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Convergent evolution of conserved mitochondrial pathways underlies repeated adaptation to extreme environments

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Extreme environments test the limits of life; yet, some organisms thrive in harsh conditions. Extremophile lineages inspire questions about how organisms can tolerate physiochemical stressors and whether the repeated colonization of extreme environments is facilitated by predictable and repeatable evolutionary innovations. We identified the mechanistic basis underlying convergent evolution of tolerance to hydrogen sulfide (H_2S)—a toxicant that impairs mitochondrial function—across evolutionarily independent lineages of a fish (*Poecilia mexicana*, Poeciliidae) from H_2S-rich springs. Using comparative biochemical and physiological analyses, we found that mitochondrial function is maintained in the presence of H_2S in sulfide spring *P. mexicana* but not ancestral lineages from nonsulfidic habitats due to convergent adaptations in the primary toxicity target and a major detoxification enzyme. Genome-wide local ancestry analyses indicated that convergent evolution of increased H_2S tolerance in different populations is likely caused by a combination of selection on standing genetic variation and de novo mutations. On a macroevolutionary scale, H_2S tolerance in 10 independent lineages of sulfide spring fishes across multiple genera of Poeciliidae is correlated with the convergent modification and expression changes in genes associated with H_2S toxicity and detoxification. Our results demonstrate that the modification of highly conserved physiological pathways associated with essential mitochondrial processes mediates tolerance to physiochemical stress. In addition, the same pathways, genes, and—in some instances—codons are implicated in H_2S adaptation in lineages that span 40 million years of evolution.

adaptive evolution | comparative physiology | ecological genomics | hydrogen sulfide | phylogenetic comparative analysis

Stephen J. Gould made a strong case for the importance of contingency in evolution, famously quipping that replaying the "tape of life" would lead to different outcomes every time (1). However, despite the unpredictability of mutations, the effects of genetic drift, and other historical contingencies, convergent evolution of phenotypic traits and their underlying genes is common, indicating that natural selection sometimes finds repeatable and predictable solutions to shared evolutionary challenges (2, 3). A major challenge that remains is the identification of the ecological, genetic, and functional factors that might determine the repeatability and predictability of evolutionary outcomes (4).

Mitochondria and their genomes provide a fascinating model to ask questions about the predictability of evolution for two reasons: 1) Mitochondrial genomes were historically thought to be a prime example of contingency evolution because alternative genetic variants were assumed to be selectively neutral (5). This paradigm has been shifting though, with mounting evidence that mitochondria—and genes encoded in the mitochondrial genome—can play important roles in adaptation, especially in the context of physiochemical stress (6). 2) Mitochondria are critical for the cellular function of eukaryotes (7). Their function is dependent on the gene products from two genomes, the mitochondrial and the nuclear (8), which interact to ultimately shape whole-organism performance. Despite extensive characterization of allelic variation in mitochondrial genomes, it often remains unclear how variation in genes that contribute to mitochondrial function translates to

Significance

Some organisms can tolerate environments lethal for most others, but we often do not know what adaptations allow them to persist and whether the same mechanisms underly adaptation in different lineages exposed to the same stressors. Investigating fish inhabiting springs rich in toxic H_2S, we show that tolerance is mediated by the modification of pathways that are inhibited by H_2S and those that can detoxify it. Sulfide spring fishes across multiple genera have evolved similar modifications of toxicity targets and detoxification pathways, despite abundant lineage-specific variation. Our study highlights how constraints associated with the physiological consequences of a stressor limit the number of adaptive solutions and lead to repeatable evolutionary outcomes across organizational and evolutionary scales.

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Data deposition: Data and code associated with biochemical and physiological analyses are available on GitHub (https://github.com/michitobler/convergent_h2s_evolution). All sequence data are available at National Center for Biotechnology Information (NCBI) BioProject (accession nos. PRJNA473350 and PRJNA608180).

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variation in physiological and organismal function. Furthermore, it is not known whether exposure to similar selective regimes may cause convergent modifications of mitochondrial genomes and emergent biochemical and physiological functions in evolutionarily independent lineages.

Extreme environments that represent novel ecological niches are natural experiments to address questions about mechanisms underlying mitochondrial adaptations and illuminate the predictability of adaptive evolution of mitochondria. Among the most extreme freshwater ecosystems are springs with high levels of H_2S, a potent respiratory toxicant lethal to metazoans due to its inhibition of mitochondrial ATP production (9). Multiple lineages of livebearing fishes (Poeciliidae) have colonized H_2S-rich springs throughout the Americas and independently evolved tolerance to sustained H_2S concentrations orders of magnitude higher than those encountered by ancestral lineages in nonsulfidic habitats (10). Here, we identify the molecular basis of an evolutionary innovation that facilitated the independent colonization of extreme environments (increased H_2S tolerance) and ask if the underlying mechanisms have evolved in convergence in disparate lineages of livebearing fishes.

