

Adaptation in sulfide spring fishes: experimental approaches

by

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Abstract

Poecilia mexicana is a live-bearing fish known for its extremophilic populations. The species has been studied for their high tolerance to hydrogen sulfide (H_2S), an environmental toxicant that occurs naturally in some freshwater springs in southern Mexico. Though studies often reduce this system to a presence or absence of H_2S , focusing entirely on H_2S is likely to generate an incomplete picture. H_2S springs are complex habitats, differing in biotic and abiotic conditions besides the presence of H_2S . For example, sulfidic habitats are characterized by hypoxia, low pH, different trophic regimes and predators, and strong energy constraints. How these other sources of selection impact the evolution of extremophile fish populations, and how they might interact with H_2S , remains largely unstudied. My research focused on two questions within the *P. mexicana* system, where one question acknowledged the complexity of environment and organism, and the other consciously stripped away complexity to isolate the direct impacts of H_2S .

My first chapter embraced environmental and organismal complexity to investigate phenotypic differences between sulfidic and nonsulfidic populations of *P. mexicana*. Though H_2S is commonly identified as the driving selective force between the two populations, not all divergent traits exhibit clear adaptive hypotheses related to this toxicant. I compared phenotypic traits between populations in the wild, then compared those same traits in first-generation individuals from a common-garden environment. Almost every trait showed significant divergence between wild sulfidic and nonsulfidic populations. Most traits remained different between populations reared in the lab, though many simultaneously presented evidence of plasticity. My results suggested that trait divergences between populations in different habitats were largely shaped by evolutionary change, and I developed hypotheses about the potential adaptive function of divergent traits in the context of the complex selective regimes in sulfide springs.

My second chapter examined H_2S responses outside of environmental and organismal context. To test what organismal responses are a direct response of H_2S alone, I analyzed the gene expression response of *P. mexicana* to an H_2S -releasing pharmaceutical compound (AP39) that targets the mitochondria. I exposed both sulfidic and nonsulfidic populations to AP39, then compared their gene expression in the liver and the gills to individuals not exposed to AP39. I

found no gene expression differences between the gill tissue of AP39-exposed and unexposed individuals. While there were significant differences in gene expression in livers, none of the expressed genes were related to H₂S tolerance or detoxification. I concluded that AP39 makes a poor model for H₂S exposure in *P. mexicana*. My findings underscore the importance of context. Exposing just the mitochondria to H₂S via a targeted compound did not evoke the same responses as exposing the whole organism via H₂S dissolved in water, as it is found in nature. Overall, my research attests that evolutionary biologists cannot ignore environmental and organismal complexity, even in systems with strong selective pressures.

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Preface

“It is interesting to contemplate a tangled bank, clothed with many plants of many kinds, with birds singing on the bushes, with various insects flitting about, and with worms crawling through the damp earth, and to reflect that these elaborately constructed forms, so different from each other, and dependent upon each other in so complex a manner, have all been produced by laws acting around us.”—C. Darwin. 1859

Darwin’s tangled bank is one of the most famous allegories in evolutionary biology and a perfect picture for the inherent complexity of nature. No organism can escape interacting with its environment, nor the evolutionary forces that are imposed as a consequence. Evolutionary biologists have spent 150 years trying to untangle these interactions. While far from finished, remarkable progress has been made, especially on the topic of adaptation. Adaptation is perhaps the core prediction of Darwin’s theory—the alteration of species by natural selection to better survive and reproduce within their environment. Evolutionary biologists have perfected the study of adaptation with a very simple experimental approach: find two environments that differ in a well-defined source of selection and then compare the populations within. This approach has been used to study adaptation to different predation regimes (Reznick 1982; Reznick et al. 2001; Heckley et al. 2022), darkness (Poulson 2001; Trontelj 2019), salinity (McCairns and Bernatchez 2010; Jones et al. 2012; DeFaveri and Merilä 2014) and a multitude of other selective pressures. However, this reductionist approach is incomplete. We know this because even in the systems with a clear-cut source of selection, populations are typically exposed to other environmental gradients that coincide with the focal selective agent. Though biologists often consider systems under similar selective pressures to be identical, nonconvergence between populations reminds us that they are not. Seemingly identical environments may vary not just in biotic and abiotic conditions, but also in the evolutionary history of the populations within, in the forces of sexual selection, and in the plain fact that selection acts on fitness and there may be multiple paths to maximize fitness in the same environmental context (Kaeuffer et al. 2012). By reducing system complexity by singling out sole sources of selection or singular organismal traits, biologists are effectively ignoring Darwin’s tangled bank and how environmental and organismal complexity shape adaptation.

Admitting complexity into our studies of evolution is, however, easier said than done. By Darwin's own admission, a tangled bank is incredibly difficult to untangle and catalogue. Environmental characteristics are multitudinous; exploring complexity requires well-characterized systems, preferably within a relatively predictable and replicated environment. Fortunately, such a system exists within the populations of *Poecilia mexicana*.

Poecilia mexicana is a live-bearing fish known for its extremophilic populations. The species has been studied for their high tolerance to hydrogen sulfide (H₂S), an environmental toxicant that occurs naturally in some freshwater springs in southern Mexico. H₂S inhibits the aerobic production of ATP by binding to complex IV of the cytochrome *c* oxidase (COX) protein in the oxidative phosphorylation (OxPhos) pathway within the mitochondria. This interrupts the OxPhos process and can lead to rapid cell death, even at micromolar concentrations (Cooper and Brown 2008). Despite the toxic effects, populations of *P. mexicana* have repeatedly colonized sulfide springs across southern Mexico. Natural sulfide springs occur in multiple river drainages in Tabasco and Chiapas, and at least four of these springs contain a lineage of locally-adapted *P. mexicana* that colonized the spring independently of other sulfidic lineages. It is commonly thought that within each drainage the nonsulfidic population colonized the sulfidic habitats, with no migration from populations in the sulfidic habitats of other drainages (but see transporter hypothesis, Schluter and Conte 2009). Despite their close relation, however, sulfidic and nonsulfidic populations exhibit striking phenotypic and genetic differences. Sulfidic populations have significantly different gene expression, even when not exposed to H₂S (Passow et al. 2017c). Sulfidic populations are phenotypically characterized by larger heads and gills (Tobler and Hastings 2011; Camarillo et al. Tobler 2020), shorter intestines (Tobler et al. 2015), different coloration (Owens et al. 2022), and smaller overall body size (unpublished data). Most notably, studies have repeatedly found a lack of gene flow between sulfidic and nonsulfidic populations in the wild, despite extremely close proximity and lack of physical migration barriers (Tobler et al. 2011; Palacios et al. 2013; Riesch et al. 2015; Plath et al. 2013).

Previous research has delved deeply into the H₂S resistance traits of *P. mexicana*. Sulfidic populations exhibit similar adaptations, among these the alteration of COX IV to lessen the inhibitory effects of H₂S, and the upregulation of pathways responsible for H₂S-detoxification. These are the key molecular changes that allow maintenance of mitochondrial function despite high H₂S concentrations (Pfenninger et al. 2014; Kelley et al. 2016; Greenway

et al. 2020). However, these mechanisms of adaptation are not identical across evolutionarily independent populations or lineages. For example, some populations rely on detoxification pathways with little to no evidence for adaptive alterations to the COX IV complex (Pfenninger et al. 2014). Overall mechanisms of adaptation across drainages are strikingly similar, but the minor nonconvergent traits belay a greater systemic complexity. The natural environmental replicates, along with the strong selective force of H₂S, lend this system to the study of convergent evolution and adaptation—and an ideal place to explore how environmental and organismal complexity factors into adaptation.

While H₂S is likely the strongest selective force in this system, it is not the only environmental factor that can potentially shape phenotypic expression and evolutionary change in sulfide spring fishes. Studies often reduce this system to the presence or absence of H₂S, but focusing entirely on H₂S is likely to generate an incomplete picture. H₂S springs are complex habitats, differing in biotic and abiotic conditions besides the presence of H₂S (Tobler et al. 2018). Sulfidic habitats are characterized by hypoxia, lower pH, and higher dissolved salt concentrations (Tobler et al. 2011). Primary production is based in chemoautotrophic bacteria (Hotaling et al. 2019), which serves as the primary food source for extremophile fish (Roach et al. 2011; Tobler et al. 2015). The toxic habitat excludes much interspecific competition and aquatic predators (Greenway et al. 2014), and organisms face significant energy constraints (Passow et al. 2017a) largely attributed to the need for aquatic surface respiration (ASR). ASR mitigates the hypoxia and H₂S toxicity, but also limits time for benthic foraging (Tobler et al. 2009). These are just a few of the complex selective forces at interplay with H₂S. How these other sources of selection impact the evolution of extremophile fish populations, and how they might interact with H₂S, remains largely unstudied. My research focused on two questions associated with environmental complexity in the *P. mexicana* system, where one question acknowledged the complexity of environment and organism, and the other consciously stripped away complexity to examine the source of H₂S-induced transcriptome responses.

My first chapter embraced environmental and organismal complexity to investigate phenotypic differences between sulfidic and nonsulfidic populations of *P. mexicana*. Though H₂S is commonly identified as the driving selective force between sulfidic and nonsulfidic populations, not all divergent traits exhibit clear adaptive hypotheses related to this toxicant. It is unclear if phenotypic differences between populations have a genetic basis at all. Some trait

differences may come from individuals developing under different environmental conditions—phenotypically plasticity that may or may not influence individual survival and reproduction. Even genetically differentiated traits might be traced to pleiotropy, genetic linkage, or adaptation to another source of selection. We cannot ask questions about phenotypic differences between sulfidic and nonsulfidic populations being adaptations without first testing whether these differences have a genetic basis. To explore which divergent traits are shaped by genetics and which are the result of plasticity, I compared phenotypic traits between sulfidic and nonsulfidic populations from the wild, and then compared those same traits in first-generation individuals from a common-garden environment. I found significant trait divergences in wild sulfidic and nonsulfidic populations. Most traits remained different between populations in the lab, though many simultaneously presented evidence of plasticity. My results suggested that trait divergences between populations were largely shaped by evolutionary change, though plasticity is surprisingly common. I concluded my analyses by developing hypotheses about the potential adaptive function of divergent traits in the context of the complex selective regimes in sulfide springs. My results suggest that, even in the presence of a strong selective pressure, phenotypic differences must be carefully assessed; making assumptions can reduce or misleading our understanding of environmental complexity and its interaction with evolutionary forces.

