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Male red throat coloration, pelvic spine coloration, and courtship behaviours in threespine stickleback

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ABSTRACT

Background: In addition to exhibiting red nuptial throat coloration, male threespine sticklebacks (*Gasterosteus aculeatus*) often possess red coloration on their pelvic spines. Although variation in throat colour has been shown to be associated with male reproductive behaviour, limited research has been devoted to the behavioural correlates of red spine colour. Like the red throat, spine colour might correlate with components of male reproductive behaviour and may also be androgen dependent, with important implications for a potential signalling function.

Hypothesis: Spine coloration, like throat coloration, is correlated with components of courtship behaviour, and also with the fish androgen 11-ketotestosterone (11KT).

Methods: To examine the role of male throat and spine colour expression in a mating context, we measured the behavioural response of nesting males to conspecific females. We analysed the relationships between throat and spine colours with reproductive behaviours as well as with 11KT.

Results: Males with more intensely red throats courted females more vigorously, whereas males with redder spines displayed relatively more aggressive behaviour during courtship. An initial analysis of a subsample of males showed that 11KT was positively associated with spine colour intensity, but not with red throats or any behaviours. Hence throat and spine colour may reflect different components of male reproductive behaviour, and 11KT is a potential mediator of spine coloration in males.

Keywords: aggression, colour, *Gasterosteus aculeatus*, 11-ketotestosterone, pelvic spine, threespine stickleback.

INTRODUCTION

Many animal species use signals, which are traits that have evolved under selection for communicative purposes (Ruxton and Schaefer, 2011), to transmit information to others. Signals

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can be both informative and manipulative [by exerting influence over the behaviour of the receiver (Ruxton and Schaefer, 2011)], and exist in a variety of forms that are used for both intra- and inter-species communication (Johnstone, 1997; Candolin, 2003). Recent work has shown that many organisms possess multiple signals (Higham and Hebets, 2013), which can be used to convey information via separate channels; for example, male frogs produce an audible croak while also visually inflating the throat sack to attract mates (Preininger *et al.*, 2013). Such studies highlight the fact that communication is often multimodal (Higham and Hebets, 2013), requiring a re-examination of now classic examples of animal signalling systems.

Carotenoid-based throat coloration of reproductively active male threespine sticklebacks has been studied extensively and shown to be an important character for female mate preference (ter Pelkwijk and Tinbergen, 1937; Milinski and Bakker, 1990; Bakker and Mundwiler, 1994) and male agonistic behaviour (Rowland, 1994; McKinnon, 1996). The red coloration is suggested to function as a signal during male–male aggressive interactions (ter Pelkwijk and Tinbergen, 1937; Bakker and Milinski, 1993; Rowland, 1994) and male-typical traits are often androgen dependent (Borg, 1994; Mayer *et al.*, 2004). More recently, red throat coloration has been observed in females (von Hippel, 1999; McKinnon *et al.*, 2000), although a functional explanation for its occurrence has not been established (Yong *et al.*, 2013, 2015, 2016; Wright *et al.*, 2015).

