Mechanisms Underlying Adaptation to Life in Hydrogen Sulfide–Rich Environments

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Abstract

Hydrogen sulfide (H_2S) is a potent toxicant interfering with oxidative phosphorylation in mitochondria and creating extreme environmental conditions in aquatic ecosystems. The mechanistic basis of adaptation to perpetual exposure to H_2S remains poorly understood. We investigated evolutionarily independent lineages of livebearing fishes that have colonized and adapted to springs rich in H_2S and compared their genome-wide gene expression patterns with closely related lineages from adjacent, nonsulfidic streams. Significant differences in gene expression were uncovered between all sulfidic and nonsulfidic population pairs. Variation in the number of differentially expressed genes among population pairs corresponded to differences in divergence times and rates of gene flow, which is consistent with neutral drift driving a substantial portion of gene expression variation among populations. Accordingly, there was little evidence for convergent evolution shaping large-scale gene expression patterns among independent sulfide spring populations. Nonetheless, we identified a small number of genes that was consistently differentially expressed in the same direction in all sulfidic and nonsulfidic population pairs. Functional annotation of shared differentially expressed genes indicated upregulation of genes associated with enzymatic H_2S detoxification and transport of oxidized sulfur species, oxidative phosphorylation, energy metabolism, and pathways involved in responses to oxidative stress. Overall, our results suggest that modification of processes associated with H_2S detoxification and toxicity likely complement each other to mediate elevated H_2S tolerance in sulfide spring fishes. Our analyses allow for the development of novel hypotheses about biochemical and physiological mechanisms of adaptation to extreme environments.

Key words: ecological physiology, evolution, extreme environments, gene expression, H_2S, *Poecilia mexicana* (Poeciliidae), RNA-sequencing.

Introduction

Extreme environments are characterized by physiochemical stressors lethal to most organisms, and their clearly defined and replicated selective regimes enable hypothesis-driven tests of organismal responses at all levels of biological organization (Waterman 1999; Bell 2012). Extremophiles can withstand such stressful conditions and provide ideal systems to study mechanisms underlying physiological adaptation (Storey KB and Storey JM 2005; Nevo 2011). In addition, they shed light into life's capacities and limitations to adapt to novel environmental conditions, and understanding the ways organisms function in the context of natural stressors provides basic insights into the biochemical, physiological, and developmental processes that govern life (Waterman 1999; Tobler et al. 2015). Hydrogen sulfide (H_2S) is a physiochemical stressor that is produced in many aquatic environments through geochemical or biological processes (Muyzer and Stams 2008). H_2S has been hypothesized to have played a

critical role in the origin of life and caused mass extinctions; accordingly, it has influenced basic physiological properties of organisms and shaped evolutionary diversification on Earth (Kump et al. 2005; Olson and Straub 2015). H_2S has a wide variety of cytotoxic effects (Beauchamp et al. 1984; Reiffenstein et al. 1992), but its toxicity primarily unfolds through the inhibition of cytochrome *c* oxidase (COX) in the mitochondrial respiratory chain, which halts ATP production (Cooper and Brown 2008). Exposure to environmental H_2S can consequently limit an organism's ability to survive and reproduce (Bagarinao 1992). Although environmental concentrations of H_2S are typically low or highly transient, high and sustained concentrations create extreme environments that are lethal for most life (Tobler and Plath 2011; Riesch et al. 2015). Few metazoans have colonized H_2S-rich habitats and evolved strategies to cope with the continuous exposure to this respiratory toxicant, giving rise to unique ecological communities in deep-sea hydrothermal vents,

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cold seeps, and freshwater sulfide springs (Van Dover 2000; Levin 2005; Greenway et al. 2014).