My second chapter examined H₂S responses outside of environmental and the whole-organism context. We know H₂S exposure invokes complex transcriptomic responses in sulfide spring fishes. Laboratory experiments that expose fish to H₂S while also standardizing all other environmental conditions suggested that this complex response is not explained by environmental complexity fish face in their natural habitats (Passow et al. 2017c). However, it is unknown what causes the remaining complexity in transcriptional responses. One leading hypothesis suggests that H₂S targets multiple sites within cells, resulting in a variety of transcriptional responses. Alternatively, complexity in transcriptional responses may stem from the cascading effects of disruption of mitochondrial function. To test the source of transcriptomic complexity, I analyzed the gene expression response of *P. mexicana* to an H₂S-releasing pharmaceutical compound (AP39) that specifically targets the mitochondria. I hypothesized that if complex transcriptome responses result from a variety of H₂S targets within the cell, gene expression changes in response to AP39 will be significantly less complex because H₂S will only be released inside the mitochondria, therefore limiting what targets it can reach. If transcriptome

responses result from effects of mitochondrial dysfunction cascading through the cell, gene expression responses should remain complex as previously seen in response to environmental H₂S exposures. I exposed both sulfidic and nonsulfidic populations to AP39, then compared their gene expression in the liver and the gills to individuals not exposed to AP39. Specifically, I asked whether AP39 invoked a differential response in gene expression compared to unexposed individuals, whether the gills and the liver expressed different genes or levels of complexity, and whether preestablished transcriptome expression differences between sulfidic and nonsulfidic populations were maintained. I found significantly reduced transcriptome differences in the gills, suggesting that transcriptome responses to H₂S may result from a variety of cellular targets. However, I found significantly complex transcriptome expression in the liver, suggesting that responses stem from the cascading effects of mitochondrial dysfunction. However, few candidate genes for H₂S tolerance were expressed in either tissue. I concluded that AP39 as it was applied makes a poor model for H₂S exposure in *P. mexicana*. These results underscore the importance of context; exposing just the mitochondria to H₂S via a targeted compound did not evoke the same responses as exposing the whole organism with H₂S dissolved in water, as it is found in nature. These results also encourage further investigation into the source of transcriptome complexity.

Overall, my research attests that environmental and organismal complexity, though often cryptic, is nonetheless impactful. An unforeseen level of plasticity impacts the phenotypes of sulfidic and nonsulfidic fish, raising questions about the influence of plasticity when present alongside significant genetic differentiation. Does plasticity augment or slow down the genetic divergence between populations? There are plenty of adaptive hypotheses for genetic divergence across traits, but future research should also consider that other environmental variables might be acting on organismal evolution, in tandem or independently from H₂S. It will be important to experimentally explore the selective pressures and functional differentiation of traits between populations and environments, in order to assess true adaptive value. Additionally, the potentially numerous targets of H₂S within cells raises more questions about its impacts beyond the mitochondria and what unseen effects it may be having on phenotypes. It is critical that future studies repeat targeted H₂S exposure trials with more detailed transcriptome detection methods such as RNAseq. This will confirm if transcriptional responses to H₂S are truly lower when H₂S exposure is confined to the mitochondria. If H₂S targets multitudinous sites within

cells, future studies must widen their scope to explore the impacts of H₂S across the entire cell, not just the mitochondria, and considered wider organismal complexity when studying the impacts of H₂S. If the environmental and organismal complexity I explored were to be ignored, many of these questions would remain shrouded in the paradigm of presence or absence of a selective force. Nothing inside an organism operates independent of the whole, and no organism operates independent of its environment; even in systems with strong selective pressures, we cannot ignore the tangled bank.

Chapter 1 - Effects of plasticity and genetic divergence in phenotypic trait expression of sulfide spring fishes

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 (Ghalambor et al. 2007; Whitman and Agrawal 2009; Pigliucci 2001). 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 (Gomulkiewicz and Kirkpatrick 1992; Lively 1986; Moran 1992; Sultan 1995; Via and Lande 1985; Whitman and Agrawal 2009; Scheiner and Levis 2021; Ernande and Dieckmann 2004). 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, 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 livebearing fishes (family Poeciliidae) that are widespread in Mexico and Central America (Palacios et al. 2016; Alda et al. 2013). Multiple populations within this species complex have independently colonized springs rich in toxic hydrogen sulfide (H₂S) in several

river drainages of the Rio Grijalva basin in southern Mexico (Tobler et al. 2011, 2018; Riesch et al. 2015; Palacios et al. 2013). H₂S 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 H₂S-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 H₂S detoxification, which allows individuals to maintain mitochondrial function and ATP production in the presence of H₂S (Greenway et al. 2020; Kelley et al. 2016; Pfenninger et al. 2014). Previous studies have indicated that physiological differences between sulfidic and nonsulfidic populations have a heritable basis (Passow et al. 2017c), 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).

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 are a wealth of other phenotypic differences between the same populations that are not clearly linked to H₂S 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 and Hastings 2011; Camarillo et al. 2020; Michael Tobler et al. 2011), 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. 2014, 2016; Riesch et al. 2010). 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 H₂S 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 H₂S 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 hypoxic and have lower pH and higher dissolved salt concentrations (Tobler et al. 2011). Primary production in sulfide springs is primarily mediated by chemoautotrophic microbes (Hotelling et al. 2019), which provide a primary food source for sulfide spring fishes (Tobler et al. 2015; Roach et al. 2011), and the toxic environment excludes most interspecific competitors and aquatic predators (Greenway et al. 2014; although predation from birds can be substantial in some springs; R. Riesch, Oran, et al. 2010; 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 H₂S 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 H₂S. For example, it is tempting to speculate that hypoxia has caused the evolution of traits associated with oxygen acquisition (Camarillo et al. 2020; Tobler et al. 2011; Barts et al. 2018), shifts in trophic resource use led to divergence in gastrointestinal tract morphology (Tobler 2008; Tobler et al. 2015), or energy constraints resulted in a reduced investment into energetically costly traits (Passow et al. 2017a; Schulz-Mirbach et al. 2016). However, common-garden experiments that raise fish from sulfidic and nonsulfidic populations under identical conditions have largely been lacking so far, raising questions about role of genetic and environmental factors in shaping 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 genetic 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 (Figure 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 (G; Figure 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 rearing environment (wild or common-garden-reared) but significantly different for populations between rearing environments (E; Figure 1B). If genetic 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 (Figure 1C). Finally, genetic and environmental factors may interact, such that the effect of the environment is dependent on the genetic background ($G \times E$). In this case, the direction of trait differences observed in the wild may change in common-garden-raised fish (Figure 1D), or trait differences may disappear (Figure 1E). Overall, we found consistent evidence that genetic differentiation contributed to phenotypic differences between sulfidic and nonsulfidic populations, but also varied environmental effects that add to or interact with the 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 (Figure 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 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 (nonsulfidic) 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 (nonsulfidic) as a reference site. Descriptions of all field sites used in this study and detailed

water chemistry data for each site have been previously 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 in June 2012 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 hour 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 were re-analyzed from previous works (Passow et al. 2015; Passow et al. 2017c). 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; Passow et al. 2017c): (1) Wild-caught fish were acclimated to standardized laboratory conditions for at least 48 hours. Fish were not fed 24 hours prior to trials to assure 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 hours. (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-second intervals. Note that all trials were conducted in absence of H_2S even for sulfidic populations, because the reactivity of H_2S with oxygen in aqueous solution affects the measurement of oxygen consumption rates (K. Y. Chen and Morris 1972), and even fish from sulfide springs face elevated mortality rates in presence of H_2S 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 /hour) 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 Figure 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 quantifications 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 hours in a Thermo Scientific Heratherm Oven (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Following the 72-hour 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).

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 how genetic background, 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 (log10-transformed) against habitat of origin (sulfidic

or nonsulfidic) and rearing environment (wild or common garden) using linear models (see Figure 1). In addition, our linear models also included three potential confounding variables: drainage (Pichucalco or Tacotalpa), sex (female or male), and blotted wet mass (log10-transformed). The inclusion of these variables was important to account for potential non-convergent evolution among replicated population pairs (indicated by 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 2009) to rank models based on Akaike Information Criteria with finite sample correction (AICc) (Johnson and Omland 2004). If more than one model exhibited $\Delta AICc < 2$, 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 log10-transformed) using GGLOT2 (Wilkinson 2011). In addition, we calculated and plotted group-specific estimated marginal means and standard errors for relevant subgroups of fish 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 gpagen() 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 square. To visualize body shape variation, we conducted a principal component analysis using the `gm.prcomp()` function on the Procrustes shape coordinates.

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 (Figures 1.4–1.6) for all traits besides eye size (no difference between sulfidic and nonsulfidic populations in the Río Tacotalpa; Figure 1.4D) and liver size (no differences between sulfidic and nonsulfidic populations in either river drainage; Figure 1.5F). 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 1.2–1.3). 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 brain size; Table 1.2). Finally, all traits also co-varied consistently with body size (Tables 1.2–1.3).

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 background 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 Figure 1.1). Note that no trait strictly followed the first or second scenario (Figure 1.1A–B), where trait environment is only shaped by either genetic background 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 genetic background and environment

Four traits—routine metabolic rate and the sizes of eyes, heart, and intestine—were shaped by both genetic background and by rearing environment, with differences between sulfidic and nonsulfidic populations being maintained in the laboratory in the same direction and magnitude (G + E; Figure 1.1C). 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 1.2).

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 1.2), this predictor variable was not included in the top model. Per unit mass, fish from sulfide springs had 26% lower routine metabolic rates (Figure 1.4A–B), and there were significant interactions between body size and habitat of origin as well as body size and drainage (Table 1.2). Habitat of origin ($\eta^2 = 0.409$) and body size ($\eta^2 = 0.358$) had the largest effect on routine metabolic (Table 1.2). Overall, these results indicate that differences in routine metabolic rate observed between fish from sulfidic and nonsulfidic populations were primarily shaped by genetic background and—to a much lesser degree—by exposure to different rearing environments (G + E; Figure 1.1C).

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 $\times$ drainage, $P < 0.001$ in Table 1.2; Figure 1.4C–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 (Figure 1.4D). The effect of habitat of origin on eye size was stronger ($\eta^2 = 0.728$) than the effect of rearing environment ($\eta^2 = 0.313$). 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 captivity (common garden environments) by an average of 49% compared to wild populations (Figure 1.4E–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 (Figure 1.4G–H). Habitat of origin had a stronger effect on intestine size ($\eta^2 = 0.717$) than rearing environment ($\eta^2 = 0.451$). In addition, males had significantly shorter intestines than females (sex, $P < 0.001$; Table 1.2 and Figure 1.5H). There was also a significant interaction term between sex and body size. 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 that fish experience in sulfidic and nonsulfidic habitats (G + E; Figure 1.1C).

Traits shaped by interactions between genetic background and environment

The best-fit models for four traits—brain size, gill size, body shape, and liver size—included significant interaction terms between habitat of origin and rearing-environment ($G \times E$, Figure 1.1D–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 1.2 and Figure 1.5B). This relative difference was maintained in captivity, but a significant interaction term arose because the magnitude of difference between wild and common-garden populations differed by habitat (Figure 1.5A–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, rearing environment had a stronger effect on gill size ($\eta^2 = 0.858$) than habitat of origin ($\eta^2 = 0.162$). 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 1.2), and females consistently had larger gills than males (Figure 1.5B).

