UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

BEHAVIORAL AND CHEMICAL MECHANISMS OF A CONDITIONAL SIGNAL IN THE GREEN SWORDTAIL (*XIPHOPHORUS HELLERII*)

A THESIS

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

Degree of

MASTER OF SCIENCE

By

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Norman, Oklahoma
2016

BEHAVIORAL AND CHEMICAL MECHANISMS OF A CONDITIONAL SIGNAL IN THE GREEN SWORDTAIL (*XIPHOPHORUS HELLERII*)

A THESIS APPROVED FOR THE
DEPARTMENT OF BIOLOGY

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Acknowledgements

I owe my success to the unwavering support from my family, my friends, and my colleagues at the University of Oklahoma. To Dr. Ingo, Schlupp, thank you for helping me expand my horizons as a scientist and be a whole lot braver. To my lab mates Ms. Shelby Burridge, Dr. Amber Makowicz, and Ms. Carolyn Burt, thank you for so much guidance and laughs. Thank you especially to Amber for providing extensive edits to this manuscript. A very big thank you to Dr. James B. Johnson for your help with statistical analyses. Thank you to Dr. Heather Ketchum and the rest of the physio gang for keeping me around and unknowingly helping me impress my family with physiology knowledge. To Dr. Rosemary Knapp, thank you for being a well of knowledge and experience for my hormone study. Thank you to Dr. Edie Marsh-Matthews for your advice on fish sedation and your patience with my many amendments. I also want to thank Dr. Knapp and Dr. Marsh-Matthews for being on my committee and providing me with great input and critiques over the course of my three years. Thank you to Dr. David McCauley for taking the time to finesse and produce the plastic swordtail models. Thank you to Dr. Lloyd Bumm for your generous assistance with color reflectance measurements. Thanks to Mr. George Martin for making the cylinders that were so vital for my experiments. Thank you to Dr. Michi Tobler and Ryan Greenway for providing the La Gloria fish stock. Thank you to Ms. Shelly Wu for being my blind in the chemical mechanism study, and for being a great friend. Thank you to Ms. Tara McDonald, Mrs. Rachel Steele, and Mr. Reid Mathews for assisting with photography and labeling, an arduous task indeed! To Momala, Farger,

and Erica, thank you for being my cheerleaders and always making the stressful times a little better, mostly with cat pictures.

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Abstract

Color polymorphisms are a common occurrence across most taxa. These differing forms of visual signals can act as a determinant for status and relative attractiveness for the choosy sex in a given species. While this provides many interesting avenues of research, we generally assume that differences in color give a signaler certain costs or advantages, depending on their phenotypic expression. In one population of the livebearing fish species *Xiphophorus hellerii,* a dimorphic trait in other populations has become a conditional, dynamic color signal. Males in this population can change their lateral stripe color to adapt to their social environment. This ability puts into question how individuals communicate within this population and amongst allopatric non-shifting populations.

Using a series of behavioral trials, we tested: 1) what mode of communication triggers color change and how male aggression is impacted 2) what preferences may exist amongst females for particular stripe color and 3) how shifting males allocate behaviors towards males and females from the same and from two allopatric populations. When comparing isolated modalities, visual communication initiated greater color change and aggression over chemical communication. However, when comparing mixed modalities there was not a clear trend. Females showed no distinct preference for a particular color stimulus, suggesting stripe color alone is not solely utilized in mate choice. We found that males do not change stripe color differentially towards different male stimuli based on population, but had a significant preference for sympatric females. Additionally, we investigated a potential chemical mechanism for stripe change, using two of the most common endogenous compounds, norepinephrine

and epinephrine, implicated in teleost color change. Both compounds failed to initiate a change, thus suggesting a different hormone or neurotransmitter may be the trigger for color expression. These findings raise further questions as to the function and production of visual cues, as well as potential prioritization of signaling.

Chapter 1

Modalities of male communication for a dynamic signal in *Xiphophorus hellerii*

Authors: Elizabeth J. Hardy, Ingo Schlupp

Abstract

Bright coloration or bold patterns serve as important visual signals to conspecifics, be it for male competition or to attract mates. These traits range in development, but it is understood that across a species or populations that the trait or polymorphisms of said trait remains the same, or static. In a single population of green swordtail (*Xiphophorus hellerii*) a trait previously thought to be static had been observed to be dynamic. Males possess the ability to change the color expression of their lateral stripe to suit their social environment. We investigated how male interactions responded to such a change in signal transmission modality by using a series of cylinders specialized for visual and/or chemical communication. Specifically, we looked at how visual or odor cues could potentially alter the magnitude and rate of color change, as well as the allocation of aggressive behaviors. We found the visual-only mode was more effective for causing color change over chemical-only or combined chemical-visual treatments. Aggression frequency and types, however, did not significantly vary by modality. These findings may offer insight into how conditional signaling impacts the behavioral interactions we expect with wild *X. hellerii*.

Keywords: modality, male-male contests, male aggression, color change

Introduction

Visual signals can be considered the physical embodiment of sexual selection, and are a common occurrence across taxa. This can take the form of exaggerated ornaments such as antlers or the enlarged claws of crabs that serve as weapons. Such ornaments are important in male-male interactions, used in settling territorial disputes and to win access to females. Other signals include areas of bright color or conspicuous patterns on the body itself, which advertise some reproductive benefit to females or serve as a badge of status for males (Barlow 1976; Thompson and Moore 1991). Vibrant colors are often associated with better health or status, as they require greater energetic input into pigmentation or avoidance of predators and parasites (Hamilton and Zuk 1982). Color and patterns are of particular interest because they can be utilized in social interactions either as a sole signal of information regarding health or status, or displayed as a precursor to further behaviors (Moretz and Morris 2006).

The animal kingdom has many examples of coloration used in intra-species interactions. Well-known cases include tropical bird species (Doucet et al. 2007), polymorphic lizard underbellies (Thompson and Moore 1991), and vibrant dimorphism of cichlids (Barlow 1976). Bright colors, contrasting patterns, and iridescence are usually associated with dominant and oftentimes larger males (Olsson 1994; Keyser and Hill 2000). Conspicuous coloration can inform rival males of the state of an individual, which can in turn impact whether or not interactions become escalate into physical contests. Male widowbirds with red collars, for example, have larger territories and participate in fewer aggressive interactions with intruders than males with less carotenoid-based coloration (Pryke et al. 2002). Coloration can also attract females into

associating with a male or his territory. A classic example is the female preference for spots of orange and iridescence in *Poecilia reticulata* (Kodric-Brown 1985). Females' sensory biases for male traits- in guppies and in other species- often contain a color component. It has been suggested that females have a preference for orange coloration as a sign of quality (Kodric-Brown 1985), or perhaps even a sensory bias for the color itself (Rodd et al. 2002).

An individual's coloration can change over the course of its life, but generally occur between the juvenile and adult stages. Coloration can also change in preparation for breeding, often referred to as nuptial coloration. Kodric-Brown (1998) referred to these types of changes in fish as seasonal or ephemeral changes. At the extreme end of the color change spectrum are species that can undergo physiological color change. Chameleon and cephalopod species are the most recognizable examples of rapid color change, but some teleost, arthropod, and amphibian species also posses this ability (Ligon and McCartney 2016). This can involve neurological or hormonal shifts that impact pigment cell expression, and can occur over a matter of minutes or even seconds.

In most cases these male color traits are considered to be static; they are developed in males across differing populations and species while retaining consistent visual information amongst them. Some exceptions do exist to the idea of presumed trait consistency, however. Polymorphisms in male coloration are not unusual (Morris et al 2003; Bond 2007; Gray and McKinnon 2007), but the ability to change between forms post-maturity appears to be relatively uncommon.

Males from the Actopan population of wild green swordtails (*Xiphophorus hellerii*) appear to have evolved a dynamic trait from one previously seen as static. *X. hellerii* is a member of the Poeciliidae family, and is a livebearing species. Green swordtails are native to the tropical climes and rapid-flowing brackish water of Central America, living in small hierarchal shoaling communities that range from Mexico to Honduras. *X. hellerii*, along with other sworded species in the genus, have been used as a model species in sexual selection research, particularly focusing on the importance of the male's caudal fin extension known as the sword.

Males' color and pattern dimorphisms can have social consequences in other populations, such as aggression or differential rates of mating (Franck et al. 2001; 2003), but no other wild *X. hellerii* population has been observed with a reversible color trait. Mature Actopan males have been observed reversibly changing the color of the lateral stripe that spans the length of the body, as described in Rhodes and Schlupp (2012). Males can change their stripe between a spectrum of light red to black within minutes. When the male appears red the black pigment cells, known as melanophores, are not being expressed over the red-pigmented erythrophores below. When displaying black, males fully express the melanophores and thus cover the red pigment cells below them.

Green swordtail dominance hierarchies have been studied at length. Body size, sword length and aggressive behaviors such as chasing, biting, and tail beats are seen as visual signals between males that can establish the social dynamics in a population (Franck and Ribowski 1989; Benson and Basolo 2006; Prenter et al 2008). Actopan males are observed as highly aggressive, even after apparent hierarchies have been

established (E.J. Hardy and I. Schlupp, personal obs.) Therefore, the question is: do these signals remain viable for communication between males in the Actopan population, wherein an additional transient visual trait could be utilized as a badge of status?

The purpose of this study is to determine the modality or combined modalities that males of *X. hellerii* use to communicate status, and how aggression may be altered based on said modalities. This could be of great importance in nature where ecosystem and social conditions are not consistent. Modality of communication can be important for how individuals proceed in behavioral interactions. For example, in *X. birchmanni*, the mechanism in which the cues are received from male conspecifics or related species impact female preference. When raised with conspecifics, *X. birchmanni* strongly preferred the chemical and visual cues of conspecific males. However, when only exposed temporarily to conspecific or heterospecific cues, olfactory stimuli results showed stronger timing effects than visual stimuli alone (Verzijden and Rosenthal 2011). In terms of male-male interactions, male stripe color might not only be a badge of status, but a warning for rival males. Males can already utilize visual signaling tactics as precursors for further aggression, such as the sigmoid curve (Beaugrand et al. 1984), but perhaps color change provides an alternative action for Actopan males. We used a series of treatments isolating and then combining separate visual and chemical cues to test male response to conspecifics. Specifically, we wanted to determine whether color change and aggression were impacted by mode of communication. We would expect if lateral stripe color were used as a signal, a difference in response over visual versus chemical treatments would be observed. Understanding how visual and

chemical cues impact potential contests can provide us with insight into how swordtail males may settle conflict in nature.

Methods

Study specimen

Adult males from the Actopan population reared at the University of Oklahoma were used for this study. These lab-raised fish are descendants of wild-caught fish collected May 2009 in the Río Actopan, near Xalapa, Mexico (Veracruz; 19◦ 25·47702' N; 096◦ 36·82764' W). Stock was kept in 250-L tanks in a greenhouse at the Aquatic Research Facility at the University of Oklahoma. Fish were transferred to laboratory housing in December 2013, and testing was conducted during the summer of 2014. Laboratory housing consisted of 37.85-75.71-L tanks under 12L/12D light conditions, with weekly water changes with deionized tap water supplemented with reef salt (Instant Ocean). No more than 4 males were housed in a tank, and all tanks contained females and juveniles to approximate natural social conditions. Fish were fed twice daily with flake food (TetraMin) or a mix of bloodworms, *Daphnia*, and brine shrimp (Hikari).

Prior to the start of trials, all males were photographed (Nikon D5200 24.1 MP CMOS Digital SLR camera) and measured in order to assign *a priori* small and large size categories. The cutoff for the large category was 41mm standard length during both sets of trials, with males ranging between 35 and 75mm long. Standard length was measured as the length from the snout to the base of the caudal fin. Sword length was measured from the base of the caudal fin to the tip of the sword. Additionally, males

were assigned a social rank based on the behavioral cues (aggressive versus hiding) and visual characteristics (size, stripe, and body colorations) while under normal housing conditions. If the male was isolated or only housed with females, it was categorized as an alpha male in order to analyze the effect of rank on color change and aggression variables. Past studies have shown isolated males tend to be more aggressive (Hannes and Franck 1983), so in this type of experimental setup it would be more appropriate to label males as alphas versus a subdominant category. The number of males within each category was comparable (9 alpha males, 6 beta males, and 11 omega males by the end of the experiment), but changed over the course of the study due to natural mortality and introduction of new fish to housing tanks.

Experimental setup

This experiment used Plexiglas cylinder treatments to isolate modes of communication between a free-swimming focal male and a stimulus male. Experimental contests, referred to as "matchups" were separated into four categories: small focal-large stimulus, large focal-small stimulus, small focal-small stimulus, and large focal-large stimulus. Matchup type and the order of the four treatments were randomized for each trial. Males were given at least 24 hours before being used again as a stimulus or focal male for subsequent trials. Before and after the trial both males' stripe colors were recorded, with a subjective color scale devised by the author as a metric. Colors were given designations based on those provided in the Microsoft Word color wheel that best matched the range of colors found on Actopan males. Colors were selected on a MacBook Pro (13-inch, 2012) on the 2011 Word software. A designation

of 1 indicated a light red to pink (R255, G68, B83), 2 was medium red (R220, G0, B0), 3 was dark red (R136, G0, B0), 4 was brown (R62, G0, B0), and a 5 was completely black. Focal males were photographed in the minute before and after the trial in a glass holding tank designed for taking photos.