H_2S toxicity and detoxification are associated with highly conserved physiological pathways in mitochondria (Fig. 1*A*) (11, 12), providing a priori predictions about the potential molecular mechanisms underlying adaptation to this strong source of selection. Toxic effects of H_2S result from binding to and inhibition of COX (cytochrome c oxidase, complex IV) in the oxidative phosphorylation (OxPhos) pathway, which contains subunits encoded in both the nuclear and the mitochondrial genomes (13). Animal cells can also detoxify low concentrations of endogenously produced H_2S via the mitochondrial SQR (sulfide:quinone oxidoreductase) pathway, which is linked to OxPhos but entirely encoded in the nuclear genome (14). We have previously shown that genes associated with both pathways are under divergent selection and differentially expressed between fish populations in sulfidic and nonsulfidic habitats (10). These include nuclear and mitochondrial genes encoding subunits of the direct toxicity target (COX) and the nuclear gene encoding the enzyme mediating the first step of detoxification (SQR) (10). Tolerance to H_2S may, therefore, be mediated by resistance (modification of toxicity targets that reduce the negative impact of H_2S), regulation (modification of physiological pathways that maintain H_2S homeostasis), or both (9).

Based on these previous results, we hypothesized that the repeated modification of enzymes in the OxPhos and SQR pathways in *P. mexicana* populations from sulfidic habitats leads to an increased ability to maintain mitochondrial function in the presence of H_2S. In the present study, we used a series of in vivo and in vitro assays to identify the functional consequences of modifications to the OxPhos and SQR pathways in evolutionarily independent population pairs of *P. mexicana* from adjacent sulfidic and nonsulfidic habitats that are situated in different river drainages. In addition, we hypothesized that convergent molecular modifications in the same pathways underlie the convergent evolution of H_2S tolerance across different lineages of poeciliid fishes. Hence, we also used phylogenetic comparative analyses of gene expression and analyses of molecular evolution to detect patterns of molecular convergence in 10 lineages of sulfide spring poeciliids and ancestors from nonsulfidic habitats.

Results and Discussion

Sulfide Spring *P. Mexicana* Exhibit a Resistant Toxicity Target. If resistance is the primary mechanism of tolerance, we would predict that COX function is maintained in the presence of H_2S in fish from sulfidic populations but not those from nonsulfidic populations. Quantification of COX function indicated that enzyme activity generally declined with increasing H_2S concentrations, but this decline was reduced in populations from sulfidic habitats (Fig. 1*B*; habitat × H_2S: $P < 0.001$, *SI Appendix*, Table S3). Even though the drainage of origin was not retained as an explanatory variable in statistical models (*SI Appendix*, Table S2), COX activity in one H_2S-tolerant population [Tacotalpa (Tac)] declined just as in nonsulfidic populations (Fig. 1*B*). The other two *P. mexicana* populations from sulfidic habitats [Puyacatengo (Puy) and Pichucalco (Pich)] maintained significant COX activity even at the highest H_2S concentrations, which should reduce the negative impact of H_2S on cellular respiration. These results are consistent with previous analyses (15) and indicate that resistance may contribute to H_2S tolerance in some populations but cannot explain the repeated evolution of H_2S tolerance by itself.

Sulfide Spring *P. Mexicana* Can Regulate Mitochondrial H_2S through Increased Detoxification. We also tested whether tolerant and intolerant populations differ in their ability to detoxify H_2S by conducting enzyme activity assays of SQR. Activity of SQR was significantly higher in mitochondria from sulfidic populations at intermediate and high H_2S concentrations (Fig. 1*C*; habitat × H_2S: $P < 0.001$ in *SI Appendix*, Table S5), likely helping fish from sulfidic habitats to maintain H_2S homeostasis during environmental exposure. To test this prediction in vivo, we used a novel mitochondria-specific H_2S-probe (MitoA) that allows for the monitoring of relative H_2S levels inside the mitochondria of living organisms (16). We measured mitochondrial H_2S concentrations in this manner using laboratory-reared fish that were exposed to varying levels of environmental H_2S. Because laboratory-reared fish were not available for the population pair from Pich, only two population pairs were used for this analysis. Overall, mitochondrial H_2S concentrations increased with environmental exposure ($P = 0.001$) and was higher in fish from nonsulfidic habitats ($P < 0.001$ in *SI Appendix*, Table S7). H_2S concentrations in mitochondria isolated from livers (Fig. 1*D*) and other organs (*SI Appendix*, Fig. S2) of fish from nonsulfidic habitats increased above control levels at all exposure concentrations. In contrast, mitochondrial H_2S concentrations in isolates of fish from sulfidic populations did not usually exceed control levels and remained lower than levels in fish from nonsulfidic habitats. Together, these results indicate that populations of *P. mexicana* from sulfidic habitats can detoxify H_2S at higher rates and, thus, regulate mitochondrial H_2S upon environmental exposure.