Fish from sulfidic habitats consistently exhibited smaller brains than those from adjacent nonsulfidic habitats, with magnitude differences between drainages (47% smaller in Pichucalco, 27% smaller in Tacotalpa) (Figure 1.5C–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 14% in captivity for both sulfidic and nonsulfidic populations, while it increased 29% in fish from the Río Tacotalpa (rearing environment $\times$ drainage, $P = 0.031$; Table 1.2 and Figure 1.5D). 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 shape 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* (Figure 1.6). Body shape differences along PC axis 1, which principally reflected sexual dimorphism, was associated with the relative position of the anal fin and dimensions of the caudal peduncle, as previously documented for poeciliid fishes (Culumber and Tobler 2017). Body shape differences along PC axis 2 pertained to the relative size of the head, with sulfide spring fishes from the wild having larger heads than fish from nonsulfidic habitats and sulfide spring fishes raised in captivity. Besides a significant main effect for habitat of origin, there was also a significant habitat-of-origin by rearing-environment interaction (Table 1.3). Visualization of principal components (Figure 1.6) 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 largely overlapped with fish from nonsulfidic habitats (both wild and captive) and not with their counterparts from wild sulfidic populations. These results suggest that population differences in body shape observed in nature are largely 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 3x smaller than sulfidic fish (Figure 1.5E–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) (Figure 1.5F). Effect size supports the larger impact of rearing environment ($\eta^2 = 0.545$) over habitat of origin ($\eta^2 = 0.266$). 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 (Figure 1.5F). 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 genetic background and environmental factors ($G \times E$, Figure 1.1D-E).

Discussion

In this study, we explored previously documented phenotypic differences between populations of *Poecilia mexicana* that reside in toxic, H₂S-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 captivity, although rearing environment also affected trait expression and sometimes interacted with the genetic background of the fish. The maintenance of phenotypic differences in the common garden suggests a genetic basis for trait variation observed in nature. Two exceptions to this general pattern were body shape, for which population differences disappeared in captivity, 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 habitat.

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; Losos 2011; Schluter 2000). 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 variation 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. 2021). 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 captivity maintained a larger gill size even though their body shape (and head size) changed plastically to match that of fish from nonsulfidic habitats. Increased gill size is likely an adaptation to the rampant hypoxia in sulfide springs. Increases in gill size are frequently observed in fish from hypoxic environments (Crampton, Chapman, and Bell 2008; Langerhans, Chapman, and Dewitt 2007; Dhillon et al. 2013), and 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 the 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). Future research will have to test whether exposure to hypoxia alone, perhaps during critical developmental stages, induce variation in head size 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 captive populations, 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 clear and convergent dietary shifts (Tobler 2008; Tobler et al. 2015); fish in nonsulfidic habitats primarily feed on detritus and algae, while those in sulfide spring 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 much 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). Both limitations in energy and oxygen availability have been documented to lead to metabolic rate suppression (Richards 2010) and the reduction of tissues with high demands in energy and oxygen, including brains (Kaufman, Marcel Hladik, and Pasquet 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 is 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 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, the genetic basis of many traits investigated in this study and the functional ramifications of trait variation that can be linked to sources of selection that coincide with the presence or absence of H_2S suggests that 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 behavior traits after at least four generations in captivity (Coffin and Tobler 2022). Second, our experiment did not use a fully factorial design; we raised all fish under normoxia, in 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, H_2S exposure, 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 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 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.

Table 1.1. 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

Table 1.2. ANOVA results for univariate traits

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

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

Table 1.3. ANOVA results for body shape

Results of analyses of variance (ANOVA) examining body shape variation. Table includes predictor terms (dependent variable), degrees of freedom (DF), sum-of-squares (SS), mean square (MS), F-ratio (F), Z-score (Z) and P-value (P). Terms with a P-value ≤ 0.05 are given in bold.

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 $\times$ Drainage	1	0.002	0.002	0.001	1.075	0.376	P = 0.350
Centroid Size $\times$ Habitat	1	0.005	0.005	0.003	2.199	1.841	P = 0.034
Centroid Size $\times$ Rearing	1	0.002	0.002	0.001	0.954	0.155	P = 0.443
Centroid Size $\times$ Sex	1	0.005	0.005	0.003	2.418	2.075	P = 0.019
Drainage $\times$ Habitat	1	0.016	0.016	0.010	7.442	4.319	P < 0.001
Drainage $\times$ Rearing	1	0.003	0.003	0.002	1.297	0.778	P = 0.218
Drainage $\times$ Sex	1	0.005	0.005	0.003	2.323	1.980	P = 0.024
Habitat $\times$ Rearing	1	0.007	0.007	0.004	3.275	2.704	P = 0.004
Habitat $\times$ Sex	1	0.005	0.005	0.003	2.128	1.761	P = 0.039
Rearing $\times$ Sex	1	0.005	0.005	0.003	2.069	1.749	P = 0.039
Habitat $\times$ Rearing $\times$ Sex	1	0.002	0.002	0.001	1.106	0.452	P = 0.328
Drainage $\times$ Rearing $\times$ Sex	1	0.002	0.002	0.001	0.919	0.063	P = 0.469
Drainage $\times$ Habitat $\times$ Sex	1	0.005	0.005	0.003	2.111	1.769	P = 0.039
Drainage $\times$ Habitat $\times$ 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					



Figure 1.1. Experimental Hypotheses

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 (G), environmental (E), additive (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 for outcomes (S = significant; NS = nonsignificant) in corresponding statistical models (see methods for details). A. A trait only affected by genetic inheritance will show no difference regardless of 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 experiencing both genetic 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 average 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 depend on the data.

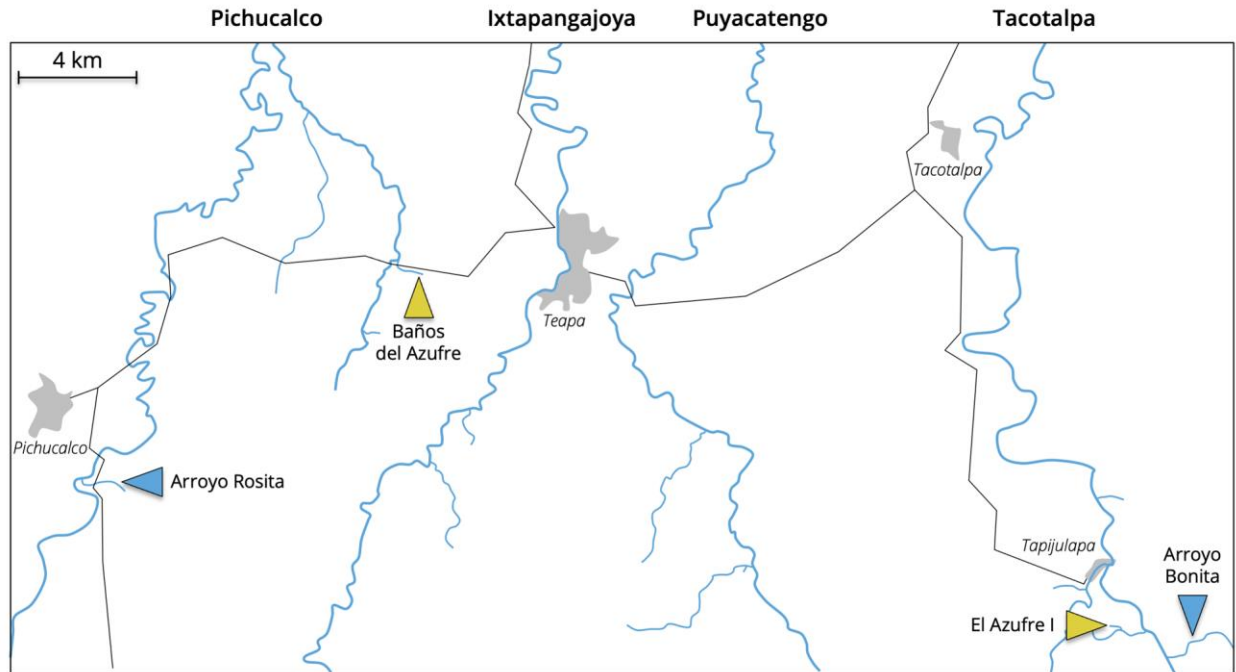


Figure 1.2. Sampling sites

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.

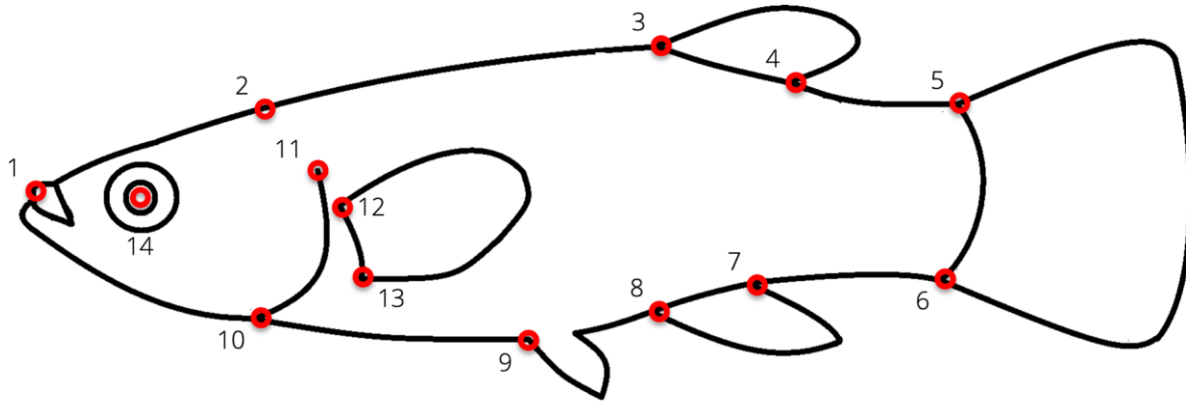


Figure 1.3. Landmarks

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.

