Adaptation to hydrogen sulfide: A case study in sulfide spring fishes

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Key points

- Hydrogen sulfide (H_2S) is a potent respiratory toxicant that can occur in high concentrations in some aquatic habitats, like freshwater sulfide springs.
- Mitochondria are central to biological responses to H_2S; H_2S blocks the mitochondrial oxidative phosphorylation (OxPhos) pathway and aerobic ATP production, and it is detoxified enzymatically through the mitochondrial sulfide:-quinone oxidoreductase (SQR) pathway.
- Multiple lineages of livebearing fishes (family Poeciliidae) have colonized sulfide springs, withstanding H_2S concentrations orders of magnitude higher than what is considered lethal for most animals. Ecological physiology studies have used these fishes to understand the evolution of tolerance to an extreme environmental stressor.
- Comparative genomic and transcriptomic studies have identified a number of candidate genes mediating adaptation to H_2S, including direct toxicity targets (cytochrome *c* oxidase, COX in OxPhos) and multiple components of the SQR pathway.
- Functional physiology studies revealed that modification of COX make the toxicity target inert to the effects of H_2S, and modification of SQR allows adapted fish to detoxify H_2S more rapidly and maintain homeostasis during environmental exposures. Together these adaptations allow sulfide spring fish to maintain mitochondrial function and produce ATP aerobically even in the presence of H_2S.
- While mitochondrial adaptations are critical for sulfide spring fishes to survive in the presence of H_2S, other physiological, behavioral, and morphological adaptations also play a role. Our understanding of adaptation is further complicated by the fact that other sources of selection—like hypoxia, resource limitation, and predation—interact with H_2S to shape trait evolution in sulfide springs.

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Glossary

Anoxia The absence of dissolved oxygen in water.

ATP Adenosine triphosphate; an organic compound that provides energy for physiological processes within cells. It is primarily produced through oxidative phosphorylation (OxPhos) and glycolysis.

Chemoautotrophy The process of oxidizing inorganic chemicals for organismal energy production.

Cold seep Area of the ocean floor where water rich in methane and hydrogen sulfide seeps through the sediment.

COX Cytochrome *c* oxidase; complex IV of the respiratory transport chain that is built from multiple protein subunits. It is a transmembrane protein embedded in the inner mitochondrial membrane, where is receives electrons from cytochrome *c* and transfers them to oxygen, ultimately producing water. COX is also the primary toxicity target of H_2S.

Deep-sea hydrothermal vent Area of the ocean floor near volcanic activity or tectonic plate boundaries, where geothermally heated water discharges. Vent water is typically enriched in H_2S and other mineral compounds.

Electron transport chain A series of protein complexes in the mitochondria that transfer electrons from organic substrates to oxygen, transporting protons across the inner mitochondrial membrane in the process. The resulting electrochemical gradient across the inner mitochondrial membrane ultimately drives aerobic ATP production.

Euxinic Euxinic conditions occur when water is anoxic and contains high concentrations of H_2S. Such conditions are rare today but still occur in highly stratified water bodies, like the Black Sea.

Homeostasis A self-regulating process by which organismal systems maintain stability despite changing environmental conditions.

Hypoxia Low concentrations of dissolved oxygen in the water. For freshwaters, the hypoxia threshold is typically around 2–3 mg of dissolved oxygen per liter of water.

Live-bearing A reproductive strategy by which live young are birthed. Fertilization in live-bearing species is internal, and embryonic and larval development occurs inside a pregnant mother.

OxPhos Oxidative phosphorylation; a mitochondrial process that uses the chemical energy released by the oxidation of nutrients to produce ATP.

Poeciliidae A family of fishes commonly known as livebearers. Note that while all species in this family (with one exception) are live-bearing, many other groups of fishes have independently evolved this reproductive strategy as well.

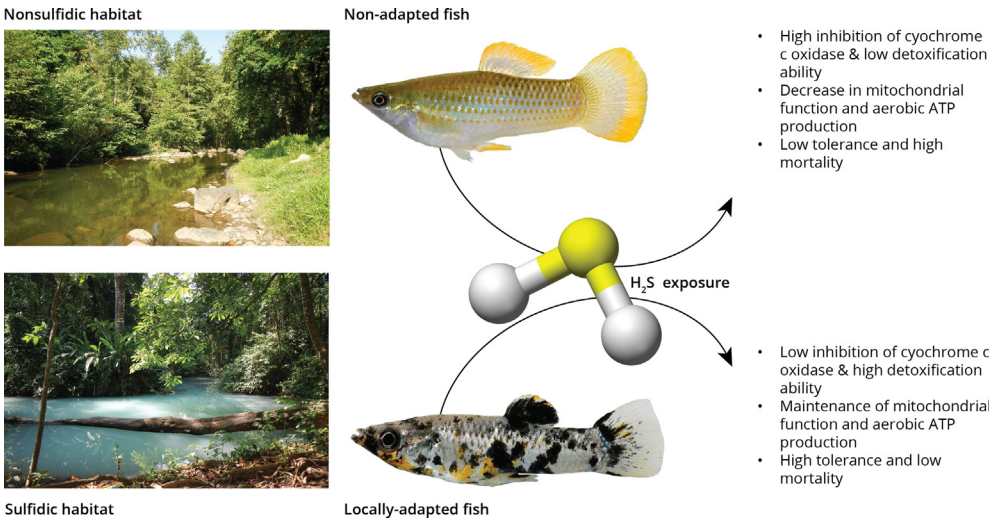
Proterozoic A geological period (eon) during the last portion of the Precambrian. It spanned the time interval from 2.5 billion to about 540 million years ago.

SQR Sulfide:quinone oxidoreductase; a protein bound to the inner mitochondrial membrane. It catalyzes the first step of the enzymatic oxidation of H_2S and feeds electrons into the quinone (coenzyme Q) pool of the electron transport chain.

Abstract

Hydrogen sulfide (H_2S) is a naturally occurring toxicant in some aquatic habitats. H_2S readily invades the cells of animals and inhibits the function of mitochondria, curtailing aerobic ATP production. Livebearing fishes (Poeciliidae) that inhabit freshwater springs rich in H_2S have been models to investigate how organisms adapt to such extreme environmental conditions. We summarize recent genomic, transcriptomic, and physiological studies that have documented how modification to the function of mitochondria—both in terms of limiting the toxic effects of H_2S and an increased ability for detoxification—contribute to the evolution of H_2S tolerance. We also discuss how H_2S interacts with other environmental stressors to shape evolution of sulfide spring fishes.

Teaching slide



Introduction

Extreme environments provide an interesting natural context to explore life's capacity to function under seemingly lethal conditions. Hydrogen sulfide (H_2S) is a gas that creates extreme environmental conditions for aquatic organisms due to its toxic properties that inhibit the function of mitochondria. Still, animals from a diverse array of phyla inhabit environments with H_2S concentrations that are orders of magnitude higher than what is considered acutely toxic for most species. In this article, we focus on a group of small, live-bearing fishes from the family Poeciliidae that have repeatedly colonized freshwater springs rich in H_2S and provide an accessible model to study the complex adaptations to this environmental stressor. We will introduce H_2S as an environmental factor and toxicant before reviewing recent genomic and physiological studies that uncovered mechanisms underlying H_2S tolerance in these fishes. We will then briefly discuss the need to embrace environmental complexity to holistically understand evolution in, and adaptation to, extreme environments.

Background

H_2S as an environmental factor

Prior to the "great oxidation event" nearly 800 million years ago, Earth's oceans were euxinic and characterized by anoxia and high concentrations of H_2S. This environment, radically different from the Earth we know today, sustained early life, and many organisms relied on H_2S as a source of energy (Olson and Straub, 2016). As oxygen levels in the atmosphere rose as a consequence of photosynthesis, H_2S—and with it many chemoautotrophic sulfur oxidizers—was gradually eliminated from most environments. Nonetheless, H_2S continues to play an important role in cellular function and signaling of metazoans, and the mechanisms regulating H_2S metabolism are highly conserved across taxa.

In today's world, H_2S remains scarce and is mostly present in aquatic habitats in which a continuous supply of H_2S counteracts its steady oxidation (Tobler et al., 2016). In some of these environments, H_2S originates from geochemical processes. Deep in the Earth's crust, water interacts with hot rock and molten lava, causing the reduction of sulfate and other sulfur species to H_2S (Jannasch and Taylor, 1984). The hot, H_2S-rich water is then released at tectonic plate boundaries, forming deep-sea hydrothermal vents, where concentrations can reach 3−110 mmol L^{-1}. These extreme environments teem with unique and endemic forms of microbial and animal life (Van Dover, 2000). In other aquatic habitats, H_2S is of biotic origin. Under anoxic conditions, some microbes can metabolize organic compounds and use sulfate as a terminal electron receptor, producing H_2S as a metabolic waste product (Muyzer and Stams, 2008). This process can cause continuously elevated H_2S concentrations in aquatic sediments and cold seeps (Giere, 2009). Disturbance of such sediments can release H_2S, leading to temporary surges in the water column even in habitats that are typically H_2S free (Reese et al., 2008). Not surprisingly, eutrophication and pollution that cause or expand anoxic zones have also been associated with increased incidence of H_2S (Achá et al., 2018).

Under natural conditions, H_2S as an environmental stressor rarely occurs alone. Most critically, it is tightly associated with reduced oxygen concentrations. Due to H_2S's propensity to react with oxygen at ambient temperatures, hypoxic environments can approach anoxia during surges of H_2S. Depending on the ecosystem context, H_2S is also correlated with a suite of other abiotic and biotic environmental factors (Tobler et al., 2016). We will return to this ecological context later in this article.

