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Social function of a variable lateral stripe in *Xiphophorus hellerii*?

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Abstract

In a single population of a livebearing fish, the green swordtail (*Xiphophorus hellerii*), a trait considered a static badge of status in males of other populations, the color of a stripe covering the lateral line, has been found to be dynamic relative to their social environment, potentially rendering this signal meaningless. Males change the color expression of their lateral stripe dynamically based on social environment. We investigated if males (n = 26) respond to visual and chemical information about other males with a color change and aggressive behavior. We found that visual information is indeed causing color change, whereas chemical information is less effective. Aggressive responses and frequency of response did not change significantly with the mode of communication. We also studied female preferences for color, but found no significant preference (n = 32). Our results lead to questions as to how interpopulation variation can affect preferences and how dynamic signaling—in this case signal transmission presumably dependent on sex and/or status—influences the behavioral interactions we might expect between *X. hellerii* in the field.

KEYWORDS

badge of status, color change, female preference, male–male contests, modality, sexual selection

1 | INTRODUCTION

Signals used in female mate choice and male–male competition are often considered costly to allow for the evolution of reliable, honest signals (Maynard Smith & Harper, 2003; Searcy & Nowicki, 2005). A large number of these signals are fixed and, once expressed during ontogeny, cannot be changed by the signaler. Notable examples include the dewlap color of tree lizard *Urosaurus ornatus* (Thompson & Moore, 1991) and the male *Passer domesticus* breast badge (Møller, 1987), amongst others. On the other hand, some signals can be dynamically altered by the sender. Males from some anuran and fish species, for example, temporarily change color or pattern based on their reproductive success or outcomes from male–male interactions (Doucet & Mennill, 2009; Korzan, Robison, Zhao, & Fernald, 2008; Sköld, Aspengren, & Wallin, 2013). Although this appears to be less common than static signals, this situation raises important questions of signal function in intraspecies interactions (Kindermann & Hero, 2016; Kodric-Brown, 1998; Rhodes & Schlupp, 2012). One such question is how one state evolves into the other and the evolutionary transition between states.

Badges of status provide an example of fixed signals. These badges indicate the social status of an individual. They determine the outcome of aggressive encounters, while saving the cost of actual fights with other individuals, and often males with a high status are preferred by females. Despite a low cost of production, they can be honest signals, as modeled by Maynard Smith, Harper, and Brookfield (1988) and Johnstone and Norris (1993) using game theory. Although these models have also been criticized, the general existence of badges of status seems widely accepted (Maynard Smith & Harper, 2003). One intriguing aspect of badges of status has been how cheating may evolve and invade a stable situation in which a badge of status has evolved to indicate dominance (Maynard Smith & Harper, 2003), essentially rendering the badge meaningless.

A potential case of cheating was described by Rhodes and Schlupp (2012) in a livebearing fish, the green swordtail, *Xiphophorus hellerii*. In this species, the color of a lateral stripe can be variable within a

population, but is fixed for a given individual. Previous work on one population of *X. hellerii* from Jalapa (Veracruz, Mexico) showed that individual males show either a black or a red stripe (Franck, Müller, & Rogmann, 2003; Zander, 1986). Males with red lateral stripes in this population are dominant over black-striped males in aggressive interactions, and females show a preference for red striped males (Franck et al., 2003). Essentially, this turns the stripe into a badge of status (Johnstone & Norris, 1993; Rhodes & Schlupp, 2012), signaling male dominance to other males and females. In the population described by Rhodes and Schlupp (2012), however, this system has apparently broken down and males can change the color of the lateral stripe depending on the social context within minutes (Figure 1).

Because badges of status are generally static traits, this raises the question if a dynamic version of such a trait would still function reliably as a badge of status, or if a successful cheater morph has evolved: All non-alpha males of the Actopan population could in theory benefit immensely from the fact that their signal is not fixed. They could be submissive (and display a black lateral line) in the presence of a dominant male (thus avoiding the cost of aggression) and switch to a red lateral line when courting a female. One ramification of this is that, eventually, this might render the male signal unreliable, and consequently, it would lose its efficacy in either female choice or male–male competition, or both.

This highlights that many signals have evolved dual functions: They can simultaneously attract females and function in male–male competition (Hunt, Breuker, Sadowski, & Moore, 2009; Kindermann & Hero, 2016). A relevant example of this is found in several species of northern swordtails, including *Xiphophorus cortezi*, which prominently display vertical bars. These bars are a dynamic signal that is both a deterrent to males and sexually attractive to females (Morris, Mussel, & Ryan, 1995). Using a phylogenetic reconstruction, Morris, Tudor, and Dubois (2007) were able to show that the female preference for the signal evolved prior to the signal gaining a function in male–male competition.

