Biological Journal of the Linnean Society, 2024, **143**, blae076
https://doi.org/10.1093/biolinnean/blae076
Advance access publication 5 September 2024
Original Article

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The evolution of sensory systems after signal change in threespine stickleback

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ABSTRACT

Sensory drive can lead to the evolution of signals that are optimized to the environment in which they are perceived. However, when environmental conditions change, the interactions between signal, environment, and receiver may also shift, leading to the evolution of a new signal optimum or more categorical shifts in sexual signals (gains or losses). We evaluated how visual systems have evolved following a change in environment and male signal, and whether visual system divergence contributes to reproductive isolation between ancestral and derived types in red and black morphs of Pacific Northwest freshwater threespine stickleback. We found that opsin sequence was tuned to enhance the perceived contrast of black fish on a red-shifted light background, whereas opsin expression was not. Further, we found no evidence for homotypic preferences or assortative mating between colour morphs; males of both morphs were equally successful in no-choice mating contexts, perhaps because black males are more vigorous courters. Together, our results suggest that habitat transitions in black stickleback have led to a shift in sensory-drive dynamics with some aspects of the visual system and behaviour evolving in response to other factors (foraging or predation) or lagging behind the evolution of opsin sequences in red-shifted environments.

Keywords: mating signals; sexual selection; visual opsin; sensory drive; male competition; colour polymorphism

INTRODUCTION

Many conspicuous animal traits, from coloration to courtship displays, pheromones, and sounds are signals used to attract mates. These signals are delivered by senders (typically males) to receivers (typically choosy females) and are thus shaped by sexual selection (Andersson 1994, Rosenthal 2017). Natural selection also shapes sexual signals (Endler 1990, Bradbury and Vehrencamp 2011), as signals and how they are perceived evolve to optimize detectability in a particular environment (sensory drive; Endler 1992), and sexual signals are conspicuous to unintended receivers like predators, parasites, and competitors (reviewed in Zuk and Kolluru 1998). Sensory drive can alter signal development, mechanisms of sensory systems, and associated behaviours (Kröger and Fernald 1994, Fuller and Travis 2004, Hofmann *et al.* 2009, Long and Houde 2010, Reichert and Ronacher 2015, Sandkam *et al.* 2016, Wright *et al.* 2018), leading to signal divergence among populations and even speciation (Boughman 2002, Seehausen *et al.* 2008, Servedio and Boughman 2017). Sensory drive dynamics may also change if environments change or animals migrate to locations where existing signals are no longer easily detected (Seehausen 1997), which can lead to the evolution of new signal optima or even categorical shifts in sexual signals (gains or losses). An outstanding question is how changes in sensory drive dynamics contribute to variation within species and the maintenance of signal polymorphisms.

Visual pigments offer a clear and well-characterized link between genotype (opsin coding sequence) and phenotype (spectral sensitivity), making vision one of the most studied sensory modalities (Kawamura and Yokoyama 1995, Yokoyama 2008). Visual pigments are composed of a heptahelical transmembrane opsin protein and a light-sensitive vitamin A derived chromophore (Shichida and Imai 1998). The wavelength at which the visual pigment maximally absorbs photons, or spectral

Received 10 May 2024; revised 1 August 2024; accepted 6 August 2024

sensitivity, is determined by the interaction between the opsin protein and the chromophore (Kawamura and Yokoyama 1995, Bowmaker 2008, Yokoyama 2008). Therefore, spectral sensitivity can be altered through structural changes, such as variation in opsin coding sequence (Yokoyama 2000, 2008) and regulatory changes, such as opsin gene expression levels (Carleton and Kocher 2001). There are many examples of visual pigments co-evolving with the light environment and reflectance of sexual signals (e.g. Bloch 2015, Cortesi *et al.* 2020, Schneider *et al.* 2020, Owens *et al.* 2022), but it is less clear how visual systems evolve in environments when colour signals are lost. Here, we use the recent and rapid switch from red to black male nuptial coloration in Pacific Northwest freshwater threespine stickleback (*Gasterosteus aculeatus*; McPhail 1969, Reimchen 1989) to investigate visual system evolution following a change in colour signal and whether signal divergence can contribute to reproductive isolation between ancestral and derived types.

Threespine stickleback have repeatedly colonized freshwater lakes, rivers, and streams in the Northern Hemisphere (McPhail 1993, Bell and Foster 1994). Most male threespine sticklebacks develop a bright red throat in the breeding season that extends from the mouth to the pelvic spines, and contrasts with a blue-black eye (Fig. 1). Marine ancestors of male freshwater sticklebacks had red throats (McLennan 1996) and female mates strongly prefer this trait (Semler 1971, Milinski and Bakker 1990, McKinnon 1995, Tinghitella *et al.* 2015). However, in many western North American freshwater rivers and streams, the red throat has been categorically lost and males instead develop 'black' or 'melanic' body coloration during the breeding season (Fig. 1; McPhail 1969, Semler 1971, Moodie 1972, Jenck *et al.* 2020, 2022). Melanic coloration likely has a genetic basis (Lewandowski and Boughman 2008, Malek *et al.* 2012, Jenck *et al.* 2022) and the black male sticklebacks we focus on in this study never develop red throats, even when fed a carotenoid rich diet (R.M. Tinghitella, personal observation). In the Pacific Northwest, the black morph appears to have evolved at least twice in coastal rivers and streams: once in Oregon, once in the Chehalis River drainage in Washington, and perhaps a third time in Conner Creek, Washington (Jenck *et al.* 2022). Evolution of black coloration and divergence among populations in Washington occurred relatively recently; ancestral marine sticklebacks colonized freshwater habitats in the Pacific Northwest following glacial retreat less than 12 000 years ago (McPhail 1994). The red and black colour morphs collected from sites in Oregon, Connor Creek, and the Chehalis River drainage in Washington are genetically distinct (Jenck *et*

