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Parallel shifts of visual sensitivity and body colouration 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 both due to differences in the water's spectral properties and correlated ecological changes.

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 and 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 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., Tobler et al. 2010; Rennison et al. 2016), although phenotypic plasticity can also induce large shifts (e.g., (Nandamuri et al. 2017; Kranz et al. 2018; Luehrmann et al. 2018)). 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 and Warren 1957; Munz 1958). But 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 coevolution 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 and 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 where sensory drive seems to explain patterns of co-evolution between shifts in female visual perception and male nuptial colouration. Previous work has attempted to understand this connection by linking the evolutionary rate of opsin genes (Bloch et al. 2015a,b) or opsin gene expression (Sandkam et al. 2015a; Brock et al. 2018) with male nuptial colouration, 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's 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 non-sulphidic populations have been documented to diverge in physiological (Greenway et al. 2020), male body colour (Zimmer et al. 2018), morphological (Tobler and Hastings 2011) and life history traits (Riesch et al. 2010b). In addition, populations in adjacent sulphidic and non-sulphidic 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 non-sulphidic habitats, including the availability of food resources (Tobler et al. 2015) and community composition (presence of predators & competitors) (Greenway et al. 2014; Tobler et al. 2015).

Sulphur containing solutions (aqueous and non-aqueous) are known to absorb wavelengths in the ultraviolet (200-360 nm) region (Okada 1963; Khan 2011). This suggests the ambient light environment may also differ between the adjacent sulphidic and non-sulphidic 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 colouration (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 non-sulphidic sites containing *Poecilia* species to ask the following questions: 1) Has there been parallel evolution in visual sensitivity and/or body pigmentation of the sulphidic and non-sulphidic ecotypes across the different drainages? 2) Has there been co-evolution between female perception and body pigmentation?

Methods

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, El Azufre) and one non-sulphidic (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 10-15 live male and female fish from each location (30 fish total per population). During transport, the live fish were held in black buckets for one to two hours 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 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, USA). During analysis we identified technical issues with our irradiance measurements, and we decided to not include analyses of environmental light spectrum (see Supplementary Online Material)

Opsin expression and spectral sensitivity

Both eyes were removed immediately after euthanasia, stored in 1 ml RNAlater (Qiagen, Netherlands), and moved to a -20 ºC 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 (Haan, Germany) using a carbide bead. Total RNA was extracted using the AurumTM 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

iScriptTM 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 ultra-pure water for RT-qPCR analysis.

To develop unique qPCR primers and probes (see Supplementary Table 1 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 et al. (2015b). 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® (Iowa, USA) 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 reverse-transcription quantitative polymerase chain reaction protocol (see Supplementary 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 Supplementary Methods for all equations used in estimation of expression). Differences in mean expression of each opsin gene between sulphidic and non-sulphidic populations were determined using linear mixed effects models with habitat type (sulphidic or non-sulphidic) as a fixed effect and drainage as a random effect (Pinheiro et al. 2013; Ben-Shachar et al. 2020). We calculated the percent variance explained by the fixed effect using MuMIn (Barton 2009; Nakagawa and Schielzeth 2013). Since proportional cone expression is sum-constrained, we also *ln*-ratio transformed our values and repeated the linear mixed effect models (Kucera and Malmgren 1998; Veen et al. 2017). We found that results from transformed and non-transformed datasets were quantitatively similar, and non-transformed 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 also used maximum absorbance values from

153 microspectrophotometry of *P. mexicana* with cone type absorbances mapped to opsin genes
154 based on proportional expression and orthologous gene sensitivity values (Körner et al. 2006).
155 Opsin proteins can be conjugated to the chromophores A_1 or A_2, which affect the shape of the
156 absorption curve. Thus, we repeated our analyses based on only A_1, only A_2 or a 50:50 mix,
157 although we believe that A_1 is most likely to be the primary chromophore because
158 microspectrophotometry found that the absorption profile of *Poecilia* visual pigments best fit the
159 A_1 chromophore template (Archer and Lythgoe 1990). These absorbance curves were summed
160 in proportion to each opsin's relative expression to get an individual spectral sensitivity curve for
161 each fish.
162
163 All statistical analyses were conducted in R (v4.1.1) using tidyverse (v1.3.0) and nlme (v3.1-
164 137) packages (Pinheiro et al. 2013; Wickham et al. 2019).
165