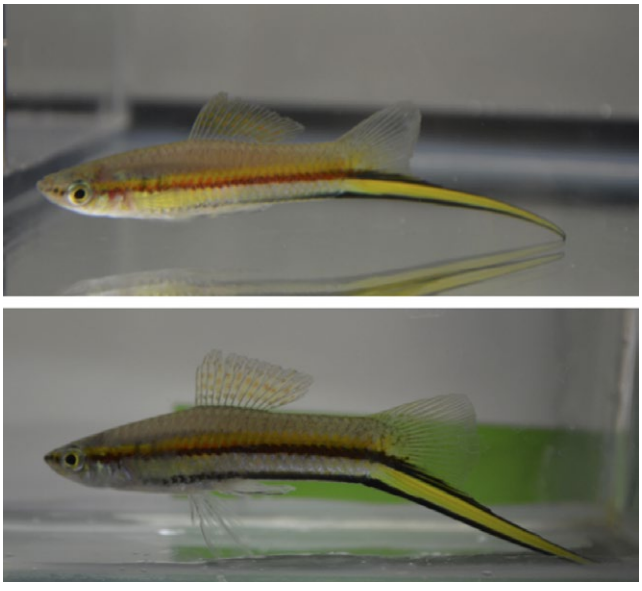

FIGURE 1 Images of an Actopan male with a red stripe and a black stripe. Photographs were taken from the same male in a Study 1 trial within 10 min of each other. [Colour figure can be viewed at wileyonlinelibrary.com]

In this study, we investigate the role of a badge of status using one particular population of *X. hellerii* from the Rio Actopan in Mexico (Rhodes & Schlupp, 2012). The initial study by Rhodes and Schlupp (2012) established the existence of the phenomenon, but did not address if the ability to change color is in any way adaptive. Furthermore, the mechanisms regulating the color change are unknown.

Overall, there is remarkable variability in color patterns in this species and it is well known that color is used in mate choice and in male–male competition. The lateral stripe can be black, red, or brownish, and in some populations, both sexes can be spotted. Furthermore, the name-giving elongated part of the caudal fin can have yellow or orange color in it. This predicts that color preferences will evolve in females exploiting the existing variability. Indeed, preferences for color are well documented in *X. hellerii* and other livebearing fishes (Franck et al., 2003; Rios-Cardenas & Morris, 2011). Many species in the genus *Xiphophorus* are model species in sexual selection research, particularly studying female preference for male swords (Basolo, 1990, 1996; Basolo & Trainor, 2002; Rosenthal & Evans, 1998; Rosenthal, Wagner, & Ryan, 2002). Because the sword adds to the size of the fish, preference for a sword is often interpreted as a preference for size (Ryan & Keddy-Hector, 1992). In addition to the sword, pigmentation patterns and spotting are known to be sexually selected visual traits in other species of *Xiphophorus* too. In *X. cortezi*, males develop melanomas that can influence female choice (Fernandez & Morris, 2008). *Xiphophorus cortezi* females, for example, have also shown preferences for male symmetry in vertical black bars on the sides of males (Morris & Casey, 1998).

Furthermore, chemical communication also plays a role in sexual selection and male–male interactions in many species (Hurst, 1990), but also in this genus for both male and females (McLennan & Ryan, 1997; Rosenthal, Fitzsimmons, Woods, Gerlach, & Fisher, 2011; Wong, Fisher, & Rosenthal, 2005). For example, in *Xiphophorus birchmanni*, the modality of cues received from male conspecifics or related species influences female preference. When raised with conspecifics, *X. birchmanni* females strongly preferred the chemical and visual cues of conspecific males. However, when only exposed temporarily to conspecific or heterospecific cues, olfactory stimuli produced stronger timing effects than did visual stimuli alone (Verzijden & Rosenthal, 2011).

Based on this background, we used the Actopan population of *X. hellerii* to test two main hypotheses. First, we investigated how males responded to color signals from other males. Previous work established that males will respond to a color change, but which sensory input is inducing color change in male Actopan swordtails remained unclear. Intuitively, one would assume that this is a purely visual signal, but other modalities might also play a role, especially chemical cues. We examined this by selectively withholding cues about an opponent from a focal male. We predicted the strongest response when both visual and chemical information was available, and the weakest based on chemical information only.

Second, we measured female responses to red and black coloration, predicting that red would be preferred if it honestly indicates dominance in this species. However, in the case of the Actopan population, males can manipulate the signal, which may render it unreliable. For this population, finding a lack of female preference could indicate that females have already evolved to ignore a previously useful signal.

