

## Article

# Variation in female aggression in 2 three-spined stickleback populations with female throat and spine coloration

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## Abstract

Despite growing interest in female ornament evolution, we still have a rudimentary understanding of female display traits relative to similar traits in males. Under one popular adaptive scenario, female ornaments are hypothesized to function in female–female competition and serve as badges of status, such that their expression is linked with elevated aggression in some cases. In this study, we investigated the relationship between 2 female ornaments—male-like red throat color and red spine coloration—and female aggression in 2 independently derived stream-resident populations of three-spined stickleback *Gasterosteus aculeatus*. Using simulated intrusions, we tested whether females with redder ornaments were generally more aggressive, and for variation in aggressive and social behaviors between the 2 populations. We found that the red intensity of the throat and spine did not predict aggression levels in either population, suggesting a limited role for both female ornaments during female–female interaction. The 2 populations exhibited different levels of aggressive behaviors, unrelated to the color patches. Our results suggest that variation in selective pressures between populations may promote interpopulation variance in aggressive behavior but not the correlation between female ornamentation and aggression, and raise the possibility that red coloration may have evolved through different mechanisms or processes in the 2 populations.

**Key words:** aggression, animal coloration, female ornaments, *Gasterosteus aculeatus*, stickleback.

Within the last decade, work on sexual selection has demonstrated that female ornaments are more common than previously thought, and can be as conspicuous as those found in males (Kraaijeveld et al. 2007; Clutton-Brock 2009; Tobias et al. 2012; Dale et al. 2015; Charmantier et al. 2017). A long-held hypothesis for female ornaments views them as correlated by-products of selection on males, and some recent studies support this perspective (Lande 1980; Dale et al. 2015; Yong et al. 2015; Charmantier et al. 2017). However, investigations of possible functions of display traits in females have often yielded evidence that they benefit females directly (Clutton-Brock 2009; Tobias et al. 2012; Flanagan et al. 2014). Resolving the relative importance of by-product and direct selection processes in

female ornament evolution remains one of the central goals of studies of female ornament evolution.

The evolution of such ornaments is in some systems associated with aggressive behaviors in the context of female–female competition (Pryke 2007, 2009; Clutton-Brock 2009; Midamegbe et al. 2011; Tobias et al. 2012). Despite some potential costs, female aggressiveness may be advantageous in a number of intrasexual contexts, including competition for rank and territories, and for access to mates (Bakker 1986; Rosvall 2011; Stockley and Campbell 2013). Moreover, agonistic behaviors are often accompanied and correlated with badges of status, that is, colorful traits whose functions include advertising competitive ability. Females bearing such conspicuous

badges tend to be more aggressive (Clutton-Brock 2009; Pryke 2009; Midamegebe et al. 2011), and badges are commonly found in mating systems with intense female–female competition and extra-pair mating (Rubenstein and Lovette 2009; Stockley and Bro-Jørgensen 2011).

Relationships between color variation and a variety of traits, including aggression, have been studied extensively in the three-spine stickleback *Gasterosteus aculeatus*, in which replicated adaptive radiations have facilitated extensive and wide-ranging studies of the evolution of male secondary sexual traits (e.g., Bakker and Milinski 1993; Rowland 1994; McKinnon 1996; Boughman 2001, 2007; Malek et al. 2012). The ancestral marine (or anadromous) stickleback form, which is hypothesized to have given rise to derived-freshwater populations, is known to be conventionally sexually dichromatic for the male-typical nuptial throat ornament (i.e., red throats are present almost exclusively in males). In a few freshwater-resident North American stream populations, however, females too have been found to exhibit red nuptial throat coloration at high frequencies (von Hippel 1999; McKinnon et al. 2000; Yong et al. 2013). Two such populations, which are the focus of this study, are found in different drainages in southern British Columbia (Little Campbell River, BC, Canada, hereafter LC) and Northern California (Matadero Creek, CA, USA, hereafter MAT). Given the distance between them and the likely postglacial origin of at least the British Columbia population, these populations have likely arisen from independent colonizations of freshwater by marine or anadromous ancestors in which females lacked red throats. To date, formal documentation of stickleback red female coloration is confined to stream-resident ecotypes from just these localities and one more (von Hippel 1999) although we have observed the trait at additional sites (McKinnon, unpublished data).