166 Estimation of body colouration

167 We smoothed reflectance measures using a rolling mean with a five nm window width and fitting
168 a spline function to the reflectance curve from 350 to 700 nm. We removed any replicate which
169 contained negative reflectance values. Reflectance measures were normalized so that the sum
170 reflectance across the measured spectrum was equal across all samples. Three replicate
171 measurements of the same region were averaged by wavelength to get a single spectrum
172 measurement for each region on each fish. To visualize how sulphidic and non-sulphidic
173 populations differed in coloration, we calculated the mean and standard error for reflectance at
174 each five nm wavelength window for each population.
175
176 Reflectance across the visual spectrum is a complex phenotype with a non-independent
177 measurement per wavelength per sample. In other systems, this type of data has been
178 represented by the relative activation of three or four visual receptors, thereby turning a visual
179 spectrum into a predicted perceived colour. In our case, the visual system is much more
180 complex, because *P. mexicana* has nine visual opsins. Instead of making assumptions about
181 colour perception, we took an agnostic approach and used a principal component analysis to
182 describe the major axis of variation in reflectance. Reflectance measures were averaged in five
183 nm windows (a total of 70 wavelength segments), and the principal component analysis was

conducted independently for each body part including all populations together. When plotted, we found that principal component one (PC1) separated samples by environment. To examine the pattern of variation, we conducted an 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 percent variance explained using a type-II ANOVA.

Parallelism of opsin expression and body colouration

To determine to what degree changes in body colouration and visual sensitivity are parallel across independent drainages, we used principal component analyses to reduce the dimensionality of the data. For body colouration, we used the mean reflectance in five 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, therefore 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 non-sulphidic 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., Stuart et al. 2017; Rennison et al. 2019). We described the direction of divergence between sulphidic and non-sulphidic 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 anti-parallelism, 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 one, divided by the number of iterations plus one. We used a permutation approach to account for the non-independence of angles between populations (Watanabe 2021).

Correlation between visual sensitivity and body colouration

We found differences in visual sensitivity, and body colouration between sulphidic and non-sulphidic 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 non-sulphidic 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 non-sulphidic population within a drainage, yielding the change in reflectance (ΔR). 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. Change in sensitivity was calculated as the difference between the median non-sulphidic and sulphidic sensitivity curves.

This resulted in two spectral quantities—sensitivity and reflectance—measuring the difference between sulphidic and non-sulphidic 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.