Stickleback fishes also possess coloration on their pelvic spines, a trait that has been observed across numerous populations and in both sexes, as well as in closely related species (McLennan, 1993, 1994; Bell and Foster, 1994; von Hippel, 1999; McKinnon *et al.*, 2000; Nordeide, 2002; Nordeide *et al.*, 2006; Hodgson *et al.*, 2013; Yong *et al.*, 2013; Amundsen *et al.*, 2015). Previous work has focused on the behavioural associations of spine display, with spine extension occurring more frequently with aggression and fighting in males (van Iersel, 1953; Symons, 1966), and the use of spines in defence against predation (Hoogland *et al.*, 1956). More recently, work has focused on the reduction/loss of such anti-predator structures (Bell *et al.*, 1993; Klepaker, 1993; Shapiro *et al.*, 2004, 2006; Chan *et al.*, 2010). To date, however, only a few studies have specifically addressed coloration of the pelvic spines. Work in a Norwegian population found that males preferred females with less red pelvic spine coloration (Nordeide, 2002), a trait that is negatively correlated with egg carotenoid levels (Nordeide *et al.*, 2006). Tests of male mate preference in a Canadian population found no evidence of preference for or against female pelvic spine colour (Wright *et al.*, 2015). Recent work by Amundsen and colleagues found that pelvic spine colour is widespread among numerous populations in northwest Europe, occurring in both sexes in all 17 sites sampled (Amundsen *et al.*, 2015). When reared in the laboratory, the authors found only limited heritability of pelvic spine colour (Amundsen *et al.*, 2015), although Yong and colleagues found an overlapping quantitative trait locus (QTL) region that contributes to both spine and throat coloration (Yong *et al.*, 2016). Finally, in brook sticklebacks (*Culaea inconstans*), red pelvic spine coloration was more common in males than females and associated with higher male condition (Hodgson *et al.*, 2013).

If pelvic spine colour is an important sexual signal, it is not unreasonable to suspect involvement of steroid hormones. Previous work has shown that secondary sexual characters of male threespine sticklebacks can be androgen dependent (Wai and Hoar, 1963; Borg, 1994; Borg and Mayer, 1995) and that the most common circulating androgen in breeding males is 11-ketotestosterone (Mayer *et al.*, 1990). 11-Ketotestosterone (11KT) has been implicated as an important mediator of male reproductive behaviour and a key driver of traits such as throat colour and kidney hypertrophy (Borg and Mayer, 1995; Mayer *et al.*, 2004). Should pelvic spine colour be a secondary sexual character, one could expect a similar role of androgens in trait expression (Mank, 2007).

In this study, we further explore the evolutionary correlates of red pelvic spine coloration, as well as throat coloration, using male threespine sticklebacks from a stream-resident population in British Columbia, Canada (McKinnon *et al.*, 2000). We report a novel relationship between the intensity of pelvic spine coloration and male aggressive behaviours during mate choice trials. Additionally, we sample a subset of males for levels of the androgen 11KT and report the correlation with spine coloration. Although the function of pelvic spine coloration has been speculated upon (Rowland, 1994), we are, to our knowledge, the first to report direct behavioural and androgen-based correlations with this trait.

METHODS

Fish collection and mate choice trials

This study is part of a larger assessment of male mate preference for female throat coloration [for a detailed description of experimental procedures, see Wright *et al.* (2015)]. Fish were collected from field sites in British Columbia, Canada (Little Campbell River, BC, Canada: 49.0321N, 122.657W) at the start and at the peak of the breeding season (late March and late May 2010, and April and June 2011) and transported to a climate-controlled facility at East Carolina University, North Carolina. Males demonstrating breeding coloration of blue eyes and red throat were isolated in 102-litre aquaria and stimulated to build nests. With the completion of the nest and the onset of courtship behaviour (zigzagging), males were tested with a series of conspecific gravid females and males (controls). Fish were presented in water-sealed, UV-transparent, Plexiglas containers and lightly anaesthetized with MS-222 to minimize movement and its possible effect on male behaviour (detailed in Wright *et al.*, 2015). Males were scored for throat colour, spine colour, and other traits at the conclusion of each presentation series. MS-222 had no visible effect on throat/spine coloration (Wright *et al.*, 2015), as spine colour does not appear to change quickly in this population, and throat coloration also appears more consistent than in some sticklebacks, especially anadromous fish. This study was performed with approval of the Animal Care and Use Committee of East Carolina University (Animal Use Protocol: AUP #D224).

Throat colour assessment

Reflectance measurements of each fish's throat were taken using an Ocean Optics Maya 2000 spectrometer and SpectraSuite software following procedures previously detailed for this population (Yong *et al.*, 2013, 2015; Wright *et al.*, 2015). Reflectance for one male was not recorded, resulting in a lower sample size for throat colour analyses ($n = 60$).