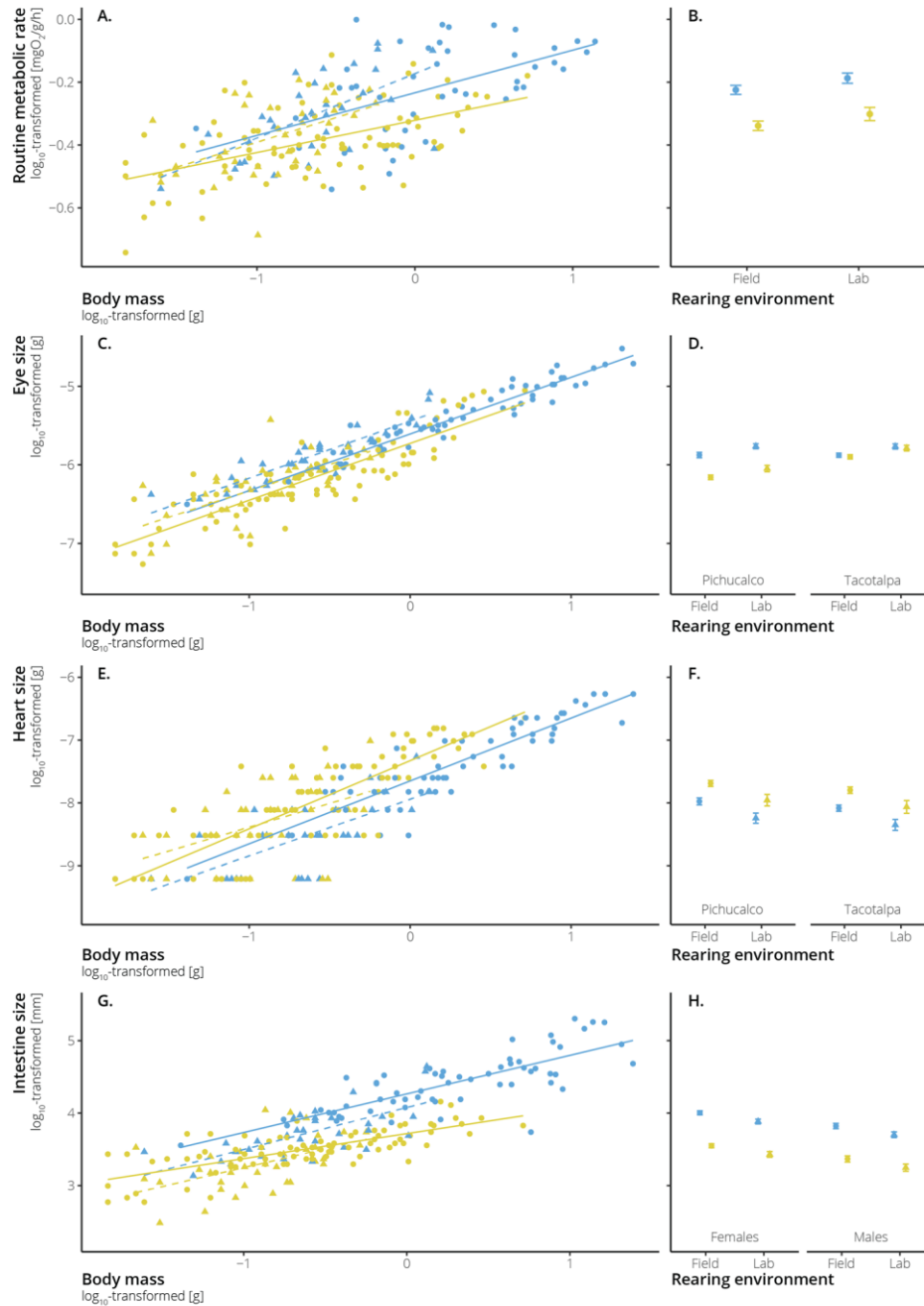


Figure 1.4. Traits influenced by genetic background and environment

Routine metabolic rate and size of eyes, heart, and intestines were shaped by both genetic background and by rearing environment. Differences between sulfidic and nonsulfidic populations were maintained in the laboratory in the same direction and magnitude, as illustrated in Figure 1C. The raw data (left) and average means (right) are visualized via scatterplot. These traits relate to prediction C in Fig. 1.

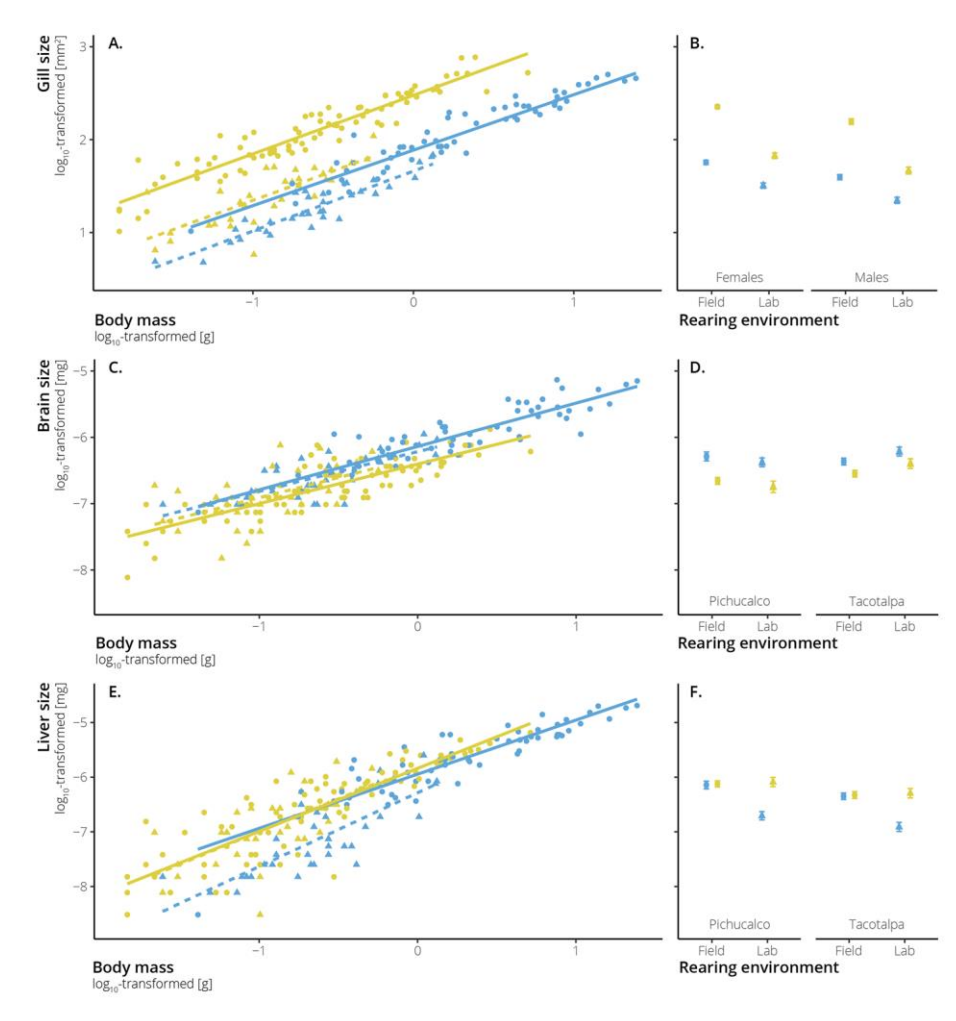


Figure 1.5. Traits shaped by interactions between genetic background and environment. Gill size, brain size, and liver size exhibited significant habitat $\times$ rearing environment interactions. The raw data (left) and average trait means (right) are visualized via scatterplot. These traits relate to predictions D–E in Fig. 1. The significance of interaction 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 & liver, D & F).

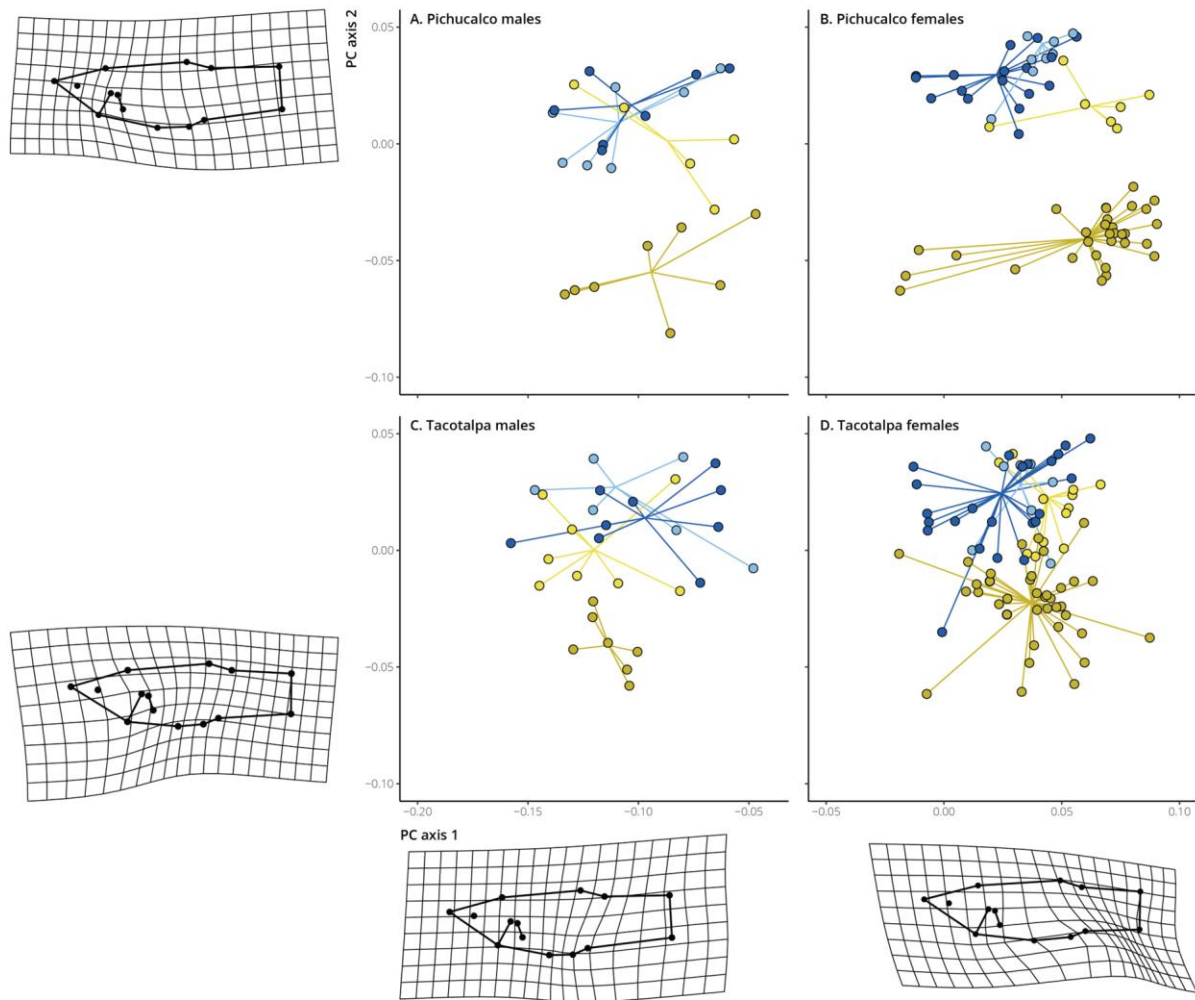


Figure 1.6. Body shape

Body shape was significantly different between wild-caught sulfidic fish (dark yellow) and all other populations, suggesting a plastic response to sulfide exposure. 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.

