



Effects of plasticity and genetic divergence in phenotypic trait expression of sulfide spring fishes

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Abstract Natural environments vary, and organisms cope with this variation in two general ways: local adaptation and phenotypic plasticity, although these strategies often overlap and interact. This study explored how local adaptation, phenotypic plasticity, and their interactions shaped phenotypic variation in populations of *Poecilia mexicana*, an extremophile fish living in adjacent but ecologically divergent habitats. By comparing populations from the wild with fish raised in a common-garden environment, we evaluated how genetic differentiation between populations and plasticity contributed to the phenotypic variation observed in nature. We quantified variation

in the size of six organs (brain, eyes, gills, heart, liver, and gastrointestinal tract), routine metabolic rate, and body shape. We found evidence for genetic differences between populations impacting the expression of the majority of traits, in addition to or in interaction with phenotypic plasticity and other predictor variables. Overall, our results suggest that trait divergence between populations was at least in part driven by evolutionary change and not just merely by plasticity induced by environmental differences between habitats. Future studies will have to rigorously test whether evolutionary divergence was caused by natural selection and what traits represent adaptations to the different ecological conditions.

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Introduction

Natural environments vary, and organisms must cope with this environmental variation to survive and reproduce. If environmental variation is spatial, coping can occur through local adaptation, where natural selection favors certain heritable phenotypes, causing genetic differentiation among populations (Endler 1986; Kawecki and Ebert 2004). Alternatively, organisms may cope with environmental variation through phenotypic plasticity, where the same genotypes can

express alternative phenotypes depending on the environment they encounter (Pigliucci 2001; Ghalambor et al. 2007; Whitman and Agrawal 2009). Though local adaptation and plasticity may exist at opposite ends of a spectrum, they are not mutually exclusive; they may co-occur and interact (Kawecki and Ebert 2004; Sasaki et al. 2019). Plasticity itself may even undergo evolution or local adaptation, such that populations vary in their ability to modulate phenotypes in response to environmental cues (Via and Lande 1985; Lively 1986; Gomulkiewicz and Kirkpatrick 1992; Moran 1992; Sultan 1995; Ernande and Dieckmann 2004; Whitman and Agrawal 2009; Scheiner and Levis 2021). Interactions between plasticity and local adaptation can complicate our understanding of the origins of phenotypic variation observed in nature, and it is not sufficient to analyze correlations between environmental variables and phenotypes if we want to understand the causes and consequences of phenotypic variation among natural populations. Instead, common-garden experiments are required to test whether population differences have a heritable basis and may represent adaptation shaped by natural selection, or whether trait differences simply arise through population-specific exposure to different environmental conditions.

This study aimed to explore how plasticity, genetic differentiation between populations, and their interactions have shaped phenotypic variation in populations of fish that live in adjacent but ecologically divergent habitats. The *Poecilia mexicana* species complex includes several closely related species of live-bearing fishes (family Poeciliidae) that are widespread in Mexico and Central America (Alda et al. 2013; Palacios et al. 2016). Multiple populations within this species complex have independently colonized springs rich in toxic hydrogen sulfide (H_2S) in several river drainages of the Río Grijalva basin in southern Mexico (Tobler et al. 2011, 2018; Palacios et al. 2013; Riesch et al. 2015). H_2S is toxic even at micromolar concentrations, and it interferes with oxidative phosphorylation (OxPhos) in mitochondria by binding to and inhibiting the function of COX (cytochrome c oxidase), which is the fourth complex in the respiratory chain (Cooper and Brown 2008; Tobler et al. 2016). Inhibition of OxPhos ultimately stops the aerobic production of ATP and can lead to rapid death (Bagarinao 1992; Reiffenstein et al. 1992). However, *P. mexicana* populations are locally

adapted to H_2S -rich environments. Compared to ancestral lineages in adjacent nonsulfidic habitats, *P. mexicana* populations in sulfide springs exhibit modified OxPhos proteins and upregulated physiological pathways that mediate enzymatic H_2S detoxification, which allows individuals to maintain mitochondrial function and ATP production in the presence of H_2S (Pfenninger et al. 2014; Kelley et al. 2016; Greenway et al. 2020). Previous studies have indicated that physiological differences between sulfidic and nonsulfidic populations have a heritable basis (Passow et al. 2017b), and there is strong genetic differentiation between populations in the different habitat types despite a lack of physical barriers that would prevent fish movement (Plath et al. 2013; Greenway et al. 2023).

While there is clear evidence for local adaptation and the role of natural selection in shaping the evolution of physiological differences between closely related populations in sulfidic and nonsulfidic habitats, there is a wealth of other phenotypic differences between the same populations that are not clearly linked to H_2S tolerance (Tobler et al. 2018). For example, compared to their ancestors in nontoxic habitats, sulfide spring fish typically exhibit larger heads and increased gill surface area (Tobler et al. 2011; Tobler and Hastings 2011; Camarillo et al. 2020), modified nervous and sensory systems (Schulz-Mirbach et al. 2016; Owens et al. 2022), changes in body coloration (Owens et al. 2022), a trophic morphology reflecting a shift from protein-poor to protein-rich diets (Tobler et al. 2015), and changes to their reproductive life history (Riesch et al. 2010b, 2014, 2016). Some of these trait differences may be an inadvertent consequence of individuals developing under fundamentally different environmental conditions—phenotypically plastic responses that may or may not impact individual performance and fitness. Some of these trait differences may be a consequence of pleiotropy if genes under selection by H_2S toxicity also impact the expression of seemingly unrelated traits. And, finally, some trait differences may actually represent adaptations in their own right. Sulfide springs and adjacent nonsulfidic habitats do not merely differ in the presence or absence of H_2S in the water, but the two habitat types are characterized by complex regimes of divergent selection, including abiotic and biotic environmental factors (Tobler et al. 2018). For example, sulfide springs are also hypoxic and have

lower pH and higher dissolved salt concentrations (Tobler et al. 2011). Primary production in sulfide springs is at least in part mediated by chemoautotrophic microbes (Hotelling et al. 2019), which provide a primary food source for sulfide spring fishes (Roach et al. 2011; Tobler et al. 2015), and the toxic environment excludes most interspecific competitors and aquatic predators (although predation from birds can be substantial in some springs; Riesch et al. 2010a; Greenway et al. 2014; Doran et al. 2022). Finally, sulfide spring fishes also face significant energy constraints (Passow et al. 2017a), because a compensatory behavior (aquatic surface respiration) that allows individuals to cope with H_2S toxicity and hypoxia also limits time for benthic foraging (Tobler et al. 2009). Trait divergence between sulfidic and nonsulfidic populations may therefore be a result of selective forces other than H_2S . For example, it is tempting to speculate that hypoxia has caused the evolution of traits associated with oxygen acquisition (Tobler et al. 2011; Barts et al. 2018; Camarillo et al. 2020), shifts in trophic resource use led to divergence in gastrointestinal tract morphology (Tobler et al. 2008, 2015), or energy constraints resulted in a reduced investment into energetically costly traits (Schulz-Mirbach et al. 2016; Passow et al. 2017a). However, common-garden experiments that raise fish from sulfidic and nonsulfidic populations under identical conditions have largely been lacking so far, raising questions about how genetic differences between populations and environmental factors shape phenotypic expression.