The mechanistic basis of physiological adaptation to high levels of H_2S remains poorly understood. Previous studies have primarily focused on organisms with endosymbiotic sulfide oxidizing bacteria, which occur in some polychaete worms and mytilid mussels (Childress and Fisher 1992; Roeselers and Newton 2012). Other studies have compared physiological processes between H_2S tolerant and nontolerant species across taxonomically disparate groups with vastly different body organizations and genomic architectures (rats vs. lugworms) and suggested that H_2S-tolerant organisms may exhibit detoxification enzymes with higher activity rates (Hildebrandt and Grieshaber 2008). Nonetheless, the diversity of H_2S's physiological effects and molecular targets (Li et al. 2011; Olson 2011) suggests that adaptation to sulfide-rich environments could include a variety of alternative or complementary modifications. The identification of such adaptive modifications largely hinges on the comparison between closely related species and populations from sulfidic and nonsulfidic environments.

Recent progress in our understanding of the physiological effects and processing of H_2S, driven primarily by biomedical research investigating the role of H_2S in disease formation and potential biomedical applications (Szabo 2007; Li et al. 2011), enables hypothesis-driven tests of the potential molecular basis underlying adaptation to H_2S-rich environments. Elevated tolerance to environmental H_2S could be driven by three, nonmutually exclusive mechanisms. First, organisms perpetually exposed to high ambient H_2S concentrations may minimize its flux from the environment into the body (Vismann 1991). H_2S is lipid soluble and accordingly able to readily pass through biological membranes and invade organismal systems (Reiffenstein et al. 1992). Adaptation to environmental H_2S may thus include modifications of the integumentary system and respiratory surfaces directly exposed to the environment. Such modifications could include changes in the structure and composition of biological membranes and molecular components involved in intercellular interactions, as well as excretion of compounds that create a barrier to H_2S diffusion.

Second, increased tolerance could be mediated by an increased capability to maintain H_2S homeostasis despite continuous influx from the environment. This would involve modification of mechanisms involved in the active elimination of H_2S. All animals are able to detoxify low levels of H_2S through enzymatic oxidation associated with the highly conserved sulfide:quinone oxidoreductase (SQR) pathway and subsequent excretion of oxidized sulfur species (typically sulfate; Hildebrandt and Grieshaber 2008; Shahak and Hauska 2008; Lagoutte et al. 2010). H_2S can also be immobilized and sequestered by methylation, sulfhydration, or reaction with methemoglobin (Bagarinao and Vetter 1992; Levitt et al. 1999; Paul and Snyder 2012). Alternatively, increased capacity for the regulation of H_2S homeostasis could be facilitated by reducing endogenous H_2S production. Sulfide is endogenously produced through reactions associated with the transsulfuration pathway and the processing of sulfur-containing amino acids (Stipanuk 2004; Stipanuk and Ueki 2011).

Third, tolerance to H_2S could be achieved through the modification of toxicity targets that make them less sensitive to adverse consequences caused by elevated endogenous concentration in the face of continuous influx from the environment. For example, COX—the primary target of H_2S toxicity—in sulfide-tolerant organisms has been documented to exhibit structural changes that reduce blocking by H_2S (Degn and Kristensen 1981; Pfenninger et al. 2014). Considering recent biomedical research that has found H_2S to play a critical role in cell signaling and to have an increasing number of putative molecular targets, including ion channels and receptors, cell signaling proteins, and enzymes involved in metabolism (Li and Moore 2008; Li et al. 2011; Olson 2011; Whiteman et al. 2011), it is not straightforward to establish a comprehensive list of potential targets. Nonetheless, mitochondria in general—and the mitochondrial respiratory chain in particular—are expected to provide primary targets of selection mediated by the presence of environmental sulfide (fig. 1A), because they include both primary targets for H_2S toxicity (COX, Cooper and Brown 2008) and are directly linked to H_2S detoxification through SQR (Hildebrandt and Grieshaber 2008). To identify molecular mechanisms potentially underlying high H_2S tolerance, we leveraged a natural system with closely related lineages that occur in environments with perpetually elevated concentrations of naturally occurring H_2S as well as in adjacent nonsulfidic habitats.