H_2S as a toxicant

Environments rich in H_2S are characterized by low biodiversity due to the sensitivity of modern life to environmental H_2S, with toxicity becoming apparent at micromolar concentrations. Through decades of toxicological and biomedical research, the biochemical and physiological effects of H_2S in metazoans are well understood (Reiffenstein et al., 1992; Truong et al., 2006). Due to its lipid solubility, H_2S readily passes through cellular membranes to invade organisms (Mathai et al., 2009), and elevated internal concentrations result in the disruption of oxygen transport, aerobic respiration, and neurological processes. These pathological effects impact organisms across levels of organization (from organelles to organ systems), demonstrating the strong selective pressure experienced by organisms exposed to H_2S.

At the cellular level, the primary toxic effect of H_2S is inhibition of cellular respiration (**Fig. 1**). Similar to cyanide, H_2S binds to cytochrome *c* oxidase (COX, Complex IV of the mitochondrial respiratory chain) and disrupts the final step of the electron transport chain (Cooper and Brown, 2008). The inhibition of COX diminishes its ability to donate electrons to oxygen even at micromolar H_2S concentrations, resulting in the failure to complete oxidative phosphorylation (OxPhos) and hampering the ability of mitochondria to generate ATP aerobically (Cooper and Brown, 2008).

H_2S may also reduce organisms' ability to acquire oxygen from their environment. H_2S binds to the porphyrin ring of both hemoglobin and myoglobin, resulting in the formation of sulfhemoglobin and sulfmyoglobin (Pietri et al., 2011). These sulfheme proteins are characterized by a reduction in oxygen affinity, which reduces the efficiency of oxygen transport to tissues (Carrico et al., 1978). This physiological pathology may exacerbate the reduced ability of mitochondria to produce ATP aerobically.

Finally, H_2S also possesses neurotoxic effects, primarily through the disruption of calcium homeostasis, modulation of calcium channels, and interaction with various neurotransmitters (Kombian et al., 1988; Garcia-Bereguiain et al., 2008). H_2S may also interact with and modify the function of a suite of cellular proteins involved in the generation and regulation of blood vessels, inflammation, and cellular signal transduction (Reiffenstein et al., 1992; Li et al., 2011). However, it is unclear if all these biochemical and physiological effects are directly caused by H_2S or indirectly as result of loss in homeostasis in response to H_2S toxicity (Olson, 2011).

H_2S as a signaling molecule

Despite its toxicity, H_2S also plays important biological functions at very low concentrations. H_2S is produced endogenously as a byproduct of the transsulfuration pathway (Stipanuk and Ueki, 2011). Through this process, the sulfur group of methionine is transferred to the thiol group of homocysteine. The enzyme cystathionine β-synthase (CBS) then condenses homocysteine and serine to form cystathione, which is hydrolyzed by cystathionine γ-synthase (CSE) to form cysteine. Desulfuration of cysteine and the release of endogenous H_2S is catalyzed by both CBS and CSE, as well as by 3-mercaptopyruvate sulfurtransferase (3-MPST).

Originally thought to be a toxic by-product of cysteine catabolism, H_2S is now appreciated as an important cellular signaling molecule (Li et al., 2011). H_2S is a regulator of vascular tone across vertebrates, indicating that this gasotransmitter serves as an oxygen sensor during hypoxia responses (Olson, 2015). In mammals, H_2S regulates metabolic suppression during hibernation (Jensen and Fago, 2021), and it is known to alter osmoregulatory capacity of zebrafish (Perry et al., 2016). Furthermore, deviations from H_2S homeostasis are linked to a number of pathologies in mammals, including cardiovascular disease, neurodegeneration, and inflammation (Li et al., 2011; Kimura, 2014). As a consequence, H_2S-releasing drugs are in development to treat a number of pathologies (Wallace and Wang, 2015).

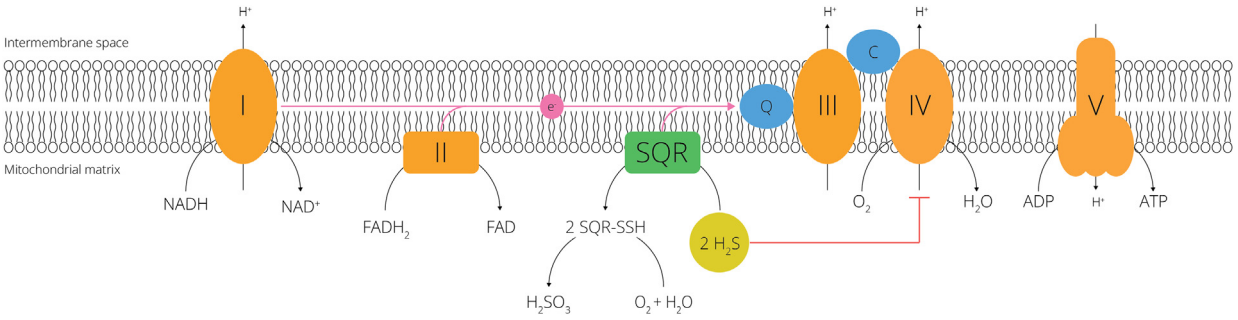


Fig. 1 The OxPhos (orange) and SQR (green) pathways in the inner mitochondrial membrane. H_2S is toxic because it inhibits complex IV (COX) of the respiratory chain. H_2S is detoxified by binding to SQR and subsequent oxidation through additional enzymatic reactions (not shown). Note that the SQR pathway is linked to OxPhos, because it feeds electrons into the quinone pool upon binding to H_2S.

H_2S regulation in eukaryotes

During the Proterozoic era, organisms relied on H_2S as an energy source, and as such much of life possesses the capability to detoxify H_2S, at least at low (i.e., nanomolar) concentrations (Olson and Straub, 2016). In eukaryotic cells, H_2S is chemically inactivated in the mitochondria via oxidation to nontoxic sulfur species, which is catalyzed first by sulfide:quinone oxidoreductase (SQR). SQR is an integral membrane protein found within the mitochondria and tightly linked to the mitochondrial respiratory chain. SQR catalyzes the oxidation of H_2S into sulfane sulfur, ultimately forming persulfide and donating two electrons into the electron transport chain via coenzyme Q (Hildebrandt and Grieshaber, 2008; Olson, 2018). From there, a suite of chemical reactions results. In the transfer of bound sulfane sulfurs to the glutathione pool, a sulfur dioxygenase (ETHE1) oxidizes the sulfane sulfurs to sulfite, which can be further oxidized to sulfate (Olson, 2018). Although all animals possess the machinery needed to detoxify H_2S (Shahak and Hauska, 2008), few species exhibit the ability to mitigate the toxic effects of this compound when exposed to environmental H_2S.

An unexpected by-product of H_2S catabolism is stimulation of ATP production, at least at low concentrations (Fu et al., 2012). As mentioned above, the oxidation of H_2S by SQR donates two electrons into the electron transport chain, providing additional fuel for oxidative phosphorylation. Additionally, H_2S inhibits the function of phosphodiesterase 2A, which increases the concentration of cyclic AMP (cAMP), additionally stimulating electron transport (Módis et al., 2013). Once endogenous concentrations of H_2S reach a critical threshold, however, these stimulatory effects become overwhelmed, and ATP production ultimately ceases because of the blocking of COX.

Ecological and evolutionary questions regarding the physiology of sulfide tolerance

As physiologists, we seek to identify the mechanisms that mediate tolerance to environmental stressors. Since H_2S regulation occurs exclusively in the mitochondria, and the molecule's primary toxicity target is embedded within the mitochondrial respiratory chain, these organelles and the physiological processes conducted within them are likely targets of selection in H_2S-rich environments. We begin by asking the questions "How have organisms adapted to chronic H_2S exposure"? and "Have different lineages of sulfide-tolerant organisms evolved in similar or different ways"? Over the past decade, researchers dedicated significant time to identify the mechanisms underlying H_2S tolerance in poeciliid fishes, and we will discuss what is currently known below.

Sulfide springs and their fishes: Models for ecological and evolutionary physiology

Distribution of sulfide springs

Freshwater sulfide springs have been documented around the globe, and some of these toxic springs have been colonized by fishes that live in adjacent non-sulfidic waters (Greenway et al., 2014). Most sulfide springs inhabited by fish have been reported from the Neotropics. A high density of springs occurs in the southern Mexican states of Tabasco and Chiapas. In this region, several tributaries of the Rio Grijalva (the Rios Tacotalpa, Puyacatengo, Ixtapangajoya, and Pichucalco; **Fig. 2**) flow in parallel from the Sierra Madre de Chiapas into the floodplains adjacent to the Gulf of Mexico. In the foothills of the Sierra Madre, all of these rivers exhibit sulfide springs, providing independent replicates of adjacent sulfidic and nonsulfidic habitats (Palacios et al., 2013). In at least one tributary (Rio Tacotalpa), sulfide springs are also located within a cave, the Cueva del Azure (Parzefall, 2001). Surface springs with high H_2S concentrations have also been documented in the states of Florida and Oklahoma in the United States, in the Dominican Republic, Costa Rica, and other Latin American countries (Greenway et al., 2014). All sulfide springs containing fish are small (typically 0.3−2 ha in surface area), and they drain into adjacent streams and rivers, such that there are no physical barriers that would prevent the movement of fish.