Sulfide Spring *P. Mexicana* Can Maintain Mitochondrial Function in the Presence of H_2S. Modification of the OxPhos and SQR pathways in *P. mexicana* suggests that mitochondrial adaptations are key to the evolution of H_2S tolerance. Therefore, mitochondrial function of fish from sulfidic habitats should be maintained upon exposure to H_2S. We tested this hypothesis by quantifying different aspects of mitochondrial function (basal respiration, maximal respiration, and spare respiratory capacity) along a gradient of H_2S concentrations using an ex vivo coupling assay. As expected, all aspects of mitochondrial function generally declined with increasing H_2S (Fig. 1*E* and *SI Appendix*, Figs. S3–S5). Comparison of mitochondrial function between adjacent populations in sulfidic and nonsulfidic habitats indicated no differences in basal respiration (*SI Appendix*, Fig. S3). However, individuals from sulfidic populations were able to maintain maximal respiration and spare respiratory capacity at higher levels compared to individuals from nonsulfidic habitats of the same river drainage (Fig. 1*E*), even though the magnitude of difference and the shape of response curves varied (*SI Appendix*; significant drainage × habitat interactions in *SI Appendix*, Tables S10–S12 and Figs. S4 and S5). These findings indicate that mitochondria of H_2S-tolerant individuals continue to produce ATP in the presence of a potent inhibitor that reduces mitochondrial function in ancestral lineages.

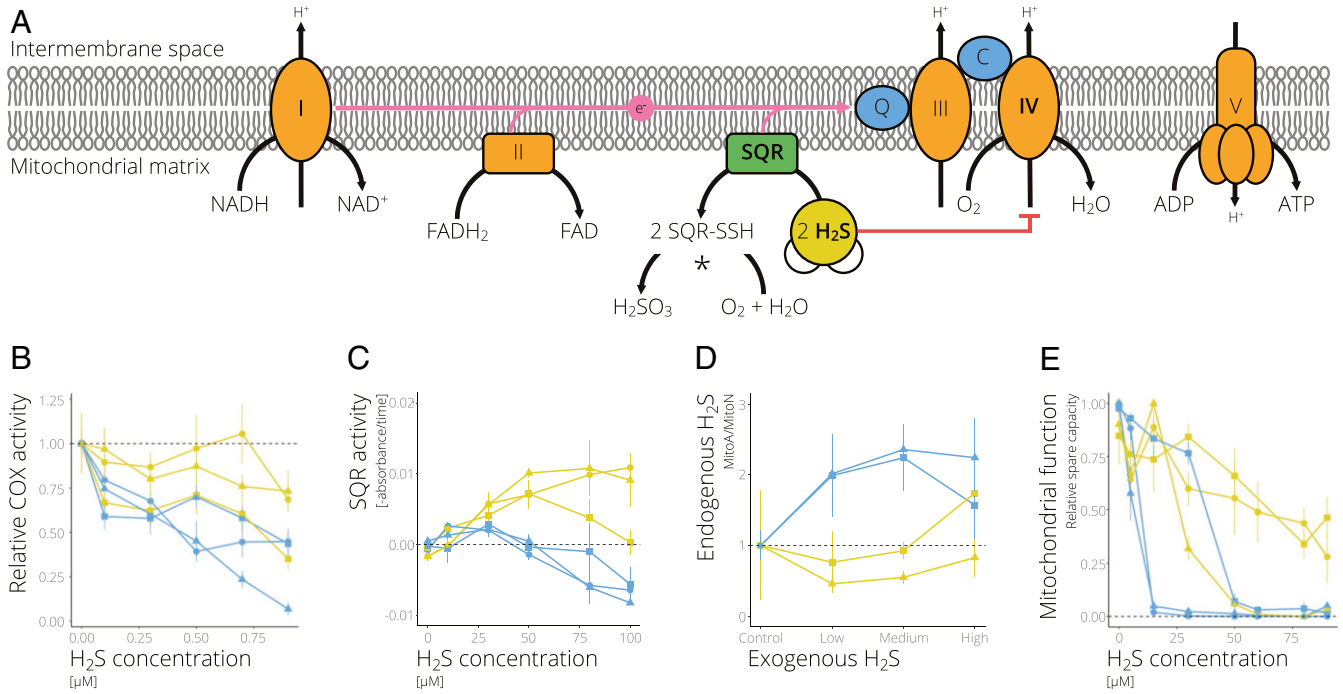

Fig. 1. (*A*) Physiological pathways associated with H_2S toxicity and detoxification are located in the inner mitochondrial membrane. H_2S inhibits OxPhos (orange enzymes, encoded by genes in the mitochondrial and nuclear genomes) by binding to cytochrome *c* oxidase (COX) (Complex IV). H_2S can be detoxified through sulfide:quinone oxidoreductase (SQR) (green enzyme, encoded by a gene in the nuclear genome) and additional enzymes (indicated by the asterisk). (*B*) Relative activity of COX upon H_2S exposure, which was primarily explained by an interaction between habitat type of origin and ambient H_2S concentration (*SI Appendix*, Tables S2 and S3). (*C*) Activity of SQR as a function of H_2S concentration, which was explained by an interaction between habitat type of origin and H_2S concentration (*SI Appendix*, Tables S4 and S5). (*D*) Relative change in mitochondrial H_2S concentrations in the liver of live fish exposed to different levels of environmental H_2S. Variation in mitochondrial H_2S levels were explained by habitat type of origin and exogenous H_2S concentration (*SI Appendix*, Tables S6 and S7). (*E*) Relative spare respiratory capacity of isolated liver mitochondria at different levels of H_2S. The interaction between habitat type of origin and drainage of origin best explained variation in spare respiratory capacity (*SI Appendix*, Tables S11 and S12). For all graphs, yellow colors denote *P. mexicana* from H_2S-rich habitats, and blue denotes *P. mexicana* from nonsulfidic habitats. Symbols stand for populations from different river drainages (■: Tac; ▲: Puy; •: Pich; see *SI Appendix*, Fig. S1).