In the present study, we leveraged knowledge about trait differentiation between replicated sulfidic and nonsulfidic populations of *P. mexicana* to ask questions about how population divergence and phenotypic plasticity contribute to shaping phenotypic variation between natural populations. Specifically, we investigated a series of morphological and physiological traits in sulfidic and nonsulfidic population pairs from two river drainages to ask how phenotypic traits differ between fish from sulfidic and nonsulfidic populations in the wild, and how these differences change when fish are reared under standardized environmental conditions in the laboratory. Based on past research (see above), we predicted significant trait differences between sulfidic and nonsulfidic populations in the wild, but the patterns of trait differentiation in laboratory-raised fish can provide insights into

the potential roles of genetic and environmental factors in phenotypic expression (Fig. 1). If phenotypic differences between populations in the wild match patterns of difference in common-garden-raised fish, then these trait differences are primarily shaped by genetic divergence between populations (G; Fig. 1A). At the opposite end of the spectrum, phenotypic expression is primarily shaped by environmental factors that induce plasticity if trait expression is identical for populations within a rearing environment (wild or common-garden-reared) but significantly different for populations between rearing environments (E; Fig. 1B). If genetic differences between populations and environmental factors both impact phenotypic expression (G + E), we expect that any differences in the wild are maintained in magnitude and direction in common-garden-raised fish, but also that trait values differ depending on the rearing environment (Fig. 1C). Finally, genetic differences between populations and environmental factors may interact, such that the effect of the environment is dependent on the population-specific genetic background (G × E). In this case, the direction of trait differences observed in the wild may change in common-garden-raised fish (Fig. 1D), or trait differences may disappear (Fig. 1E). Overall, we found consistent evidence that genetic differentiation between populations contributed to phenotypic differences between sulfidic and nonsulfidic populations, but also varied environmental effects that add to or interact with the population-specific genetic background of individuals.

Methods

Study system and collection sites

We sampled populations of the *P. mexicana* species complex from sulfide springs and nearby nonsulfidic streams in the Mexican states of Tabasco and Chiapas (Fig. 2). The two spring complexes used in this study are located in the foothills of the Sierra Madre de Chiapas, each occurring in a separate tributary (Ríos Pichucalco and Tacotalpa) of the Río Grijalva basin. These tributaries are separated by mountains in their upper reaches, where the sulfide springs occur, but widely interconnected in the floodplains of Tabasco where they join the mainstem of the Río Grijalva. The sulfide



Fig. 1 Potential outcomes of trait comparisons between fish from sulfidic (yellow) and nonsulfidic habitats (blue) and from wild-caught (W) and common-garden-reared (C) populations. Comparisons may reflect genetic differences between populations (G), environmental differences between rearing environments (E), additive effects (G + E), or interaction (G × E) effects on traits. The top panels represent a graphical representation of different scenarios, while the bottom panels provide a summary of outcomes (S = significant; NS = nonsignificant) in corresponding statistical models (see methods for details). **A** A trait only affected by genetic differences between populations will show no difference regardless of the rearing environment. **B** A trait only affected by environmental factors will show no

difference between habitats but will vary by rearing environment. **C** A trait affected by both genetic differences between populations and environmental influences will exhibit differences between habitats, and these relative differences will be maintained in the laboratory environment, though actual trait phenotypes will shift between field and laboratory. **D** A case where the interaction between habitat and rearing environment averages out, leaving only the interaction as significant. **E** Any trait with a significant interaction term between habitat and rearing environment where the trait means between field and laboratory populations do not even out. The significance of habitat and rearing environment depends on the data

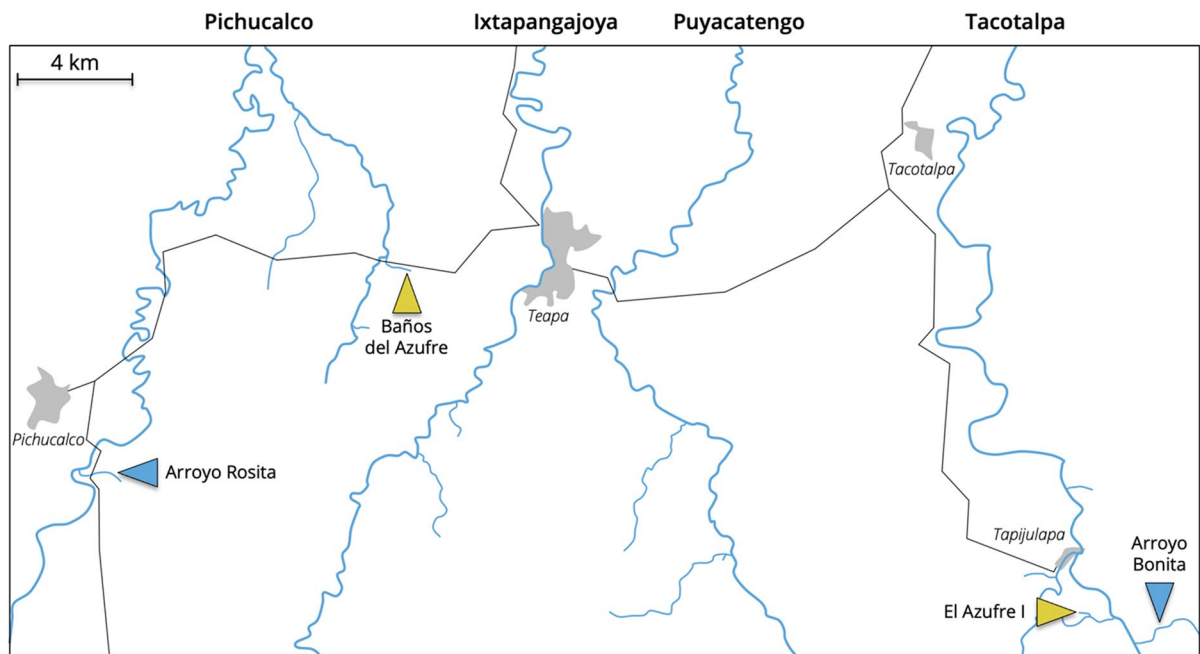


Fig. 2 Maps of the study region, including four major tributaries of the Río Grijalva. Sampling sites of *Poecilia* spp. from sulfidic (yellow) and nonsulfidic habitats (blue) were located in

the Ríos Pichucalco and Tacotalpa. Grey areas represent major towns in the study region, and black lines represent major roads, provided for visual orientation

springs in the Río Tacotalpa drainage are inhabited by locally adapted populations of *P. mexicana* (Tobler et al. 2008), and we sampled fish from El Azufre I (sulfidic) and used Arroyo Bonita (non-sulfidic) as a reference site. Fish in the sulfide springs of the Río Pichucalco drainage have been described as a distinct species, *P. sulphuraria* (Álvarez del Villar 1948), endemic to sulfide springs (Tobler and Plath 2009; Palacios et al. 2013). We sampled *P. sulphuraria* from the Baños del Azufre (sulfidic) and used Arroyo Rosita (non-sulfidic) as a reference site. Descriptions of all field sites used in this study and detailed water chemistry data for each site have previously been published (Tobler et al. 2011; Palacios et al. 2013; Plath et al. 2013; Greenway et al. 2014; Culumber et al. 2016). All wild specimens used for analyses were collected using seines and transported to a local field station (Centro de Investigación e Innovación para la Enseñanza y el Aprendizaje in Teapa, Tabasco) to measure routine metabolic rates (see below). After metabolic rate trials, fish were euthanized with buffered MS222 and fixed in a 10% formaldehyde solution for subsequent dissections (see below).

Common-garden experiment

To establish captive colonies for the common-garden experiment, adult fish were collected by seine and transported to the laboratory. After a 5-day acclimation period, multiple breeding pairs of each population were set up in 40-L tanks. All fish were kept in dechlorinated, nonsulfidic water at temperatures between 24 and 26 °C and subjected to a 12/12-h light/dark cycle. Fish were fed ad libitum with commercially available fish flake food (American Brine Shrimp Company, Ogden, UT, USA), frozen bloodworms, and *Cyclops*, as well as herbivore aquatic gel diet (Mazuri, PMI Nutrition, Shoreview, MN, USA) enriched with highly unsaturated fatty acids (Selco, INVE Aquaculture, Salt Lake City, UT, USA). Following parturition, offspring were moved to new 40-L tanks and raised to adulthood in family groups according to the same maintenance protocol. After reaching maturity, we quantified routine metabolic rates and then fixed specimens in formalin as described for the wild-caught fish.