All trials were conducted in a clear 10-gallon tank with all sides but the one facing the observer covered by white plastic boards to prevent additional visual distractions to the focal male. The stimulus male was placed in prior to the start of the trial, into either a clear cylinder with no holes (deemed the "visual-only" treatment), a cylinder with small holes ("small chemical+visual"), a cylinder with larger holes but with total open space equivalent to the previous cylinder ("large chemical+visual"), and the final treatment ("free swim"), which allowed the males to move freely to engage in all modalities, including the tactile communication available to both males. Cylinders were made from clear plastic panels connected with silicone sealant. The stimulus male was given a 5-minute acclimation period before the focal male was introduced. Entry of the focal male into the tank marked the start of a 10-minute observation period.

During the trials the observer noted the color changes (using the 5-point scale) and time of change of the focal male. Most trials were recorded using a high-definition camera (Nikon D5200 24.1 MP CMOS Digital SLR). Aggressive behaviors were counted while watching video playback, but only time of initial aggression and the instigator of said aggression were noted at the time of observation. Four aggressive behaviors were counted during each trial: the bite, chase, sigmoid or "s-curve," and the side-by-side posture (Franck and Ribowski 1987; Franck and Ribowski 1989). The four behaviors were chosen based on descriptions by Beaugrand et al. (1984). Chases were

defined as a rapid rush towards the stimulus male, as the male could not perform a full chase when separated by the cylinders.

After each trial, both males were removed and the tank and cylinders were cleaned with soap and water, followed by hydrogen peroxide, to reduce potential remaining chemical signals from previous trials (McLennan and Ryan 1999). Fresh water was added prior to each treatment, maintaining salinity level (averaging 800 ±50 mS) and temperature (averaged 27°C ± 2°) within a consistent range throughout the experiment. The same timing and recording protocol was used for each of the four treatments. "Chemical-only" trials were conducted at a later date, using a black opaque plastic cylinder with small holes, and the same procedures. It was not feasible to recreate matchups for the chemical-only trials, so new randomized trials were required. The University of Oklahoma's Institutional Animal Care and Use Committee approved protocol and animal handling for this experiment (R15-014).

Data Analysis

Data analysis for color change and aggression data was conducted in SPSS (version 17.0.0, 2008 SPSS Inc.). A total of 27 trials was completed for the four-treatment set, but one trial set was discarded due to the death of the focal fish shortly following the trial. A total of 26 additional "chemical-only" trials was conducted following the initial four-treatment sets. During the chemical-only trials seven additional males were added to the pool of subjects to account for mortality. The chemical only trials were analyzed using an independent-sample t-test with the "visual-

only" data set from the four-treatment experiment used to compare isolated modes of communication.

Color change

To examine differences in color expression and aggression in the four-treatment set, we used a repeated-measures ANOVA. Cylinder type was used as the factor, and normalized size differences (√arc (sin) transformation function) between males and differences in housing rank were used as covariates. The "free swim" treatment data were not used for the repeated-measures ANOVA. The dependent variables were 'net change in focal male stripe color from the start of each 10-minute trial to the finish of the trial' ("TXNetChange"), and 'time (s) to first observable focal male color change' ("TXFirstChange").

Aggression

Aggressive behaviors were quantified by the total time displaying aggressive behaviors as well as the numbers of each type of behavior. However, due to a lack of independence amongst these behaviors, we used a Principal Component Analysis for each cylinder treatment to create components. In this experiment males often demonstrated a suite of behaviors in quick succession, therefore separating behaviors into independent variables for analysis would not be realistic. Accidentally, video recordings were not completed for all trials, with eight trials consequently lacking aggression data. As was done for the color change data, we used repeated-measures ANOVA with cylinders as a factor, the normalized size and housing rank as our

covariates, and the PCA components as the dependent variables. Out of the 26 "chemical-only" trials there was only a single bite during the final matchup, therefore aggression was not analyzed for this trial set.

Results

Qualitative Observations

Stripe color changes are rapid, and a full shift from red to black or vice versa can occur within minutes or even faster (Rhodes and Schlupp (2012) & E. J. Hardy, personal obs.). Males displaying shades of red are generally thought to be more dominant, and in lab tanks only one male will usually stay red, after initial aggressive bouts during introductions of new males. Males in isolation will also stay red in solitary housing conditions. Stressful situations such as handling or losing aggressive bouts with conspecifics will instigate changes to darker stripe colors, ranging from a dark red to brown to black. Males in poor health will display darker stripe colors as well, which suggests red coloration may provide a signal to conspecifics of body condition or aggressiveness. In contrast, introduction of a female will trigger a change to red in most males in isolation, regardless of size.

Color Change

From the 26 four-treatment trials, 17 males displayed some degree of color change in the visual only and large "chemical+visual" treatments, and 14 males in the small "chemical+visual" treatment (Table 1). Of the 26 chemical trials, only 15 had any color change. Some interesting trends did appear when examining the rank of the

stimulus males relative to the focal males and net change. In the four-treatment trials where males were of equal rank (n=6), three matchups showed focal males shifting towards lighter colors/red, two did not change, and one became darker but only during the large "chemical + visual" treatment. When stimulus males were of a lower rank (n=9) eight of the focal males shifted to lighter/red colors, and the remaining male did not change color. In the trials with higher-ranked stimulus males (n=11) results were more evenly distributed, with four focal males becoming darker/black, four lighter/red, and three with no change.

The trends in the chemical only trials were not consistent with those of the previous trial set. In the equally ranked matchups (n=14), two focal males became lighter/red, and five became darker/black. Surprisingly, for matchups with lower ranked stimulus males (n=7), only three focal males changed, and each became darker. In the matchups with higher-ranked stimulus males (n=5), all males changed to darker/black stripes.

All repeated-measures ANOVAs for cylinder effects on net change in color were not significant (Table 2), with or without covariates. The "visual-only" cylinder stood out as having the greatest mean net change (=0.96 $\pm$ 1.22 s, indicating a shift up/down the color scale), while the "chemical+visual" cylinders and free swim control had both very similar and lower values when compared to the "visual-only" treatment. For the time to first change variable there was a significant effect with normalized size difference as a covariate ($F_{1,23}$ =4.87, p=0.01) (Table 2). With both normalized size difference and housing rank difference as covariates cylinder effect was non-significant ($F_{1,23}$ =2.83, p= 0.063), and the normalized size difference effect was significant ($F_{1,23}$

=4.87, p=0.01). "Visual-only" treatments took longer on average for the first change (=97.5 ± 124.02 s), whereas "small chemical+visual" treatment took the shortest time (=42.08 ± 52.5 s) (Table 1). When compared to "visual-only" treatment, the chemical trial net change was barely significant ($F_{2,50}$ =3.99, p=0.05), but the span of time to first change was not.

Aggression

In the four-treatment experiment 15 of the 26 males displayed some manner of aggressive behavior (Figure 1). Bites were the most frequent behavior (average = 5.97 ± 13.87 per trial), followed by the sigmoid display (average = 2.17 ± 6.1). Sigmoid displays are generally a precursor behavior to escalated aggression, and are more common when males are similar in size (Franck and Ribowski 1989). Amongst the treatment types the "visual-only" cylinder had a greater average (= 11.69 ± 21.55) combined aggression total than any other treatment. Interestingly the "free swim" control, which allows for all avenues of communication, had the lowest aggression average (average = 6.42 ± 14.51). Of the two mixed-mode cylinders the small chemical cylinder showed greater average aggression.

Principal Components Analysis produced 4 components to cover 100% of the variance. The first two components accounted for about 99% of the variance as dependent variables for the repeated-measures ANOVA. PC1, which had heavy loadings on bites and sigmoid displays, was not significant across cylinder treatments with or without covariates. PC2, with loadings in the negative range for bites and side-by-side displays, and sigmoid displays in the positive range were also not significant.

Discussion

Despite the ambiguous trends amongst combined modalities, we can at least confirm that visual stimuli are a primary source for male response (i.e. color change). "Visual-only" cylinders elicited both the greatest amount of change and required the longest time for change. This is not a completely unexpected result, as visual displays and ornaments are valuable communication tools for swordtails in male contest and for attracting females. We can also see that a high hierarchical position and male size can be important for male interaction. For contests with focal males of larger size, we can predict shifts to red. In cases where stimulus males were of equal or larger size, males were more unpredictable.

This is not to say chemical or olfactory cues are not being perceived and used as well. A number of studies have discussed chemical communication amongst poeciliids (McLennan and Ryan 1997; Wong et al. 2005; Fisher and Rosenthal 2006). In our case, chemical cues alone do not appear to be able to instigate aggressive behaviors and the observed color change based on stimulus size was inconsistent. The lack of clear-cut differences between mixed modality treatments may be a byproduct of the experimental set up, or it could reiterate visual stimuli as the most reliable source of intraspecific information.

A lack of significant aggression trends provides us with further questions. Even when male size and rank were used in analyses aggression factors were not significant, which brings into question how hierarchies can be established in the Actopan population. It may be possible that stripe color is used as a primary signal for status but does not factor into aggression like we see in other populations of swordtails. Franck

and Ribowski (1989), found that green swordtail aggression omega males took the offensive more often than alpha males in contests with unfamiliar males. Aggression type didn't significantly vary by rank or comparison of size in our data, and omega focal males actually tended to have fewer aggressive behaviors. This could be due to potential error in the designation of rank. However, frequencies of aggression types were similar to that in the aforementioned study. This may then connect back to the fact that this is a dynamic trait, and suggests that males, regardless of size or temporary rank, can endure risks from instigating aggression more often in order to gain dominance. This concept is not unheard of, particularly in fish species. Cichlid species *Astatotilapia burtoni* males can utilize color change into behaviors to settle territorial disputes (Korzan et al. 2008). In the future, examining this population in the field could provide us with more insight into potential differences in social structure compared to most populations of *X. hellerii*. Based on our findings, it is clear that the lateral stripe color is a visual cue, but it appears that the characteristics of both the signaler and receiver impact how this color change signal is initiated.

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Tables

Table 1- Descriptive statistics of cylinder effects on net change in color and time of first observed color change. Note that the "chemical-only" treatment was a separate experiment. Mean color change in the positive range indicates a lighter stripe, while negative values indicate a trend towards darker colors.

Cylinder Treatment (n=26)	Mean color change	First Change (sec)
Visual Only	0.96 $\pm$ 1.216	97.50 $\pm$ 124.017
Visual + Small Chemical	0.69 $\pm$ 1.05	42.08 $\pm$ 52.495
Visual + Large Chemical	0.65 $\pm$ 1.441	60.69 $\pm$ 129.926
Control (Free Swim)	0.69 $\pm$ 1.123	66.35 $\pm$ 131.49
Chemical Only	-0.50 $\pm$ 0.762	75.62 $\pm$ 150.734

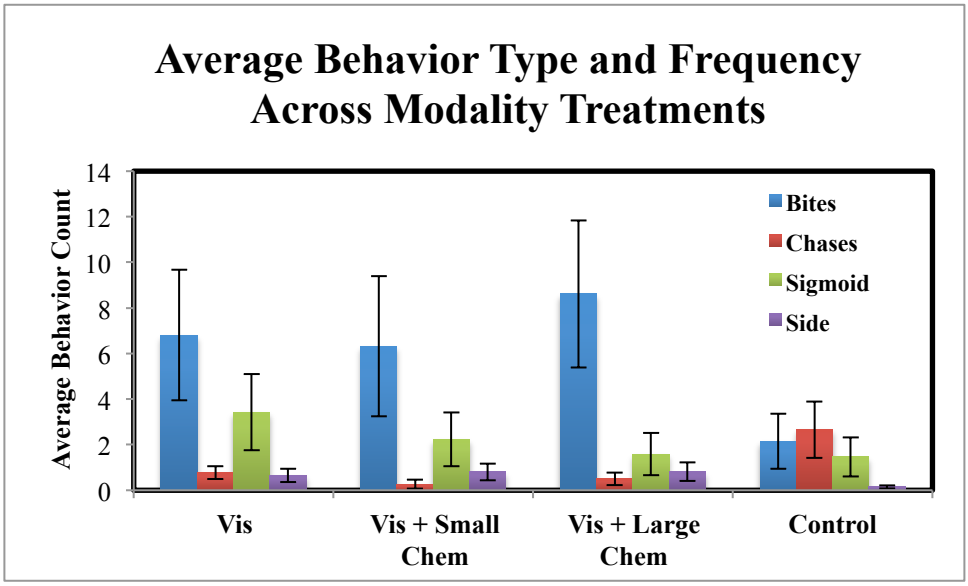
Table 2- Results from repeated-measures ANOVA for the cylinder treatments (excluding "chemical-only", which was tested via t-test). The table shows the F- and p-values for all iterations of the ANOVA for cylinder effects (n=26).

Cylinder Effect (r.m. ANOVA)	F			p		
		w/ rank	w/ size diff.		w/ rank	w/ size diff.
Net Change	2.617	1.809	2.100	0.078	0.176	0.131
First Change	2.831	0.339	4.867	0.063	0.797	0.01

Figure Legend

Fig. 1- Average (+ S.E.) number of aggressive behaviors across cylinder treatment. Note that there were no aggressive behaviors in the "chemical-only" treatment.

Figure 1


Average Behavior Type and Frequency Across Modality Treatments
Average Behavior Count
14
12
10
8
6
4
2
0
Bites
Chases
Sigmoid
Side
Vis
Vis + Small Chem
Vis + Large Chem
Control

Chapter 2

Lack of female preference for a visual signal in a conditionally signaling population of *Xiphophorus hellerii*

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Abstract

Female preference has been established as the key driving force for sexually selected visual traits displayed by males (Andersson 1994). These traits can be consistent across a species, or specialized by population depending on a variation of biotic factors. In a population of green swordtail (*Xiphophorus hellerii*) males can reversibly change their lateral stripe color in response to social environment. Females possess preference for particular coloration in other polymorphic populations, but it is unknown whether a conditionally signaling population maintains the same preference. We used two experiments using red and black colored stimuli, either a color strip or a model male, to test female association time. In both cases females failed to show a significant preference, even when separated by size. This leads to further intriguing questions as to how inter-population variation can impact preferences.