2 | METHODS

We conducted two different experiments to test our hypotheses; The first (Study 1) was a male–male interaction experiment, whereas the other study (Study 2) and its preliminary experiment (see Supporting Information) used classical binary choice tests to investigate female preferences.

2.1 | Study specimens

We used laboratory-raised descendants of fish collected in 2009 in the Río Actopan, near Xalapa, Mexico (Veracruz; N19°25′07.4″; W96°37′02.3″). The original site was visited on July 2, 2009 and was essentially unchanged from a previous visit in 1996. The Río Actopan is roughly 20 m wide at the field site and lies on the outskirts of a small village. Collections were made upstream and downstream of a small bridge. Water flow was high, and water temperature was 27°C. At this site, *X. hellerii* was associated with two other livebearing fishes, *Poeciliopsis gracilis* and *Poecilia sphenops* (McCoy, Syska, Plath, Schlupp, & Riesch, 2011). Fishes were transported back to Norman, Oklahoma, in aerated and filtered coolers. In Norman, the fish were maintained as randomly outbred populations in 250- to 1,000-L greenhouse tanks with flow-through water at the Aquatic Research Facility (ARF) of the Department of Biology (University of Oklahoma). We estimate that the population has been in the laboratory for 7–10 generations. For testing, some fish were transferred to indoor housing in Dec. 2013, with Study 1 conducted in the summer of 2014. In the laboratory, fishes were kept in 37.85- to 75.71-L tanks with gravel substrate and filtration under 12-L/12-D light conditions. They received weekly water changes with deionized tap water supplemented with reef salt (Instant Ocean) (Makowicz & Schlupp, 2015). Fish were fed twice daily with flake food and a mix of frozen bloodworms, *Daphnia*, and brine shrimp naupliae. Fish were transferred to laboratory housing at least a week before testing in order to acclimate them to laboratory conditions. Prior to Study 1, no more than four males were housed in laboratory tanks, and all tanks contained females and juveniles to approximate natural social conditions. A week prior to the experiment, males were randomly chosen for either isolated tanks or male-only tanks limited to three fish to ease the ranking process. Females were housed in mixed-sex tanks until at least a week preceding Study 2, which was conducted in the spring of 2016. Generally, we would expect females to show increased interest in males and mating if deprived of contact. At the conclusion of both studies, fish were returned to their stock tanks.

2.2 | Experimental setup—Study 1: male communication study

1. Pre-trial treatment: Prior to the start of Study 1, all males were photographed (Nikon D5200 24.1 MP CMOS Digital SLR camera) and their standard length (SL) measured (mm) to assign individuals a priori to small and large size categories. Standard length was measured as the length from the snout to the base of the caudal peduncle. Males >41 mm SL were designated as large (and alpha males), with males in the sample population ranging between 35 and 75 mm long. Additionally, males were assigned a social rank by a single observer based on behavioral cues (winning of fights) and visual characteristics (size, stripe, and body coloration) while under normal housing conditions (Beaugrand, Caron, & Comeau, 1984). We found this assessment to be reliable, but did not formally conduct a repeatability study. Males that chased shoal members, attempted sigmoid displays, biting, or female guarding were considered to be alphas. Males that hid in sheltered parts of the tank or fled from male interaction were labeled omegas. Males with intermediate behaviors were given a beta designation. If the male was isolated or only housed with females, it was categorized as an alpha male. Past studies have shown that isolated males tend to be more aggressive and escalate confrontations more frequently (Hannes & Franck, 1983); hence, we labeled such males as alpha males. It should be noted that dominance status is plastic and depends on the current social conditions (Rhodes & Schlupp, 2012). The number of males within each category was comparable (9 alpha males, 6 beta males, and 11 omega males), but fluctuated over the course of the study due to natural mortality and introduction of new fish to housing tanks.
2. Experimental protocol: Experimental trials were separated into four categories: small focal-large stimulus, large focal-small stimulus, small focal-small stimulus, and large focal-large stimulus. Treatment order was randomized for each trial. Males were given at least 24 h before use as a stimulus or focal male for subsequent trials. Males' stripe colors were photographed and scored in the minute before and after the trial while kept in a glass holding tank, with a color scale devised by E.H. as a dependent variable. There is no precedent for analyzing this color trait; therefore, a subjective measurement was used. Color scores were given designations based on the Microsoft Word color wheel best matching the range of colors found in Actopan males. Colors were selected on a MacBook Pro (13-inch, 2012) in 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 black.