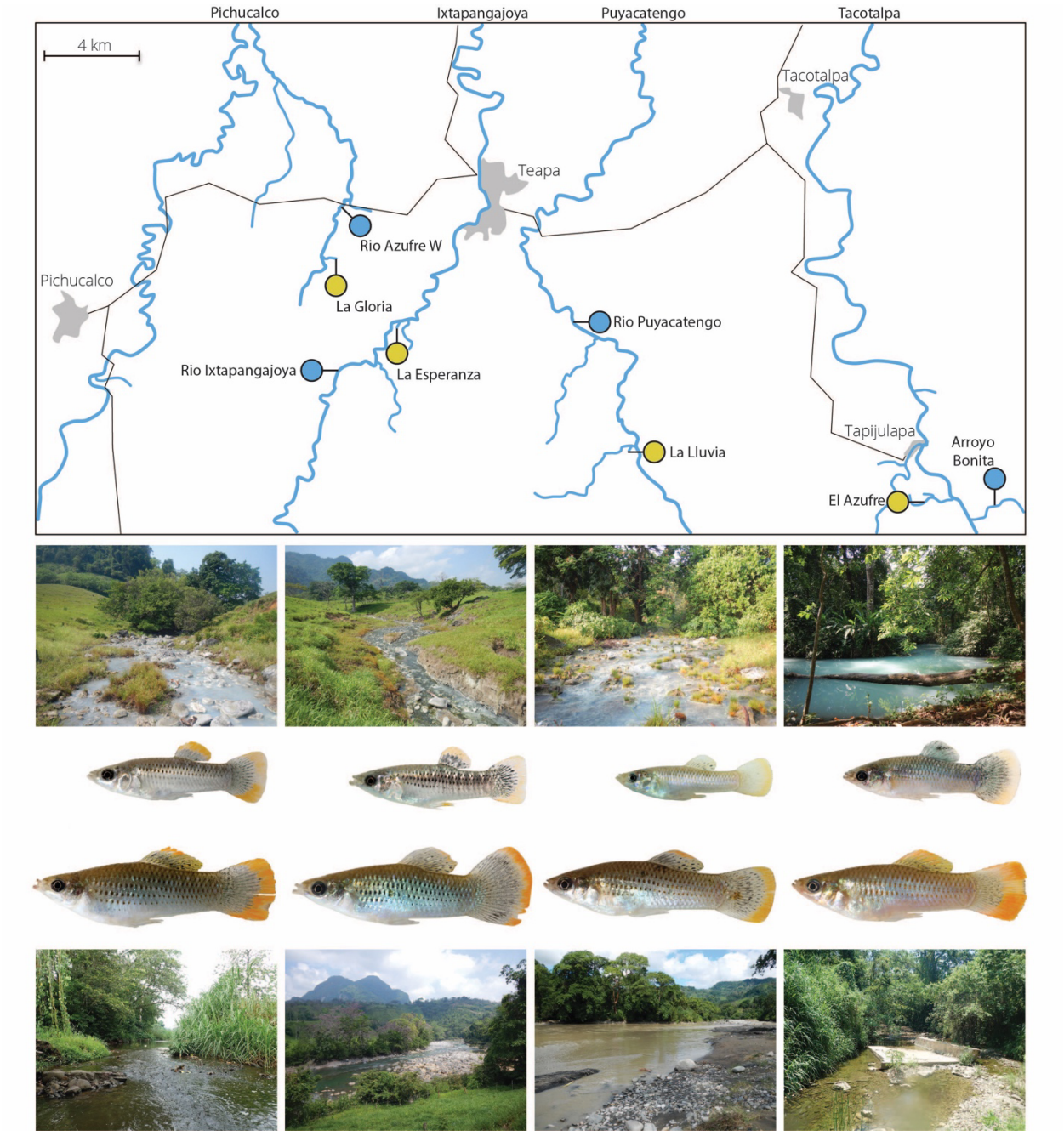

Figure 1. Map of the study region including the four drainages with paired sulphidic (yellow) and non-sulphidic (blue) sites. Photos show sulphidic habitats and *P. mexicana* (top row) non-sulphidic habitats and *P. mexicana* (bottom row) for each of the drainages. All photos were taken in late May or early June toward the end of the dry season.

Results

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 (Supplementary Figure 1). We compared proportional expression of opsins between sulphidic and non-sulphidic environments, while controlling for drainage, and found significant ($p < 0.05$) 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 (Supplementary Figure 2). 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 non-sulphidic populations.