Quantification of routine metabolic rates

Data for routine metabolic rates used here were previously published (Passow et al. 2015, 2017a), and in this paper, we re-analyzed these data to address the hypotheses outlined in the introduction. Routine metabolic rates were quantified using closed chamber respirometry, tracking oxygen consumption rates through time. This approach has been widely used to quantify metabolic costs associated with a variety of traits and environmental conditions (Haney and Nordlie 1997; Seibel and Drazen 2007; Pirozzi and Booth 2009). In brief, we used the following procedures (Passow et al. 2015, 2017a): (1) wild-caught fish were acclimated to standardized laboratory conditions for at least 48 h. Fish were not fed 24 h prior to trials to ensure measurements were conducted on post-absorptive individuals (Timmerman and Chapman 2004). (2) Individuals were haphazardly chosen and placed into opaque 500-mL respirometry bottles. Bottles were then placed in a water bath to minimize temperature fluctuations. For acclimation to experimental conditions, fish were left undisturbed in the bottles with continuous aeration for at least 12 h. (3) After acclimation, bottles were flushed with fresh aerated water to remove metabolic waste products that could affect metabolism (Timmerman and Chapman 2004) and capped with a lid that had a hole drilled in the top to allow for the insertion of a YSI ProODO optical dissolved oxygen probe (YSI Inc., Yellow Springs, OH, USA). Plumber's putty was fitted around the probe to prevent gas exchange during trials. Probes were set to measure the dissolved oxygen concentration at 10-s intervals. Note that all trials were conducted in absence of H₂S even for sulfidic populations, because the reactivity of H₂S with oxygen in aqueous solution affects the measurement of oxygen consumption rates (Chen and Morris 1972), and even fish from sulfide springs face elevated mortality rates in presence of H₂S without access to the water surface (Plath et al. 2007). (4) After the termination of a trial, individuals were weighed, sexed, and euthanized for further analyses. For each trial, we removed outliers (random readings of zero oxygen) that were caused by instrument error. We also removed data points from the first 60 min of each trial, as the flushing of the bottle with fresh water and the installation of the probe may have caused erratic fish activity (Timmerman and Chapman 2004). Because fish metabolic

rates may be affected by reduced ambient oxygen concentrations (Steffensen 1989), we only included data points measured at dissolved oxygen saturations $\geq 70\%$. Routine metabolic rate (in mgO_2/h) was then calculated for each individual as the product of volume of water in the bottle and the slope of a regression between oxygen concentration and time (mean $R^2=0.99$).

Quantification of body shape

To quantify variation in body shape, we performed a geometric morphometric analysis (Zelditch et al. 2004). For all individuals, we took lateral photographs with a Canon EOS 400D digital camera (Canon USA Inc., Lake Success, NY, USA) mounted on a copy stand. We digitized 14 morphological landmarks on each image (see Fig. 3 for landmark descriptions) using the software program tpsDig (Rohlf 2004). Because the focal distance varied among specimens (to allow for maximum resolution irrespective of specimen size), we size-corrected all pictures by digitizing a 10-mm distance on a size standard in each picture and resizing landmark coordinates based on the number of pixels per millimeter. The size-corrected coordinates from these digitized landmarks were extracted and used as input variables for analyses.

Dissections and quantification of organ sizes

Each specimen was dissected, and we isolated the liver, heart, brain, both eyes, intestinal tract, and

the gill arches on both sides of the body. To quantify variation in the sizes of the liver, heart, brain, and eyes, samples were placed into individual tin weighing boats and dried at 60°C for 72 h in a Thermo Scientific Heratherm Oven (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Following the 72-h drying period, the weights of each organ (excluding hearts) were measured on an OHAUS Adventurer Pro scale (OHAUS Corporation, Parsippany, NJ, USA). Due to their extremely small size, hearts were measured using a Mettler-Toledo XP6U microbalance (Mettler-Toledo, LLC, Columbus, OH, USA). We also measured the length of the intestinal tract, which is tubular and without a discrete stomach in *Poecilia*.

To approximate the size of the gills, the four gill arches of the left branchial basket were placed on a microscope slide and photographed alongside a 5-mm size standard using a Canon EOS Rebel T5i digital camera (Canon USA Inc., Lake Success, NY, USA) mounted on a stereomicroscope. The area of each arch (as defined by its outline) was measured using the image processing software ImageJ. All photographs were scaled by quantifying the pixels per millimeter using the 5-mm size standard. We acknowledge that this is a relatively crude metric of gill size, as functional variation in gill size (i.e., respiratory surface area) is dependent on both macrostructures (e.g., filament length) and microstructures (e.g., number and surface area of lamellae). Nonetheless, studies on other fish have indicated that the area of a gill arch is highly correlated with other metrics used to quantify gill surface area (Langerhans et al. 2007).

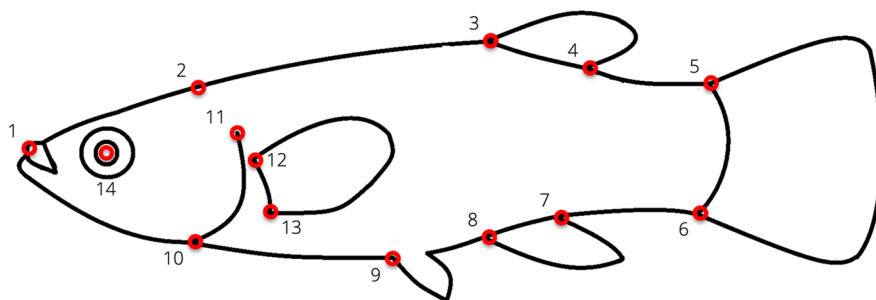


Fig. 3 Landmarks used to characterize variation in body shape: (1) the tip of the upper jaw, (2) the postero-dorsal corner of the head, (3) the anterior and (4) posterior insertions of the dorsal fin, (5) the dorsal and (6) ventral insertions of the caudal fin, (7) the posterior and (8) anterior junctions of the anal fin, (9) the

anterior junction of the pelvic fin, (10) the bottom of the head where the operculum breaks away from the body outline, (11) the dorsal endpoint of the opercular bone, (12) the dorsal and (13) ventral insertions of the pelvic fin, and (14) the center of the pupil

Data analysis

We compared trait variation between populations from sulfidic and nonsulfidic habitats and between wild-caught and common-garden-raised fish to gain a better understanding of how habitat of origin, environmental factors, and their potential interactions shape phenotypic expression in *P. mexicana*. All analyses were conducted in R version 4.3.1 (R Core Team 2023). We regressed measurements of trait expression ($\log_{10}$ -transformed) against the habitat of origin (sulfidic or nonsulfidic) and rearing environment (wild or common garden) using linear models (see Fig. 1). In addition, our linear models also included three potential confounding variables: drainage (Pichucalco or Tacotalpa), sex (female or male), and blotted wet mass ($\log_{10}$ -transformed). The inclusion of these variables was important to account for potential non-convergent evolution among replicated population pairs (indicated by the significance of the drainage term) (Kaeuffer et al. 2012), sexual dimorphism, and sex-specific responses (indicated by significance of the sex term) (Culumber and Tobler 2017), or allometric effects (indicated by significance in of the body mass covariate) (Pélabon et al. 2014). To limit model complexity and avoid model overfitting, we only included two and three-way interactions among variables, and we used model selection to identify combinations of predictor variables that best explained variation in our response variables. To do so, we used the dredge() function from the MuMIn package (Bartoń 2023) to rank models based on Akaike Information Criteria with finite sample correction (AIC_c) (Johnson and Omland 2004). If more than one model exhibited $\Delta AIC_c < 2$, we used the model.avg() function from the MuMIn package to calculate conditional averages for model coefficients (Grueber et al. 2011). If a single model was supported, we calculated χ^2 values and significance levels based on the top model (Table 2). We also calculated the partial η^2 value for each variable in a trait's top model using the eta_squared() function of the EFFECTSIZE package (Ben-Shachar et al. 2020). Note that in traits for which conditional averages were used for model coefficients, some variables were absent from the top model and thus do not have partial η^2 values. To visualize data, we plotted trait expression against body mass (both $\log_{10}$ -transformed) using GGPlot2 (Wickham 2009). In addition to the raw

data, we also calculated and plotted group-specific estimated marginal means and standard errors for relevant subgroups by using the Effect() function from the EFFECTS package based on the top model for each trait (Fox and Weisberg 2018).