Spine colour assessment

Pelvic spine colour scores were determined from standardized photographs (Fig. 1) using Adobe Photoshop CS3 as done previously with this population (Yong *et al.*, 2013; Wright *et al.*, 2015). To calculate maximum spine coloration, the left spine was divided into eight equal, pre-determined sections and the RGB values were recorded at the most intense red spot within each section. Individual RGB values were taken for each section, and standardized to an 18% grey card present in all pictures to obtain new, standardized RGB (R_{Stand}, G_{Stand}, B_{Stand})

Fig. 1. Variation in pelvic spine coloration of stream-resident male threespine sticklebacks.

values. Red spine intensity (I_{Red}) for each section was then calculated by dividing $\mathrm{R}_{\mathrm{Stand}}$ by the sum of $\mathrm{R}_{\mathrm{Stand}}$, $\mathrm{G}_{\mathrm{Stand}}$, and $\mathrm{B}_{\mathrm{Stand}}$ (Nordeide *et al.*, 2006; Yong *et al.*, 2013; Amundsen *et al.*, 2015; Wright *et al.*, 2015). The Max I_{Red} score of each male ($n = 61$) was used in all subsequent analyses; average I_{Red} followed the same general patterns.

Hormone sampling and extraction

In a subset of males ($n = 16$), we measured baseline values of 11KT using the waterborne hormone sampling approach (Hirschenhauser *et al.*, 2004; Lorenzi *et al.*, 2006; Sebire *et al.*, 2007, 2009) and tested whether hormone levels were associated with trait colour expression. We favoured waterborne hormone sampling because it provides a non-invasive method to estimate systemic hormone levels, and has been extensively validated and replicated in a variety of fishes, including sticklebacks (Sebire *et al.*, 2007, 2009). These studies have confirmed a positive relationship between waterborne and systemic levels of 11KT in male sticklebacks (Sebire *et al.*, 2007, 2009).

Males were individually placed in a sterile sampling container with 100 mL of pure water for 30 minutes. During the procedure, fish behaved very calmly, exhibited little movement, and remained at the bottom of the container. Fish were sampled opportunistically following the completion of the entire mate choice presentation series (detailed in Wright *et al.*, 2015) so as not to disrupt mate choice trails. After the waterborne sampling, males were gently returned to social housing tanks. All waterborne hormone samples were stored at –20°C until extraction.

Blood- and waterborne hormones were extracted and measured using C18 solid-phase extraction (Sepak cartridges 3 mm, Waters) and enzyme immune assay (EIA) specific to 11KT (Cayman Chemicals) respectively, following protocols thoroughly detailed in Lorenzi *et al.* (2006). The 11KT kit has been previously validated for sticklebacks (Kitano *et al.*, 2011), and was additionally assessed for parallelism of serial dilution curve with the standard curve ($t_{12} = 0.064$, $P = 0.86$). In brief, all samples were first run through the cartridges, whose sorbent packing retained the hormone samples. Hormones were then subsequently eluted with HPLC grade methanol, and the solution was then evaporated using a gentle stream of nitrogen (Evap-ORac, Cole Parmer) or a Speedvac concentrator (Jouan RC 10-10, Thermo Fisher). Dried hormones were reconstituted with the supplied EIA buffer (waterborne hormone pellets in 350 μL). Samples were further diluted to 1:80 to fit on the standard curve. All samples were thoroughly mixed for 1 hour using a multi-tube vortexer, and 50 μL of each sample was plated and run in duplicate followed by acetylcholinesterase tracer and antiserum, in line with the kit's instructions. All plates were placed on an orbital shaker and incubated at 4°C for 18 hours. Post incubation, Ellman's reagent was added to each sample, and the samples were incubated for an additional 90 minutes at 4°C for the assay to develop. Plates were read at 405 nm on a microplate reader (Opsys MR microplate reader, Dynex Technologies, Chantilly, VA). Optical density of each sample was compared to the best standard curve ($r^2 > 0.98$). The cross-reactivities for 11KT were as follows: 11-ketotestosterone (100%), testosterone (<0.01%), adrenosterone (2.9%), 4-androsten-11β, 17β-diol-3-one (0.01%). After accounting for the dilution factors, all sample concentrations were expressed in $\text{pg} \cdot \text{mL}^{-1}$, and subsequently log-transformed for statistical analyses.

