

The relative influences of sexual and natural selection upon the evolution of male nuptial colouration in the brook stickleback, *Culaea inconstans*

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Summary

- (1) Previous research suggests that the temporal cycling of nuptial colour in a population of brook stickleback, *Culaea inconstans*, from the Atlantic lineage mirrors that described for its close relative, the three-spine stickleback, *Gasterosteus aculeatus*.
- (2) We report an alternative cycling of nuptial colour in a population of brook sticklebacks from Nebraska corresponding to the Mississippian lineage. Males in this population do not assume characteristic black nuptial dress during courtship activities but do become highly melanic during the final days of fry guarding.
- (3) We also report the first evidence of the plesiomorphic nuptial barring pattern in female brook sticklebacks.
- (4) The loss of the nuptial signal during courtship and its maximal expression during parental care likely reflects fluctuating relative influences of sexual and natural selection vectors and suggests that at least two pathways of colour evolution are operating within the stickleback family.
- (5) Geographic variability in the nuptial colour signal of the brook stickleback may have significant implications for mate recognition, should allopatric divergent populations be brought back into contact.

Keywords: stickleback, nuptial colour, *Culaea inconstans*, *Gasterosteus aculeatus*.

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Introduction

The sexually dimorphic colouration displayed by many species of freshwater fish is often the product of a complicated interaction between sexual and natural selection. For example, male guppies, *Poecilia reticulata*, develop a colour signal of black, orange and blue spots on a silver grey background. Generally, females preferentially mate with males that exhibit more and larger orange spots (Endler, 1983; Kodric-Brown, 1985; Houde, 1987), but the trait and preference often vary geographically with respect to predation pressure. Males from high predation environments typically have fewer spots and exhibit a reduction in patch size (Endler, 1978, 1980) while corresponding females prefer duller males (Breden & Stoner, 1987; Stoner & Breden, 1988; Houde & Endler, 1990; Endler & Houde, 1995) and/or show a reduction in mate preference when the risk of predation is increased (Godin & Briggs, 1995). Geographic variation in the male trait and the female preference are correlated (Houde & Endler, 1990) and a reduction in the intensity of the female preference for conspicuous males reflects the balance between natural and sexual selection (Endler, 1978; Godin & Briggs, 1995).

Male threespine sticklebacks, *Gasterosteus aculeatus*, develop an equally complicated nuptial signal, although their colours are presented in larger swathes of red, blue and black and are labile, rather than fixed. When given the option, female threespine sticklebacks usually prefer to spawn with more intensely red males, possibly because intensity reflects male condition, dominance status, parental abilities and/or resistance to some types of parasites (reviewed in Candolin, 2000). Threespine stickleback lineages that have been selectively bred for dominance, however, experience a moderate increase in levels of male aggression and colour, which is quickly countered by increasing female infertility (natural selection) and decreasing courtship success by the brightest and most aggressive males (intersexual selection). The extent to which the male signal can be elaborated is thus determined by the strength of the female preference matched against the strength of all the opposing selection vectors (Bakker, 1986; Ward & FitzGerald, 1987).

McLennan (et al., 1988; McLennan, 1991, 1996) examined the relative influences of three different selection vectors, intersexual, intrasexual and natural selection, on the evolution of male nuptial colouration in the stickleback family Gasterosteidae. Based on the relationship between the origin

and elaboration of the colour signal and of behaviours associated with different selective regimes (e.g., courtship behaviours and intersexual selection), she hypothesized that intersexual selection, reinforced by natural selection during paternal care, were the dominant forces favouring the evolution of the male signal once it originated. As discussed above, numerous researchers have documented female preference for intensely coloured males, so the phylogenetic hypothesis simply corroborated something that we already knew. But those patterns also predicted something novel: if the development of the male signal across the breeding cycle reflected the relative importance of the three selection vectors, then colour should be at its lowest level during nest-building, with a peak during courtship and a second but lower peak during fry guarding. This phylogenetic prediction was subsequently confirmed for an anadromous population of threespine sticklebacks (McLennan & McPhail, 1989a). Field observations have also corroborated the prediction for various threespine stickleback populations from North America and Europe (van Iersel, 1953; Milinski & Bakker, 1990; Bakker 1994; McKinnon, 1994).

To demonstrate that the phylogenetic patterns are telling us something about underlying evolutionary processes, we need to test pattern-based hypotheses for more than one species. The nuptial signal of the brook stickleback, *Culaea inconstans* is simpler than that of the threespine stickleback. It consists of a deep jet-black body, fins and spines, and a black vertical bar over a golden iris (Winn, 1960; Reisman & Cade, 1967; McKenzie, 1969a, b). Observations in the field and in the lab have suggested that males are most intensely coloured during courtship, as predicted (Hall, 1956; Winn, 1960; Reisman & Cade, 1967; McKenzie, 1969a). An investigation of colour cycling in a population of brook sticklebacks from southern Ontario revealed a pattern identical to that described for the threespine stickleback; colour intensity is low during the territory acquisition/nest-building stage, peaks during courtship activities, declines in the early stages of parental care and reaches a second, but lower, peak with the emergence of fry from the nest (McLennan, 1993a). The study also showed that males increase the intensity of their signal more to the presence of a courting female than to a rival male during the courtship stage of the cycle. At all other stages, males react in the same way to both male and female intruders. These results imply that 'black' serves as a threat signal during territory acquisition, parental care, and during courtship when a rival appears. It is only during the courtship of a receptive female

that the male achieves his most intense colour, as sexual motivation replaces aggression.

Recent phylogeographic analysis has suggested that *Culaea inconstans* comprises two, and possibly three, distinct lineages. The two oldest clades are hypothesized to have originated sometime during the Pliocene and to have sheltered in different refugia during the Pleistocene glaciations (Mississippian versus Atlantic) (Mattern, 2005). Cycling of male colour has been documented for a population corresponding to the Atlantic lineage (McLennan, 1993a) and in this paper, we report the results of a study documenting male colour changes across a complete breeding cycle for a population from the Mississippian lineage. We ask two questions: (1) is the pattern of colour change the same as that reported for the Atlantic brook stickleback lineage? (2) If not, how have the relative contributions of intersexual, intra-sexual and natural selection changed? We discuss the results with reference to the plesiomorphic pattern of colour cycling described for the Gasterosteidae (McLennan, 1996) and suggest that there may be two pathways of colour evolution operating in the stickleback family.