Due to its multivariate nature, body shape data was analyzed with an alternative approach. We first conducted a generalized Procrustes analysis, as implemented in the gpa() function from the GEOMORPH package (Adams et al. 2013), which aligned landmark coordinates to remove effects of translation, rotation, and scale and calculated a consensus shape and centroid sizes. Aligned coordinates were then analyzed with a Procrustes ANOVA using the procD.lm() function from the GEOMORPH package. Procrustes ANOVA uses a permutation approach to assess the statistical significance of predictor variables. As for analyses described above, we included habitat of origin, rearing environment, drainage, sex, and centroid size (as a metric of body size), as well as all two and three-way interactions. To calculate significance levels, we used 9,999 permutations and type III sums of squares. To visualize body shape variation, we conducted a principal component analysis using the gm.prcomp() function on the Procrustes shape coordinates (Table S1).

Results

Overall, we analyzed 258 individual fish, but we dropped 17 samples because they had missing data for at least one of the traits ($N=241$ for all analyses; see Table 1 for details). As expected based on previous research, we found significant trait differences between fish from sulfidic and nonsulfidic populations in the wild (Figs. 4, 5, and 6) for all traits besides eye size (no difference between sulfidic and nonsulfidic populations in the Río Tacotalpa; Fig. 4D) and liver size (no differences between sulfidic and nonsulfidic populations in either river drainage; Fig. 5F). Accordingly, habitat of origin was retained as a predictor variable in all best-fit models, and with the exception of liver size, this main effect was also significant for all traits (Tables 2 and 3). Similarly, rearing-environment was included as a predictor variable in all models, indicating that environmental variation also impacts trait expression in this system (although environmental effects were not statistically significant for

Table 1 List of field sites and sample sizes after removal of outliers

Drainage	Site	Habitat type	<i>N</i> field total	<i>N</i> lab total	<i>N</i> field males	<i>N</i> lab males	<i>N</i> field females	<i>N</i> lab females
Pichucalco	Baños del Azufre	Sulfidic	39	12	8	5	31	7
	Arroyo Rosita	Nonsulfidic	25	18	7	7	18	11
Tacotalpa	El Azufre I	Sulfidic	44	22	7	8	37	14
	Arroyo Bonita	Nonsulfidic	34	16	9	7	25	9

brain size; Table 2). Finally, all traits also co-varied with body size (Tables 2 and 3).

Even though our focal predictor variables were consistently included in the top models, there were some substantial differences in terms of what other predictor variables and what interaction effects explained variation in specific traits, indicating that genetic differences between populations and environmental factors do not affect all traits equally. In the following sections, we describe our detailed findings trait by trait, grouping different traits based on the scenarios outlined in the introduction (see Fig. 1). Note that no trait strictly followed the first or second scenario (Fig. 1A, B), where trait expression is only shaped by either genetic differentiation between populations or environmental factors, although—as we will see—brain size may be the exception to this, as rearing environment was included in the top models but did not turn out to be statistically significant.

Traits shaped by habitat of origin and environment

Four traits—routine metabolic rate and the sizes of eyes, heart, and intestine—were shaped by both habitat of origin and by rearing environment, with differences between sulfidic and nonsulfidic populations being maintained in the laboratory in the same direction and magnitude (G+E; Fig. 1C). Although these traits exhibited the same patterns in terms of the effects of our focal variables, there were differentially affected by confounding variables, which warrants a more detailed discussion of each trait separately (Table 2).

Routine metabolic rate differed significantly between fish from sulfidic and nonsulfidic habitats (habitat of origin, $P < 0.001$), but even though rearing environment was marginally significant in the model averaging ($P = 0.05$; Table 2), this predictor variable was not included in the top model. Per unit mass, fish from sulfide springs had 26% lower routine metabolic rates (Fig. 4A, B), and there were significant interactions between body size and habitat of

origin as well as body size and drainage (Table 2). Habitat of origin ($\eta_p^2 = 0.409$) and body size ($\eta_p^2 = 0.358$) had the largest effect on routine metabolic (Table 2). Overall, these results indicate that differences in routine metabolic rate observed between fish from sulfidic and nonsulfidic populations were primarily shaped by habitat-of-origin and—to a much lesser degree—by exposure to different rearing environments (G+E; Fig. 1C).

Fish from sulfidic environments generally had smaller eyes than those from nonsulfidic habitats, although this difference was much more pronounced in populations from the Río Pichucalco (51% smaller) than those from the Río Tacotalpa (5% smaller) (habitat×drainage, $P < 0.001$ in Table 2; Fig. 4C, D). In addition, fish raised in the common garden exhibited significantly larger eyes (33%) than their counterparts from the wild, irrespective of habitat of origin or drainage (Fig. 4D). The effect size of habitat of origin on eye size was larger ($\eta_p^2 = 0.728$) than that of rearing environment ($\eta_p^2 = 0.313$). There was also a significant interaction term between drainage and body size.

Fish from sulfidic habitats also had 52% larger hearts than fish from nonsulfidic habitats, and heart size consistently decreased in the common garden environment by an average of 49% compared to wild populations (Fig. 4E, F). Note that there were also significant differences in heart size between the sexes, with females having larger hearts than males, and between river drainages, with fish from the Río Pichucalco having larger hearts than those from the Río Tacotalpa (not shown).

Finally, there were significant differences in intestinal tract length, with fish from sulfidic habitats having 69% shorter intestines than those from nonsulfidic habitats, and fish in captivity having 27% shorter intestines than those in the wild (Fig. 4G, H). Habitat of origin had a stronger effect on intestine size ($\eta_p^2 = 0.717$) than rearing environment ($\eta_p^2 = 0.451$). In addition, males had significantly shorter intestines than females (sex, $P < 0.001$; Table 2 and Fig. 5H). There was also a significant interaction term between sex and body size.