Overall, our quantitative analyses indicate clear patterns of convergence in functional physiological traits associated with H_2S tolerance. Nonetheless, further inspection of the results also reveals lineage-specific patterns (especially in H_2S-dependent COX activity and mitochondrial respiration), indicating that evolutionary responses across lineages are similar but not necessarily identical. These idiosyncrasies are consistent with the results of previous comparative transcriptome analyses, which revealed a large number of genes that are under selection or differentially expressed in just a subset of lineages in addition to genes that are consistently differentially expressed and under selection across all lineages (17–19). Based on their functions, the OxPhos and SQR pathways undoubtedly include some major-effect genes influencing H_2S tolerance in different populations of *P. mexicana* but tolerance—as an emergent physiological trait—is a complex trait impacted by other genes as well. In the future, quantitative genetic analyses will be required to understand how other loci contribute to tolerance within each lineage and how population-specific patterns of genetic differentiation might shape variation in functional physiology evident in our data.

Convergence among *P. Mexicana* Populations Is Shaped by Selection on De Novo Mutations and Standing Genetic Variation. The convergent evolution of H_2S tolerance in *P. mexicana* begs questions about the origin of adaptive alleles (20). On microevolutionary scales, convergence may be a consequence of the repeated assembly of related alleles into different genomic backgrounds either through selection on standing genetic variation or introgression (21, 22). However, the epitome of convergent evolution is, arguably, the independent origin of adaptive mutations at the same locus that lead to consistent functional outcomes (23). To identify convergence at a genomic level, we resequenced whole genomes of multiple *P. mexicana* individuals from sulfidic and nonsulfidic habitats. Analyzing phylogenetic relationships among *P. mexicana* populations (with *Poecilia reticulata* as an outgroup) using 13,390,303 single nucleotide polymorphisms (SNPs) distributed across the genome confirmed three independent colonization events of sulfide springs and distinct evolutionary trajectories for sulfide spring populations in different drainages (Fig. 2*A*), as inferred by previous studies (24). If adaptive alleles arose separately through de novo mutation in each sulfide spring population, we would expect that putative adaptive alleles mirror these relationships as previously documented for H_2S-resistance alleles in mitochondrial *COX* subunits (15). However, patterns of divergence (*SI Appendix*, Fig. S6) and local ancestry were highly variable across the genome. Classifying local patterns of genetic similarity using a hidden Markov model and a self-organizing map allowed us to identify genomic regions in which ancestry patterns deviate from the genome-wide consensus, including multiple regions with a strong signal of clustering by ecotype (sulfidic vs. nonsulfidic populations). Such clustering by ecotype occurred in less than 1% of the genome (*SI Appendix*, Fig. S7) but included genomic regions encoding key genes associated with H_2S detoxification (e.g., SQR and ETHE1, Fig. 2*B* and Dataset S2). Clustering by ecotype indicates a monophyletic origin of putatively adaptive alleles at these loci that are shared across independent lineages of sulfide spring *P. mexicana* as a consequence of selection on standing genetic variation or introgression (25), although the latter scenario is less likely

considering the geographic barriers and strong survival selection against migrants from sulfidic to nonsulfidic habitats (26). Consequently, multiple mechanisms—not just selection on de novo mutations (19)—played a role in the convergent evolution of H_2S tolerance in *P. mexicana*.

Convergent Modifications of Toxicity Targets and Detoxification Pathways Are Evident on Macroevolutionary Scales. While selection on standing genetic variation and introgression can contribute to convergent evolution on microevolutionary scales, adaptive alleles are unlikely to be shared among lineages on macroevolutionary scales due to high phylogenetic and geographic distances separating gene pools (27). The absence of convergence in molecular mechanisms at broader phylogenetic scales might indicate the importance of contingency in evolution as asserted by Gould (3). In contrast, the presence of convergence would indicate that fundamental constraints limit the number of solutions for a functional problem (28).

We used phylogenetic comparative analyses of gene expression and analyses of molecular evolution to detect patterns of molecular convergence in 10 lineages of sulfide spring poeciliids and ancestors in nonsulfidic habitats (*SI Appendix*, Fig. S1). This included members of five genera that span over 40 million years of divergence and occur in different biogeographic contexts (*SI Appendix*, Fig. S1). We found evidence for convergence in both gene expression and sequence evolution. Variation in overall gene expression was strongly influenced by phylogenetic relationships (Fig. 3*A*). However, 186 genes exhibited significant evidence for convergent expression shifts in sulfide spring fishes (Fig. 3*B* and Dataset S3), segregating lineages based on habitat type of origin, irrespective of phylogenetic relationships (Fig. 3*C*). The only outlier was *Limia sulphurophila*, which clustered with nonsulfidic lineages despite significant expression differences with its sister, *Limia perugiae*. Functional annotation indicated that genes with convergent expression shifts were enriched for biological processes associated with H_2S detoxification (SQR pathway, Fig. 3*D*), the processing of sulfur compounds, and H_2S toxicity targets in OxPhos (*SI Appendix*, Fig. S8 and Table S14).

