ORIGINAL ARTICLE

Phenotypic correlates of pelvic spine coloration in the threespine stickleback (*Gasterosteus aculeatus*): implications for function and evolution

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Abstract

Animal color patches may be static or plastic in expression and concealable or continuously visible, yet the evolution and function of these aspects of coloration have seldom been studied together. We investigated such color pattern elements using the threespine stickleback (*Gasterosteus aculeatus*). Despite a rich history of study of stickleback nuptial color pattern evolution, disagreement persists regarding selection pressures and function and only limited research has addressed the role of pelvic spine coloration, a potentially important, and substantially concealable, color pattern element. We investigated (i) whether male pelvic spine (along with throat and body) coloration is relatively static or plastic across the reproductive cycle, (ii) when pelvic spines are raised versus concealed across behavioral contexts, and (iii) associations between color patches and behavior in males. We found no significant variation in spine color across reproductive stages whereas body color was more plastic and intensely red during courtship and egg/fry care. Conspicuousness of pelvic spine coloration instead varied behaviorally, through increased erection frequency during social interactions and in response to a model predator. Spine erection frequency was positively associated with behaviors that enhance spine color visibility, i.e., flees and leads to nest. These findings suggest that stickleback use pelvic spines to display an intensely red color patch facultatively, either as a complement to similar body coloration or possibly as a substitute.

Significance statement

The interplay between color patches that are either readily concealable or always visible has been little studied, particularly in organisms with patches on a single individual that differ in capacity for concealment, such as the threespine stickleback (*Gasterosteus aculeatus*). While much work has been done on the evolution of coloration in stickleback, little has addressed patterns of expression and evolutionary functions of pelvic spine color, which can be facultatively concealed. We evaluated the expression characteristics of male spine coloration within the spawning period and how spine color relates to other color patches. Our work also examines the relationship between pelvic spine erections and presentation of spine color patches and raises the possibility of pelvic spine color being naturally selected and functioning across different behavioral contexts.

Introduction

Explaining animal ornaments is a longstanding problem in evolutionary biology and conspicuous color patterns have been studied extensively in this regard. Natural and sexual selection have often been investigated as if they act in straightforward opposition in ornament evolution, yet they can also interact in other ways (see Smith and Harper 2003; Cuthill et al. 2017). Functions served by animal color patches include crypsis, aposematic "warnings" for predator deterrence (Harvey and Paxton 1981; Endler 1984, 1992), and socially/sexually selected "display" ornaments to

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intimate rivals and/or attract mates (e.g. Endler 1984; Houde 1987; McKinnon 1995; Kim and Velando 2014; Johnson and Candolin 2017). In some cases, single color patches may serve multiple functions (e.g. Bakker and Milinski 1993).

Color patches can be static, i.e., uniformly expressed over time, or plastic in expression where the intensity and/or area of the color patch changes over relatively short timeframes (Cuthill 2007; Duarte et al. 2017). Animals may also conceal static color patches, potentially reducing some costs associated with them, and only present them in contexts where they provide a benefit. For example, deimatic displays (behaviors used to frighten a predator and deter attack) and flash coloration (exposure of previously hidden conspicuous coloration) may only be presented in response to predators when animals quickly and suddenly expose conspicuous color patches through purposeful movement (Umbers et al. 2015, 2017; Loeffler-Henry et al. 2018).

The subject of the present study, the threespine stickleback, *Gasterosteus aculeatus*, comprises a species complex with extensive color variation within and between the sexes and among populations (Bell and Foster 1994; McKinnon and Rundle 2002). Stickleback are cryptic through much of the year, and may adjust their coloration in response to variation in background color and illumination (Brock et al. 2017; Tibblin et al. 2020). Males exhibit nuptial coloration during the breeding season, usually including iridescent blue eyes and orange-red coloration along the throat, jaw and/or lateral portions of the body (for simplicity, we will henceforth refer to all color patches that contain orange-red coloration as "red") (Bell and Foster 1994). Within the breeding season, male color varies across the reproductive cycle, which has four main stages: (i) nest building, (ii) mate attraction, (iii) egg guarding, and (iv) fry guarding. Males typically become conspicuously colored during courtship and coloration may persist during egg and fry care and defense (McLennan and McPhail 1989; McKinnon 1996; Candolin and Tukiainen 2015).

Red nuptial coloration in male stickleback has long been considered a sexually selected trait, functioning as a cue for female mate choice and a badge of status during male-male competition (Rowland 1982, 1984; Milinski and Bakker 1990; Bakker and Milinski 1993; Bakker and Mundwiler 1994). However, some evidence points to functions beyond sexual selection, such as for nest and offspring defense or in social situations (McKinnon 1996; Candolin and Tukiainen 2015). Indeed, reports of an increase in coloration occurring between the courting and parental stages (Scott and Foster 2000; Candolin and Tukiainen 2015; but see McLennan and McPhail 1989), when males are no longer attracting females, highlight functionality beyond signaling to potential mates. A more comprehensive evaluation of color pattern evolution in stickleback may help to reconcile the multiplicity of possible functions and views in the literature, especially through investigations of other color patches. The red color patch found along pelvic spines, which can be substantially concealed, has been little studied to date relative to red coloration elsewhere along the body (though see, e.g. Nordeide 2002; Yong et al. 2013; Amundsen et al. 2015; Wright et al. 2016) but may be important in this context.

Pelvic spines are thought mainly to function in defense against predators but may also be important for signaling in social contexts (van Iersel 1953; Symons 1966; Moodie 1972; Huntingford 1976; Bell and Foster 1994), possibly across several species of Gasterosteidae in addition to *G. aculeatus*. Conspicuous pelvic spine coloration has been noted in males of *Pungitius pungitius* (Morris 1958; Herczeg et al. 2010), *Gasterosteus wheatlandi* (Ostlund-Nilsson et al. 2006), and *Culaea inconstans* (Hodgson et al. 2013); in *Apeltes quadracus* (e.g. Rowland 1974) red color is found exclusively in the spines.

Stickleback pelvic spines articulate with the skeleton and rest flush against the body until erected. Unlike dorsal spines (with rare exceptions: O. Seehausen, pers. comm. 2019), threespine stickleback pelvic spines usually contain an orange-red patch of color that is strongest along the inner-ventral surface and associated membranes; it is relatively inconspicuous until the pelvic spines are extended away from the body. Male and female threespine stickleback possessing colored pelvic spines have been found in anadromous and freshwater populations across North America and Europe (Nordeide 2002; Yong et al. 2013; Amundsen et al. 2015; Kroken et al. 2021). The few published works assessing the function of spine coloration in *G. aculeatus* have found that (i) males often exhibit redder spines than females, although female color is typically substantial (Yong et al. 2013; Amundsen et al. 2015); (ii) spine color is present throughout the year but is most intense during the spawning season (Amundsen et al. 2015); (iii) males court females with drab colored pelvic spines more than red spines (Nordeide 2002); (iv) males with redder spines perform more aggressive courtship behaviors (Wright et al. 2016); and (v) overall, redness along pelvic spines does not significantly affect behavior towards dummies with varying red intensities along the pelvic spine (Kroken et al. 2021). These results argue for investigating hypotheses of both natural and sexual selection concerning the functions of stickleback pelvic spine coloration, as spine color is linked both to potentially naturally selected patterns (such as its presence in both males and females) and sexually selected patterns (intensity of color is greatest during the breeding season).

In the present study, we seek to elucidate the likely function(s) of spine coloration by evaluating how spine color is expressed through the reproductive cycle and the contexts in which pelvic spines are raised and made more conspicuous. A secondary objective is to evaluate changes in red nuptial coloration across the reproductive stages in

additional color patches, i.e., the lateral body and throat, and possible associations between these color patches, spine coloration and behavior. Since pelvic spine coloration can be readily hidden, relative to body coloration, we hypothesized that spine color would vary relatively little across reproductive stages in comparison with red color patches along the throat and lateral body. Further, if pelvic spines are indeed important in specific interaction types, we hypothesized that pelvic spine use would vary across contexts, with more frequent pelvic spine erection/presentation in those interactions where it is most important.