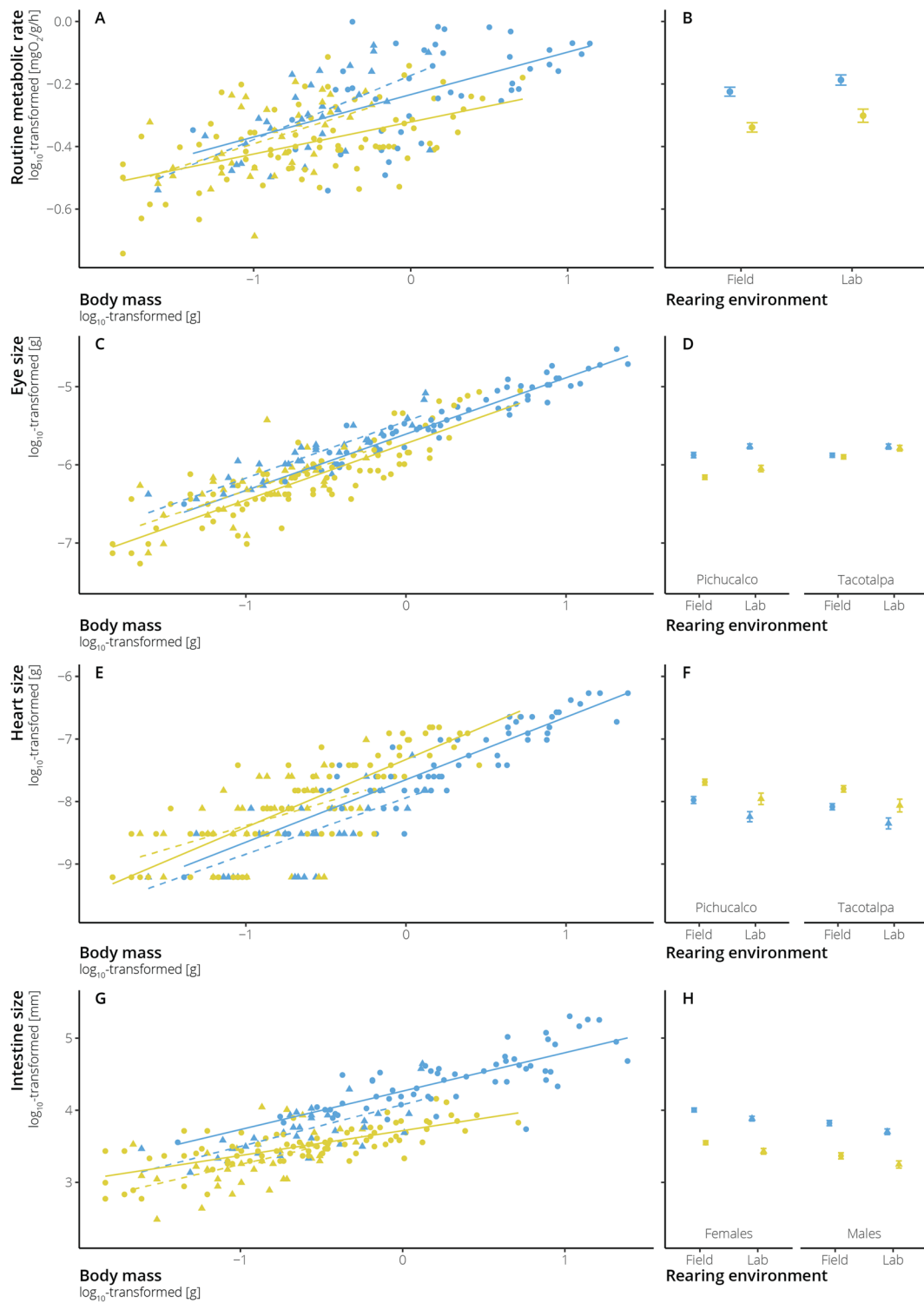


Fig. 4 Routine metabolic rate and sizes of eyes, heart, and intestines were shaped by both habitat of origin and by rearing environment. Differences between sulfidic and nonsulfidic pop-

ulations were maintained in the laboratory in the same direction and magnitude, as illustrated in Fig. 1C. The raw data (left) and estimated marginal means (right) are visualized via scatterplots

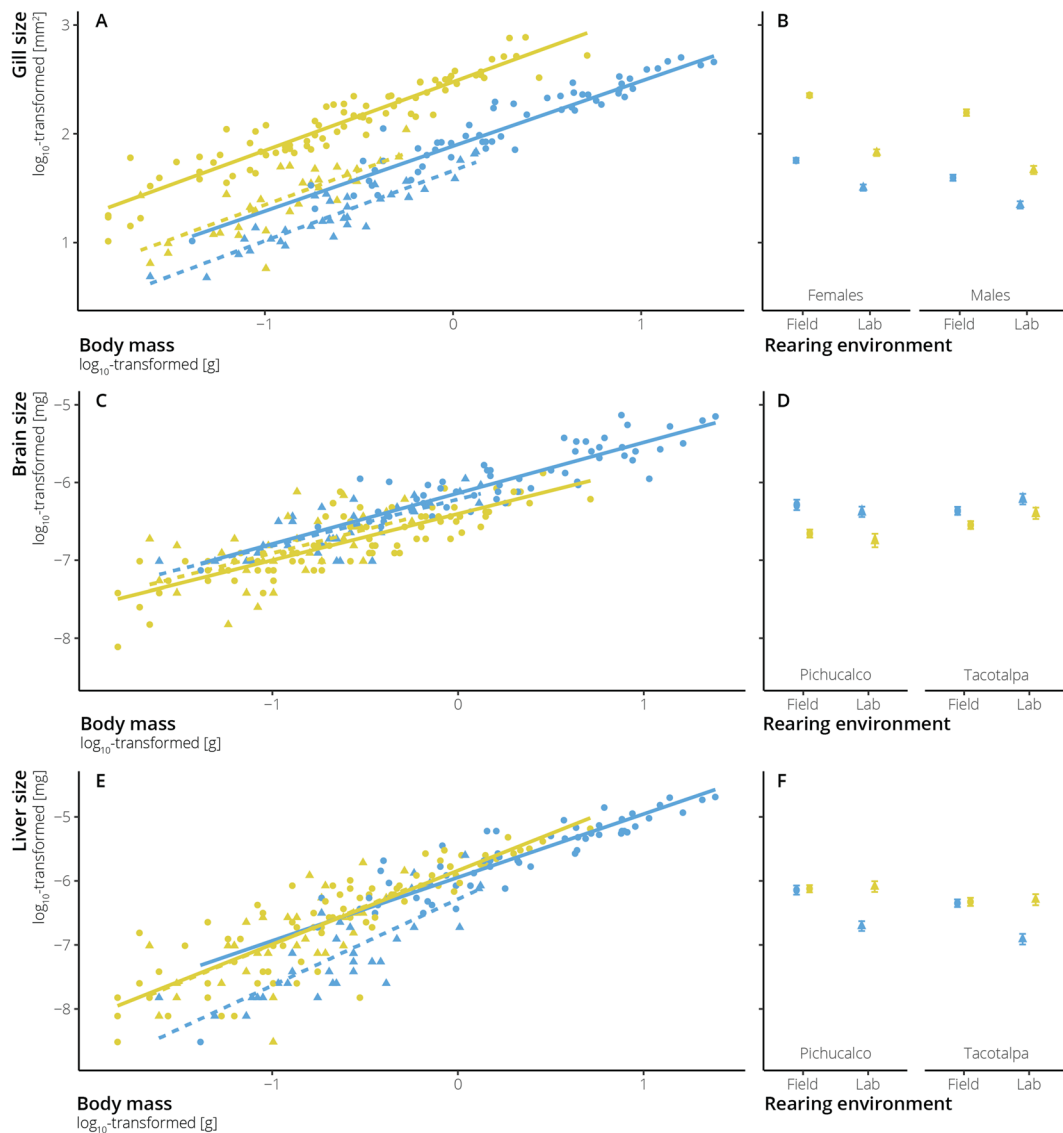


Fig. 5 Gill size, brain size, and liver size exhibited significant habitat of origin $\times$ rearing environment interactions. The raw data (left) and estimated marginal means (right) are visualized via scatterplots. These traits relate to predictions D–E in Fig. 1.

Overall, the results for measurements of routine metabolic rates and the sizes of eyes, hearts, and intestines indicated that differences between sulfidic and nonsulfidic populations observed in the wild generally persist in captivity, even if the rearing environment—and other variables—also affect the expression of these traits to some degree. Hence, population differences observed in the wild are not merely a consequence of plastic responses to different environmental conditions

The significance of interactions was driven by magnitude differences in trait means between sulfidic and nonsulfidic fish (gills, **B**), or differing responses to common-garden conditions mediated by plasticity and habitat of origin (brain and liver, **D** and **F**)

that fish experience in sulfidic and nonsulfidic habitats (G+E; Fig. 1C).