We also identified 11 genes with elevated nonsynonymous to synonymous substitution rates across the phylogeny, including three mitochondrial genes that encode subunits of H_2S's toxicity target (*COX1* and *COX3*) and OxPhos complex III (*CYTB*; Dataset S4). Most amino acid substitutions in *COX1* and *COX3* occurred in a lineage-specific fashion, but convergent substitutions across clades occurred at six codons in *COX1* and two codons in *COX3* (Fig. 4). These findings suggest that modifications of H_2S toxicity targets and detoxification pathways are not only critical in the evolution of H_2S tolerance in *P. mexicana*, but also they have evolved in convergence in other lineages that were exposed to the same source of selection.

Conclusions

We capitalized on past evolutionary genetics studies that compared *P. mexicana* populations from sulfidic and nonsulfidic environments (10) to test hypotheses about functional ramifications of genetic differences and their impact on organismal performance. As predicted, we found that the repeated evolution of H_2S tolerance in independent *P. mexicana* populations is mediated both by modifications of a direct toxicity target (causing increased resistance to H_2S) and a pathway involved in detoxification (causing an increased ability to regulate mitochondrial H_2S). Similar modifications to COX and SQR have been hypothesized to mediate H_2S adaptation in other groups of organisms (29–31), but the evolutionary context and the consequences for mitochondrial function in these cases remain unknown. Overall, our analyses indicated that closely related populations can exhibit substantial differences in what we assume to be highly conserved physiological pathways associated with the function of mitochondria. Modification of mitochondrial processes, consequently, can be critical in mediating adaptation to different environmental conditions on microevolutionary scales, underscoring the long overlooked role of mitochondria in adaptive evolution (6).

Our comparative transcriptome analyses across a broader sampling of sulfide spring fishes further indicated that colonization of novel niches with extreme environmental conditions can arise through the convergent modification of conserved physiological pathways. The convergent evolution of high H_2S tolerance across species is the result of repeated and predicted modifications of the same physiological pathways, genes, and—in some instances—codons associated with mitochondrial function.

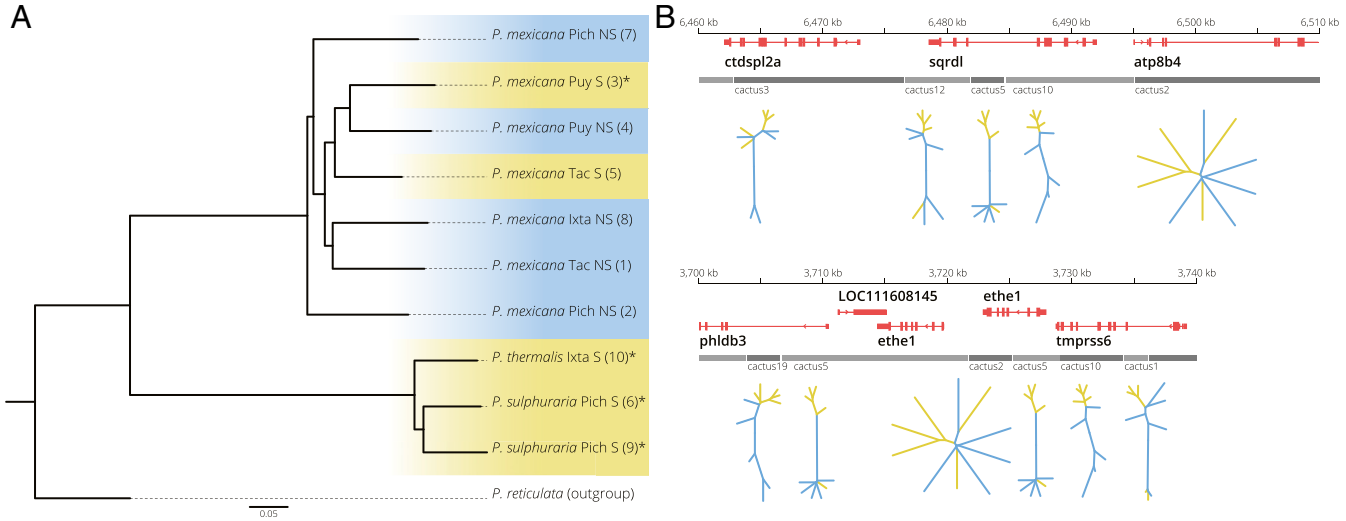

Fig. 2. (*A*) Phylogeny of different populations in the *P. mexicana* species complex (with *P. reticulata* as an outgroup) based on genome-wide SNPs. Colors indicate sulfidic (yellow) and nonsulfidic (blue) lineages. (*B*) Local ancestry patterns around genes encoding two enzymes involved in H_2S detoxification, *SQR* and *ETHE1*. Gray bars represent the local ancestry pattern (cactus) associated with each region. Unrooted trees represent local ancestry relationships with sulfidic lineages colored in yellow and nonsulfidic lineages colored in blue. Cacti 10 and 19 show clear clustering by ecotype. In cacti 1, 5, and 12, four of five sulfidic individuals cluster together.