Online Supplement Results for model ranking of each univariate trait, obtained using the dredge() function from the MUMIN package (Bartoń 2009). Models were limited to one- and two-way interaction terms. Measurements of organ size were used as dependent variables in these linear models. Predictor variables included habitat of origin (sulfidic or nonsulfidic), rearing environment (wild or common garden), drainage (Pichucalco or Tacotalpa), sex (female or male), and blotted wet mass (log10-transformed). Models are ranked based on Akaike Information Criteria with finite sample correction (AICc) (Johnson and Omland 2004). This online supplement is provided in the submitted Excel file.

Chapter 2 - The effects of an H₂S-releasing drug (AP39) on gene expression of sulfide-adapted fish (*Poecilia mexicana*, Poeciliidae)

Introduction

Hydrogen sulfide (H₂S) is a potent respiratory toxicant that naturally occurs in some aquatic ecosystems. When present in sustained and high concentrations, H₂S creates extreme environmental conditions lethal for most forms of animal life (Malone Rubright et al., 2017; Riesch et al., 2015). The nearly-universal toxicity of H₂S is tied to its interference with a highly conserved physiological pathway, oxidative phosphorylation (OxPhos), which is responsible for aerobic ATP production in the mitochondria (Saraste, 1999). H₂S blocks electron flow in the mitochondrial respiratory chain by binding to cytochrome c oxidase (COX, complex IV) and disrupts the electrochemical gradient across the inner mitochondrial membrane that drives aerobic ATP production (Cooper and Brown, 2008). Despite the toxicity, some animals have adapted to habitats rich in H₂S. These organisms tolerate environmental concentrations orders of magnitude higher than closely related populations and species in adjacent nonsulfidic habitats (Tobler et al., 2016).

Among H₂S-tolerant species are livebearing fishes of the family Poeciliidae. These species have repeatedly colonized freshwater springs rich in H₂S throughout the Neotropics and evolved modifications to physiological pathways governing mitochondrial function (Greenway et al., 2014; Tobler et al., 2018). Modifications include amino acid substitutions in COX that render the toxicity target inert to H₂S and increased detoxification of H₂S through enzymatic oxidation, which is mediated by the sulfide:quinone oxidoreductase (SQR) pathway (Greenway et al., 2020; Pfenninger et al., 2014). Genes associated with COX and the SQR pathway are clear targets of selection and significantly upregulated in fish inhabiting H₂S-rich habitats (Kelley et al., 2016; Ryan et al., 2023). However, locally-adapted populations exhibit a wide variety of transcriptional differences from adjacent nonsulfidic populations, including genes that have no clear ties to H₂S responses (Brown et al., 2018; Greenway et al., 2020; Kelley et al., 2016; Passow et al., 2017a). Some gene expression differences between sulfidic and nonsulfidic populations can be explained by the complex environmental differences between habitat types; besides the presence of H₂S, sulfide springs are also characterized by severe hypoxia, reduced

pH, elevated salinity, and a host of biotic environmental differences (Tobler and Plath, 2011; Tobler et al., 2018). However, complex transcriptional differences were also observed in laboratory experiments with *Poecilia mexicana*, a widely studied species because of its genetically and phenotypically distinct population pairs that have repeatedly diverged across adjacent sulfidic and nonsulfidic habitats. In fish raised under nonsulfidic common-garden conditions, exposure to H₂S elicits differential expression of genes in multiple organs and over short time periods (Passow et al., 2017b; Tobler et al., 2014). Even though populations from sulfidic and nonsulfidic habitats differ significantly in how they modulate gene expression upon H₂S exposure, differentially expressed genes are not merely associated with OxPhos and the SQR pathways but include a variety of other biological processes, including glycolysis and gluconeogenesis, redox homeostasis and oxidative stress, cell cycle regulation, as well as cell signaling and gene regulation (Passow et al., 2017b).

There are two alternative hypotheses about how environmental H₂S exposure alone can elicit complex transcriptional changes in *P. mexicana*. (1) Beyond impacting energy metabolism, studies in biomedical models have identified a multitude of physiological effects of H₂S. These include interactions with cell signaling that affect cell cycle, inflammation, and cellular defenses, as well as interactions with ion channels and receptors underpinning responses in the cardiovascular and nervous systems (Li et al., 2011; Olson, 2011; Olson, 2012). Hence, complex transcriptional responses may arise because H₂S has multiple molecular targets and interferes with many physiological processes at once, including transcription factors like signal transducer and activator of transcription 3 (STAT3), nuclear factor erythroid 2-related factor 2 (Nrf-2), and nuclear factor kappa-light-chain-enhancer (NF-κB), which can have wide-ranging effects on gene expression throughout the genome. (2) Alternatively, complexities in gene expression responses may be a consequence of disruptions to mitochondrial homeostasis. The effects of mitochondrial dysfunction may cascade through the cell when H₂S interferes with primary toxicity targets and shuts down aerobic ATP production. In this case, H₂S would not directly interfere with a multitude of physiological targets, but it is H₂S-induced mitochondrial dysfunction and the associated oxidative stress that elicits sweeping changes in gene expression. Indeed, disruption of mitochondrial function has been linked to a variety of pathologies in biomedical research (Bhandary et al., 2012; Murphy et al., 2016; Pieczenik and Neustadt, 2007; Sorrentino et al., 2018).

In the present study, we tested these alternative hypotheses by leveraging pharmacological tools that allow for the targeted manipulation of endogenous H₂S concentrations. H₂S-releasing compounds build on the role of H₂S as a cell signaling molecule and are designed to deliver H₂S inside cells or specific organelles (Caliendo et al., 2010; Kashfi and Olson, 2013; Powell et al., 2018; Szabo, 2007). Though originally conceived to explore medical applications of H₂S, these compounds also provide a potential tool for studying responses to H₂S, especially in non-model systems like *P. mexicana* with adapted (H₂S-tolerant) and non-adapted (H₂S-susceptible) populations. Since H₂S-releasing compounds bypass traditional exposure sites, like the gills and skin in fishes, and allow previously unachievable accuracy to manipulate endogenous H₂S concentrations (Caliendo et al., 2010; Gemici et al., 2015), they are uniquely suited to test the alternative hypotheses developed above. Specifically, we used AP39 [10-oxo-10-(4-(3-thioxo-3H-1,2-dithiol5yl)phenoxy)decyl]triphenylphosphonium bromide], which was designed to deliver and release H₂S specifically inside the mitochondria (Szczesny et al., 2014). If H₂S has many direct targets in cells, we predict that H₂S release by AP39 severely reduces the complexity of transcriptional responses in exposed fish compared to those documented in previous studies using environmental H₂S exposures, because elevated H₂S is restricted to the mitochondria. Alternatively, if the complexity of transcriptional responses is a consequence of loss of mitochondrial function that cascades through the cell, we predict that at least some of the complexity in gene expression changes is maintained, perhaps with upregulation of genes involved in anaerobic metabolism and oxidative stress. We analyzed gene expression in two population pairs of *P. mexicana* and in both liver and gill tissues. Although gene expression differences are most pronounced in gills when fish are exposed to environmental H₂S (because they are directly exposed to the toxic water; Passow et al., 2017a), we hypothesized that liver tissues may exhibit more differentially expressed genes because they are a common site of detoxification for endobiotic and xenobiotic toxicants. We also hypothesized that *P. mexicana* from nonsulfidic habitats would exhibit stronger transcriptional responses because they have an overall lower H₂S tolerance and fail to maintain homeostasis at lower exposure concentrations (Greenway et al., 2020; Tobler et al., 2011).

Methods

Origins and maintenance of experimental animals

All experiments were conducted using *Poecilia mexicana*, including two population pairs from adjacent sulfidic and nonsulfidic habitats. Laboratory populations were originally sourced from the Puyacatengo or Tacotalpa river drainages in the foothills of the Sierra Madre de Chiapas, both of which are separate tributaries of the Río Grijalva (Palacios et al. 2013; Tobler et al. 2011). Sites in the Puyacatengo include La Lluvia springs (sulfidic) and a downstream tributary to the mainstem of the Puyacatengo river (nonsulfidic), those in the Tacotalpa drainage included El Azufre I (sulfidic) and Arroyo Bonita (nonsulfidic). All stocks used in this study were housed under common-garden conditions for several generations. All fish were kept in dechlorinated, non-sulfidic water at temperatures between 24 and 26 °C, subjected to a 12/12-hour light/dark cycle, and provided with a standardized diet of commercially available fish flake food (American Brine Shrimp Company, Ogden, UT, USA), frozen bloodworms and Cyclops copepods, 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).

Experimental exposure

All individuals used in the experiment were adult females assigned to one of four treatment groups. Two treatment groups functioned as controls: group 1 received no injection (no-injection control), and group 2 received an intraperitoneal injection of 50 µl of phosphate-buffered saline (PBS; injection control). The two treatment groups received phosphate-buffered saline with AP39 at different concentrations: group 3 received 50 µl of PBS with 0.03895 µM of AP39, and group 4 received 50 µl of PBS with 0.0779 µM of AP39. While the exact rate of H₂S release from AP39 is unknown, it does occur in a dose-dependent manner, such that doubling dose will double the effective concentrations inside the mitochondria (Le Trionnaire et al. 2014). Overall, six individuals of each population were assigned to each of the four treatment groups (total N = 4 populations × 4 treatments × 6 replicated = 96).

For the injection, females were sedated by submersing them in a buffered solution of MS222 (20 mg/l). They were then immediately transferred to 1-L recovery containers with

aerated water, where they remained for 6 hours. The same amount of time induced significant changes in gene expression when *P. mexicana* was exposed to environmental H₂S (Passow et al. 2017c; Tobler et al. 2014). After the 6-hour incubation period, fish were euthanized by pithing and dissected; liver and gill tissues were isolated and stored in RNAlater at –80 °C until further processing.

RNA extraction, library prep, and sequencing

Tissues were thawed on ice and then homogenized using an Omni Bead Ruptor 96 using a new 2.8-mm ceramic bead per sample. Total RNA was extracted using the Macherey-Nagel NucleoSpin RNA kit and then quantified using the Qubit High Sensitivity RNA kit. RNA integrity and size distribution were checked for a random subset of 11 samples using a Fragment Analyzer. RNA concentrations were normalized to 50 ng/μl before being shipped to the University of Texas Genomic Sequencing and Analysis Facility Center for TagSequencing. Libraries were prepared following the protocol by Lohman et al. (2016) and sequenced on two lanes of a NovaSeq 6000.