Keywords: Sexual selection, female preference, color change

Introduction

Female preference for male traits plays a vital role in the genesis of male ornaments and coloration. From Darwin (1871), Fisher (1930), and other fellow scientists who shaped sexual selection theory to the present, this concept has been evident for more than a century (Andersson 1994). Females base their preference on benefits they may gain from more "attractive" males, oftentimes the showier or most heavily ornamented of the available mature males (Kodric-Brown 1993). Benefits can be more tangible and direct, such as food, territory, or other environmental resources. For example, males from wild fowl populations of *Gallus gallus domesticus* use food offerings as a part of their courtship rituals with nearby females (Pizzari 2003). In a lab setting, females of two species of cichlids (*Pundamilia* sp.) chose nesting sites based on the quality of a male's territory rather than being swayed by a preference for conspecific male coloration (Dijkstra et al. 2008). This preference has been confirmed previously in the field as well, as species with social structures highly based on territoriality show females selecting males according to territory quality (Andersson 1994). Males that can both provide a benefit for the female while energetically maintaining colorful expressions in the form of mating signals is more likely to be chosen (Hamilton and Zuk 1982).

Additionally, males that have the preferred version of a visual signal may provide indirect (genetic) benefits to the female in terms of increasing fitness as well (for a comprehensive review refer to Andersson and Simmons 2006). This can work in tandem with the direct benefits previously mentioned as an indicator of preferential genetic traits. Females can choose mates based on indicator mechanisms (Maan et al.

2006) or pre-existing biases (Basolo 1995), leading to greater fecundity or the increased likelihood of offspring surviving. A male able to maintain higher levels of homeostatic health can allocate more energy into visual traits and can presumably pass this advantage on to future male offspring, known as the the "sexy son hypothesis" (Weatherhead and Robertson 1979). This can be an even more valuable tool for female selection in environments with high predation or parasitism (Zahavi 1975; Maan et al. 2006).

There is evidence of sexual selection for visual traits across the animal kingdom (Andersson 1994). Fish provide some of the most well known models of sexual selection theory, including cichlids (Seehausen and van Alphen 1998), sticklebacks (Milinski and Bakker 1990), and guppies (Kodric-Brown 1985). Livebearing poeciliids, including guppies and members of the genus *Xiphophorus*, have been used widely as model systems for the study of sexual selection for visual traits and ornaments (Basolo 1990; Rosenthal and Evans 1998; Morris et al. 2003).

The swordtail (*Xiphophorus hellerii*) is one of the most well studied species within the genus *Xiphophorus*, and was even described by Darwin (1871). This livebearing species is native to Central America, but has become a popular aquarium fish. The caudal sword found on wild-caught males in *X. hellerii* and other swordtail species is the most heavily studied of the genus' sexually selected traits, and was noted in Darwin's observations of the green swordtail. Studies on this trait have shown that a longer sword cannot only attractive (Basolo 1990), but can also enhance the perception of body size (Rosenthal and Evans 1998). Basolo (1996) determined that this preference is a product of a pre-existing bias in females for larger perceived body size

in swordless *Xiphophorus* species (i.e., platyfish). In addition to sword length, the coloration of the sword itself can impact female preference (Basolo and Trainor 2002). Of course, these preferences can be lost depending on biotic factors such as predation or population structure (Rosenthal et al. 2002).

In addition to the sword, pigmentation patterns and spotting can also be sexually selected visual traits. *Xiphophorus cortezi* males develop oncogene-based melanoma or black marks that can impact female choice, with distinctions varying down to an individual or population level (Fernandez and Morris 2008). *Xiphophorus cortezi* females have also shown preferences for male symmetry in the placement of vertical black bars on the sides of male conspecifics (Morris and Casey 1998). Some species even have ornaments that reflect in the ultraviolet spectrum, a characteristic visible to conspecifics, but not to the human eye or to *Astyanax mexicanus*, a species known to prey upon *X. cortezi* (Cummings et al. 2003).

While Darwin did note the importance of the male sword, he did not put much focus on the *Xiphophorus* lateral stripe, and it has not been as extensively studied. We do know, however, it can be utilized as a signal in intra- and intersexual communication. One population of *X. hellerii* found in the Río Chachalacas in Xalapa, Veracruz, Mexico has male color dimorphism that impacts inter- and intrasexual interactions (Zander 1986; Franck et al. 2003). Males possessing lateral red stripes in this population are believed to be more dominant males with black stripes in aggressive interactions, and females show a preference for red as well (Franck et al. 2003).

In a different population from the Actopan region of Veracruz, that same dimorphism in male visual signaling has developed into a dynamic badge of status

(Rhodes and Schlupp 2012). The ability of males to change their lateral stripe color brings into question the validity of stripe color as a reliable signal in the context of mating. It is important to note that Actopan females do not posses this ability to change color. Red males in the Xalapa population are heterozygous dominant for stripe color, whereas black males are recessive. Franck et al. (2003) suggested color preference could be adaptive for females in order to avoid producing offspring with the lethal homozygous dominant genotype (Zander 1986). This brings into question how mate choice may be impacted in a population like Actopan, where the coloration of a male is not only conditional but also controlled by the individual's social environment.

If mature males can, regardless of size and rank within the Actopan population, alter this trait, does it still transmit the same information to Actopan females? Red males in the Xalapa population do tend to be larger, and even in male-male contests smaller males that were red have been observed defeating black males (Franck et al. 2003). Lab-reared Actopan males in the presence of other males will form social hierarchies (see Beaugrand et al. 1984 for more information on *X. hellerii* social dynamics). However, when given the chance to interact with a female, a subdominant male who stays black in the presence of larger or more aggressive males will turn red (Rhodes and Schlupp 2012). Though larger sized males usually stayed red longer, all males -regardless of size (i.e. weight or standard length) - appear to be capable of shifting back and forth between the colors. These alterations are based on the social environment and possibly status of their health.

In order to test the significance of conditional signaling on female preference we used two versions of binary choice tests to measure association with red versus

black stimuli. Based on past studies, we would expect red to be preferable in other populations with polymorphisms. In the case of the Actopan population, any male can manipulate the signal, so we cannot yet assume females rely on it as a cue for preference. Males advertise themselves to females via complex mating displays like other populations, but are also capable of changing their stripe to red or almost pink in color. Therefore, we would expect that males use this as a signal to stimulate a visual preference in the female. We hypothesize that if a preference for color exists, females will display said preferences through a difference in how they allocate their time in distinct preference zones. We conducted two experiments- the first using a simplified color stimuli, the second using decoys- to test for preference between red and black. A divergence from the expected preference for red may suggest the preference has been altered by the ephemeral nature of the trait in Actopan males.

Methods

Study specimen

Lab-reared females from the Actopan population were used for both experiments in this study. The Actopan population originated from the Río Actopan, near Xalapa, Mexico (Veracruz; 19◦ 25·47702' N; 096◦ 36·82764' W). The fish were originally collected in May of 2009 and brought to the University of Oklahoma. Stock was reared in 250-1000 L tanks in the Aquatic Research Facility, as well as laboratory housing composed of 37.85-75.71-L tanks. In greenhouse conditions fish were fed two or three times a week and were kept under natural light conditions. Lab housing was kept under 12L/12D light conditions, with weekly water changes. Specific housing

details are outlined in Makowicz and Schlupp (2015). Fish were transferred to laboratory housing at least a week before testing in order to acclimate to lab conditions. Females were housed in mixed-sex tanks until the weeks preceding both experiments. Females can be identified based on the lack of long caudal extension known as the sword and often will often have a dark ventral brood spot between anal and caudal fin. Fish were fed twice daily with flake food (TetraMin) or a mix of bloodworms, *Daphnia*, and brine shrimp (Hikari).

Experimental setup

Experiment 1: Color Strip

The first experiment examined female preference for color utilizing isolated color stimuli. Females (n=44) were tested in a 75.7-L tank (61×39×30cm) separated using permanent marker into three zones: a central "neutral" zone, and a preference zone on opposing sides (Figure 1). Tank salinity (averaging 800 ±50 ppm) and temperature (averaged 27 °C ± 2°C) were kept within a consistent range throughout the experiment. Four white Plexiglas panels surrounded the sides and bottom of the tank, with the exception of the side facing the observer. The left and right panels had either a black or red colored strip of paper (approximately 6.5 × 2 cm) fixed to it to serve as color stimuli. The back panel in the back of the tank had equidistant strips of each color, placed one in each preference zone. The colored strips were switched after each replication of the trial to test for side bias (see Landmann et al 1999).

Live males were not used as stimuli in either experiment, due to stripe color inconsistency during pilot trials. Video stimuli, while successful for measuring

response to behaviors (Trainor and Basolo 2000), were also not used due to potential distortion of color from the swordtails' visual spectrum. Swordtails do not perceive color in the same spectral range as human; therefore we had to rule out this experimental method to increase our chances of interpretable results (Rosenthal 1999; Oliveira et al. 2000).

Prior to the start of a trial, each female was put into a size category based on her standard length, which was measured prior to being introduced to the experimental setup. Standard length was measured from the snout to the base of the caudal fin. Lengths ranged from 28-62mm, with females smaller than 35mm placed in the small group, 36-43mm in the medium group, and those above 44mm into the large group. Group size was later used as a covariate in our data analysis. A complete trial consisted of two parts, 5 minutes each to prevent side bias. A clean, open-bottomed plastic cylinder containing the focal female was placed directly in the center of the tank (between the two stimulus zones in neutral section) for the 5-minute acclimation period. At the end of this period the cylinder was removed and the 5-minute timed observation period begins (trial 1).

Female association time (s) within each of the two preference zones was recorded using stopwatches. Any time the female passed into a zone with more than half of her body the timer for that respective zone started. Once the first trial replicate (part 1) was concluded, the female was placed back into the cylinder and another 5-minute acclimation period began. Once acclimated, a second observation period (part 2) was conducted, with identical protocol but the color stimuli were switched. The sums from each trial (parts 1 and 2) for each female were then used for data analysis.

Experiment 2: Model Test

To attempt to provide a more realistic color stimulus we created a set of male swordtail models. Two plastic models were created via 3-D printer, using a digital swordtail template purchased on a 3-D model website (www.turbosquid.com). Dimensions from the template were altered to best fit the size of an average *X. hellerii* male in the Actopan population. Acrylic paint (Grumbacher Academy Acrylics codes C026, C134) was used to create the appearance of males expressing red or black stripes. Unfortunately, the visual spectrum for swordtails is not identical to that of humans (Watson et al. 2010). To create a similar color stimulus similar to that of a live male we used spectrophotometry to measure reflectance of both the paint samples and live male stripes. Our goal in this was to match the colors and wavelength range reflected by an actual living male that a female swordtail may encounter.

Paint and male stripe reflectance data (Figures 2 and 3) were gathered using an Ocean Optics HR-2000 spectrometer, connected to Spectra Suite software (Ocean Optic Inc.). The fiber optic cable was fed into a camera attachment on a Nikon Eclipse ME 600 microscope. Reflectance measurements were taken for each of four potential red paint samples, which were painted onto a sample sheet of the same plastic material used for three-dimensional models. Stripe color reflectance was measured while individuals were sedated with clove oil and submerged in a glass petri dish with water. Multiple stripe measurements were taken per male during a single sedation period.

Reflectance data were analyzed using the summary function in R-Studio package 'pavo' (Maia et al. 2016). Male data were averaged to approximate the best potential red paint for the range of male stripe colors expressed. We compared

brightness, chroma, and hue data (see the 'pavo' user manual for descriptions) of each paint sample. We selected a paint that matched the average values closest to that of male spectral data (Table 1). The selected color was then painted onto the model as a stripe mimicking that of live males and sealed with a waterproofing coating.

The models were affixed to the outside of the tanks and switched in between trials like in the previous experiment. Size categories were slightly adjusted based on female availability. Small females were 31mm or shorter, medium females were 32-38 mm and large female were 39 mm or larger in standard length. Standard length ranged from 25-53 mm. The Institutional Animal Care and Use Committee at the University of Oklahoma approved animal handling and experimental protocol for this study (R15-014).

Data Analysis

Experiment 1: Color Strip

Trials from 44 females were used for analysis in the first experiment. All analyses were performed using SPSS software (version 17.0.0, 2008 SPSS Inc.). For the initial color strip experiment, strength of preference was calculated using a paired t-test. We used total time (s) in zones, as well as percentage of time and normalized time as potential dependent variables. Percentage of time was calculated as the total in a particular zone divided by the sum of time spent in both preference zones. Data was then normalized using √arc (sin) transformation. Any trials where female showed a side-bias were excluded from the analysis. Side bias was calculated by dividing the sum of all time spent in the left region over the total time spent in preference zone

during the two replicates. Using this calculation, a value of less than 15% or higher than 85% indicated a bias for the right or left side, respectively (McCoy et al. 2008; Makowicz at al. 2016). In a post-hoc analysis, female size was used as a factor in a one-way ANOVA, with the three aforementioned preference variables as dependent variables.

Experiment 2: Model Test

A total of 32 trials was used for the analysis of the second experiment. Sample sized diminished between experiments due to availability of mature females and notably frequent side biases. Again, strength of preference was calculated via paired t-test in SPSS. We used the same three dependent variables for both the t-test and post-hoc one-way ANOVA with a female size factor.