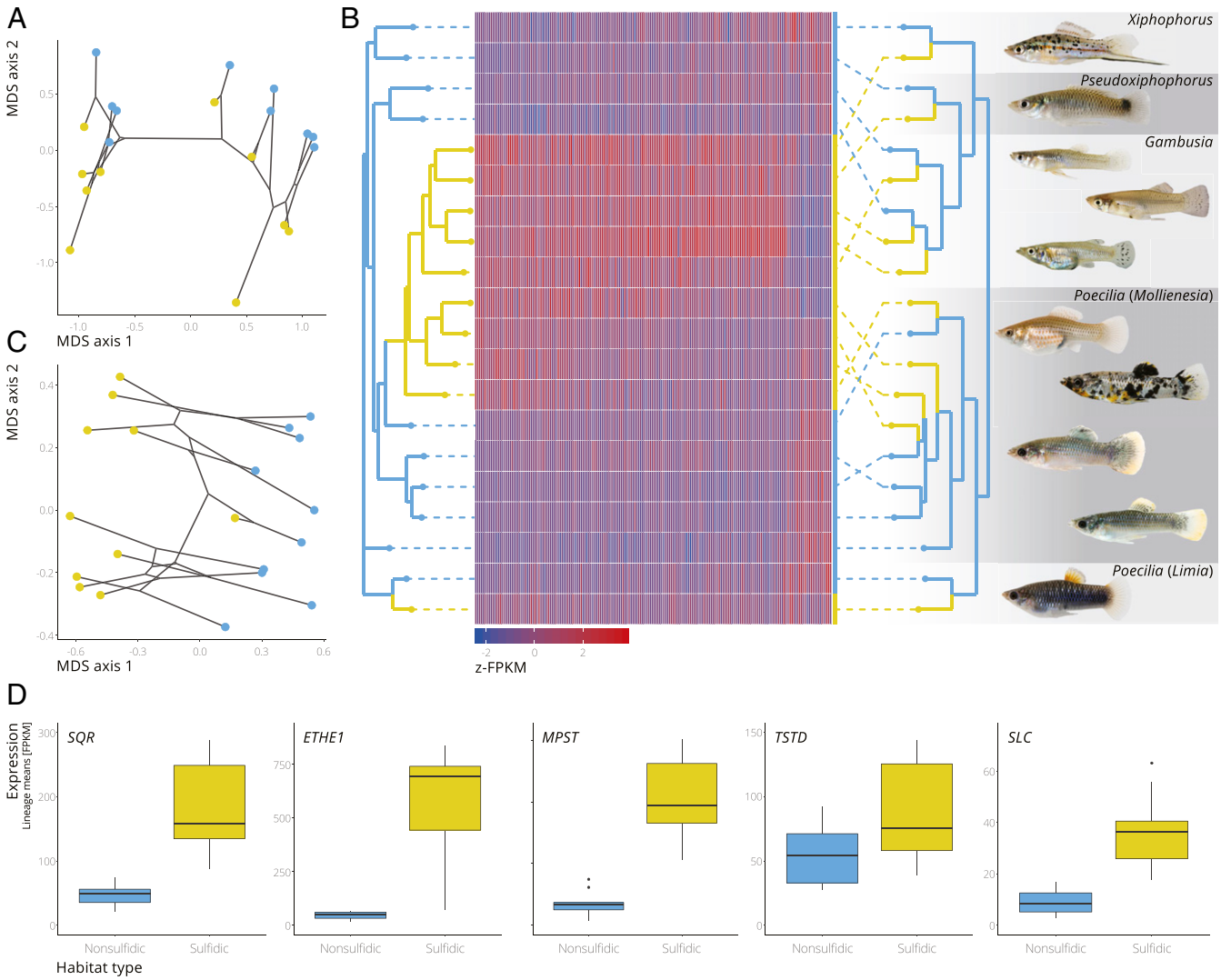

Fig. 3. (*A*) Multidimensional scaling (MDS) plot of overall gene expression patterns across 20 lineages of poeciliid fishes. Black lines represent phylogenetic relationships among lineages; color represents habitat type of origin (yellow: sulfidic; blue: nonsulfidic). (*B*) Expression variation of 186 genes with evidence for convergent expression shifts (*z*-transformed fragments per kilobase of transcript per million mapped reads). Colors represent expression levels as indicated by the scale. The neighbor-joining tree on the left groups lineages based on expression similarity. The cladogram on the right shows the phylogenetic relationship among lineages. Pictures on the side are examples of sulfide spring fishes (from top to bottom): *Xiphophorus hellerii*, *Pseudoxiphophorus bimaculatus*, *Gambusia holbrooki*, *G. sexradiata*, *G. eurystoma*, *Poecilia latipinna*, *P. sulphuraria* (Pich), *P. mexicana* (Tac), *P. mexicana* (Puy), and *Limia sulphurophila*. (*C*) MDS plot of the expression of 186 genes with evidence for convergent expression shifts. (*D*) Boxplots with mean expression levels of different components of the SQR pathway across lineages from sulfidic (yellow) and nonsulfidic (blue) habitats.