The intensity of male stickleback red coloration has repeatedly been found to be positively correlated with male aggressiveness to a standardized stimulus (such as a dummy or a conspecific in a small container: Rowland 1984; McLennan and McPhail 1989; Wright et al. 2016) as well as with dominance in pairwise interactions (Bakker and Sevenster 1983; Baube 1997; McKinnon 1996). In our investigations till date, we have not found a relationship between female red intensity and dominance (Yong et al. 2015), but we have not tested for a relationship with aggressiveness toward a standardized stimulus; the latter tests are important because they may be more sensitive than experiments in which fish fully interact, because dominance and aggression are not always correlated (e.g., Rowland 1989; Baube 1997) and because the negative dominance result is surprising in light of findings for males. In addition, our behavioral studies so far have been confined to one population and drawing definitive conclusions from studies of single populations is problematic in a species that shows as much geographic variation as the three-spined stickleback (Bell and Foster 1994). Inclusion of additional populations also enables the initiation of potentially informative comparative investigations. It is additionally important to note that some field observations of sticklebacks suggest that aggression and dominance may sometimes be beneficial to females, to facilitate access to nesting males, or maintain feeding territories (MacLean 1980).

Another orange-red color patch is gaining increasing attention in stickleback research, on the posterior portion of pelvic spines and associated membranes (Nordeide 2002; Hodgson et al. 2013; Amundsen et al. 2015). The spine color patch seems almost ubiquitous (Amundsen et al. 2015; Yong et al. 2015), and earlier work suggests that erected pelvic spines might function during social displays as indication of elicited aggression; males with erected spines have been observed to often charge at conspecifics (van Iersel 1953). In addition, we have found that

male spine color intensity predicts attack behavior aimed at females during courtship trials (Wright et al. 2016). Spine color intensity is often positively correlated with that of the throat, with which it shares to some extent a genetic basis (Yong et al. 2013, 2016).

Our goals in the present study were to 1) examine the relationship between variation in female throat color, spine color, and aggressive response to a standardized stimulus between and within populations, and 2) evaluate possible differences in aggressive and social behavior between our 2 study populations, which are hypothesized to have independently evolved red female throat coloration (Yong et al. 2013). Regarding the latter goal, the prediction was that if the female color patches generally evolved in the context of intrasexual competition and aggression, females from the population with the most intense throat and spine coloration should behave more aggressively.

## Materials and Methods

### Stickleback collection and maintenance

Three-spined sticklebacks were collected during the breeding season in 2 years and stream locations: LC in April 2010 (49.012 N, –122.624 E) and MAT in June 2012 (37.393 N, –122.162 E). Fish were shipped to our laboratory at East Carolina University (ECU), where they were housed in conditioned community tanks, as detailed in Yong et al. (2015). All fish were maintained on a 16 L:8 D photoperiod at 16 °C. All fish were fed twice daily with brine shrimp and bloodworm (chironomid larvae), and allowed to acclimate to laboratory conditions for at least 1 month before any behavioral trials were conducted. All experimental procedures were approved by the ECU IACUC (Protocol #AUP 224a).

### Aggression experiment: simulated territorial intrusion

To test for variation in female aggression, we used a simulated territorial intrusion previously validated in stickleback (Bakker 1986; Sanogo et al. 2012). Behavioral experiments for each population were conducted during the breeding season (April–July), and within the same year in which they were collected. Resident females (LC:  $n = 23$ ; MAT:  $n = 20$ ), varying in throat color intensity from each population were haphazardly selected from community tanks, and individually isolated for 42 h in an experimental 21-L tank (41 × 20 1/4 × 25 1/2 cm) containing a UV transparent chamber (15 × 7 × 7 cm) hung inside and on the back of the tank. As the focus was on the resident's response to the visual presence of the intruder, the chamber was sealed, eliminating the potential transmission of water and chemical cues. The designated isolation period was previously validated to promote territorial behavior and enhanced aggression in females (Yong et al. 2015). All females were nongravid. After the isolation period and at the start of the behavioral trial, an intruding female of the same population with duller throat coloration was introduced into the chamber. Dull-throated females were primarily used as natural and standardized stimuli. The chamber prevented physical contact between the females, and allowed us to focus mainly on the resident's behavioral response. Resident and intruder females were size-matched (up to ~5% difference in standard length). The behavioral trial was video-recorded (Sony Handycam Digital Camcorder-HDR-XR 500), and the behaviors of the resident female directed at the intruder were scored using Noldus Observer v7 (Noldus Information Technology, Leesburg, VA). We quantified *latency to respond to the intruder* (in seconds), *number of approaches* (within one body length of the intruder's chamber),