Results

Experiment 1: Color Strip

Females on average showed a slightly higher preference for the black strips (53.8%, versus 46.2%), but this was not significant (t_{43}=-1.09, p=0.28, n=44). All dependent variable comparisons proved to be non-significant. When analyzed separately by size, small (n=14) and large (n=13) females showed greater preference for black (mean preference=58% and 60.5%, respectively). Medium-sized females showed the opposite preference, with a 54.8% preference for red (Table 2). However, when female size was integrated in a post-hoc one-way ANOVA the results were still not significant ($F_{2,43}$=2.55.2, p= 0.09, n=44).

Experiment 2: Model Test

Unlike with the simplified color strip experiment, females tested with models showed a slightly higher preference for red (53%) versus black (47%), however it proved again to be insignificant (t_{31}= 0.8, p= 0.43, n=32). Interestingly preference percentages essentially reversed between the two experiments. Total time, percentage preference, and normalized preference were all insignificant. Post-hoc analysis also confirmed that size was not a significant factor for preference. Medium females (n=11) showed the highest preference for red at an average 53.8%, while small (n=12) and large (n=9) females showed a slightly lower and comparable average (both at approximately 52.5%) (Table 2).

Discussion

Females from the Actopan population, based on these two experiments, appear to lack a preference for the isolated male characteristic of stripe color. We would expect females in swordtail populations with polymorphic males to choose the brighter or more elaborate male. Based on previous research on female preference for body size and sword presence (Rosenthal and Evans 1998), we would have expected females to prefer larger, generally red shifting males. However, females from the Actopan population expressed near to equal preferences for either red or black male morphs in their experimental environment. There are a multitude of possible explanations for this absence of preference. We do not believe our experimental setup was at fault, especially using model stimuli. Females did respond to the models, at times darting

rapidly in response to first viewing a model. Models have been successfully used in other color-related fish studies (Phamduy et al. 2014, Anderson et al. 2016).

It may be that females require more complex visual stimuli than simply color. For example, females could rely on sword length or physical mating display in tandem with stripe color. In this case a red stripe would provide a contrasting pattern for the yellow and black coloration of the sword, versus a more similar black stripe. Using video-playback, Basolo and Trainor (2002) showed that female green swordtails show greater response to complete male swords over their components, so it could be that stripe color alone is not a complete signal for Actopan females. Preference can also be context-dependent, so if females prefer a "rare-type" male morph for genetic benefits (Royle et al. 2008), choosing solely based on stripe would not be effective in this population.

A further intriguing possibility is that females have lost a color preference due to the conditional nature of the signal in this particular population. In polymorphic populations of *X. pygmaeus*, females do not show a preference for male color morph regardless of rarity, making visual traits or behaviors other than color of importance (Baer et al 1995). Dominant males remain red in aggressive contests, so while this remains a signal of aggression, it may no longer be attractive to females. Female guppies avoid repeat matings with males and will discriminate against other males with a similar appearance (Eakley and Houde 2004). Perhaps swordtail females in this population use a similar tactic and disregard color traits as attractive characteristics. There is much left to be discovered about female preference in the Actopan population,

as well as what the magnitude of variation might be hidden amongst the natural populations in this species' range.

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Tables

Table 1- Average scores for paint and male lateral line reflectance data. Variables and their units are described in the R-package "pavo" user manual.

	S1 UV	S1 violet	S1 blue	S1 green	S1 yellow	S1 red	H1	H2	H3	H4	H5	B2	B3
C026	0.147	0.157	0.102	0.165	0.162	0.265	642	685	416	-0.913	189	178.683	496.118
C027	0.085	0.091	0.054	0.165	0.261	0.472	618	669	404	-0.893	593	0.261	0.472
C029	0.085	0.091	0.066	0.142	0.207	0.475	627	669	408	-0.818	593	313.923	1791.3
C095	0.101	0.107	0.065	0.17	0.245	0.417	618	669	404	-0.921	593	261.876	1343.68
Male Avgs	0.183	0.196	0.107	0.159	0.151	0.184	833	833	511	-1.051	832	150.277	490.92

Table 2- Average (+S.E.) female percentage preference for color in preference zones, separated by size category. In the stripe experiment groups were: small (<35mm) , medium (36-43mm) and large (>44mm). For the male model experiment groups were: small (<31mm), medium (32-38 mm), and large (>39mm).

	Exp. 1-Stripe		**Exp. 2-Model**	
	Mean Percent Preference (%)		**Mean Percent Preference (%)**	
	Red	**Black**	**Red**	**Black**
small	42.0 $\pm$ 4.3	58.0 $\pm$ 4.3	52.5 $\pm$ 7.0	47.4 $\pm$7.0
medium	54.8 $\pm$ 5.1	45.2 $\pm$ 5.1	53.8 $\pm$ 7.4	46.2 $\pm$7.4
large	39.5 $\pm$ 6.3	60.5 $\pm$ 6.3	52.6 $\pm$ 4.0	47.4 $\pm$4.0

Figure Legend

Fig. 1-Representation of testing tank with preference zones. Layout remains the same for both experiments. Note that preference zones were switched in trial 2 of each female to detect side bias. The circle in the center represents the focal female.

Fig. 2- Screenshot of the reflectance of paint samples. Each line represents the reflectance from a paint swatch painted on the same plastic as the models. The 100% reflectance was scaled to a white reference (spectralon) in air.

Fig. 3- Screenshot of male reflectance data. Each individual line represents one reading from the lateral stripe of one of three males. Note males were measured in multiple areas of the stripe. Fish were measured under water, therefore there were additional reflection/transmission losses that reduced reflectivity. Those additional losses are uniform across the spectrum.

Figure 1

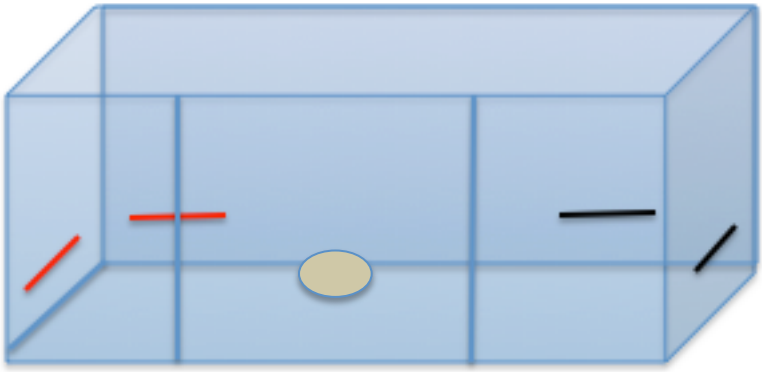


Figure 2

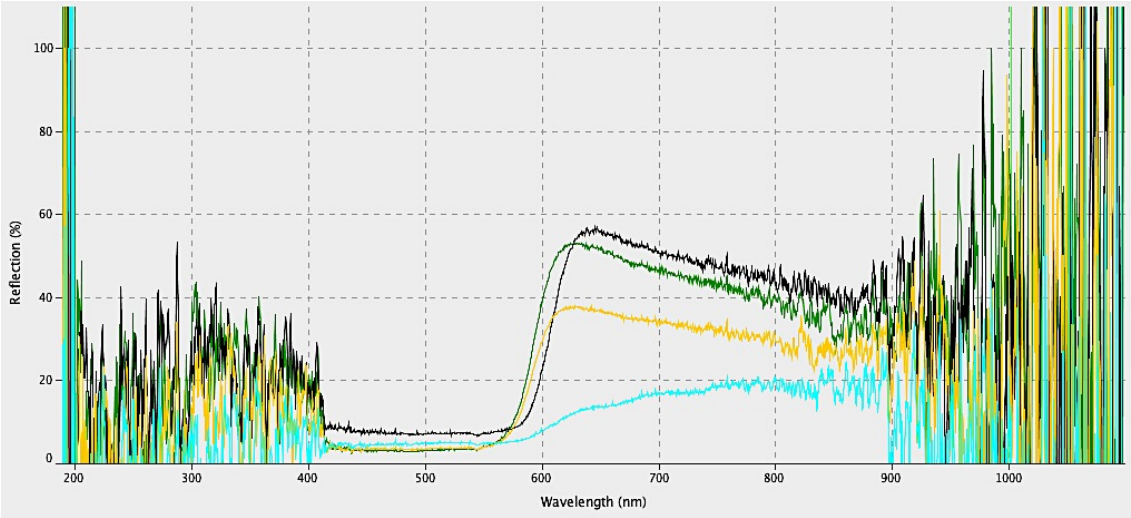
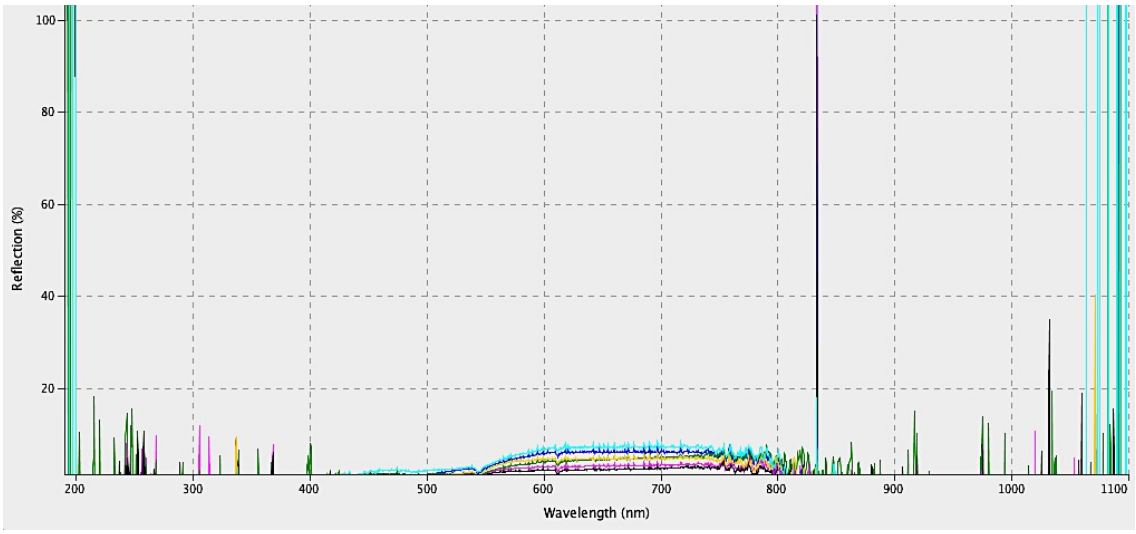

Reflection (%)
100
80
60
40
20
0
200
300
400
500
600
700
800
900
1000
Wavelength (nm)

Figure 3


Reflection (%)
100
80
60
40
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200
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400
500
600
700
800
900
1000
1100
Wavelength (nm)

Chapter 3

A proposed chemical mechanism for a conditional signal in *Xiphophorus hellerii*

Authors: Elizabeth J. Hardy, Ingo Schlupp, and Rosemary Knapp

Abstract

Physiological color change is a rapid alteration in pigment aggregation or dispersal, signaling social status or health. This is a complex process, carried out by a number of different pathways in amphibian, reptile, and fish species. The literature on fish examines the effect of endocrine or neurological pathways on the black pigment cell, known as a melanophore. There is no single endogenous compound that consistently causes the same types of changes in all fish species. However, adrenergic pathways, using the hormone epinephrine and the neurotransmitter norepinephrine, are common stimuli for melanin aggregation in many fish species. This study uses epinephrine and norepinephrine to test a novel color-changing trait in *Xiphophorus hellerii*. Males in one observed population have the ability to rapidly expand and contract the melanophores on their lateral stripe, but the chemical mechanism causing the response is unknown. We investigated relative melanophore expression by using topical application of epinephrine and norepinephrine at varying concentrations. Additionally, we added a beta-blocker (propranolol) to test our observed effects of adrenergic compounds. We found that melanophore pigment dispersed in all treatments, but did not reverse once the drug was flushed from the skin. The addition of

a beta-blocker did not change the results, revealing that lateral stripe color change does not rely on a beta-adrenergic pathway.

Keywords: color change, melanophores, epinephrine, norepinephrine

Introduction

Color change is one of the most striking forms of visual communication in the animal kingdom. In general terms, color change is classified into two forms: morphological and physiological change. Morphological change is associated with stages of development and maturity, halting once an individual reaches adulthood (see Fujii 1969 review). This is the type we think of most often in cases such as avian plumage (Figuerola and Senar 2005) or mammalian fur patterns (Duncan and Goldman 1984). The distribution of pigment in these cases provide social cues indicating status or maturity level, as well as distinguishing between members of a population. For example, house sparrows (*Passer domesticus*) use black throat patches as a social signal. Males with larger black patches are more dominant and often engage in more fights (Møller 1987). Stickleback and cichlid males undergo a shift to brighter nuptial coloration during the breeding seasons in order to attract females and settle contests with rivals (Rowland 1989; Seehausen and Schluter 2005).

Physiological changes are color shifts associated with more with dynamic internal and external stimuli. Chameleons and cephalopods are probably the most recognized examples of physiological color changers, but it has also been observed in arthropods (Umbers et al. 2014), fish (Fujii 2000), and amphibians (King et al. 1994), just to name a few. Unlike morphological color changes, these are not permanent changes in pigmentation expression and distribution. Instead, a change in health or social environment instigates rapid but temporary alteration in colors or patterning. Many types of rapid change occurring in these taxa are due to neurological processes

(Mäthger and Hanlon 2007; Stuart-Fox and Moussalli 2008), but slower-acting hormonal fluctuations can also play a role.