All trials were conducted in a clear 37.85-L tank with all but the observer's side covered by white plastic boards to prevent visual distractions to the focal male. Using a variety of smaller prism-shaped Plexiglas containers, we created treatments that limited information transmission

between males (Makowicz, Tiedemann, Steele, & Schlupp, 2016). We did this to examine which sensory modalities might be involved in regulating color change of the lateral stripe. A stimulus male was placed into either a clear container (8 cm × 8 cm × 35.5 cm inner dimensions) with no holes (for the "visual-only" treatment), a container with small holes (0.3 mm in diameter) ("visual+small chemical"), a container with fewer, yet larger holes (0.6 mm in diameter), and no container ("open field"), that allowed the males to move freely to engage in all modalities, including tactile, and close range chemical communication. We assumed that both prisms with holes would allow chemical information to diffuse from the prism to the main tank and that the larger holes would also allow for tactile information based on the lateral line system of these fishes (Rüschenbaum & Schlupp, 2013). The stimulus male was given a 5-min acclimation period in the container prior to the introduction of the focal male. Entry of the focal male into the tank marked the start of a 10-min observation period. The order of presentations was randomized.

During the trials, the observer subjectively noted the color changes (using the previously mentioned 5-point scale) and time of change for 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 initial aggression time and the instigator of aggression were noted at the time of observation. Four aggressive behaviors were measured: bites, chases, sigmoid or "s-curves," and the side-by-side posture (Franck & Ribowski, 1987, 1989), in which males evaluate their opponent's size. The four behaviors were chosen based on descriptions by Beaugrand et al. (1984). Chases were defined as a rapid rush toward the stimulus male, as the male could not perform a full chase when separated by the container.

At the trial's conclusion, both males were removed and the tank and containers were cleaned with soap and water, followed by hydrogen peroxide, to reduce residual chemicals from previous trials (McLennan & Ryan, 1999). New water was added prior to each treatment, maintaining salinity level (mean 800 ± 50 μS) and temperature (mean 27 ± 2°C) throughout the experiment. The timing and recording protocol was the same for all four treatments. In addition, "chemical-only" trials were conducted in the fall of the same year, using a black opaque plastic container with small holes, with the procedure described above again using randomized trials. The University of Oklahoma's Institutional Animal Care and Use Committee approved the protocol and animal handling for this experiment as well as the female preference experiments (R15-014).

2.3 | Experimental setup—female preference study

2.3.1 | Study 2: dummy test

A preliminary experiment testing female preference for simple color stimuli showed no significant trends (see Supporting Information); therefore, in an attempt to provide a more realistic color stimulus, we created a set of male swordtail dummies. Live males were not used as stimuli due to stripe color inconsistency during pilot trials. Video stimuli, while successful for measuring response to behaviors (Trainor & Basolo, 2000), were also not used due to potential distortion of color from the swordtails' natural visual spectrum. Swordtails do not perceive color in the same spectral range as humans; therefore, we ruled out this method to increase the likelihood of interpretable results (Oliveira et al., 2000; Rosenthal, 1999; Watson, Lubieniecki, Loew, Davidson, & Breden, 2010). Two plastic dummy models of identical size (40 mm SL) were created via 3D printer, using a digital swordtail template purchased on a 3D model website (www.turbosquid.com). Dimensions from the template were chosen to match the population mean of male size (SL = 41 mm) for the Actopan population. Acrylic paint (Grumbacher Academy Acrylics codes C026, C134) was used to recreate the appearance of males expressing red or black stripes. To create a color stimulus relatively similar to a live male, we used spectrophotometry to measure the reflectance of both paint samples and live male stripes. The process is described in detail in the Supporting Information. Our goal was to match the colors reflected by living males in the range of wavelengths that a female swordtail can perceive.

Reflectance data (Figures 2 and 3; see Supporting Information for additional data) were analyzed using the summary function in the R-Studio package "pavo" (Maia, Bitton, & Eliason, 2016). Male data were averaged to approximate the best potential red paint for the range of expressed male stripe colors. Variables of interest included brightness, chroma, and hue data (see Supporting Information for "pavo" descriptions) of each paint sample. We selected paint with mean values closest to the values of male spectral data (Table 1). The color was then painted onto the dummy as a stripe mimicking that of live males and sealed with a waterproofing coating, which did not appear to alter the color and should not have impacted the reflectance.

