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Parallel shifts of visual sensitivity and body coloration in replicate populations of extremophile fish

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Abstract

Visual sensitivity and body pigmentation are often shaped by both natural selection from the environment and sexual selection from mate choice. One way of quantifying the impact of the environment is by measuring how traits have changed after colonization of a novel habitat. To do this, we studied *Poecilia mexicana* populations that have repeatedly adapted to extreme sulphidic (H_2S-containing) environments. We measured visual sensitivity using opsin gene expression, as well as body pigmentation, for populations in four independent drainages. Both visual sensitivity and body pigmentation showed significant parallel shifts towards greater medium-wavelength sensitivity and reflectance in sulphidic populations. Altogether we found that sulphidic habitats select for differences in visual sensitivity and pigmentation. Shifts between habitats may be due to both differences in the water's spectral properties and correlated ecological changes.

KEYWORDS
adaptation, colour patterning, fish, sexual selection, sulphide spring, vision

1 | INTRODUCTION

Patterns of parallel and convergent evolution are strong evidence of the action of natural selection, as it is unlikely that drift would lead to the same phenotype evolving in multiple independently derived populations or species (Schluter & Nagel, 1995). Due to vision's central role in predation avoidance, mate choice and foraging, it is predicted to be under strong natural and/or sexual selection in many species (Endler, 1992). Indeed, work in a variety of systems has indicated that shifts in the visual system do evolve repeatedly (O'Quin et al., 2010; Rennison et al., 2016; Torres-Dowdall et al., 2017). These shifts have often been found to be largely genetically determined (e.g., Rennison et al., 2016; Tobler et al., 2010), although phenotypic plasticity can also induce large shifts (e.g., Kranz et al., 2018; Luehrmann et al., 2018; Nandamuri et al., 2017). Yet, identification of the ecological factors and functional mechanisms shaping evolutionary shifts in visual sensitivity has proven difficult. The visual system is predicted to evolve to roughly match the availability of wavelengths to maximize photon catch and contrast detection through natural selection (Clarke, 1957; Denton & Warren, 1957; Munz, 1958). However, even in cases where there is some evidence of matching over portions of the visual spectrum, overall shifts in visual sensitivity remain largely unexplained by hypotheses related to background matching (e.g., Rennison et al., 2016).

Apart from natural selection, sexual selection may also be playing a role in determining visual sensitivity. Shifts in visual sensitivity are often accompanied by differences in body pigmentation and colour-based mate choice. The sensory bias and sensory drive hypotheses

attempt to explain patterns of co-evolution between male signals and female perception. These hypotheses suggest that male sexual signals should become tuned to match the sensitivity of the female's sensory system to optimize attractiveness (Boughman, 2002; Fuller et al., 2005). Sensory bias posits that mate signals should match sensory perception and is exemplified in taxa such as swordtail fish (Basolo, 1990) and tungara frogs (Ryan & Rand, 1990). In contrast, sensory drive integrates natural selection and proposes that while signals and perception should match, both are constrained and influenced by the environment. African cichlids (Seehausen et al., 2008) and threespine stickleback (Boughman, 2001) are among the few systems in which sensory drive seems to explain patterns of co-evolution between shifts in female visual perception and male nuptial coloration. Previous work has attempted to understand this connection by linking the evolutionary rate of opsin genes (Bloch et al., 2015; Bloch Price et al., 2015) or opsin gene expression (Brock et al., 2018; Sandkam, Young, Breden, Bourne, et al., 2015) with male nuptial coloration, or with ambient light (Fuller et al., 2004, 2005). To tease apart the most common ecological mechanisms that drive shifts in body pigmentation and visual sensitivity, further studies are needed. For example, when signalling conditions seem relatively benign, such as in a habitat where ambient light is generally broad spectrum and/or differences in ambient light are subtle between habitats, it is unclear whether the selective pressure is strong enough to drive spectral matching.

Poecilia fish inhabiting sulphide springs in Mexico are a phenomenal example of convergent evolution (Tobler et al., 2018). These fish have evolved to survive in the presence of hydrogen sulphide (H_2S), a potent respiratory toxicant (Tobler et al., 2016), and adaptation has been repeated in multiple independently colonized locations (Tobler et al., 2011). Sulphidic and nonsulphidic populations have been documented to diverge in physiological (Greenway et al., 2020), male body colour (Zimmer et al., 2018), morphological (Tobler & Hastings, 2011) and life history traits (Riesch et al., 2010). In addition, populations in adjacent sulphidic and nonsulphidic habitats are reproductively isolated and exhibit very low levels of gene flow despite a lack of physical barriers that would prevent fish movement (Plath et al., 2013). Aside from the presence of H_2S, the colonized habitats also vary in other ecological properties compared to the ancestral nonsulphidic habitats, including the availability of food resources (Tobler et al., 2015) and community composition (presence of predators and competitors) (Greenway et al., 2014; Tobler et al., 2015).

Sulphur-containing solutions (aqueous and nonaqueous) are known to absorb wavelengths in the ultraviolet (200–360 nm) region (Khan, 2011; Okada, 1963). This suggests the ambient light environment may also differ between the adjacent sulphidic and nonsulphidic locations and may drive shifts in visual sensitivity and/or body pigmentation. Based on previous work showing some signs of visual sensitivity differences between environments (Körner et al., 2006), and differences in male coloration (Zimmer et al., 2018), we predict that there will be parallel evolution in these traits. We surveyed four independently colonized drainages with paired sulphidic and nonsulphidic sites containing *Poecilia* species to ask the following questions: (i) Has there been parallel evolution in visual sensitivity and/or body pigmentation of the sulphidic and nonsulphidic ecotypes across the different drainages? (ii) Has there been co-evolution between female perception and body pigmentation?