proportion of time spent inspecting the intruder (duration of time spent within one body length of the intruder's chamber/total trial duration), number of bites, bites while in proximity (bite number/approach duration). Behavioral trials generally lasted about 30 min, but up to 35 min in a few trials, that is, 5 trials, for the LC population. To control for the variation in trial time duration across trials, we calculated behavioral rates per minute.

### Throat color measurement

Immediately after the behavioral trial, females were measured for red throat intensity or “red throat chroma” as detailed in Yong et al. (2013, 2015). Throat coloration was only sampled once and after the trial, as it was previously found that color intensity does not significantly change before or after a social interaction (Yong et al. 2015). The reflectance of the throat was measured using an Ocean Optics Maya spectrometer (Ocean Optics Inc., Dunedin, FL), where measurement was taken from 2 to 3 spots (0.8 mm in diameter) along the midline of the throat, deliberately selected to yield maximum red throat. Then, we incorporated the spectrometry data into a physiological model of stickleback vision to estimate stickleback visual perception of red throat coloration. The relative quantum catches for each cone (UV, SWS, MWS, LWS) were calculated and used to calculate Cartesian coordinates ( $x$ ,  $y$ ,  $z$ ) to obtain the maximum (red) throat chroma, based on the Euclidean distance from the achromatic center in a tetrahedral space; this measure was used for subsequent analyses.

### Spine color measurement

Spine color was quantified from standardized digital photographs, each including a gray card, taken after the behavioral trial and using methods detailed in Yong et al. (2013) and Wright et al. (2016). In brief, we divided a digital image of the left spine into 8 equal sections and calculated the red chroma of each, using Adobe Photoshop CS3, as standardized R divided by the sum of standardized R, G, and B. We used the maximum among these 8 initial chroma in subsequent analyses, as “red spine chroma.”

### Statistical analyses

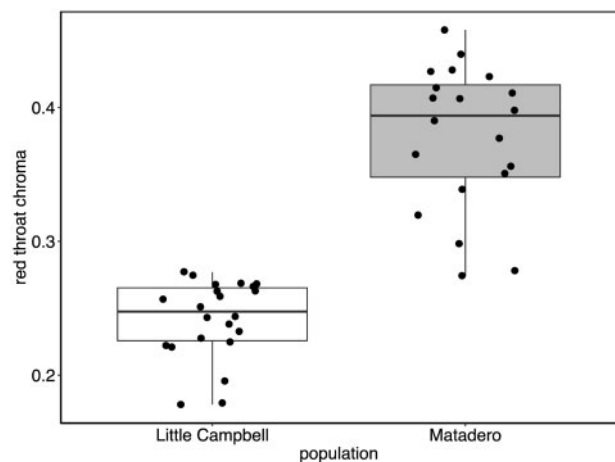
Assumptions of residual normality were checked by visually inspecting the residuals for all models. If residual normality was met, we used linear models to examine the relationship between color patches and bites between populations, where either red throat or spine chroma and population (LC vs. MAT) were treated as fixed effects, and bite rate [ $\log(n+1)$  transformed] as the response variable. Standard length was first included in the model as a continuous variable, but was removed as it did not approach significance. In some cases, model residuals did not conform with normality and data transformation did not improve the situation; in such instances, nonparametric univariate tests were used to test for differences in color and behaviors (e.g., bites, approaches, approach duration, and bites while in proximity) between the 2 populations. We acknowledge that the year, in which the trials were conducted, and population are statistically confounded (i.e., each population was tested in separate years), and thus cannot tease apart the separate effects of year and population. In light of this, our comparisons between populations are interpreted with caution. All statistical analyses were performed in the R environment v. 3.3.2 (R Core Team 2016).