Statistical analyses

Principal components analysis (PCA) on the correlation matrix of male response variables was used to obtain a single composite variable of male mating behaviour using 'zigzags', 'bites', 'latency', and 'time in proximity' to female [PCA described in greater detail in Wright *et al.* (2015)]. These four behaviours were chosen to account for the two common male courtship actions, 'zigzags' and 'bites', while also allowing for a measure of interaction time, 'time in proximity' to the female, as well as how quickly the male responded (moved one body length in the direction of the test female), 'latency'. Results of PCA using these four behaviours showed that PC1 loads positively with zigzags, bites, and time in proximity, and negatively with latency (PC1: 51.9% of the variation) and thus serves well as a general measure of male courtship vigour (males respond quickly to introduced females, spend large amounts of time in proximity to females, and perform high numbers of zigzags and bites). PC2 loads positively with bites, latency, and time in proximity, and negatively with zigzags (PC2: 21.8% of the variation) and thus is indicative of a differential male response, perhaps a measure of aggressive behaviour (males respond much slower to introduced females, but then spend a lot of time in proximity to females, while exhibiting a high number of bites and very few zigzags). Both PC1 and PC2 were used in subsequent analyses and supplemented with two key individual behaviours underlying these composite traits (bites and zigzags). Regression analyses were used to analyse the relationship between behaviour, throat and spine colour intensity, and 11KT levels. When applicable, year was included as a covariate to control for potential differences between the 2010 and 2011 fish collections (year was not included in the hormone analyses because all assays were performed in 2011).

RESULTS

Relationship between male traits and courtship behaviour

Multiple regression analysis of male behaviour with male colour traits (throat/spine colour) and year revealed significant relationships between male colour scores and behaviour. Mean male courtship (PC1) was significantly influenced ($R^2 = 0.23$, $F_{3,58} = 5.85$, $P = 0.001$) by throat colour ($\beta = 9.66$, $P = 0.015$; Fig. 2a) and pelvic spine colour ($\beta = 4.63$, $P = 0.022$; Fig. 2b) but not year ($\beta = -0.45$, $P = 0.11$). Multiple regression of mean PC2 scores proved marginally non-significant ($R^2 = 0.103$, $F_{3,58} = 2.22$, $P = 0.095$) but pelvic spine colour did have a significant influence ($\beta = 3.03$, $P = 0.034$), while throat colour ($\beta = -3.51$, $P = 0.20$) and year ($\beta = 0.29$, $P = 0.15$) were non-significant. Mean number of zigzags was significantly influenced ($R^2 = 0.18$, $F_{3,58} = 4.301$, $P = 0.0083$) by male throat colour only ($\beta = 15.53$, $P = 0.0008$), while mean number of bites was significantly influenced ($R^2 = 0.17$, $F_{3,58} = 4.05$,