Methods

Stickleback collection and maintenance

Adult male and female threespine stickleback were collected near White Rock, British Columbia during the beginning of the breeding season (May, 2017 and May, 2018). Using minnow traps, anadromous fish were collected from a downstream site of the Little Campbell River (49.016N, 122.778W) and transported to our lab at East Carolina University (Greenville, NC, USA). Immediately upon arrival, fish were housed in our aquatic facility in mixed-sex 99-liter tanks (92 cm × 33 cm × 33 cm in size) at approximately 15–20 fish per tank and allowed to acclimate for 2 weeks. Tanks each contained an air stone, aquarium filter, and 1 cup of limestone rocks. All aquaria were placed under natural-spectrum mimicking fluorescent lights (Lumicrhome® Full Spectrum Plus, Lumiram Electric Co, Larchmont, NY, USA) for a 15-h photoperiod at a temperature of 17–20°Celsius. Fish were fed bloodworms (chironomid larvae) and brine shrimp twice daily. All experimental trials took place from June to August 2017 and 2018.

Experimental tank design

To conduct the experimental trials, four large tanks (212 L, approximately 123 cm × 54 cm × 32 cm in size) were set up, each with a removable, opaque plexiglass divider that separated the tanks into two sides. Tanks were covered on three sides with sheets of brown paper in order to visually isolate individuals within the tanks and approximate natural habitat background coloration. Nesting materials (one 6″ clear plastic plant saucer filled ¾ full with sand and topped with sphagnum moss), an artificial plant, limestone aquarium rocks, and a single sponge filter were placed on each side of the divided tanks (Fig. S1). To further visually isolate experimental tanks, large black plastic sheets were suspended in front of all shelving units containing experimental tanks and only removed during times of observation and videotaping.

Holding tanks, control tanks, and control trials

Forty-five liter tanks (approximately 52 cm × 26 cm × 32 cm in size) were set up for either control fish trials or to house fish already used in the experiment. The four "Control Tanks" were maintained and set up under the same guidelines as experimental tanks but were smaller in size because only a single control male was present in each tank. Tanks were visually isolated by brown paper placed between them. Because control fish were used to assess color variation in the absence of either stickleback or predators, the tanks were set up to parallel standard housing conditions. Black plastic sheeting was again placed around the shelving units during times of experimentation and only removed for observation or behavioral analysis.

Control males were not given nesting supplies and were not exposed to other fish. Control males in trials were presented with a single UV-transparent plexiglass box of the same design presented to experimental males (outlined further below), following the same timing protocols, though with no stimulus fish present.

Experimental trials

Initiation and stage 1: nest building

Males showing signs of breeding coloration, i.e., blue eyes and some red body coloration, were used in experimental trials. Pairs of males were selected from different housing tanks and size matched to within 2–3 mm. For each, mass, standard length, and visual color scores were collected and any unique markings noted. Reflectance measurements were taken at two spots along the throat and standardized photographs of the lateral body and ventral surface of the left pelvic spine were taken (details below). Pairs of males were then placed into assigned experimental tanks, one on each side.

Following a 24–36-h acclimation period, a gravid female, housed in a custom-built UV-transparent plexiglass container, was introduced to each male for 30 min daily, beginning when the male oriented himself to the female while within ~10 cm of the container (indicating that the male has recognized the female's presence). Female presentations continued for up to 5 days. Gravid females were selected at random from the housing tanks and not used in any immediate mating trials. If after 5 days neither male built a nest, stimulus females were placed directly into the experimental tank (free swimming) for up to 60 min daily. These interactions were closely monitored the entirety of the time (out

of sight of the fish) to ensure that females did not lay eggs and to prevent injury if aggressive interactions occurred. If no recognizable nest was built in the following 2 days, a second female was introduced directly into the tank during stimulus interactions. If after 8–10 days neither male successfully built a nest ($n = 10$ pairs), or a male died ($n = 1$), the trial was canceled. The first male within each pair to successfully build a nest became the "parental male" and was the only one allowed to spawn, while the other male became the "rival male." The first day a nest was identified concluded stage 1.

Stage 2: courtship (mating trials)

The first day post nest-build, a gravid stimulus female was introduced in a plexiglass container into the parental male's tank for 30 min and videotaped. Three hours after this stimulus female interaction, the parental male was removed from his tank for color measurements. On the second day post nest-build, an ovulating female was selected from the housing tanks and weighed, then placed into the parental male's tank. Courtship trials concluded once spawning occurred, or 2 h elapsed. If spawning was unsuccessful, a second attempt was made at least 1 h later with a different female. This protocol was repeated as needed (for up to 3 additional days) until the male spawned. If after 4 days spawning had not occurred, the experimental trial ended ($n = 1$), and data were not used. Each mating interaction was videotaped (see below for more detailed methods). The day eggs were deposited concluded stage 2.

Stage 3: egg guarding

On the 4th day post egg-laying, a gravid stimulus female was again introduced to the parental male, while housed in a plexiglass container, for 30 min and videotaped. After 3 h, the male was removed, and a third round of spectrophotometry and photographs were taken. The following day, the middle barrier separating the two sides of the large experimental tank was removed, permitting both males to interact for the first time. Males were able to swim freely for 2 h and were videotaped. The middle barrier was then replaced with each male on their respective sides of the tank. Stage 3 continued until fry appeared by the nest.

Stage 4: fry guarding

On day 1 post-fry appearance, the parental male was presented with a contained gravid stimulus female for 30 min and videotaped. After 3 h, each parental male was taken for the final round of spectrophotometry and photographs. The next day, the middle barrier was again removed and male-male interaction occurred for 2 h while being videotaped.

Immediately afterward, rival males were removed from the tank, leaving the parental male alone.

Finally, to explore behaviors towards a perceived predator, we performed simulated attacks on the parental male on day 3 of stage 4 using a manually manipulated rainbow trout model, as in related experiments with stickleback (Kozak and Boughman 2012, 2015). Following a similar protocol to Kozak and Boughman (2015), we randomly selected one of two 10-inch rainbow trout models (Platinum Naturals Trout Body, Castaic Swimbaits, Trenton, TX) for use in each trial. A trout scent (rainbow trout scent bait, Pro-Cure, Salem, OR, USA) was applied to the head of the trout model to elicit olfactory cues of predation before each exposure (Kozak and Boughman 2015). Each model was connected at the nose to a wooden dowel using monofilament fishing line and a swivel, which was then connected to a 3 ft. handle, allowing it to be manually operated from some distance from the tank. Simulated attacks consisted of two parts (i) models were guided rapidly towards nesting males, stopping abruptly two inches away from where they were located, then (ii) models were guided gently above the males for 3 s and away from the male to allow for another "attack." Simulated attacks occurred 15 times per nesting male and were videotaped in their entirety. All parental males were removed from their tanks 10 min post model predator interaction and euthanized using a lethal dosage of MS-222, concluding the experimental trial. After parental males were removed from their tanks and euthanized, the trout models were rinsed thoroughly in 100% ethanol to remove any trace of scent, then dried, allowing them to be reused in subsequent trials. Over the next 2 days, all fry (and any unhatched eggs) were collected from the tank, euthanized using MS-222 and preserved in 10% Neutral Buffered Formalin.

Photography, spectrophotometry, and videography-gathering data

Spectrophotometry and photography

In order to measure color along the threespine stickleback, males had to be removed from their tanks four times throughout the experiment (i.e., initial measurement (stage 1), stage 2, stage 3, and stage 4). Males were collected using dip nets and placed into a 1L container with water from their own tank, with a unique container for each fish. All spectrophotometric measurements of throat tissue were acquired within ~60 s of removal from the tank, and all photographs of the lateral body and pelvic spine were made next within another ~60 s. We did not observe any qualitative change in coloration along any part of the body during the brief period that fish coloration was being measured.

