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Correlates of red throat coloration in female stickleback and their potential evolutionary significance

Lengxob Yong[1]*, Ruqing Guo[1,2]*, Daniel S. Wright[1], Samantha A. Mears[1], Michele Pierotti[1] and Jeffrey S. McKinnon[1]

[1]*Department of Biology and Center for Biodiversity, East Carolina University, Greenville, North Carolina, USA and* [2]*Department of Biology, Nanjing University, Nanjing, Jiangsu, China*

ABSTRACT

Background: In two stream-resident populations of threespine stickleback (*Gasterosteus aculeatus*), females often exhibit male-typical red throat coloration. These fish inhabit the Little Campbell River (British Columbia) and Matadero Creek (California). An anadromous population that lacks such coloration also inhabits the Little Campbell River. Anadromous character states are usually considered ancestral in this system. Theory suggests that ornaments such as red throat coloration can be favoured in some social contexts if they convey information about individual quality. Correlations with a second carotenoid-based ornament, red pelvic spine coloration, may also affect the information conveyed by throat colour.

Question: Within and between populations, how is red throat coloration in females associated with the quality/fitness-related traits condition, body size, age, and growth, and with red pelvic spine coloration?

Methods: In 2010–2012, we measured female throat coloration and evaluated its relationships with condition, body size, age, growth, and pelvic spine coloration.

Results: Throat red intensity was positively correlated with body size in both stream-resident populations. Analyses of one stream population suggest the most intensely red females grow fastest, but older individuals also exhibit more intense throat coloration. We did not observe correlations between throat red intensity and body condition. Red spine coloration was often positively correlated with both throat colour and body size within stream populations. In contrast, the transition from putatively ancestral anadromous character states to derived stream-resident states involved a reduction in spine red intensity, but an increase in throat coloration.

Keywords: colour, condition dependence, female ornament, *Gasterosteus aculeatus*, natural selection, sexual selection, threespine stickleback.

*These authors contributed equally to this work.

Correspondence: J.S. McKinnon, Department of Biology and Center for Biodiversity, East Carolina University, Greenville, NC 27858, USA. e-mail: mckinnonj@ecu.edu

INTRODUCTION

The evolution of conspicuous female ornaments has become an increasingly important and contentious topic for evolutionary biologists (Darwin, 1871; Andersson, 1994; Amundsen, 2000; Clutton-Brock, 2009; Nordeide *et al.*, 2012). Indeed, mounting evidence has shown that female ornaments – either male-typical or specific in form to females – abound in many taxa and in some cases are as conspicuous as in males, or more so (Amundsen, 2000; Amundsen and Forsgren, 2001; Weiss, 2006; Baldauf *et al.*, 2011, Prudic *et al.*, 2011; Tobias *et al.*, 2012). However, in contrast to males, in which conspicuous ornamental traits are overwhelmingly attributed to sexual selection, explanations for the presence and evolution of female ornaments have included both adaptive and non-adaptive hypotheses. The main adaptive hypotheses are female–female competition ['social selection' (West-Eberhard, 1983; Baldauf *et al.*, 2011; Tobias *et al.*, 2012)] and male mate choice (Amundsen and Forsgren, 2001; Chenoweth *et al.*, 2007). Alternatively, the main non-adaptive hypothesis is based on a genetic correlation between the sexes in expression of the trait, combined with sexual selection on the male trait (Lande, 1980; Amundsen, 2000; Clutton-Brock, 2009; Cardoso and Mota, 2010). Although relevant studies are now accumulating, results are mixed, and additional studies are warranted.

Correlations between female ornaments and female condition or components of fitness may shed light on their potential role as signals of mate quality (Weiss, 2006) and may also indicate the extent to which they have been subjected to strong selection, especially sexual selection (Amundsen and Forsgren, 2001; Boughman, 2007). Studies of birds and fish have shown that variation in a female ornament may reflect aspects of phenotypic or genotypic quality (Amundsen *et al.*, 1997; Amundsen, 2000; Amundsen and Forsgren, 2001; Weiss, 2006; Kraaijeveld *et al.*, 2007; Simons *et al.*, 2012). For instance, redder bills in female zebra finches (*Taeniopygia guttata*) have been found to correlate with increased survival and greater reproductive output (Simons *et al.*, 2012). Among fishes, female two-spotted gobies (*Gobiusculus flavescens*) express vibrant, carotenoid-rich eggs through pigmented but somewhat translucent skin, which may advertise reproductive quality to males (Amundsen and Forsgren, 2001; Svensson *et al.*, 2005, 2009). These studies suggest that female ornaments can be reliable indices of phenotypic quality in relation to reproductive fitness, and may evolve condition dependence under inter-sexual selection.

The relationships between different colour-based ornaments may be important in elucidating how ornaments evolve and co-evolve, and the information they convey (Moller and Pomiankowski, 1993; Candolin, 2003; Bro-Jorgensen, 2010). Multiple ornaments may separately convey different messages or altogether signal one message to the receiver, such as different aspects of condition or overall condition of an individual, respectively. Different signals may also be more effective in different contexts/environments. Where different ornaments share a common pigment such as carotenoids, negative correlations among elements may indicate that the pigment could be limiting and traded off among colour patches or other ornaments (Nordeide *et al.*, 2006, 2012; Svensson and Wong, 2011; Hill and Johnson, 2012). Alternatively, positive correlations may suggest that individuals simply vary in their availability of pigments for allocation to ornaments; or there may be no relationship among different ornate colour patches, which simply evolve independently (Moller and Pomiankowski, 1993; Grether *et al.*, 2004).