In southern Mexico, small livebearing fish of the *Poecilia mexicana* species complex (Poeciliidae) have repeatedly colonized H_2S-rich freshwater springs in at least three river drainages (fig. 1B and C; Palacios et al. 2013). Sulfide spring populations are locally adapted and characterized by phenotypic modifications, including physiological, morphological, and life history traits that have largely evolved in convergence (Tobler and Hastings 2011; Tobler et al. 2011; Pfenninger et al. 2014; Riesch et al. 2014). In addition, sulfide spring populations are undergoing ecological speciation, and there is significant—albeit varying—genetic differentiation between sulfidic and adjacent nonsulfidic populations despite small geographic distances and a lack of physical barriers preventing fish movement (Plath et al. 2013; Pfenninger et al. 2015). Reproductive isolation between sulfidic and nonsulfidic populations is primarily mediated by natural and sexual selection against maladapted migrant individuals (Plath et al. 2010, 2013).

The availability of evolutionarily replicated pairs of closely related populations in sulfidic and nonsulfidic environments provides a unique opportunity for comparative studies of organismal responses to continuous disruptions of H_2S homeostasis. We contrasted variation in gene expression to test hypotheses about the potential molecular underpinnings of H_2S tolerance. We focused on gill tissues, because they are in direct contact with the toxic environment, mediate a variety of physiological processes involved in the maintenance of homeostasis (Evans and Claiborne 2006; Evans et al. 2011), and exhibit strong transcriptional responses upon exposure to H_2S (Tobler et al. 2014). We addressed four key questions: 1) Do levels of gene expression vary between proximate
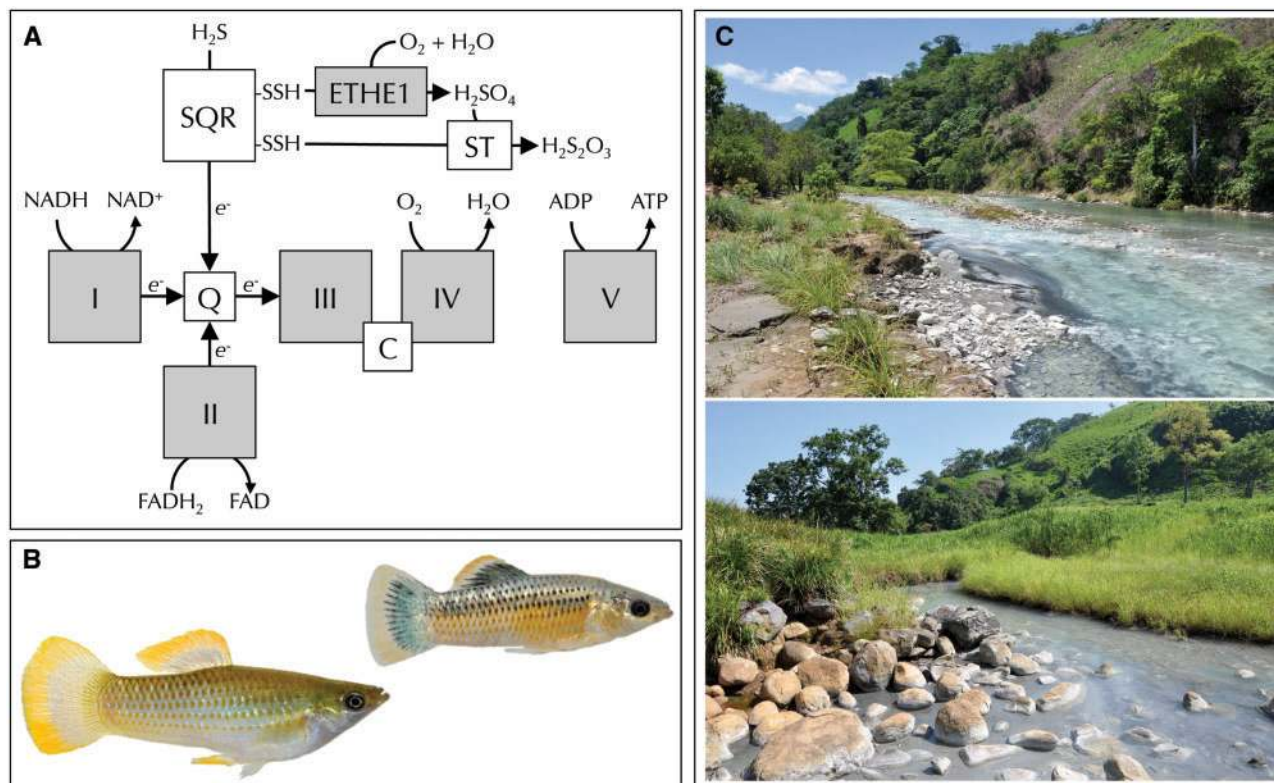
Fig. 1. (*A*) Primary enzymes involved in the oxidative phosphorylation (OXPHOS) pathway of the mitochondrial respiratory chain, which represents a nexus of H_2S toxicity and detoxification. H_2S is toxic because it blocks COX (complex IV) of the respiratory chain. H_2S is detoxified in mitochondria through pathways linked to the respiratory chain. Specifically, sulfide oxidation to thiosulfate is mediated by SQR, a sulfur dioxygenase (ETHE1), and a sulfur transferase (ST). SQR feeds electrons into the respiratory chain, which are ultimately transferred to oxygen by complex IV. (*B*) Representative specimens of the *Poecilia mexicana* species complex, including a male from a nonsulfidic population (left) and a male from a sulfide spring populations (right). (*C*) Representative sulfide springs in the Rio Puyacatengo (top) and Rio Pichucalco (bottom) drainages.