Reflectance spectra of male throats were gathered using a Maya 2000 spectrometer (Ocean Optics Inc., Dunedine, FL,

USA) with a broad-spectrum, UV-Vis, illumination source (Newport Co., Irvine, CA, USA). A fiber-optic probe was attached to the Maya spectrometer and situated into a 60 mm Macro lens (Coastal Optics) mounted on a tripod. The lens was situated at a 90° angle to the body of the fish, about 10 cm above fish, with the light source at a 45° angle. Fish were placed ventral side up into a custom sponge made to stabilize the fish while leaving the ventral side exposed. All reflectance measurements were standardized using a Spectralon™ white standard and recorded using OceanView (V 1.6.3, Ocean Insight, USA) software. Reflectance measurements ~1 mm in diameter were taken at two spots along the midline of the ventral throat. Wavelengths between 350 and 700 nm were analyzed.

Two photographs were taken of each fish, of the lateral body and of the ventral surface of the left pelvic spine, using a digital camera (Cannon DS 126171) mounted on a copy stand directly above the fish. The fish was illuminated with Solux Halogen lamps (Tailor Lighting Inc., Rochester, NY, USA) on either side of the camera and angled at 45°. All fish were photographed against an 18% gray card for standardization. Immediately upon removal from the water, fish were gently dabbed to prevent water interference and placed onto the gray card for the lateral body photo. Fish were then moved to a custom-angled sponge that allowed for a ventral view of the erected pelvic spine surface at a 90° angle to the camera. The left pelvic spine was manually erected and held in place using a size 1 specimen pin.

Videography

A digital HD video camera (Sony HDR-XR500V) was positioned on a tripod approximately 1 m in front of the tanks depending on the context (i.e., ensuring the whole visual field of interest was in view). Videos were recorded for the following durations for each context: (i) stimulus female fish -15 min, (ii) mating -until egg deposit occurred, up to 120 min maximum, (iii) male-male -120 min, and (iv) model predator – length of time required to complete 15 simulated attacks (~5–8 min).

Measurement of color and behavioral analysis

Spectrophotometry-based measurement of color

Reflectances were analyzed using the pavo 2.4.0 package in R (Maia et al. 2019), interpolated to yield 1 nm intervals from 350 to 700 nm. The stimulation of stickleback-specific visible and UV wavelength sensitive cones were then calculated in the above range (Rush et al. 2003; Yong et al. 2013; Stuckert et al. 2019) set to ideal, even-illumination and a wavelength-independent background, in order to provide quantum catches from each cone type used in matrix

calculations of hue (h.theta, h.phi) and saturation/chroma (r.vec, the distance from achromatic center). Data from the two spots were averaged to produce the final r.vec value for each fish at each stage. The r.vec measurement will from here forward be referred to as "throat chroma." In total, four of 100 possible mean throat chroma measurements were excluded from analyses due to death ($n = 1$) or technical failures ($n = 3$).

Photography-based measurements of color: spine and lateral coloration

Photographic methods for measuring stickleback coloration have provided useful insight into color expression (e.g., Candolin 1999; Clarke and Schluter 2011; Candolin and Tukiainen 2015; Jenck et al. 2020), and are the best methodological approaches to use when spectrophotometric measurements are not feasible, such as in the measurement of multiple, localized small points along the stickleback body and pelvic spine. Previous work has shown chroma values of red coloration using photographic and spectrophotometric measurements to be moderately-to-strongly correlated (Yong et al. 2013).

Red color intensity (I_R) scores were generated using Adobe Photoshop (version 21.2.4) (Adobe Systems, San Jose, CA, USA) for predetermined points along the pelvic spines and lateral body. Color measurements were corrected relative to the gray card in order to obtain the necessary standardized RGB values (R_{Stand}, G_{Stand}, B_{Stand}) by dividing the individual red (R), green (G), and blue (B) values for each point by the mean RGB values from four points along the gray card standard. Red color intensity for each point was then generated by dividing R_{Stand} by the sum of R_{Stand}, G_{Stand}, $B_{Stand.}$ (Nordeide et al. 2006; Yong et al. 2013; Amundsen et al. 2015; Wright et al. 2015). Final I_R values, which were used as a measure of color, were generated for each spot along the lateral body and pelvic spine, separately.

To assess pelvic spine coloration, spines were divided into nine equal sections relative to spine length. RGB values were recorded at fourteen points within the first 8 sections (section 9 was excluded as it contained only the distal bony tip). All points selected for measurement were along the ventral portion of the spines (Fig. S2). The mean spine I_R for each photo was calculated and recorded. In total, seven of 100 possible pelvic spine color measurements were excluded from analyses due to fish death ($n = 1$) or technical failures ($n = 6$).

To assess coloration along the body, I_R was measured at five areas known to commonly exhibit orange-red nuptial coloration: Lower jaw, Operculum, Pectoral plate, Ventral area between the lower jaw and pelvic spines (VPS), and Lateral plates (McLennan and McPhail 1989). Red intensity values were generated for nine total spots, each defined

using anatomical landmarks and/or photoshop generated guidelines (Fig. S3). Mean red intensity values were taken for the lower jaw (spots 1–3), operculum (spots 4–5), and lateral plate (spots 8–9). In total, nine of 100 possible color measurements were excluded from analyses due to fish death ($n = 1$) or technical failures ($n = 8$).

Video behavioral analyses

Behavioral videos were analyzed using BORIS (Behavioral Observation Research Interactive Software, version 7.9.6) (Friard and Gamba 2016). Prior to the start of analyses, videos ($n = 131$) were separated into groups by observation type (i.e., context) and renamed using randomly designated codes to prevent observer bias (i.e., the analyses were blind). An Ethogram was generated to include 10 point behaviors (scored for each occurrence) and three state behaviors (scored over time) with no modifiers (van Iersel 1953; Wootton 1971; McKinnon 1996; McLennan and Ward 2006). Not all behaviors could occur in every context, so only behaviors that could occur were scored (Table S1).

Data collection began at the point of first direct orientation by the parental male towards either the stimulus box (control), stimulus female inside a stimulus box, ovulating female, conspecific rival male, or model predator depending on the social context, and lasted 15 min (or less if the duration of interaction lasted fewer than 15 min). Mating stage videos were scored through 5 s post successful deposition of eggs into the male's nest, while predator videos were scored through 5 s post final simulated attack. All videos were scored by a single investigator (CMA) upon completion of all experimental trials using blinded methods to prevent observer bias. Any video with a glitch (i.e., error in loading, error in recording, or maintained visibility errors) or premature end ($n = 4$) was excluded from the data set and not analyzed.

All behaviors were calculated on a *per minute* basis to ensure comparable results across all fish and contexts, with the length of video analysis used as the metric of time. Qualitative assessment was performed by scanning raw data manually to determine which behaviors were performed often enough to warrant inclusion; retained behaviors were then $log(1 + x)$ transformed to improve the normality of the distributions. These transformed values were used for all statistical analyses.

Statistical analyses

Principal components

All statistical analyses were conducted in JMP v. 14.1.0 and 15.0.0 (SAS Institute Inc., Cary, NC, 1989–2022). Within JMP, the 'multivariate methods' module was used to calculate all principal components, using correlations. Principal components were used as a tool to generate a single composite variable for lateral coloration, as well as for male behaviors within each class of unique behaviors by context, including (i) stimulus female, (ii) mating (courtship), (iii) male-male, and (iv) control. Only behaviors that occurred with sufficient frequency (i.e., median above 0) within this subset were included in the PCA (Table S2). All subsequent analyses in this research used PC1 values from each of the various behavioral and color PCAs outlined above.

For the stimulus female ($n = 42$) PCAs, behaviors were chosen to account for common stickleback behaviors involved in the courting, nest guarding, and fry guarding stages of the reproductive cycle that did not require direct interaction with another fish: 'bite/bump,' 'pelvic spine erection,' 'leads to nest,' 'time spent at nest,' and 'time spent at stimulus box.' One behavior, 'zigzag', was excluded from analysis due to limited occurrences. PC1 loaded positively with 'bite/bump,' 'pelvic spine erection,' and 'time spent at stimulus box,' while negatively with 'leads to nest' and 'time spent at nest' (PC1: 44.6% of variation), and thus served well as a measure of the behavior of a male in proximity to a stimulus fish; males spent large amounts of time in close proximity to the stimulus box while performing bites/bumps and erecting their pelvic spines, while behaviors performed away from the nest, i.e., leads to nest and time spent at nest, were positively associated. PC1 values were used in all subsequent analyses.