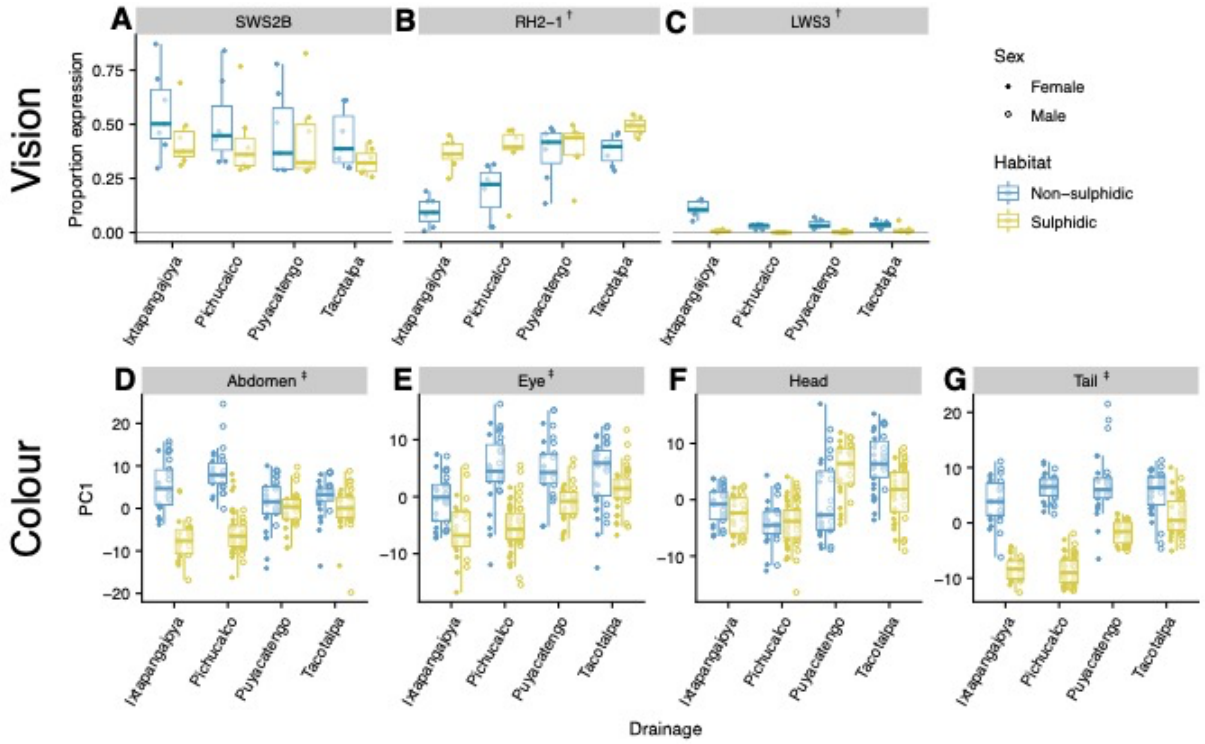

Figure 2: Parallel phenotypic differentiation in vision and body colour between environments. A-C: Proportion opsin gene expression for genes differentially expressed between environments. D-G: Principal component 1 scores for body colour. Box area contains the middle two quantiles. † indicates p-value < 0.05 from linear mixed effect model testing the effect of environment. ‡ indicates p-value < 0.05 from ANOVA for the effect of environment.

Table 1. Differences in mean proportion cone opsin expression between sulphidic and non-sulphidic populations. 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 non-transformed data.

Gene	λ_{max} *reticulata* (nm)	λ_{max} *mexicana* (nm)	Difference in expression (+/- STE)	$F_{1,51}$	Effect size 95% C.I.	Proportion variance explained	p (transformed p)
LWS-1	571	563	1.78E-5 (4E-5)	0.20	[-0.21, 0.33]	<0.01	0.65 (0.47)
LWS-2	516	563	7.9E-5 (2E-4)	0.25	[-0.33, 0.20]	<0.01	0.62 (0.32)
LWS-3	519	563	0.05 (0.01)	50.45	**[-0.80, -0.45]**	**0.37**	**< 0.0001 (< 0.0001)**
LWSr (LWS-4)	NA	NA	8.9E-6 (6E-5)	0.02	[-0.28, 0.24]	<0.01	0.89 (0.16)
RH2-1	516	537	0.14 (0.03)	25.03	**[0.29, 0.68]**	**0.53**	**< 0.0001 (<0.0001)**
RH2-2	476	461	0.002 (0.005)	0.19	[-0.20, 0.31]	<0.01	0.66 (0.08)
SWS1	353	349	0.01 (0.02)	0.71	[-0.36, 0.15]	0.01	0.41 (0.12)
SWS2A	438	461	0.001 (0.001)	1.04	[-0.39, 0.13]	0.02	0.31 (0.53)
SWS2B	408	403	0.08 (0.04)	3.72	[-0.52, 0.01]	0.06	0.06 (0.29)

Body colouration

Principal component analysis was effective at reducing 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; Supplementary Figure 3).

In most cases, the first principal component separated samples by habitat type (sulphidic vs. non-sulphidic) (Figure 2D-H). We further probed the sequential effect of drainage, habitat, and sex using an ANOVA of PC1 and found that the habitat type explained the most variation in abdomen, eye, and tail colouration (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 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 non-sulphidic environments than sulphidic ones, and the opposite pattern for long wavelengths (Supplementary Figure 4).

Table 2: The analysis of variance investigating the effects of drainage, habitat and sex on PC1 of four body colouration regions. Df = degrees of freedom for ANOVA. PVE = Proportion variance explained. Bolded p-values < 0.05.