2 | METHODS

2.1 | Sample collection

Specimens of *Poecilia mexicana* were collected from four drainages in the Río Grijalva basin (from west to east: Pichucalco, Ixtapangajoya, Puyacatengo and Tacotalpa). In each drainage, we sampled fish from one sulphidic (La Gloria springs, La Esperanza springs, La Lluvia springs and El Azufre) and one nonsulphidic (Rio El Azufre west branch, Rio Ixtapangajoya, Rio Puyacatengo at Vicente Guerrero and Arroyo Bonita) population (Figure 1). Ten female individuals were sampled from each population and euthanized using MS222 for opsin expression analysis. Reflectance measurements were taken from 10 to 15 live male and female fish from each location (30 fish in total per population). During transport, the live fish were held in black buckets for 1–2 h before spectral measurements were collected. Three replicated measurements were taken from each of four body locations (top of head, behind the eye, abdomen and tail). Due to technical constraints, we only measured reflectance in fully opaque body regions. Measurements taken at partially transparent body parts, particularly the fins, produced inconsistent measurements between replicates. This means that our tail measurement is of the caudal peduncle and not the caudal fin. At each of the eight locations where we collected fish, we also measured the in situ spectral conditions from 351 to 700 nm. Irradiance measurements of side-welling light were taken at depths of 0, 10, 20 and 30 cm (maximal depth) at five or six sites within each sampling location using a cosine corrector attached to a spectrophotometer (Ocean Optics). During analysis we identified technical issues with our irradiance measurements, and we decided to not include analyses of environmental light spectrum (see Supporting Information).

2.2 | Opsin expression and spectral sensitivity

Both eyes were removed immediately after euthanasia, stored in 1 ml RNAlater (Qiagen), and moved to a −20°C freezer for up to a month until RNA was extracted. Left and right eyes were pooled for each individual. The pooled eyes were homogenized in a Retsch mm 400 Mixer Mill using a carbide bead. Total RNA was extracted using the Aurum Total RNA Fatty and Fibrous Tissue (BioRad), which included a DNase I incubation step. The concentration and purity of the extracted RNA was assessed on a NanoDrop spectrophotometer (Thermo Scientific). Synthesis of cDNA was accomplished using the iScript cDNA Synthesis Kit (Bio-Rad), and 1000 ng of RNA was used as the input for the cDNA synthesis of each sample. The resulting cDNA was diluted 1:100 in ultrapure water for RT-qPCR

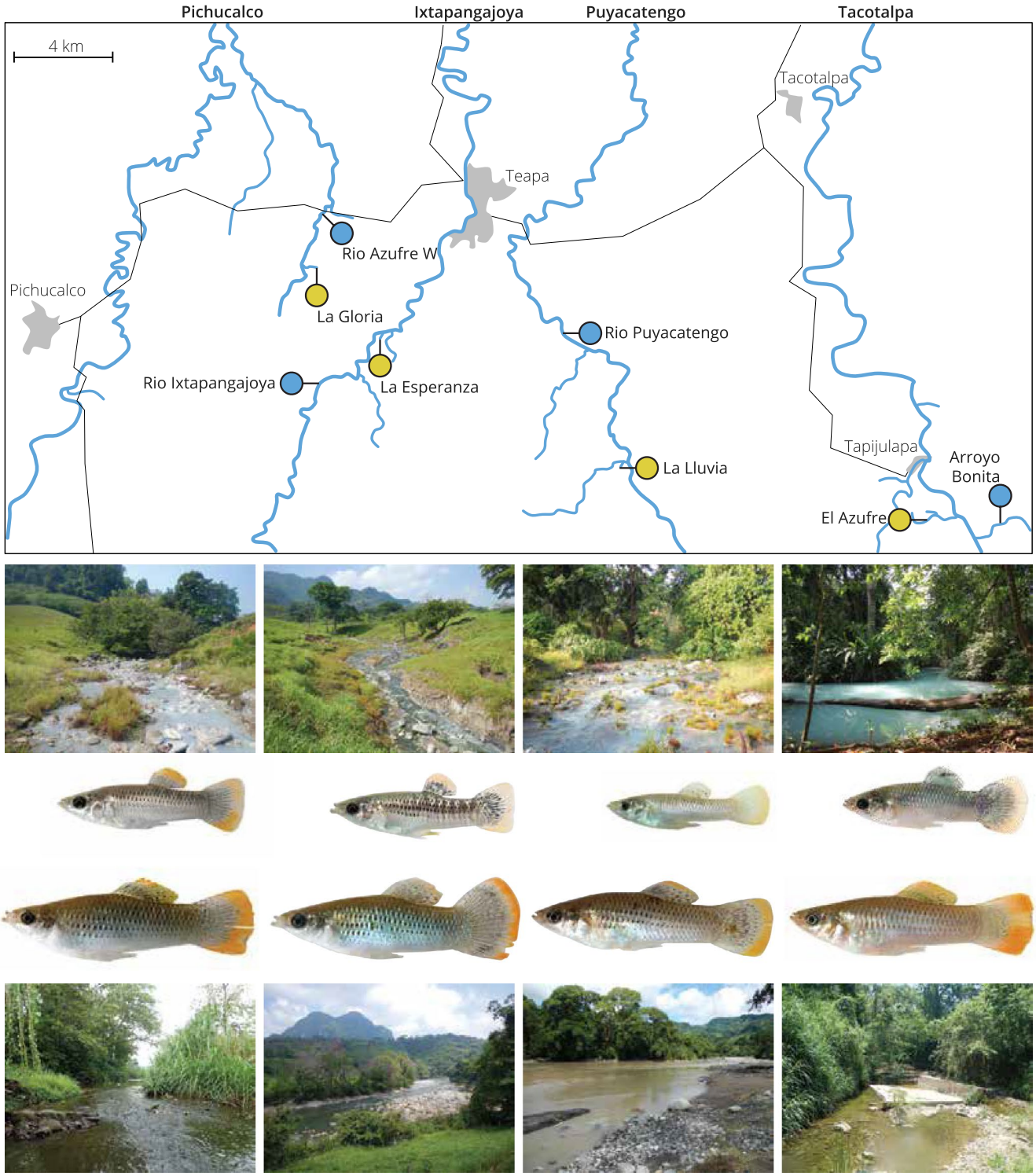

FIGURE 1 Map of the study region including the four drainages with paired sulphidic (yellow) and nonsulphidic (blue) sites. Photos show sulphidic habitats and *Poecilia mexicana* (top row) nonsulphidic habitats and *P. mexicana* (bottom row) for each of the drainages. All photos were taken in late May or early June toward the end of the dry season [Colour figure can be viewed at wileyonlinelibrary.com]

(reverse-transcription quantitative polymerase chain reaction) analysis.