Using a standard binary choice test design, females (n = 32) were tested in a 75.7-L tank (61 cm × 39 cm × 30 cm) divided into three equal zones using permanent marker: a central "neutral" zone, and a

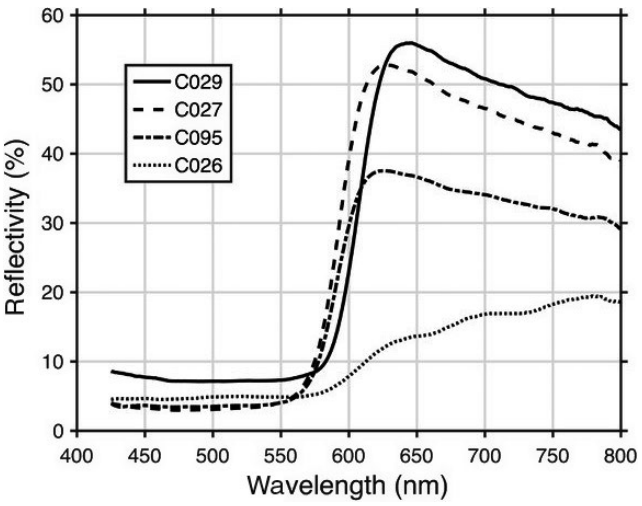

FIGURE 2 Reflectance of paint samples. Each line and code represent the reflectance from a paint swatch (Grumbacher Academy Acrylics) painted on the same plastic as the fish models. The 100% reflectance was scaled to a white reference (spectralon) in the air. Paint C026 was the paint sample used in the study

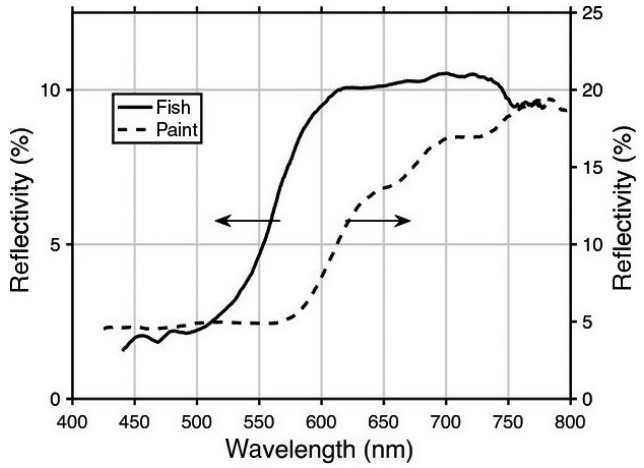

FIGURE 3 Reflectance spectra comparison. The averaged spectrum from different spots on the lateral stripe of two individuals is compared to the reflectance spectrum of the paint used in this study

preference zone on each opposing side with a dummy male affixed to the outside of the tank. Tank salinity (average ± *SE*: 800 ± 50 μS) and temperature (average 27 ± 2°C) were kept within a consistent range throughout the experiment. Four white plastic panels surrounded the sides and bottom of the tank, with the exception of the side facing the observer. The dummies were switched after each replication of the trial to test for side bias (see Landmann, Parzefall, & Schlupp, 1999). Hence, a complete trial consisted of two parts, 5 min each to prevent side bias. A clean, open-bottomed plastic container containing the focal female was placed directly in the center of the tank (in the neutral section) for the 5-min acclimation period. At the end of this period, the container was removed and the 5-min timed observation period began. We used association time to measure female preferences.

Prior to the start of a trial, each female was binned into a size category based on her SL, which was measured prior to the experiment. Small females were 31 mm or shorter, medium females were 32–38 mm, and large females were 39 mm or larger in SL. Standard length ranged from 25 to 53 mm.

Time (s) within each of the two preference zones was recorded using stopwatches. The time for a respective zone started as the female passed into a preference zone with more than half of her body. After the first trial replicate (part 1) was concluded, the female was returned to the container and another 5-min acclimation period began. Once acclimated, a second observation period (part 2) started, with identical protocol but the color stimuli switched. The sums from each trial (parts 1 and 2) for each female were then used for data analysis.

3 | DATA ANALYSIS

3.1 | Male communication study

Unless stated otherwise, data analysis for color change and aggression data was conducted in SPSS (version 17.0.0, 2008 SPSS Inc.). A total of 27 trials were completed for the four-treatment set, but one trial set was discarded due to the death of the focal fish shortly following the trial. In addition, 26 "chemical-only" trials were conducted at a later time. This was done because the first approach did not result in conclusive findings, and we wanted to test an additional hypothesis that color changes might be induced by chemical communication only. During these 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

TABLE 1 Average scores for paint and male lateral line reflectance data

Reflectance measurements	Samples				
	C026	C027	C029	C095	Male avgs
S1 UV	0.147	0.085	0.085	0.101	0.183
S1 violet	0.157	0.091	0.091	0.108	0.196
S1 blue	0.102	0.054	0.066	0.065	0.107
S1 green	0.165	0.165	0.142	0.170	0.159
S1 yellow	0.162	0.261	0.207	0.245	0.151
S1 red	0.265	0.472	0.475	0.417	0.184
H1	642	618	627	618	833
H2	685	669	669	669	833
H3	416	404	408	404	511
H4	−0.913	−0.893	−0.818	−0.921	−1.051
H5	189	593	593	593	832
B2	178.683	0.261	313.923	261.876	150.277
B3	496.119	0.472	1,791.300	1,343.680	490.919

Variables and their units are described in detail in the R-package "pavo" user manual. S1 values refer to the contribution of different wavelength ranges to total brightness, H values refer to hue (H1 = peak wavelength, H2 = bmaxneg, H3 = Rmid, H5 = bmax), and B values refer to brightness (B2) and intensity (B3).