This convergence at multiple levels of biological organization is likely a consequence of constraint because the explicit biochemical and physiological consequences of H_2S limit the ways organisms can cope with its toxicity (32, 33). Due to these constraints, molecular convergence is not only evident on microevolutionary scales where selection can repeatedly assemble related alleles into different genomic backgrounds, but also on macroevolutionary scales including lineages separated by over 40 million years of evolution.

That said, there is an inordinate amount of genetic and gene expression variation that seemingly varies idiosyncratically across different lineages. In comparative analyses of highly quantitative traits (such as H_2S tolerance), there is an inherent bias to emphasize the importance of shared modifications in adaptation, while we tend to dismiss lineage-specific patterns as noise. But how lineage-specific genetic and gene expression variation interacts with molecular mechanisms that have evolved in convergence remains largely unknown for most study systems. So, if we replayed the tape of life, the same characters may make an appearance in the same setting, but the overall plots may still unfold in very different ways when many characters are part of the story.

Methods

The following sections provide a synopsis of the procedures used in this study. Detailed materials and methods are provided in the *SI Appendix*.

Sampling. Samples of *P. mexicana* for comparative biochemical and physiological analyses were collected from three population pairs from the Tac, Puy, and Pich drainages in Mexico, each including evolutionarily independent H_2S-tolerant and ancestral, intolerant populations (*SI Appendix*, Table S1) (34). With the exception of measurements of mitochondrial H_2S levels, which were conducted with common-garden-reared individuals, all assays were conducted with specimens collected in the field. For macroevolutionary analyses of convergence, we collected specimens from multiple

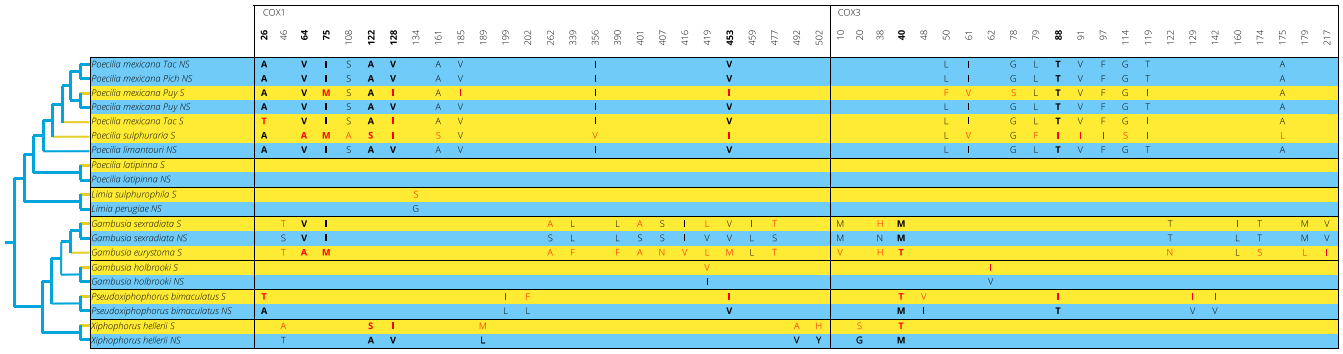

Fig. 4. Amino acid differences in *COX1* and *COX3* between lineages from sulfidic (yellow) and nonsulfidic (blue) habitats. Derived amino acids are shown in red. Bold letters indicate codons with convergent amino acid substitutions in different clades (separated by black horizontal lines) of sulfide spring fishes. Numbers above represent the position in the amino acid sequence.

species that have independently colonized H_2S-rich springs in the United States, Mexico, and the island of Hispaniola, as well as from geographically and phylogenetically proximate lineages in nonsulfidic habitats. Our sampling included populations of *P. latipinna* and *G. holbrooki* in Florida; populations of the *P. mexicana* species complex (including *P. mexicana*, *P. sulphuraria*, and *Poecilia limantouri*), the *G. sexradiata* species complex (including *G. sexradiata* and *G. eurystoma*), *P. bimaculatus,* and *X. hellerii* in Mexico; as well as populations of the *Limia perugiae* species complex (*L. perugiae* and *L. sulphurophila*) in the Dominican Republic (*SI Appendix*, Table S1).