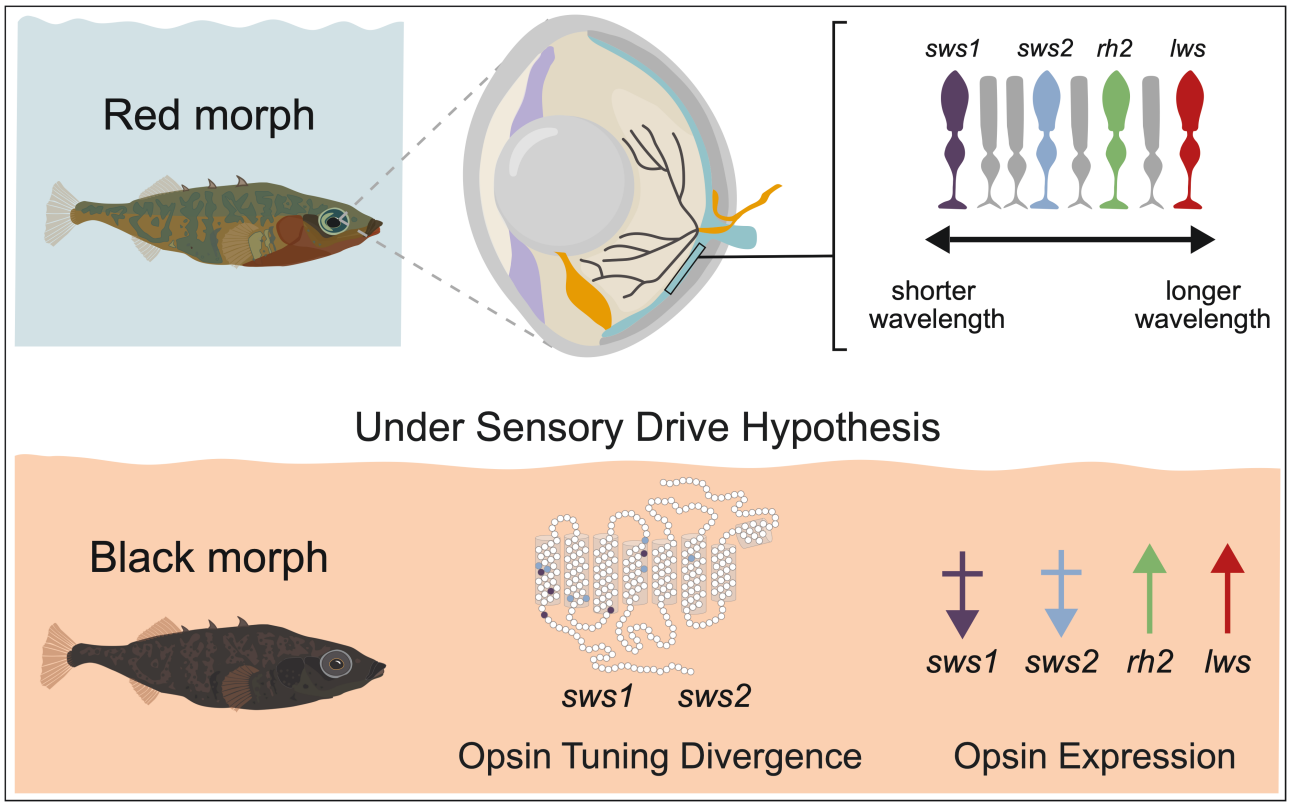

Figure 1. Overview of expectations under a sensory drive model. If black male nuptial colour and its perception is optimized to be perceived in red-shifted light environments, then we expect to see greater divergence in spectral tuning sites in short wavelength genes, relatively decreased or similar levels of gene expression in short wavelength genes (represented by downward arrows with horizontal stroke), and relatively increased levels of gene expression in long wavelength genes (represented by upwards facing arrows) relative to the ancestral red male nuptial colour morph, as depicted here. Alternatively, if male signal evolves in response to other selection pressures (e.g. predators, foraging) or there is a lag between opsin gene sequence and expression evolution, then we might see some combination of divergence in visual traits. Abbreviations for genes are: *sws1* (short-wavelength sensitive opsin 1), *sws2* (short-wavelength sensitive opsin 2), *rh2* (rhodopsin-like 2), and *lws* (long-wavelength sensitive opsin).

al. 2022), suggesting there is reduced gene flow among colour morphs and the potential for local adaptation of visual systems. Red males tend to be found in fast moving rivers with full spectrum light environments, and black males are found in slow moving, tannin-rich streams in which the light environment is red-shifted (see also Reimchen 1989, Scott 2001, Jenck *et al.* 2020, 2022). In most locations, including those studied here, one morph or the other is found, though there remain some regions where both can be found (Jenck *et al.* 2022). It has been predicted that in red-shifted light environments black males may stand out to the female stickleback visual system, leading to selection for black coloration and female mating preferences that favour black males (Reimchen 1989, Boughman 2001). Alternatively, black coloration may have evolved through relaxed selection from female mating preferences and/or in response to other selective pressures (e.g. predation, foraging).

Whether divergence in visual systems and signals impacts assortative mating in these fish is unclear. Early studies found no evidence for assortative mating between allopatric populations of red and black fish (McPhail 1969, McKinnon 1995) but some evidence for assortative mating in one region of sympatry in Conner Creek (Scott 2004). More recently, in simulated secondary contact, both red and black females from allopatric populations in Washington directed more courtship behaviours towards red than black males even under red-shifted light (Tinghitella *et al.* 2015), suggesting the ancestral preference for red is retained in both colour morphs and expressed similarly under alternate lighting environments. Interestingly, several lines of evidence suggest that male competition may reduce interbreeding between red and black fish. Male sticklebacks compete vigorously for territories where they build nests, court females, and care for offspring (Van Iersel 1953). In laboratory-based mixed-morph assemblages, black males biased their aggression toward red males, resulting in red males receiving more aggression overall (Tinghitella *et al.* 2015). Such a pattern of aggression may allow black males to exclude red males from preferred breeding sites (particularly brighter red males; Tinghitella *et al.* 2018b), enhancing habitat use differences and reducing gene flow between morphs (a mechanism reviewed in Tinghitella *et al.* 2018a).

If the black nuptial coloration in male sticklebacks evolved to be visible in red-shifted light environments, as suggested (Boughman 2001, Jenck *et al.* 2020), then we would predict that both male and female visual systems and mating preferences will have diverged in black stickleback populations such that there is a match between visual sensitivities, light environments, and the signals of the 'home' morph. Likewise, we would predict homotypic preferences (males preferentially court same colour type females and females direct more interest behaviours towards same colour type males) and positive assortative mating (more within than between colour type matings; Fig. 1). Alternatively, if the black nuptial coloration is evolving in response to other pressures or there is a lag in the evolution of black nuptial coloration as a signal via sensory drive, then there may be a mismatch between visual systems, light environment, and mating preferences. To test these hypotheses, we quantified visual system divergence by comparing opsin coding sequence and opsin gene expression among allopatric freshwater stickleback morphs and sexes. We then tested the role of visual system divergence in assortative mating by colour morph (female preference and male courtship). Altogether, we consider how the evolution of visual system, signal, and behaviour contribute to the maintenance of the red and black colour morphs.