Threespine stickleback fish (*Gasterosteus aculeatus*) exhibit striking sexual pigmentation that often diverges among populations. This diversity has contributed to their emergence as one of the best studied model organisms in behaviour, ecology, evolution, and more recently evolutionary genetics (Bell and Foster, 1994; Boughman, 2001; Peichel *et al.*, 2001; McKinnon and Rundle, 2002;

Kingsley *et al.*, 2004; Cresko *et al.*, 2007). Sexual dichromatism is a widely observed attribute of sticklebacks, with males typically expressing orange-red nuptial throat coloration (Pelkwijk and Tinbergen, 1937; Milinski and Bakker, 1990; Boughman, 2001). However, this male-typical coloration has also been documented in females of some freshwater populations, in which it is likely a derived state because the trait is overwhelmingly lacking in the ancestral anadromous and marine populations (von Hippel, 1999; McKinnon *et al.*, 2000; but we have recently learned of an apparent exception: J. Willacker and F. von Hippel, personal communication, 2012). To date, the evolutionary processes responsible for red female throats remain unclear.

Throat coloration may be an indicator of female quality, as this ornament has previously been linked with larger body size in females (McKinnon *et al.*, 2000). Large body size may be advantageous for females, and can have positive impacts on survival and reproductive success, such as overwintering survival (Sogard, 1997) and fecundity (Wootton, 1973), respectively. In taxa in which male mate choice is present, including fishes, males often prefer female ornaments that are correlated with indices of female fecundity, like body size (Herdman *et al.*, 2004; Byrne and Rice, 2006; McKinnon *et al.*, 2012). Alternatively, larger size could have costs, such that large individuals may be more visible to predators and/or require more resources to support their body (Blanckenhorn, 2000). Other findings suggest that, at least in males, a redder throat can be an indicator of better condition and lower parasite load, raising the possibility that the female ornament provides some analogous information (Milinski and Bakker, 1990; Barber *et al.*, 2000; Boughman, 2007). Unlike dorsal mottling, female throat intensity does not seem to be linked with readiness to spawn (Rowland *et al.*, 1991; von Hippel, 1999; McKinnon *et al.*, 2000). Because only male sticklebacks care for eggs after they are laid (Wootton, 1984; but see Blouw, 1996), it would make sense that males should at least sometimes be choosy, and preferences for dorsal mottling, large body size, and other traits have been documented (Rowland, 1989; Rowland *et al.*, 1991; McLennan, 1995; McKinnon *et al.*, 2012). However, findings concerning possible male preference with regard to female throat colour have yet to be presented.

In addition to the red throat, male and female sticklebacks sometimes have orange-red coloration on the pelvic spines. Studying the pelvic spine colour patch in female sticklebacks, Nordeide *et al.* (2006) found a negative relationship between red intensity and one (possible) aspect of reproductive quality, carotenoid allocation to eggs. This may explain why males in the same population preferred to court females with drab spines (Nordeide, 2002). The trade-off between ornaments and eggs suggests that the female ornament is not a signal of quality, but may instead be a non-adaptive by-product of a genetic correlation with, and selection for, red intensity in males (Nordeide *et al.*, 2006). This study is noteworthy because although the pelvic spines of sticklebacks have been extensively studied (Reimchen and Nosil, 2004; Shapiro *et al.*, 2004, 2006; Chan *et al.*, 2010), spine colour patterns have rarely been assessed (but see Hodgson *et al.*, 2013). Indeed, we know surprisingly little about the evolution of spine colour, given the long history of study of threespine stickleback colour patterns (Bakker, 1993; McLennan, 2007). In females with red throat coloration, red spine colour is also important to study since correlations between different colourful ornaments that share the same pigment basis have not been assessed in sticklebacks, to our knowledge, and minimally in other taxa; yet such correlations may play an important role in the evolution of both colour patches.

The principal goal of the present study is to investigate whether orange-red throats in female sticklebacks are associated with components of female fitness and/or indicators of quality, including body size, growth rate, condition, and age. A second objective is to evaluate whether, and how, throat intensity is correlated with intensity of a previously

described female ornament, red spine coloration (Nordeide, 2002), both between and within populations. While stream-resident females were the focus of the investigation, we also included an anadromous population and stream-resident males for comparative purposes.

METHODS

Fish collection and maintenance

Using minnow traps and seine nets, male and female stream-resident sticklebacks were collected during the breeding season from two creeks, the Little Campbell (LC: British Columbia, Canada, 49.0321N, 122.657W) and Matadero (MAT: California, USA, 37.393N, 122.162W). LC stream stickleback were sampled in late March/April in 2010–2012, whereas LC anadromous fish were sampled downstream (49.016N, 122.779W) in June 2011 (Hagen, 1967). MAT stickleback were sampled June–July in 2010 and 2012. All females were sampled so as to maximize variation in female body size and red throat coloration among retained fish.

To examine components of female quality/fitness in relation to female red throats, we used two types of collection: 'captive', which included LC and MAT stream and LC anadromous ecotypes, and 'field', which only included LC stream fish. 'Captive' fish were transported to our aquatic facility and held under natural spectrum-mimicking fluorescent light (Lumichrome® Full Spectrum Plus, Lumiram Electric Co., Larchmont, NY, USA) and photoperiod at 17–20°C. They were kept in 102-litre tanks at an approximate density of 15 fish per tank, and fed bloodworms (chironomid larvae) and brine shrimp twice a day. They were allowed to acclimate to laboratory conditions for 2 weeks prior to any measurements. 'Field' fish were euthanized upon capture with a lethal dose of MS-222, frozen in liquid nitrogen, and shipped overnight on dry ice to our laboratory for further analysis. Samples are summarized in Table 1. All animal procedures were approved by the East Carolina University Animal Care and Use Committee (Protocol AUP D#224a).

Table 1. Collection type, population, collection year, and sample size, by sex

				Sex	
Collection	Population	Ecotype	Year	M	F
Captive	Little Campbell	Anadromous	2011	N.A.	40
		Stream	2010	30	155
		Stream	2011	32	140
	Matadero	Stream	2010	14	55
		Stream	2012	9	45
Field	Little Campbell	Stream	2010	—	36
		Stream	2011	—	16
		Stream	2012	—	30

Red throat chroma and measures of female quality/fitness

Although captive fish were primarily collected for behavioural studies (D.S. Wright *et al.*, in preparation; L. Yong *et al.*, in preparation), we were able to collect data for the present study alongside the behavioural work. Females were measured for standard length, body mass, red throat reflectance, and assessed for reproductive status. Fish were photographed under standardized conditions without being sedated. LC males (allowed to build nests) and MAT males (non-nesting) were also included for comparison. To quantify the red coloration of the throat and spine, we used two methods: reflectance spectrophotometry and Adobe Photoshop with digital photographs, respectively (see 'Spectrophotometry-based measurement of red throat chroma' and 'Photoshop measurements of red spine chroma').