		Abdomen	*Eye*	*Head*	*Tail*
Drainage (Df = 3)	*F-value*	1.82	21.5	45.1	8.58
	p-value	0.14	**$1.52*10^{-12}$**	**$8.84*10^{-24}$**	**$3.06*10^{-21}$**
	PVE	1.2	13.4	32.0	14.0
Habitat (Df = 1)	*F-value*	146.0	118.0	1.47	437.5
	p-value	**$2.66*10^{-27}$**	**$3.84*10^{-23}$**	0.22	**$5.26*10^{-59}$**
	PVE	32.6	24.5	0.3	52.1
Sex (Df = 1)	*F-value*	18.7	20.5	8.58	6.49
	p-value	**$2.18*10^{-5}$**	**$8.74*10^{-6}$**	**$3.69*10^{-3}$**	**0.01**
	PVE	4.2	4.3	2.0	0.8

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 colouration (Figure 3A). We used the loadings of the first two principal components to quantify the degree of parallelism in the overall direction of divergence between sulphidic and non- sulphidic population pairs. We found significant parallelism across the four independent replicates for opsin expression (mean θ =

32.0°, range: 13.4° - 58.2°, p = 0.011; Figure 3). There was also significant parallelism in the direction of divergence in body colouration between replicate sulphidic and non-sulphidic populations for the tail (mean θ: 25.0°, range 2.1° - 49.2°, p = 0.016), abdomen (mean θ: 22.6°, range: 0.14° - 42.6°, p = 0.016), and eye (mean θ: 22.3°, range: 1.1° - 44.2°, p = 0.007). There was no evidence for parallelism in the direction of divergence for the head colouration (mean θ: 106.6°, range: 44.8° - 163.9°, p = 0.768; 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 was analysed separately (results not shown).