MATERIAL AND METHODS

Opsin protein-coding sequence and gene expression analysis

During the summers of 2017 and 2018, we collected 46 fish from six locations on the Pacific coast of Washington, which included 13 red individuals from full spectrum light environments (Wishkah River, Satsop River, and Chehalis River) and 33 black individuals from red-shifted light environments (Connor Creek, Vance Creek, Black River, and Scatter Creek) (Supporting information, Table S1; Jenck *et al.* 2020). We collected all fish using unbaited galvanized steel funnel traps, and after identifying their sex and colour morph visually, we humanely euthanized them by decapitation and dissected out both eyes, placing them into RNAlater (ThermoFisher Scientific, Waltham, MA, USA). We sampled each site on a single day, retrieved all fish from traps at ~17:00 local time, and dissected eyes by 20:30, reducing the variation in opsin expression that might stem from the diel pattern of light (see Supporting information, Table S1). In the laboratory, we dissected the retinas and preserved them in fresh RNAlater until we extracted total RNA using a Qiagen RNeasy Micro Kit (Qiagen, Germantown, MD, USA). We synthesized complementary DNA (cDNA) using SuperScript® IV Reverse Transcriptase (ThermoFisher Scientific, Waltham, MA) according to the manufacturer's protocol.

Threespine sticklebacks have four cone opsin genes incorporated into visual pigments that allow them to see different wavelengths of light (Fig. 1): *sws1* (UV, short-wavelength sensitive opsin 1), *sws2* (blue, short-wavelength sensitive opsin 2), *rh2* (green, rhodopsin-like 2), and *lws* (red, long-wavelength sensitive opsin) (Yokoyama 2008). For a subset of individuals ($N = 5$ for black morphs; $N = 4$ for red morphs, see Supporting information, Table S1) we amplified all four opsin genes (*sws1*, *sws2*, *rh2*, and *lws*) from cDNA using gene-specific primers (Supporting information, Table S2; Shao *et al.* 2014) in 25 μL-volume reactions containing 1× PCR Buffer, 1.5 mM $MgCl_2$, 0.3 mM dNTPs, 0.4 μM of each primer, 0.05 U of Phusion High-Fidelity DNA Polymerase (New England BioLabs, Ipswich, MA), and 500 ng of cDNA. For all four genes, we used the following cycling parameters: 95 °C for 1 min, followed by 35 cycles of 95 °C for 30 s, 60 °C for 40 s, and 72 °C for 1 min, with a final extension of 72 °C for 5 min. We then cleaned our PCR products using ExoSAP-IT Express (ThermoFisher Scientific, Waltham, MA) and sequenced our products on an Applied Biosystems 3730 genetic analyzer at GeneWiz (Azenta Life Sciences, Chelmsford, MA). We assessed raw sequence data for quality and cleaned it in Geneious Prime v.2022.1.1 (https://www.geneious.com).

To estimate divergence among opsin sequences within sticklebacks and more broadly across fishes, we created gene datasets for the four genes that included other teleost fish from GenBank (Supporting information, Table S3). We generated multiple alignments for each gene dataset in Geneious Prime v.2022.1.1 using MAFFT, and then inferred Maximum Likelihood (ML) gene trees for each opsin gene using RAxML (Stamatakis 2014) under the general time reversible nucleotide substitution model

with gamma distributed rate variation among sites (GTR+G+I) with 10 000 bootstrap replicates and rooting the trees with *Neoceratodus fosteri* (lungfish).

To identify potential genetic and functional variation (i.e. spectral tuning sites) we generated separate alignment datasets for each of the four focal genes aligned to the bovine *RH1* gene (GenBank accession number NM001014890), and opsin sequences from the annotated whole genome of *G. aculeatus* (GCA_016920845.1) and from marine red stickleback (the ancestral state; from Supporting information, Table S3; Shao *et al.* 2014). We generated separate alignments for our nine individuals and the aforementioned reference sequences using the MAFFT algorithm implemented in Geneious Prime v2022.1.1. We then identified amino acid sites that differed between our individual red and black morph individuals and the reference sequences that may be involved in spectral tuning (Yokoyama 2008). All amino acid residues were numbered based on the bovine *RH1* sequence.

To compare levels of opsin gene expression between red and black fish and between males and females, we used established qRT-PCR methods (Carleton and Kocher 2001). Briefly, we tested the amplification efficiency and melting curve of specific primer pairs for qRT-PCR (Supporting information, Table S2; Shao *et al.* 2014) with 10-fold serial dilutions of the templates (0.01 pg to 1 ng), with three replicates for each gene and sample. Each reaction contained 1 ng of cDNA, 0.5 mM of each primer, and 1× GoTaq qPCR Master Mix (Promega, Madison, WI) in a final volume of 20 μL. We performed each reaction using an ABI QuantStudio 3 Real-Time PCR System (Applied Biosystems Inc.) with the following steps: one cycle of 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. We performed a melting curve analysis for each PCR product and calculated opsin gene expression as a fraction of the total cone opsin gene expression in an individual following the equation in Carleton and Kocher (2001). We compared data from two groups (black vs. red; male vs. female) using the Shapiro–Wilk normality test ($P > 0.05$), followed by a Mann–Whitney U test. Because black sticklebacks collected from the Chehalis River drainage (Vance Creek, Black River, and Scatter Creek) all appear closely related genetically and likely reflect a single origin of black nuptial colour, whereas the black sticklebacks from Conner Creek are distinct (Jenck *et al.* 2022), we also compared colour and drainage (Chehalis red vs. Chehalis black vs. Conner Creek black), and sex and drainage (Chehalis male vs. Chehalis female vs. Conner Creek male vs. Conner Creek female) using Kruskal–Wallis tests for both comparisons. To determine which comparisons among the Kruskal–Wallis tests were significant, we used a *post hoc* Dunn's test. We used a False Discovery Rate (FDR; Benjamini and Hochberg 1995) correction to account for multiple comparisons for all tests.