Within the LC captive collection, we used two distinct sub-samples of females to determine whether variation in red throat coloration was associated with body condition and reproductive readiness. For the first sample ($n = 29$), we estimated body condition by calculating the residuals of a regression of the natural log ($\log_e$) of body mass without eggs (BM_{log}) on log standard length (SL_{log}), controlled for year of collection (Jakob *et al.*, 1996; Boughman, 2007). In the second sample ($n = 36$), females were monitored over a short period (approximately 10–15 days) and colour-assayed twice, both at the non-gravid and ovulated stages. The order in which throat colour measurements were taken (i.e. ovulated or non-gravid first) was randomized and approximately balanced.

Field-collected females were thawed in the laboratory, and reflectance measurements of their throats were taken (Table 1, see 'Field' section). Although we did not measure the colour in field-collected fish prior to freezing to compare pre- and post-freezing colours, previous studies have shown that carotenoid pigmentation and concentration in the tissues of salmonids remain relatively stable for at least 6 weeks after being frozen at −80°C (No and Storebakken, 1991; Sheehan *et al.*, 1998). For further validation, we measured the throat reflectance of captive fish before (while alive) and after freezing (post-mortem) under similar conditions (e.g. −80°C), which revealed that throat coloration remained stable, with no significant differences resulting from freezing (paired t-test: $t_{16} = 1.492$, $P = 0.1551$; $r = 0.84$, $P < 0.0001$). We also measured standard length and body mass (after egg-stripping), and extracted and measured otoliths to calculate estimates of age and indices of growth (see 'Otolith preparation and measurements').

Photography

For each fish, we photographed the throat and left spine. For the throat, fish were placed on a sponge with their ventral side up. To make sure that the whole throat area was included, an area extending from the tip of the mouth to the pelvic girdle was photographed. For the spine photo, fish were positioned with their spine extended, with the spine set facing and parallel to the camera. All fish were photographed against a grey card (18%) background. The field collection was photographed using a Nikon D50 camera and speedlight flash (SB600: Nikon Inc.); the captive collection was photographed with a Canon Powershot A1100IS (Canon USA Inc., Lake Success, NY, USA) mounted on a copy stand with daylight-mimicking Solux Halogen lamps (Tailor Lighting Inc., Rochester, NY, USA) angled at 45°.

Spectrophotometry-based measurement of red throat chroma

Reflectance spectra of female red throats were measured using a Maya 2000 spectrometer (Ocean Optics Inc., Dunedine, FL, USA) coupled with a broad-spectrum illumination source (Newport Co., Irvine, CA, USA). To capture throat reflectance, a fibre-optic probe attached to the spectrometer was fitted into a Coastal Optics® 60 mm Macro lens through a macro tube extension, which was mounted on a tripod at a 90° angle and stabilized 13 cm above the fish. Fish were placed under the lens on a sponge with their ventral side up, and the light source was aimed at a 45° angle. All reflectance measurements were standardized with a white standard, Spectralon™, and were taken from 2–3 throat spots, approximately 0.8 mm in diameter, deliberately chosen to yield maximum red throat chroma. Measurements covered the UV and visible spectrum range at 0.24 nm wavelength intervals and were recorded using Spectrasuite (Ocean Optics, Dunedine, FL, USA). Spectral data were averaged across 3 nm intervals over 349–703 nm, and these averages were used in subsequent calculations.

To quantify female red throat coloration (hereafter 'throat chroma') or the spectral purity or saturation of the colour patch, a physiological model of stickleback vision was used to approximate stickleback-specific colour perception (Rush *et al.*, 2003; Endler and Mielke, 2005; Pike *et al.*, 2011; Rick *et al.*, 2011). This approach allowed us to estimate red colour intensity based on differences in the relative stimulation of cones in the stickleback eye. We used the spectral sensitivity curves for each type of stickleback cone – ultraviolet (UV), short (S), medium (M), and long (L) wavelength – calculated using an established nomogram (Rowe *et al.*, 2004; Rush *et al.*, 2003). These sensitivity curves were strongly correlated ($r = 0.998$) with those described in Pike *et al.* (2011).

We calculated absolute quantum catches for each individual by multiplying the reflectances by cone-specific spectral sensitivities at specific wavelengths (349–703 nm) and summing across wavelengths for each cone. Although some studies have factored in the constant standard illuminant D65 as a measure of ambient irradiant spectrum, we omitted this value in our calculations because our final measures of throat chroma intensity with and without the ambient irradiance were very highly correlated ($r = 0.997$). Then, we divided cone-specific absolute quantum values by the sum of all absolute quantum catches to obtain a relative quantum catch value for each cone ($Q_{\mathbf{Relat.\ UV}}$, $Q_{\mathbf{Relat.\ S}}$, $Q_{\mathbf{Relat.\ M}}$, and $Q_{\mathbf{Relat.\ L}}$). The relative quantum catch values of all cones were used to calculate Cartesian coordinates, x, y, and z, with which maximum throat chroma intensity was calculated based on the Euclidean distance from the achromatic centre in a tetrahedral colour space (Endler and Mielke, 2005; Pike *et al.*, 2011). We used the maximum value obtained from the 2–3 spots assayed for each fish as the 'throat chroma' value. The use of maximum values in this context is a longstanding practice in studies of stickleback coloration (e.g. Bakker, 1993; Frischknecht, 1993; McKinnon *et al.*, 2000; Bakker *et al.*, 2006), and maximum values were strongly correlated with average values in our study (e.g. $n = 293$, $r = 0.9524$). Moreover, statistically significant correlations, and other relationships, between chroma and other variables were always in the same direction for average and maximum values in our data sets.