Studies of fish coloration are of particular interest, because these changes run the gamut of mechanisms. There have been a number of studies on fish color change in the past century. Color cells, known as chromatophores, are located throughout the body, but can be seen most prominently in the scales and skin of fish. Color or pattern changes can provide cryptic advantages for those living in in predator-ridden environments (Donnelly and Whoriskey 1991), as well as provide signals to dispute territories (Korzan et al. 2008), or to attract females (Kodric-Brown 1998).

Much of the teleost literature focuses on the black pigment cells, known as melanophores. How these melanophores are dispersed and expressed impacts what visual signals are projected to surrounding conspecifics or predators. For example, *Oryzias latipes* can adjust the innervation and expression of melanophores over the course of a few weeks to match the light conditions of its habitat, producing a mechanism for being cryptic in its environment (Sugimoto and Oshima 1995). Other color cells, such as erythrophores (red), xanthophores (yellow), leucophores (white), and structural iridophores can provide a diverse palette for fish to communicate their status and health. In cichlids species with hierarchal or territorial social systems males use patterns and vividness of coloration to advertise their position. After being in aggressive or territorial contests, male *Astatotilapia burtoni* will change color depending on the outcome of the interaction. Within seconds the victorious male is brightly colored with a dark bar near the eye, while the loser reverts to a more cryptic,

female-like coloration (Fernald 2009; Maruska and Fernald 2010). These changes are also accompanied by shifts in blood levels of hormones.

The mechanisms behind the types of changes mentioned above vary in their neurological and hormonal involvement. Chromatophores can have direct innervation (Fujii and Novales 1969), suggesting rapid, neurological pathways. Autonomic neurons can release neurotransmitters to bind to pigment cells, triggering a fast reaction (Fujii and Miyashita 1976). Chromatophores can also possess receptors that respond to slower blood-borne hormone release pathways (Fujii 1969). Much of the color change research examines hormonal pathways, with a variety of endogenous compounds being implicated as triggers for change. Common hormones in color change literature are melanin-concentrating hormone (MCH) (Oshima et al. 2001), melanin-stimulating hormone (MSH) (Negishi and Obika 1980), and adrenergic monoamines such as epinephrine (Miyashita and Fujii 1975). In generalized terms, the select hormone would be secreted by a given endocrine gland, attach to specific receptors on the pigment cell, and trigger a signal transduction pathway that disperses or aggregates pigment.

The green swordtail (*Xiphophorus hellerii*) is not considered a color changing fish species, outside of the development of the lateral stripe that occur in both sexes upon maturity, and the development of the sword in males. *X. hellerii* is a well-known model species in the field of sexual selection (Ryan et al. 1990; Basolo 1990; Meyer et al 1994), but outside of genetic studies of color inheritance (Gordon 1937; Goodrich et al. 1941; Culumber 2014), color change has not been extensively investigated. Males in a single population from Actopan, Veracruz, Mexico have been observed with the

ability to change their lateral stripe color (Rhodes and Schlupp 2012). This color change is reversible and dependent on the health and social environment of the male. Dominant males or males in the presence of females will often change their stripe to a vibrant red or almost pink, whereas subdominant males or males in stressful situations like handling will darken their stripe to brown or black. The mechanism by which males shift stripe color is not known due to its novel nature in this species.

In Actopan males the melanophores are located atop the red erythrophores in the fish's skin (E. J. Hardy, personal ob.) (Figure 1). We expect that endogenous compounds may attach to the melanophore receptors and trigger expansion or aggregation of black pigment, revealing the erythrophores below. One of the most common hormones associated with melanophore expression is epinephrine, as well as the neurotransmitter norepinephrine (Fujii and Miyashita 1975). Melanophores in other teleosts have alpha and beta-adrenergic receptors that when bound by epinephrine or norepinephrine can cause cellular responses (Grove 1969; Fernando and Grove 1974). The effects of these compounds are not consistent between all teleosts, however (Fujii 1969). Based on the literature, we propose that the skin of male Actopan swordtails will react to the introduction of norepinephrine or epinephrine, causing a change in melanophore expression. Furthermore, using a beta-blocker should prohibit the changes seen from these compounds. If melanophores are in fact controlled via adrenergic receptors, we would expect pigment to move in the presence of adrenergic compounds, and reverse this movement once the drug was removed. Additionally, if a beta-blocker were applied, this movement would be prevented- regardless of the presence of an adrenergic compound.

Methods

Study specimen

Adult males from the Actopan population reared at the University of Oklahoma were used for this study. These lab-raised fish are descendants of wild-caught fish collected May 2009 in the Río Actopan, near Xalapa, Mexico (Veracruz; 19◦ 25·47702' N; 096◦ 36·82764' W). Stock was kept in 250-L tanks in a greenhouse at the Aquatic Research Facility at the University of Oklahoma. Fish were transferred to laboratory housing in December 2013 and testing was conducted during the summer of 2015. Laboratory housing consisted of 37.85-75.71-L tanks under 12L/12D light conditions, with weekly water changes. No more than 4 males were housed in a tank, and all tanks contained females and juveniles to approximate natural social conditions. Fish were fed twice daily with flake food (TetraMin) or a mix of bloodworms, *Daphnia*, and brine shrimp (Hikari).

Hormone preparation

Norepinephrine and epinephrine solutions were prepared at 6 different concentrations, ranging from 10^{-4} to 10^{-9}M. The solutions were created in clean 1 oz. amber vials using teleost ringers and measured volumes of 1g powdered (+/-)-norepinephrine (+)-bitartrate salt and 5g powdered (-)-Epinephrine (Sigma Aldrich). All drugs were kept refrigerated and in light-sensitive conditions. A collaborator assigned the letter codes (A-M) to allow for blind testing of each concentration. Codes were not revealed until after all trials in the initial experiment were concluded.

Experimental setup

Experiment 1: Norepinephrine and epinephrine

Individual males (n= 12) were placed in a 0.5-L small plastic tank (23 x15 x 16.5 cm) for a five- minute acclimation period in water with an average salinity level of 800ppm. After this period the male was transferred to a tank containing treated water and approximately 0.10mL clove oil (Healing Solutions). Once the male showed signs of being anesthetized (decreased gill movement, body remaining stationary when touched), it was moved to a petri dish positioned under a dissecting microscope (Olympus model SZX2-ILLT). The petri dish was lined with a wet paper towel to provide moisture during testing. A portion of the paper towel covered the head and operculum to prevent drying. The microscope was connected to a SPOT Advanced Imaging system (Diagnostic Instruments, Inc.) with a feed on a MacBook 4 laptop (Apple). At the time of transfer to the tray, photographs of the entire body (magnification 1.6X) and a magnified view of the lateral stripe (region of stripe located between pectoral and anal fins, magnification 11.2X) were taken.

Prior to application of the drug, a Kimwipe tissue was used to gently remove excess water in the stripe region, carefully avoiding the removal of the mucus layer. At this time 0.25mL of one of the six epinephrine or six norepinephrine treatments was applied via a plastic 1mL pipette. Drops were applied along the length of the stripe, from the end of the operculum until the end of the caudal peduncle. Once the drug was fully applied, a timer was started and a first treatment photo was taken after 15 seconds. Photos were taken every 15 seconds until reaching two minutes of exposure (totaling of 8 pictures). After the 2-minute mark, a "flush" of tank water was applied via pipette to

rinse the drug from the skin. Flush was simply a rinse of the treated water like that used in housing tanks. Post-flush photos were taken every 15 seconds for one minute, starting 15 seconds after flush application with a final full-body picture taken before the fish was returned to the tank. Once in the travel/recovery tank it was monitored until it displayed normal ventilation rates and swam steadily.

An additional trial with both treatments was used to attempt to mimic the potential signaling mechanism of norepinephrine triggering epinephrine release. Only the 10^{-7}M concentrations were used for both drugs, as these caused some of the most drastic changes in melanophore expression based on dose-response data (Figure 2). Anesthesia protocols were the same as previously described. Norepinephrine was first applied, and after two minutes the epinephrine was introduced. The skin was rinsed after an additional two minutes. Pictures were taken every 15 seconds for the four minutes of drug exposure, and the minute following flush application.

Experiment 2: Beta-blocker

In order to confirm the effects seen in the previous experiment, we added a beta-blocker into the protocol that we would expect to inhibit the changes from adrenergic compounds. Propranolol is a commonly used beta-blocker in human medicine, but it has also been used for environmental toxicity experiments in fish. A preparation of 1.5g/L (+/-)-propranolol hydrochloride solution was used prior to exposure to epinephrine or norepinephrine. Concentration and enantiomer structure were selected based on work done with similar fish size and use (Huggett et al. 2002; Stanley et al. 2006).

Melanophore response was measured in 10 isolated males. Males in the control trial were sedated in the same fashion as the previous experiment and received a "treatment" of deionized water treated with reef salt (Instant Ocean) applied to the skin. Trials with epinephrine and norepinephrine also followed the procedures of first experiment. Once again both drugs were in 10^{-7}M concentrations. In propranolol trials males were sedated and treated with the propranolol solution for one minute before exposure to an epinephrine or norepinephrine treatment. The treatment period lasted for two minutes and then the stripe was flushed and measured for one final minute. Males were given time to recover before returning to housing tanks. The Institutional Animal Care and Use Committee at the University of Oklahoma approved both experiments and animal handling (R15-015).

Data Analysis

Experiment 1: Norepinephrine and Epinephrine

Photos were scored on a categorical scale by the investigator and a naïve observer to minimize potential biases. Scores were assigned and averaged for: 1) the photo of the stripe prior to drug application, 2) the photo two minutes after drug application, and 3) one minute after flush application. Given the minimal apparent changes, scoring was limited to a score of more (+ values), equal (0), or less (-values) melanophore expression. Three males died during the span of experiment 1 and their data was not used for analysis.

Experiment 2: Beta-blocker

Scoring methods followed the same format as Experiment 1. For the trials with propranolol, four pictures were used for scoring, with the additional photo being the one-minute after propranolol exposure. The same naïve observer was used for this experiment to maintain consistency. One male died prior to the completion of the full experiment and was not used in the analysis.

Results

Experiment 1: Norepinephrine and epinephrine

All concentrations of epinephrine and norepinephrine on average caused change to darker stripes (i.e. melanophore dispersal) when scored after two minutes of exposure (n=9). When calculating 95% confidence intervals there was considerable overlap between treatments, suggesting the difference in mean score was not significant between treatment types of concentration (Figure 2). The flush scores were all close to values of zero (no change in melanophore expression), and the confidence intervals again overlapped considerably (Figure 2).

The trial with a combination of both norepinephrine and epinephrine showed a darkening after the initial application of norepinephrine. However, on average subsequent applications of the epinephrine and flush did not show further changes. There was far less overlap between the norepinephrine treatment confidence intervals and the other two solutions, suggesting that further studies may be needed.

Experiment 2: Beta-blocker

Once again, the average male melanophore scores (n=9) across all trials indicated increased expression, even in the presence of a beta-blocker (Figure 3). Means for the epinephrine and norepinephrine trials with propranolol application were lower than those of the drug-only or control trials. However, the overlap of 95% confidence intervals indicated this was not a significant difference. Flush scores across treatments also overlapped and all were close to zero, indicating a lack in change after the skin was rinsed (Figure 3).

Discussion

The results from both experiments indicate that the adrenergic monoamines, epinephrine and norepinephrine, are not triggering the color change in Actopan swordtails. To confirm this finding, a future study with a hypodermic application of the drugs should be completed in the future. Some suggest a beta-adrenergic reaction would cause greater dispersal, as cyclic adenosine monophosphate (cAMP) levels would increase with activation of adenylate cyclase and phosphorylate proteins to trigger pigment dispersal (Nery and Castrucci 1997). The ineffectiveness of the beta-blocker propranolol makes it less likely, however, it is a beta-adrenergic receptor-based change. To further negate this, a study by Negishi et al. (1982) found that *X. maculatus* and *X. hellerii* melanoma actually see a decrease in cAMP after epinephrine exposure. Additionally, these cells can also have alpha-adrenergic receptors (Fujii and Miyashita 1975), indicating that while we can likely rule out a beta-adrenergic receptor mechanism, the same endogenous compounds could be binding in a separate pathway. In guppies this alpha-receptor mechanisms caused melanophore pigment aggregation.

The increased melanophore expression observed could be the product of one of an array of hormones, neurotransmitters, or ion changes in Actopan males. Fish literature identifies melanocyte-stimulating hormone (MSH) and adrenocorticotropic hormone (ACTH) as dispersing agents, but their impacts vary by species, and can actually cause expansion of other pigment cell types (Fujii 2000). Stress responses are often tied with skin darkening in fish species. One study found that *Salmo trutta* had higher blood cortisol levels when stressed, and interestingly also had greater MSH levels (Sumpter et al. 1985). Swordtail males turn black when triggered by social descent or poor health; therefore melanophores may be expressed in association with stressful conditions and hormone release. It is worth noting that more dominant X. *hellerii* males tend to have lower adrenocortical activity than their subdominant rivals (Scott and Currie 1980), possibly providing an intriguing explanation for male darkening during aggressive contests.

As previously stated, chromatophores can be controlled neurologically as well. Some studies have implicated the adrenergic compounds we used as part of these systems (Fujii and Novales 1969). Male *Haplochromis burtoni* have cranial nerves that specifically innervate an area around the eye, producing an eyebar via melanophore expression (Muske and Fernald 1987). To further complicate matters, combination endocrine and neurological controlled mechanisms do occur, in some cases even on the same pigment cells (Nery and Castrucci 1997).

There is still much left to be investigated in this system. The inherent difficulty in studying mechanisms of color change is that there is so much variation in the compounds and resulting pigment movement. Mechanisms can be much more complex

than one hormone binding to a single receptor type. Many hormones can have opposing results across species, or shifts in concentration can reverse the action of pigment cells. For example, in one study using different concentrations of MCH, tilapia species *Oreochromis niloticus* showed black pigment aggregation in lower-dose treatments, but then showed pigment dispersal at higher dosages. In the same study the medaka (*Oryzias latipes*) only had aggregation regardless of dosage (Oshima et al. 2001).