For the mating context ($n = 15$) PCA, behaviors included both social behaviors, i.e., 'pelvic spine erections' and 'bite/bump,' and courtship behaviors, i.e., 'leads to nest' and 'time spent at nest.' Since the mating context allowed for direct contact with the female, three additional common behaviors, 'zigzag,' 'chase' and 'flee,' were initially addressed, though ultimately excluded from PCA analysis due to severely limited occurrence. PC1 loaded positively with all behaviors (PC1: 43.4% of variation), and thus provided a measure of overall vigor of male mating behavior. PC1 values were used in all subsequent analyses.

For the male-male context ($n = 29$) PCA, all the social and territorial behaviors were initially assessed since males were permitted to interact directly with a rival male, but only 'bite/bump,' 'pelvic spine erection,' 'charge,' 'circle fight,' and 'time spent at nest' were included due to limited occurrence of other scored behaviors (Table S2). Since no females were present during this interaction, no mating-specific behaviors were included. The behavior 'flee' was excluded from the PCA due to limited occurrence in some males but included in later specific analyses assessing the relationship between pelvic spine erections and the use of behaviors that could expose pelvic spine color. PC1 loaded positively with all included behaviors (PC1: 37.03% of variation) and thus served as a measure of overall vigor of male-male behavior.

PC2 loaded positively with 'bite/bump,' 'pelvic spine erection,' and 'circle fight,' but negatively with 'total time at nest' (PC2: 33.07% of variation); i.e., there was a positive association between all point behaviors but negative association with the state behavior. PC1 values were used in all subsequent analyses.

Lastly, for the control context ($n = 30$) PCA, all social behaviors that could occur without direct interaction with another fish were assessed, including 'bite/bump,' 'pelvic spine erection,' 'charge,' and 'time spent at stimulus box.' The 'head down threat' behavior was excluded due to non-occurrence. Since control males were not able to build nests, no territorial behaviors were addressed. Results from this PCA showed that PC1 loaded positively with 'bite/bump' and 'time spent at stimulus box,' but negatively with 'pelvic spine erection' and 'charge' (PC1: 43.295% of variation). This provides a measure of control male behaviors as most behaviors were performed at low frequencies, but males would often bite/bump the empty stimulus container when exploring it before swimming away. PC1 values were used in all subsequent analyses.

For lateral coloration, PCAs were calculated for experimental and control males separately using the same 9 spots along the lateral body. For the nesting male lateral coloration ($n = 53$ different measurements along 14 males, with $n = 477$ total spots) PCA, PC1 loaded positively with all 9 points (PC1: 63.47% of variation), and thus serves as a general measure of lateral coloration intensity. In results for control male lateral coloration ($n = 38$ different measurements along 10 males, with 342 total spots) PC1 loaded positively with spots along the operculum, pectoral plate, VPS, and lateral plates, but negatively with all three spots along the lateral jaw (PC1: 35.54% of variation) and is thus indicative of a general pattern of color expression in fish without any social or reproductive stimulation. PC1 values from both the experimental and control male PCAs were used in subsequent analyses.

Assessing variation in color and behavior across reproductive stages and behavioral contexts, and the relationship between red intensity and behavior

We employed linear mixed effect models for the majority of tests in order to control for multiple measures and different collection years by using the 'Fit Model' function in JMP. The mixed effect models that included multiple measurements from the same fish (i.e., any test with multiple reproductive stages being analyzed) contained fish ID as a random effect. To control for differences that may exist across collection years, mixed models that examined experimental fish also contained year as a random effect (control fish were only collected in 2018 so analyses that included only control males excluded year as an effect). The variables of color and/

or behavior were always assessed as dependent or response variables as outlined below.

To address how color and behaviors varied across reproductive stages, repeated measures analysis of variance (ANOVA) was used. Models contained a single response variable (i.e., color or behavior), fish ID as a random effect, year as a random effect, and reproductive stage as a fixed effect. To determine relationships between color patches, i.e., pelvic spine color, throat color, and PC1 lateral body color, as well as coloration and size, regression analyses were run across all experimental males (3 total analyses) and control males (3 total analyses) with fish ID and year as random effects within the model.

To address the relationship between each of the color patches and PC1 behaviors/pelvic spine erections, regression analyses were again employed for each applicable context type (i.e., female stimulus, mating female, rival male, control, and predator). Models for experimental contexts included fish ID and year as random effects (21 total analyses), while control contexts included only fish ID as a random effect (6 total analyses). To address the relationship between the number of pelvic spine erections and individual behaviors of interest (i.e., 'flee' and 'leads to nest'), regressions were run for each applicable context type (4 total analyses). In these analyses, fish ID and year were defined as random effects, color (if applicable) as a predictor variable, and behavior as the dependent variable. To address how PC1 behaviors and pelvic spine erections varied across reproductive stages, ANOVA was employed with Fish ID (control and experimental contexts) and year (experimental contexts only) as random effects. Lastly, to address how the number of pelvic spine erections compared across experimental contexts (i.e. stimulus female, mating, male-male, and predator), ANOVA was utilized with fish ID and Year as random effects, behavioral context as a fixed effect, and pelvic spine erections as the response variable. Because control fish all came from the same year and were a different set of fish than experimental males, un-pooled two sample T-tests were run to garner rudimentary statistical results about the relationship between the control context and all other experimental contexts (4 total analyses). For these tests, mean number of pelvic spine erections for each male within each of the behavioral contexts was calculated and used as values for comparison within the T-tests.

To address the use of pelvic spine erections in relation to behaviors that could expose pelvic spine coloration across contexts, i.e., leads to nest (stimulus female and mating contexts) and flees (male-male and predator contexts), the number of pelvic spine erections performed within ± (i.e., plus or minus) 2 and 5 s of either a lead or flee were quantified, as well as the number of leads or flees that were performed within ± 2 and 5 s of a pelvic spine erection. Results for patterns within ± 5 s (i.e., pelvic spine erections

performed within 5 s before or after either a flee/lead or vice versa) were more comprehensive than $\pm$ 2 s, so only 5 s results were analyzed further and presented here. To assess how these behaviors were used across reproductive stages, repeated measures ANOVAs were performed for the stimulus female and male-male contexts (which are the only two contexts with multiple measures of the same individual), with fish ID and year as random effects, reproductive stage as a fixed effect, and the quantified behavior as the response variable. Lastly, percentages of pelvic spine erections performed within $\pm$ 5 s of either a lead or flee and percentages of leads or flees performed within $\pm$ 5 s of a pelvic spine erection were calculated from videos in which the focal behavior was performed (i.e., 'pelvic spine erections' for the question of pelvic spine erections performed within $\pm$ 5 s of a flee/lead OR 'flees/leads' for the question of the number of flees/leads performed within $\pm$ 5 s of a pelvic spine erection). These percentage calculations serve as a complement to the regressions previously mentioned assessing the relationship between pelvic spine erections and flees and leads.

We did not correct for multiple comparisons as all analyses were planned a priori to address specific, complementary questions with the minimal number of possible comparisons per question (see Rothman 1990; Perneger 1998 for discussions about multiple comparison correction).

