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6-25-2020

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### Recommended Citation

Greenway, Ryan; Barts, Nick; Henpita, Chathurika; Brown, Anthony P; Arias Rodriguez, Lenin; Rodríguez Peña, Carlos M; Arndt, Sabine; Lau, Gigi Y; Murphy, Michael P; Wu, Lei; Lin, Dingbo; Tobler, Michael; Kelley, Joanna L; and Shaw, Jennifer H., "Convergent evolution of conserved mitochondrial pathways underlies repeated adaptation to extreme environments" (2020). *PCOM Scholarly Papers*. 2066.  
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# Convergent evolution of conserved mitochondrial pathways underlies repeated adaptation to extreme environments

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Edited by David M. Hillis, The University of Texas at Austin, Austin, TX, and approved May 21, 2020 (received for review March 9, 2020)

**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<sub>2</sub>S)—a toxicant that impairs mitochondrial function—across evolutionarily independent lineages of a fish (*Poecilia mexicana*, Poeciliidae) from H<sub>2</sub>S-rich springs. Using comparative biochemical and physiological analyses, we found that mitochondrial function is maintained in the presence of H<sub>2</sub>S 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<sub>2</sub>S 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<sub>2</sub>S 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<sub>2</sub>S 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<sub>2</sub>S 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<sub>2</sub>S, we show that tolerance is mediated by the modification of pathways that are inhibited by H<sub>2</sub>S 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.

Author contributions: R.G., N.B., C.H., M.T., J.L.K., and J.H.S. designed research; R.G., N.B., C.H., L.A.R., C.M.R.P., S.A., G.Y.L., M.P.M., L.W., D.L., M.T., J.L.K., and J.H.S. performed research; L.A.R., C.M.R.P., S.A., G.Y.L., M.P.M., L.W., D.L., M.T., J.L.K., and J.H.S. contributed new reagents/analytic tools; R.G., N.B., C.H., A.P.B., M.T., J.L.K., and J.H.S. analyzed data; and R.G., N.B., M.T., and J.L.K. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

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Data deposition: Data and code associated with biochemical and physiological analyses are available on GitHub ([https://github.com/michtobler/convergent\\_h2s\\_evolution](https://github.com/michtobler/convergent_h2s_evolution)). All sequence data are available at National Center for Biotechnology Information (NCBI) BioProject (accession nos. [PRJNA473350](https://ncbi.nlm.nih.gov/submit/) and [PRJNA608180](https://ncbi.nlm.nih.gov/submit/)).

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This article contains supporting information online at <https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2004223117/-DCSupplemental>.

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<sub>2</sub>S, 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<sub>2</sub>S-rich springs throughout the Americas and independently evolved tolerance to sustained H<sub>2</sub>S 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<sub>2</sub>S tolerance) and ask if the underlying mechanisms have evolved in convergence in disparate lineages of livebearing fishes.

H<sub>2</sub>S toxicity and detoxification are associated with highly conserved physiological pathways in mitochondria (Fig. 1A) (11, 12), providing a priori predictions about the potential molecular mechanisms underlying adaptation to this strong source of selection. Toxic effects of H<sub>2</sub>S 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<sub>2</sub>S 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<sub>2</sub>S may, therefore, be mediated by resistance (modification of toxicity targets that reduce the negative impact of H<sub>2</sub>S), regulation (modification of physiological pathways that maintain H<sub>2</sub>S 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<sub>2</sub>S. 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<sub>2</sub>S 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.