No choice mating assays

To test the hypothesis that mating is positively assortative (by colour morph) we conducted standardized no choice courtship trials with wild caught threespine sticklebacks from two populations, one that contains only the black morph (Black River) and one that contains only the red morph (Chehalis River; Supporting information, Table S1). We collected fish in the summers of 2014, 2015, and 2016 using unbaited galvanized steel minnow traps and returned them to the laboratory at the University of Denver by air. In the laboratory we housed fish in visually isolated 110 L tanks (77 cm × 32 cm × 48 cm) in a temperature and photoperiod controlled room set to 17 °C and a 15 h:9 h light:dark schedule at the beginning of the breeding season. We adjusted temperature and photoperiod conditions throughout the breeding season so that they tracked those occurring in the field. We housed the animals, separated by sex and colour morph, at densities of no more than 30 fish per tank (following Tinghitella *et al.* 2018b) and fed them a mixture of frozen bloodworms (*Chironomus spp.*) and brine shrimp (*Artemia spp.*) daily *ad libitum*.

Prior to courtship trials, we isolated males in 110 L nesting tanks outfitted with a sand-filled tray, half a flower pot for cover, a gravel pack, a plastic plant, and pieces of aquatic plant material for nest building. In previous works we found that red-shifted lighting environments did not change mating or competitive behaviours relative to full spectrum light (they preferred red males regardless of lighting; Tinghitella *et al.* 2015), so these trials were conducted under full spectrum light. To induce nesting, we enticed males by placing a gravid female in a jar into their tanks for 10 min once per day. We checked male nesting tanks daily for complete nests and considered nests to be complete when they had a clear entry and exit hole and when the male was seen swimming through the nest completely. We also checked female population tanks daily for gravid females with distended bellies.

We randomly paired gravid females with males who had completed nest building in no choice courtship trials in a 2 × 2 fully factorial design [red × red ($N = 52$), red × black ($N = 15$), black × red ($N = 14$), black × black ($N = 13$); male colour type listed first]. In each trial we released the gravid female into the male's nesting tank after a 2 min acclimation period during which the female was contained in an opaque releasing container inside the male's tank. Immediately after releasing the female we began recording the timing and frequency of all male and female courtship behaviours using JWatcher (Blumstein *et al.* 2006) for 20 min or until the female entered the male's nest. For male behaviours we recorded approaches, bites, chases, dorsal pricks, leads, rubs, shows, thrus, and zigzags. For female behaviours we recorded approaches, angles, headups, follows, examines, and enters. All behaviours are defined in Tinghitella *et al.* (2015).

We ran all statistics related to the courtship trials in JMP Pro v.15 (SAS Institute Inc. 2018) and constructed all related figures in R v.2022.07 (R Core Team 2020). We first asked whether female stickleback from southwest Washington prefer red or black males in courtship and whether that depends on their home population (whether they were collected in Chehalis River which contains only the red morph or Black River which contains only the black morph). The basic model structure included female population, male morph, and their interaction as main effects. We used two metrics to assess whether female sticklebacks from red and black populations show preferences for interacting with or mating with one of the two colour morphs: female interest behaviours/min (approaches + angles + head ups) and nest entry (yes/no). In both models, the female population × male morph interaction allowed us to test whether female responses to the two male colour morphs depend on the population (river) from which the female originated. Evidence for homotypic preferences would come from a higher rate of

female interest behaviours directed towards males of colour found in the female's home population, and evidence for assortative mating would come from higher within population than between population mating rates, both of which would be captured in the interaction effects in our models.

Having found little evidence for assortative mating, we were then interested in whether the courtship behaviour of males of the two colour morphs differed generally and/or whether male behaviour differed when they were paired with females from Chehalis River vs. Black River. If so, these differences in behaviour may explain how males of the two colour types achieve nearly equal mating success with females from locations where red males or black males predominate. The basic model structure was the same as for the female focused models, except that female approaches/minute was included as a covariate to account for the possibility that female initiation of courtship was responsible for male behaviour. The outcome variable was male courtship vigour (courtship behaviours/minute).

RESULTS

Opsin sequence and expression divergence

We sequenced partial coding sequences (approximately 800 bp for *lws*, 950 bp for *rh2*, 900 bp for *sws1*, and 960 bp for *sws2*) from retinal mRNA for all four of our opsin genes for both red and black morphs. Using multiple alignments, we explored opsin divergence between the two stickleback morphs and in relation to other teleost fish (Fig. 2a), and also surveyed the amino acid site differences between stickleback morphs that may play a role in changing spectral sensitivity (Fig. 2b). Short wavelength genes (*sws1* and *sws2*) exhibited clear divergence between red and black populations, resolving two shallowly diverged clades between the morphs (Fig. 2a; Supporting information, Fig. S1), whereas long/medium wavelength genes (*lws* and *rh2*) exhibited divergence, but less resolution between red and black populations (Supporting information, Fig. S1).

We identified amino acid substitutions between individuals of both morphs ($N = 9$) and the *G. aculeatus* and marine red reference sequences in all four of the investigated opsin genes at sites known in other vertebrates to tune spectral sensitivity of visual pigments (Yokoyama 2008), as well as some sites that have not been evaluated for tuning spectral sensitivity (Fig. 2b; Supporting information, Table S4). No amino acid differences were identified between individual samples of the same morph or different populations for any of the four opsins. On the *lws* opsin, three amino acid substitutions occurred between the red and black morphs, all in the transmembrane regions. Two of these substitutions (49 and 52) were observed at known tuning sites in other opsins (*rh2* in zebrafish; Chinen *et al.* 2005b, *sws1* in primates; Hunt *et al.* 2007), but not in *lws*, while the third (50) was not known as a spectral tuning site (Fig. 2b). On the *rh2* opsin, only one amino acid substitution occurred between morphs in the transmembrane region at site 255 (Fig. 2b). Substitutions at this site (I255V) have recently been shown to have potential effects on spectral sensitivity; however, it may only cause shifts in spectral sensitivity when combined with substitutions at other sites (Yokoyama and Jia 2020), which do not vary between our morphs. On the *sws1* opsin, one amino acid substitution occurred at site 154 in the transmembrane region between morphs (Fig. 2b), but it is not known to affect spectral sensitivity. *sws2* exhibited the greatest number of amino acid differences, with seven in total. Five of the sites occurred in the transmembranes (40, 96, 97, 109, 161), and two occurred in the N-terminus tail (33) and the C2 loop (150). Sites 40, 97, and 109 all faced the retinal binding pocket of the opsin protein, therefore most likely conferring a function effect (Carleton *et al.* 2005). Two of the amino acid changes occurred at known *sws2* spectral tuning sites (97 and 109) and one occurred at a known *RH1* spectral tuning site (96; Fig. 2b). The substitutions S97C and A109G have been reported to result in a -17 nm and -2 nm shift, respectively, in bluefin killifish (*Lucania goodei*) *sws2* (Yokoyama *et al.* 2007). We saw the opposite amino acid substitutions (C97S and G109A) in black stickleback morphs, indicating a potential red-shift in *sws2* in red-shifted environments.