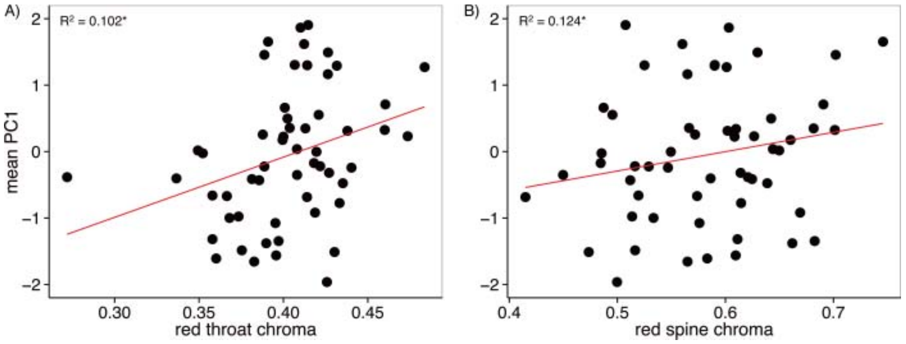

Fig. 2. There was a significant, positive relationship between mean male courtship behaviour, PC1, and (A) red throat and (B) red pelvic spine colour. Statistical significance: *$P < 0.05$.

Table 1. Slope estimates (± standard error) of multiple regression analyses

	PC1	PC2	Zigzags	Bites
Throat colour	9.66 ± 3.89*	−3.51 ± 2.75	15.53 ± 4.43***	6.13 ± 3.87
Pelvic spine colour	4.63 ± 1.98*	3.03 ± 1.39*	−0.73 ± 2.25	5.35 ± 1.96**
Year	−0.45 ± 0.28	0.29 ± 0.20	−0.31 ± 0.32	−0.11 ± 0.28

Statistical significance: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

$P = 0.011$) by pelvic spine colour only ($\beta = 5.35$, $P = 0.008$; Fig. 3b). For both zigzags and bites, all other predictors were non-significant ($P > 0.11$). There was no significant relationship between throat and spine colour ($R^2 = 0.01$, $P = 0.279$), neither score differed between years ($P > 0.145$), and no behaviours were associated with standard length ($P > 0.14$ in all cases). Results of multiple regressions are summarized in Table 1.

Male spine colour intensity is associated with 11KT levels

Males with higher levels of 11KT displayed brighter red spine colour ($R^2 = 0.28$, $F_{1,14} = 5.56$, $P = 0.033$; Fig. 4b), but not more intensely red throats ($R^2 = 0.001$, $F_{1,14} = 0.014$, $P = 0.905$; Fig. 4a). We did not detect any relationships between 11KT levels and measured behaviours ($P > 0.3$ for all behaviours).

DISCUSSION

In this study, male threespine sticklebacks with more intense throat coloration displayed more vigorous courtship behaviour, as indicated by the composite male courtship behaviour (PC1) and zigzags. Red pelvic spine colour was also associated with PC1 scores, but not with zigzags. Instead, pelvic spine colour was the only significant predictor influencing mean PC2 scores. When looking only at the mean number of bites, male red pelvic spine colour was a good indicator, but red throat coloration was not. In the subsample of males analysed for waterborne androgens, baseline values of 11KT were positively associated with red pelvic spine colour, but not male red throats or behaviours.