Measurements of Enzyme Activity. Measurements of COX and SQR activities were conducted using isolated mitochondria from three *P. mexicana* population pairs and followed previously established methods. COX activity was quantified by measuring a decrease in absorbance at 550 nm resulting from the protein's oxidation of reduced cytochrome *c* (35, 36). Samples were assayed along a gradient of H_2S concentrations between 0 and 0.9 μM. SQR activity was quantified by measuring the reduction of decyl-ubiquinone (37) along a gradient of H_2S concentrations between 0 and 100 μM. Variation in enzyme activity was analyzed using linear mixed-effects models to estimate the effects of habitat type of origin (sulfidic vs. nonsulfidic), drainage, and H_2S concentration. Alternative models were assessed by using Akaike information criteria with finite sample correction (AIC_C) (38).

Measurements of Mitochondrial H_2S. H_2S exposure experiments and measurement of mitochondrial H_2S were conducted using common-garden-raised F1 individuals from two population pairs (Tac and Puy). We used a mitochondria-specific H_2S probe (MitoA) that can be injected into living organisms, where it accumulates within mitochondria due to its unique chemical structure (16). Inside mitochondria, MitoA reacts with H_2S and is converted to MitoN. The ratio of MitoN/MitoA can be quantified using liquid chromatography with tandem mass spectrometry and serve as a metric of mitochondrial H_2S. We used a MitoA protocol specifically validated for *P. mexicana* (39). Fish injected with MitoA were exposed to different environmental H_2S concentrations for 5 h. Gill, liver, brain, and muscle tissues were sampled from fish and used for the quantification of MitoN/MitoA ratios. Standardized MitoN/MitoA ratios were analyzed using linear mixed-effects models with habitat type of origin, drainage, and ambient H_2S concentration as predictor variables. Alternative models were assessed by using AIC_C.

Measurements of Mitochondrial Function. Mitochondrial function was measured in mitochondria isolated from livers from three *P. mexicana* population pairs. Mitochondrial function was assayed using a Seahorse XFe96 Extracellular Flux Analyzer (Agilent Technologies, Santa Clara, CA), which allows for the quantification of oxygen consumption rates (OCR) of isolated mitochondria in 96-well plates (40). Measuring mitochondrial OCR in the presence of different substrates and inhibitors allows for the quantification of a variety of mitochondrial functions (41), and we measured basal respiration, maximal respiration, and spare respiratory capacity as indicators of mitochondrial function across a range of H_2S concentrations between 0 and 90 μM. To compare responses in mitochondrial function to H_2S between sulfidic and nonsulfidic populations, we employed a drug response analysis where separate *n*-parameter logistic regressions were fit for each mitochondrial isolate with the metrics of mitochondrial function (basal respiration, maximal respiration, and spare capacity) as dependent variables and H_2S concentration as an independent variable (42). For each model, we quantified the area under the curve (AUC) based on Simpson's rule, and higher AUC values represent an increased ability to maintain mitochondrial function in the presence of H_2S. AUC values inferred for different mitochondrial isolates were then used as a dependent variable in linear models with habitat type of origin and drainage as predictor variables. Alternative models were assessed by using AIC_C.

Comparative Genomics and Local Ancestry Analysis. To test hypotheses about the origin of putatively adaptive alleles, we reanalyzed data from Brown et al. (25), which included whole-genome sequences from all sites known to harbor H_2S-tolerant populations of the *P. mexicana* species complex ($n = 5$) as well as adjacent nonsulfidic habitats ($n = 5$; *SI Appendix*, Table S1). Raw reads were mapped to the *Xiphophorus maculatus* reference genome (43), and we called SNPs using the Genome Analysis Toolkit (44). Local ancestry patterns were identified with Saguaro (45), which uses a combination of a hidden Markov model and a self-organizing map to build cacti (matrices of pairwise genetic distance between samples) that describe phylogenetic relationships among samples in specific genomic regions. Saguaro was executed for 29 iterations, which resulted in 30 cacti describing local ancestry patterns for segments of the genome.

Comparative Transcriptomics, Phylogenetic Comparative Analyses, and Molecular Evolution. For comparative transcriptomic analyses, we isolated RNA from gills for 5 to 6 individuals each from 10 sulfidic and 10 nonsulfidic lineages distributed across multiple genera in the family Poeciliidae. Raw reads from transcriptome sequencing were mapped to the *P. mexicana* reference genome (46), and the number of RNA-sequencing reads mapped to each gene was determined for each individual using cufflinks (47). To analyze patterns of gene expression across lineages, we used a phylogenetic comparative approach for analyzing gene expression variation to explicitly account for the effects of evolutionary relationships. Specifically, we used individual-level expression data of the top 5,000 expressed genes as dependent variables in expression variance and evolution (EVE) models that identify genes exhibiting convergent shifts in expression upon the colonization of sulfidic habitats based on a phylogeny of focal taxa (48, 49). To identify genes with potential structural or functional changes in lineages from sulfidic habitats, we analyzed patterns of molecular evolution in protein coding genes included in the analysis of gene expression. For each gene, we generated a consensus sequence for all exons across individuals from the same populations and then used branch models implemented in the program codeml from the PAML package to test for evidence of positive selection (50).

Data Availability. Data and code associated with biochemical and physiological analyses are available on GitHub (github.com/michitobler/convergent_h2s_evolution). All sequence data are available at the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov under BioProject) (accession nos. PRJNA473350 and PRJNA608180).

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