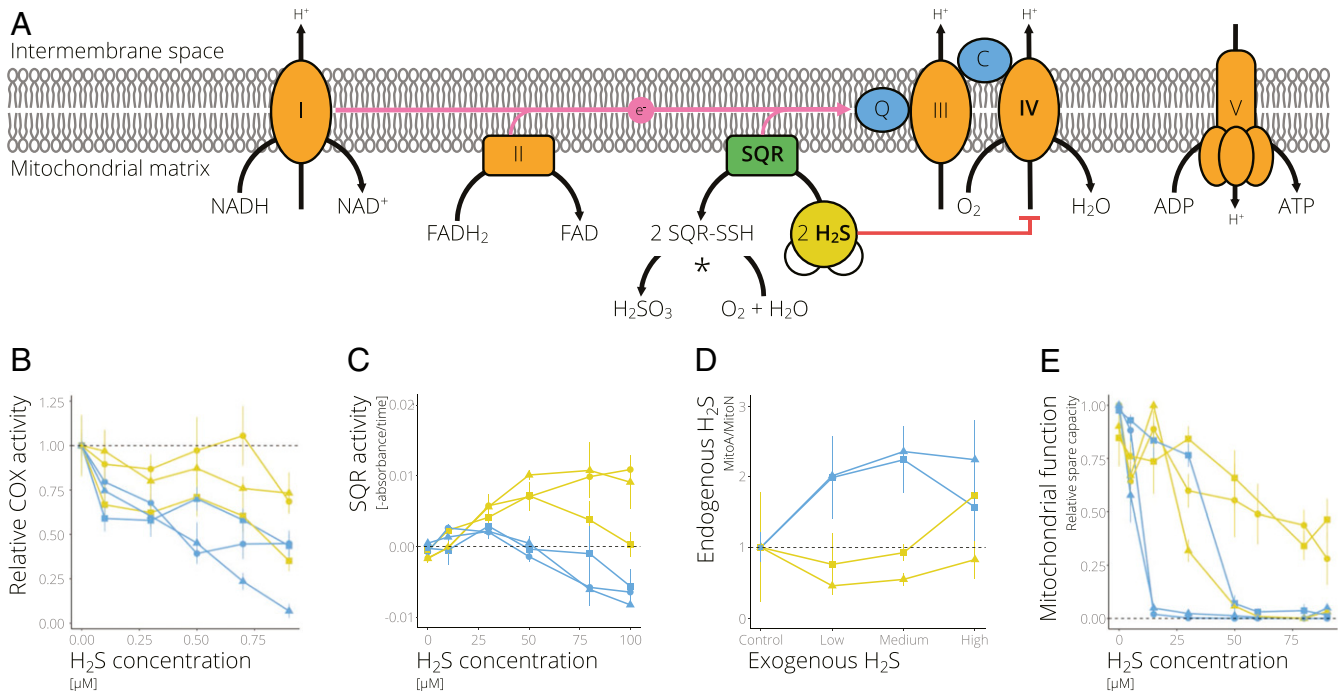
## Results and Discussion

**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<sub>2</sub>S in fish from sulfidic populations but not those from nonsulfidic populations. Quantification of COX function indicated that enzyme activity generally declined with increasing H<sub>2</sub>S concentrations, but this decline was reduced in populations from sulfidic habitats

(Fig. 1B; habitat × H<sub>2</sub>S:  $P < 0.001$ , *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<sub>2</sub>S-tolerant population [Tacotalpa (Tac)] declined just as in nonsulfidic populations (Fig. 1B). The other two *P. mexicana* populations from sulfidic habitats [Puyacatengo (Puy) and Pichucalco (Pich)] maintained significant COX activity even at the highest H<sub>2</sub>S concentrations, which should reduce the negative impact of H<sub>2</sub>S on cellular respiration. These results are consistent with previous analyses (15) and indicate that resistance may contribute to H<sub>2</sub>S tolerance in some populations but cannot explain the repeated evolution of H<sub>2</sub>S tolerance by itself.