There are striking morphological variations across *X. hellerii* populations in terms of color and patterns. Since only one population can change stripe color, it does bring into question what physiological differences may exist between other swordtail populations. Future findings on color change mechanisms may help us further understand more complex arrangements of population dynamics and environmental adaptations.

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Figure Legend

Fig. 1- Photo of a section of magnified lateral stripe (11.2X).

Fig. 2- Dose-response data (+S.E.) for the six concentrations of epinephrine and norepinephrine solutions and swordtail male (n=9) melanophore expression.

Fig. 3- Average (+S.E.) melanophore expression (n=9) with epinephrine and norepinephrine treatments, and with a flush applied. High concentrations are 10^{-5}M, middle are 10^{-7} M, and low are 10^{-9}M. Positive values indicate melanophore dispersal, and negative indicates aggregation. Values near zero indicate no change.

Fig. 4- Average (+S.E.) melanophore expression (n=9) with epinephrine, norepinephrine (w/ and w/o propranolol), and with a flush applied. Positive values indicate melanophore dispersal, and negative indicates aggregation. Values near zero indicate no change.

Figure 1

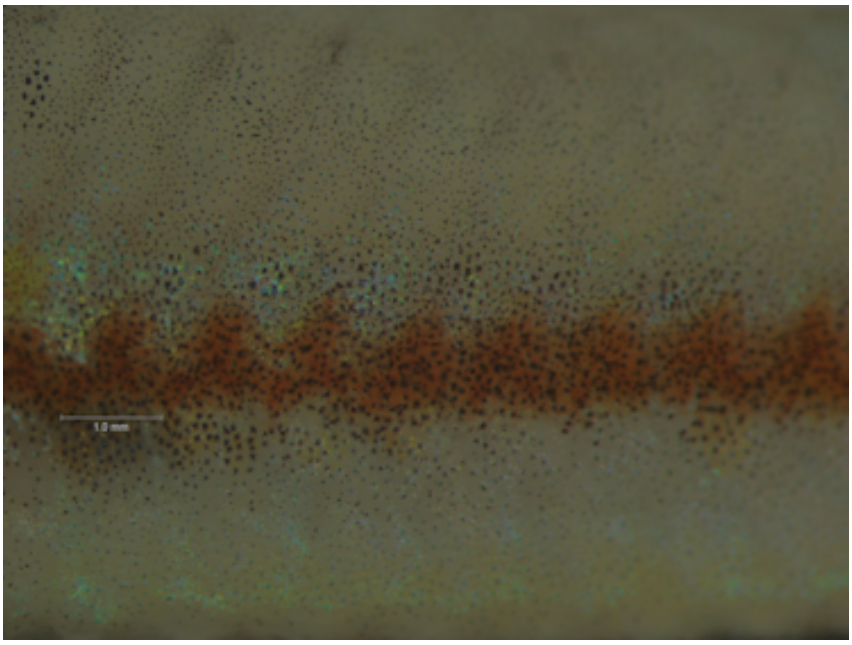
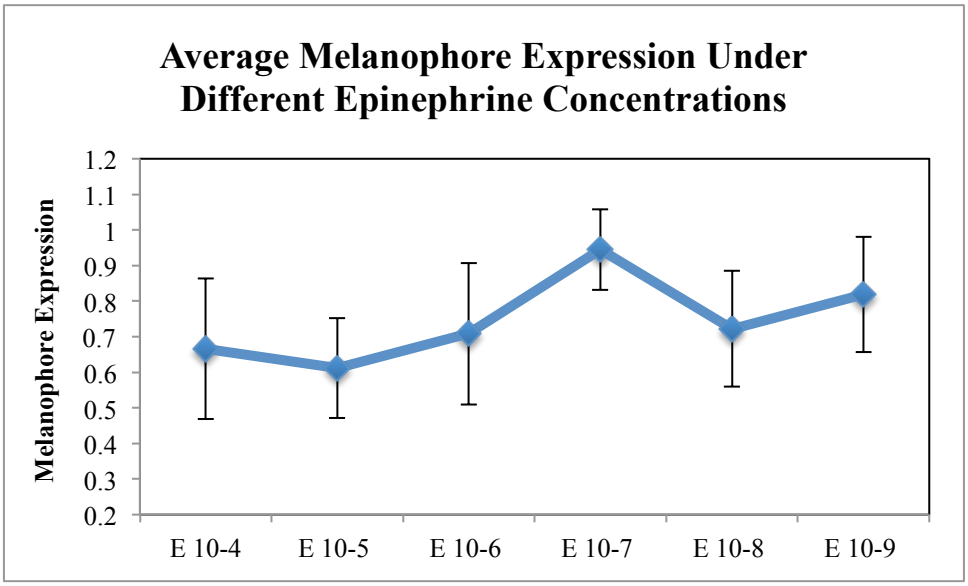


Figure 2


Average Melanophore Expression Under Different Epinephrine Concentrations
Melanophore Expression
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
E 10-4
E 10-5
E 10-6
E 10-7
E 10-8
E 10-9

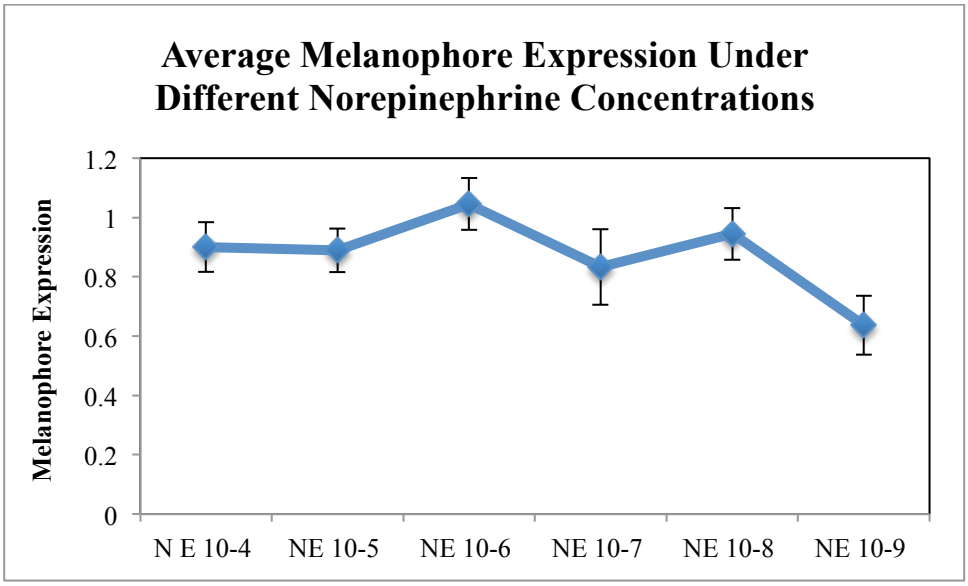
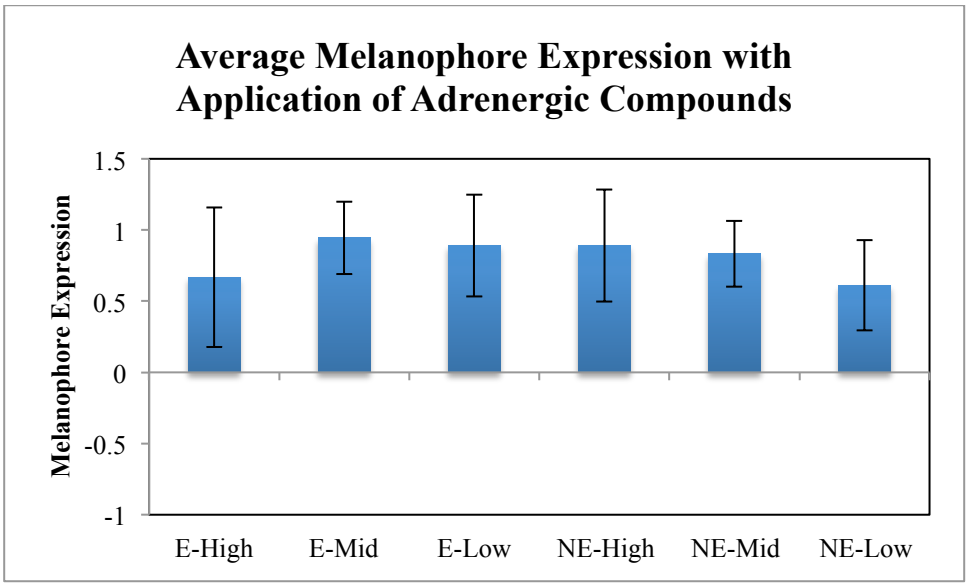

Average Melanophore Expression Under Different Norepinephrine Concentrations
Melanophore Expression
1.2
1
0.8
0.6
0.4
0.2
0
N E 10-4
NE 10-5
NE 10-6
NE 10-7
NE 10-8
NE 10-9

Figure 3


Average Melanophore Expression with Application of Adrenergic Compounds
Melanophore Expression
1.5
1
0.5
0
-0.5
-1
E-High
E-Mid
E-Low
NE-High
NE-Mid
NE-Low

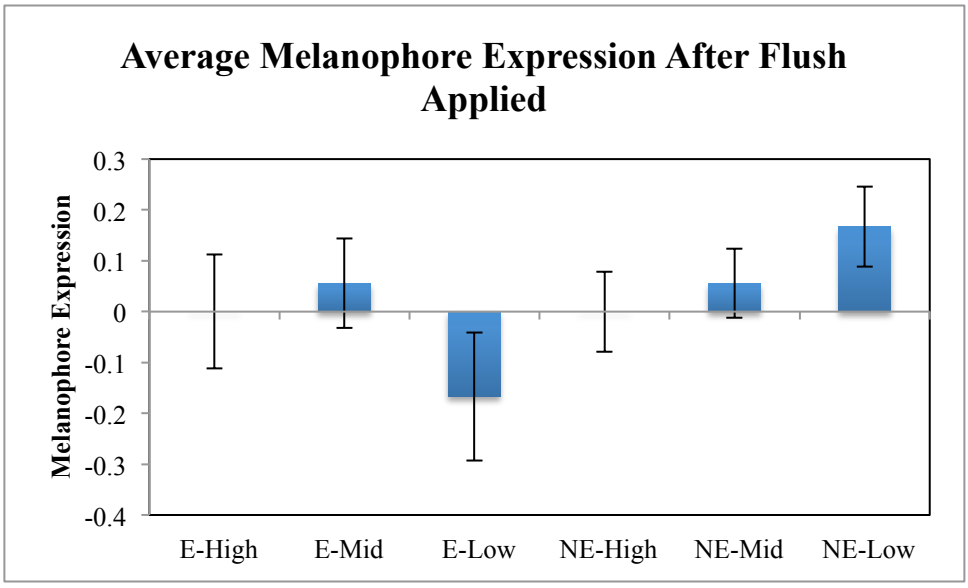
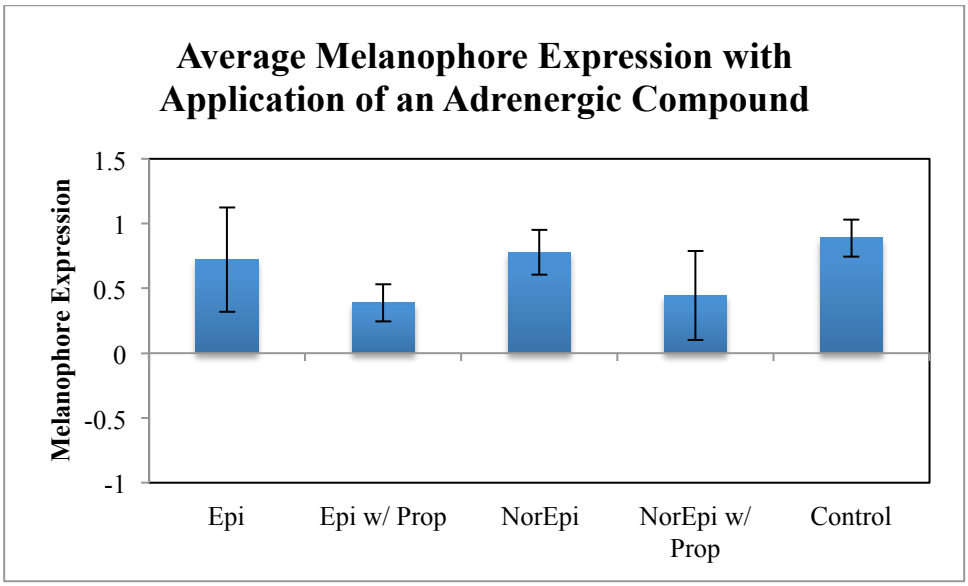
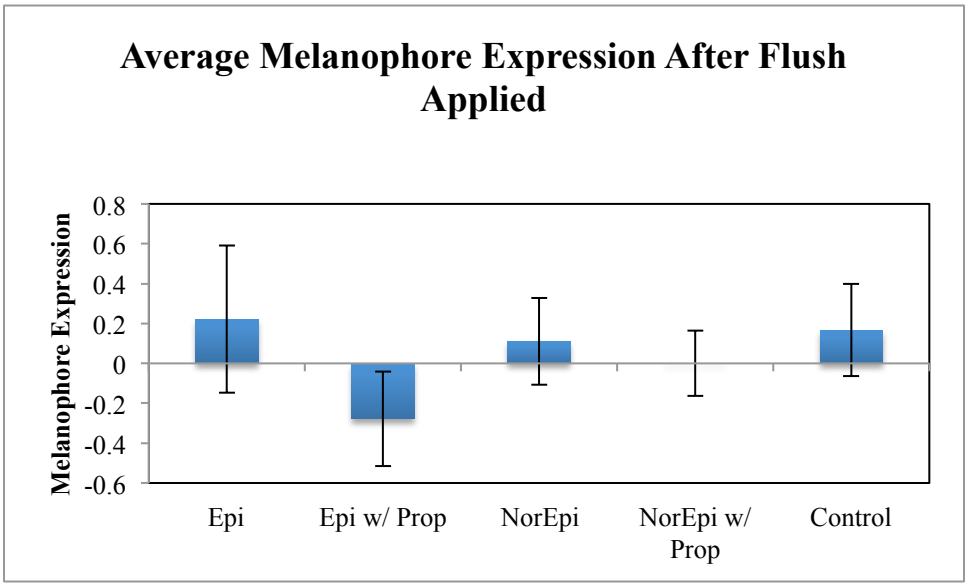