Photoshop measurements of red spine chroma

Spine coloration was assessed from images using Adobe Photoshop CS3 (Adobe Systems, San Jose, CA, USA) approximately following Frischknecht (1993), because spine colour

patches were too small to be captured accurately with the spectrometry system. We previously validated this alternative method by comparing red throat chroma measured with Photoshop and spectrometry. Throat chroma values for the two methods were moderately-to-strongly correlated ($r = 0.64$–0.79), suggesting that this method is valid for assessing spine coloration. All spine coloration measurements were corrected relative to the grey card where the average red (R), blue (B), and green (G) colour values for the grey area were first calculated for each image.

Using Photoshop, the left spine was divided into eight equal, predetermined sections, and the RGB values for each landmark were obtained from the most intense red spot within each. We sectioned the spine relative to its length, from the point where it meets the pelvic girdle to its tip. Individual R, G, and B values were taken for each section, and divided by their respective mean grey value to obtain new standardized RGB (R_{Stand}, G_{Stand}, B_{Stand}) values. Red spine intensity (I_{Red}) for each section was then calculated by dividing R_{Stand} by the sum of R_{Stand}, G_{Stand}, and B_{Stand} (Nordeide *et al.*, 2006). Consistent with spectrophotometry-based analyses, we used the highest I_{Red} as our measure of 'spine chroma' (to differentiate from 'throat chroma').

Otolith preparation and measurements

To evaluate age and growth rate, we measured the seasonal bands and radius of the sagittal otolith of females from the field collection. Sagittal otoliths were first extracted, mounted with glue on a glass slide, ground and polished to expose the core, and examined under a compound microscope (Leica Camera AG, Solms, Germany). Because sizes of the left and right sagittal otolith are strongly correlated, we haphazardly selected one. To estimate the age (years) of the fish, we examined the seasonal bands of the otoliths (Behm *et al.*, 2010), counting the number of white and dark bands from the core to the edge, where a dark band indicated a summer and a white band signified a winter. Thus, from the core, two clear dark seasonal bands separated by a white band would suggest that the fish was at least 1 year old. We then estimated growth rate by first measuring the maximum radius (nm) of the sagittal otolith. To obtain indices of growth rate, we used two subsequent methods. The first (hereafter relative growth), which is commonly used, involved calculating the ratio of fish standard length (mm) to the measured radius of the sagittal otolith (e.g. Behm *et al.*, 2010). The second method was employed to additionally account for the effects of age (indicated by bands) and the time of collection of the fish, and consists of the residuals (hereafter growth residuals) of a fitted model in which standard length was regressed on maximum radius of the sagittal otolith, annual age, and collection year. All main effects and three interaction terms were significant, accounting for 77% of variance (age: $F_{1,72} = 16.9211$, $P = 0.0001$; otolith radius: $F_{1,72} = 12.8663$, $P = 0.0006$; collection year: $F_{2,72} = 15.1206$, $P < 0.0001$; collection year × otolith radius interaction: $F_{2,72} = 4.4547$, $P = 0.0006$; collection year × age interaction: $F_{2,72} = 4.3186$, $P = 0.0169$; otolith radius × age interaction: $F_{2,72} = 4.1086$, $P = 0.0464$). The two methods were significantly correlated ($r = 0.60$, $P < 0.0001$).

Statistical analyses

We analysed captive and field collections separately to control for differences in fish handling and methods. For the captive fish collection, we combined samples within each population to test for general population and sex differences in body size, red throat

chroma, and spine chroma using one- and two-way analyses of variance (ANOVAs). Non-parametric tests (i.e. Kruskal-Wallis and Wilcoxon) were used on data that did not show homogeneity of variance. We used *t*-tests (or Wilcoxon for non-parametric tests) with sequential Bonferroni correction for multiple pairwise comparisons on a data set (Rice, 1989). We report the uncorrected *P*-values for pairwise comparisons within ANOVAs; all those reported as significant remain so after Bonferroni correction. We used generalized linear models, univariate and multivariate, to test whether female throat chroma was associated with body size, body condition, and spine chroma, controlling where appropriate for year effects. Unless noted otherwise, non-significant interaction terms were removed from the final multifactor analyses presented here.

RESULTS

Captive fish: patterns in body size, red throat chroma, spine chroma, and dimorphism among populations

Females of the LC and MAT stream ecotypes were both significantly smaller than females of the anadromous ecotype, and LC females were larger than MAT females (ANOVA: $F_{2,432} = 192.77$, $P < 0.0001$; $P < 0.0001$ for all pairwise comparisons; Table 2). Among the stream populations, LC sticklebacks were generally bigger, and females were larger than males (two-way ANOVA, population: $F_{1,475} = 397.1599$, $P < 0.0001$; sex: $F_{1,475} = 51.9271$, $P < 0.0001$). Although data on LC anadromous males are not presented here, females are known to be larger than males in that population as well (Kitano *et al.*, 2011).

Testing for differences in red throat chroma between female populations revealed that both LC and MAT females had higher throat chroma than anadromous females (ANOVA: $F_{2,434} = 80.90$, $P < 0.0001$; $P < 0.0001$ for both pairwise comparisons; Fig. 1). For stream populations, MAT sticklebacks had overall more intense red throat chroma, and the chroma was higher in males than in females (two-way ANOVA, population: $F_{1,475} = 6.7531$, $P = 0.0096$; sex: $F_{1,475} = 157.8328$, $P < 0.0001$; population × sex interaction: $F_{1,475} = 8.0035$, $P = 0.0049$); however, male and female chroma exhibited substantial overlap. As suggested by the interaction term, patterns varied with sex: MAT females exhibited higher red throat chroma than LC females, despite similar throat chroma between corresponding males (*t*-tests, females: $t_{393} = 6.334$, $P < 0.0001$; males: $t_{82} = -0.13648$, $P = 0.8918$).