Environmental conditions in sulfide springs

Despite the close proximity, the environmental conditions within sulfide springs and their spring runs are starkly different from adjacent nonsulfidic habitats. Due to the continuous water discharge, H_2S concentrations within the springs are sustained and high. Close to the spring heads, H_2S concentrations can reach over 1500 $\mu mol\ L^{-1}$ (Greenway et al., 2014), which is orders of magnitude higher than what is considered acutely toxic for most aquatic organisms. Although H_2S concentrations are relatively stable throughout the day and across seasons, there is considerable spatial variation within springs (Culumber et al., 2016). H_2S concentrations tend to decrease with increasing distance from spring heads because of volatilization and reaction with oxygen. H_2S-oxidation leads to non-toxic sulfur species in the water, including sulfate and colloidal sulfurs that create a milky turbidity with light blue coloration (**Fig. 2**).

Besides the presence of acutely toxic concentrations of H_2S, sulfide springs are also characterized by hypoxia, which is often exacerbated during the warm hours of the day (Lukas et al., 2021). In addition, sulfide springs tend to be warmer, have a lower pH, and a higher salt content than adjacent nonsulfidic habitats (Greenway et al., 2014). The stark differences in abiotic environmental conditions are also reflected in the composition of biological communities. Sulfide springs exhibit diverse environmental microbiomes dominated by extremophile microbes, including a diverse set of sulfur oxidizers and reducers similar to those found in deep-sea hydrothermal vents (Hotaling et al., 2019). These microbial communities form thick mats in sulfide springs, and at least part of the primary production in these ecosystems is derived from chemoautotrophy (Roach et al., 2011). In contrast, animal

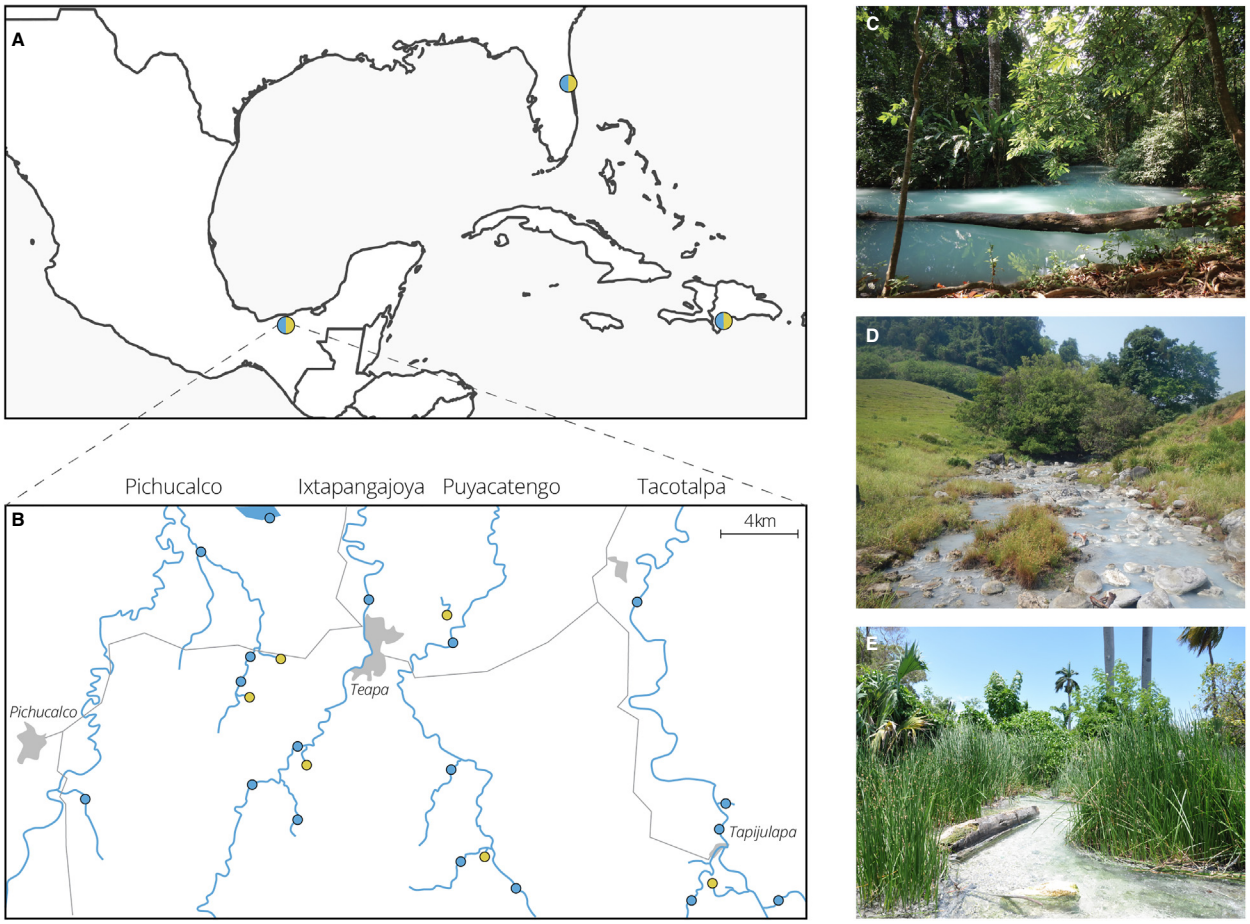


Fig. 2 Geographic overview of the most commonly studied sulfide springs in Mexico, the USA, and the Dominican Republic (A) as well as a magnification of the region in Mexico (B). Sulfide springs are indicated by yellow dots, adjacent nonsulfidic habitats with blue dots. The photographs on the right side are examples of sulfide springs, including El Azufre in Mexico's Tacotalpa drainage (C), La Lluvia spring in Mexico's Puyacatengo drainage (D), and La Zurza spring in the Dominican Republic (E).

communities in sulfide springs are depauperate, with only few species persisting in significant numbers due to the inhospitable conditions (Greenway et al., 2014). Common inhabitants of sulfide springs include chironomid larvae (Diptera) and giant water bugs (Belostomatidae, Heteroptera; Greenway et al., 2014). In terms of fishes, sulfide spring inhabitants are found in the families Synbranchidae (one species in Mexico), Cyprinodontidae (one species in Mexico and three species in Iran), Anablepidae (one species in Argentina), and Poeciliidae (18 species distributed across the Americas; Greenway et al., 2014; Aguilera et al., 2019). Some species are only transient occupants of sulfide springs. For example, juveniles of the air-breathing synbranchid *Ophisternon aenigmaticum* occasionally occupy sulfide springs, presumably to avoid predation and to feed. However, most sulfide spring fishes represent locally adapted populations or endemic species restricted to these unique environments. Among these extremophile specialists, members of the family Poeciliidae have been studied in most detail.

Sulfide spring poeciliids

The family Poeciliidae includes about 270 small-bodied species that are distributed from North Carolina in the United States south to Argentina, including some of the Caribbean Islands. All species have internal fertilization and give birth to live young (Greven, 2011). Some species within the family are ecological opportunists, have broad physiochemical tolerances, and inhabit a variety of marginal habitats (Meffe and Snelson, 1989). Not surprisingly, these characteristics have facilitated the colonization of novel habitats, and several species—including mosquitofish, guppies, and swordtails—are now considered global invasives (Stockwell and Henkanaththegedara, 2011).

Poeciliids have colonized sulfide springs in the United States, Mexico, Costa Rica, Panama, Venezuela, and the Caribbean Island of Hispaniola (Fig. 3). Perhaps best studied are the mollies in the *Poecilia mexicana* species complex (subgenus *Mollienesia*), which inhabit the sulfide springs in southern Mexico (Palacios et al., 2013, 2016). Sulfide springs have been colonized at least three times independently within this complex. Two colonizations in the Rio Tacotalpa and Rio Puyacatengo have occurred relatively recently