## Results

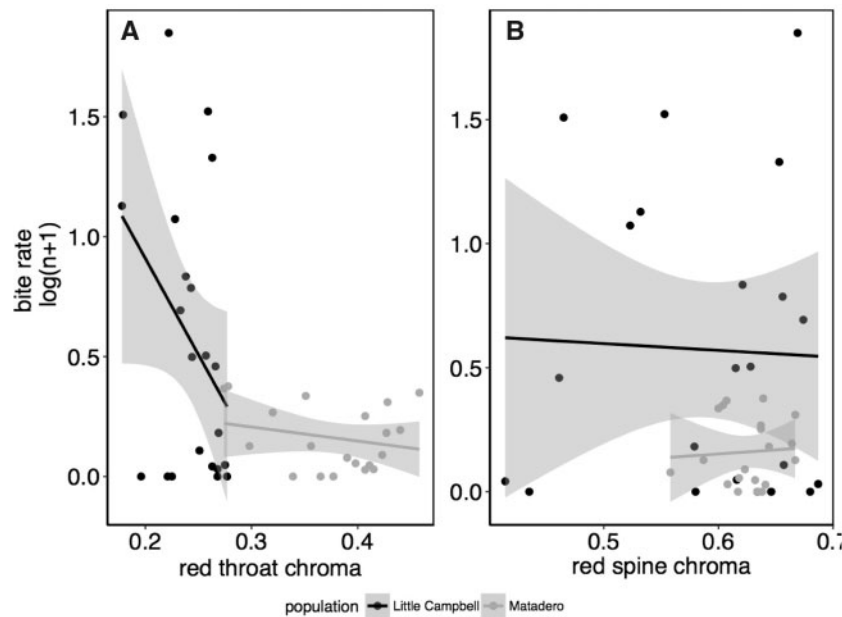
Females generally responded to the intruders within 5 min by orienting toward or approaching them, with no significant differences between the populations ( $W=245$ , latency:  $P=0.541$ , Mann–Whitney U test). One LC female showed no behavioral response during the trial, and was removed from further analyses. As previously documented, MAT females had more intense red throat coloration than LC females ( $W=2$ ,  $P=9.48 \times 10^{-8}$ , Mann–Whitney U test, Figure 1), but spine color intensity did not differ between populations ( $W=189.5$ ,  $P=0.449$ , Mann–Whitney U test).

While there is a negative trend for the relationship between throat color and bite rate, the linear models revealed that the intensity of red throat and spine coloration were not significantly associated with the resident's bite rate during the territorial intrusion in either population (throat chroma:  $F_{1,38}=0.127$ ,  $P=0.127$ , population:  $F_{1,38}=10.05$ ,  $P=0.003$ , interaction:  $F_{1,38}=4.27$ ,  $P=0.045$ , Figure 2A; spine chroma:  $F_{1,38}=0.039$ ,  $P=0.84$ , population:  $F_{1,38}=8.56$ ,  $P=0.005$ , interaction:  $F_{1,38}=0.023$ ,  $P=0.881$ , Figure 2B). Both linear models explained 42% and 44% of the variation, respectively. Although a significant interaction term was present for throat color, further within population analyses revealed no significant association between bite rate and throat color (LC:  $P=0.071$ ; MAT:  $P=0.323$ ), or spine color (LC:  $P=0.93$ ; MAT:  $P=0.82$ ). Similarly, we found no relationships between either throat or spine chroma with bite rate whereas in proximity (throat chroma:  $F_{1,38}=0.024$ ,  $P=0.878$ , population:  $F_{1,38}=16.97$ ,  $P=0.0002$ , interaction:  $F_{1,38}=0.05$ ,  $P=0.826$ ; spine chroma:  $F_{1,38}=0.17$ ,  $P=0.682$ , population:  $F_{1,38}=17.01$ ,  $P=0.0002$ , interaction:  $F_{1,38}=0.004$ ,  $P=0.947$ ). Relationships between throat color and other behaviors, that is, latency to respond, approaches, and proportion of time spent, were also nonsignificant ( $P>0.22$ ).