There were significant differences in mean spine chroma between females of the different populations (Kruskal Wallis, $\chi^2 = 101.79$, $P < 0.0001$, Fig. 2). In contrast to the pattern for throat chroma, mean spine chroma was higher in LC anadromous females than in both LC and MAT females (Wilcoxon test: $P < 0.0001$ for both pairwise comparisons), but no differences were detected between the two stream populations ($P = 0.1883$). For just stream

Table 2. Body sizes [standard length (mm) ± S.E.] by population and sex in the captive collection

Sex	LC stream	MAT stream	LC anadromous
F	58.60 ± 0.38	46.20 ± 0.49	62.44 ± 0.29
M	53.05 ± 0.45	43.08 ± 0.58	N.A.

Note: LC = Little Campbell, MAT = Matadero.

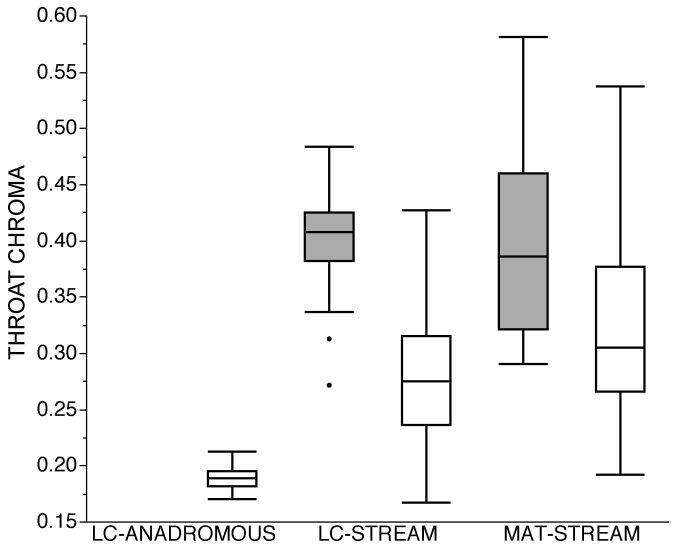
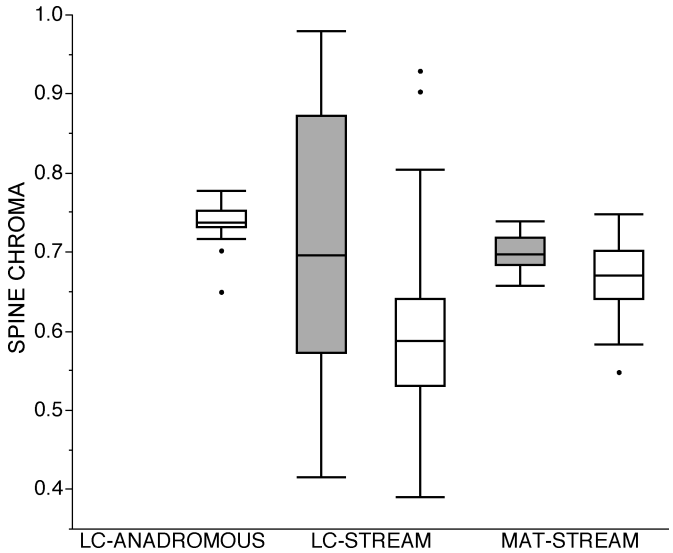


Fig. 1. Boxplots of throat chroma variation as a function of population for males (grey boxes) and females (open boxes; no data for LC anadromous males). Boxes include 50% of the data (first and third quartiles); central horizontal lines (second quartile) represent medians, and the ends of whiskers represent the minimum and maximum values. Data points outside the whisker range are statistical outliers and are represented by solid circles. LC-ANADROMOUS = Little Campbell anadromous; LC-STREAM = Little Campbell stream; MAT-STREAM = Matadero stream.



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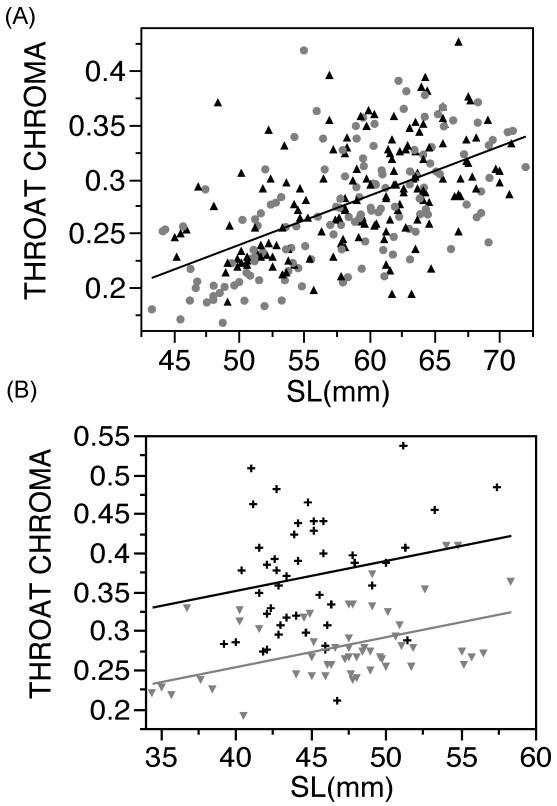

Fig. 3. Relationship between body size and red throat chroma in captive (A) Little Campbell and (B) Matadero females. In the Little Campbell (A), black triangles represent the 2010 collection, whereas grey circles represent the 2011 collection, with no differences between years. In the Matadero (B), the grey inverted triangles and regression line are for the 2010 collection, whereas the crosshairs and dark line are for 2012. SL = standard length.

populations, mean spine chroma was higher in males than in females (Wilcoxon test, LC: $\chi^2 = 31.229$, $P < 0.0001$; MAT: $\chi^2 = 6.1396$, $P = 0.0132$). Overall, sexual dichromatism in spine chroma appeared less pronounced than for throat chroma.

Captive fish: relationships between female red throat chroma and indicators of phenotypic quality/fitness, within stream-resident populations

In both LC and MAT (stream-resident) populations, female body size and red throat chroma were positively associated, such that large females had higher red throat chroma (LC, body size: $F_{1,292} = 47.06$, $P < 0.0001$; year: $F_{1,292} = 2.50$, $P = 0.1147$; MAT, body size: $F_{1,97} = 3.19$, $P = 0.0019$; year: $F_{1,97} = 68.56$, $P < 0.0001$) (Fig. 3A and B, respectively). As confirmed by the significant year effect, female throat chroma was more intense in 2012 in the MAT population.