Mapping and count matrix generation

Reads from the same sample were merged before quality control. Read quality was assessed prior to and post trimming using FastQC v0.11.9 (Andrews, 2010) and then summarized using MultiQC v1.13a (Ewels et al. 2016). The Perl script tagseq_clipper.pl (https://github.com/z0on/tag-based_RNAseq) was used to clip the 5' leader introduced from library preparation and to remove PCR duplicates identified as reads with the same degenerate header and first 20 bases of the sequence (or Ns in this region). CutAdapt v2.10 (Martin 2011) was used to filter quality scores less than 15, remove adaptors and polyAs, and reads less than 25 base pairs (bp) in length. Filtered and trimmed reads were mapped to the *Poecilia mexicana* reference genome (GCF:001443325.1; Warren et al., 2018) with an appended mitochondrial sequence (addition of mitochondrial genome to reference) (Pfenninger et al. 2014) using STAR v2.7.9a (Dobin et al. 2013). The reference genome's corresponding GTF file was converted to a GFF file using the GEMMaker script gffread (Pertea and Pertea 2020). The reference genome was then indexed using STAR. Finally, reads were mapped and quantified using the –quantMode

GeneCounts parameter in STAR, and a gene count matrix was generated using a bash script `generate_counts_matrix.sh` (Liao et al. 2014).

Differential gene expression analyses

Differential gene expression analysis was conducted with DESeq2 v1.34.0 (Love, Huber, and Anders 2014) in R 4.2 (R Core Team, 2023). First, to determine if there was a significant difference between our control and PBS injection control, and between our AP39 half and AP39 full injections, the count matrix was split by tissue, and samples with an average Pearson's correlation value of less than 0.6 across all genes after a variance stabilizing transformation were removed. Additionally, a sample that was labeled liver but clustered with gill tissue was removed before differential gene expression analysis. The `plotPCA` function in DESeq was used to perform and visualize a principal components analysis. Due to the lack of differentially expressed genes between the two control groups and the two AP39 groups (see results for details), we compared the response of the AP39 full treatment to the control group. Genes with a Benjamini and Hochberg (1995) adjusted P-value of less than 0.05, and an average log₂-fold change greater than 1 or less than -1 were considered differentially expressed between treatment groups.

Annotation

After identifying upregulated and downregulated genes for each contrast, differentially expressed genes (DEG) were compared among populations in a series of Venn diagrams. Genes in the intersections (i.e., DEG shared among all populations, just between the sulfidic populations, and just between the nonsulfidic populations) were annotated with UniProt accession numbers, which were then used to conduct a Gene Ontology (GO; Ashburner et al. 2000; Gene Ontology Consortium, 2021) enrichment analysis using GOrilla (Eden et al. 2007; Eden et al. 2009). GO terms associated with biological processes and an FDR q-value < 0.001 were then investigated for potential links to H₂S toxicity and detoxification.

Results

Issues with RNA extraction, cDNA library construction, and sequencing required us to drop some samples from the analyses, and the final sample sizes included were $N = 23$ for the sulfidic Tacotalpa population and $N = 22$ for the nonsulfidic Tacotalpa population, as well as $N = 20$ for the sulfidic Puyacatengo population and $N = 22$ for the nonsulfidic Puyacatengo population (overall $N = 87$). For the remaining samples, TagSequencing resulted in 3.0–8.3 million reads per sample across two sequencing lanes. After trimming, 1.2–4.5 million reads were retained for each sample. Mapping rates ranged from 84.9–90.0%, with a range of 1.1–4.0 million reads aligned per sample (Table S2.1). Due to the four-fold variation in the number of reads, initial analyses indicated strong effects of library size on variation in gene expression (indicated by significant correlations between library size and the first two principal component axes of the counts matrix). This effect was likely caused by an increased chance of detecting lowly expressed genes in larger libraries. To remove biases arising from library size, we down-sampled libraries with more than 4 million reads by randomly selecting 4 million reads from the original pool.

As expected, patterns of gene expression differed significantly between gills and livers (Figure 1). However, the number of differentially expressed genes was comparatively small when comparing the two controls (no injection vs. injected with PBS; 0–63 differentially expressed genes; Table S2) and when comparing the two AP39 treatments (low vs. high concentration; 0–12 differentially expressed genes; Table S3). For our final analyses, we consequently focused on comparing fish from the no-injection control and the high-AP39 treatment (Figure S1). This comparison provided the clearest results, and the results of other contrasts were qualitatively similar.

Differences in AP-39 responses between gills and the liver

Gene expression changes in response to AP39 exposure were much more pronounced in the liver compared to the gills (an average of 1706 vs. 10 differentially expressed genes) (Figure 2.2). In the liver, 1424 genes were differentially expressed in the sulfidic population from Tacotalpa (765 upregulated, 659 downregulated), 766 in the nonsulfidic population from Tacotalpa (439 upregulated, 327 downregulated), 1932 in the sulfidic population from Puyacatengo (1019

upregulated, 913 downregulated), and 2702 in the nonsulfidic population from Puyacatengo (1260 upregulated, 1442 downregulated). In contrast, only 3 genes were differentially expressed in the gills of fish from the sulfidic Tacotalpa population (2 upregulated, 1 downregulated), 1 in the nonsulfidic Tacotalpa population (0 upregulated, 1 downregulated), 22 in the sulfidic Puyacatengo population (10 upregulated, 12 downregulated), and 12 in the nonsulfidic Puyacatengo population (5 upregulated, 7 downregulated). Overall, this finding contrasts starkly with gene expression responses during environmental exposures of H₂S, which elicit stronger changes in the gills opposed to the liver.

Gene expression responses in the gills

Gene expression responses in the gills were not only sparse, but they were also highly idiosyncratic across populations. With the exception of a single gene, all differentially expressed genes were unique to a single population (Figure 2.1). The only exception was the gene OLFM4, which was upregulated in fish from the sulfidic population from the Tacotalpa drainage and the nonsulfidic population from the Puyacatengo drainage. OLFM4 encodes for Olfactomedin-4, a protein involved in cell growth, differentiation, and apoptosis. Neither OLFM4 nor differentially expressed genes that were unique to specific populations had known connections to H₂S responses or mitochondrial function. Overall, the lack of consistent changes in gene expression in the gills may suggest that the few differentially expressed genes are largely spurious.

Gene expression responses in the liver

In contrast to the gills, gene expression changes in response to AP39 exposure in the liver were much more pronounced, and intersecting the lists of differentially expressed genes among populations revealed shared responses that could shed some light into commonalities and differences in physiological responses. We particularly focused on three intersections: (1) Genes that were differentially expressed in the same direction in all four populations, representing shared, ancestral responses common to fish from both sulfidic and nonsulfidic habitats, (2) genes that were differentially expressed in the same direction in fish from both sulfidic populations (but not the nonsulfidic ones), indicating potentially adaptive responses to H₂S that have evolved upon colonization of sulfidic habitats, and (3) genes that were differentially expressed in the

same direction in fish from both nonsulfidic populations (but not the sulfidic ones), indicating potentially maladaptive responses to H₂S that have been selected against upon colonization of sulfidic habitats.

256 genes were differentially expressed (159 up- and 87 downregulated) in all populations, representing a shared response sulfidic and nonsulfidic populations have inherited from their common ancestor. There was no evidence for differential expression of genes associated with the primary toxicity target in (OxPhos) or H₂S detoxification via the SQR pathway, although both are located in the mitochondria where AP39 released H₂S. However, GO-enrichment analysis indicated modulation of biological processes that have been linked to H₂S exposure or mitochondrial function. For example, upregulated genes were disproportionately associated with controlling protein localization to the mitochondria and protein transport into mitochondrial matrix (Figure S2.2, Table S2.2). There also was evidence for upregulation of general stress responses, including NF- κ B signaling, which regulates transcription in response to cellular stress, and other biological processes associated with transcription and translation. Evidence for stress responses also come from a plethora of GO terms associated with responses to damaged and misfolded proteins, including ubiquitination that targets proteins for degradation, protein transport, endoplasmic-reticulum-associated protein degradation, and other processes that contribute to the function of the endoplasmic reticulum during oxidative stress. Finally, there was evidence for the upregulation of genes associated with the function of the immune system (T-cell receptor signaling, antigen processing and presentation, and leucocyte activation) and the regulation of different cell types in the blood. Downregulated genes were primarily associated with the metabolism of fatty acids and vitamins, bile production and secretion, as well as responses to xenobiotics and drug metabolism (Figure S2.3, Table S2.2).

126 genes were differentially expressed (64 up- and 62 downregulated) in both sulfidic populations (one from Tacotalpa, one from Puyacatengo) but not in the nonsulfidic populations. Again, we found no evidence that genes associated with OxPhos or the SQR pathway were differentially expressed, but GO enrichment analyses revealed several biological processes that have previously been associated with H₂S responses (Figure S2.4, Table S2.3). Upregulated genes unique to fish from the sulfidic populations were largely associated with Acyl-CoA biosynthesis. Acyl-CoA are coenzymes that metabolize fatty acids, providing substrates for the citric acid cycle and the formation of ATP. Other significant GO terms included prenylated

protein catabolic process, intracellular transport, and regulation of cell size. There was no evidence for significant enrichment of GO terms in the downregulated genes.

Finally, 83 genes were differentially expressed (32 up- and 51 downregulated) in fish from both nonsulfidic populations (one from Tacotalpa, one from Puyacatengo) but not in the sulfidic populations. As before, there was no evidence for differential expression of genes associated with OxPhos and the SQR pathway. However, upregulated genes were significantly enriched in metabolic processes of noncoding and ribosomal RNAs, including the maturation of SSU-rRNA that has been implicated in oxidative stress responses (Figure S2.5, Table S2.4). Downregulated genes were enriched in GO terms describing the regulation of miRNAs, the regulation of neuron projection, and the organization of photoreceptors (Figure S2.6, Table S2.4).

Discussion

In this study, we investigated gene expression responses to targeted H₂S exposure in the mitochondria using a pharmacological compound (AP39). We compared the gene expression response of gill and liver tissues between two population pairs of *Poecilia mexicana*: two adapted to high environmental concentrations of H₂S and corresponding ancestral populations still susceptible to elevated environmental H₂S. AP39-induced gene expression was much more pronounced in the liver than the gills, contrasting previous studies that focused on responses to environmental H₂S exposure (Passow et al., 2017a). While AP39-induced gene expression responses in the gills were much simpler (in terms of number of differentially expressed genes) than during environmental exposures, responses in the liver was similarly complex during environmental and pharmacological exposures (Passow et al., 2017a; Passow et al., 2017b). Consequently, the simple gene expression responses in the gills were consistent with the hypothesis of H₂S having a multitude of targets throughout the cell, while the complex responses in the liver suggested that H₂S-induced stress and mitochondrial dysfunction likely have cascading effects for gene expression throughout the cell.