Traits shaped by interactions between habitat of origin and environment

The best-fit models for four traits—brain size, gill size, body shape, and liver size—included significant

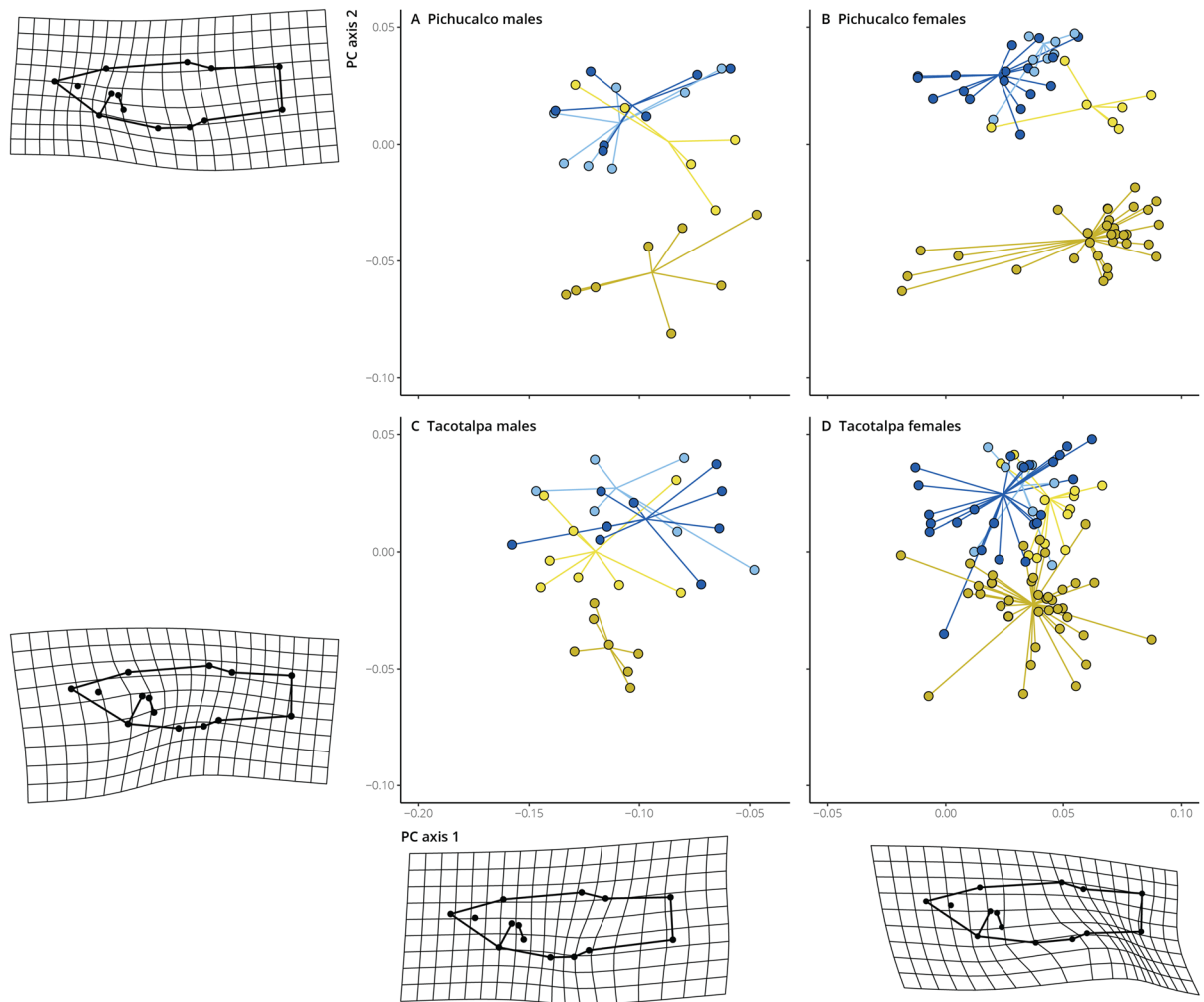


Fig. 6 Body shape was significantly different between wild-caught sulfidic fish (dark yellow) and all other populations, suggesting a plastic response to sulfide spring environments. Body shape was quantified via generalized Procrustes analysis and analyzed with Procrustes ANOVA, then visualized via

principal component analysis. Resulting graphs are presented split by sex and drainage. Note that the scales between the x and the y axes differ, since PC axis 1 explains 60% of variation in body shape, and PC axis 2 only explains 14% of variation (see Table S1)

interaction terms between habitat of origin and rearing-environment ($G \times E$, Fig. 1D, E). However, the direction of the interaction term differed across traits.

Fish from sulfidic habitats exhibited gills over three times as large as those from nonsulfidic habitats (Table 2 and Fig. 5B). This difference was maintained in captivity, but a significant interaction term arose because the magnitude of the difference was smaller in common-garden as opposed to wild populations (Fig. 5A, B). Both sulfidic and nonsulfidic fish had smaller gills in captivity than in the wild, but this decrease was more pronounced in sulfidic fish (68%

smaller) than nonsulfidic fish (42% smaller). Overall, the rearing environment had a stronger effect on gill size ($\eta_p^2 = 0.858$) than the habitat of origin ($\eta_p^2 = 0.162$). Hence, the significant interaction term between habitat of origin and rearing-environment in this case might reflect variation in plasticity. Note that gill size was also affected by sex and a sex by body size interaction (Table 2), and females consistently had larger gills than males (Fig. 5B).

Fish from sulfidic habitats consistently exhibited smaller brains than those from adjacent nonsulfidic habitats, with magnitude differences between

Table 2 Results of analyses of variance (ANOVAs) testing the effect of habitat of origin, rearing environment, drainage of origin, sex, and size (mass) on univariate physiological traits of *Poecilia mexicana* and *Poecilia sulfuraria*