To develop unique qPCR primers and probes (see Table S1 for sequences), each opsin of the nine cone opsin genes (LWS-1, LWS-2, LWS-3, LWSr, RH2a, RH2b, SWS1, SWS2a and SWS2b) was sequenced using primers developed by Sandkam, Young, Breden, et al. (2015). Based on these sequencing results, we designed probe and primer sets for RT-qPCR. For each gene, one of the primers and/or the RT-qPCR probe spanned an intron, which allowed us to avoid amplification of genomic DNA. We used the PrimeTime qPCR 5′

Nuclease Assays from Integrated DNA Technologies for each of the targeted genes. The assays used had a double-quenched probe with 5′ 6-FAM dye, internal ZEN and 3′ Iowa Black FQ Quencher. Using our custom primers and probes, we measured the expression of visual opsins in female fish using a standard RT-qPCR protocol (see Supporting Information Methods for full details).

Each gene's expression was normalized against the total cone opsin expression such that each gene's expression was represented as a percentage of the total cone opsins (see Supporting Information Methods for all equations used in estimation of expression). Differences in mean expression of each opsin gene between sulphidic and nonsulphidic populations were determined using linear mixed effects models with habitat type (sulphidic or nonsulphidic) as a fixed effect and drainage as a random effect (Ben-Shachar et al., 2020; Pinheiro et al., 2013). We calculated the per cent variance explained by the fixed effect using MUMIN (Barton, 2009; Nakagawa & Schielzeth, 2013). Since proportional cone expression is sum-constrained, we also ln-ratio transformed our values and repeated the linear mixed effect models (Kucera & Malmgren, 1998; Veen et al., 2017). We found that results from transformed and nontransformed data sets were quantitatively similar, and nontransformed are easier to interpret, so we present figures using proportions. Although the translation between opsin expression and visual perception is complicated through both protein production and neuronal pathways, opsin expression and visual perception are correlated (Sakai et al., 2018). Therefore, in the absence of specific parameters, we also translated opsin expression proportions into a spectral sensitivity measure using a simplifying assumption that opsins contribute additively. For each opsin, *o*, we calculated a spectral sensitivity curve S_o (350–700 nm) using the absorbance templates from Govardovskii et al. (2000) and estimates of the wavelength of maximum absorbance from Kawamura et al. (2016). Additionally, we used maximum absorbance values from microspectrophotometry of *P. mexicana* with cone type absorbances mapped to opsin genes based on proportional expression and orthologous gene sensitivity values (Körner et al., 2006). Opsin proteins can be conjugated to the chromophores A_1 or A_2, which affect the shape of the absorption curve. Thus, we repeated our analyses based on only A_1, only A_2 or a 50:50 mix, although we believe that A_1 is most likely to be the primary chromophore because microspectrophotometry found that the absorption profile of *Poecilia* visual pigments best fits the A_1 chromophore template (Archer & Lythgoe, 1990). These absorbance curves were summed in proportion to each opsin's relative expression to get an individual spectral sensitivity curve for each fish.

All statistical analyses were conducted in R (version 4.1.1) using the tidyverse (version 1.3.0) and nlme (version 3.1-137) packages (Pinheiro et al., 2013; Wickham et al., 2019).

2.3 | Estimation of body coloration

We smoothed reflectance measures using a rolling mean with a 5-nm window width and fitting a spline function to the reflectance curve from 350 to 700 nm. We removed any replicate which contained negative reflectance values. Reflectance measures were normalized so that the sum of reflectance across the measured spectrum was equal across all samples. Three replicate measurements of the same region were averaged by wavelength to get a single spectrum measurement for each region on each fish. To visualize how sulphidic and nonsulphidic populations differed in coloration, we calculated the mean and standard error for reflectance at each 5-nm wavelength window for each population.

Reflectance across the visual spectrum is a complex phenotype with a nonindependent measurement per wavelength per sample. In other systems, this type of data has been represented by the relative activation of three or four visual receptors, thereby turning a visual spectrum into a predicted perceived colour. In our case, the visual system is much more complex, because *P. mexicana* has nine visual opsins. Instead of making assumptions about colour perception, we took an agnostic approach and used a principal component analysis (PCA) to describe the major axis of variation in reflectance. Reflectance measures were averaged in 5-nm windows (a total of 70 wavelength segments), and the PCA was conducted independently for each body part including all populations together. When plotted, we found that principal component 1 (PC1) separated samples by environment. To examine the pattern of variation, we conducted an analysis of variance (ANOVA), testing for the effects of drainage, habitat type and sex on PC1, separately for each body part. For each parameter (i.e., drainage, habitat type, and sex), we compared the full model containing all parameters against a model without that parameter but containing all other parameters using the anova() command in R to test if including each parameter significantly improved the model. Using the full model, we extracted the percentage variance explained using a type-II ANOVA.

2.4 | Parallelism of opsin expression and body coloration

To determine to what degree changes in body coloration and visual sensitivity are parallel across independent drainages, we used PCAs to reduce the dimensionality of the data. For body coloration, we used the mean reflectance in 5-nm windows as the trait values for the PCA, as described above. For visual sensitivity, we used proportion opsin expression as the trait response variable. This presents a problem for PCA because proportion values are constrained to sum to 1, so we used a robust PCA for compositional data (Templ et al., 2011). We found that the first two principal components explained a majority of the variation in each trait, so we used them as input for a multivariate vector-based analysis that describes the direction of divergence between pairs of populations (Bolnick et al., 2018). In this analysis, each vector represents the direction of divergence in colour or opsin expression between the sulphidic and nonsulphidic ecotypes. A small angle between the divergence vectors of two independent ecotype pairs represents a highly similar pattern of divergence (greater

parallelism). A 90° angle would indicate no parallelism in the pattern of divergence, and a large angle (closer to 180°) indicates an opposing direction of divergence. This vector-based approach has previously been used to estimate parallelism in phenotypes or genotypes between populations diverging repeatedly across similar environments (e.g., Rennison et al., 2019; Stuart et al., 2017). We described the direction of divergence between sulphidic and nonsulphidic ecotypes within each drainage using a vector connecting the mean position (centroid) of individuals of one ecotype to the mean position of individuals of the other ecotype. We estimated the angle (θ, in degrees) between the divergence vectors of each ecotype pair (from each drainage) and calculated the average angle between all pairs of populations for a single trait. This resulted in six pairwise combinations. To assess parallelism, we then tested whether the average angle between divergence vectors of different ecotype pairs was smaller than expected. To do this, we used a permutation approach where, for 1000 iterations, we shuffled ecotype status (breaking any correlation between the variable and environment), while retaining population groupings and calculated the average angle between the six pairwise population comparisons. This provided a null-distribution of average angles in the absence of an ecotype effect. Next we compared the average angle we calculated (with potential ecotype effect) against this distribution. We are specifically interested in parallelism (average angle $< 90°$), and not antiparallelism, so our p value is one-tailed and determined by the number of permutations with an average angle smaller than the observed average angle plus 1, divided by the number of iterations plus 1. We used a permutation approach to account for the nonindependence of angles between populations (Watanabe, 2021).