Materials and methods

Study animals

Adult brook stickleback were collected in minnow traps from Sutherland Creek, Nebraska (41°08'13N, 101°07'28W) in May 2004 as they moved from deeper over-wintering waters into the shallow breeding areas. Sutherland Creek is moderately vegetated and steep banked, with shallow, clear water flowing gently over soft silt and pebble substrate, between adjacent farmlands. Fish were immediately transported back to the University of Toronto in chilled, continually aerated water. Once there, they were maintained in a large opaque tank with continual flow-through of dechlorinated water. To slow the rate of sexual maturation, fish were held under winter conditions (10–12°C, 12 h : 12 h light-dark cycle) and high population density (Reisman 1961; Reisman & Cade, 1967). Reproductive behaviour was further discouraged by a lack of suitable nest-building materials and substrate, although large sticks and stones were provided for shelter.

Individuals were removed as needed from the winter holding tank and placed in 58-liter community tanks, lined with 2.5 cm of fine gravel, fitted

with a charcoal filter and aeration system, and covered on three sides with light green paper. These tanks were maintained under a 16 h light : 8 h dark regime, a constant temperature of 16°C, and low density (three fish per tank), all of which promote breeding (Winn, 1960; Reisman & Cade, 1967). Each tank was supplied with nesting material consisting of soft moss and algae and one or more aquatic plants. Under these conditions, fish usually became reproductively active within three days.

Individual male brook sticklebacks were introduced directly from the community tanks into 58-liter aquaria lined with 2.5 cm of fine gravel and fitted with a charcoal filter and aeration system in one corner. Two sides of each tank were covered with light green paper and a third with a photographic backdrop of aquatic vegetation. To minimize disturbance, a green curtain was hung in front of each tank and removed during filming. Each tank was supplied with *Hygrophila* rooted in a peat-filled 10 cm diameter plastic dish, and an abundance of soft algae, grasses, and twigs collected from a nearby marsh. All males who successfully completed a breeding cycle began nest building in the *Hygrophila* within 72 hours. Males that failed to build a nest within five days were replaced.

The reproductive cycle

Following the experimental protocol of McLennan (1993a), territorial male brook sticklebacks were presented with captive male and female intruders at six successive stages of the breeding cycle. For each presentation, a nuptially coloured, sexually mature male or female stimulus fish was placed in a 12 × 5.5 cm glass jar, capped with fine mesh to permit the exchange of chemical cues (McLennan, 2004, 2005). The size of the jar restricted excess movement, minimizing the behavioural response of the intruder. Presentation order of the male and female intruder was randomized and each male was permitted a three hour rest between trials. All presentations were videotaped for five minutes using a SONY digital handycam. The results of the behavioural analysis are reported in a previous paper (Ward & McLennan, 2006).

Male sticklebacks have four distinct breeding phases, broadly categorized as territory acquisition and nest building, courtship, egg-guarding and fry guarding (van Iersel, 1953; Winn, 1960; Sevenster, 1961; Reisman & Cade, 1967; Kynard, 1978). Fry guarding may be subsequently broken into three

additional sub-stages corresponding to the age and development of the fry in the nest (McLennan, 1993a). Presentations were therefore made at the following six stages:

- (i) Territory acquisition and nest building phase (Day 2): The male is engaged in nest construction.
- (ii) Courtship phase (Day 3): The nest is complete and the male actively courts attending females. Following presentations on this day, males were permitted to spawn with a gravid female.
- (iii) Egg-guarding phase (Day 6): The offspring are approximately half-way through embryonic development and the male spends most of his time fanning (aerating) the eggs and removing those that are damaged or diseased (McKenzie, 1974).
- (iv) Fry hatching phase (Day 10): The newly hatched fry are immobile within the nest. The parental male begins to tear apart the nest and construct a loosely tangled algal nursery.
- (v) Post-nursery construction phase (Day 12): The nursery is complete and the fry have been transferred to the new structure.
- (vi) Free-swimming fry phase (Day 17): The fry begin attempting to leave the nursery but the male actively retrieves them in his mouth and returns them to the nursery.

Colour scoring

The intensity of body colour, fin colour and spine colour, as well as the distribution of melanophores and the development of eye-bars, were scored prior to, and immediately after, each presentation. An intensity value ranging from 0 (least intense) to 10 (most intense) was assigned over five bodily regions (Figure 1) by comparing body hue with the grayscale colour series in the *Naturalist Colour Guide* (Smithe, 1975). Summing the values produced an overall color value ranging from 0 (no colour) to 50 (intensely black). Because colour distribution is frequently irregular, the distribution of melanophores at each of the five regions was categorized as silver (no melanophores apparent), speckled or solid (no silver present).

In all fins, the melanophores are concentrated along the fin rays and occasionally scattered upon the interradial membranes (McLennan, 1993a). Fin colour and spine colour were recorded on a scale of 0-2 corresponding to no colour (0), speckled melanophore distribution (1) and solid black colour (2).

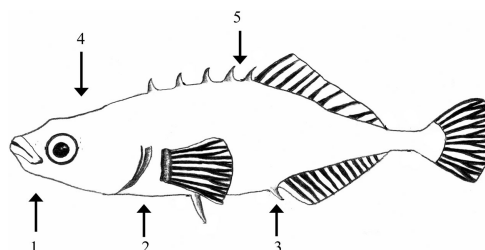


Figure 1. Five regions in which colour was scored for both intensity and distribution of melanophores. (1) Lower jaw and throat; (2) ventral surface from throat to pelvic spines; (3) ventral-lateral surface from midway between pelvic and anal spines to caudal fin; (4) dorsal-lateral surface from snout to a point above the pelvic spines; (5) dorsal lateral surface from the point above the pelvic spines to the caudal fin. (Modified from McLennan, 1993a).

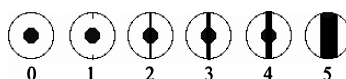


Figure 2. Intensity scores assigned to eye-bar state in order of increasing development (Modified from McLennan, 1993a).