**Sulfide Spring *P. Mexicana* Can Regulate Mitochondrial H<sub>2</sub>S through Increased Detoxification.** We also tested whether tolerant and intolerant populations differ in their ability to detoxify H<sub>2</sub>S by conducting enzyme activity assays of SQR. Activity of SQR was significantly higher in mitochondria from sulfidic populations at intermediate and high H<sub>2</sub>S concentrations (Fig. 1C; habitat × H<sub>2</sub>S:  $P < 0.001$  in *SI Appendix, Table S5*), likely helping fish from sulfidic habitats to maintain H<sub>2</sub>S homeostasis during environmental exposure. To test this prediction in vivo, we used a novel mitochondria-specific H<sub>2</sub>S-probe (MitoA) that allows for the monitoring of relative H<sub>2</sub>S levels inside the mitochondria of living organisms (16). We measured mitochondrial H<sub>2</sub>S concentrations in this manner using laboratory-reared fish that were exposed to varying levels of environmental H<sub>2</sub>S. 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<sub>2</sub>S 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<sub>2</sub>S concentrations in mitochondria isolated from livers (Fig. 1D) and other organs (*SI Appendix, Fig. S2*) of fish from nonsulfidic habitats increased above control levels at all exposure concentrations. In contrast, mitochondrial H<sub>2</sub>S 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<sub>2</sub>S at higher rates and, thus, regulate mitochondrial H<sub>2</sub>S upon environmental exposure.

**Sulfide Spring *P. Mexicana* Can Maintain Mitochondrial Function in the Presence of H<sub>2</sub>S.** Modification of the OxPhos and SQR pathways in *P. mexicana* suggests that mitochondrial adaptations are key to the evolution of H<sub>2</sub>S tolerance. Therefore, mitochondrial function of fish from sulfidic habitats should be maintained upon exposure to H<sub>2</sub>S. We tested this hypothesis by quantifying different aspects of mitochondrial function (basal respiration, maximal respiration, and spare respiratory capacity) along a gradient of H<sub>2</sub>S concentrations using an ex vivo coupling assay. As expected, all aspects of mitochondrial function generally declined with increasing H<sub>2</sub>S (Fig. 1E 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. 1E), 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<sub>2</sub>S-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<sub>2</sub>S toxicity and detoxification are located in the inner mitochondrial membrane. H<sub>2</sub>S inhibits OxPhos (orange enzymes, encoded by genes in the mitochondrial and nuclear genomes) by binding to cytochrome c oxidase (COX) (Complex IV). H<sub>2</sub>S 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<sub>2</sub>S exposure, which was primarily explained by an interaction between habitat type of origin and ambient H<sub>2</sub>S concentration (*SI Appendix, Tables S2 and S3*). (C) Activity of SQR as a function of H<sub>2</sub>S concentration, which was explained by an interaction between habitat type of origin and H<sub>2</sub>S concentration (*SI Appendix, Tables S4 and S5*). (D) Relative change in mitochondrial H<sub>2</sub>S concentrations in the liver of live fish exposed to different levels of environmental H<sub>2</sub>S. Variation in mitochondrial H<sub>2</sub>S levels were explained by habitat type of origin and exogenous H<sub>2</sub>S concentration (*SI Appendix, Tables S6 and S7*). (E) Relative spare respiratory capacity of isolated liver mitochondria at different levels of H<sub>2</sub>S. 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<sub>2</sub>S-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<sub>2</sub>S tolerance. Nonetheless, further inspection of the results also reveals lineage-specific patterns (especially in H<sub>2</sub>S-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<sub>2</sub>S 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<sub>2</sub>S 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. 24), 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<sub>2</sub>S-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<sub>2</sub>S detoxification (e.g., SQR and ETHE1, Fig. 2B 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<sub>2</sub>S 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. 3A). However, 186 genes exhibited significant evidence for convergent expression shifts in sulfide spring fishes (Fig. 3B and *Dataset S3*), segregating lineages based on habitat type of origin, irrespective of phylogenetic relationships (Fig. 3C). 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<sub>2</sub>S detoxification (SQR pathway, Fig. 3D), the processing of sulfur compounds, and H<sub>2</sub>S toxicity targets in OxPhos (*SI Appendix, Fig. S8 and Table S14*).