2.5 | Correlation between visual sensitivity and body coloration

We found differences in visual sensitivity, and body coloration between sulphidic and nonsulphidic populations, so we next asked if these shifts were correlated across the visual spectrum. For example, is decreased short-wavelength sensitivity in sulphidic populations accompanied by decreased short-wavelength reflectance? We answered this question by taking a spectrum-wide approach described fully in the supplementary material of Rennison et al. (2016). We used a statistic to quantify the association between the shift in spectral sensitivity, and changes to body reflectance between sulphidic and nonsulphidic populations across all wavelengths for each drainage. For each population, we constructed reflectance curves by calculating at each wavelength (λ) the median reflectance per population per body part. At each wavelength, we then subtracted the median value of the sulphidic population from the median value of the nonsulphidic population within a drainage, yielding the change in reflectance (ΔR). The change in spectral sensitivity (ΔS) was calculated similarly; for each population, we calculated the median sensitivity at each wavelength using the proportions of opsin expression and maximal sensitivity assuming an A_1 chromophore. The change in sensitivity was calculated as the difference between the median nonsulphidic and sulphidic sensitivity curves.

This resulted in two spectral quantities—sensitivity and reflectance—measuring the difference between sulphidic and nonsulphidic populations in each drainage. For reflectance, we have four different measures for the four body parts recorded. We chose pairs of spectral quantities and calculated the correlation coefficient (r) between them. For example, a positive r indicates that regions of the spectrum with increased sensitivity also have increased reflectance. We tested if r was significantly different from zero (no relationship) for each combination, using drainage as our unit of replication in a single-sample two-sided t test. We repeated this analysis using our two different sets of gene wavelength of maximum sensitivity and three chromophore proportions.

3 | RESULTS

3.1 | Opsin expression and visual sensitivity

In all samples, opsin expression was predominantly violet-sensitive SWS1, blue-sensitive SWS2B, green-sensitive RH2-1 and green-sensitive LWS-3 (Figure S1). We compared proportional expression of opsins between sulphidic and nonsulphidic environments, while controlling for drainage, and found significant ($p < .05$) differences between populations from different habitat types in RH2-1 and LWS-3, and a strong trend of differences in SWS2B expression (Figure 2a–c; Table 1). For these three genes, the direction of divergence in opsin expression was repeated across the four independent drainages.

Based on opsin expression, we calculated sensitivity curves for all samples (Figure S2). Inferred sensitivity peaked around 438 and 516 nm, corresponding to the three highly expressed genes, although these peaks differed depending on the source of λ_{max} values used. Due to the consistent differences in opsin expression, we found generally more long-wavelength sensitivity in sulphidic populations and more short-wavelength sensitivity in nonsulphidic populations.

3.2 | Body coloration

Principal component analysis was effective at reducing the dimensionality of our reflectance spectrum measures. For each body part, the first two principal components (PCs) explained between 85.4% and 92.9% of the total variation (Table 2; Figure 3a; Figure S3). In most cases, the first principal component separated samples by habitat type (sulphidic vs. nonsulphidic) (Figure 2d–h). We further probed the sequential effect of drainage, habitat and sex using an ANOVA of PC1 and found that habitat type explained the most variation in abdomen, eye and tail coloration (Table 2). In all cases, sex played a relatively small role in explaining variation of the first PC, although we note that our measurements did not include the dorsal or