When we compared opsin expression between colour morphs we found higher levels of *rh2* mRNA in red individuals compared to black individuals ($W = 50$, adj. $P < 0.01$) and higher levels of *sws1* mRNA in black individuals compared to red individuals ($W = 329$, adj. $P = 0.02$; Fig. 2c). We compared opsin expression between sexes and found no significant differences (Fig. 2c). To examine the differences between colour and sex between drainages, we conducted Kruskal–Wallis (KW) tests with a *post hoc* Dunn's test to determine which comparisons were significant. We found significantly higher levels of mRNA in *rh2* (KW $\chi^2 = 16.34$, d.f. = 2, adj. $P < 0.01$) and *sws1* (KW $\chi^2 = 7.86$, d.f. = 2, adj. $P = 0.04$) for comparisons of colour morphs between drainages. Pairwise comparisons using Dunn's test indicated that Chehalis red morphs were observed to be significantly different for *rh2* compared to both Chehalis black individuals (adj. $P < 0.01$) and Conner Creek black individuals (adj. $P < 0.01$), and that Chehalis red morphs were observed to be significantly different for *sws1* compared to Chehalis black individuals only (adj. $P = 0.02$). No other comparisons were statistically significant. We also found significantly higher levels of mRNA in *lws* (KW $\chi^2 = 17.59$, d.f. = 3, adj. $P < 0.01$) and *rh2* (KW $\chi^2 = 9.23$, d.f. = 3, adj. $P = 0.05$) for comparisons of sex between drainages. Pairwise comparisons using Dunn's test indicated that Chehalis females were observed to be significantly different from both Conner Creek females (adj. $P < 0.01$) and Chehalis males (adj. $P < 0.01$) for *lws*, and that Conner Creek females were observed to be significantly different from both Chehalis females (adj. $P = 0.03$) and Conner Creek males (adj. $P = 0.05$). No other comparisons were statistically significant. Supporting information, Tables S5–S7 include all statistics.

Testing for assortative mating and behavioural differences in courtship

We first asked whether females prefer one male morph over another, and whether female preferences depend on the population the females are from or the interaction of male morph and female population. We found no interaction between female population and male colour morph on the number of female interest behaviours ($F = 0.418$, d.f. = 1, $P = 0.52$), and females from both tested populations performed similar overall rates of interest behaviours ($F = 0.869$, d.f. = 1, $P = 0.35$). However,