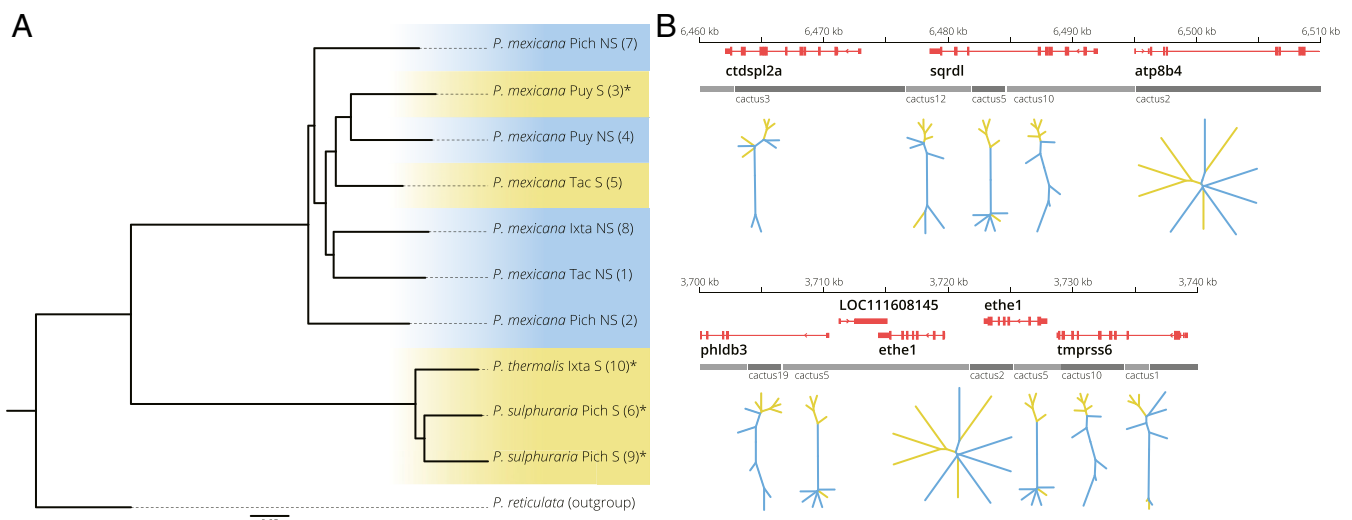
We also identified 11 genes with elevated nonsynonymous to synonymous substitution rates across the phylogeny, including

three mitochondrial genes that encode subunits of H<sub>2</sub>S'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<sub>2</sub>S toxicity targets and detoxification pathways are not only critical in the evolution of H<sub>2</sub>S tolerance in *P. mexicana*, but also they have evolved in convergence in other lineages that were exposed to the same source of selection.