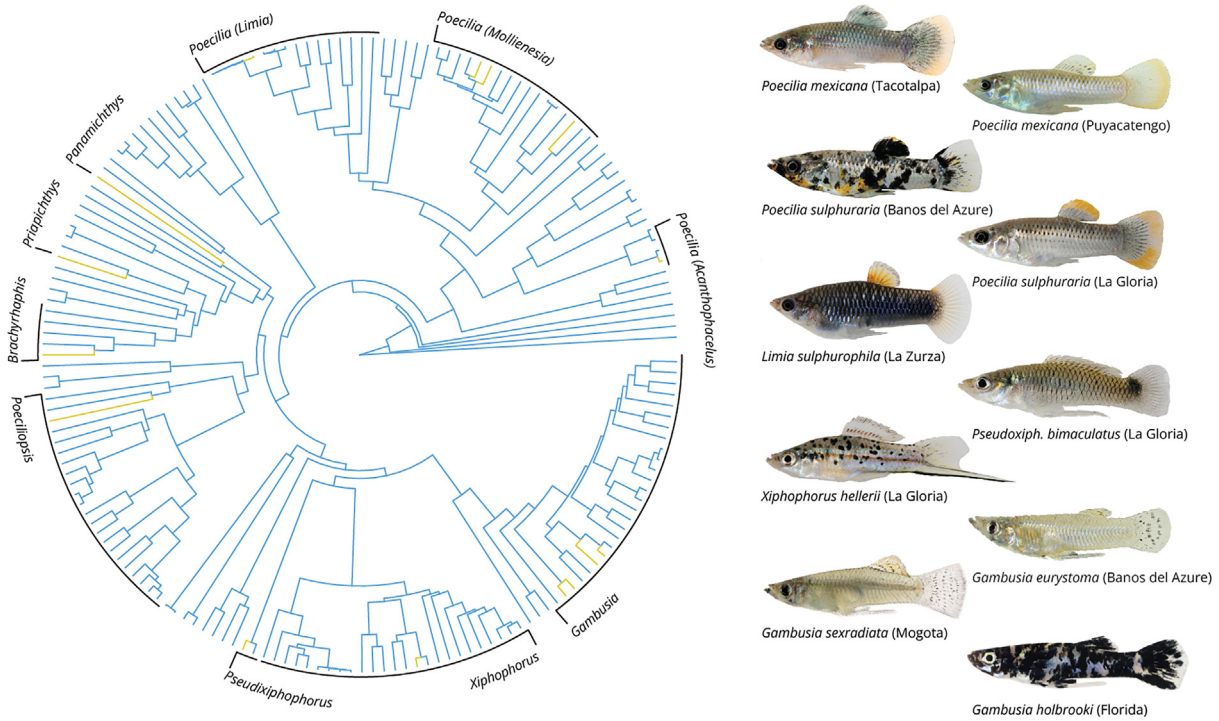


Fig. 3 Phylogeny of the family Poeciliidae with genera containing sulfide spring lineages (yellow branches) highlighted. Photographs depict select sulfide-spring lineages, including four populations from the *P. mexicana* complex in Mexico, *L. sulphurophila* from the Dominican Republic, *Pseudoxiphophorus bimaculatus*, *Xiphophorus hellerii*, *Gambusia eurystoma*, and *G. sexradiata* (all from Mexico), and *G. holbrooki* from Florida (USA).

(<10,000 years ago; Brown et al., 2018), and sulfide spring populations in these drainages are classified as divergent, locally-adapted populations of *P. mexicana*. In contrast, a single colonization event is associated with the sulfide springs in the Rios Ixtapangajoya and Pichucalco (Palacios et al., 2013). In these drainages, sulfide spring colonization occurred about 18,000 years ago (Greenway et al., 2021), and the sulfide spring populations have been described as distinct and highly endemic species: *Poecilia thermalis* (for the Ixtapangajoya population) and *Poecilia sulphuraria* (including two distinct populations, Baños del Azufre and La Gloria, in the Pichucalco). Note that another lineage in the *P. mexicana* complex has also colonized a sulfide spring in Costa Rica (Culumber, Greenway, and Tobler; unpublished data), but this lineage has not been studied in detail yet.

Several other poeciliid species have also colonized sulfide springs in southern Mexico and co-exist syntopically with members of the *P. mexicana* complex (**Fig. 3**). The sulfide spring endemic *Gambusia eurystoma* co-exists with *P. sulphuraria* at the Baños del Azufre (Culumber et al., 2016), and divergent populations of *Pseudoxiphophorus bimaculatus* and *Xiphophorus hellerii* co-exist with *P. sulphuraria* at La Gloria (Greenway et al., 2020). In addition, a population of *Gambusia sexradiata* inhabits a distinct sulfide spring (Mogote de Puyacatengo) downstream of sulfide springs inhabited by *P. mexicana* in the Rio Puyacatengo drainage (Greenway et al., 2020).

Outside of Mexico, a putative sulfide spring endemic, *Limia sulphurophila*, has been described from the Dominican Republic (Greenway et al., 2020). At least one other species, *L. perugiae*, occurs in habitats with elevated H_2S concentrations adjacent to sulfide springs (Tobler, unpublished data). Finally, there are a number of sulfide spring populations of more widespread species. In the United States, populations of *Gambusia holbrooki* and *Poecilia latipinna* have colonized sulfide springs in Florida (Riesch et al., 2016), and a population of *Gambusia affinis* occurs in a sulfide spring in Oklahoma (Tobler and Hastings, 2011). In Venezuela, a population of *Poecilia (Acanthopacelus) cf. reticulata* also inhabits a sulfide-rich spring (Tobler and Hastings, 2011).

Additional species have been reported in association with sulfide springs, but we don't yet know whether they actually occupy microhabitats with elevated H_2S concentrations, and if so, whether they exhibit any trait divergences compared to adjacent populations in nonsulfidic habitats. Such species include *Brachyrhaphis roseni* (Panama), *Poecilia formosa* (Mexico), *Poeciliopsis elongata* (Panama), *Priapichthys annectens* (Costa Rica), and *Panamichthys panamensis* (Panama) (Greenway et al., 2014).

In summary, a diversity of poeciliid species is associated with toxic sulfide springs. Even though several species endemic to these extreme environments have been formally described based on morphological analyses, it is important to note that nearly all these species are closely related to ancestral populations in adjacent nonsulfidic habitats. Despite their phenotypic distinctness, all sulfide spring endemics are part of species complexes in which species boundaries are poorly delineated and introgressive hybridization is ongoing (Jourdan et al., 2016; Palacios et al., 2016; Weaver et al., 2016).

Why sulfide spring poeciliids make for a great model

Sulfide spring poeciliids offer several advantages as study systems for the investigation of physiological adaptation in explicit ecological and evolutionary contexts: (1) Unlike other habitats characterized by high concentrations of H_2S (e.g., deep-sea hydrothermal vents and cold seeps), sulfide springs are easily accessible for research. Because they are nested within river networks, they also provide spatially isolated replication across multiple scales (within and among river basins). (2) The relatively recent colonization of sulfide springs and the presence of ancestral populations in adjacent nonsulfidic waters facilitates microevolutionary comparisons among populations in contrasting habitats, including laboratory experiments that disentangle effects of genetic differentiation and phenotypic plasticity on the expression of physiological traits. This approach has primarily been used to investigate adaptation to sulfide springs in the *P. mexicana* species complex (see Tobler et al., 2018 for a review). (3) The presence of evolutionary replication within species, among species of the same genus, and among species in different genera facilitates macroevolutionary comparisons to address questions about evolutionary repeatability and predictability in different biogeographic contexts.

Exploring molecular mechanisms underlying the evolution of H_2S tolerance

A fundamental question about sulfide spring adaptation is how some poeciliid fishes can tolerate H_2S concentrations that are orders of magnitude higher than what is acutely toxic for their ancestors in nonsulfidic habitats. Early studies indicated such population differences in H_2S tolerance using exposure and translocation experiments (Tobler et al., 2009b, 2011). Compensatory behaviors seem to play a crucial role in avoiding the toxic conditions, as sulfide spring fish spend much time conducting aquatic surface respiration (ASR), during which they skim the air-water interface that has higher oxygen and lower H_2S concentrations (Tobler et al., 2009a; Lukas et al., 2021). In absence of ASR, short-term survival even for individuals from H_2S-adapted populations declines significantly (Plath et al., 2007).

While the importance of behavior in H_2S tolerance was recognized early, it was less clear how physiological processes were different between fish from sulfidic and nonsulfidic habitats. High-throughput sequencing technologies that allow for the genome-wide quantification of DNA sequence variation and gene expression provided tools for an unbiased exploration of physiological differences between populations in sulfidic and nonsulfidic habitats and among different lineages of sulfide spring fishes.

A priori hypotheses

In theory, adaptation to toxic levels of H_2S can be accomplished through multiple, non-mutually exclusive mechanisms (see Tobler et al., 2016 for a comprehensive review). These hypotheses provide important context for the interpretation of genomic and transcriptomic analyses:

- *Avoidance and exclusion:* H_2S readily crosses biological membranes. Nonetheless, organisms may be able to reduce the flux of H_2S from the environment into the body, either by seeking out microhabitats with lower H_2S concentrations, changing oxygen acquisition strategies, or through structural modifications to the integument or respiratory surfaces that exclude H_2S from the body.
- *Regulation:* Organisms may be able to regulate endogenous H_2S concentrations and keep them at low levels despite constant influx from the environment. Increased regulatory capacity may stem from upregulation of physiological pathways involved in sulfide oxidation (i.e., the SQR pathway introduced above) or from downregulation of pathways involved in endogenous H_2S production (i.e., transsulfuration pathway). H_2S-oxidation may also be mediated by endosymbiotic microbes.
- *Mitigation:* Organisms may simply conform to elevated H_2S concentrations in the environment and mitigate the toxic effects either by modifying toxicity targets and making them inert to the effects of H_2S, or by relying on alternative pathways that fulfill tasks impaired by H_2S. For example, since H_2S blocks aerobic ATP production by inhibition of COX, the effects of H_2S could be mitigated by modification of COX or by upregulation of anaerobic ATP production.

Genetic variation in protein coding genes

A variety of studies have quantified genetic variation in sulfide spring fishes using different methodologies, including RNA sequencing (Brown et al., 2018; Greenway et al., 2020), sequencing of mitochondrial genomes (Pfenninger et al., 2014), genomic sequencing of pooled samples (Pfenninger et al., 2015), as well as targeted (Ryan et al., 2023) and whole-genome resequencing of individual fish (Greenway et al., 2021). Most of these studies have been devoted to sulfide spring populations within the *P. mexicana* species complex, but some have also included a broader taxon sampling of sulfidic and nonsulfidic lineages across the family Poeciliidae. Although there is not a single gene that has consistently been implicated in playing a role in H_2S adaptation across all lineages investigated to date, there are several patterns emerging.