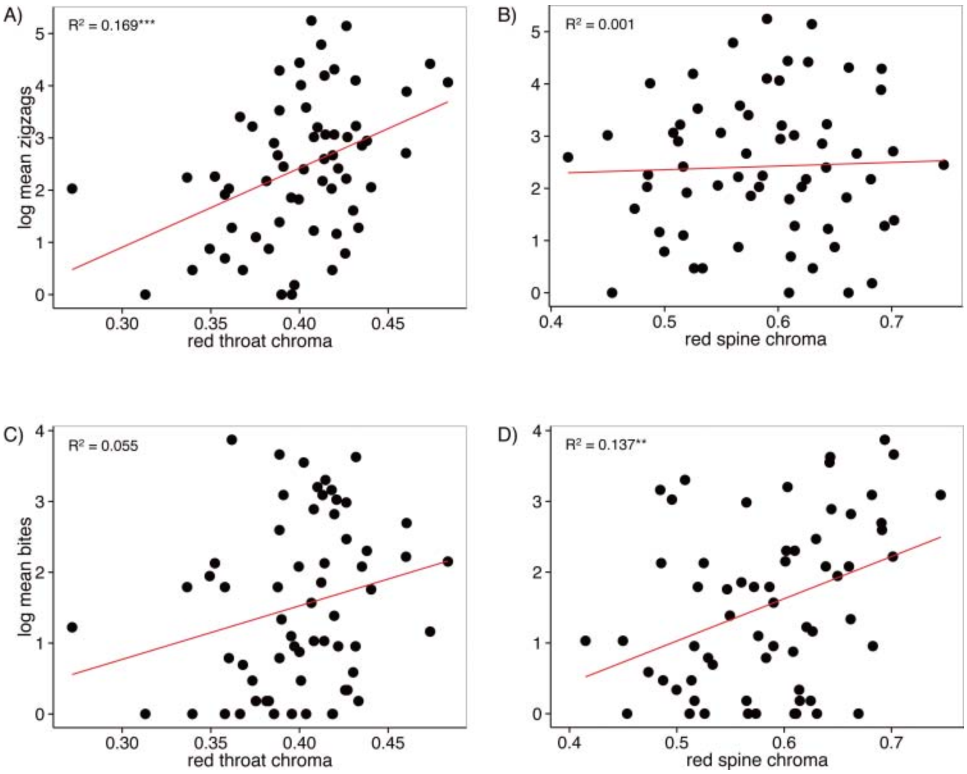

Fig. 3. Linear regression showed a significant positive correlation between the mean number of zigzags and throat colour (A) but not mean number of zigzags and pelvic spine colour (B). The relationship between the mean number of bites and throat colour was not significantly correlated (C) but that between mean number of bites and spine colour was (D). Statistical significance: $^{**}P < 0.01$; $^{***}P < 0.001$.

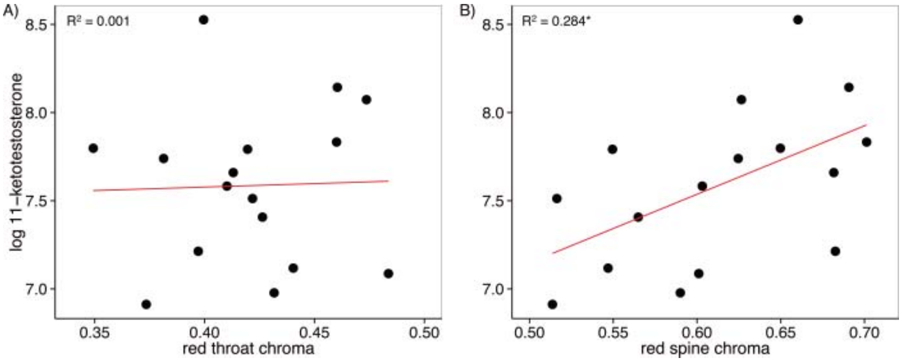

Fig. 4. Regression analyses show (A) no relationship between male throat colour and 11KT but a (B) significant positive correlation between 11KT and pelvic spine colour. Statistical significance: $^{*}P < 0.05$.

Novel pelvic spine–behaviour relationship

Our results confirm the previously reported relationship between male coloration and courtship behaviour (McLennan and McPhail, 1989; Rowland, 1994), as evidenced by the relationship between throat colour and PC1/zigzag scores. However, the positive correlation between red pelvic spines and male behaviour is new. Spine colour has only been specifically examined in a few recent studies, and we are, to our knowledge, the first to report this relationship. Previous studies have assessed pelvic spine coloration for its prevalence and heritability (Amundsen *et al.*, 2015), for its role in mate choice (Nordeide, 2002; Wright *et al.*, 2015), and for its implications for carotenoid allocation (Nordeide *et al.*, 2006); however, none have specifically tested associations between pelvic spine coloration and male behaviour. Work in brook sticklebacks did show that pelvic spine colour is more common in males, and that individuals with more colourful spines had higher condition scores (Hodgson *et al.*, 2013), but there has been no direct examination of behaviour–trait relationships. We show that males with redder spines display more aggressive courtship and that spine colour correlates positively with 11KT (discussed further below). Recent work has highlighted the continued interest in stickleback colour signals (Candolin and Tukiainen, 2015; Hiermes *et al.*, 2016), even calling into question the long-held assumptions regarding the selective pressures shaping them (Candolin and Tukiainen, 2015). Our work suggests that the potential signalling role of stickleback spine coloration deserves further research.