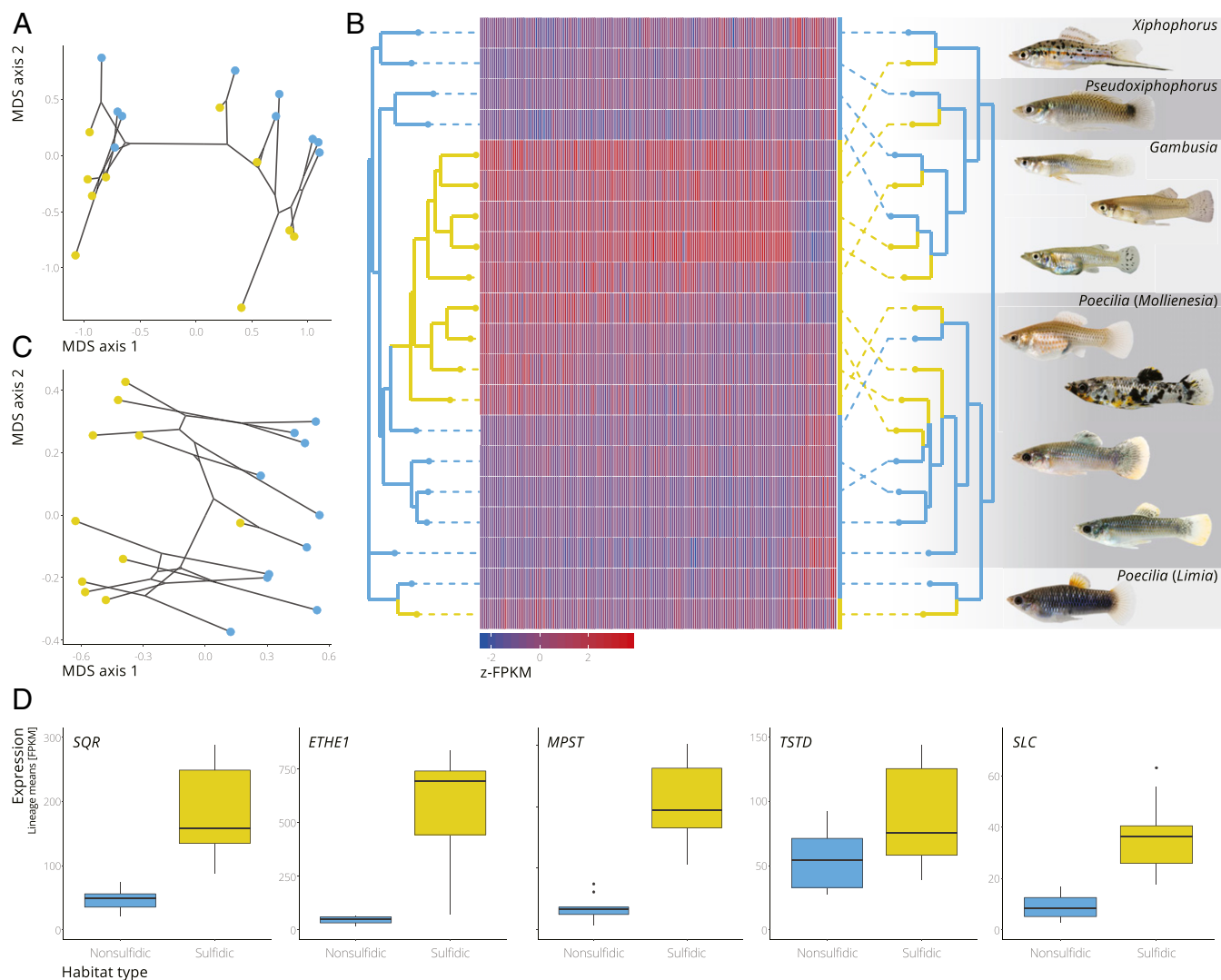
## Conclusions

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<sub>2</sub>S tolerance in independent *P. mexicana* populations is mediated both by modifications of a direct toxicity target (causing increased resistance to H<sub>2</sub>S) and a pathway involved in detoxification (causing an increased ability to regulate mitochondrial H<sub>2</sub>S). Similar modifications to COX and SQR have been hypothesized to mediate H<sub>2</sub>S 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<sub>2</sub>S 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<sub>2</sub>S 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





**ACKNOWLEDGMENTS.** We thank the Centro de Investigación e Innovación para la Enseñanza y Aprendizaje (CIEA), O. Cornejo, T. Morgan, D. Petrov, R. Rohlfs, and N. Rohnner for their help. This work was supported by grants from the NSF (IOS-1463720, IOS-1557795, IOS-1557860, and IOS-1931657 to J.H.S.,

J.L.K., and M.T.), the US Army Research Office (W911NF-15-1-0175, W911NF-16-1-0225 to J.L.K. and M.T.), the Medical Research Council UK (MC\_U105663142 to M.P.M.), and a Wellcome Trust Investigator Award (110159/Z/15/Z to M.P.M.).

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