	Habitat	Rearing	Habitat × rearing	Habitat × size	Habitat × drainage	Rearing × size	Rearing × sex	Rearing × drainage	Size	Sex	Drainage	Sex × size	Drainage × size
Metabolic rate	$\chi^2 = 5.416$ $P < 0.001$ $\eta_p^2 = 0.409$	$\chi^2 = 1.992$ $P = 0.0464$		$\chi^2 = 3.478$ $P < 0.001$ $\eta_p^2 = 0.057$		$\chi^2 = 2.033$ $P = 0.042$			$\chi^2 = 6.234$ $P < 0.001$ $\eta_p^2 = 0.358$	$\chi^2 = 1.975$ $P = 0.050$ $\eta_p^2 = 0.026$	$\chi^2 = 0.386$ $P = 0.699$ $\eta_p^2 = 0.138$		$\chi^2 = 2.449$ $P = 0.014$ $\eta_p^2 = 0.009$
	$\chi^2 = -5.833$ $P < 0.001$ $\eta_p^2 = 0.728$	$\chi^2 = 3.762$ $P < 0.001$ $\eta_p^2 = 0.313$			$\chi^2 = 4.460$ $P < 0.001$ $\eta_p^2 = 0.055$				$\chi^2 = 16.626$ $P < 0.001$ $\eta_p^2 = 0.821$		$\chi^2 = 0.432$ $P = 0.666$ $\eta_p^2 = 0.089$		$\chi^2 = 2.916$ $P = 0.004$ $\eta_p^2 = 0.040$
Heart size	$\chi^2 = 4.693$ $P < 0.001$ $\eta_p^2 = 0.121$	$\chi^2 = 3.095$ $P = 0.002$ $\eta_p^2 = 0.409$				$\chi^2 = -2.650$ $P = 0.009$ $\eta_p^2 = 0.034$			$\chi^2 = 18.738$ $P < 0.001$ $\eta_p^2 = 0.649$	$\chi^2 = -2.200$ $P = 0.029$ $\eta_p^2 = 0.015$	$\chi^2 = -2.019$ $P = 0.045$ $\eta_p^2 = 0.214$		
	$\chi^2 = 12.362$ $P < 0.001$ $\eta_p^2 = 0.717$	$\chi^2 = 3.130$ $P = 0.002$ $\eta_p^2 = 0.451$		$\chi^2 = 4.340$ $P < 0.001$ $\eta_p^2 = 0.085$					$\chi^2 = 14.376$ $P < 0.001$ $\eta_p^2 = 0.572$	$\chi^2 = 3.759$ $P < 0.001$ $\eta_p^2 = 0.075$	$\chi^2 = 1.987$ $P = 0.047$	$\chi^2 = 2.327$ $P = 0.020$ $\eta_p^2 = 0.026$	
Gill size	$\chi^2 = 19.130$ $P < 0.001$ $\eta_p^2 = 0.162$	$\chi^2 = 6.840$ $P < 0.001$ $\eta_p^2 = 0.858$	$\chi^2 = -6.005$ $P < 0.001$ $\eta_p^2 = 0.142$						$\chi^2 = 31.300$ $P < 0.001$ $\eta_p^2 = 0.848$	$\chi^2 = -5.940$ $P < 0.001$ $\eta_p^2 = 0.132$		$\chi^2 = -3.094$ $P = 0.002$ $\eta_p^2 = 0.045$	
	$\chi^2 = 3.585$ $P < 0.001$ $\eta_p^2 = 0.614$	$\chi^2 = 0.932$ $P = 0.351$ $\eta_p^2 = 0.290$	$\chi^2 = 1.800$ $P = 0.072$	$\chi^2 = 1.374$ $P = 0.170$	$\chi^2 = 1.875$ $P = 0.061$ $\eta_p^2 = 0.013$			$\chi^2 = 2.155$ $P = 0.031$ $\eta_p^2 = 0.027$	$\chi^2 = 16.242$ $P < 0.001$ $\eta_p^2 = 0.688$		$\chi^2 = 0.082$ $P = 0.935$ $\eta_p^2 = 0.084$		
Liver size	$\chi^2 = 0.249$ $P = 0.803$ $\eta_p^2 = 0.266$	$\chi^2 = -5.542$ $P < 0.001$ $\eta_p^2 = 0.545$	$\chi^2 = 4.683$ $P < 0.001$ $\eta_p^2 = 0.098$						$\chi^2 = 20.303$ $P < 0.001$ $\eta_p^2 = 0.736$	$\chi^2 = -3.473$ $P < 0.001$ $\eta_p^2 = 0.044$	$\chi^2 = -3.420$ $P < 0.001$ $\eta_p^2 = 0.321$		

Terms included from the best-supported model(s) for each trait. Models generated via model selection, limited to one- and two-way interaction terms

Table 3 Results of analyses of variance (ANOVA) examining body shape variation

Dependent variable	DF	SS	MS	Rsq	F	Z	P
Centroid size	1	0.006	0.006	0.003	2.664	2.246	P=0.013
Drainage	1	0.003	0.003	0.002	1.175	0.567	P=0.285
Habitat	1	0.008	0.007	0.004	3.381	2.728	P=0.003
Rearing	1	0.003	0.003	0.002	1.160	0.565	P=0.287
Sex	1	0.010	0.010	0.006	4.588	3.350	P<0.001
Centroid size×drainage	1	0.002	0.002	0.001	1.075	0.376	P=0.350
Centroid size×habitat	1	0.005	0.005	0.003	2.199	1.841	P=0.034
Centroid size×rearing	1	0.002	0.002	0.001	0.954	0.155	P=0.443
Centroid size×sex	1	0.005	0.005	0.003	2.418	2.075	P=0.019
Drainage×habitat	1	0.016	0.016	0.010	7.442	4.319	P<0.001
Drainage×rearing	1	0.003	0.003	0.002	1.297	0.778	P=0.218
Drainage×sex	1	0.005	0.005	0.003	2.323	1.980	P=0.024
Habitat×rearing	1	0.007	0.007	0.004	3.275	2.704	P=0.004
Habitat×sex	1	0.005	0.005	0.003	2.128	1.761	P=0.039
Rearing×sex	1	0.005	0.005	0.003	2.069	1.749	P=0.039
Habitat×rearing×sex	1	0.002	0.002	0.001	1.106	0.452	P=0.328
Drainage×rearing×sex	1	0.002	0.002	0.001	0.919	0.063	P=0.469
Drainage×habitat×sex	1	0.005	0.005	0.003	2.111	1.769	P=0.039
Drainage×habitat×rearing	1	0.004	0.004	0.003	1.997	1.665	P=0.051
Residuals	190	0.419	0.002	0.244			
Total	209	1.717					

The table includes predictor terms (dependent variable), *DF* degrees of freedom, *SS* sum-of-squares, *MS* mean square, *F* F-ratio, *Z* Z-score, *P* *P* value. Terms with a *P* value ≤ 0.05 are given in bold

drainages (47% smaller in Pichucalco, 27% smaller in Tacotalpa) (Fig. 5C, D). While population differences were maintained in common-garden-reared fish, the direction of change was different for the populations in different river drainages. In fish from the Río Pichucalco, brain size decreased by 14% in captivity for both sulfidic and nonsulfidic populations, while it increased by 29% in fish from the Río Tacotalpa (rearing environment×drainage, $P=0.031$; Table 2 and Fig. 5D). Hence, the significant interaction term in this case arose from fish from different river drainages responding differently to common-garden conditions.

Fish from sulfidic and nonsulfidic habitats also had significantly different body shapes in the wild. This difference was evident for both males and females, even though there is strong sexual dimorphism in body shape in *P. mexicana* (Fig. 6). Body shape differences along PC axis 1, which principally reflected sexual dimorphism (females had higher scores than males), was associated with the relative position of the anal fin and dimensions of the caudal peduncle (Fig. 6), as previously documented for *P. mexicana* and other poeciliid fishes (Tobler et al. 2008, 2011;

Culumber and Tobler 2017). Body shape differences along PC axis 2 pertained to the shape and relative size of the head as well as the shape of the trunk and caudal peduncle (Fig. 6). Sulfide spring fishes from the wild had lower scores along the second axis, reflecting larger heads, a more compact trunk and, a narrower caudal peduncles, while fish from nonsulfidic habitats and sulfide spring fishes raised in captivity had higher scores (smaller heads, more elongated trunk, and deeper caudal peduncles). These body shape differences corroborate the results of previous studies on *P. mexicana* and other sulfide spring fishes (Tobler et al. 2011; Tobler and Hastings 2011). Besides a significant main effect for the habitat of origin, there was also a significant habitat-of-origin by rearing-environment interaction (Table 3). Visualization of principal components (Fig. 6) indicates that this interaction term arose because body shapes differed between sulfidic and nonsulfidic populations in the wild, but these differences largely disappeared in captivity; common-garden-reared fish from sulfidic habitats overlapped with fish from nonsulfidic habitats (both wild and captive) but not with their counterparts from wild sulfidic populations. These results

suggest that population differences in body shape observed in nature are primarily a consequence of phenotypic plasticity, and these differences disappear once fish are exposed to shared environmental conditions.

Finally, our results indicated that there were minimal differences in liver size in the wild (5% difference between sulfidic and nonsulfidic populations), but clear differences between sulfidic and nonsulfidic populations arose in the lab, with nonsulfidic fish exhibiting livers over three times smaller than sulfidic fish (Fig. 5E, F). The liver size of sulfidic fish raised in a common garden was similar to that of wild-caught sulfidic individuals (7% change), while captive fish from nonsulfidic environments showed a dramatic decline in liver size (69% decrease) (Fig. 5F). Effect size supports the larger impact of rearing environment ($\eta_p^2 = 0.545$) over habitat of origin ($\eta_p^2 = 0.266$). This decline was present in populations from both river drainages investigated here, although fish from the Río Tacotalpa overall had slightly smaller livers than those from the Río Pichucalco (Fig. 5F). There was also a significant sex effect on liver size, with females consistently having larger livers than males (not shown). Overall, these results illustrate that liver size in these *P. mexicana* populations is shaped by complex interactions between habitat of origin and environmental factors ($G \times E$, Fig. 1D, E).