It is important to emphasize that the nature of gene expression responses differed markedly between our current study that leveraged pharmacological tools and previous studies that relied on environmental exposures. Key genes that have been identified as candidates underlying adaptation to H₂S—including those associated with the primary toxicity target (COX

in OxPhos) and with enzymatic H₂S detoxification (SQR pathway)—did not respond to AP39 exposure, even though they are among the top differentially expressed genes in the gills during environmental exposures (Greenway et al., 2020; Kelley et al., 2016; Passow et al., 2017a; Passow et al., 2017b). Most changes in gene expression in this study were instead related to cellular stress, oxidative stress response, immune system response, drug metabolism, and pathways linked to H₂S as a signaling molecule. Overall, our study sheds light into the origins of gene expression differences previously documented among natural populations of *P. mexicana* residing in different habitat types and in laboratory experiments using environmental H₂S exposures. At the same time, we also uncovered limitations of H₂S-releasing drugs for the study of adaptation mechanisms in extremophile fishes.

What is the site of H₂S detoxification?

Previous studies of gene expression responses to H₂S have suggested that the gills are a major site of H₂S detoxification in *P. mexicana* and other sulfide spring fishes (Greenway et al., 2020; Passow et al., 2017a; Passow et al., 2017b). Expression differences between sulfidic and nonsulfidic populations are not only more pronounced in the gills than in the livers (Passow et al., 2017a), but they also include key genes with a priori predictions in terms of the biochemical and physiological effects of H₂S. Genes directly or indirectly associated with H₂S toxicity (OxPhos and energy metabolism) and detoxification (SQR and allied pathways) are consistently among the top differentially expressed genes (Greenway et al., 2020; Kelley et al., 2016; Passow et al., 2017b). The present study draws stark contrasts to these previous results.

H₂S exposure mediated by AP39 elicited much more pronounced gene expression changes in the liver than in the gills. Consequently, it is not only the elevated level of H₂S in the mitochondria but also the nature of exposure that critically shapes organismal responses to this toxicant. The high transcriptional response to environmental H₂S exposure in the gills may be simple consequence of their direct contact to the ultimate source of H₂S, while cells in organs within the body are more shielded. This finding highlights our superficial understanding about the physiology of H₂S tolerance, and future studies need to investigate what cells in the gills (or in the blood that circulates through them) are mediating the H₂S response and responsible for the bulk of H₂S detoxification. Recent advances in single-cell sequencing may provide a critical tool to tackle this question (Nawy, 2014). Alternatively, the lack of a pronounced transcriptional

response in the gills during AP39 exposure may not be due to a lack of specialized cells in the gills, but rather a consequence of H₂S release tied to a xenobiotic (AP39), and xenobiotics are primarily processed in the liver (Chen et al., 2019; Lohr et al., 1998). Hence, future studies wanting to leverage the power of such pharmacological compounds will need to first assess how the drug is distributed throughout the body, and whether it is reasonable to assume equal accumulation and release of H₂S throughout different organs and cell types. The development of mitochondrial H₂S probes that can quantify in vivo levels of H₂S within the organelle may be leveraged for this purpose (Arndt et al., 2017; Lau et al., 2019).

Why was there no signal of classical H₂S response genes?

As mentioned previously, transcriptomic profiling of fish from sulfidic and nonsulfidic populations in natural and experimental settings has been instrumental to identifying genes associated with OxPhos and the SQR pathways, which are currently considered prime candidates in mediating the elevated H₂S tolerance and adaptation of sulfide spring populations (Greenway et al. 2020; Kelley et al. 2016; Passow et al. 2017c). The critical role of these pathways has been ascertained in functional physiology studies, showing that fish from sulfide spring populations are able to detoxify H₂S at a higher rate, less susceptible to detrimental effects of elevated endogenous concentrations of H₂S, and able to maintain mitochondrial function in presence of H₂S (Barts 2020; Greenway et al. 2020; Pfenninger et al. 2014). Why then did genes associated with these pathways not respond to AP39 exposure? One explanation we already alluded to is that gills could be the primary site of the detoxification response, but AP39 may not have reached those tissues in sufficient quantities. An alternative explanation is that the realized concentrations of H₂S within the mitochondria was simply not high enough, because we faced several constraints when administering AP39 to our experimental fish: (1) *P. mexicana* are small-bodied fish, with most individuals weighing approximately 1 g. Due to the small body size, we were only able to administer up to 50 µl of PBS in a peritoneal injection. (2) AP39 has limited solubility in PBS, which—in conjunction with the limited injection volume—constrained the amount of drug we were able to administer. Considering how tolerant *P. mexicana* are to environmental exposure of H₂S (even nonadapted individuals from nonsulfidic habitats have a higher H₂S tolerance than many other freshwater fishes), the limited amount of H₂S released by AP39 may simply have failed to reach the threshold necessary to elicit the predicted response.

For comparison, in cultures of mouse endothelial cells, the AP39 begins to inhibit basal oxygen consumption rate around 0.3 μM (Szczesny et al., 2014), and the maximum dose we were able to administer in our fish was only about a quarter of this concentration. Future research consequently needs to explore alternative compounds that allow for higher doses to be administered despite the small size of the fish (Caliendo et al. 2010; Kashfi and Olson 2013).

What pathways responded to AP39, and is there evidence for adaptive differences?

The primary pathways that responded to AP39 in the liver were related to general cellular stress responses, oxidative stress, and H_2S as a cell signaling molecule. Many GO terms were associated with oxidative stress in the endoplasmic reticulum, immune system function, and regulation of different cell types in the blood. H_2S was likely not the direct source of oxidative stress; although it can be a prooxidant, it must be present at higher concentrations than encountered in this study (Olas 2017). The upregulation of the NF- κB suggests that H_2S may be acting to counteract oxidative stress in this study. NF- κB is a commonly identified target of H_2S when acting as a signaling molecule (Li et al. 2011); NF- κB signaling regulates transcription in response to cellular stress, inflammation, and immunity (Gilmore 2004). It is unclear if NF- κB was upregulated in response to cellular stress or upregulated due to elevated H_2S concentrations, or both. Even if not directly related to oxidative stress via NF- κB , H_2S is also known act as an antioxidant, reducing ROS and oxidative stress (Olas 2017). If H_2S is not the source of the observed oxidative stress responses, that begs the question of what is. One potential source of the observed oxidative stress is the activation of cytochrome P450, which is associated with the metabolism of xenobiotic drugs (such as AP39) in the liver (Monostory and Vereczkey 1995; Pentiuk et al. 2004). Past research suggests that cytochrome P450 produces reactive oxygen species and oxidative stress (Hrycay and Bandiera 2015; Lewis 2002; Pentiuk et al. 2004). This may explain the downregulation of drug metabolic processes in the liver (Figure S2.6). Indeed, other organisms have evolved changes in cytochrome P450 in response to potentially negative effects of its upregulation in response to xenobiotic pollutants (Whitehead et al. 2017). Overall, these responses suggest that AP39 did release an impactful amount of H_2S in the liver, but not enough to induce inhibition of the OxPhos pathway and ATP production.

Most of the pathways that responded to AP39 in the liver were shared between all populations (256 genes), with comparatively fewer being unique to sulfidic (126 genes) or nonsulfidic (83 genes) populations. If these unique genes are adaptive/maladaptive, then future studies will have to test how so, because their links to H₂S remain unclear. Genes directly related to H₂S adaptation that were previously identified in similar analyses, OxPhos and the SQR pathway, are definitively missing in the results of this study. Therefore, there is no evidence for differential responses between sulfidic and nonsulfidic populations that may be indicative of direct adaptation to coping with the toxicity of H₂S. There are two potential explanations for this result: (1) Adaptive differences are not present in the liver due to lack of natural exposure to H₂S, or (2) all the caveats mentioned above apply, especially concerning exposure concentrations. Adaptive differences may only become evidence at more substantive H₂S concentrations.

Why should we continue to explore the power of pharmacological tools to study physiological adaptation?

Over two decades of research have investigated the evolution of H₂S tolerance in *P. mexicana* based on environmental H₂S exposure. While this method accurately represents the ecological context, it limits our ability to learn about the mechanisms underlying elevated H₂S tolerance, and how tissue- and cell-specific processes may contribute to it. H₂S-releasing drugs, which were designed to explore the medical applications of H₂S via exposure of specific tissues, cells, and even organelles (Caliendo et al. 2010; Kashfi and Olson 2013), provide an opportunity to address those problems, and are a perfect example of how pharmacological tools allow researchers to manipulate specific environmental factors with unprecedented power and control. Successful employment of pharmacological methods would allow us to disentangle the complex physiological and organismal responses we observe in nature. Just as gene editing allows us to isolate the effects of a single gene or nucleotide in organismal phenotypes, pharmacological tools allow us to control for a variety of interactions and contexts that are otherwise impossible to untangle.

This study identified previously unknown variation in H₂S response sites with *P. mexicana*, and uncovered novel questions about the nature of H₂S detoxification. Our findings suggest that H₂S-induced stress and mitochondrial dysfunction likely have cascading effects for

gene expression throughout the cell, rather than H₂S having a multitude of targets throughout the cell, though our results come with several caveats. The study we present here is rife with unanswered questions and potential complications, offering a glimpse into the many unforeseen obstacles encountered in the endeavor of using novel tools to understand organismal responses to physiochemical stressors. We undertook this study precisely because it is a novel approach to a well-characterized system, and novel approaches beget novel questions. For example, as of this study, we cannot conclude what the primary site of H₂S detoxification is, nor why. Figuring out how to deliver a higher concentration of H₂S to the mitochondria would allow us to explore if such exposure would evoke gene expression responses akin to those observed in environmental exposure, and where that expression occurs. Although this first venture into this area has raised more questions than it answered, this is an avenue that ecological and evolutionary physiologists should continue to entertain and explore.