Finally, eye bar development was assigned a value from 0 (no eye bar) to 5 (well developed vertical barring) (Figure 2). Colour was also scored on each non-presentation day to assess the endogenous cycling of body colour in the absence of conspecific stimulation.

Results

Of the initial twenty-three males selected for breeding trials, seven failed to build nests and five spawned with a gravid female but did not successfully raise the eggs to hatching. Consequently, results during the nest-building and courtship stages are based upon the sixteen males that successfully spawned with a female and results during the parental care stages are based upon the eleven males who completed the breeding cycle. Of those eleven males, eight were guarding one clutch, and three were guarding two clutches. Mann-Whitney U tests confirmed that there were no significant differences in the colour intensity of these two groups at any stage of the breeding cycle [male intruder: nest ($z = -1.23$, $p = 0.28$), court ($z = -0.31$, $p = 0.78$), egg-guarding ($z = -0.20$, $p = 0.92$), fry hatch ($z = -1.02$, $p = 0.31$), nursery moved ($z = -0.20$, $p = 0.92$), free-swimming fry ($z = -0.41$, $p = 0.78$);

female intruder: nest ($z = 0.00$, $p = 1.00$), court ($z = -0.10$, $p = 0.92$), egg-guarding ($z = 0.00$, $p = 1.00$), fry hatch ($z = -1.03$, $p = 0.38$), nursery moved ($z = -0.83$, $p = 0.50$), free-swimming fry ($z = -1.46$, $p = 0.19$), nor was there a difference during the pre-parental stages in the colour intensity of males who completed the breeding cycle versus those whose clutches failed [male intruder: nest ($z = -0.28$, $p = 0.83$), court ($z = -0.91$, $p = 0.38$); female intruder: nest ($z = -0.23$, $p = 0.83$), court ($z = -0.40$, $p = 0.74$)]. All males were subsequently pooled and statistical analysis undertaken via non-parametric Wilcoxon sign rank tests, unless otherwise stated.

Temporal cycling in the intensity and distribution of body colour

Territorial male brook sticklebacks all displayed a silver or lightly speckled lower jaw and ventral surface and individual dorsolateral hue ranged from a speckled, olive-undertoned light gray to a speckled, moderate gray (Figure 3). The colour response of a territorial male towards either a male or female intruder is constant across the nest-building and courtship stages, as well as across the fry hatching and post-nursery construction stages. The transition from the courtship to the egg-guarding stage, however, is accompanied by a significant increase in body colour intensity (male intruder: $z = -1.96$, $p = 0.05$; female intruder: $z = -2.22$, $p = 0.03$). Despite the low intensity values recorded, a comparison of mean before-interaction and after-interaction colour scores confirmed that the focal males did respond to the conspecific stimulation with an increase in colour intensity at each stage of the breeding cycle [male intruder: nest ($z = -3.30$, $p = 0.001$), court ($z = -3.18$, $p = 0.001$), egg-guarding ($z = -2.26$, $p = 0.02$), fry hatch ($z = -2.69$, $p = 0.007$), nursery moved ($z = -2.37$, $p = 0.02$), free-swimming fry ($z = -2.67$, $p = 0.008$); female intruder: nest ($z = -2.70$; $p = 0.007$), court ($z = -2.92$; $p = 0.003$), egg-guarding ($z = -2.43$; $p = 0.02$), fry hatch ($z = -2.38$, $p = 0.02$), nursery moved ($z = -2.20$, $p = 0.03$), free-swimming fry ($z = -2.52$, $p = 0.01$)], suggesting that low intensity values across the majority of breeding stages are characteristic for the population as a whole. Once the fry begin to leave the nest, males achieve characteristic deep black dorsolateral colouration, a solid black lower jaw and a speckled ventral surface from the operculum to a point approximate to the pelvic spines (Figure 3). In this final stage of parental care, body colour is

Table 1. Results of Wilcoxon sign rank tests of differences in body colour scores in response to either a male or female intruder (pre-parental stages: $N = 16$; parental care stages: $N = 11$).

Breeding cycle stage	Free swimming fry			
	Male intruder		Female intruder	
	z	p	z	p
Nest	-2.86	0.004	-2.94	0.003
Courtship	-2.93	0.003	-2.94	0.003
Egg-guarding	-2.93	0.003	-2.93	0.003
Fry Hatch	-2.93	0.003	-2.93	0.003
Nursery Moved	-2.81	0.005	-2.94	0.003

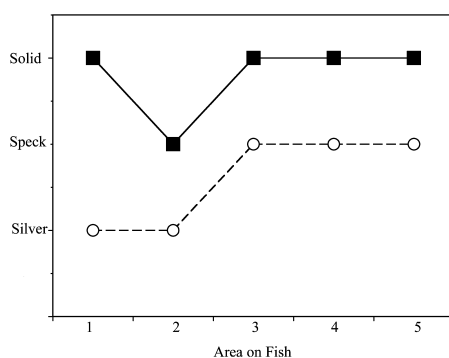
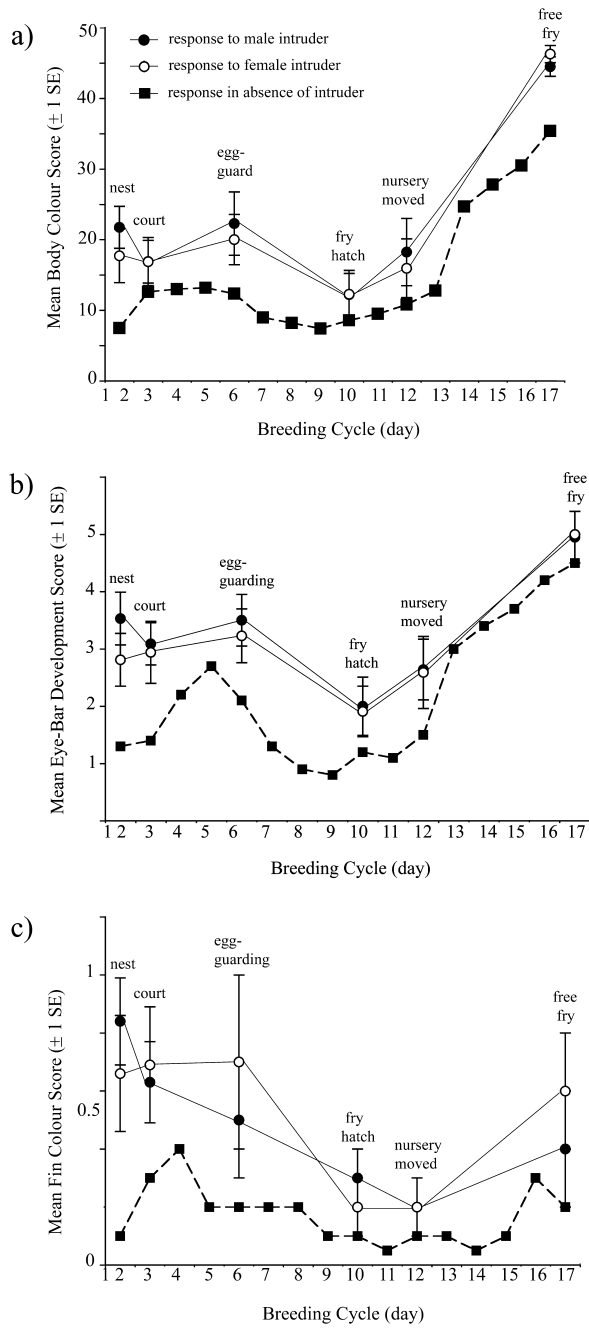