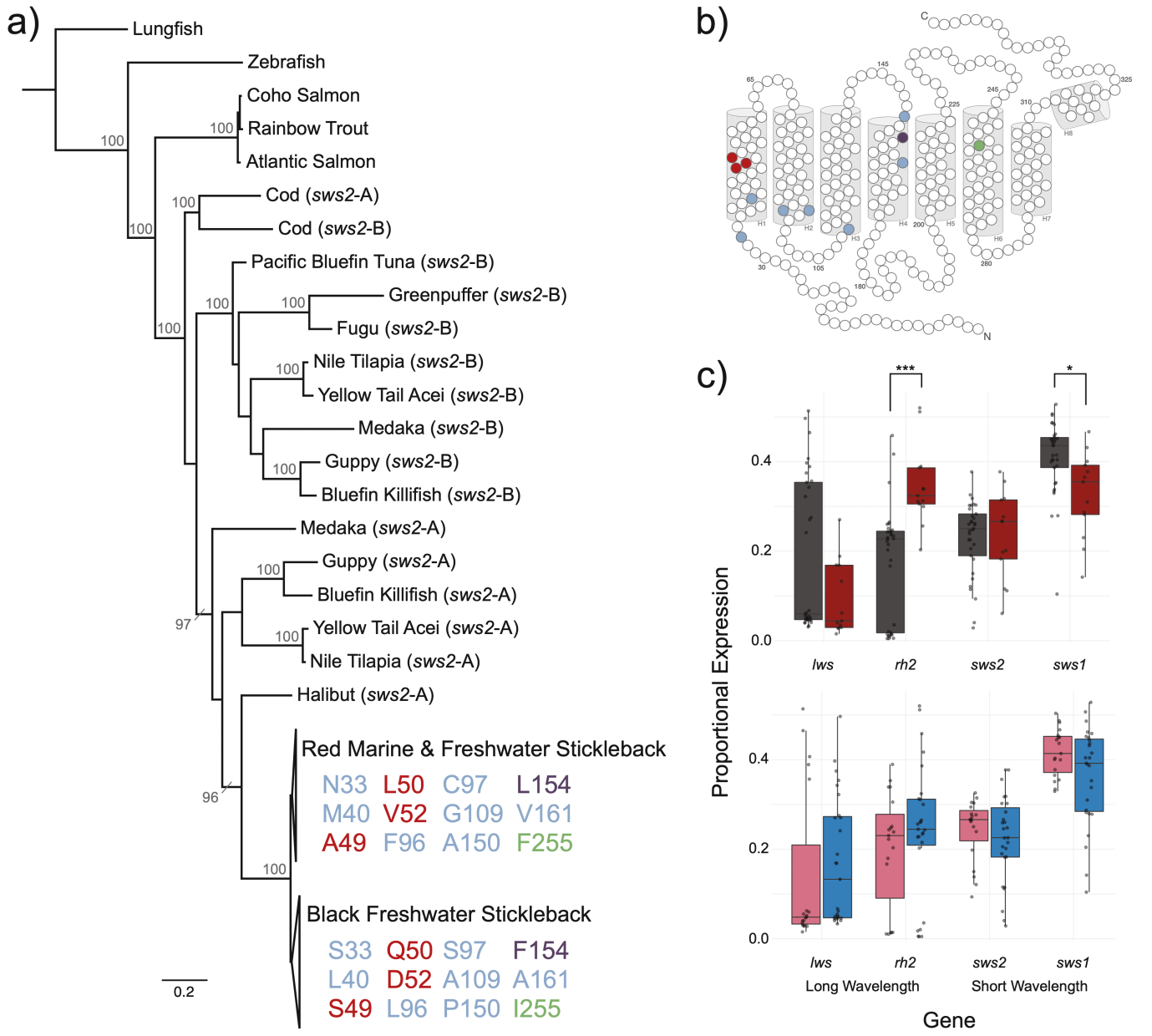

Figure 2. Opsin variation and expression. a, Maximum likelihood gene tree for *sws2* (other individual gene trees in Supporting information, Fig. S1). Specific amino acid changes between stickleback morphs for all four opsins are listed under their respective morphs on the gene tree (*lws* = red, *rh2* = green, *sws1* = blue, *sws2* = purple). Numbers on nodes indicate bootstrap probabilities (> 90) with 10 000 replicates. b, schematic representation of bovine rhodopsin (*RH1*) with variable sites for each opsin gene (*lws* = red, *rh2* = green, *sws1* = blue, *sws2* = purple) identified between stickleback morphs. c, opsin mRNA levels between colour morphs (top) and sex (bottom). *** $P < 0.0001$, * $P < 0.01$. Bar colour in the top graph indicates morph colour (black morph = black vs. red morph = red) and in the bottom graph indicates sex (male = blue vs. female = pink).

females from both populations performed more interest behaviours towards red males than black males (F = 3.920, d.f. = 1, $P = 0.05$), suggesting they prefer red over black males as mates (Fig. 3a; Table S8), consistent with previous work in these populations (Tinghitella *et al.* 2015).

We found no clear evidence for assortative mating. Whether females entered the nest of a courting male or not did not depend on male colour morph (Likelihood Ratio [LR] $\chi^2 = 3.180$, d.f. = 1, $P = 0.08$), female population (LR $\chi^2 = 0.940$, d.f. = 1, $P = 0.33$), or their interaction (LR $\chi^2 = 2.772$, d.f. = 1, $P = 0.10$; Fig. 3b; Table S8). Even after controlling for female initiation of courtship (approaches/min; which did differ across female populations, with females from Chehalis River initiating more courtship interactions; F = 8.36, d.f. = 1, $P < 0.01$), black males performed more courtship behaviours per minute than red males (F = 16.936, d.f. = 1, $P < 0.01$). Males of both colour types also tended to perform more courtship behaviours when paired with Chehalis River females than Black River females (F = 3.881, d.f. = 1, $P = 0.05$; Fig. 3c; Table S8). There was no interaction between male colour morph and female population on male courtship behaviours (F = 0.026, d.f. = 1, $P = 0.87$). The greater courtship vigour of black males who lack ancestral

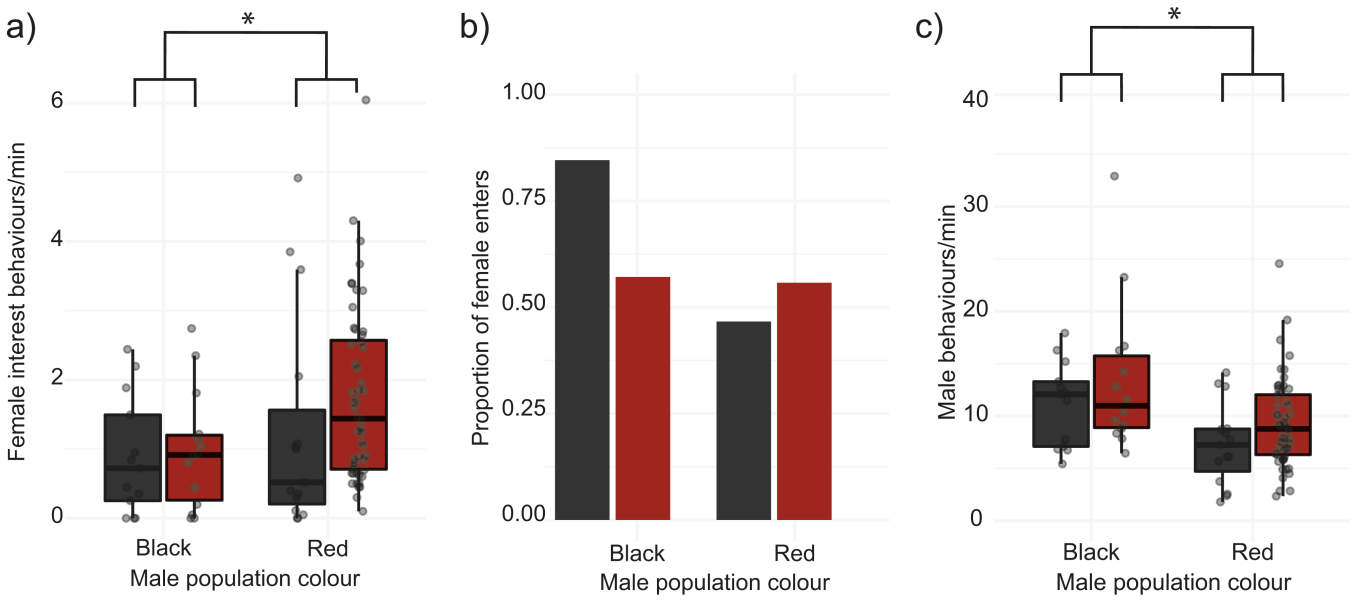

Figure 3. Assortative mating and behavioural differences. Bar colour indicates the colour of males in the population where the female is from. Key outcomes are that during courtship females express more interest behaviours towards red than black males (a), yet both male colour types are equally likely to spawn successfully (b), perhaps because black males are more vigorous courters (c). * $P < 0.05$.

nuptial coloration and are less preferred by females during courtship (see above) may explain the relatively equal rates of mating across trial types.