Results

Coloration across reproductive stages and relationships between color patches

The lateral body, ventral throat and pelvic spines exhibited different patterns of red intensity across reproductive stages and between experimental and control males. Lateral body coloration (PC1) did not significantly differ across stages in control fish ($F_{3,24.81} = 1.400$, $P = 0.2662$) but did in experimental fish ($F_{3,36.73} = 12.294$, $P < 0.0001$; Fig. 1a). Tukey multiple comparisons of experimental males revealed stage 1 to be significantly lower than all other stages (1:2 $P = 0.0003$; 1:3 $P < 0.0001$; 1:4 $P = 0.0006$), while stages 2, 3, and 4 were not significantly different from each other (2:3 $P = 0.8937$; 2:4 0.9994; 3:4 0.8515). Ventral throat chroma also varied significantly across stages in experimental fish ($F_{3,38.71} = 5.117$, $P = 0.0044$) but not control fish ($F_{3,27} = 0.730$, $P = 0.5433$). The pattern of color changes along the throat mirrored those of the lateral body (Fig. 1b). A Tukey multiple comparison test in experimental males revealed stage 1 to be significantly lower than in stages 3 and 4 (1:3 $P = 0.0080$; 1:4 $P = 0.0215$), but found no other significant stage differences (1:2 $P = 0.6698$; 2:3 $P = 0.1505$; 2:4 $P = 0.2951$; 3:4 $P = 0.9822$). Spine coloration did not vary significantly across reproductive stages

in either experimental males ($F_{3,36.27} = 1.552$, $P = 0.2178$) or control males ($F_{3,27} = 2.380$, $P = 0.0918$) (Fig. 1c).

Analyses of the relationship between the different color patches along the stickleback body (i.e., between pelvic spine color and body color, body color and throat chroma, and spine color and throat chroma in experimental and control males) revealed significant associations between the different color patches in experimental fish, but not control fish. There was a strong positive relationship between PC1 lateral coloration and spine coloration in experimental males ($F_{1,39.76} = 7.108$, $P = 0.0110$; Fig. 2a), while almost no relationship was detected in control males ($F_{1,33.35} = 0.0690$, $P = 0.7944$; Fig. 2b). Similarly, PC1 lateral coloration was positively associated with throat chroma in experimental fish ($F_{1,50.59} = 18.1097$ $P < 0.0001$; Fig. 2c), but not in control fish ($F_{1,35.81} = 0.035$, $P = 0.8525$; Fig. 2d). No significant relationship was detected between pelvic spine color and throat chroma in either experimental fish ($F_{1,42.53} = 0.8786$, $P = 0.3539$; Fig. 2e) or control fish ($F_{1,34.65} = 0.195$, $P = 0.6615$; Fig. 2f).

Behaviors and correlates across stages and contexts

PC1 for male behavior in the female stimulus context showed significant overall variation across reproductive stages ($F_{2,26.58} = 3.8636$, $P = 0.0337$), with Tukey pairwise comparisons revealing lower values in stage 4 relative the other stages, though differences only approached significance (2:4 $P = 0.0616$; 3:4 $P = 0.0556$; 2:3 $P = 0.9976$; Fig. S4a). No significant differences were found in male PC1 behaviors across reproductive stages in either the presentation of rival male ($F_{1,11.67} = 2.7657$, $P = 0.1229$; Fig. S4b) or the control male behaviors ($F_{2,18} = 0.1771$, $P = 0.8392$; Fig. S4c).

For the social presentations of a stimulus female, the number of pelvic spine erections by experimental males decreased steadily across reproductive stages ($F_{2,27.41} = 6.5719$, $P = 0.0047$), with Tukey pairwise comparisons showing a statistically significant difference between stages 2 and 4 ($P = 0.0034$), but not between stage 3 and the others (2:3 $P = 0.2802$; 3:4 $P = 0.1037$; Fig. S5a). In contrast, pelvic spine erections did not vary significantly for the male-male context ($F_{1,12.05} = 2.8599$, $P = 0.1165$; Fig. S5b) or control contexts ($F_{2,18} = 0.453$, $P = 0.6428$; Fig. S5c).

ANOVA assessment of pelvic spine erections across experimental behavioral contexts (i.e., stimulus female, mating, male-male, and model predator) showed significant differences ($F_{3,83.55} = 11.5814$, $P < 0.001$; see Fig. 3 for boxplots of all contexts). Tukey pairwise comparisons revealed that pelvic spine erections were performed significantly less in the stimulus female context as compared to the others ($P \leq 0.0495$), but no other significant distinctions ($P \geq 0.0997$); see Table S3 for data on all pairwise comparisons.

Fig. 1 Patterns of color change for each of the three color regions across reproductive stages (i.e. nest building (1), mating (2), egg guarding (3) and fry guarding (4)) in experimental and control males: Lateral Color (**a**), throat chroma (**b**), and spine color (**c**). Boxes indicate the inter quartile range (IQR), with the central line depicting the median and the whiskers extending to 1.5*IQR, and outliers indicated as dots above/below the whiskers
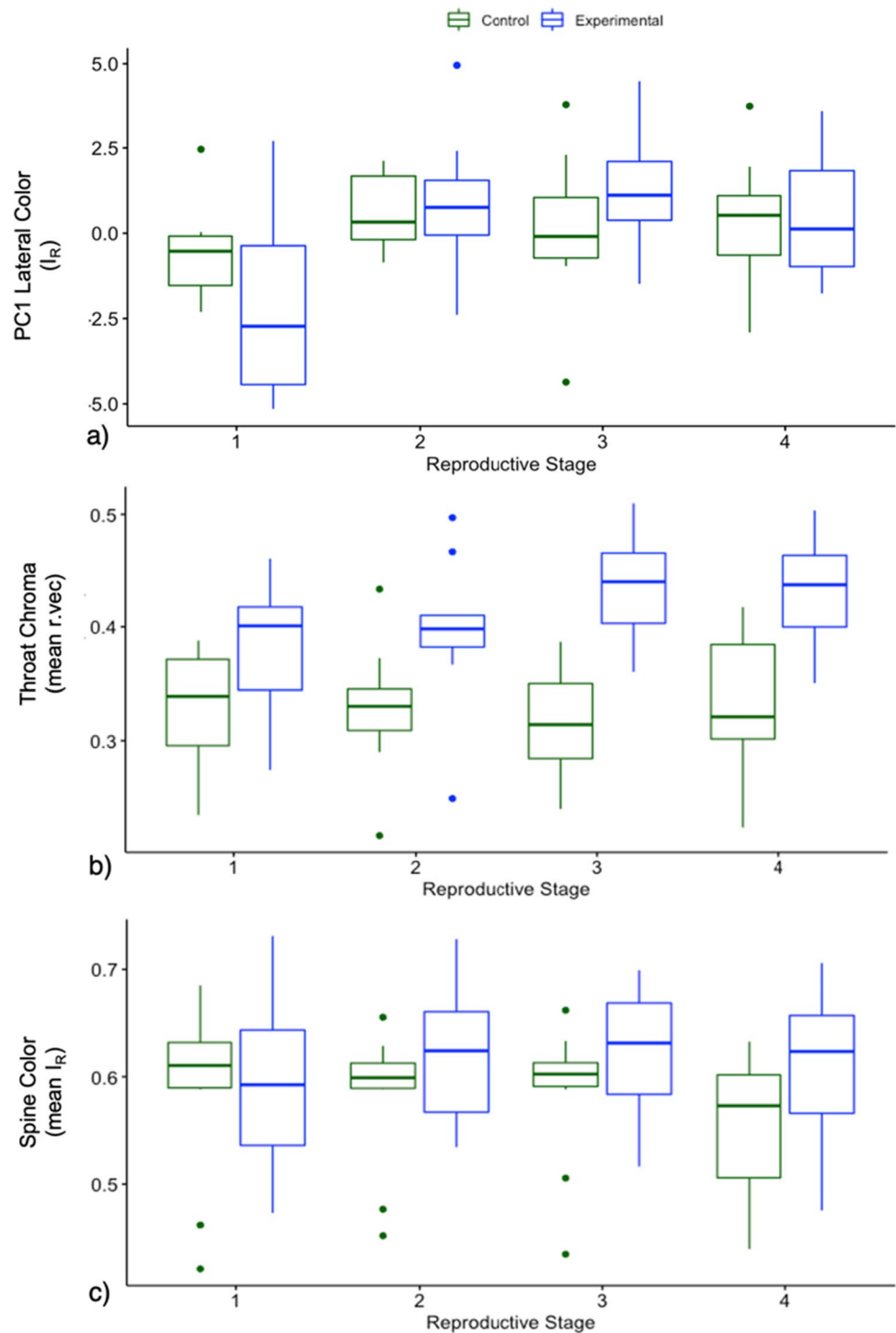
Two sample *T*-tests revealed that the number of pelvic spine erections varied significantly between control and all experimental contexts ($P \leq 0.0007$ in all cases; see Table S4), with control males performing significantly fewer overall.