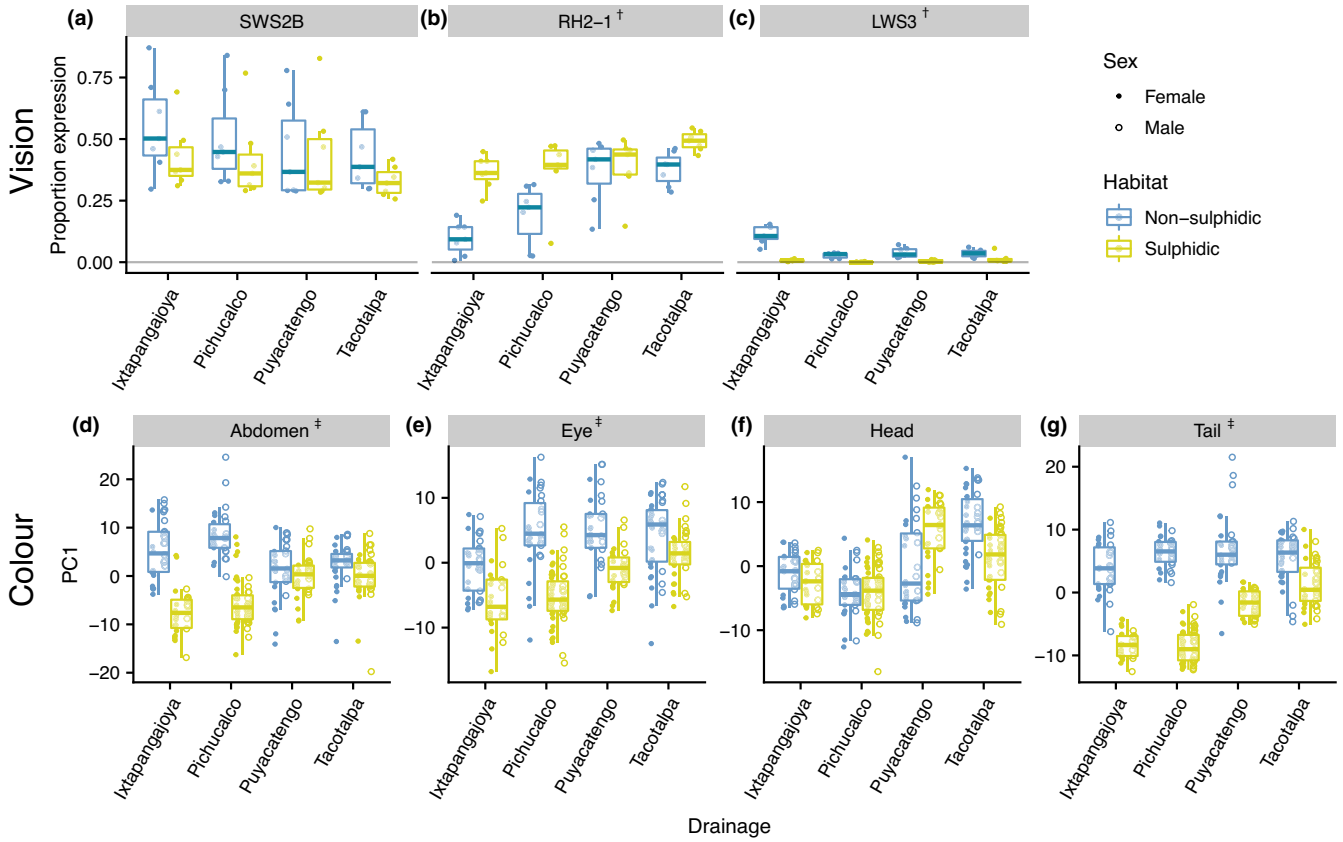

FIGURE 2 Parallel phenotypic differentiation in vision and body colour between environments. (a–c) Proportion of opsin gene expression for genes differentially expressed between environments. (d–g) Principal component 1 scores for body colour. Box area contains the middle two quantiles. †$p < .05$ from a linear mixed effect model testing the effect of environment; ‡$p < .05$ from ANOVA for the effect of environment [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Differences in mean proportion cone opsin expression between sulphidic and nonsulphidic populations

Gene	λ_{max} *reticulata* (nm)	λ_{max} *mexicana* (nm)	Difference in expression (±*SE*)	$F_{1,51}$	Effect size 95% confidence interval	Proportion variance explained	*p* (transformed *p*)
LWS-1	571	563	1.78E−5 (4E−5)	0.20	[−0.21, 0.33]	<0.01	.65 (.47)
LWS-2	516	563	7.9E−5 (2E−4)	0.25	[−0.33, 0.20]	<0.01	.62 (.32)
LWS-3	519	563	0.05 (0.01)	50.45	**[−0.80, −0.45]**	**0.37**	**<.0001 (<.0001)**
LWSr (LWS-4)	NA	NA	8.9E−6 (6E−5)	0.02	[−0.28, 0.24]	<0.01	.89 (.16)
RH2-1	516	537	0.14 (0.03)	25.03	**[0.29, 0.68]**	**0.53**	**<.0001 (<.0001)**
RH2-2	476	461	0.002 (0.005)	0.19	[−0.20, 0.31]	<0.01	.66 (.08)
SWS1	353	349	0.01 (0.02)	0.71	[−0.36, 0.15]	0.01	.41 (.12)
SWS2A	438	461	0.001 (0.001)	1.04	[−0.39, 0.13]	0.02	.31 (.53)
SWS2B	408	403	0.08 (0.04)	3.72	[−0.52, 0.01]	0.06	.06 (.29)

Note: Results of linear mixed effect models using proportional cone expression. *p*-values included for ln-ratio-transformed cone expression are quantitatively similar to results on nontransformed data. *p*-values < .05 are in bold type.

caudal fins, where orange, yellow and black pigmentation is sexually dimorphic. From examining the spectrum, we found that for body parts that differed based on environment (the abdomen, eye and tail), there was generally more reflectance of short wavelengths in nonsulphidic environments than in sulphidic environments, and the opposite pattern for long wavelengths (Figure S4).

3.3 | Parallel phenotypic change

We used a vector-based analysis of PCA space to quantify the degree of parallelism between pairs of populations for opsin expression and body coloration (Figure 3a). We used the loadings of the first two principal components to quantify the degree of parallelism

TABLE 2 The analysis of variance investigating the effects of drainage, habitat and sex on PC1 of four body coloration regions

		Abdomen	Eye	Head	Tail
Drainage ($df = 3$)	*F*-value	1.82	21.5	45.1	8.58
	p-value	.14	$\mathbf{1.52 \times 10^{-12}}$	$\mathbf{8.84 \times 10^{-24}}$	$\mathbf{3.06 \times 10^{-21}}$
	PVE	1.2	13.4	32.0	14.0
Habitat ($df = 1$)	*F*-value	146.0	118.0	1.47	437.5
	p-value	$\mathbf{2.66 \times 10^{-27}}$	$\mathbf{3.84 \times 10^{-23}}$	0.22	$\mathbf{5.26 \times 10^{-59}}$
	PVE	32.6	24.5	0.3	52.1
Sex ($df = 1$)	*F*-value	18.7	20.5	8.58	6.49
	p-value	$\mathbf{2.18 \times 10^{-5}}$	$\mathbf{8.74 \times 10^{-6}}$	$\mathbf{3.69 \times 10^{-3}}$	**.01**
	PVE	4.2	4.3	2.0	0.8

Note: *p*-values < .05 are in bold type.

Abbreviations: *df*, degrees of freedom for ANOVA; PVE, proportion of variance explained.