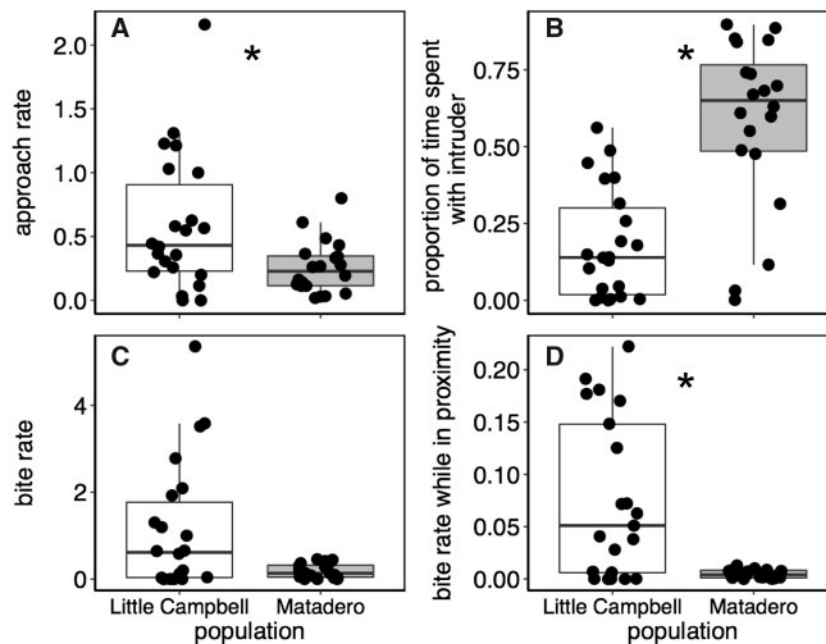
As there was a significant effect of population on bite rate, we conducted further tests between populations, revealing other considerable behavioral differences. Although MAT females had more intense throat color, they did not generally exhibit higher levels of agonistic behaviors. LC females made more frequent approaches to the intruder, but spent shorter periods of time close to them, whereas MAT females spent more time in proximity overall ( $W=307.5$ , approach:  $P=0.0284$ ;  $W=59$ , proportion of time spent with intruder:  $P=5.294 \times 10^{-5}$  Mann–Whitney U test, Figure 3A, B). While LC and MAT females did not differ statistically



**Figure 1.** Boxplots showing differences in red throat color between populations. Plots show median and 25<sup>th</sup>–75<sup>th</sup> percentiles.



**Figure 2.** Differences in the relationship between aggressive behavior and (A) red throat color and (B) spine color. The black and gray lines represent the Little Campbell and Matadero populations, respectively, in (B). Gray areas represent 95% confidence intervals.



**Figure 3.** Behavioral differences between stickleback populations. Boxplots show median and 25th–75th percentiles. \* denotes significance at  $P < 0.05$  level.

in bite rates ( $W = 284$ ,  $P = 0.108$ , Mann–Whitney U test, Figure 3C), LC females engaged in more biting per unit time than MAT females when in close proximity to the intruder ( $W = 312$ ,  $P = 0.0078$ , Mann–Whitney U test, Figure 3D), suggesting that LC females mainly approach intruders to attack them, relative to MAT females.

## Discussion

Ornamental traits often evolve in the context of social competition, including aggression and dominance, and are linked with behavioral

variation. In our study populations, in which females often possess the red throats typical of males of most populations, females responded aggressively toward the intruder but the color intensities of the throat and spine were not significantly correlated with aggression, and the trend (though nonsignificant) was for females with redder throats generally to exhibit reduced aggression. These results complement and corroborate our earlier findings, in which red-throated females were not more aggressive or dominant during a dyadic social interaction (Yong et al. 2015). Also, we observed behavioral differences in aggression between the 2 populations, but these too failed to indicate a positive relationship between red



coloration and aggression. In fact, LC females, whose red throats were the less intense of the 2 populations, exhibited more frequent aggression when in close proximity to the intruder whereas MAT individuals spent more time close to the intruder, much as LC females dominated, and differed from, anadromous females in an earlier study (Yong et al. 2015).