Discussion

In this study, we explored previously documented phenotypic differences between populations of *Poecilia mexicana* that reside in toxic, H_2S -rich springs and adjacent nonsulfidic habitats and set out to test if differences observed in the wild were maintained if fish are reared in the laboratory under shared environmental conditions. For most traits, we found that differences documented in the wild were maintained in the common-garden environment, although the rearing environment also affected trait expression and sometimes interacted with the habitat of origin of the fish. The maintenance of phenotypic differences in the common garden suggests genetic differences between sulfidic and non-sulfidic populations influence trait variation observed in nature. Two exceptions to this general pattern were body shape, for which population differences disappeared in the

common garden, and liver size, for which the shared environment induced population differences not observed in nature. Overall, our results suggest that phenotypic differences observed between populations in sulfidic and nonsulfidic habitats are not merely a consequence of phenotypic plasticity induced by the environmental differences between habitat types. The fact that trait divergences at least in part have a genetic basis indicates that they may represent examples of evolution by natural selection. However, future studies will need to test whether and how trait differences between populations affect the fitness of individuals in their respective habitats.

Most trait differences between populations were not only maintained in captivity but—with the exception of variation in eye size—they were also present in similar fashions in both river drainages. Since springs in the Río Pichucalco and Río Tacotalpa drainages were colonized independently by lineages within the *P. mexicana* species complex (Tobler et al. 2011; Ryan et al. 2023), shared traits in sulfide spring populations are a consequence of evolutionary convergence, which is widely interpreted as evidence for adaptation (Endler 1986; Schluter 2000; Losos 2011). Nonetheless, not all convergent evolution is caused by natural selection (Losos 2011), and rigorously testing hypotheses about adaptation requires direct measurements of selection or assessment of trait function. For several traits investigated here, hypotheses about trait function and fitness implications have been previously developed.

Perhaps best investigated are variations in gill size and body shape, and increases in gill size and head size have been widely documented in sulfide spring fishes even beyond *P. mexicana* (Tobler and Hastings 2011; Greenway et al. 2023). The increased size of the head was typically assumed to be required to make space for bigger gills, but the current study sheds doubt on that interpretation because sulfide spring fish raised in a common garden maintained a larger gill size even though their body shape (including head size) changed plastically to match that of fish from nonsulfidic habitats. Increased gill size is frequently observed in fish from hypoxic environments (Langerhans et al. 2007; Crampton et al. 2008; Dhillon et al. 2013) and likely represents an adaptation to the rampant hypoxia in sulfide springs. In concordance with this finding, sulfide spring fishes also exhibit selection on oxygen transport proteins that

may be associated with hypoxia (Barts et al. 2018). Although variation in head size does not seem to be caused indirectly by selection on gill size, it likely also functions in oxygen acquisition in hypoxic environments; larger head size is associated with larger buccal volumes and increased ventilation capacity, such that sulfide spring fishes are able to pump higher volumes of water across their gills (Camarillo et al. 2020). In addition, variation in body shape (especially the shape of the caudal peduncle) between sulfidic and nonsulfidic populations has also been associated with variation in swimming performance (Camarillo et al. 2020). Future research will have to test whether exposure to hypoxia alone, perhaps during critical developmental stages, induces variation in head size and other aspects of body shape in sulfidic and nonsulfidic populations of *P. mexicana*, and whether such plasticity might vary between populations from the different habitat types.

Variation in gastrointestinal tract length was also maintained in the common-garden environment, even though this trait is highly plastic in other *Poecilia* species (Scharnweber et al. 2011). Shorter intestines, as observed in sulfide spring fishes, are typically associated with more protein-rich diets (Kramer and Bryant 1995). Indeed, colonization of sulfide springs by *P. mexicana* has been accompanied by convergent dietary shifts (Tobler et al. 2008, 2015); fish in nonsulfidic habitats primarily feed on detritus and algae, while those in sulfide springs feed on the abundant biofilms of chemoautotrophic bacteria and chironomid larvae. The consistency of trait divergence with other fishes and the heritable nature of variation in intestine length suggests that divergence was caused by natural selection and represents an adaptation to a novel trophic niche.

For other traits, the functional ramifications and potential adaptive values of population differences are less clear. Reductions in routine metabolic rates, brain size, and eye size in sulfide spring fishes were previously interpreted in the context of the expensive tissue hypothesis (Aiello and Wheeler 1995). Limitations in both energy and oxygen availability lead to metabolic rate suppression (Richards 2010) and the reduction of tissues with high demands in energy and oxygen, including brains (Kaufman et al. 2003; Tsuboi et al. 2015) and eyes (Moran et al. 2015). While reduction of metabolic rates and expensive tissues may be adaptive for sulfide spring fishes, this

scenario also implies trade-offs in organismal function. However, the potential costs associated with trait reductions in sulfide spring fishes remain unknown, and future research will have to test whether reductions in brain size are associated with limitations in cognition or reduction in eye size with limitations in sensory perception.

The expensive tissue hypothesis would also have predicted a reduction in the heart size of sulfide spring fishes, but instead, they exhibited larger hearts than their relatives from nonsulfidic habitats. We speculate that increased heart size may be associated with hypoxia adaptation. Preliminary data have indicated that sulfide spring fishes have a higher hematocrit (percentage of red blood cells in the blood) than nonsulfidic fish (Barts and Tobler, unpublished data), and hematocrit is positively correlated with the viscosity of the blood (Picart et al. 1998; Eckmann et al. 2000). Bigger hearts may be required to effectively transport more viscous blood through the body.

Overall, our results suggest that many traits investigated in this study are shaped by genetic differences between populations, and the functional ramifications of trait variation can be linked to sources of selection that coincide with the presence or absence of H_2S . Hence, trait divergences between populations in different habitat types are likely a consequence of evolution by natural selection. Still, we caution that our study may have overestimated the contributions of genetic variation and underestimated the contributions of environmental variation as a consequence of our experimental design. First, all experiments on common-garden-reared fish were conducted with fish born to wild-caught mothers. Hence, we cannot exclude that maternal effects have influenced trait expression in our captive populations, especially considering significant differences in epigenetic modification between fish from sulfidic and nonsulfidic populations remain stable across generations (Kelley et al. 2021). It is important to note, however, that another recent study, which worked with long-term laboratory stocks derived from the Río Tacotalpa populations, still found significant population differences in life history and behavioral traits after at least four generations in captivity (Coffin et al. 2023). Second, our experiment did not use a fully factorial design; we raised all fish under normoxia, in the absence of H_2S , and with ad libitum access to food high in protein. Hence, we do not know whether and to what degree fish from nonsulfidic habitats might

plastically adjust trait expression when exposed to stress associated with hypoxia, H₂S exposure, and resource scarcity. Experimentally manipulating environmental conditions in the laboratory will be critical in future studies to better understand phenotypic plasticity in this system and how it may have shaped the evolutionary responses of populations during the colonization of extreme environments.

In summary, the majority of analyzed traits that exhibited significant differences between fish from sulfidic and nonsulfidic populations in the wild also maintained those differences in the laboratory, even though rearing-environment and other predictor variables also impacted trait expression. While the heritability of trait variation is requisite for evolution by natural selection, and there are clear hypotheses about the adaptive value of trait differences in the context of environmental variation in our study system, future studies will have to rigorously test for adaptation by directly measuring selection or documenting how organismal function differs between populations and environmental contexts.

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Data availability All data of this study is deposited on <https://datadryad.org/>.

Declarations

Ethics approval All procedures used in this study were approved by the Institutional Animal Care and Use Committee at Oklahoma State University (protocol number; AS-1015).

Conflicts of interest The authors declare that they have no competing interests.

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