First, closely related lineages in adjacent sulfidic and nonsulfidic habitats often have starkly different mitochondrial genomes, and there is clear evidence for positive selection on mitochondrially encoded subunits of the primary toxicity target (cox1 and cox3) and other OxPhos protein complexes (Pfenninger et al., 2014; Brown et al., 2018; Greenway et al., 2021). Although DNA sequence evolution in mitochondrially encoded genes is expected to lead to correlated changes in the nuclear genome (Hill,

2020), there is no evidence for consistent selection nuclear-encoded OxPhos subunits, perhaps suggesting that H_2S adaptation does not hinge on mito-nuclear coevolution (Greenway et al., 2021; Ryan et al., 2023). Although positive selection on mitochondrially-encoded OxPhos genes is common in sulfide spring lineages, there are several sulfide spring populations that appear to lack modifications to COX, including the Tacotalpa population in the *P. mexicana* species complex, *P. latipinna*, *L. sulphurophila*, and *G. holbrooki* (Pfenninger et al., 2014; Greenway et al., 2020).

Second, there is some evidence for positive selection on genes associated with sulfide oxidation, including SQR, ETHE1, and other components of the SQR pathway (Pfenninger et al., 2015; Brown et al., 2018; Ryan et al., 2023). Most evidence for selection on H_2S detoxification genes comes from species in the *P. mexicana* species complex, while analyses at a broader phylogenetic scale did not consistently detect these genes as potential candidates for adaptation (Greenway et al., 2020, 2021).

Third, as predicted based on H_2S's impact on oxygen transport proteins, some lineages of sulfide spring fishes exhibit selection on hemoglobin genes, but not on myoglobin (Barts et al., 2018; Greenway et al., 2021). Different evolutionary lineages vary in the type of hemoglobin gene under selection, leading to idiosyncratic evolutionary patterns despite a shared source of selection (Barts et al., 2018). Similar lineage-specific patterns of selection have been documented in genes associated with energy metabolism, oxidative stress responses, and the immune system (Brown et al., 2019; Greenway et al., 2021; Ryan et al., 2023), although the potential adaptive functions of these changes are not always clear.

Overall, analyses of DNA sequences have revealed candidate genes of H_2S adaptation that align with a priori hypotheses associated with regulation and mitigation. However, it is important to emphasize that genomic patterns of selection and the genetic basis of H_2S adaptation appear to be highly variable among lineages. In a family-wide analysis with 10 independent sulfide-spring lineages, evidence for positive selection was largely restricted to mitochondrially-encoded genes, and even then, nonsynonymous substitutions were not consistently present in all lineages (Greenway et al., 2020). Similarly, population genomic analyses in three lineages that inhabit the same sulfide spring revealed very limited evidence for selection on the same genomic regions outside of the mitochondria, and none of the nuclear regions with consistent evidence for selection were associated with genes that have clear ties to H_2S toxicity or detoxification (Greenway et al., 2021). These results indicate that the genetic underpinnings of the convergent evolution of elevated H_2S tolerance vary substantially across evolutionarily independent lineages, which could be related to the polygenic nature of tolerance (i.e., there appear to be multiple ways to tweak physiological pathways to achieve elevated H_2S tolerance). The exception to these general patterns can be found in the *P. mexicana* species complex, where convergence is also evident at the genomic level (Brown et al., 2019; Ryan et al., 2023). Genomic convergence across these lineages, however, did not arise from selection on *de novo* mutations in the same genes or genomic regions, but from selection on standing genetic variation in ancestral populations and potentially gene flow among lineages that independently colonized sulfide springs.

Gene expression and regulatory evolution

Changes in H_2S tolerance can not only arise through mutations in protein-coding regions of the genome, but also through the differential regulation of relevant genes. A series of studies have compared patterns of gene expression between sulfidic and nonsulfidic lineages, at first through qPCR (Tobler et al., 2014a) and eventually with transcriptomics (Kelley et al., 2016; Greenway et al., 2020). As for the genomic analyses described above, comparisons of gene expression have been conducted both at micro and macroevolutionary scales. Unlike in genomic analyses, however, there are very clear patterns of convergent evolution in gene expression patterns no matter what the phylogenetic scale of analysis is. As expected, genes associated with H_2S detoxification (SQR pathway), aerobic and anaerobic metabolism, and oxidative stress responses are consistently upregulated in sulfide spring lineages (Kelley et al., 2016; Greenway et al., 2020).

Differential expression in relevant genes is not merely a consequence of different H_2S exposure histories in natural populations. Common-garden experiments have shown that sulfide spring populations have higher expression of H_2S detoxification genes than those from nonsulfidic populations, even when reared under identical, nonsulfidic conditions (Passow et al., 2017b). In addition, H_2S exposure experiments in the laboratory have also indicated that sulfide spring fish have a heightened ability to upregulate relevant genes when they do encounter the toxicant (Passow et al., 2017b). Thus, variation in gene expression between sulfidic and nonsulfidic populations in nature is not just a consequence of plasticity, but evolution has changed mechanisms of gene regulation that cause differences in the constitutive expression and the H_2S-inducibility of candidate genes. This notion is supported by the fact that there is greater overlap between genes that are under selection and differentially expressed than expected by change (Brown et al., 2018).

In the light of these findings, the grand question is what changes in gene regulatory mechanisms have created the convergent patterns of gene expression. While comparative studies across different lineages remain missing, some have started to explore gene regulatory mechanisms in *P. mexicana*. For example, there are differences in the expression levels of microRNAs that act as post-transcriptional regulators of gene expression (Kelley et al., 2021a). MicroRNAs that target mRNAs involved in H_2S detoxification are significantly downregulated in sulfidic populations, which likely slows down the degradation of mRNAs and might increase the production of the corresponding proteins. Furthermore, a study examining patterns of epigenetic modifications throughout the genome found significant differences in DNA methylation between populations from different habitats, including in genomic regions containing genes associated with H_2S detoxification (Kelley et al., 2021b). Many differentially methylated regions remained consistent in wild-caught and laboratory-reared fish, indicating that epigenetic transgenerational stability could play a role in adaptation.

Functional physiology of sulfide adaptation

Genomic and transcriptomic analyses revealed potential targets of H_2S toxicity and detoxification that are under selection, differentially expressed, or both in fishes inhabiting sulfide-rich environments. While studies relying on high-throughput sequencing technologies often assume that selection on or differential expression of candidate genes ultimately impact physiological function, few studies explore the functional consequences of changes in DNA sequences or gene expression in non-model organisms. We have used genomic and transcriptomic data to test hypotheses about differences in the biochemical function of proteins and the physiological function of organelles and organisms between sulfidic and nonsulfidic populations.

H_2S resistance and modification of COX

One possible mechanism of adaptation to H_2S-rich environments is modification of the primary toxicity target, COX, that allows for the continued function of the enzyme in the presence of H_2S. Genetic analyses have indicated positive selection on two COX subunits (cox1 and cox3), and protein modeling has suggested that nonsynonymous substitutions potentially affect the structure and function of this enzyme (Pfenninger et al., 2014). Specifically, some amino acid substitutions are in highly conserved regions of the protein and appear to trigger conformational changes in COX1 that block the access of H_2S to the reactive core of the protein (Pfenninger et al., 2014).

Interestingly, patterns of selection on COX vary among different sulfidic populations of *P. mexicana*, such that some H_2S-tolerant populations are predicted to have a structurally modified COX that may reduce its affinity for H_2S (e.g., Pichucalco and Puyacatengo drainages) while others do not (e.g., Tacotalpa drainage). Two different studies tested this prediction by measuring COX function across a gradient of H_2S concentrations (Pfenninger et al., 2014; Greenway et al., 2020). As expected, COX activity rapidly declined in presence of H_2S in fish from nonsulfidic populations (that is the toxic effect). In contrast, sulfidic populations from the Pichucalco and Puyacatengo drainages, which experience selection in amino acid positions that alter COX structure, both exhibit an H_2S-resistant COX that maintains enzyme activity in the presence of H_2S. Meanwhile, sulfidic fish from the Tacotalpa drainage lacking evidence of selection on COX subunits exhibit the same pattern of activity as observed in non-sulfidic fishes. The results of these studies confirm that positive selection on COX genes have the predicted functional ramifications. In addition, adaptation to H_2S does not require the evolution of an H_2S-resistant COX; in the Tacotalpa population, H_2S resistance may instead rely on an alternative mechanism, such as detoxification mechanisms that maintain low endogenous concentrations of H_2S regardless of environmental exposure.

H_2S *regulation and modification of SQR*

Genomic and transcriptomic analyses also indicated potential modification of SQR and other enzymes in the SQR pathway. SQR catalyzes the initial step in the oxidation of H_2S, and studies in other animals already suggested that an increased capacity to oxidize H_2S into less toxic forms could mediate adaptation to H_2S-rich environments (Ma et al., 2012). Functional studies on SQR activity in sulfidic populations of *P. mexicana* have found similar results (Greenway et al., 2020). The activity of SQR in sulfidic populations from three different river drainages (Pichucalco, Puyacatengo, and Tacotalpa) was significantly higher than that of nonsulfidic fishes at intermediate and high H_2S concentrations. This increase in SQR activity was correlated with an increased ability to regulate endogenous H_2S concentrations inside the mitochondria of multiple organs, when fish are exposed to environmental H_2S (Greenway et al., 2020). In fish from nonsulfidic populations, H_2S inside mitochondria generally increased as environmental concentrations of H_2S increased, suggesting that their ability to detoxify H_2S is limited, and they must conform to the external environment in respect to H_2S. In contrast, mitochondria in fish from sulfidic populations maintained low concentrations of H_2S, rarely exceeding control concentrations without H_2S in the environment. Together, these results suggest that H_2S tolerance in *P. mexicana* is in part mediated by an increased ability to detoxify and regulate H_2S inside mitochondria upon environmental exposure. Considering that SQR and other genes associated with this pathway are frequently under selection and consistently differentially expressed in sulfide spring fishes, increased capacity for detoxification may be a ubiquitous adaptation necessary for survival in H_2S-rich environments.