TABLE 2 Descriptive statistics for container effects on net change in color and time of first observed color change (n = 26)

Treatment	Mean color change	First change (s)
Visual only	0.96 ± 1.216	97.50 ± 124.017
Visual + small chemical	0.69 ± 1.05	42.08 ± 52.495
Visual + large chemical	0.65 ± 1.441	60.69 ± 129.926
Control (open field)	0.69 ± 1.123	66.35 ± 131.49
Chemical only	−0.50 ± 0.762	75.62 ± 150.734

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 toward darker colors.

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

3.1.1 | Color change

We used a repeated-measures ANCOVA to examine differences in color expression and aggression in the four-treatment set. Container type was used as a factor, and normalized size differences (√arc (sin) transformation) between males and differences in housing rank (high, equal or low-rank stimuli) were used as covariates. The "open-field" treatment data were not used for the repeated-measures ANCOVA. The dependent variables were "net change in focal male stripe color from the start of each 10-min trial to the finish of the trial" ("TXNetChange"), and "time (s) to first observable focal male color change" ("TXFirstChange").

3.1.2 | Aggression

Aggressive behaviors were quantified by the total time displaying said behaviors, as well as the numbers of occurrences of each behavior type. However, due to a lack of independence amongst these behaviors, we used a preparatory principal component analysis (PCA) for each container treatment to create independent principal 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 ANCOVA with container type as a factor, the normalized size and housing rank as our covariates, and the PCA components as the dependent variables. Of the 26 "chemical-only" trials, there one instance of aggressive behavior (a bite) during the final trial; therefore, aggression was not analyzed for this set of trials.

3.2 | Female preference study

3.2.1 | Study 2: dummy test

A total of 32 trials were conducted in this experiment. Preferences were analyzed via a paired t test in SPSS. We measured three dependent variables for both the paired t test and post hoc one-way ANOVA with female size as factor. Total time (s) in preference zones, as well as percentage of time and normalized time, were used as dependent variables. Percentage of time was calculated as the total time in a particular zone divided by the sum of time spent in both preference zones. Data were then normalized using √arc (sin) transformation. Any trials in which the female showed a side bias (more than 85% of time on one side; McCoy et al., 2011; Makowicz et al., 2016) were excluded from analysis. Eighteen trials were excluded due to side bias, but a binomial test indicated that side biases were distributed randomly between the two sides.

4 | RESULTS

4.1 | Male communication study

4.1.1 | Qualitative observations

Stripe color changes are rapid, and a full shift from red to black or vice versa can occur within minutes. Males displaying shades of red are generally thought to be more dominant, and in our tanks only one male usually showed a red stripe after initial aggressive bouts during introductions of new males. Males maintain a red stripe in isolated housing conditions. Handling by the experimenter or losing aggressive bouts with conspecifics typically leads to changes to darker stripe colors, ranging from a dark red to brown to black. Males in poor health display darker stripe colors as well, which suggests that red coloration may be a costly signal to conspecifics of good body condition or aggressiveness. In contrast, the introduction of a female triggers a change to red in most males in isolation, regardless of size.

4.1.2 | Color change

In the 26 four-treatment trials, 17 focal males displayed some degree of color change in the visual-only and "visual+large chemical" treatments, and 14 males in the "visual+small chemical" treatment (Table 2), indicating a limited role for chemical communication in inducing color change. In the "chemical-only" trials, 15 of 26 males displayed color change. Some interesting trends did appear upon examination of stimulus rank relative to the focal males and net color change. In the four-treatment trials with males of equal rank (n = 6), three trials showed focal males shifting toward lighter colors/red, two went unchanged, and one became darker but only during the "visual + large chemical" 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. In the trials with higher-ranked stimulus males (n = 11), results were more evenly distributed, with four focal males becoming darker/black, four becoming lighter/red, and three showing no change.

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

TABLE 3 Results from repeated-measures ANCOVA for the container treatments (excluding "chemical-only", which was tested via paired *t* test)

	F			*p*		
	w/o covariate	w/rank	w/size diff.	w/o covariate	w/rank	w/size diff.
Net change	2.617	1.809	2.100	.078	.176	.131
First change	2.831	0.339	4.867	.063	.797	.01

The table shows the *F*- and *p*-values for all iterations of the ANCOVA for container effects (n = 26). Covariates were difference in housing rank and net difference in size.