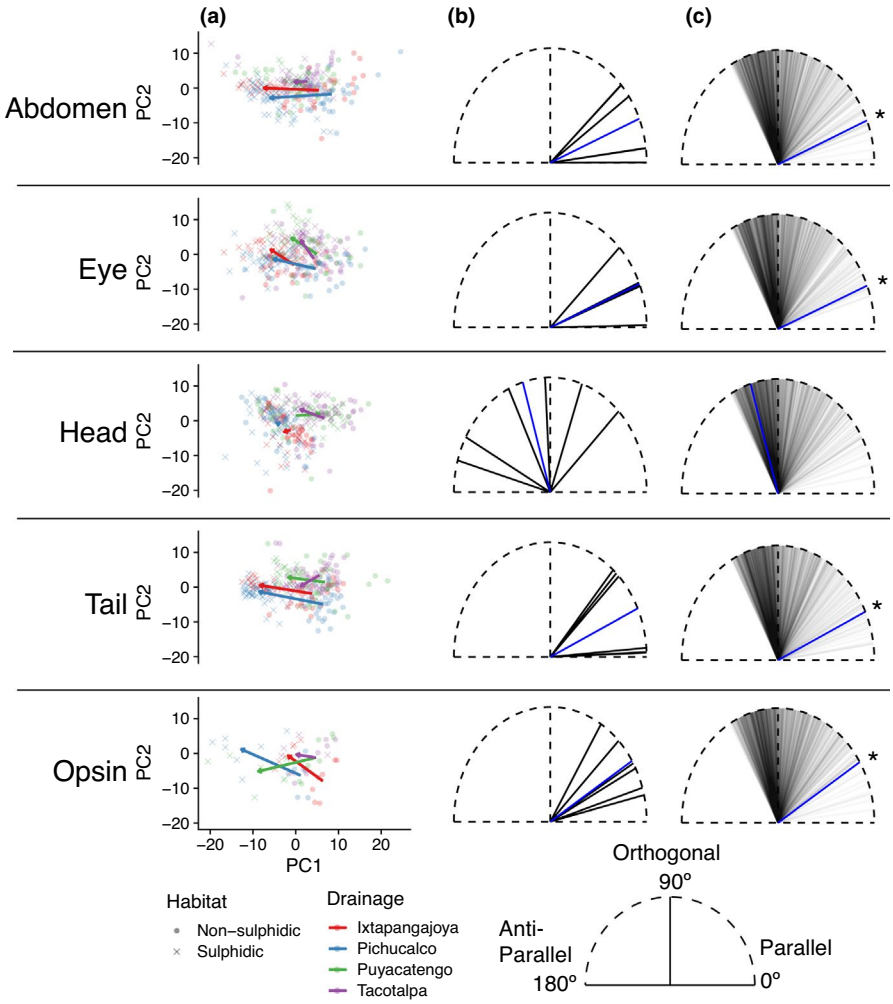

FIGURE 3 Parallel trait evolution. (a) Principal component analysis of body coloration and opsin expression. The arrows show the shift in mean value from nonsulphidic to sulphidic populations in each drainage. (b) Pairwise angle between evolutionary trajectories for vision and body colour phenotypes. Each line represents a pair of drainages. (c) Mean evolutionary trajectory angles for vision and body colour phenotypes for 1,000 permutations. The blue line represents the empirical mean angle; *$p < .05$ [Colour figure can be viewed at wileyonlinelibrary.com]

in the overall direction of divergence between sulphidic and nonsulphidic population pairs. We found significant parallelism across the four independent replicates for opsin expression (mean θ:32.0°, range: 13.4–58.2°, $p = .011$; Figure 3). There was also significant parallelism in the direction of divergence in body coloration between replicate sulphidic and nonsulphidic populations for the tail (mean θ:25.0°, range: 2.1–49.2°, $p = .016$), abdomen (mean θ:22.6°, range: 0.14–42.6°, $p = .016$), and eye (mean θ:22.3°, range: 1.1–44.2°, $p = .007$). There was no evidence for parallelism in the direction of divergence for the head coloration (mean θ:106.6°, range: 44.8–163.9°, $p = .768$; Figure 3). The degree of parallelism tended to be greater for body reflectance than for opsin expression. The magnitude of parallelism also varied among pairwise population comparisons for each trait. The results of the vector-based parallelism

analysis were consistent when male and female pigmentation were analysed separately (results not shown).

3.4 | Correlations between visual sensitivity and body coloration

We found that visual sensitivity and body coloration for body parts with parallel trait evolution were generally positively correlated (i.e., abdomen, eye and tail), especially for body regions with parallel colour shifts, indicating that increased visual sensitivity tended to be associated with increased reflectance of body pigmentation (Figure 4). This pattern was robust to the predicted chromophore proportion or the λ_{max} (Table S2). This analysis used drainage as a unit of replication, so sample sizes were small ($n = 4$) and correlations were only marginally significantly different from zero ($.05 < p < .09$).

4 | DISCUSSION

4.1 | Parallel phenotypic shifts

Repeated shifts in the phenotypes and/or genotypes of organisms that have independently colonized new environments provide strong evidence for the action of natural selection and suggest adaptive value (Schluter & Nagel, 1995). In our survey of four independent drainages containing sulphidic and nonsulphidic populations of Atlantic mollies, we found consistent differences in the opsin gene expression levels and body colour between the two ecotypes. We see repeated differences in the expression of the LWS-3 (λ_{max} 519 nm) and RH2-1 (λ_{max} 516 nm) opsins, as well as the SWSB (λ_{max} 353 nm) opsin with marginal significance. Differences between the ecotypes in the expression of the other six opsins appear to be drainage-specific. Together, the differences in opsin expression between sulphidic and nonsulphidic populations are predicted to translate into differences in the overall visual sensitivity, and perhaps discriminatory ability, of the two ecotypes (Figure S3). Functionally, the parallel shifts in visual sensitivity appear to have reduced short-wavelength sensitivity, while comparatively increasing medium-wavelength sensitivity of sulphidic populations. These patterns are robust to assumptions about λ_{max} values as well as chromophore usage. Our results are somewhat consistent with previous measures of visual sensitivity in the same system. Microspectrophotometry results found very few of the longest wavelength sensitivity cones (Körner et al., 2006); these correspond to the LWS-1 opsin which is expressed at very low levels in our study. In another study, opsin expression was quantified using generic primers for gene families (instead of specific gene copies, as used here) for surface and cave mollies and found relatively consistent amounts of RH2 and LWS opsins (Tobler et al., 2010). In our work, we found generally much higher RH2 expression, although proportions were equal in some samples.