Figure 3. Distribution of melanophores across the body corresponding to: (○) the nest-building, courtship, egg-guarding, fry hatch and post-nursery construction phase; (■) the final stage of parental care corresponding with the emergence of fry from the nest.

significantly more intense than at any of the preceding stages (Table 1; Figure 4a). In the absence of conspecific stimulation, the endogenous cycling of body colour is lower in intensity, but shows a similar pattern of temporal cycling (Figure 4a).

There is no difference in the intensity of a territorial male's colour response towards a male, versus a female, intruder at any stage of the breeding cycle (nest: $z = -1.02$, $p = 0.31$; court: $z = -0.11$, $p = 0.91$; egg-guarding: $z = -0.36$, $p = 0.72$; fry hatch: $z = -0.17$, $p = 0.87$; nursery moved: $z = -0.98$, $p = 0.33$; free-swimming fry: $z = -1.13$, $p = 0.26$), although Levene equality of variance tests indicate that the range of colour scores in courting males is narrower in response to a female intruder during the courtship stage ($F = 4.65$, $p = 0.04$). At all other stages of the breed-



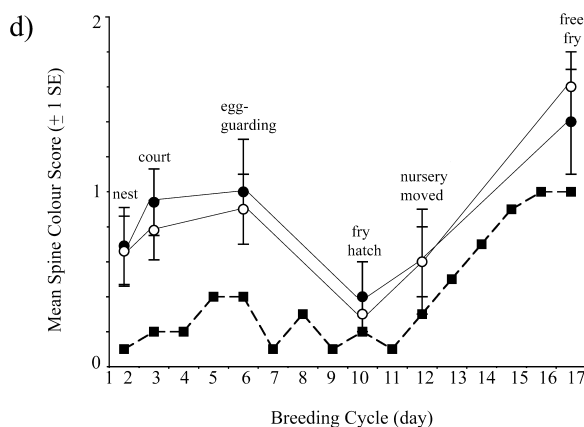


Figure 4. Mean total scores corresponding to changes in intensity over the breeding cycle for male *Culaea inconstans* ($N = 11$): (a) body colour; (b) degree of eye-bar development; (c) caudal fin colour; (d) pelvic spine colour. For all graphs, (○) = response to a female intruder; (●) = response to a male intruder. Dashed line = score recorded on each non-presentation day.

Table 2. Results of Wilcoxon sign rank tests in the degree of eye-bar development in response to either a male or female intruder (pre-parental stages: $N = 16$; parental care stages: $N = 11$).

Breeding cycle stage	Free swimming fry			
	Male intruder		Female intruder	
	z	p	z	p
Nest	-2.38	0.02	-2.68	0.01
Courtship	-2.68	0.01	-2.23	0.03
Egg-guarding	-2.38	0.02	-2.54	0.01
Fry Hatch	-2.69	0.01	-2.81	0.01
Nursery Moved	-2.67	0.01	-2.54	0.01

ing cycle, the range of inter-male variation does not differ significantly with respect to the sex of the intruder (nest: $F = 1.28$, $p = 0.27$; egg-guarding: $F = 1.70$, $p = 0.21$; fry hatch: $F = 0.16$, $p = 0.70$; nursery moved: $F = 0.44$, $p = 0.52$; free-swimming fry: $F = 0.00$, $p = 0.99$). Variation in male body colour scores is generally extensive throughout the breeding cycle until the final stage of parental care. Once the fry begin attempting to leave the nest, inter-male variation in hue intensity decreases, with the majority of males corresponding to the highest score interval.

Temporal cycling in the intensity of eye bar development

Figure 4b depicts the mean eye-bar development scores in response to a male or female intruder across the breeding cycle. A comparison of the mean scores indicates that eye-bar development does not differ significantly with respect to the sex of the intruder at any stage of the breeding cycle (nest: $z = -1.64$, $p = 0.10$; court: $z = -0.36$, $p = 0.72$; egg-guarding: $z = -1.32$, $p = 0.19$; fry hatch: $z = -0.06$, $p = 0.95$; nursery moved $z = -0.58$, $p = 0.57$; free-swimming fry: $z = -1.0$, $p = 0.32$). Eye intensity is moderate and constant across the nest building, courtship and egg-guarding stages. In response to both a male and female intruder, the transition between egg-guarding and fry hatching is accompanied by a significant decrease in eye-bar development (male intruder: $z = -2.53$, $p = 0.01$; female intruder: $z = -2.33$, $p = 0.02$). After the eggs hatch, eye-bar development gradually increases to peak levels, corresponding to the emergence of fry from the nest. Eye-bar development is significantly greater during the final stage of parental care than it is during any of the preceding breeding stages (Table 2; Figure 4b).

The range of eye-bar development values is wide in response to both a male and female intruder throughout the breeding cycle until the emergence of free-swimming fry, when eye-bar development becomes restricted to the highest intensity categories. During the final stage of parental care, eye-bars are wide and well developed. In extreme cases, the iris may become entirely black.