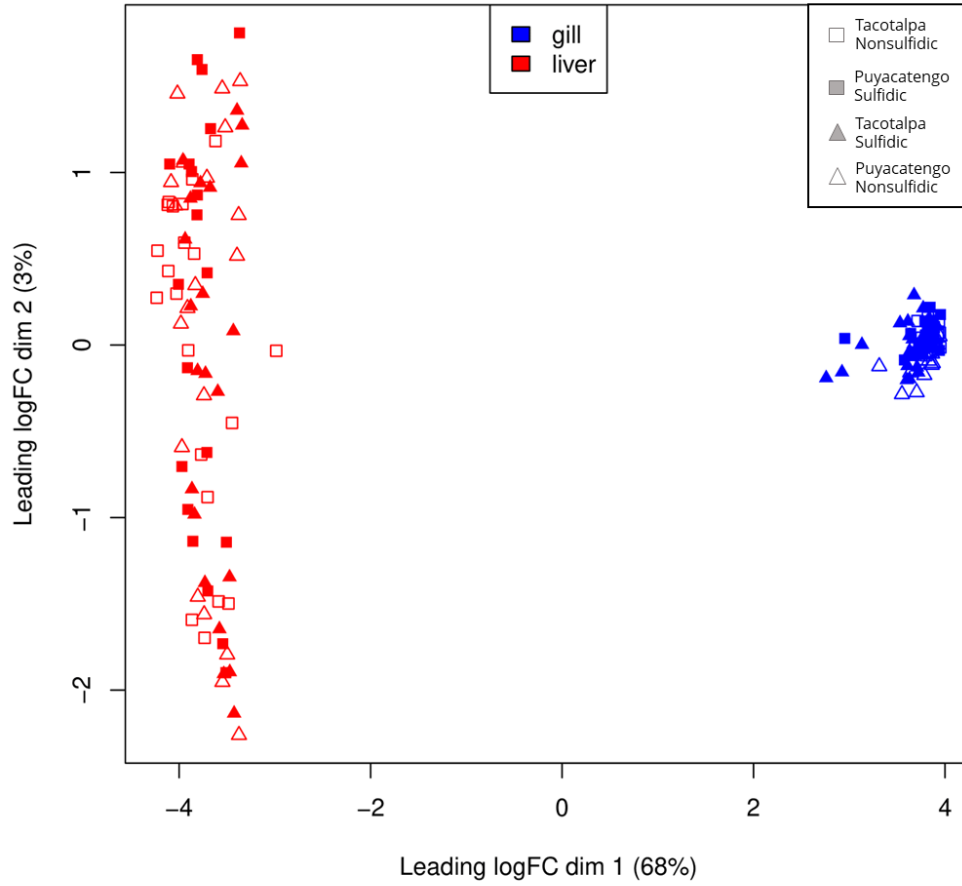


Figure 2.1. MDS plot of gene expression in liver and gills

An MDS plot showing patterns of gene expression in the liver and gill tissue of all four populations. The clear separation between liver and gill tissues confirms that the organs exhibit significantly different transcriptional responses.

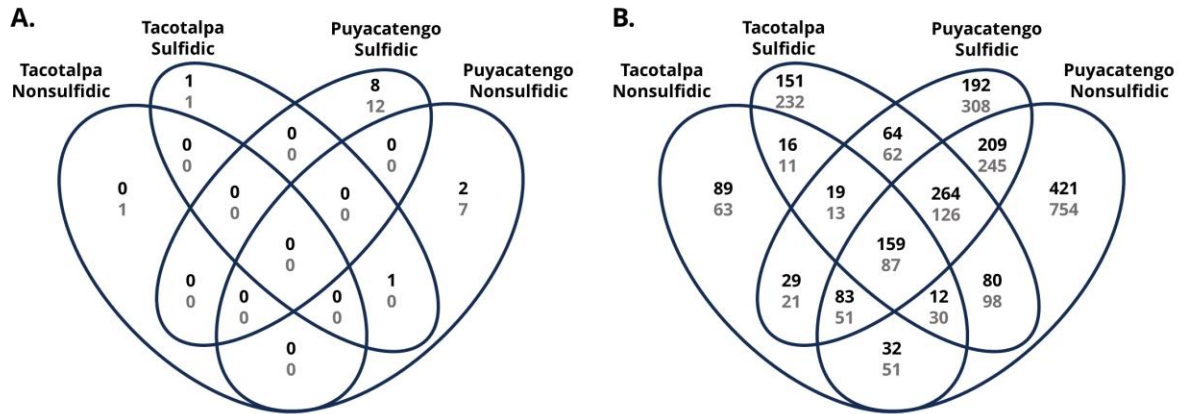


Figure 2.2. Venn diagram of gene expression counts in liver and gills

Venn diagram depicting the number of differentially expressed genes between sulfidic and nonsulfidic populations for both the Tacotalpa and the Puyacatengo river drainage in gill (A) and liver (B). Values that are bolded are upregulated and those in grey are downregulated.

Table S2.1. Mapping and alignment averages for all populations.

Every column contains the mean and standard deviation across all individuals in that population, by millions of reads. Mapped sequences refer to how many fragments were identified by TagSequencing methods, which were then trimmed down to eliminate incomplete or artificially duplicated reads. Alignment refers to how many identified reads were successfully aligned to the reference genome, as well as what percentage of reads were successfully aligned.

Population	Sequences mapped (millions)	Sequences mapped post-trim (millions)	Sequences aligned (millions)	Alignment percentage
Tacotalpa Sulfidic	5.41 ± 0.94	3.17 ± 0.49	2.76 ± 0.41	86.94 ± 0.86
Tacotalpa Nonsulfidic	5.38 ± 0.91	3.01 ± 0.52	2.62 ± 0.44	87.04 ± 0.84
Puyacatengo Sulfidic	5.41 ± 0.93	3.28 ± 0.45	2.84 ± 0.41	86.87 ± 0.96
Puyacatengo Nonsulfidic	5.47 ± 0.96	3.15 ± 0.47	2.73 ± 0.40	87.00 ± 0.90

Table S2.2. DEGs for all populations

A list of all differentially expressed genes between control and AP39 high concentration treatments that were shared between the four experimental populations. This is provided in a separate file, TableS2.allDEGs.liver.csv.

Table S2.3. DEGs in only sulfidic populations

A list of all differentially expressed genes between control and AP39 high concentration treatments that were unique to the experimental populations tolerant to sulfide. This is provided in a separate file, TableS3.sulfideDEGs.liver.csv.

Table S2.4. DEGs in only nonsulfidic populations

A list of all differentially expressed genes between control and AP39 high concentration treatments that were unique to the experimental populations not tolerant to sulfide. This is provided in a separate file, TableS4.nonsulfideDEGs.liver.csv.

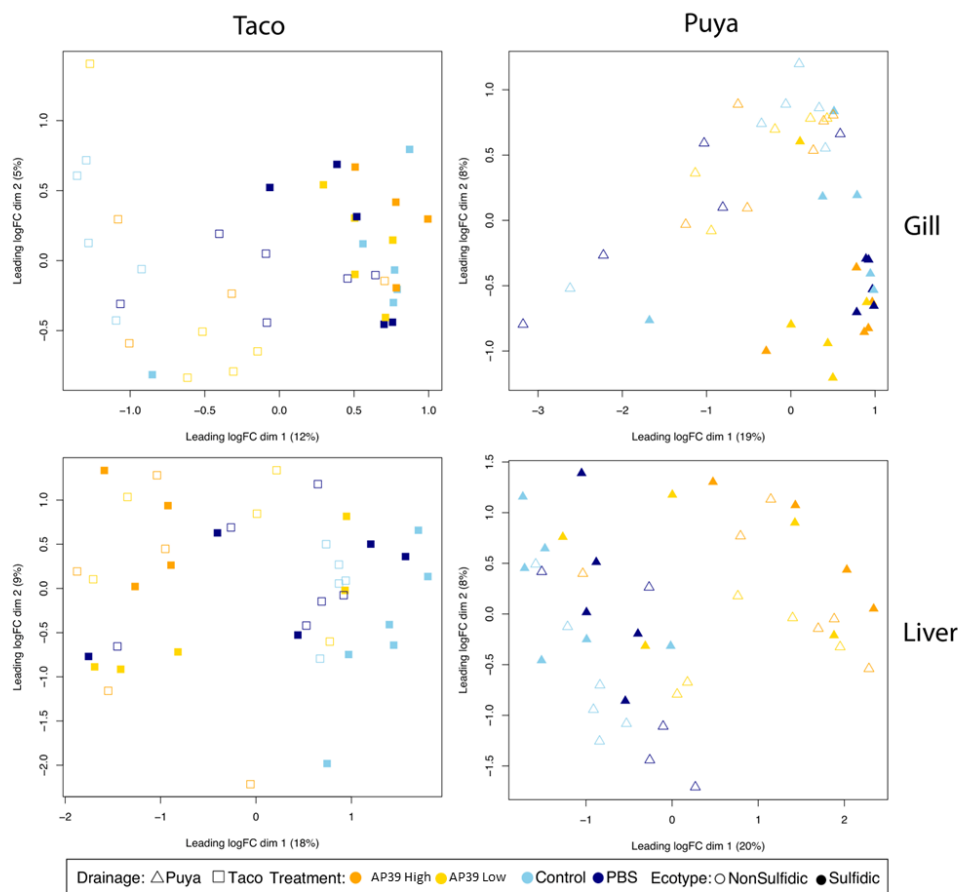


Figure S 2.1. MDS plot of gene expression response by tissue and drainage

MDS plot of gill (top) and liver (bottom) tissue in response to AP39 treatment from two drainages, Tacotalpa (left) and Puyacatengo (right). Color represents treatment and fill represents ecotype.

Figure S2.2. GOenrichment map for all upregulated DEGs

GO enrichment map for all differentially expressed genes between control and AP39 high concentration treatments that were upregulated and shared between the four experimental populations. Shading indicates level of enrichment, with more color corresponding to a lower FDR q-value. This is provided in a separate file, Figure2.GOenrichment.allDEGs.up.liver.png.

Figure S2.3. GO enrichment map for all downregulated DEGs

GO enrichment map for all differentially expressed genes between control and AP39 high concentration treatments that were downregulated and shared between the four experimental populations. Shading indicates level of enrichment, with more color corresponding to a lower

FDR q-value. This is provided in a separate file,
FigureS3.GOenrichment.allDEGs.down.liver.png.

Figure S2.4. GO enrichment map for upregulated DEGs in only sulfidic populations

GO enrichment map for all differentially expressed genes between control and AP39 high concentration treatments that were upregulated and unique to the experimental populations tolerant to sulfide. Shading indicates level of enrichment, with more color corresponding to a lower FDR q-value. This is provided in a separate file,
FigureS4.GOenrichment.sulfideDEGs.up.liver.png.

Figure S2.5. GO enrichment map for upregulated DEGs in only nonsulfidic populations

GO enrichment map for all differentially expressed genes between control and AP39 high concentration treatments that were upregulated and unique to the experimental populations not tolerant to sulfide. Shading indicates level of enrichment, with more color corresponding to a lower FDR q-value. This is provided in a separate file,
FigureS5.GOenrichment.nonsulfideDEGs.up.liver.png.

Figure S2.6. GO enrichment map for all downregulated DEGs in only nonsulfidic populations

GO enrichment map for all differentially expressed genes between control and AP39 high concentration treatments that were downregulated and unique to the experimental populations not tolerant to sulfide. Shading indicates level of enrichment, with more color corresponding to a lower FDR q-value. This is provided in a separate file,
FigureS6.GOenrichment.nonsulfideDEGs.down.liver.png.

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