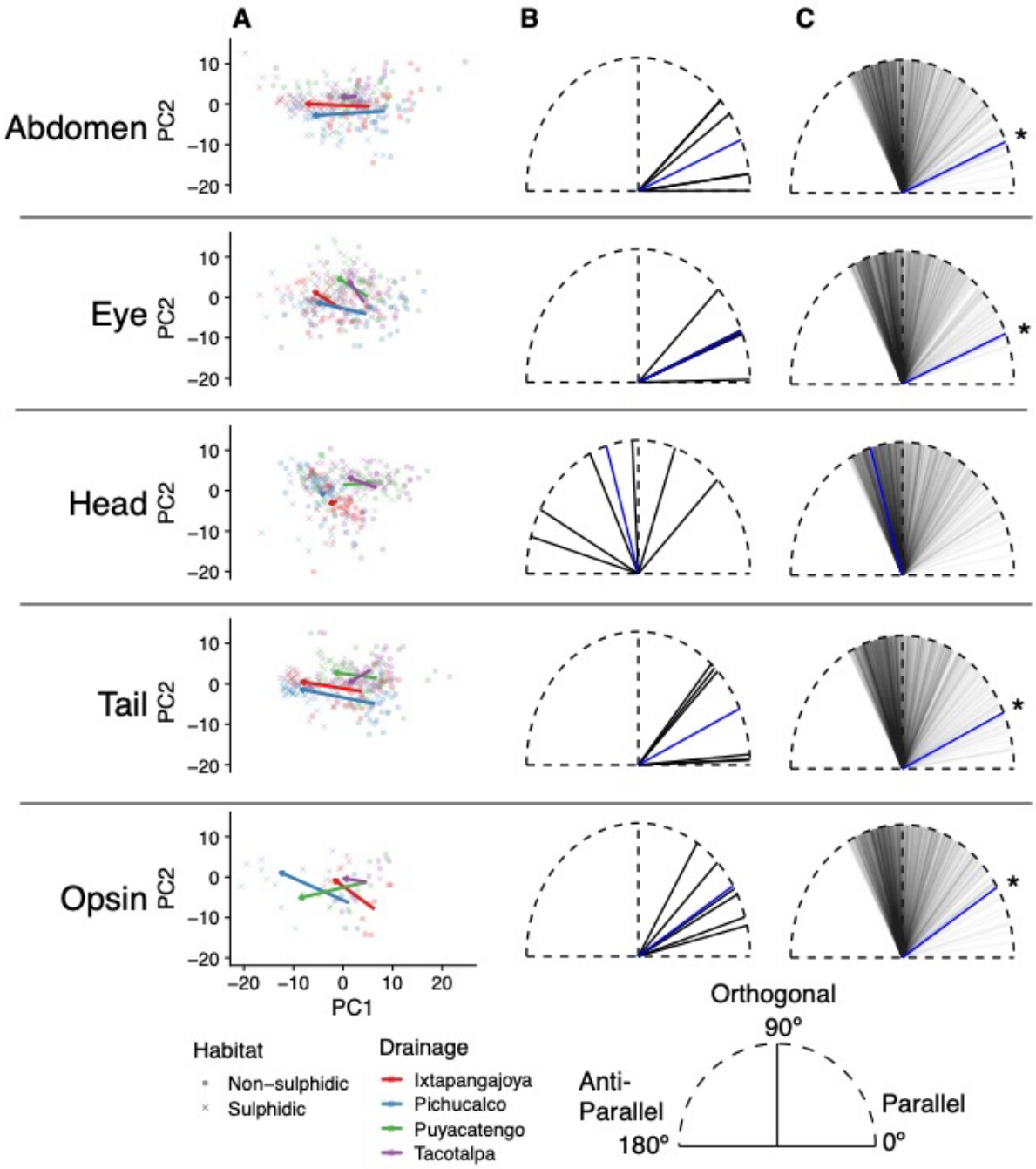

Figure 3: Parallel trait evolution.

A: Principal component analysis of body colouration and opsin expression. The arrows show the shift in mean value from non-sulphidic 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 1000 permutations. The blue line represents the empirical mean angle and * indicates $p < 0.05$.

Correlations between visual sensitivity and body colouration

We found that visual sensitivity and body colouration 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} (Supplementary Table 2). This analysis used drainage as a unit of replication, therefore sample sizes were small (n=4) and correlations were only marginally significantly different from zero ($0.05 < p < 0.09$).