Average Melanophore Expression After Flush Applied
Melanophore Expression
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4
E-High
E-Mid
E-Low
NE-High
NE-Mid
NE-Low

Figure 4


Average Melanophore Expression with Application of an Adrenergic Compound
Melanophore Expression
1.5
1
0.5
0
-0.5
-1
Epi
Epi w/ Prop
NorEpi
NorEpi w/ Prop
Control


Average Melanophore Expression After Flush Applied
Melanophore Expression
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
Epi
Epi w/ Prop
NorEpi
NorEpi w/ Prop
Control

Chapter 4

Comparison of male aggression and courtship behaviors in a conditionally signaling population of *Xiphophorus hellerii*

Authors: Elizabeth J. Hardy, James B. Johnson, and Ingo Schlupp

Abstract

Polymorphisms of coloration are common across most taxa. These differing forms of visual signals can act as determinants for status and relative attractiveness for the choosy sex. While this provides many interesting avenues of research, we can generally assume that differences in color provide an individual certain costs or advantages depending on phenotypic expression. In one population of the green swordtail (*Xiphophorus hellerii*) what is a dimorphic stripe color trait in other populations has become a conditional signal. Males in this population change their lateral stripe color to adapt to their social environment, putting into question how individuals respond to members of their species without the shifting trait. Using a series of behavioral trials, we tested how shifting males allocate behaviors to males and females from the same and from two different allopatric populations, and then what variances might emerge in female preference. We found that males do not change stripe color differentially towards different population-based male stimuli, but do prefer associating with sympatric females. This raises further questions regarding the importance of visual cues and the prioritization of signaling.

Keywords: color change, male aggression, mating displays, visual communication, polymorphism

Introduction

It is generally assumed that across a species' populations that behaviors and preferences are largely consistent. However, when differences in visual signals do arise they can provide scientists with additional insights into a species' adaptive ability and the power of sexual selection. One of the more obvious differences we see in nature is color polymorphism. We see examples of color polymorphisms across a number of taxa, from reptiles (Sinervo et al. 2001), mammals (Hoekstra et al. 2004), birds (Lowther 1961), arthropods (Roderick and Gillespie 1998), and fish (Seehausen et al. 1999). Much of the anuran species community has variation in color and pattern (Hoffman and Blouin 2000). Visual signal polymorphisms are most often found in males of species with some degree of competition for mates and territories (Sinervo et al. 2001; Dijkstra et al. 2008).

Fish provide many model systems for studying sexually selected color traits, many which would be considered polymorphic within a given species or some of its populations. These traits can be important in both mating contexts (Eakley and Houde 2004) and in antagonistic conspecific interactions (Dijkstra 2008). Male guppies possess bright orange and iridescent pigmentation, which can provide males an advantage for mating opportunities in certain contexts (Kodric-Brown 1985). Sticklebacks have nuptial coloration that shifts in intensity based on the stage of the breeding season (McLennan and McPhail 1989). Cichlids use transient physiological color change to assert social status (Korzan et al. 2008).

Color polymorphisms can create more complex forms of interaction, especially when the species utilizes other visual signals to communicate status or attractiveness.

For example, in a population of *Xiphophorus hellerii* in Jalapa, Mexico, males are dimorphic for color on the lateral stripe expressed along the body (Franck et al. 2003). This species generally uses body size (Beaugrand et al. 1991; Rosenthal and Evans 1998), the caudal fin extension known as the sword (Basolo 1990; Benson and Basolo 2006), and a suite of behaviors (Schlosberg et al. 1949; Beaugrand et al. 1984) to settle male-male contests or attract females. In this case, however, a male's stripe color serves both as a signal directed at females for mating opportunities and as a badge of status between males. More specifically, males with red lateral stripes are generally more dominant and preferred by females. Consequently, this fitness advantage is accompanied by an increased cost of aggression by rival males. Males with black lateral stripes are subject to less aggression but are not seen mating as often (Franck et al. 2003).

While this polymorphism provides new avenues for visual signaling, it still holds true in this species and many others that the mechanism of signaling is consistent. Variation in patterns and color are not unusual in the genus *Xiphophorus*, and *X. hellerii* has been observed with intra-population polymorphisms in melanophore (black pigment cell), black spot sizes (Franck et al. 1998), as well as differences in sword color and lateral stripe structure (Gordon 1937). However, the following study addresses an exception to the assumption of consistent signaling.

One population of *X. hellerii,* found in the Río Actopan in Veracruz, Mexico, has an unusual trait unobserved in all other wild populations. Actopan males are able to rapidly express red or black lateral stripes via dispersal of pigment cells on the skin and scales (Rhodes and Schlupp 2012). The costs and benefits documented in Franck et al.

(2003) appear to remain in part constant in the Actopan population. Larger, more dominant males tend to stay red or turn red while courting. Males that turn black are often smaller or turn black after losing an aggressive contest.

Males utilize their ability to change stripe color amongst members of their own population, but we were interested in whether such color changes also occur when in the presence of males and females from other populations of *X. hellerii*. This study addressed two questions: 1) Will males with conditional signaling alter aggression when presented with males from the same versus an allopatric population? 2) Do males show a preference via association time and type/frequency of behaviors with females from the same versus allopatric population? Given that Actopan males have an apparently novel trait to utilize as a visual signal, we would expect differential expression of behaviors and colors based on how the receiver initially responds.

Methods

Study specimen

Fish from the Actopan population reared at the University of Oklahoma were used for this study. These lab-raised fish were descendants of wild-caught fish collected May 2009 in the Río Actopan, near Xalapa, Mexico (Veracruz; 19◦ 25·47702' N; 096◦ 36·82764' W). Stock was kept in 250-L tanks in a greenhouse at the Aquatic Research Facility at the University of Oklahoma. Fish were transferred to laboratory housing in December 2013. Laboratory housing consisted of 37.85-75.71-L tanks under 12L/12D light conditions, with weekly water changes. No more than 4 males were housed in a tank, and all tanks contained females and juveniles to approximate natural

social conditions. Fish were fed twice daily with flake food (TetraMin) or a mix of bloodworms, *Daphnia*, and brine shrimp (Hikari).

Experiment 1: Male-male aggression

The first experiment was designed to examine how Actopan males may allocate aggressive behavior towards males from the same or from two separate *X. hellerii* populations. Males from these additional populations do not have observed conditional signaling like that of the Actopan males. "Doce Millas" fish were purchased from the Xiphophorus Stock Center at Texas State University in December 2013. This strain was originally collected in 2001 from the Río Junapan drainage in Oaxaca, Mexico (17° 16' 46.9194" N, -95° 4' 16.32" E). "La Gloria" fish were wild-caught from a sulfidic spring, part of the Pichucalco Drainage of the Río Grijalva in Chiapas, Mexico (17° 31' 55.2" N, -93° 0' 54.36" E, for more information refer to Culumber et al. 2014).

Focal males were placed in a 75.7-L tank containing a stimulus male in a clear plastic cylinder. Based on pilot study results, 5-minute trials were deemed adequate for observing male aggression. Males were exposed to three stimulus males, one from each of the previously mentioned populations. The order of stimulus male presentation was selected randomly. Male stimuli were size matched within 3mm standard length in each trial. Actopan males were tested only with three replicates per trial. Actopan males could be used as both focal and stimulus males but had at least 24 hours before being used again.

Prior to the start of each trial replicate, the focal male was placed in the tank inside a plastic cylinder placed at the side of the tank. The male was then given a 5-

minute acclimation period. An additional empty cylinder was placed at the center of the tank during this time. After the acclimation period, a stimulus male was introduced to the clear cylinder in the center of the tank. At this time, the focal male was released and the trial started. The trial was recorded using a high-definition camera (Nikon D5200 24.1 MP CMOS Digital SLR).

The observer monitored and recorded color change of the focal male's lateral stripe at minute intervals, using a 5-point color scale established by E.H. in a previous study (see Chapter 1). Colors were given designations based on colors provided in Microsoft Word software that best matched the range of colors found on Actopan males. A score of 1 indicates a light red to pink (R255, G68, B83), 2 is medium red (R220, G0, B0), 3 is dark red (R136, G0, B0), 4 is brown (R62, G0, B0), and a 5 is black. After the first replicate the focal male was returned to the side cylinder and the first stimulus male removed. The second and third replicates followed the same protocol as described above. After a full trial set was completed the tank and cylinders were cleaned thoroughly with soap and hydrogen peroxide, to reduce the risk of biasing future males via chemical cues (McLennan and Ryan 1999).

Focal male aggressive behaviors were measured after the trial using video playback. Behaviors measured were: bite, chase, sigmoid display, and side-by-side posture. These behaviors have been used in much of the swordtail literature focused on aggressive interactions (Thines and Heuts 1968; Franck et al. 1985; Franck and Ribowski 1987). The four behaviors were chosen from behaviors described in Beaugrand et al. (1984). However, given that focal males could not interact completely

with the stimulus males, a chase was counted as any sudden rush towards the male in the cylinder.

Experiment 2: Male preference

The second experiment was a test of male preference, focusing on association time and mating behaviors with females from sympatric or allopatric populations. Males were given a binary choice test (repeated for side-bias) with size-matched females from Actopan and one of the two other populations. Stimulus females were selected randomly and were within 3-mm standard length of each other (n=30). Males were only tested as a focal male once (with a two-part trial), while females could be used as stimuli up to once every 24 hours.

Testing was conducted in a 75.7-L glass tank with delineations on the glass, separating the tank into three sections. One clear cylinder was placed in each of the left and right sections, with stimulus females introduced during the acclimation period. The high-definition video camera (Nikon D5200 24.1 MP CMOS Digital SLR) was placed in front of the tank to record the two replicates per trial. The focal male was placed in the middle "neutral zone" in a clear cylinder and allowed to acclimate for 5 minutes. After the acclimation period, the male was released from the cylinder and allowed to swim freely in the tank. At this time, the trial began and video recording was started. Trials were 5 minutes long, based on the adequate number of displays initiated in 5-minute pilot tests.

Color change was recorded per minute for the duration of the trial. Scoring protocol was the same as that in experiment 1. Once the first replicate was complete,

the male was returned to the cylinder in the neutral zone and the second acclimation period started. The stimulus females switched preference zones to control for potential male side bias. After the acclimation period, the second replicate was recorded using the same protocol as for the first replicate. After a full set of trials was completed, the water was replaced and cylinders were cleaned with soap and hydrogen peroxide to limit chemical communication between trials.

The male's courtship displays and behaviors were tallied during viewing the video recordings. Behaviors quantified were: gonopodial flexing, sigmoid "s-curve" displays, backwards swimming, and lateral display (male places himself perpendicular to the female and shakes). These behaviors have been described in the behavior literature for *X. hellerii* (Schlosberg et al. 1949; Rosenthal and Garcia De León 2006).

Data Analysis

Experiment 1: Male-male aggression

We used a repeated-measures ANOVA to test how stimulus male type influences net change in color and how long it takes for males to first change color. The net change in color from the start to the end of the trial, as well as the time (s) that the male showed observable color change, were dependent variables. Male swordtails often use a suite of behaviors in quick succession. Therefore, it was necessary to create Principal Component Analysis scores to create components that best fit the fishes' activity

Experiment 2: Male preference

Association time and behavioral data were analyzed via a paired samples t-test. The identity of the first behavior was analyzed using a binomial test. To account for the connectedness between mating behaviors, we used a PCA to create components for behavioral dependent variables. Males showing side-bias (spending >85% or <15% in one preference zone) were discarded from analysis.

Results

Experiment 1: Male-male aggression

Using the color change data from the aggression experiment (n=28) (Table 1), we found that the time of the first change observable color change was not significant ($F_{2,25}$= 1.19, p= 0.322), even with the stimulus male size groupings as a covariate ($F_{2,25}$= 1.66, p= 0.210). The net change in male stripe color also did not differ based on stimulus male, with ($F_{2,22}$= 0.250, p= 0.781) or without ($F_{2,22}$= 0.52, p= 0.781) the stimulus male size as a covariate. Average color scores at the start and completion of the trial tended to be similar across all stimulus treatments.

Two components were used from those extracted from the aggression PCA analysis, and covered approximately 90% of the variance in the data set (Figures 1 and 2). The first component, when used as a dependent variable, proved to be non-significant. However, the second component, which accounted for about 21% of the variance, was significantly influenced by the stimulus male size ($F_{2,25}$= 3.35, p= 0.043) This component was most heavily loaded with sigmoid displays in the negative range and bites and side-by-side displays to a lesser extent in the positive range. The average score for males with an Actopan stimulus was negative (average= -0.051), indicating

males performed more sigmoid displays with the males from their own population. When presented with La Gloria males the average shifted towards bites and side displays. Doce Millas males did not have as strong a skew towards either loading.