11KT and pelvic spine colour

To the best of our knowledge, the finding that 11KT is positively correlated with pelvic spine colour is also novel. Previous work has shown that male nuptial coloration in sticklebacks can be androgen dependent (Wai and Hoar, 1963; Borg, 1994; Borg and Mayer, 1995; Kurtz *et al.*, 2007), but work has been limited to characters relevant to breeding (i.e. throat coloration and kidney hypertrophy). Our results indicate that males with redder pelvic spines have higher 11KT levels, but the same relationship was not observed with red throats. Additionally, 11KT does not correlate with any male behaviours. This is surprising given the fact that 11KT has been implicated as an important mediator of reproductive behaviour and secondary sexual characters (Mayer *et al.*, 2004). 11-Ketotestosterone is known to differ throughout the male nesting cycle of threespine sticklebacks, being highest in the sexual/courtship phase (Mayer and Páll, 2006). Males in this study were tested only in the breeding season and never allowed to mate (i.e. always in the sexual phase), so one might expect 11KT levels to correlate positively with male courtship behaviour and throat coloration. However, this was not the case. We postulate two potential explanations for this lack of association: (1) In the present study, only a subset of males was opportunistically assayed for androgens at the completion of the presentation series (so as not to interfere with behavioural scoring of mate choice trials). Perhaps systematic, repeated measures of 11KT with more individuals would be more revealing. (2) Pre-test measures of coloration and androgens were not performed (again, so as not to interfere with mate choice tests). Males were simply selected for nuptial coloration (red throat and blue eyes) and then scored for traits at the conclusion of mate choice trials. Perhaps the change in coloration and the associated change in 11KT levels with the onset of breeding behaviour (zigzags) would be more informative. Nonetheless, the results presented here provide a first glimpse into a physiological correlate, and possibly cause, of pelvic spine coloration in sticklebacks.

Moving forward, manipulative studies should be adopted to fully explore the interaction of spine colour with 11KT. And given the small sample of fish in the androgen analyses, replication is also important.

CONCLUSION

The results presented here show that red throats and red pelvic spines may signal different aspects of the male behaviour repertoire, namely courtship versus aggression (or at least more aggressive courtship), and that spine colour may be influenced by 11KT. Given the absence of any relationship between throat and spine coloration in males (i.e. no apparent trade-offs), we postulate that red pelvic spines may act as a separate colour cue, potentially functioning in conjunction with typical male courtship behaviour. However, such speculation is based solely on mate choice encounters using stream-resident fish; tests of the role of spine coloration outside of mate choice are lacking. For example, since spine coloration is associated with aggression, it may serve as an anti-predator mechanism, especially when pelvic spines are extended (i.e. redder spines and more aggression may lead to a lower likelihood of being attacked or preyed upon). Although not addressed in this study, spine coloration could also be related to the extent of defensive armour, which has recently been shown to correlate with certain behaviours – boldness and activity (De Winter *et al.*, 2016). Furthermore, marine sticklebacks often possess brightly coloured pelvic spines – indeed, more than in stream residents (Yong *et al.*, 2013) – but correlates of spine coloration in marine fish have been little studied. The novel relationship between spine colour, behaviour, and androgens suggests new opportunities for studying signal evolution and communication in sticklebacks.

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