DISCUSSION

Sensory drive predicts that the environment shapes the ways that signals are produced and received. Our goal was to understand how visual systems, signals, and behaviour evolve in response to changed environmental conditions and how altered sensory drive dynamics contribute to the maintenance of signal polymorphisms. We used a complementary approach of genetic and behavioural analyses to determine if black male nuptial colour in sticklebacks and its perception is optimized to be perceived in red-shifted light environments. We compared sequence and gene expression divergence in candidate visual genes for two colour morphs of freshwater threespine sticklebacks that occupy different light environments. We then tested whether divergence in these visual systems is associated with assortative mating in secondary contact. We expected to observe greater divergence in spectral tuning sites in short wavelength genes, relatively decreased or similar levels of gene expression in short wavelength genes, and relatively increased levels of gene expression in long wavelength genes relative to the ancestral red male nuptial colour morph, in turn leading to homotypic mating preferences between morphs. Overall, we found some evidence that opsin gene sequence and expression levels have diverged in red and black sticklebacks. However, we found that females of both colour morphs prefer the ancestral red male type and that there is no evidence of assortative mating between morphs in secondary contact. Together, our results suggest that habitat transitions in black stickleback have led to a shift in sensory-drive dynamics with some aspects of the visual system and behaviour evolving in response to other factors (foraging or predation) or lagging behind the evolution of opsin sequences in red-shifted environments.

Divergence in visual systems after colour loss

The transition from red to black nuptial colour in freshwater stickleback may disrupt sensory drive dynamics by changing the mechanism of signalling. Because black body coloration does not give off any reflectance (Caivano 2022), females are not perceiving the reflectance of light alone off a black fish as a signal. Instead, black male nuptial colour may act as a signal in tannic, red-shifted water by creating a high chromatic contrast against darker backgrounds and it is this contrast that is perceived by other fish (Marchetti 1993, Boughman 2001, Fuller *et al.* 2005, Seehausen *et al.* 2008, Brock *et al.* 2017). The contrast hypothesis has been corroborated in black morph sticklebacks inhabiting blackwater lakes in the Haida Gwaii archipelago of British Columbia, Canada. In British Columbia, blackwater lakes are almost 'nocturnal' (red-shifted to the point that there are only small amounts of downwelling red light (Reimchen *et al.* 2013, Reimchen 1989, Flamarique *et al.* 2013, Marques *et al.* 2017). Sticklebacks in these lakes have spectral tuning changes to *sws2* that shift peak sensitivity towards longer wavelengths, and increased *lws* expression that maximizes the sensitivity to background light (Flamarique *et al.* 2013, Marques *et al.* 2017). Similar patterns of expression and spectral tuning have been observed in bluefin killifish which have been attributed to chromatic contrast that inhabit shallower, red-shifted waters (Fuller and Travis 2004, Fuller *et al.* 2005, Mitchem *et al.* 2018). Thus, if black male nuptial coloration and its perception is optimized in tannic waters, we would expect to see greater divergence in black stickleback spectral tuning sites in short wavelength genes (*sws1* and *sws2*) and relatively increased levels of gene expression in long wavelength genes (*lws* and *rh2*; Fig. 1).

In the freshwater threespine stickleback populations that we studied, divergence in spectral tuning sites matches what we would expect if the stickleback visual system adapted to perceiving black through a high contrast with red-shifted light. Most notably, we observed amino acid changes at spectral tuning sites that shift vision to a longer wavelength in *sws2* in black stickleback populations. Similar amino acid changes in *sws2* are well documented in several fish species that are adapted to dark water environments (Shand *et al.* 2002, 2008, Yokoyama *et al.* 2007, Marques *et al.* 2017). We also see more resolved phylogenetic divergence (two distinct lineages between red and black populations) in short wavelength genes, especially *sws2* (Fig. 2; Supporting information, Fig. S1), compared to long wavelength gene divergence. This suggests that these short wavelength genes may be more rapidly evolving to the different light environments of red and black populations. While we also found several amino acid changes in the other three opsin genes, the effects of these changes on spectral sensitivity of the visual pigment are unknown (Chinen *et al.* 2005a, Yokoyama 2008, Yokoyama and Jia 2020).

In comparison to protein-coding divergence, opsin gene expression divergence does not directly match our expectations based on sensory drive leading to high contrast black colour. We observed higher expression of *sws1* (a gene that is found in visual pigments most sensitive to short wavelengths that are typically violet/UV wavelengths) in black populations in tannic water (Fig. 2c). This finding is contrary to the contrast hypothesis, which posits that we should observe higher expression in longer wavelength genes. This may indicate that the black colour of these populations is not tuned to their visual systems, and instead the visual systems are being used to better perceive predators or for foraging in the red-shifted environment (Fuller *et al.* 2010, Rick *et al.* 2012, Maan *et al.* 2017). UV light has been shown to play a major role in foraging in threespine sticklebacks (Boulcott and Braithwaite 2005, Rick *et al.* 2012). Thus, it may be possible that sticklebacks in red-shifted waters have relatively increased *sws1* expression to compensate for what little UV light might be available. It is also possible that the higher expression of *sws1* is because there is more light available in the shallow red-shifted streams where we find black freshwater stickleback in Washington, compared to the blackwater lakes in British Columbia, but this remains to be tested. Black populations in red-shifted waters may also encounter more crayfish, a common predator of sticklebacks (R.M. Tinghitella, personal observation). However, crayfish are not known to have UV markings (Schuster 2020) and, therefore, may be perceived in some other way by sticklebacks in these red-shifted environments. *sws1* expression in other fish has also been observed to vary with seasonality, wherein there is higher visibility of UV light/colour in darker water environments during certain seasons that correlates with higher expression of *sws1* (Stieb *et al.* 2017). However, this seems less likely in our study because we collected fish in early summer, which would be after the period of elevated turbidity (in the spring during heavy rains) and before the period of elevated tannicity/red-shifting (in the autumn (fall)/winter when leaf litter gives off more tannins in the water). Moreover, though there is some evidence that opsin gene expression varies with environment and physiology in the threespine stickleback (Shao *et al.* 2014, Rennison *et al.* 2016, Marques *et al.* 2017, Veen *et al.* 2017), the degree of plasticity may be minor and of limited functional significance (Flamarique *et al.* 2013).

There may also be alternative ways that red and black freshwater stickleback visual systems have diverged. For example, we observed higher expression of *rh2* (a gene that is found in visual pigments most sensitive to medium/long wavelengths that are typically green/yellow wavelengths) in red morphs. Increased expression of *rh2* in red morphs may aid in their perception of colour, specifically the contrast between the blue eye and red throat, and could play a role in female stickleback preferences for males that have higher contrast between the eye and the throat (Flamarique *et al.* 2013). Patterns of increased *rh2* expression have also been observed in damselfish species and are attributed to abilities to discriminate between colours (Stieb *et al.* 2016, 2017, Luehrmann *et al.* 2018).