Temporal cycling in the intensity of fin colour

In response to both a male and female intruder, the caudal fin is significantly lighter in colour intensity than either the dorsal or the anal fin during the courtship (male intruder: caudal/dorsal: $z = -2.43$, $p = 0.02$; caudal/anal: $z = -2.43$, $p = 0.04$; female intruder: caudal/dorsal: $z = -2.12$, $p = 0.03$; caudal/anal: $z = -2.12$, $p = 0.03$) and free-swimming fry stages (male intruder: caudal/dorsal: $z = -2.12$, $p = 0.03$; caudal/anal: $z = -2.27$, $p = 0.02$; female intruder: caudal/dorsal: $z = -2.07$, $p = 0.04$; caudal/anal: $z = -2.07$, $p = 0.04$) and significantly lighter than the anal fin after nursery construction (male intruder: $z = -2.06$, $p = 0.04$; female intruder: $z = -2.06$, $p = 0.04$). Additionally, in response to a male intruder, the caudal fin is significantly lighter than the anal fin during the courtship stage

Table 3. Results of Wilcoxon sign rank tests in the intensity of fin colouration in response to either a male or female intruder (pre-parental stages: $N = 16$; parental stages: $N = 11$).

Dorsal fin	Male intruder							
	Nest-build		Courtship		Egg-guard		Free Fry	
	z	p	z	p	z	p	z	p
Fry hatch	-2.26	0.02	-2.26	0.02	NS	NS	NS	NS
Nursery moved	-2.05	0.04	-2.02	0.04	NS	NS	NS	NS
Free fry	-2.27	0.02	NS	NS	NS	NS	NS	NS
	Female intruder							
Fry hatch	-2.03	0.04	-2.38	0.02	NS	NS	-2.57	0.01
Nursery moved	NS	NS	-2.21	0.03	-2.13	0.03	-2.16	0.03
Caudal fin	Male intruder							
	Nest-build		Courtship		Egg-guard		Free Fry	
	z	p	z	p	z	p	z	p
Fry hatch	-2.21	0.03	-2.06	0.04	NS	NS	NS	NS
Nursery moved	-2.07	0.04	-2.12	0.03	NS	NS	NS	NS
Free fry	-2.23	0.03	NS	NS	NS	NS	NS	NS
	Female intruder							
Fry hatch	-2.03	0.04	-2.23	0.03	-2.12	0.03	NS	NS
Nursery moved	-2.03	0.04	-2.46	0.02	-2.06	0.04	NS	NS
Anal fin	Male intruder							
	Nest-build		Courtship		Egg-guard		Free Fry	
	z	p	z	p	z	p	z	p
Fry hatch	-2.34	0.02	-2.26	0.02	NS	NS	NS	NS
Nursery moved	-2.05	0.04	NS	NS	NS	NS	NS	NS
Free fry	-2.33	0.02	NS	NS	NS	NS	NS	NS
	Female intruder							
Fry hatch	-2.06	0.04	NS	NS	-2.60	0.01	-2.57	0.01
Nursery moved	NS	NS	NS	NS	-2.18	0.03	-2.01	0.04

($z = -2.07$, $p = 0.04$). In response to both a male or female intruder, all fins are lightly tinged during the nest-building and courtship stages. The caudal fin flushes a creamy white while the fry are nest bound. The dorsal and anal fins remain speckled through egg-guarding, then lose colour during the first

Table 4. Results of Wilcoxon sign rank tests of differences in spine colour scores in response to either a male or female intruder (pre-parental stages: $N = 16$; parental stages: $N = 11$).

Breeding cycle stage	Free swimming fry			
	Male intruder		Female intruder	
	z	p	z	p
Nest	NS	NS	-2.69	0.01
Courtship	NS	NS	-2.59	0.01
Egg-guarding	NS	NS	-2.21	0.03
Fry Hatch	-2.41	0.02	-2.98	<0.01
Nursery Moved	-2.07	0.04	-2.52	0.01

days of fry guarding, when the dorsal fin also becomes a conspicuous creamy white colour. Once the young begin to leave the nest, all fins become lightly speckled again (Table 3; Figure 4c).

Temporal cycling in the intensity of spine colour

Dorsal spines are significantly darker than anal spines and pelvic spines during the nest-building/territory acquisition stage in response to a male intruder (dorsal/pelvic: $z = -2.0$; $p = 0.05$; dorsal/anal: $z = -2.8$, $p = 0.01$) and significantly darker than anal spines following nursery movement ($z = -2.13$, $p = 0.03$). There are no other differences between the dorsal, anal and pelvic spines. Subsequent temporal analysis was therefore conducted upon pelvic spine colour. The general trends found for the intensity of spine colour mirror those reported for other components of the nuptial signal (Figure 4d). In response to an intruder of either sex, the intensity of spine colouration during the nest-building, courtship and egg-guarding stages is moderate and constant and the transition to fry care is accompanied by a significant decrease in spine colour intensity (male intruder: $z = -2.03$, $p = 0.04$; female intruder: $z = -2.22$, $p = 0.03$). In the latter stages of parental care, spine colour intensity gradually increases, achieving peak levels with the emergence of fry from the nest. In response to a female intruder, spine colour during the last stage of parental care is significantly more intense than at any of the preceding stages and is significantly more intense than the previous two parental care stages when the territorial intruder is male (Table 4; Figure 4d).

The female nuptial signal

A total of twenty-eight females were observed during spawning in the experimental tanks ($N = 25$) and in the stock tanks ($N = 3$). Of the twenty-five females who spawned in the experimental tanks, fourteen laid eggs that were subsequently raised to fry by the male. All of these females developed very pale pinkish-gold dorsal coloration with extremely prominent vertical bars extending from the dorsal midline downward and terminating in the region of abdominal distention. The bars in the Nebraska population appeared more similar to the barring expressed by *Gasterosteus aculeatus* females, than the swirled pattern described for the population of brook sticklebacks from southern Ontario (McLennan, 1994a).