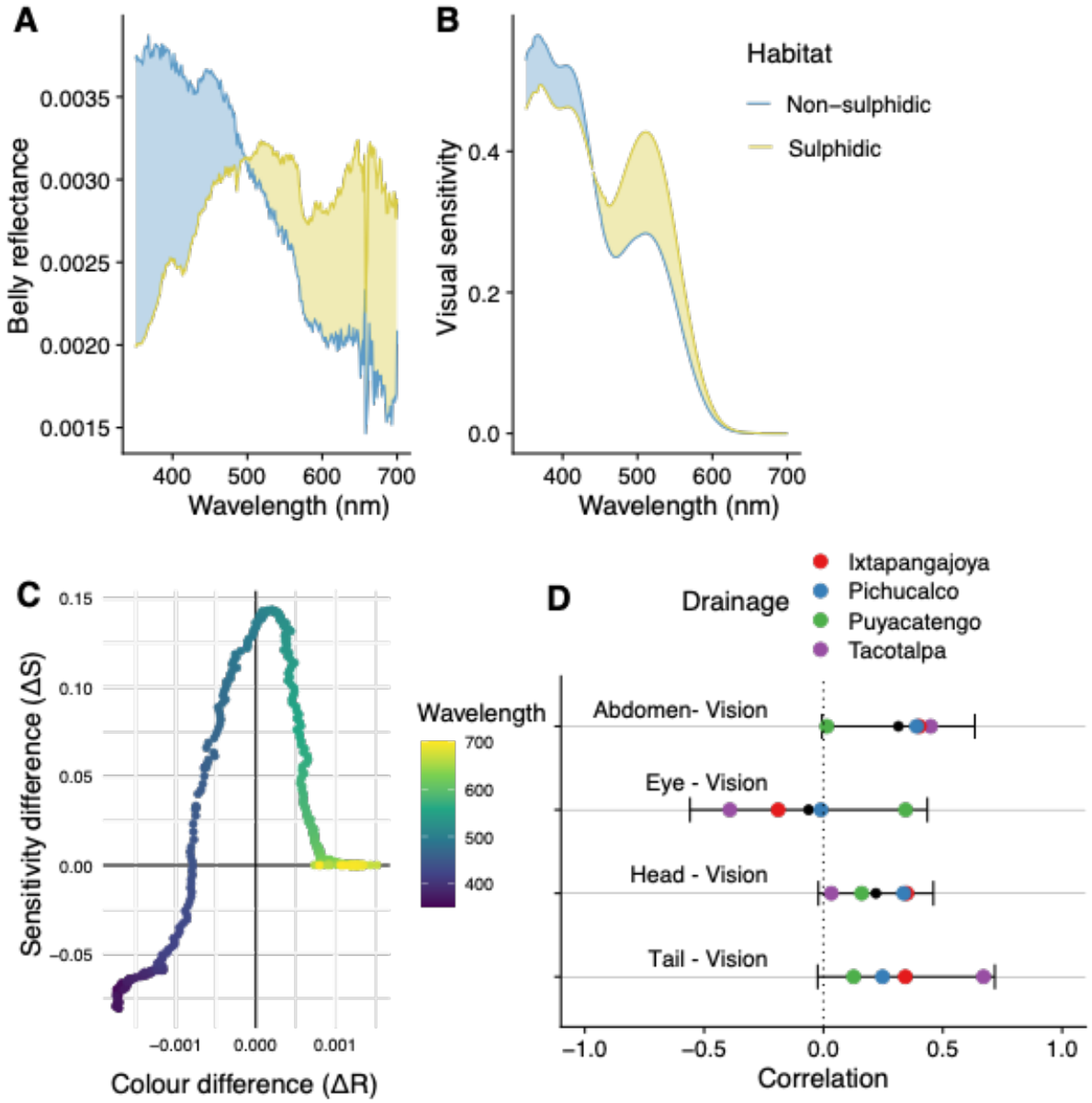

Figure 4: Correlation between body colouration and visual sensitivity.

A: Example median normalized belly colour for sulphidic and non-sulphidic populations of a single drainage. The difference between reflectance (ΔR) is highlighted. B: Example median inferred visual sensitivity between sulphidic and non- sulphidic populations of a single drainage. C: The correlation between the example ΔS and ΔR. D: Correlation values for each drainage using *P. reticulata* λ_{max} values and 100% A1 chromophore. The black dot indicates mean of correlations and error bars include 95% confidence interval. * indicates one-sided t-test p-value < 0.05 (not present).

Discussion

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 suggests adaptive value (Schluter and Nagel 1995). In our survey of four independent drainages containing sulphidic and non-sulphidic 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) 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 non-sulphidic populations are predicted to translate into differences in the overall visual sensitivity, and perhaps discriminatory ability, of the two ecotypes (Supplementary Figure 3). 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 very lowly expressed 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.

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's 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 colouration 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 and Yan 2019); cardinalfish (Luehrmann et al. 2018)]. Plasticity seems key for responses to short term or small-scale variation in light environment (Stieb et al. 2016; Veen et al. 2017; Kranz et al. 2018). 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 non- sulphidic ecotypes is due to a combination of genetically determined factors and phenotypic plasticity. Similarly, skin colouration 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 non-heritable 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, likely some parallel and some non-parallel. 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 the Tacotalpa drainage does not only contain non-sulphidic and sulphidic ecotypes, but *P. mexicana* have also colonised and adapted to a non-sulphidic and a sulphidic cave (Tobler et al. 2008). Cave populations are characterised by regressive evolution of body pigmentation and eye function, including reduced opsin gene expression (Tobler et al. 2010; McGowan et al. 2019), 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.

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 colouration traits, there were positive correlations between shifts in visual sensitivity and shifts in body

pigmentation in 3 out of 4 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 Supplementary Online Material). Based on the properties of dissolved sulphur, we expect greater absorbance, 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 non-sulphidic 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 non-sulphidic 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 divergence 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 non-sulphidic 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 non-sulphidic habitats differ in their food webs, fish communities, and levels of bird predation (Riesch et al. 2010a; 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 non-sulphidic 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.

Conclusions

We surveyed the divergence of spectral sensitivity and body pigmentation for four replicate population pairs of mollies inhabiting sulphidic and non-sulphidic 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.

Data availability

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

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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 lab 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.

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