All repeated-measures ANCOVAs evaluating the effect of the treatment on net color change were not significant (Table 3). As an exploratory analysis, we ran the test with and without covariates (difference in housing rank and size difference), but the results were unaffected. The "visual-only" treatment stood out as having the greatest mean net change ($\bar{x}$ = 0.96 ± 1.22 *SD*), but also showed the slowest response time. Interestingly, the "visual+chemical" treatments and the "open-field" treatment had both very similar and lower values when compared to the "visual-only" treatment. Time to first change showed a significant effect with normalized size difference as a covariate ($F_{1,23}$ = 4.87, p = .01; Table 3). A post hoc comparison showed that the "visual-only" treatment was significantly different from the "visual + small chemical" when examining treatment effects on first change (LSD, p = .03) and nearly significant for net change (p = .052). All other post hoc pairwise comparisons proved non-significant. With both normalized size difference and housing rank difference as covariates, "container" was non-significant ($F_{1,23}$ = 2.83, p = .063, observed power = 0.6), but the normalized size difference had a significant effect ($F_{1,23}$ = 4.87, p = .01, observed power = 0.8). Males in "visual-only" treatments took longer on average for the first change ($\bar{x}$ = 97.5 ± 124.02 s), whereas "visual+small chemical" treatments took the shortest time ($\bar{x}$ = 42.08 ± 52.5 s; Table 2). When compared to the "visual-only" treatment, the chemical trial net change was barely significant ($F_{2,50}$ = 3.99, p = .05).

4.1.3 | Aggression

In the four-treatment experiment, 15 of the 26 males displayed aggressive behavior (Figure 4). Bites were the most common behavior ($\bar{x}$ = 5.97 ± 13.87 per trial), followed by sigmoid display ($\bar{x}$ = 2.17 ± 6.1). Sigmoid displays are generally a precursor behavior to escalated aggression and are more often displayed when males are similar in size (Franck & Ribowski, 1989). Amongst the treatment types, males in the "visual-only" treatment had a greater average ($\bar{x}$ = 11.69 ± 21.55) combined aggression total than males in any other treatment. Interestingly, males in the open-field control, which allows for all avenues of communication, had the lowest aggression average ($\bar{x}$ = 6.42 ± 14.51). Of the two mixed-mode treatments, the small chemical container showed greater average aggression.

The first two principal components of the PCA accounted for ca. 99% of the variance and were subsequently used as dependent variables for the repeated-measures ANCOVA. PC1, which had strong loadings on bites and sigmoid displays, was not significant across 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, was also not significant.

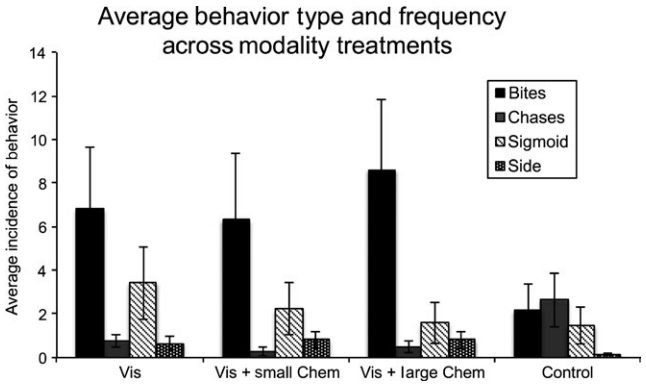

FIGURE 4 Average (+*SE*) number of aggressive behaviors across container treatments. Note that there were no aggressive behaviors in the "chemical-only" treatment. vis, visual (container is clear); chem, chemical (container has holes)

4.2 | Female preference study

4.2.1 | Study 2: dummy test

Females tested with dummy males showed a slightly higher preference for the red (53%) vs. black (47%) dummy (t_{31} = 0.8, p = .43, n = 32). Total time, percentage preference (Figure 5), and normalized preference were all non-significant. Post hoc analysis also confirmed that size was not a significant factor in female preference. Medium females (n = 11) chose red an average 53.8% of the time, and small (n = 12) and large (n = 9) females showed choices of 52.5% and 52.6%, respectively.