Using a subsample from the LC collection, we found no relationship between red throat chroma and body condition ($r^2 = 0.00$, $n = 29$, $P = 0.9881$; condition data available only for LC fish).

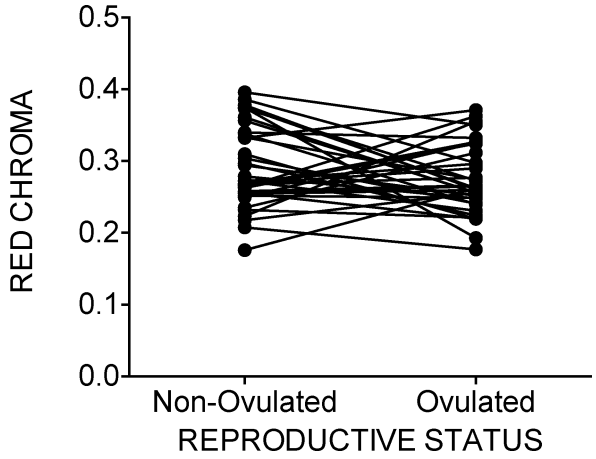

Fig. 4. Throat chroma differences between ovulated and non-ovulated stages in captive LC females.

In a separate LC sample, females were assayed repeatedly when ovulated or not ovulated. Throat chroma did not vary significantly based on ovulation (paired *t*-test: $t_{35} = -1.59$, $P = 0.1198$, $n = 36$; Fig. 4).

Captive fish: relationship between throat chroma and spine chroma

In the LC population, females with higher chroma throats also had more intensely red spines, with no significant differences between years of collection (throat chroma: $F_{1,292} = 16.67$, $P < 0.0001$; year: $F_{1,292} = 1.06$, $P = 0.3039$; Fig. 5A). Body size was also associated with spine chroma (body size: $F_{1,291} = 2.47$, $P = 0.0141$; year: $F_{1,291} = 1.87$, $P = 0.1723$). Regressing spine chroma on both body size and throat chroma, only throat chroma was significant (throat chroma: $F_{1,291} = 18.922$, $P < 0.0001$; body size: $F_{1,291} = 1.0234$, $P = 0.3126$; year: $F_{1,291} = 1.0053$, $P = 0.3169$).

In the MAT population, there were significant main effects, and an interaction of female throat chroma and year, on spine chroma (throat chroma: $F_{1,95} = 17.57$, $P < 0.0001$; year: $F_{1,95} = 38.38$, $P < 0.001$; throat chroma × year interaction: $F_{1,95} = 11.42$, $P = 0.0011$; Fig. 5B), and thus we also analysed the 2010 and 2012 data separately. Female throat chroma was only significantly correlated with spine chroma in 2010 (2010: $r^2 = 0.17$, $P = 0.0022$; 2012: $r^2 = 0.001$, $P = 0.7826$). When analysing body size and female throat chroma together for each year, both body size and throat chroma had significant effects on spine chroma in 2010 (body size: $F_{1,50} = 7.4914$, $P = 0.0086$; throat chroma: $F_{1,50} = 4.6778$, $P = 0.0354$). In 2012, body size was marginally associated with spine chroma, whereas throat chroma had no effect (body size: $F_{1,41} = 3.9680$, $P = 0.0531$; throat chroma: $F_{1,41} = 0.0003$, $P = 0.9858$).

LC field collection: relationships between female throat chroma, body size, condition, and spine chroma

In the field collection, female throat chroma was again associated with body size, with significant variation in throat chroma also accounted for by year of collection (body size: $F_{1,78} = 24.70$, $P < 0.0001$; year: $F_{2,78} = 7.82$, $P = 0.0008$). Similar to captive laboratory fish, throat chroma was not indicative of body condition (throat chroma: $F_{1,68} = 1.165$, $P = 0.2841$; year: $F_{2,68} = 0.1592$, $P = 0.8532$; Fig. 6). Also as in the previous data sets, female

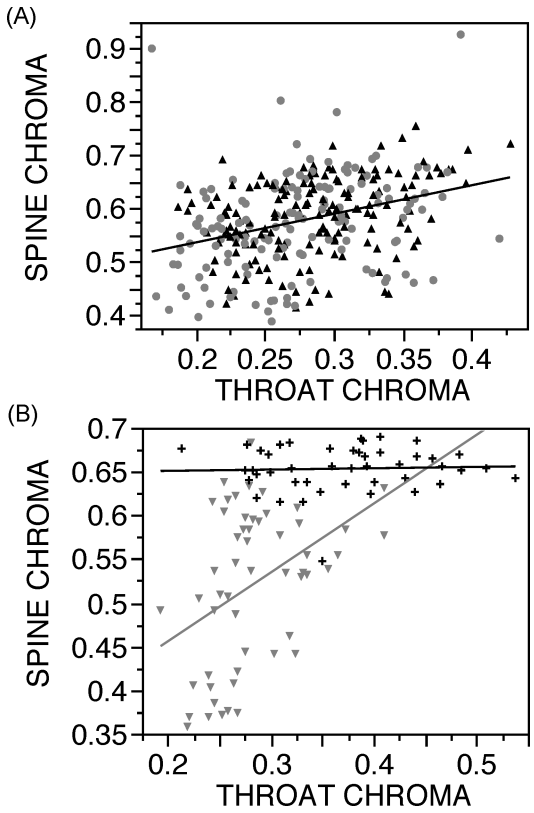

Fig. 5. Relationship between female throat chroma and spine chroma in captive (A) Little Campbell and (B) Matadero females. In the Little Campbell (A), black triangles represent 2010, whereas grey circles represent 2011, with no significant differences between years. In the Matadero (B), the inverted grey triangles and regression line correspond to the 2010 collection, whereas the crosshairs and black line are for 2012.