Discussion

Brook stickleback males from Nebraska do not develop the pattern of colour change across the breeding cycle that was predicted from the phylogenetic hypothesis (McLennan et al., 1988; McLennan, 1991, 1996). Rather than developing peak intensity during courtship, these males were moderately coloured for almost the entire cycle, boosting their signal to the 'characteristic' deep velvety black only in the final stages of parental care when the fry became free swimming (Table 5). We do not think that this pattern is an artefact of the small sample size or the test conditions because (i) males that spawned with a female but did not successfully raise offspring ($N = 5$) responded in a manner similar to successful males during courtship interactions, yet later failed to express the intense nuptial signal in the absence of viable offspring, and (ii), we did see spawnings in large stock tanks set up to provide an adequate supply of receptive females. Males built nests and raised offspring in those tanks; even in the presence of a rival none of those males developed intense black colour during courtship. Components of this unusual pattern have been reported from populations of melanic threespines on the west coast of North America. Males from the Queen Charlotte Islands, the Chehalis River system in Washington State and Pescadero Creek, California do not reach peak intensity until the latter stages of parental care, and were also described as being low-medium intensity during courtship (Moodie, 1972; von Hippel, 1999; Scott & Foster, 2000).

Table 5. Comparison of the male mosaic nuptial signal in populations from Nebraska and Ontario at each stage of the breeding cycle in response to differently sexed intruders (Tooley Creek: nest-building $N = 14$, remaining stages $N = 24$. Data from McLennan, 1994b).

	Nebraska Mean (SE)		Tooley Creek, Ontario Mean (SE)		Difference between populations (Mann-Whitney U test)	
	Male	Female	Male	Female	Male	Female
					z	p
Nest-building						
Body	21.75 (2.96)	17.71 (3.22)	14.86 (1.60)	14.50 (1.41)	-1.61	0.11
Eye-Bar	3.53 (0.46)	2.81 (0.46)	2.57 (0.36)	0.86 (0.25)	-1.91	0.06
Fins	0.84 (0.15)	0.63 (0.17)	1.36 (0.17)	0.71 (0.16)	-2.23	0.04
Spines	0.97 (0.18)	0.72 (0.16)	1.57 (0.14)	1.14 (0.18)	-2.50	0.02
Courtship						
Body	16.91 (3.34)	16.88 (2.44)	29.50 (2.36)	46.63 (0.46)	-2.88	0.003
Eye-Bar	3.09 (0.37)	2.94 (0.54)	3.67 (0.18)	4.88 (0.07)	-1.43	0.17
Fins	0.66 (0.14)	0.69 (0.16)	1.58 (0.12)	0.96 (0.04)	-3.97	<0.001
Spines	1.03 (0.18)	0.88 (0.16)	1.79 (0.85)	2.00 (0.00)	-3.54	0.001
Egg-guarding						
Body	22.27 (4.49)	20.0 (3.56)	26.33 (2.65)	31.96 (3.90)	-0.80	0.43
Eye-Bar	3.50 (0.45)	3.23 (0.47)	3.75 (0.25)	3.71 (0.34)	-0.41	0.71
Fins	0.48 (0.18)	0.68 (0.25)	1.46 (0.15)	1.46 (0.16)	-3.25	0.001
Spines	1.27 (0.22)	1.14 (0.24)	1.75 (0.11)	1.71 (0.11)	-2.34	0.05

Table 5. (Continued).

	Nebraska		Tooley Creek, Ontario		Difference between populations (Mann-Whitney <i>U</i> test)				
	Mean (SE)		Mean (SE)		Male		Female		
	Male	Female	Male	Female	<i>z</i>	<i>P</i>	<i>z</i>	<i>P</i>	
Fry hatch									
Body	12.23 (3.14)	12.27 (2.96)	27.79 (2.35)	28.21 (2.16)	-3.28	0.001	-3.43	<0.001	
Eye-Bar	2.0 (0.51)	1.91 (0.44)	3.75 (0.25)	3.79 (0.27)	-2.66	0.009	-3.19	0.001	
Fins	0.27 (0.10)	0.18 (0.10)	1.46 (0.13)	1.42 (0.15)	-4.15	<0.001	-4.00	<0.001	
Spines	0.73 (0.20)	0.45 (0.14)	1.79 (0.10)	1.75 (0.09)	-4.11	<0.001	-4.66	<0.001	
Nursery Moved									
Body	18.25 (4.76)	15.95 (4.16)	35.25 (1.98)	36.17 (1.97)	-3.02	0.002	-3.68	<0.001	
Eye-Bar	2.64 (0.53)	2.59 (0.63)	3.67 (0.27)	3.71 (0.32)	-1.65	0.12	-1.36	0.20	
Fins	0.23 (0.12)	0.23 (0.12)	1.58 (0.13)	1.58 (0.13)	-4.27	<0.001	-4.27	<0.001	
Spines	1.64 (0.20)	1.50 (0.23)	1.79 (0.08)	1.75 (0.09)	-3.97	<0.001	-3.82	<0.001	
Free-swimming fry									
Body	44.50 (1.38)	46.27 (1.21)	40.13 (1.19)	40.54 (0.97)	-1.62	0.12	-3.32	0.001	
Eye-Bar	4.95 (0.05)	5.00 (0.00)	3.92 (0.21)	4.04 (0.23)	-3.95	<0.001	-3.52	0.001	
Fins	0.41 (0.18)	0.55 (0.20)	1.63 (0.13)	1.50 (0.13)	-3.91	<0.001	-3.33	0.001	
Spines	1.36 (0.28)	1.64 (0.28)	1.79 (0.08)	1.79 (0.08)	-0.54	0.71	-1.18	0.37	

