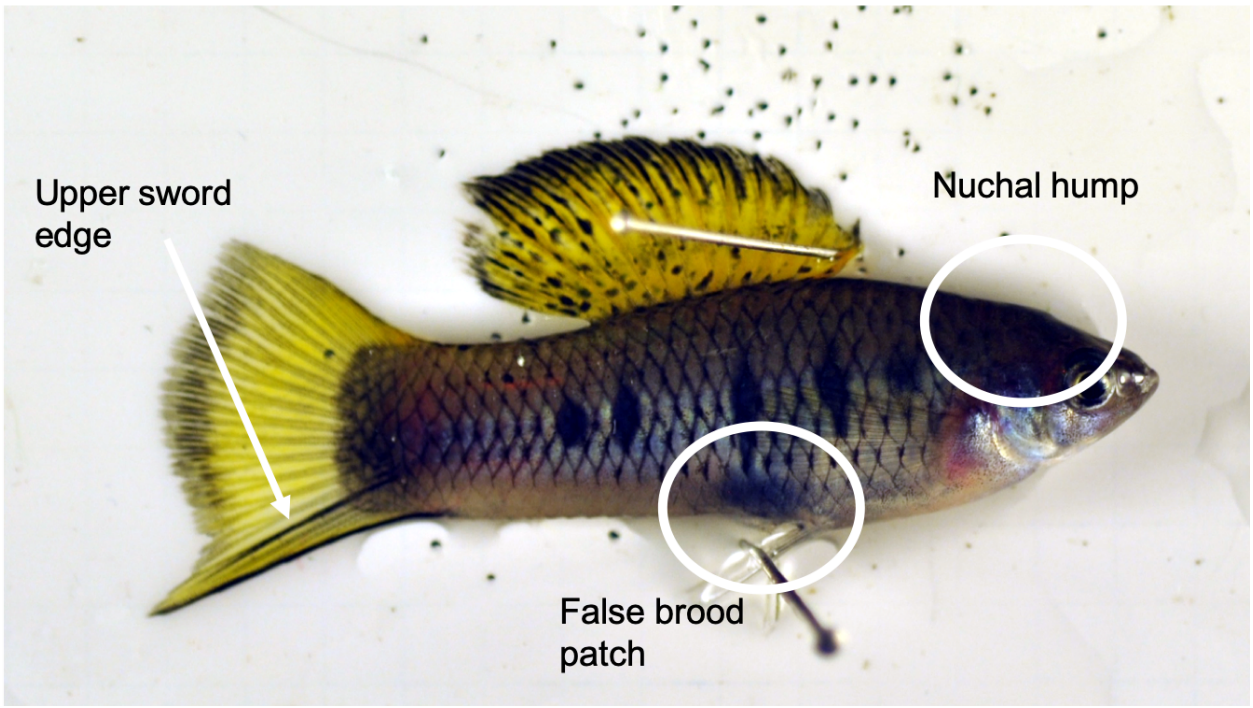

Figure S3. Traits scored as a binary (presence/absence) for QTL mapping in chapter IV. Traits include: fatty cephalic deposit (nuchal hump), and the melanin patterns, false brood patch, and dorsal sword pigmentation (upper sword edge).

Table S1. Eigenvalues and proportion of variance explained by principal components for common garden (*X. birchmanni*, F_1, and F_2) standardized male morphometrics.

	PC1	PC2	PC3	PC4	PC5	PC6
Eigenvalue	2.0075	1.6175	1.1101	0.6177	0.46938	0.17783
Proportion Explained	0.3346	0.2696	0.1850	0.1029	0.07823	0.02964
Cumulative Proportion	0.3346	0.6042	0.7892	0.8921	0.97036	1.00000

Table S2. Principal components analysis loading scores for common garden (*X. birchmanni*, F_1, and F_2) standardized male morphometrics.

Measurements	PC1	PC2	PC3	PC4	PC5	PC6
Body depth	-0.6523	0.97206	-0.89112	0.3753	-0.67201	0.109351
Sword extension	0.8673	0.93830	-0.10479	-1.0440	-0.18805	-0.001718
Dorsal width	-1.3964	0.06887	0.68653	-0.3733	-0.00381	0.451123
Dorsal height	-1.4692	0.51747	0.08397	-0.2447	0.18937	-0.489686
Gonopodium length	0.4381	0.63266	1.33783	0.3696	-0.48302	-0.130133
Peduncle depth	0.2839	1.40735	0.01948	0.3789	0.73723	0.142095

Table S3. Summary statistics for principal components analysis of common garden (*X. birchmanni*, F_1, and F_2) and wild collection (*X. birchmanni* and *X. malinche* standardized male morphometrics. Eigenvalues and proportion of variance explained by principal components.

	PC1	PC2	PC3	PC4	PC5	PC6
Eigenvalue	0.01261	0.009722	0.0008181	0.0003141	0.000191	7.733e-05
Proportion Explained	0.53139	0.409601	0.0344690	0.0132334	0.008045	3.258e-03
Cumulative Proportion	0.53139	0.940994	0.9754630	0.9886964	0.996742	1.000e+00

Table S4. Principal components analysis loading scores for common garden (*X. birchmanni*, F_1, and F_2) and wild collection (*X. birchmanni* and *X. malinche*) standardized male morphometrics.

Measurements	**PC1**	**PC2**	**PC3**	**PC4**	**PC5**	**PC6**
Body depth	-0.07848	-0.050226	0.16094	0.062254	0.0609391	-0.008960
Sword extension	0.88021	0.165071	0.02658	0.002339	0.0009058	-0.003006
Dorsal width	-0.17685	0.765491	-0.01833	0.008634	0.0167858	0.005240
Dorsal height	-0.10171	0.135168	0.13774	-0.028036	-0.0756566	-0.023044
Gonopodium length	0.01043	0.001545	-0.05971	0.123682	-0.0442138	-0.012206
Peduncle depth	0.01002	-0.012750	0.06234	0.021222	-0.0279605	0.065176