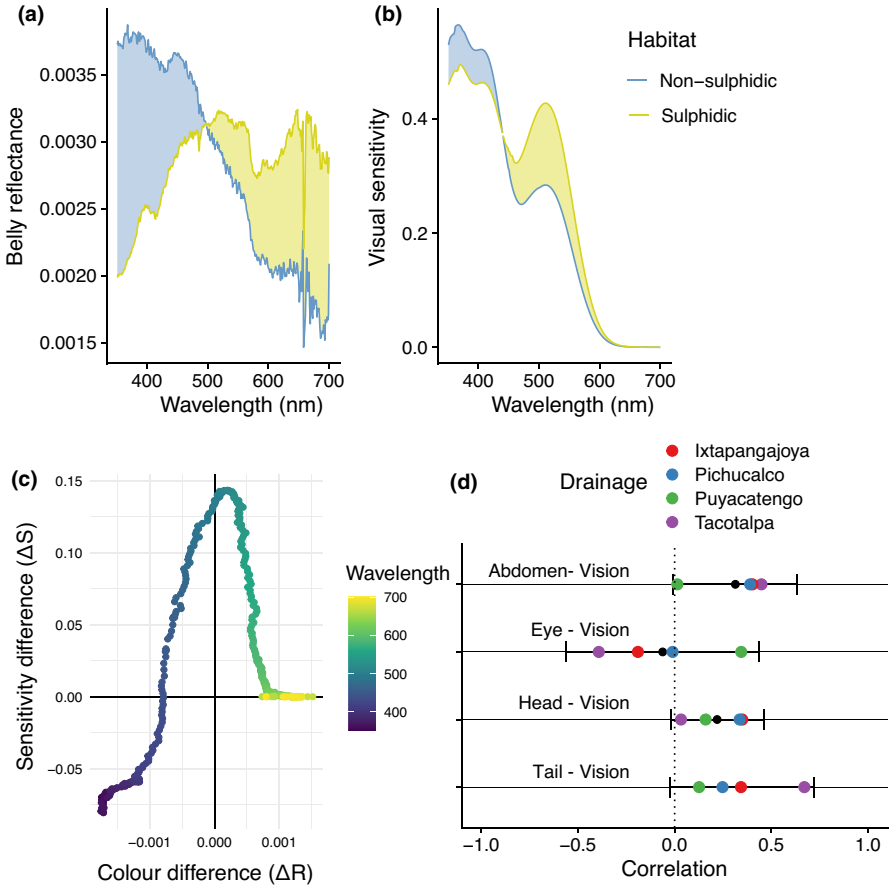

FIGURE 4 Correlation between body coloration and visual sensitivity. (a) Example median normalized belly colour for sulphidic and nonsulphidic populations of a single drainage. The difference between reflectance (ΔR) is highlighted. (b) Example median inferred visual sensitivity between sulphidic and nonsulphidic populations of a single drainage. (c) The correlation between the example ΔS and ΔR. (d) Correlation values for each drainage using *Poecilia reticulata* λ_{max} values and 100% A1 chromophore. The black dot indicates mean of correlations and error bars include 95% confidence interval. *One-sided *t*-test $p < .05$ (not present) [Colour figure can be viewed at wileyonlinelibrary.com]

Parallel shifts in opsin expression have previously been described in several fish species, including African cichlids (O'Quin et al., 2010), threespine stickleback (Rennison et al., 2016) and Neotropical Midas cichlids (Torres-Dowdall et al., 2017). This suggests that the forces shaping opsin expression (and correspondingly spectral sensitivity) are often consistent across habitat transitions. Shifts in body colour reflectance followed a similar pattern, with sulphidic populations reducing short-wavelength reflectance while increasing medium- and long-wavelength reflectance of patches behind the eye, the tail and abdomen. Previous work in three of the four drainages examined here found male body colour differed between sulphidic and non-sulphidic habitats (Zimmer et al., 2018). They noted that fin colour, a trait not measured in our study, covaried with body size, highlighting the role that social status and dominance can play in phenotype. Given that sulphidic and non sulphidic populations can differ in body size—sulphidic adults are typically smaller—it is possible that some of the variation in body colour is mediated through differences in body size (Passow et al., 2017). That being said, the parallel body coloration shifts observed are shared between males and females, so are unlikely to be a product of sexual selection or differences in frequencies of different male types (e.g., sneakers vs. large dominant males).

Shifts in pigmentation and opsin expression between sulphidic and non sulphidic molly ecotypes could be due to genetic and/or plastic changes. Previous work in fish has shown that variation in opsin expression between fish occupying different light regimes can be largely heritable (e.g., in threespine stickleback [Flamarique et al., 2013; Rennison et al., 2016]; damselfish [Stieb et al., 2016]; Atlantic mollies [Tobler et al., 2010]) or largely plastic (e.g., in African cichlids [Nandamuri et al., 2017]; red shiner [Chang & Yan, 2019]; cardinalfish [Luehrmann et al., 2018]). Plasticity seems key for responses to short-term or small-scale variation in light environment (Kranz et al., 2018; Stieb et al., 2016; Veen et al., 2017). Experimental work in guppies suggests that heritable change in opsin expression may require several generations of exposure to a differential light environment (Kranz et al., 2018). It is likely that the differences in opsin expression we observe between sulphidic and nonsulphidic ecotypes is due to a combination of genetically determined factors and phenotypic plasticity. Similarly, skin coloration in fish has both plastic and heritable variation, so the observed parallel shifts may be partially due to shared environmental differences, such as diet (Nilsson Sköld et al., 2013). Future efforts should work toward quantifying the relative contribution of heritable and nonheritable change.

The angle (magnitude) of parallelism was variable across traits and among the four drainages. A small angle between two divergence vectors indicates a very similar pattern of divergence between two independent ecotype pairs. We found that four of the five traits surveyed diverged in a significantly parallel manner across the independent drainages, with the most similar patterns of divergence across drainages occurring in tail, abdomen and eye reflectance (average angles of divergence were 25°, 22° and 23°, respectively). The pattern of divergence in opsin expression, while significantly parallel, was slightly less parallel than that seen for the three pigmentation, traits with an average angle of 32° between pairwise vectors. This suggests that the selective forces shaping patterns of differentiation in body pigmentation are perhaps more consistent among drainages than those affecting opsin gene expression or that the genetic architecture of body pigmentation is more constrained, leading to greater parallelism. The vector-based approach used here has been previously applied in threespine stickleback to quantify the pattern of parallel evolution of morphological traits. For comparison, quantification of morphological parallelism (based on 84 phenotypic traits) across 16 replicate stream and lake ecotype pairs of threespine stickleback revealed multivariate angles ranging from 30° to 135° between any two ecotype pairs (Stuart et al., 2017). These angles are not directly comparable because our analysis focused on parallelism of a single trait, while Stuart et al. reported parallelism across all traits, probably some parallel and some nonparallel. Nevertheless, this suggests the parallelism of pigmentation in sulphide spring mollies is strong relative to that described for other phenotypes.