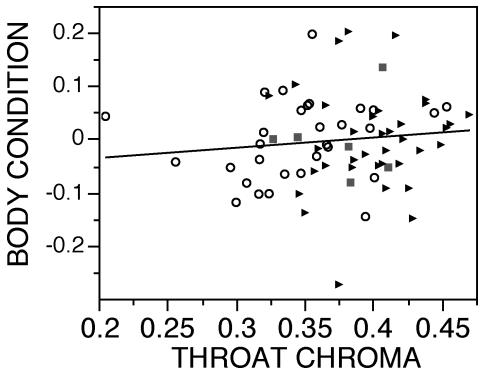

Fig. 6. Relationship (non-significant) between throat chroma and body condition in field-collected females. Each symbol type represents a distinct collection (black triangles = 2010; black squares = 2011; open circles = 2012).

throat chroma was positively, but not significantly, correlated with spine chroma (throat chroma: $F_{1,78} = 1.2714$, $P = 0.2630$; year: $F_{2,78} = 52.5702$, $P < 0.0001$), whereas body size was significantly associated with spine chroma (body size: $F_{1,78} = 4.3620$, $P = 0.040$; year: $F_{2,78} = 65.8673$, $P < 0.0001$).

LC field collection: relationships between female throat chroma, age, and growth

To test whether age was associated with red throat chroma and might contribute to the relationship between throat chroma and body size, we regressed throat chroma on age and the covariate collection year, revealing that 2-year-old females had higher throat chroma, with significant variation between collection years (age: $F_{1,78} = 8.637$, $P = 0.0043$; collection year: $F_{2,78} = 16.1648$, $P < 0.0001$). When body size was added to the model, the effect of age became non-significant ($F_{1,77} = 0.00034$, $P = 0.9536$), whereas both collection year and body size remained significant (collection year: $F_{2,77} = 7.7129$, $P = 0.0009$; body size: $F_{1,77} = 14.284$, $P = 0.0003$), suggesting that body size, rather than age, had a more direct impact on throat chroma.

To test whether growth rate was associated with red throat chroma, we regressed throat chroma on the two measurements of growth rate: relative growth rate and growth residuals. Relative growth rate and collection year had significant effects on throat chroma (relative growth rate: $F_{1,78} = 9.5842$, $P = 0.0027$; collection year: $F_{2,78} = 9.2254$, $P = 0.0003$; Fig. 7). The general pattern is robust, as throat chroma was also positively associated with growth residuals (growth residuals: $F_{1,78} = 5.6028$, $P = 0.0204$; collection year: $F_{2,78} = 21.6590$, $P < 0.0001$), suggesting that females with red throats had a faster growth rate.

DISCUSSION

Before the present study, red female throat coloration had been reported to occur at a substantial frequency in at least two populations of threespine stickleback, but little was known about potentially important correlates of female coloration that have been documented in some other vertebrate systems (Amundsen *et al.*, 1997; Amundsen, 2000; Amundsen and Forsgren, 2001; Weiss, 2006; Kraaijeveld *et al.*, 2007; Doutrelant *et al.*, 2008). Here we confirm the positive correlation between throat chroma and body size for the Little Campbell stream-resident

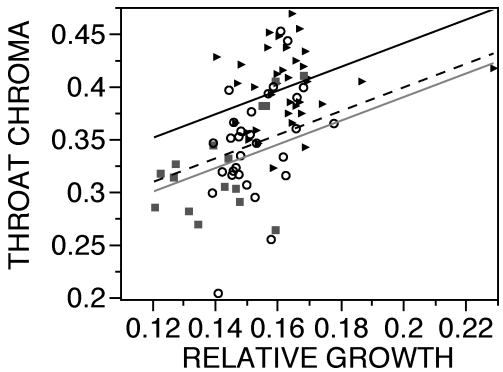

Fig. 7. Relationship between relative growth rate and throat chroma. Different lines and symbols represent distinct collections (black triangles and solid dark line = 2010; black squares and solid grey line = 2011; open circles and dashed black line = 2012).

population and document a similar pattern, although somewhat inconsistent, in a population from Matadero Creek, California. Female ornaments do not covary with a standard measure of body condition, which may indicate weak sexual selection on female throat colour; however, analyses using otoliths as indicators of age suggest that the body size–throat chroma relationship can be partly attributed to a rapid growth rate in more intensely coloured individuals. Within populations, spine chroma was positively correlated (although sometimes not significantly) with female throat colour as well as with body size – but at a macroevolutionary level the pattern was reversed between the anadromous ancestral state and derived stream states.

The body size–throat chroma relationship was previously described by McKinnon *et al.* (2000), but was not significant for all the data sets considered there and the spectrophotometry measures in that study were exclusively from the human visible spectrum. The current analyses reveal that the relationship between the two traits is indeed present in both stream populations, although again somewhat inconsistently. It is potentially mediated in part by an increased growth rate in females with higher throat chroma, although older females are also more intensely coloured. At present we can only speculate as to why red-throated females would be the fastest growing. It is possible that females with superior underlying vigour and health may have sufficient resources to both grow fast and invest in red throat ornamentation, much as in condition-dependent models (Blount *et al.*, 2000; Hill and Johnson, 2012). Alternatively, those individuals who have experienced a more carotenoid-rich diet, whether owing to chance, preference, or foraging ability, may both grow faster and develop more colourful traits (Craig and Foote, 2001; Ohlsson *et al.*, 2002; Karino and Haijima, 2004; Svensson and Wong, 2011). Biard *et al.* (2006) showed that supplementing carotenoids in the diets of blue tits resulted in larger growth and body size in offspring. The same effect was found in salmonids (Torrisen, 1984).