Because black sticklebacks collected from the Chehalis River drainage are genetically distinct from those from Conner Creek (Jenck *et al.* 2022), we also compared opsin expression divergence for colour and sex between drainages. Again, we observed differences in expression in *rh2* and *sws1* between colour morphs when comparing Chehalis red individuals to both Chehalis black and Conner Creek black individuals. We also observed differences in expression in *lws* and *rh2* between sexes in both drainages; however, it is unclear how meaningful these results are given that our sampling is uneven by sex when drainages are split.

Overall, if the black male nuptial colour evolved or is maintained by sensory drive, then we would expect visual systems to be tuned to perceiving the contrast of black bodies against red-shifted backgrounds. We find some evidence for this in opsin gene sequence divergence, but not in opsin gene expression. This suggests that the two modes of visual system divergence may differ and be uncoupled following the transition to a new light environment. Instead, the visual system may be evolving in response to other necessary functions within their habitat (e.g. predator avoidance, foraging) or components of the visual system and black nuptial colour signal are evolving at different rates.

Little evidence for homotypic preferences or assortative mating

Along with the effects of environmental variation on signal polymorphism and visual system evolution, sensory drive can shape behaviours like mating preferences and decisions. In a system in which sensory drive contributes to visual system and genetic divergence, we would expect to find mating-related behaviours and patterns that favour 'home' morphs. Here, we expected to find evidence for homotypic female mating preferences and assortative mating within colour morphs. In previous work (behavioural trials conducted in group settings) females from allopatric red and black populations both preferred red over black males during early courtship and black morph males biased their male competition behaviours towards red males (Tinghitella *et al.* 2015). Here, we used no choice courtship trials, rather than interactions in group settings, to once again assess female mating preferences, test for courtship behaviour differences between male morphs, and test the hypothesis that mating is assortative

by colour. We found that (i) consistent with previous work, females from allopatric red and black sites direct more courtship interest behaviours to red males, (ii) black males court more vigorously than red males regardless of the home population of the female with whom they interact, and (iii) there is no evidence for assortative mating by colour. Two additional trends are of note. First, females of the red morph tended to direct more interest behaviour towards red than black males, whereas females of the black morph did not appear to discriminate between male types at all (Fig. 3a), though this difference was not significant. Additionally, both male morphs tended to court slightly more towards red females (Fig. 3c), though this was not significant either. Overall, females respond to red coloration more positively than black, suggesting they retain the ancestral preference for red, despite the complete loss of red coloration in the black morph (Tinghitella *et al.* 2015; Fig. 3). How then do black males achieve reproductive success? Our behavioural results suggest that while black males lack the preferred sexual signal, they may compensate for that by being more vigorous courters than red males, leading to near equal rates of success in courtship (enters; Fig. 3). Intriguingly, this mechanism (relatively more vigorous courtship) is similar to that used by crickets that have recently lost their ancestral sexual signal to achieve reproductive success (Fitzgerald *et al.* 2022). Finally, the lack of evidence for assortative mating indicates that, as with opsin sequences and gene expression above, behaviour is uncoupled from sensory system evolution. It is important to note, however, that our results are only for one red population and one black population, and that all fish tested were from allopatric locations. Testing for assortative mating in contact zones may be particularly illuminating if the selective pressure to develop preferences for local males is weak in regions where encountering the alternate morph is highly unlikely. Indeed, Scott (2004) found assortative mating in the contact zone within Conner Creek. Tests of assortative mating under red-shifted light environments may also be illuminating, although Tinghitella *et al.* (2015) previously found no effect of red-shifted side-welling light on red and black morph courtship or competition behaviour.

CONCLUSION

Our goal was to understand how visual systems have evolved following changed environmental conditions and the evolution of a new colour morph, and whether visual systems and signal divergence contribute to reproductive isolation between black and red sticklebacks. To do this we integrated results of visual system divergence and visually based behaviours in response to the phenotypic expression of male coloration in populations of freshwater threespine stickleback. We found that opsin sequences may be tuned to enhance the perceived contrast of black fish on a red-shifted background, consistent with sensory drive in changed (red-shifted) environments. However, opsin expression did not match our expectations and we found no evidence for homotypic preferences or assortative mating between colour morphs. Our results suggest that sensory drive components are thus decoupled in black freshwater stickleback populations. Sensory drive is predicted to play an important role in the divergence of mating systems among allopatric populations that inhabit distinct environments (Boughman 2002, Ravigné *et al.* 2009). While we do not see a clear pattern of divergent sensory systems among red and black freshwater sticklebacks, we still find evidence of genetic divergence between the morphs (Jenck *et al.* 2022). How that genetic divergence is maintained remains an open question and we encourage future work to investigate, for instance, preferences and assortative mating in regions of sympatry where both red and black male morphs co-occur and selection for divergent mating preferences and decisions might be stronger.

SUPPLEMENTARY DATA

Supplementary data is available at *Biological Journal of the Linnean Society* online.

ACKNOWLEDGEMENTS

We would like to thank Clara Jenck, Whitley Lehto, Ross Minter, and Preston Hibbs for assistance collecting and dissecting fish, Brooke Washburn, Bea Clack, and Vance Imhoff for assistance with lab work, and members of the University of Denver Ecology and Evolutionary Biology group for feedback on this research. The findings and conclusions in this article are those of the authors and do not necessarily represent the views of the U.S. Fish and Wildlife Service.

COMPETING INTERESTS

The authors declare that they have no competing interests.

FUNDING

This work was supported by a PROF grant from the University of Denver to R.M.T. and E.L.L. Personnel on this project were supported by National Science Foundation Grants to R.M.T. (IOS 1846520 and DEB 2012041), E.L.L. (DEB 2012041 and IOS 2015976), and T.J.F. (DBI 2208825).

DATA AVAILABILITY

All sequences were deposited in NCBI GenBank (accession numbers will be added upon review and acceptance of manuscript) (Supporting Information, Table S3). We have included a repository on Dryad (https://doi.org/10.5061/dryad.w0vt4b8vt) that includes the following: results files for qRT-PCR, alignment files used for spectral tuning and divergence analyses, and behavioural data.

ETHICS

All methods involving animals were approved by the University of Denver IACUC (883302-9) and collection was permitted by the Washington Department of Fish and Wildlife (permits 14-078, 15-198, 16-208, 17-134, 18-173).

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