Mitochondrial function in the presence of H_2S

Given the evidence that both OxPhos and SQR pathways are under selection and exhibit functional differences between fish from sulfidic and nonsulfidic populations, the mitochondria of H_2S-tolerant *P. mexicana* should be able to maintain respiration in the presence of H_2S. This predication can be tested by measuring oxygen consumption rates of isolated mitochondria along a gradient of H_2S concentrations (Greenway et al., 2020). While there was no evidence for differences in basal respiration rates between fish from sulfidic and nonsulfidic habitats, the maximal respiratory rates and spare respiratory capacities of mitochondria isolated from sulfidic fish were consistently higher in presence of H_2S compared to mitochondria from nonsulfidic fish. Although, this pattern held for all *P. mexicana* populations that were investigated (Pichucalco, Puyacatengo, and Tacotalpa), there was also considerable variation in the spare respiratory capacity across the different sulfidic populations, potentially indicating varying levels of tolerance. Altogether, these data support the hypothesis that H_2S -tolerant populations can maintain aerobic ATP production even in the presence of H_2S.

Organismal respiration

Modification of mitochondrial respiratory function may have consequences for organismal respiratory rates and has been identified as a basis for adaptive variation in aerobic performance in high altitude deer mice (Scott et al., 2018). Measuring organismal metabolic rates in the presence of H_2S is challenging due to the molecule's competitive consumption of oxygen in aquatic environments and its corrosive nature. To date, no study has successfully measured the metabolic rate of fishes in presence of environmental H_2S. However, under nonsulfidic conditions, closed chamber respirometry revealed a convergent reduction in routine metabolic rates in *P. mexicana* populations from sulfidic habitats, which was in part driven by the smaller body size of fish inhabiting H_2S-rich springs (Passow et al., 2017a). These differences in metabolic rates were present in both wild-caught and laboratory-reared individuals, potentially suggesting a genetic component to metabolic differences (Passow et al., 2017a). However, intermittent-flow respirometry revealed no statistically significant differences in basal or maximal metabolic rates between fish of the same populations (Barts, 2020). Hence, variation in mitochondrial function appears to have little effect on the aerobic scope of fishes from these habitats under nonsulfidic conditions, and behavioral differences (e.g., activity patterns) may at least in part shape differences in routine metabolic rates documented by Passow et al. (2017a).

Interestingly, H_2S-tolerant fish are able to maintain aerobic respiration at lower ambient O_2 concentrations than nonsulfidic ones (Barts, 2020). This finding indicates differences in how fish from sulfidic and nonsulfidic populations adjust whole-organism metabolism in response to hypoxia. So, while variation in OxPhos enzymes may not result in different metabolic rates under normal conditions (i.e., normoxic and nonsulfidic), it may support maintenance of organismal metabolism under periods of environmental stress, such as low dissolved oxygen. Future experiments that use H_2S-releasing drugs to circumnavigate experimental constraints in measuring metabolic rates in the presence of environmental H_2S may shed light into whether fish from sulfidic and nonsulfidic populations differ in their ability to maintain metabolic rates upon H_2S exposure.

Physiological adaptation in the context of organismal and environmental complexities

Research on sulfide spring fishes has documented how modification of mitochondrial function contributed to the evolution of increased H_2S tolerance and local adaptation to these extreme environments. Reducing H_2S adaptation to mitochondrial physiology, however, would represent an oversimplification of the ecological physiology of sulfide spring fishes and the evolutionary mechanisms that have caused their differentiation from ancestral populations in adjacent nonsulfidic habitats. For example, even fish from sulfidic habitats suffer from increased mortality if they do not have access to the water's surface and cannot perform ASR (Plath et al., 2007; Tobler et al., 2009a). Hence, modifications of physiological processes are clearly not sufficient to explain survival in H_2S-rich environments, but how physiology interacts with behavior—and potentially other traits—remains poorly investigated.

A key part of the problem of understanding adaptation to extreme environments is that reducing the source of selection to the presence and absence of H_2S does not reflect the ecological realities of these systems. Most prominently, the water of sulfide springs is also severely hypoxic (in some instances approaching oxygen concentrations less than 0.5 mg L^{-1}), and reduced oxygen availability likely interacts with the presence of H_2S, a toxicant that directly impacts oxygen transport and aerobic metabolism. The need for efficient oxygen acquisition has impacted trait evolution in sulfide spring fishes independently of H_2S. For example, sulfide spring populations exhibit larger heads, which are correlated with higher gill surface areas (Tobler et al., 2011) and increased ventilation efficiency (Camarillo et al., 2020). How adaptations to H_2S and hypoxia interact remains largely unknown. Some physiological modifications could have synergistic effects (e.g., upregulation of anaerobic metabolism is likely beneficial in the context of hypoxia and suppression of aerobic metabolism by H_2S). In contrast, other traits may have antagonistic effects. For example, increases in gill surface area may increase oxygen acquisition in the hypoxic environment, but it may also increase the flux of H_2S into the body. Disentangling the cause-and-effect relationships of these cooccurring stressors remains a major challenge in our understanding of physiological adaptation.

Reliance on ASR, irrespective of whether it is an adaptation to hypoxia and H_2S, further has profound impacts that intersect with biotic sources of selection. Since sulfide spring fish allocate a large portion of their time budget to ASR, there are significant limitations for feeding in these benthic foragers (Tobler et al., 2009a). The trade-off between surface breathing and benthic feeding constraints individual energy budgets in sulfide spring fish, and they are in much worse nutritional conditions than fish inhabiting nonsulfidic environments (Tobler, 2008). Consequently, energy limitation has been hypothesized to be an important source of selection in this system that could explain the evolution of morphology (e.g., reduction in brain size; Schulz-Mirbach et al., 2016), physiology (e.g., reduction of routine metabolism; Passow et al., 2017a), behavior (e.g., reduction of aggression; Bierbach et al., 2012), and life histories (e.g., reduction in fecundity and increase in offspring size; Riesch et al., 2014). At least in some sulfide springs, the high frequency of ASR also interacts with predation, both from insect and bird predators (Tobler et al., 2007; Lukas et al., 2021). In response, *P. sulphuraria* at the Baños del Azufre and *P. thermalis* have evolved complex collective behaviors, creating waves at the water surface that reduce the success of aerial predators (Doran et al., 2022).

Over the years, researchers have investigated a wide variety of environmental differences between adjacent sulfidic and nonsulfidic habitats and how such differences might impact phenotypic evolution. Beyond the examples already mentioned, there are changes in trophic resource use upon colonization of sulfide springs, which are reflected in jaw and intestinal tract modifications (Tobler, 2008; Tobler et al., 2015). Sulfide springs exhibit unique ambient light regimes, which are correlated with variation in the

expression of visual pigments and body coloration (Owens et al., 2022). And, sulfide spring fish are exposed to unique communities of parasites, which may shape the evolution at MHC genes and other loci involved in immune function (Tobler et al., 2014b).

Undoubtedly, evolution in and adaptation to the extreme environmental conditions found in sulfide springs is not just a reflection of the presence of toxic H_2S. But as we are gaining at least a glimpse of the importance of correlated sources of selection, our understanding how different sources of selection interact and how modification of different traits might impact the function of organisms in their environment remains largely unexplored. Thus, admitting the complexity of environments and organismal design into our studies of adaptation is a critical frontier in ecological and evolutionary physiology.

Conclusions

Sulfide spring fishes have the ability to survive under environmental conditions that are lethal to most other animals. Comparative genomic and transcriptomic analyses have allowed for the identification of potential candidate genes that may underlie the evolution of H_2S tolerance, including genes associated with OxPhos that represent direct toxicity targets and genes associated with pathways that catalyze the oxidation of H_2S to nontoxic species of sulfur that can be excreted. Functional physiological analyses of candidate genes and pathways then confirmed the predicted functional ramifications, including inert toxicity targets and increased detoxification ability. Consequently, sulfide spring fishes are able to maintain mitochondrial function and aerobic ATP production under conditions that are highly deleterious for closely related, ancestral populations from nearby nonsulfidic habitats. Overall, this body of research indicates that evolution of tolerance to novel environmental stressors does not require fundamental innovation (e.g., new genes or new physiological pathways); rather, to survive in the presence of H_2S, sulfide spring fishes have tweaked some of the most ancient physiological pathways (OxPhos and SQR) that are otherwise highly conserved among metazoans and other eukaryotes.

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See Also: Anaerobic metabolism in fish; Deep-sea fishes and their extreme adaptations; Energy consumption: Metabolism; Mechanisms of ion transport in freshwater fishes; The osmorespiratory compromise.

References

Achá, D., Guédron, S., Amouroux, D., Point, D., Lazzaro, X., Fernandez, P.E., Sarret, G., 2018. Algal bloom exacerbates hydrogen sulfide and methylmercury contamination in the emblematic high-altitude Lake Titicaca. Geosci. J. 8, 438.

Aguilera, G., Terán, G.E., Mirande, J.M., Alonso, F., Rometsch, S., Meyer, A., Torres-Dowdall, J., 2019. Molecular and morphological convergence to sulfide-tolerant fishes in a new species of *Jenynsia* (Cyprinodontiformes: Anablepidae), the first extremophile member of the family. PLoS One 14, e0218810.

Barts, N., 2020. Comparative Physiology and the Predictability of Evolution in Extreme Environments. Kansas State University.