The similarity of the selective landscape appears to be variable with certain drainages exhibiting a more unique pattern of divergence (or lack of divergence) than the others. Within a trait, there was often considerable difference in the angles of pairwise divergence vectors. For example, the angle between pairs of vectors describing divergence in tail reflectance ranged from 3° to 62° and from 32° (parallel) to 159° (antiparallel) for head reflectance. Interestingly, comparisons involving the Tacotalpa drainage tended to have larger angles than those based on the other three drainages across all traits. This may in part be explained by the fact that not only does the Tacotalpa drainage contain nonsulphidic and sulphidic ecotypes, but also that *Poecilia mexicana* have also colonized and adapted to a nonsulphidic and a sulphidic cave (Tobler et al., 2008). Cave populations are characterized by regressive evolution of body pigmentation and eye function, including reduced opsin gene expression (McGowan et al., 2019; Tobler et al., 2010), and are connected to the sulphidic surface population investigated here by low levels of gene flow (Tobler et al., 2008). Hence, introgression of alleles from populations exhibiting different selective environments (i.e., the absence of light) might contribute to the unique evolutionary trajectory of the Tacotalpa population.

4.2 | Correlated shifts between sensitivity and reflectance

Given our finding of parallel shifts in pigmentation and opsin expression, we sought to determine whether the two traits were co-evolving. Across the four drainages and four coloration traits, there were positive correlations between shifts in visual sensitivity and shifts in body pigmentation in three out of four comparisons. This pattern was found for abdomen, eye and tail reflectance (although all were only marginally significant), suggesting that these three pigmentation traits and spectral sensitivity may be co-evolving. In general, this pattern was driven by increased sensitivity and reflectance in middle- and long-wavelength spectra for sulphide populations.

The matching of spectral shifts between body colour and visual sensitivity may suggest that both are responding to a shared environmental selective force, for example ambient light as mediated by water quality. Although we attempted to measure water transmission, we encountered technical issues (see Supporting Information). Based on the properties of dissolved sulphur, we expect greater absorbance of, and therefore less available, short-wavelength light, but available light is also affected by the amount of dissolved organic material, which may differ between environments. Future studies in this system should measure light transmission in sulphidic and nonsulphidic locations at multiple times of the year. During the wet season, turbidity increases with flow and more dramatic visual changes to water clarity are present; sulphidic waters usually acquire a blue, milky turbidity, while nonsulphidic waters shift to warmer earth-tones (exemplified in Figure 1).

Sensory drive and sensory bias models have been used to explain correlated patterns of divergence of sexual signals and sensory systems. These models predict positive correlations between female perception, male sexual signals and the signalling environment (in the case of sensory drive) (Boughman, 2001). Here, we found that divergences of body reflectance in several body regions are indeed accompanied by matched shifts in spectral sensitivity of female fish and correlate with parameters that describe the signalling environment. However, sensory drive and sensory bias models often consider sexually dimorphic traits (Boughman, 2002; Seehausen et al., 2008). Curiously, we find that male *and* female fish exhibit similar phenotypic patterns for the pigmentation traits included in our study and correspondingly have similar patterns of trait divergence and matching. Molly populations often exhibit sexual dimorphism in pigmentation (Figure 1). One possible reason why we did not find sexual dimorphism in pigmentation is that male nuptial colours are flexible and can be lost between capture and measurement due to stress. Additionally, sexually dimorphic pigmentation patterns may primarily be on the dorsal and caudal fins which were not measured in this study due to measurement issues with background reflectance through transparent fin tissues. Nevertheless, it is possible that females exhibit preferences towards certain pigment patterns, as female preference evolution has been seen in other contexts, which leaves the possibility that sexual selection contributes to divergence of these traits between sulphidic and nonsulphidic populations (Plath et al., 2006). Further experimental work will be required to explicitly test whether there is evidence for variation in female preference for pigmentation traits. Such tests will be pivotal in evaluating whether this system exemplifies sensory drive or sensory bias.

Ecological processes aside from sensory drive/bias may also explain the putatively adaptive shifts in both visual capacity and pigmentation. Sulphidic and nonsulphidic habitats differ in their food webs, fish communities and levels of bird predation (Riesch, Oranth, et al., 2010; Tobler et al., 2015). Different predator communities could affect overall predation risk and correspondingly the need for crypsis. Previous work has documented the evolution of behavioural changes in response to these changed predation pressures (Lukas et al., 2021). Differential diet between habitats could also affect pigmentation and opsin expression between sulphidic and nonsulphidic populations, through genetic and/or plastic changes, as has been suggested for guppies (Grether et al., 2001; Sandkam et al., 2016). Experimental work isolating these different agents of selection, which are correlated in nature, and testing the role of plastic environmental effects will be required to determine the most proximate mechanisms underlying our observed patterns.

5 | CONCLUSIONS

We surveyed the divergence of spectral sensitivity and body pigmentation for four replicate population pairs of mollies inhabiting sulphidic and nonsulphidic habitats. We find robust evidence of parallel shifts in opsin gene expression and body pigmentation. Both spectral sensitivity and body colour have generally positively correlated shifts across the visual spectrum, suggesting the possibility of a shared selective pressure such as a change in ambient light. The parallel phenotypic shifts across four independent populations supports the hypothesis that these are adaptive, although plasticity cannot be ruled out. Further work will be required to determine whether both natural and sexual selection contribute to the observed patterns and what specific selective agents contribute to differential fitness.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest for this work.

AUTHOR CONTRIBUTIONS

D.J.R., G.LO. and M.T. conceived of the project. D.J.R. and M.T. collected samples and environmental measurements. G.L.O., D.J.R. and D.R.M conducted molecular laboratory work. G.L.O., D.J.R. and T.V. analysed resulting data. L.A.R facilitated the field collections. G.L.O. and D.J.R. wrote the manuscript with input from all authors.

DATA AVAILABILITY STATEMENT

All data and code are available on github at https://github.com/djrennison/sulphide_molly (Owens et al., 2021).

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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