There was a significantly positive relationship between the number of leads to nest and pelvic spine erections by experimental males in response to both a stimulus female ($F_{1,35.31} = 5.9012$, $P = 0.0204$; Fig. 4a) and during courtship ($F_{1,13} = 7.611$, $P = 0.016$; Fig. 4b), the only two contexts in which leads could be performed. Regression analyses of the relationship between flees and pelvic spine erections in experimental males also revealed a significantly positive relationship in response to only a model predator ($F_{1,11} = 9.2503$, $P = 0.0112$; Fig. 4c) though not towards a rival male ($F_{1,26.33} = 0.3070$, $P = 0.5842$; Fig. 4d), which were the only contexts that flees could be scored.
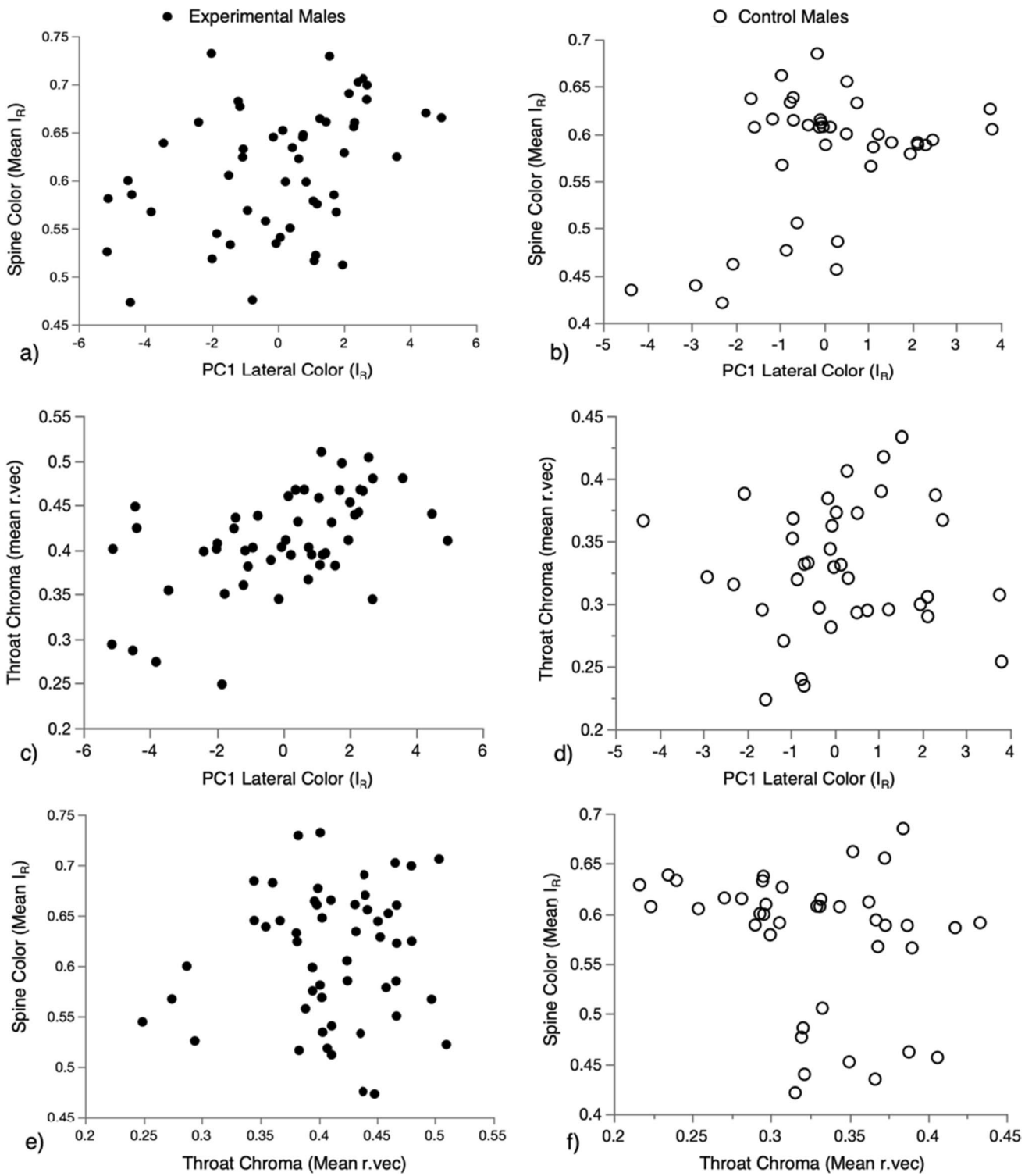
Fig. 2 Regression plots of relationships between the three color patches of interest in experimental and control males, including between (**a–b**) Spine color and PC1 lateral body color, (**c–d**) PC1 lateral color and throat color, and (**e–f**) spine color and throat color.

Only two relationships between color patches were found to be significant (a and c) in experimental males, while no relationships were significant in control males

Fig. 3 Mean number of pelvic spine erections across contexts. Results revealed notable use of pelvic spine erections across all behavioral interactions, including both social and anti-predator. In contrast, almost no pelvic spine erections were performed by control males. Boxes indicate the inter quartile range (IQR), with the central line depicting the median and the whiskers extending to 1.5*IQR, and outliers indicated as dots above/below the whiskers
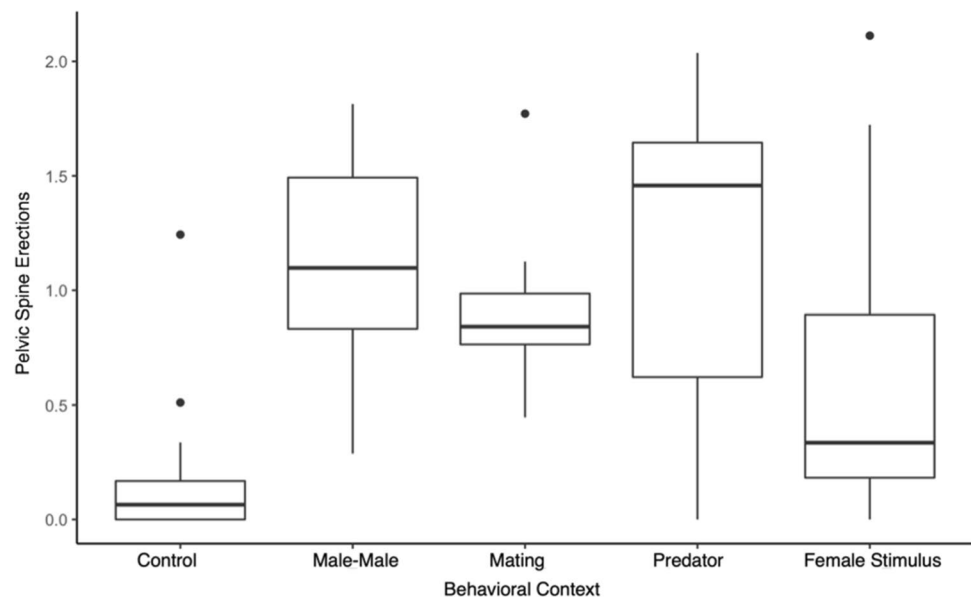
Pelvic spine erections and flees/leads performed closely in time

The percentages of pelvic spine erections performed within ± 5 s of a flee/lead (i.e., 5 s before or after), as well as flees/leads performed within ± 5 s of a pelvic spine erection, differed across behavioral contexts (see Table 1 for ± 5 s results). The highest number of pelvic spine erections performed within ± 5 s of a flee/lead occurred during interactions with a model predator (flee) or during mating (lead). For the reciprocal relationship, i.e., flees/leads performed within ± 5 s of a pelvic spine erection, the highest percentages occurred during interaction with a rival male or model predator (both flees), respectively. Results from repeated measures ANOVA tests of the number of pelvic spine erections performed within ± 5 s of a lead/flee and number of leads performed within ± 5 s of a pelvic spine erections, for both the stimulus female and male-male behavioral contexts, can be found in the supplemental material (Fig. S6, S7).