Barts, N., Greenway, R., Passow, C.N., Arias-Rodriguez, L., Kelley, J.L., Tobler, M., 2018. Molecular evolution and expression of oxygen transport genes in livebearing fishes (Poeciliidae) from hydrogen sulfide rich springs. Genome 61, 273–286.

Bierbach, D., Klein, M., Sassmannshausen, V., Schlupp, I., Riesch, R., Parzefall, J., Plath, M., 2012. Divergent evolution of male aggressive behavior: another reproductive isolation barrier in extremophile poeciliid fishes? Int. J. Evol. Biol. 2012, 148745.

Brown, A.P., Arias-Rodriguez, L., Yee, M.-C., Tobler, M., Kelley, J.L., 2018. Concordant changes in gene expression and nucleotides underlie independent adaptation to hydrogen-sulfide-rich environments. Genome Biol. Evol. 10, 2867–2881.

Brown, A.P., McGowan, K.L., Schwarzkopf, E.J., Greenway, R., Rodriguez, L.A., Tobler, M., Kelley, J.L., 2019. Local ancestry analysis reveals genomic convergence in extremophile fishes. Philos. Trans. R. Soc. Lond. B Biol. Sci. 374, 20180240.

Camarillo, H., Arias Rodriguez, L., Tobler, M., 2020. Functional consequences of phenotypic variation between locally adapted populations: swimming performance and ventilation in extremophile fish. J. Evol. Biol. 33, 512–523.

Carrico, R.J., Blumberg, W.E., Peisach, J., 1978. The reversible binding of oxygen to sulfhemoglobin. J. Biol. Chem. 253, 7212–7215.

Cooper, C.E., Brown, G.C., 2008. The inhibition of mitochondrial cytochrome oxidase by the gases carbon monoxide, nitric oxide, hydrogen cyanide and hydrogen sulfide: chemical mechanism and physiological significance. J. Bioenerg. Biomembr. 40, 533–539.

Culumber, Z.W., Hopper, G.W., Barts, N., Passow, C.N., Morgan, S., Brown, A., Arias-Rodriguez, L., Tobler, M., 2016. Habitat use of two extremophile, highly endemic, and critically endangered fish species (*Gambusia eurystoma* and *Poecilia sulphuraria*; Poeciliidae). Aquat. Conserv. Mar. Freshw. Ecosyst. 26, 1155–1167.

Doran, C., Bierbach, D., Lukas, J., Klamser, P., Landgraf, T., Klenz, H., Habedank, M., Arias-Rodriguez, L., Krause, S., Romanczuk, P., Krause, J., 2022. Fish waves as emergent collective antipredator behavior. Curr. Biol. 32, 708–714.e4.

Fu, M., Zhang, W., Wu, L., Yang, G., Li, H., Wang, R., 2012. Hydrogen sulfide (H_2S) metabolism in mitochondria and its regulatory role in energy production. Proc. Natl. Acad. Sci. U. S. A 109, 2943–2948.

Garcia-Bereguiain, M.A., Samhan-Arias, A.K., Martin-Romero, F.J., Gutierrez-Merino, C., 2008. Hydrogen sulfide raises cytosolic calcium in neurons through activation of L-type Ca^{2+} channels. Antioxid. Redox Signal. 10, 31–42.

Giere, O., 2009. Meiobenthology: The Microscopic Motile Fauna of Aquatic Sediments, second ed. Springer, Berlin.

Greenway, R., Arias-Rodriguez, L., Diaz, P., Tobler, M., 2014. Patterns of macroinvertebrate and fish diversity in freshwater sulphide springs. Diversity 6, 597–632.

Greenway, R., Barts, N., Henpita, C., Brown, A.P., Arias Rodriguez, L., Rodríguez Peña, C.M., Arndt, S., Lau, G.Y., Murphy, M.P., Wu, L., Lin, D., Kelley, J.L., Shaw, J.H., Tobler, M., 2020. Convergent evolution of conserved mitochondrial pathways underlies repeated adaptation to extreme environments. Proc. Natl. Acad. Sci. U. S. A. 117, 16424–16430.
Greenway, R., Brown, A.P., Camarillo, H., Delich, C., McGowan, K.L., Nelson, J., Arias-Rodriguez, L., Kelley, J.L., Tobler, M., 2021. Convergent Adaptation and Ecological Speciation Result from Unique Genomic Mechanisms in Sympatric Extremophile Fishes.
Greven, H., 2011. Gonads, genitals, and reproductive biology. In: Evans, J.P., Pilastro, A., Schlupp, I. (Eds.), Ecology and Evolution of Poeciliid Fishes. The University of Chicago Press, Chicago, IL, pp. 3–17.
Hildebrandt, T.M., Grieshaber, M., 2008. Three enzymatic activities catalyze the oxidation of sulfide to thiosulfate in mammalian and invertebrate mitochondria. FEBS J. 275, 3352–3361.
Hill, G.E., 2020. Mitonuclear compensatory coevolution. Trends Genet. 36, 403–414.
Hotaling, S., Quackenbush, C.R., Bennett-Ponsford, J., New, D.D., Arias Rodriguez, L., Tobler, M., Kelley, J.L., 2019. Microbial diversity in replicated hydrogen sulfide-rich streams. Microb. Ecol. 77, 559–573.
Jannasch, H.W., Taylor, C.D., 1984. Deep-sea microbiology. Annu. Rev. Microbiol. 38, 487–514.
Jensen, B.S., Fago, A., 2021. Sulfide metabolism and the mechanism of torpor. J. Exp. Biol. 224, jeb215764.
Jourdan, J., Krause, S.T., Lazar, V.M., Zimmer, C., Sommer-Trembo, C., Arias-Rodriguez, L., Klaus, S., Riesch, R., Plath, M., 2016. Shared and unique patterns of phenotypic diversification along a stream gradient in two congeneric species. Sci. Rep. 6, 38971.
Kelley, J.L., Arias-Rodriguez, L., Patacsil Martin, D., Yee, M.-C., Bustamante, C.D., Tobler, M., 2016. Mechanisms underlying adaptation to life in hydrogen sulfide-rich environments. Mol. Biol. Evol. 33, 1419–1434.
Kelley, J.L., Desvignes, T., McGowan, K.L., Perez, M., Rodriguez, L.A., Brown, A.P., Culumber, Z., Tobler, M., 2021a. microRNA expression variation as a potential molecular mechanism contributing to adaptation to hydrogen sulphide. J. Evol. Biol. 34, 977–988.
Kelley, J.L., Tobler, M., Beck, D., Sadler-Riggleman, I., Quackenbush, C.R., Arias Rodriguez, L., Skinner, M.K., 2021b. Epigenetic inheritance of DNA methylation changes in fish living in hydrogen sulfide-rich springs. Proc. Natl. Acad. Sci. U. S. A. 118, e2014929118.
Kimura, H., 2014. Production and physiological effects of hydrogen sulfide. Antioxid. Redox Signal. 20, 783–793.
Kombian, S.B., Warenyica, M.W., Mele, F., Reiffenstein, R.J., 1988. Effects of acute intoxication with hydrogen sulfide on central amino acid transmitter systems. Neurotoxicology 9, 587–596.
Li, L., Rose, P., Moore, P.K., 2011. Hydrogen sulfide and cell signaling. Annu. Rev. Pharmacol. Toxicol. 51, 169–187.
Lukas, J., Auer, F., Goldhammer, T., Krause, J., Romanczuk, P., Klamser, P., Arias-Rodriguez, L., Bierbach, D., 2021. Diurnal changes in hypoxia shape predator-prey interaction in a bird-fish system. Front. Ecol. Evol. 9, 151.
Ma, Y.-B., Zhang, Z.-F., Shao, M.-Y., Kang, K.-H., Shi, X.-L., Dong, Y.-P., Li, J.-J., 2012. Response of sulfide-quinone oxireductase to sulfide exposure in the echiuran worm *Urechis unicinctus*. Mar. Biotechnol. 14, 245–251.
Mathai, J.C., Missner, A., Kugler, P., Saparov, S.M., Zeidel, M.L., Lee, J.K., Pohl, P., 2009. No facilitator required for membrane transport of hydrogen sulfide. Proc. Natl. Acad. Sci. U. S. A. 106, 16633–16638.
Meffe, G.K., Snelson, F.F., 1989. An ecological overview of poeciliid fishes. In: Meffe, G.K., Snelson, F.F. (Eds.), Ecology and Evolution of Lifebearing Fishes (Poeciliidae). Prentice Hall, New Jersey, pp. 13–31.
Módis, K., Panopoulos, P., Coletta, C., Papapetropoulos, A., Szabo, C., 2013. Hydrogen sulfide-mediated stimulation of mitochondrial electron transport involves inhibition of the mitochondrial phosphodiesterase 2A, elevation of cAMP and activation of protein kinase A. Biochem. Pharmacol. 86, 1311–1319.
Muyzer, G., Stams, A.J.M., 2008. The ecology and biotechnology of sulphate-reducing bacteria. Nat. Rev. Microbiol. 6, 441–454.
Olson, K.R., 2018. H_2S and polysulfide metabolism: conventional and unconventional pathways. Biochem. Pharmacol. 149, 77–90.
Olson, K.R., 2015. Hydrogen sulfide as an oxygen sensor. Antioxid. Redox Signal. 22, 377–397.
Olson, K.R., 2011. The therapeutic potential of hydrogen sulfide: separating hype from hope. Am. J. Physiol. Regul. Integr. Comp. Physiol. 301, R297–R312.
Olson, K.R., Straub, K.D., 2016. The role of hydrogen sulfide in evolution and the evolution of hydrogen sulfide in metabolism and signaling. Physiology 31, 60–72.
Owens, G.L., Veen, T., Moxley, D.R., Arias-Rodriguez, L., Tobler, M., Rennison, D.J., 2022. Parallel shifts of visual sensitivity and body coloration in replicate populations of extremophile fish. Mol. Ecol. 31, 946–958.
Palacios, M., Arias-Rodriguez, L., Plath, M., Eifert, C., Lerp, H., Lamboj, A., Voelker, G., Tobler, M., 2013. The rediscovery of a long described species reveals additional complexity in speciation patterns of poeciliid fishes in sulfide springs. PLoS One 8, e71069.
Palacios, M., Voelker, G., Arias Rodríguez, L., Mateos, M., Tobler, M., 2016. Phylogenetic analyses of the subgenus *Mollienesia* (*Poecilia*, Poeciliidae, Teleostei) reveal taxonomic inconsistencies, cryptic biodiversity, and spatio-temporal aspects of diversification in Middle America. Mol. Phylogenet. Evol. 103, 230–244.
Parzefall, J., 2001. A review of morphological and behavioural changes in the cave molly, *Poecilia mexicana*, from Tabasco, Mexico. Environ. Biol. Fish. 62, 263–275.
Passow, C.N., Arias-Rodriguez, L., Tobler, M., 2017a. Convergent evolution of reduced energy demands in extremophile fish. PLoS One 12, e0186935.
Passow, C.N., Henpita, C., Shaw, J.H., Quackenbush, C.R., Warren, W.C., Schartl, M., Arias-Rodriguez, L., Kelley, J.L., Tobler, M., 2017b. The roles of plasticity and evolutionary change in shaping gene expression variation in natural populations of extremophile fish. Mol. Ecol. 26, 6384–6399.
Perry, S., Kumai, Y., Porteus, C.S., Tzaneva, V., Kwong, R.W.M., 2016. An emerging role for gasotransmitters in the control of breathing and ionic regulation in fish. J. Comp. Physiol. B 186, 145–159.
Pfenninger, M., Lerp, H., Tobler, M., Passow, C., Kelley, J.L., Funke, E., Greshake, B., Erkoc, U.K., Berberich, T., Plath, M., 2014. Parallel evolution of cox genes in H_2S-tolerant fish as key adaptation to a toxic environment. Nat. Commun. 5, 3873.
Pfenninger, M., Patel, S., Arias-Rodriguez, L., Feldmeyer, B., Riesch, R., Plath, M., 2015. Unique evolutionary trajectories in repeated adaptation to hydrogen sulphide-toxic habitats of a neotropical fish (*Poecilia mexicana*). Mol. Ecol. 24, 5446–5459.
Pietri, R., Roman-Morales, E., Lopez-Garriga, J., 2011. Hydrogen sulfide and heme proteins: knowledge and mysteries. Antioxid. Redox Signal. 15, 393–404.
Plath, M., Tobler, M., Riesch, R., Garcia de Leon, F.J., Giere, O., Schlupp, I., 2007. Survival in an extreme habitat: the role of behaviour and energy limitation. Naturwissenschaften 94, 991–996.
Reese, B.K., Anderson, M.A., Amrhein, C., 2008. Hydrogen sulfide production and volatilization in a polymictic eutrophic saline lake, Salton Sea, California. Sci. Total Environ. 406, 205–218.
Reiffenstein, R.J., Hulbert, W.C., Roth, S.H., 1992. Toxicology of hydrogen sulfide. Annu. Rev. Pharmacol. Toxicol. 1992, 109–134.
Riesch, R., Plath, M., Schlupp, I., Tobler, M., Brian Langerhans, R., 2014. Colonisation of toxic environments drives predictable life-history evolution in livebearing fishes (Poeciliidae). Ecol. Lett. 17, 65–71.
Riesch, R., Tobler, M., Lerp, H., Jourdan, J., Doumas, T., Nosil, P., Langerhans, R.B., Plath, M., 2016. Extremophile Poeciliidae: multivariate insights into the complexity of speciation along replicated ecological gradients. BMC Evol. Biol. 16, 136.
Roach, K.A., Tobler, M., Winemiller, K.O., 2011. Hydrogen sulfide, bacteria, and fish: a unique, subterranean food chain. Ecology 92, 2056–2062.
Ryan, K., Greenway, R., Landers, J., Arias-Rodriguez, L., Tobler, M., Kelley, J.L., 2023. Selection on standing genetic variation mediates convergent evolution in extremophile fish. Mol. Ecol. 32 (18), 5042–5054.
Schulz-Mirbach, T., Eifert, C., Riesch, R., Farnworth, M.S., Zimmer, C., Bierbach, D., Klaus, S., Tobler, M., Streit, B., Indy, J.R., Arias-Rodriguez, L., Plath, M., 2016. Toxic hydrogen sulphide shapes brain anatomy: a comparative study of sulphide-adapted ecotypes in the *Poecilia mexicana* complex. J. Zool. 300, 163–176.
Scott, G.R., Guo, K.H., Dawson, N.J., 2018. The mitochondrial basis for adaptive variation in aerobic performance in high-altitude deer mice. Integr. Comp. Biol. 58, 506–518.