Experiment 2: Male preference

Association time indicated Actopan male preference for Actopan females (n=20). Both percentage preference (Figure 3) and normalized preference scores (calculated using an √arc (sin) transformation) were significant, while total association time (in seconds) was barely non-significant. The average male preference for Actopan females was 63%, while preference for other female types was only 37% (t_{19}= 2.18, p= 0.042).

Actopan males displayed to one or both females in 31 of the 40 replicates (Figure 4). Males displayed in the region containing the Actopan female first in 17 of the 31 replicates. This was not significant, however, based on the binomial test (p=0.72). The PCA created four components, two of which accounted for 92.5% of the variance. However, when tested as dependent variables, neither of these PCA components were significant (PCA1: t_{19}= 0.76, p= 0.46; PCA2: t_{19}= 1.04, p= 0.31).

Discussion

Based on our results, it appears that green swordtail males from the Actopan population are more discerning of interactions with females than with males of different populations. Actopan males preferred associating with Actopan females, but we did not see a significant difference in allocation of behaviors to females from different

populations. In terms of male interaction, we did not see color change used differentially as we had expected if males were discerning some difference based on male source population. This was surprising given that the stimulus males from Doce Millas and La Gloria cannot manipulate color signaling like their Actopan counterparts can. It may simply be that color change is used primarily to amplify other signals males may be producing via displays and other traits like body size. This may explain why we saw some difference in behaviors but not in color change. An additional study testing for female preference for lateral stripe color using our allopatric stock would provide more insight into the function of the lateral stripe in the context of mating.

The lack of chemical cues may also have altered the focal males' responses. In a separate study (see Chapter 1) we did not see a difference in aggression or net color change across modality types. However, that study only tested Actopan males with stimulus males from their own population. It may be that allopatric males and females emit chemical signals that differ from those of the Actopan fish. One previous study has examined preference for odor cues in other *Xiphophorus* species and found that male *X. birchmanni* preferred female conspecifics and closely related species over allopatric, more distantly related species (Wong et al. 2005). It is also worth noting that in this same study, males did not show a visual preference for a particular type of female.

The source of the Actopan's shifting mechanism is still unknown, but it provides us with many questions regarding its purpose in social interactions. This trait could provide males with an additional pathway for advertising status in a more transient fashion. It could be that male-driven hierarchies are not as stable as we see in other

natural and lab-raised populations of *X. hellerii*. Often males of similar size engage in more aggressive battles (Beaugrand et al. 1991), thus color change enables shoaling individuals a way to signal the initiation of interaction. What remains clear here is that much more investigation is required to firmly determine the behavioral, and perhaps evolutionary, advantage to conditional signaling in this species.

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Tables

Table 1- Results from repeated-measures ANOVA (n=28) testing the effect of stimulus male population on Actopan male lateral stripe color change. Results are presented for analyses with and without stimulus group size covariate.

Stimulus Population Effect (r.m. ANOVA)	**F**		**p**	
		w/ stim group size		**w/ stim group size**
Net Change	0.522	0.250	0.601	0.781
First Change	1.185	1.662	0.322	0.210

Figure Legend

Fig. 1- Average (+ S.E.) aggressive behaviors across male stimulus types. One focal male accounted for most of the side-by-side displays shown in the La Gloria column.

Fig. 2- Average (+ S.E.) loadings for the second PCA component across stimulus population types.

Fig. 3- Average (+ S.E.) percentage male preference between Actopan and allopatric female stimuli

Fig. 4- Average (+ S.E.) mating behaviors across female stimulus types.

Figure 1

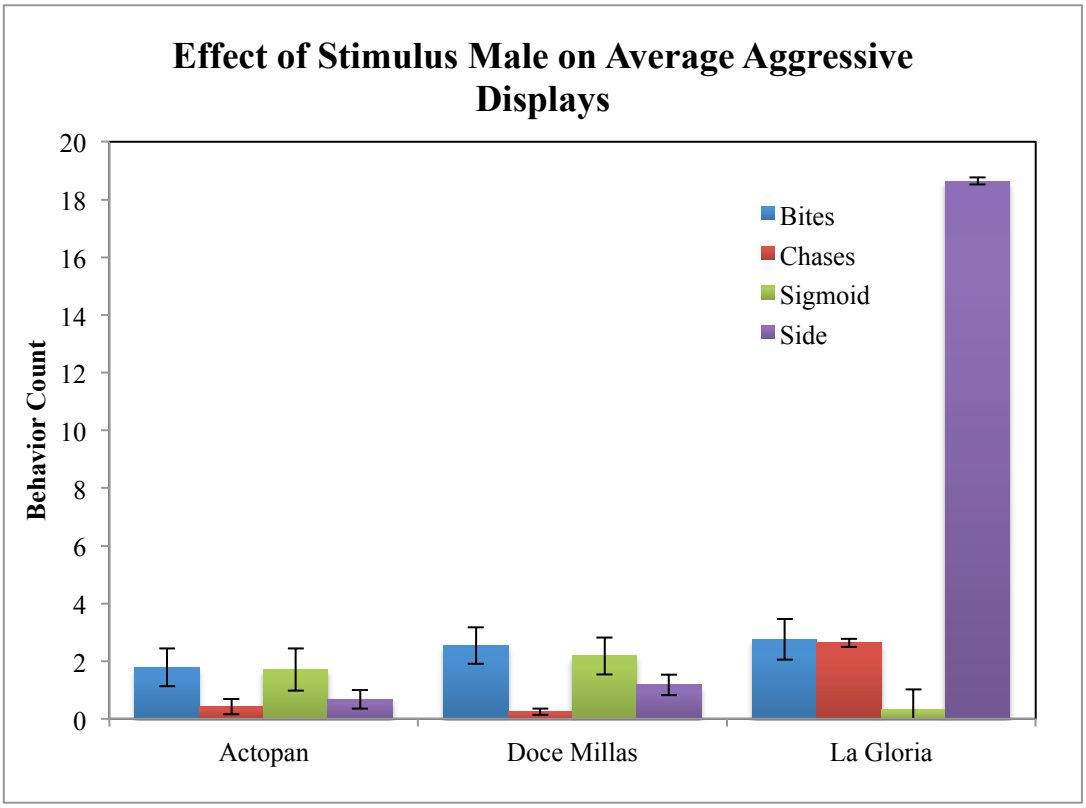

Effect of Stimulus Male on Average Aggressive Displays
Behavior Count
20
18
16
14
12
10
8
6
4
2
0
Bites
Chases
Sigmoid
Side
Actopan
Doce Millas
La Gloria

Figure 2

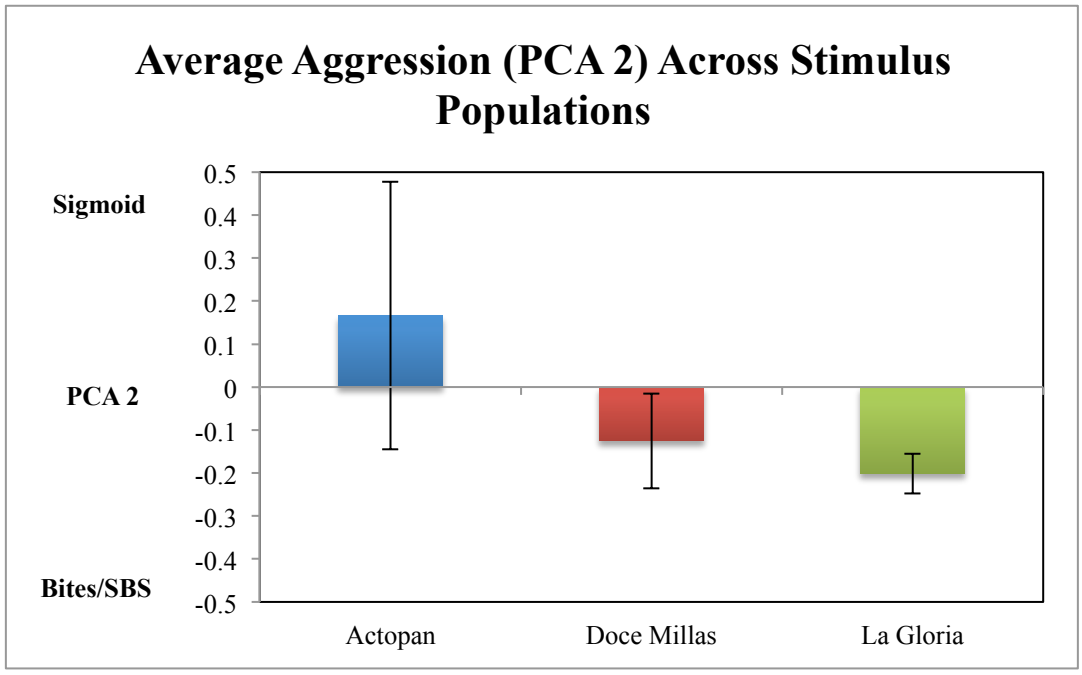

Average Aggression (PCA 2) Across Stimulus Populations
Sigmoid
PCA 2
Bites/SBS
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4
-0.5
Actopan
Doce Millas
La Gloria

Figure 3

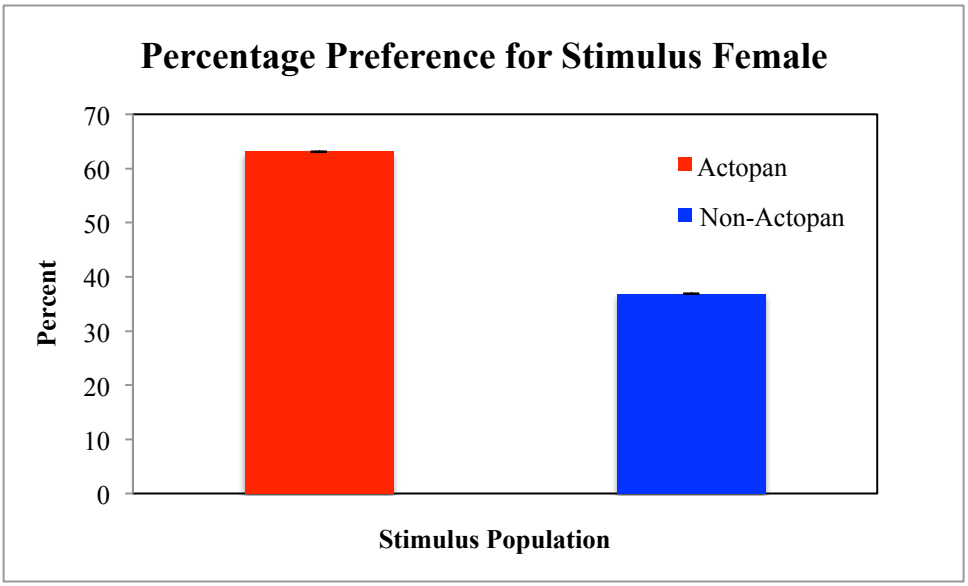
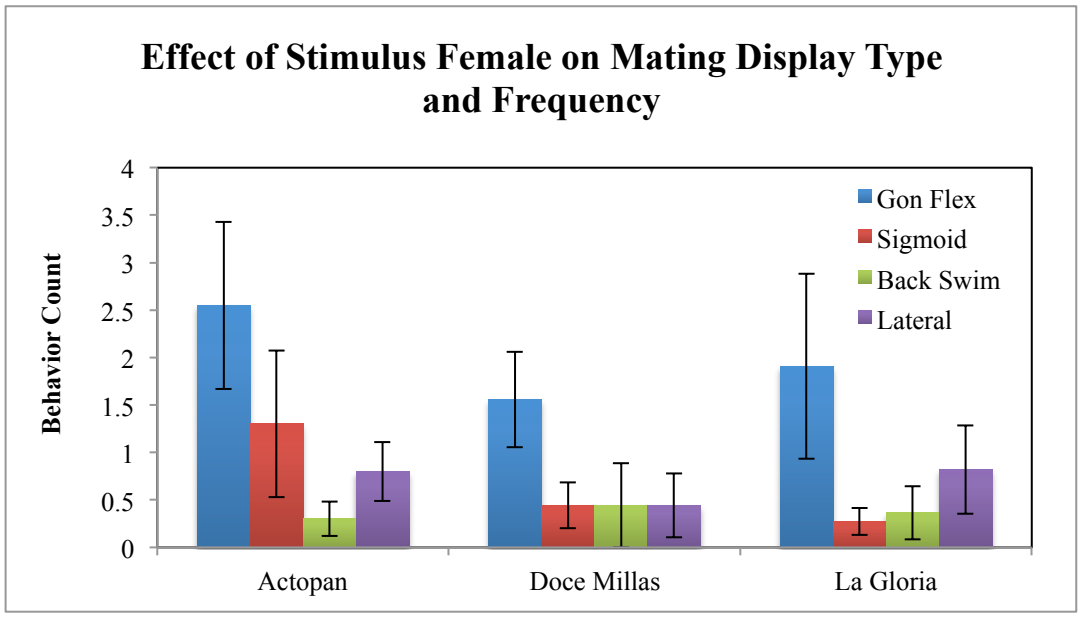

Percentage Preference for Stimulus Female
70
60
50
40
30
20
10
0
Percent
Actopan
Non-Actopan
Stimulus Population

Figure 4


Effect of Stimulus Female on Mating Display Type and Frequency
Behavior Count
4
3.5
3
2.5
2
1.5
1
0.5
0
Gon Flex
Sigmoid
Back Swim
Lateral
Actopan
Doce Millas
La Gloria