Associations between color(s) and behavior(s)

Regression analyses run across all applicable contexts, i.e., female stimulus, mating, male-male, model predator, and control, showed no significant relationship between spine coloration and number of pelvic spine erections ($P \geq 0.2069$; Table S5), nor between lateral body coloration and number of pelvic spine erections ($P \geq 0.0949$; Table S6). No significant relationship was detected between throat chroma and number of pelvic spine erections in the majority of contexts ($P \geq 0.1881$; Table S7), with the exception of a negative relationship in the female stimulus context ($F_{1,35.43}$

$= 5.3871$, $P = 0.0262$). Further, no significant relationship was found between PC1 behaviors and either (i) spine color ($P \geq 0.0512$; Table S8), (ii) lateral body color ($P \geq 0.5341$; Table S9), or (iii) throat chroma ($P \geq 0.244$; Table S10) across any behavioral context.

Lastly, regression analyses of male coloration and body size in experimental males revealed no relationship between size and pelvic spine color ($F_{1,11.95} = 1.2145$, $P = 0.2921$), PC1 lateral color ($F_{1,11.13} = 0.0019$, $P = 0.9657$), or throat chroma ($F_{1,11.63} = 0.1152$, $P = 0.7404$). The same analyses in control males revealed body size to be significantly positively associated with spine color ($F_{1,8} = 16.588$, $P = 0.0036$), but not throat chroma ($F_{1,8} = 0.0023$, $P = 0.9628$) or lateral body color ($F_{1,8.49} = 5.0460$, $P = 0.0530$).

Discussion

We investigated the expression of male stickleback spine, throat and lateral body coloration across reproductive stages and behavioral contexts, comparing how ornaments that differ in plasticity and potential for concealment are utilized. We found that spine color is more static than throat and lateral body color. We also found a positive association between the red color intensity of the pelvic spines and lateral body. Pelvic spine erection frequency varied across behavioral contexts, with males erecting their spines more when interacting with conspecifics or a model predator than when in isolation or in a constrained social context. In addition, the frequency of pelvic spine erections was positively associated with rates of 'lead to nest' and 'flee,' behaviors which may enhance spine color visibility. Together, these
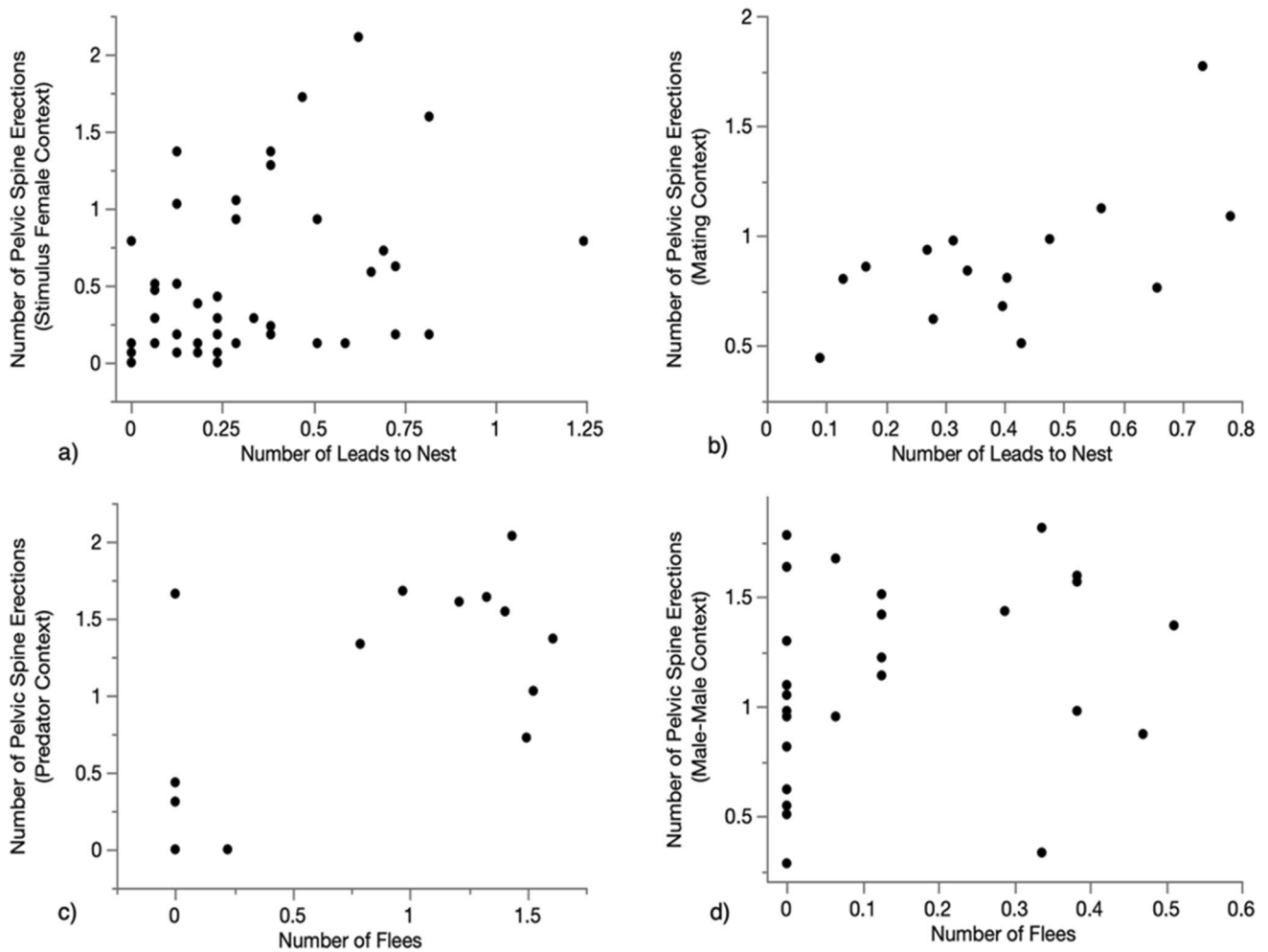
Fig. 4 Plots showing a significantly positive association between the number of pelvic spine erections and leads to nest in the (**a**) stimulus female and (**b**) mating contexts, as well as between the number of pelvic spine erections and flees in the (**c**) predator context. No significant relationship was found between the number of pelvic spine erections and flees in (**d**) the male-male context

Table 1 Percentages of pelvic spine erections performed within ± 5 s of either a lead or flee and percentages of leads/flees performed within ± 5 s of a pelvic spine erection. Flees and leads to nest were analyzed as these behaviors would position the fish in front of the other in such a way as to make spine color especially conspicuous when the pelvic spines were erected. Percentages calculated include only videos from each behavioral context where the main behavior of interest (i.e. either pelvic spine erection or lead/flee) occurred

Behavioral context	Behavior performed: leads or flees	Percentage of pelvic spine erections performed within 5 s of a flee/lead		Percentage of leads/flees performed within 5 s of a pelvic spine erection	
		n	Mean % ± SEM	n	Mean % ± SEM
Female stimulus	Leads to nest	40	6.35 ± 1.68	38	9.75 ± 3.10
Mating	Leads to nest	15	18.84 ± 4.23	15	37.28 ± 5.47
Male-male	Flees	29	9.47 ± 2.54	14	82.56 ± 8.08
Predator	Flees	12	48.25 ± 10.36	10	53.47 ± 9.80

results suggest that spine coloration may allow facultative expression of a red ornament and may serve functions across social behavioral contexts.