5 | DISCUSSION

In the present study, we investigated the responses of males and females to a signal, a colored stripe in males of the green swordtail, *X. hellerii*. In the population we studied this signal may have become

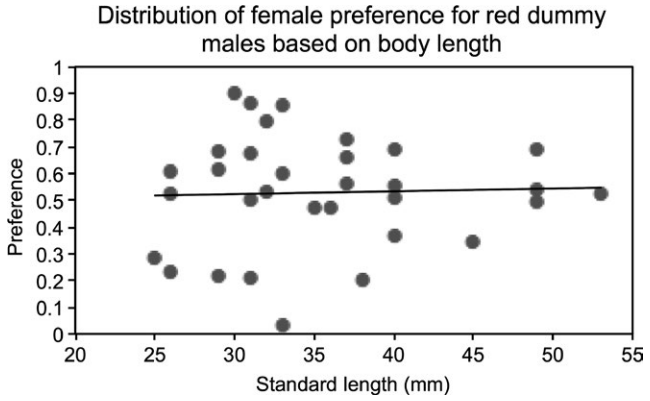

FIGURE 5 Scatterplot of female preference percentage for a red stimulus, according to female size category. Dummy male experiment groups were as follows: small (<31 mm), medium (32–38 mm), and large (>39 mm)

unreliable, as it evolved from a fixed badge of status to a dynamic signal that males express depending on social context. We find that only males do respond to the signal as predicted, and only partially. We were able to confirm that the color change described for the Actopan population is a visual signal and that using vision as sensory channel alone induces the strongest male response. Additional information provided via chemical information induces a faster color change in focal males, but decreases the magnitude of change. Chemical information alone appears ineffective, although it is otherwise used widely in swordtails and other Poeciliids (Fisher & Rosenthal, 2006; McLennan & Ryan, 1997; Wong et al., 2005). We do not think that the method used here led to the lack of response, because in a different study using similar containers and investigating clonal recognition in the Amazon molly, females were able to recognize kin using all modalities investigated here (Makowicz et al., 2016). This strong role of visual communication is not a completely unexpected result, as visual displays are known to be important in swordtails, both in male contests and for acquiring mates.

The findings from our experiment focusing on male–male aggression were less clear: Even when male size and rank were included in our analyses, aggression and color change were not connected in a clear way. Based on previous work by Franck and Ribowski (1989), we would have predicted clear responses to red or black stripes, but in our data set rank or relative size does not significantly account for variation in aggression type, and omega focal males actually exhibited fewer aggressive behaviors. While this could be due to potential error in the designation of rank, frequencies of types of aggression are similar to those in a previous study (Rhodes & Schlupp, 2012). We cautiously interpret this as evidence that lateral stripe color in the Actopan population, may not provide reliable information about male rank and resource holding potential, and may thus have lost its function as an honest badge of status. We do not know what may have caused the evolution of this loss of function.

In addition, in our female preference experiments, females from the Actopan population lack a preference for a red lateral stripe. We had initially predicted a color preference based on studies using a nearby population where the polymorphism for red and black stripes is genetically fixed (Franck et al., 2003). Furthermore, based on previous female preference research of body size and sword presence (e.g., Rosenthal & Evans, 1998), we predicted that females would prefer larger, generally red colored males. There are multiple possible explanations for this absence of preference, but this finding can also be interpreted as loss of function the stripe as reliable signal. We do not believe our experimental setup was at fault. We used artificial stimuli due to difficulties with creating experimental setups using live stimuli, but females did respond to the dummy males, at times darting rapidly in response to first viewing a model. In addition, dummies have been successfully used in other color-related fish studies of responses to color (e.g., Anderson, Jones, Moscicki, Clotfelter, & Earley, 2016; Phamduy, Polverino, Fuller, & Porfiri, 2014), with positive results. In a study using Jacky dragons, and computer animated dynamic stimuli for interactive video playback, Van Dyk and Evans (2008) showed the importance of social responses to dynamic stimuli, a property that the static dummies in our study lacked.

One potential explanation is that females may 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, vs. a more similar black stripe. Using video playback, Basolo and Trainor (2002) showed that female green swordtails show greater response toward complete male swords over their components. Hence, it is conceivable that stripe color alone does not provide a sufficient signal for Actopan females to evaluate males. Preference can also be context-dependent, and if females prefer a "rare-type" male morph for genetic benefits (e.g., Royle, Lindström, & Metcalfe, 2008), choosing solely based on stripe would not be effective in this population.

However, a more intriguing possibility is that Actopan females have lost a color preference due to the dynamic nature of the signal in this 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 more valuable (Baer, Dantzker, & Ryan, 1995). Dominant males remain red in aggressive contests, indicating that red remains partly a signal of aggression, but it may no longer be attractive to females. Overall, we speculate that in the Actopan population of the green swordtail, a former badge of status might have lost its function as an honest signal. In the same genus, Morris et al. (2007) were able to establish that female usage of a dynamic signal in males, vertical bars, predated male usage of the same signal. Here we show that females are also the first to discount a signal that has lost its value.

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SUPPORTING INFORMATION

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