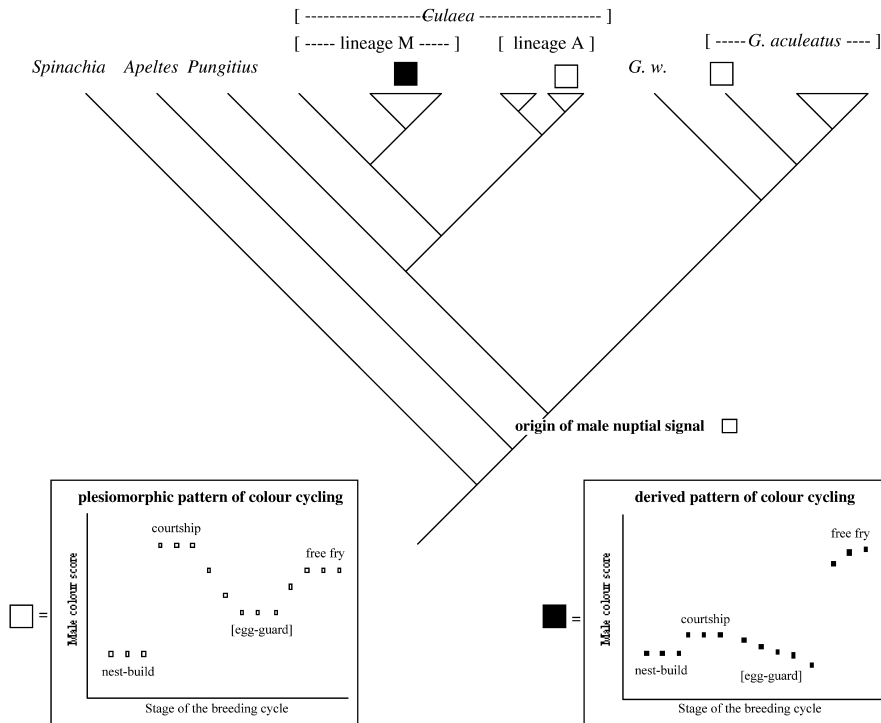


Figure 5. Patterns of colour cycling mapped above phylogenetic tree for the Gasterosteidae. Tree modified from Mattern & McLennan (2004) by expanding *Culaea* to show the two lineages. Plesiomorphic pattern of nuptial colour cycling hypothesized at point of origin for male colour based on macroevolutionary analysis (McLennan, 1991), then corroborated in *G. aculeatus* (anadromous population, representative from putative ancestor to freshwater populations, including melanistic populations with derived colour cycling) and *C. inconstans* (lineage A, southern Ontario). Derived colour cycling reported from lineage M (Nebraska) in this paper. *G.* = *Gasterosteus*; *w.* = *wheatlandi*.

So, must every gasterosteid population show the predicted pattern to corroborate the phylogenetic hypothesis? Not necessarily. If the phylogenetic patterns are telling us something about the influence of different selective vectors at the point of origin for a trait, then it is possible that the relative importance of those vectors has shifted in particular populations. What is required is that the predicted (plesiomorphic) pattern has been retained in enough species/populations to reconstruct the history of origin and change (Brooks & McLennan, 2002 and references therein). Because the populations that show the unusual patterns are all derived within their respective species (*G. aculeatus*, freshwater populations derived ultimately from an

anadromous ancestor: Reimchen, 1989; Scott, 2001; *C. inconstans*: Mattern, 2005: Figure 5), the phylogenetic hypothesis is not yet refuted. Data from the numerous *Pungitius* species would help resolve the situation further.

McLennan (1996) suggested that the plesiomorphic pattern be used to identify populations that had diverged from the historical background of colour cycling. Interpreting the results of our study in that context indicates that two pathways of signal evolution are operating in the stickleback family, reflecting the fluctuating relative influences of sexual and natural selection (Figure 5). The plesiomorphic pattern highlights the importance of intersexual selection in the success of the male nuptial signal at its point of origin. The second (derived) pattern described here suggests that the selective forces shaping the evolution of the black body signal in some populations of three-spine and brook sticklebacks have shifted from predominantly mate choice to parental care (von Hippel, 1999). Has the strength of intersexual selection decreased, has the strength of natural selection during fry care increased, or is there another factor involved in producing this derived pattern?

Reduction of the colour signal during courtship

Unlike carotenoids, melanins are synthesized as a by-product of amino acid catabolism, not extracted from prey (Fox, 1976). The relative ease with which fish synthesize melanin pigments and the ephemeral nature of their expression (reviewed in Kodric-Brown, 1998) suggests that a melanin-based signal may not reflect male condition with the same reliability as carotenoid-based colouration (Gray, 1996; Hill & Brawner, 1998; Badyaev & Hill, 2000; Senar et al., 2003). Given this, and the fact that most brook stickleback males are capable of developing the same intensely black signal, either during courtship (McLennan, 1993a) or during fry guarding (McLennan, 1993a; results, this study), it seems unlikely that melanin-based signals will transmit much information useful to mate discrimination. Black is, however, a conspicuous signal in the shallow, vegetated (green-shifted) and tea-stained (red-shifted) waters commonly inhabited North American gasterosteids (Reimchen, 1989; Scott 2001, 2004). Intersexual selection may thus be involved in the evolution of black nuptial colouration at the level of mate recognition and detection (Baube et al., 1995; McDonald et al., 1995) even if melanin pigments are not as costly to produce as carotenoids.

If black is not involved in mate discrimination, then the strength of intersexual selection on the signal is reduced compared with intersexual selection

on red in threespines. This means that the development of signal may be more sensitive to forces opposing signal amplification, the most obvious of which is predation. The conflict between maximizing the probability of finding a mate and minimizing the probability of predation is well documented in a variety of species (reviewed in Magnhagen, 1991) including threespine sticklebacks (Moodie, 1972; Whoriskey & FitzGerald, 1985). Depending upon the genetic material available for selection to work on, less conspicuous males (Endler, 1978, 1980, 1982; Moyaho et al., 2004) and reduced female preferences for conspicuous traits (Rosenthal, 2000, 2001; Kingston et al., 2003) may evolve in response to high predation risk. The South Platte River in Nebraska is home to a variety of predaceous fish, including various species of bass and trout, walleye, yellow perch, pike and pickerel, as well as numerous piscivorous birds, including loons, gulls, terns, pelicans, herons, and kingfishers (Sidle & Faanes, 1997 and references therein; S. Schainost, Nebraska Game and Parks Commission, unpubl. data).

Although Nebraskan males did not develop intense courtship colour, they did vigorously court the proffered female (mean = 15.18 pummel/five min: Ward & McLennan, 2006) and the latter typically entered the nest within a minute. Clearly then, intense colour is not a prerequisite to successful mating in this population, although males are capable of becoming intensely black. To study this interplay between the two selective forces further, we need to compare predation pressure on breeding males and the strength of female attraction to dark black in the Nebraskan and Tooley Creek, southern Ontario (plesiomorphic colour pattern) populations. We expect to discover that either the strength of attraction is decreased in the Nebraskan populations under the same or stronger predation regime than in Tooley Creek or the strength of attraction is the same across both populations but predation is more intense in Nebraska.