Shahak, Y., Hauska, G., 2008. Sulfide oxidation from cyanobacteria to humans: sulfide-quinone oxidoreductase (SQR). In: Sharkey, T.D., Eaton-Rye, J. (Eds.), Advances in Photosynthesis and Respiration. Springer, Heidelberg, pp. 319–335.

Stipanuk, M.H., Ueki, I., 2011. Dealing with methionine/homocysteine sulfur: cysteine metabolism to taurine and inorganic sulfur. J. Inherit. Metab. Dis. 34, 17–32.

Stockwell, C.A., Henkanaththegedara, S.M., 2011. Evolutionary conservation biology. In: Evans, J.P., Pilastro, A., Schlupp, I. (Eds.), Ecology and Evolution of Poeciliid Fishes. University of Chicago Press, Chicago.

Tobler, M., 2008. Divergence in trophic ecology characterizes colonization of extreme habitats. Biol. J. Linn. Soc. 95, 517–528. Oxford University Press (OUP).

Tobler, M., Hastings, L., 2011. Convergent patterns of body shape differentiation in four different clades of poeciliid fishes inhabiting sulfide springs. Evol. Biol. 38, 412–421.

Tobler, M., Henpita, C., Bassett, B., Kelley, J.L., Shaw, J.H., 2014a. H_2S exposure elicits differential expression of candidate genes in fish adapted to sulfidic and non-sulfidic environments. Comp. Biochem. Physiol. Mol. Integr. Physiol. 175, 7–14.

Tobler, M., Kelley, J.L., Plath, M., Riesch, R., 2018. Extreme environments and the origins of biodiversity: adaptation and speciation in sulphide spring fishes. Mol. Ecol. 27, 843–859.

Tobler, M., Palacios, M., Chapman, L.J., Mitrofanov, I., Bierbach, D., Plath, M., Arias-Rodriguez, L., de León, F.J.G., Mateos, M., 2011. Evolution in extreme environments: replicated phenotypic differentiation in livebearing fish inhabiting sulfidic springs. Evolution 65, 2213–2228.

Tobler, M., Passow, C.N., Greenway, R., Kelley, J.L., Shaw, J.H., 2016. The evolutionary ecology of animals inhabiting hydrogen sulfide-rich environments. Annu. Rev. Ecol. Evol. Syst. 47, 239–262.

Tobler, M., Plath, M., Riesch, R., Schlupp, I., Grasse, A., Munimanda, G.K., Setzer, C., Penn, D.J., Moodley, Y., 2014b. Selection from parasites favours immunogenetic diversity but not divergence among locally adapted host populations. J. Evol. Biol. 27, 960–974.

Tobler, M., Riesch, R., Tobler, C.M., Plath, M., 2009a. Compensatory behaviour in response to sulfide-induced hypoxia affects time budgets, feeding efficiency, and predation risk. Evol. Ecol. Res. 11, 935–948.

Tobler, M., Riesch, R., Tobler, C.M., Schulz-Mirbach, T., Plath, M., 2009b. Natural and sexual selection against immigrants maintains differentiation among micro-allopatric populations. J. Evol. Biol. 22, 2298–2304.

Tobler, M., Scharnweber, K., Greenway, R., Passow, C.N., Arias-Rodriguez, L., García-De-León, F.J., 2015. Convergent changes in the trophic ecology of extremophile fish along replicated environmental gradients. Freshw. Biol. 60, 768–780.

Tobler, M., Schlupp, I., Plath, M., 2007. Predation of a cave fish (*Poecilia mexicana*, Poeciliidae) by a giant water-bug (Belostoma, Belostomatidae) in a Mexican sulfur cave. Ecol. Entomol. 32, 492–495.

Truong, D.H., Eghbal, M.A., Hindmarsh, W., Roth, S.H., O'Brien, P.J., 2006. Molecular mechanisms of hydrogen sulfide toxicity. Drug Metab. Rev. 38, 733–744.

Van Dover, C.L., 2000. The Ecology of Deep-Sea Hydrothermal Vents. Princeton University Press, Princeton.

Wallace, J.L., Wang, R., 2015. Hydrogen sulfide-based therapeutics: exploiting a unique but ubiquitous gasotransmitter. Nat. Rev. Drug Discov. 14, 329–345.

Weaver, P.F., Cruz, A., Johnson, S., Dupin, J., Weaver, K.F., 2016. Colonizing the Caribbean: biogeography and evolution of livebearing fishes of the genus *Limia* (Poeciliidae). J. Biogeogr. 43, 1808–1819.