Whatever the underlying cause, the positive relationships between throat chroma, body size, and growth suggest that throat colour may provide information to conspecifics, or reinforce that provided by body size. Female throat coloration could advertise viability and enhanced quality/fitness, which may be important in the contexts of both intra- and inter-sexual encounters. Because larger individuals are often more dominant and successful in territorial interactions (e.g. Aubin-Horth *et al.*, 2007), displaying a correlated conspicuous trait may be advantageous for deterring rivals and acquiring mates and resources, especially since red throat coloration has been associated with dominance in males (Bakker and Sevenster, 1983). In addition, through its relationship with body size, the female red throat could advertise fecundity, a trait often preferred by males (Wootton, 1973; Pélabon *et al.*, 2003). Previous studies have shown that males often prefer large females because such females usually have larger clutch sizes and in some studies heavier eggs (Côte and Hunte, 1989; Rowland, 1989; Kraak and Bakker, 1998; Dosen and Montgomerie, 2004). Considering the throat chroma–body size relationship, it might be the case that red throat coloration acts as an indirect signal of size or acts in concert with body size to indicate reproductive output of females.

Spine colour was also correlated with body size and red throat chroma within most samples, but patterns varied across populations and years. Overall, the positive relationship between throat and spine colour makes intuitive sense, since both ornaments are composed of carotenoids (Wedekind *et al.*, 1998; Nordeide *et al.*, 2006). This finding is nevertheless noteworthy because carotenoids are often suggested to be rare and limiting, and their differential allocation between functions can involve trade-offs, especially in females (Fitzpatrick *et al.*, 1994; Blount *et al.*, 2000). Also studying sticklebacks, Nordeide *et al.* (2006) found that females with

redder spines have less carotenoid-rich eggs, which may influence males to prefer females with drab spines (Nordeide, 2002). Similarly, ornamented females in Arctic charr tend to produce offspring of lower viability (Janhunen *et al.*, 2011). However, the trade-off hypothesis rests on the assumption that carotenoids are in chronically short supply (Svensson and Wong, 2011). Thus, the presence of a positive relationship between the two ornaments may mean: (1) only high-quality females (or those with high carotenoid availability) can allocate carotenoid resources to both ornaments without impairing other physiological functions, suggesting that the ornaments are condition-dependent, or (2) trade-offs may be present, but may involve carotenoid allocation elsewhere, such as to eggs (Nordeide *et al.*, 2012). Based on our field samples, the lack of condition dependence of female ornament expression in the present study does not support the first interpretation. However, it is possible that a carotenoid-rich environment, perhaps available only to some individuals, could facilitate the simultaneously strong expression of female throat and spine coloration in some individuals. The substantial between-year variation in coloration for some of our data sets is arguably consistent with such an interpretation. We will present findings on carotenoid allocation to eggs and colour pattern elements elsewhere.

Female sexual ornaments have sometimes been implicated in signalling reproductive readiness (Amundsen, 2000; Amundsen and Forsgren, 2001; Weiss, 2006; Doutrelant *et al.*, 2008). However, female red throat intensity does not appear to play a role in signalling readiness to spawn in the LC population. We observed no differences in throat chroma between ovulated and non-ovulated females, confirming the findings of McKinnon *et al.* (2000).

The lack of association between female throat chroma and body condition suggests that the role of sexual selection in the evolution of the female ornament may be limited in the LC population. In another stickleback study, Boughman (2007) argued that variation in condition dependence in sexual traits can be used as a 'signature' of how sexual selection may be acting, such that a positive relationship between condition and a sexual trait would imply that sexual selection on the trait is strong. In that study, male red throat intensity in limnetic sticklebacks was strongly sexually selected and exhibited a strong relationship with body condition. Thus, the lack of association between female ornaments and condition dependence in our study suggests that sexual selection on the ornaments may be weak, as found in males of benthic and anadromous stickleback populations (Boughman, 2007). Although it is possible that being held in a laboratory setting could have affected the body condition of captive LC fish, the fact that condition and throat chroma were also not associated in field-collected fish suggests that the absence of a throat chroma–condition relationship is likely real.

Condition can be defined as a pool of resources that are used to maintain traits that enhance fitness and thus could be depleted because of their allocation to one trait at the cost of others (Rowe and Houle, 1996). In this study, therefore, condition could be reflected in body size rather than in body condition, considering that throat chroma is correlated with body size but not with body condition. Frischknecht (1993) showed that a negative relationship between changes in condition and body size in sticklebacks may be due to how energy is allocated between growth and condition – and the optimal allocation may depend on gender and context. From this perspective, female throat chroma could very well be condition-dependent.

The red throat and reduced spine chroma in the stream female populations are likely derived traits because anadromous females tend to have inconspicuous throats but more intense spine colour. This reversal of the relationship found within the anadromous ecotype

is particularly interesting because evolution occurred in different directions for the two traits during the transition to freshwater. Increased throat chroma and decreased spine chroma beg the question of whether there could have been a carotenoid trade-off, at the macroevolutionary scale, between spine and throat during adaptation to freshwater. Here it is important to emphasize that we know very little about the function of coloration on spines, for example whether it might be positively sexually selected in some circumstances or might serve to enhance spine conspicuousness to predators and deter predation. The intriguing correlations between throat and spine coloration both within and between populations highlight the importance of further study of the evolution of spine coloration and its co-evolution with throat pigmentation, for male as well as female stickleback (see also Hodgson *et al.*, 2013).

In conclusion, our findings suggest that female red throat coloration may be more common in stickleback than previously appreciated, and extend earlier work showing that the trait is not unique to males (von Hippel, 1999; McKinnon *et al.*, 2000). The additional documentation of red-throated females in the Matadero population suggests that the trait may be more common in stream-resident than other populations and has likely evolved repeatedly. However, further surveys of populations in which the female red throat coloration may be present are required to confirm these points. With several potential study populations as well as a complete genome, the threespine stickleback now provides a powerful model for studying longstanding genetic predictions regarding the evolution of female or mutual ornaments and sexual dimorphism (Nordeide *et al.*, 2012). QTL analysis and whole-genome expression have already proven to be successful for studying the genetic basis of many complex traits in stickleback (Peichel *et al.*, 2001; Miller *et al.*, 2007; Chan *et al.*, 2010; Greenwood *et al.*, 2012), and are likely to offer insights on whether mutual ornaments in both sexes are the result of shared or different genetic architectures. Testing these predictions using QTL mapping is currently under way, and will ultimately allow us to uncover the genetic mechanisms responsible for the development and evolution of female red throat coloration in stickleback.

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