Role of nuptial colouration during parental care

An increase in both colour and aggression towards intruders during the parental interval has been reported for both brook (Hall, 1956; Winn, 1960; Reisman & Cade, 1967; McLennan, 1993a; Ward & McLennan, 2006) and three-spined sticklebacks (Segaar, 1961; Black, 1971; Moodie, 1972; Huntingford, 1977; Kynard, 1978; McLennan & McPhail, 1989a, b; Bakker, 1994; Scott & Foster, 2000). In the early days of fry emergence, the male continues to defend his territory, guarding the fry and returning any errant

individuals to the nest. During this time the young are particularly vulnerable to predation (McPhail, 1969; Moodie, 1972; FitzGerald, 1991; Foster, 1994). Nuptial colouration functions at this time as a threat signal that reliably transmits information about the aggressive motivational state of the parental male (Tinbergen, 1948; Rowland, 1982b, 1984, 1994). The information is reliable because a common physiological pathway underlies the cycling of colour and aggression (reviewed in Bakker, 1986 for carotenoids); melanocytes express cell-surface receptors for androgens, which simultaneously influence both the intensity of colouration and aggressive behaviour (Rohwer & Rohwer, 1978; Kodric-Brown, 1998). This pathway is not the whole story however, because during fry guarding the creamy white or lightly tinged fins are produced in startling contrast to the black body. Interestingly, the same thing happens during courtship in populations whose males develop their most intense signal at that stage (McLennan, 1993a). Evidently there is a physiological connection between maximum pigment expansion within body melanocytes and contraction within fin melanocytes, although what that mechanism might be is currently unknown.

Why would males become more conspicuous, and thus risk the consequences of predation, during fry guarding? The high energetic costs of parental care (Chellappa et al., 1989; Chellappa & Huntingford, 1989; Wootton, 1994; Smith & Wootton, 1999) and the short breeding season (Winn, 1960; Reisman & Cade, 1967) means that, for any given parental male, the young that emerge from the nest will probably represent his entire reproductive output (Sargent et al., 1995; Perrin, 1995; Kraak et al., 1999). It is thus not surprising that males in all threespine stickleback and brook stickleback populations recorded to date increase their colour signal during fry guarding. What we are seeing in the Nebraska population is the retention and enhancement of the plesiomorphic 'fry guarding peak' in the colour cycle. It only looks unusual because of the reduction in male courtship colour.

Female colour

Nuptial colouration has been reported in female sticklebacks, including *Pungitius pungitius* (Morris, 1952), *Gasterosteus wheatlandi*, *Apeltes quadracus* (Rowland, 1974; photograph in McInerney, 1969), *Gasterosteus aculeatus* (McLennan & McPhail, 1989a; Rowland et al., 1991; von Hippel, 1999) and *Culaea inconstans* (McLennan, 1993b, 1994a, b, 1995). By signaling her

sexual receptivity, a female may stimulate courtship from more males and thereby reduce the costs associated with searching for and comparing potential suitors. A choosy male may benefit from this information by decreasing the energy and time lost courting a non-receptive female and reducing the probability that she will raid his nest (Rowland, 1982a; Rowland et al., 1991; McLennan, 1995). The dark, vertical dorsal barring observed in the Nebraskan females differs from the swirled reticulate patterns reported for other brook stickleback females (McLennan, 1994a) and is plesiomorphic within the stickleback family (McLennan, 1996, 2000). It would be interesting to see whether male-directed assortative mating, based on alternative female nuptial colouration patterns, exists in *C. inconstans*. At the present time we think this is unlikely, given that the female colour signal seems to signal sex and reproductive status, rather than species (McLennan, 1996).

Conclusion

The expression of male nuptial colouration in the brook stickleback is context dependent and, in this population, has shifted function from mate choice to threat signaling aggressive motivation during parental care. The reduction of the male colour signal during courtship interactions could have significant implications for mate recognition in *Culaea*. If Nebraskan females come into contact with populations in which the male expresses his peak signal during courtship, they may either (1) fail to recognize an intensely coloured male as a potential mate or (2) prefer that male because they retain the plesiomorphic preference for intense colour, despite its absence in their own population. The first scenario requires that the strength of the female preference for an elaborate male trait has actually decreased over time. Such decreases are expected according to runaway selection models, which do not specify a direction to evolution (Fisher, 1930; Lande, 1981; Kirkpatrick, 1982; Pomiankowski, 1988) and under which the coevolution of the male trait and female preference is subject to both stochastic and selective forces, making it common for coevolving traits and preferences to drift in phenotypic space (Miller & Todd, 1993). When the female is getting more than just 'trait genes' from the male, however, the relationship between the male trait and some marker of quality, such as vigour, dominance etc., will oppose decreasing the strength of the preference. The involvement of sensory bias in initiating (and maintaining)

the preference-elaborate trait interaction (Ryan, 1990a, b, 1997; Ryan et al., 1990) will also oppose decreasing preference strength. In fact, in nature there are very few examples of female preference for the less intense version of a male character (Ryan & Keddy-Hector, 1982). Could these Nebraskan brook sticklebacks be such an example? Experiments are currently underway to answer this question.

The second explanation, the retention of the female preference for an elaborate male trait in the absence of that trait, has been invoked to explain the female swordtail *Xiphophorus pygmaeus*' preference for large sworded heterospecific males, even though conspecific males are small and swordless (Ryan & Wagner, 1987; Morris et al., 1996; Hankison and Morris, 2002). Indeed, response to plesiomorphic cues is thought to be one of the main reasons why so many North American freshwater fish hybridize so readily following anthropogenic interference such as the accidental or intended introduction of non-native species or the connection of previously separate river drainages.

At the moment we do not know whether the two different male colour cycles and the structure of the female nuptial signal are lineage specific. It would be interesting to document colour changes for more basal members of each clade, then map those changes onto the phylogenetic tree for the entire species. Further research and appropriate mate choice tests investigating the implications of nuptial colour reduction are also necessary to clarify the evolutionary status of this unusual brook stickleback population.

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