Static spine and plastic body color

Pelvic spine coloration does not vary significantly over the reproductive cycle in males and is therefore less plastic than

throat and body color. This finding is broadly consistent with the only other study of spine color across time (Amundsen et al. 2015), which found only minor variation between the early and post-spawning season periods (May–Oct). In addition, observations of these color patches during times of stress (e.g., during times of prolonged transport and/or handling) have revealed greater stability of pelvic spine than body red color intensity (Amundsen et al. 2015; Wright et al. 2016; CMA, personal observation). Together, these lines of evidence reveal patterns of stickleback red coloration relative to different areas of the body that are not widely appreciated. As well, spine color appears to be less dimorphic in expression than throat color, which is infrequently present in both sexes (McKinnon et al. 2000; Amundsen et al. 2015; Yong et al. 2016), indicating a possible difference in selection and/or function between the color patches despite similarity of hue.

Because spine color is largely hidden at rest, it is likely that spine color patches are under different evolutionary and functional constraints than red patches along the body. The ability to fold spines and reduce their conspicuousness may enable them to retain high red coloration at lower cost. Because spine color is present in several species of Gasterosteidae (Bigelow and Schroeder 1953; Rowland 1974; McLennan 1996; Ostlund-Nilsson et al. 2006; Hodgson et al. 2013), and several Gasterosteids are known to use pelvic spine erections in both courtship and/or threat displays (van Iersel 1953; Morris 1958; Reisman 1963; McInerney 1969; McKenzie 1969; Rowland 1974; Ward and McLennan 2006), spine color's role in sexual, social, and/or defensive interactions may be ancestral in the family. Certainly, it is widespread.

We found significant positive relationships, for experimental males, between color of the spine and body, as well as the body and throat, though not between spine and throat. Color patches along the throat and body have typically been considered together in terms of their roles in both sexual and social contexts (Rowland 1984; McLennan and McPhail 1990; Candolin and Tukiainen 2015). The positive association between redness along the body and spines in males may be due to closely related functions, where red along the body is conspicuous when seen from the front or side, while red along the pelvic spines augments body color and is especially visible when seen from behind and/or swimming away. Static red spine coloration may also enable a stickleback to produce a conspicuous display early in reproduction before body color is extensive. However, while the positive associations between color patches are consistent with some previous research (Yong et al. 2013, 2016), their generality awaits confirmation since populations may vary in nuptial color hue, the pattern of expression across reproductive stages, and sexual dichromatism (Scott and Foster 2000; Foster et al. 2008; Amundsen et al. 2015). Stickleback

examined here were all males from a population highly sexually dimorphic in red color expression along the body, though less so in the spines.

Pelvic spine erections and associated behaviors with conspecifics

Our work revealed pelvic spines to be erected in all experimental interaction types, both social and with a predator, but almost not at all within the socially isolated control males. Pelvic spine erections were utilized when interacting with a free-swimming female during courtship and, to a lesser degree (likely owing to the constraint on interactions of the plexiglass box), the enclosed stimulus female. For the enclosed stimulus female interaction, the most standardized presentation in our study, pelvic spine erection frequency peaked during stage 2 (i.e., courtship stage), then fell steadily across successive stages, pointing to pelvic spine erections functioning during courtship and loosely following similar trends detected by Symons (1966) of a decrease in spine erections while approaching females after the courtship phase. We also uncovered a positive association between the number of pelvic spine erections and leads to nest by males in both mating and enclosed stimulus female contexts and a higher percentage of pelvic spine erections performed within $\pm$ 5 s of a lead to nest within the mating context. These results are also consistent with a role for pelvic spines during mate attraction, as are previous findings of a female preference for pelvic spine symmetry (e.g., Mazzi et al. 2003, 2004). It is possible that erecting the spines, while attempting to lead a female to a nest, presents additional red color to potential mates and draws attention to the courting male, increasing the chances of successful courtship (e.g., Milinski and Bakker 1990; Bakker and Milinski 1993; Bakker and Mundwiler 1994).

Compared to controls, pelvic spine erection rates by nesting males were also elevated during interactions with a rival male. Pelvic spine erections have been suggested to occur as a signal of aggressiveness during social displays (van Iersel 1953) or in response to fright and/or arousal (Symons 1966). Social interactions by a parental male occur with females but also conspecific males, in which the parental male is often aggressively defending his territory from invaders and rivals (Rowland 1982; Bakker 1994) or fleeing attack. We did not find a significant association between the number of pelvic spine erections and number of flees when interacting with a conspecific male. However, a high percentage (82.6 $\pm$ 8.1% SE) of flees during male-male interactions were performed within 5 s of a pelvic spine erection, possibly suggesting the importance of displaying the spines to a rival while swimming away. This may be to appear larger, make the defensive weapon more visible, simply make the male harder to attack, or display the red color patch along the pelvic spines

and signal aggression. These possibilities are not mutually exclusive and possibly all are relevant.

Has spine color evolved for increased conspicuousness to predators?

Pelvic spines in threespine stickleback are rigid, sharply pointed, and function in defensive situations by making these small fish larger (in terms of the gape needed to ingest them), harder to eat, and more threatening to potential predators (Hoogland et al. 1956; Reimchen 1994). Stickleback have the ability to lock their spines in place (Hoogland 1951; Reimchen 1983), which creates a sturdy anti-predator defense, particularly during prolonged attack. Predators such as pike and perch consequently have much greater difficulty eating stickleback with pelvic spines erected and learn to leave stickleback alone while still eating other fish that lack spines (Hoogland et al. 1956; Morris 1958). However, stickleback can also switch their pelvic spines between erect and not-erect rapidly, which allows them to use these defensive weapons to signal aggression or defensive abilities, but still swim and maneuver normally when erect spines are not needed.

The significant relationship we observed between pelvic spine erections and flees during interactions with a model predator supports assertions by van Iersel (1953) of such an association. As previously discussed, red coloration along the spines is relatively inconspicuous when the pelvic spines are held against the body, but especially visible from behind when spines are raised. Together with increased frequency of spine raising when a model predator is presented, this raises the possibility that pelvic spine coloration has evolved, at least in part, to enhance spine conspicuousness to predators. The presence of spine coloration in both sexes, though sometimes reduced in females (Nordeide 2002; Amundsen et al. 2015; Wright et al. 2016; Yong et al. 2016), is consistent with this hypothesis, especially since females do not have to defend nests or compete as intensely for mates. Notably, males are reported to preferentially court females with less red coloration along the pelvic spines (Nordeide 2002). Experimental tests of the predation hypothesis of spine color evolution are called for in light of these observations.

The present evidence does not allow for discrimination between the hypothesis that spine color has evolved for use in anti-predator displays versus alternative interpretations. While the possibility is intriguing that red coloration on spines functions both in social interactions with conspecifics and as part of an anti-predator display, spine color could have evolved mainly in the context of social interactions (but see Kroken et al. 2021) and simply have little cost when spines are erected by a fleeing fish that has already been sighted by a predator. Similarly, reduced spine dichromatism, relative to body color, is consistent with red spines being favored by an agent of natural selection such as predation. However, reduced costs of spine color, owing to being concealable, may result in a net overall benefit of red spines in females even if only favored weakly by sexual or social selection. Taken a step further, this logic can also account for weak dichromatism even if red spine color only benefits males, through reduced costs to females.

Conclusions

Our results provide novel evidence of the relatively static nature of pelvic spine coloration and its association in expression intensity with other color patches along the male stickleback's body. Further, we have documented the occurrence of pelvic spine erections across social and predation contexts, as well as significant associations between pelvic spine erections and behaviors that would expose pelvic spine color while swimming away (i.e., 'flees' and 'leads to nest'). Our findings are consistent with pelvic spine coloration functioning in sexual/social contexts and possibly also as an anti-predator defense mechanism, although experimental tests of function are needed.

Declarations

Ethics approval This study complied with and followed rules governed by the Institutional Animal Care and Use Committee (IACUC) at East Carolina University (Animal Use Protocol #D349). This work was possible because of approval by the British Columbia Ministry of Forests, Lands, and Natural Resource Operations for fish collection (Permit Numbers MRNA17-262956 and MRSU18-290075).

Consent for publication The authors of this work consent to publication of this manuscript in Behavioral Ecology and Sociobiology

Conflict of interest The authors declare no competing interests.

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