Our results are also unexpected in light of studies of male stickleback that used the territorial intrusion paradigm. When confronted with an intruder, males with brighter red throats typically display more aggression, especially in freshwater stream ecotypes (Bakker and Milinski 1993; Bakker 1994; Rowland 1994). Even in marine ecotypes, Rowland (1984) and McLennan and McPhail (1989) have shown that more intensely colored males can exhibit higher levels of attack. Because both MAT and LC populations are from stream habitats, males would be predicted to behave in a similar way. Our laboratory has previously found that throat color intensity in LC males is a positive predictor of courtship intensity during mating trials (Wright et al. 2016). In the same study, Wright et al. (2016) reported that males with more intense spine coloration bit females more often during mating trials, but we did not detect any associations between spine coloration and female aggressive behaviors in the present investigation. It is possible that color patches do not covary with behaviors consistently across the sexes and populations, and that the different social contexts (intra vs. intersexual) can affect these relationships.

Although rates of female agonistic behaviors were not readily predicted by color intensity in the throat or spine within our study populations, we did find notable interpopulation differences in aggressive and other social behaviors. We also observed that the throat color difference between female populations in this study was greater than observed by Yong et al. (2013), which may be explained by the time of the breeding season, or the year, during which fish were collected. Whereas LC females were collected earlier in the season (April), MAT females were collected later (June). Regardless, MAT females typically express the brightest throat color of the 2 populations, yet generally bit less once in proximity to intruders, which suggests that MAT females are less aggressive. Conversely, LC females, whose throat intensity was on average lower, tended to bite more, especially when in close proximity to the intruder. We also observed differences in the amount of behavioral variability exhibited, where LC females exhibited greater variation. Taken together, the inconsistent trend between the 2 populations suggests that among population color differences are unlikely to be mediated principally via agonistic social behaviors. Rather, differences in agonistic behaviors and color may evolve somewhat independently at least in some cases. Consistent with patterns in this study, stream females from the LC population also engage in more agonistic encounters than do LC anadromous females (Yong et al. 2015), which are thought to possess ancestral character states relative to the stream LC females. This supports our earlier supposition that selection for female aggressiveness may be relatively favored in the LC stream habitat. Such differences in behavior among stickleback populations are not uncommon and often have been attributed to ecological variation (Bell 2005; Bell and Sih 2007).

Based on the findings of this study together with previous work, nonadaptive mechanisms may play the major role in male-typical throat color evolution in female stickleback. Indeed, quantitative loci mapping of the red throat and spine in the MAT population confirms that coloration in both sexes is due to a similar, possibly shared genetic architecture and potential pleiotropy between traits (Yong et al. 2016), consistent with a byproduct process. However, despite an apparently shared genetic basis between the sexes for

these color traits, it appears that color and behavior might not covary consistently between the sexes, being less coupled in females. Whether these differences in correlations between traits might have an adaptive aspect is an open question.

We acknowledge potential limitations. First, the effects of population and year are confounded, which might partly influence some of the behavioral differences observed between the 2 populations. The year of collection could have included unaccounted variation in environmental conditions, in which, for instance, interspecific competition or density were variable. However, it is worth noting that all fish from both populations were sampled at approximately the same time of the year and during the breeding season, and tested within the same timeframe as to minimize variation in the experimental design. Also, given the known documented variation in aggression and other behaviors among stickleback populations (Bakker 1994; Rowland 1994), it is likely the observed behavioral variation is primarily owing to population differences. Nevertheless, we advise some caution as we cannot definitely rule out the possibility of a year effect in the absence of replicated behavioral data across years. Concerning a different aspect of our study, it is possible that other results could have been obtained if different female stimuli were used. For instance, intruders with redder throats might have elicited attacks from the resident fish, as the trait is often considered a releaser of aggression in territorial individuals (Rowland 1982). However, Rowland (1982) found the opposite effect, with red leading to reduced attacks (also see Wright et al. 2015), whereas in other studies, the intruder's coloration had no effect on the resident's aggressive response (Peeke et al. 1969; McKinnon and McPhail 1996).

In conclusion, our study reveals substantial differences in aggressive behavior between female stickleback from 2 populations in which female possess red throats and spines, and provides complementary evidence that the female traits are not associated with aggressive behavior and thus unlikely serve as badges of status. Our study populations provide an example of how female ornaments may not necessarily evolve through adaptive processes, and could arise as byproducts of a shared genetic architecture with, and selection on, males.

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## Authors' contributions

L.Y. and J.S.M. conceived the experiment and wrote the manuscript. L.Y. and B.L. conducted the behavioral trials.

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