population pairs residing in sulfidic and nonsulfidic habitats? 2) Have gene expression patterns in replicated sulfide spring populations changed in convergence? 3) Is there any evidence for potentially adaptive changes in gene expression? 4) Do shared differentially expressed genes provide any insights about potential mechanisms underlying sulfide tolerance?

Results and Discussion

Transcriptome Assembly

Sequencing the gill tissue transcriptomes on an Illumina Hiseq platform yielded over 265 million reads for 35 individual fish collected from sulfidic and nonsulfidic habitats in the Tacotalpa, Puyacatengo, and Pichucalco river drainages (supplementary table S1 and fig. S1, Supplementary Material online). Nonsulfidic populations and sulfide spring populations in the Tacotalpa and Puyacatengo drainages nominally belong to the widespread species *P. mexicana*; the sulfide spring population in the Pichucalco drainage has been described as a highly endemic, distinct species (*P. sulphuraria*) (Palacios et al. 2013). Mapping against the reference genome of *Xiphophorus maculatus* (Schartl et al. 2013), a closely related species in the same family (Poeciliidae), resulted in a *P. mexicana* gill transcriptome that was composed of 35,468 transcripts from 29,143 unique loci (supplementary table S2, Supplementary Material online).

Based on comparison with the *X. maculatus* reference annotation set, 8,503 loci were putatively unique to *P. mexicana*. When limiting the analyses to the longest transcript from each locus, the transcriptome size was 71,518,404 bp, with an N50 of 3,694 bp and a genome size of approximately 860 Mbp based on C-value estimates (Rasch et al. 1970). The longest transcript was 66,752 bp in length, stemming from the gene coding for the largest known protein (Titin; Opitz et al. 2003). This indicated that our analysis effectively captured even long transcripts present in the transcriptome. A BLAST search found that 22,566 of the 29,143 unique loci (77.4%) had matches in the SwissProt database (see supplementary table S3, Supplementary Material online, for complete annotation individual transcripts). These represented 17,021 unique SwissProt records, 13,127 of which were associated with a Gene Ontology (GO) term (Gene Ontology Consortium 2004).

Variation in Gene Expression

Hierarchical cluster analysis of the top 10,000 expressed genes recovered three major clusters, which did not primarily correspond to a geographical pattern in gene expression that could be related to our sampling scheme (i.e., clustering by river drainage). Instead, individuals clustered largely by habitat type (fig. 2). Individuals from nonsulfidic habitats appeared in a single cluster. Although there was some geographic
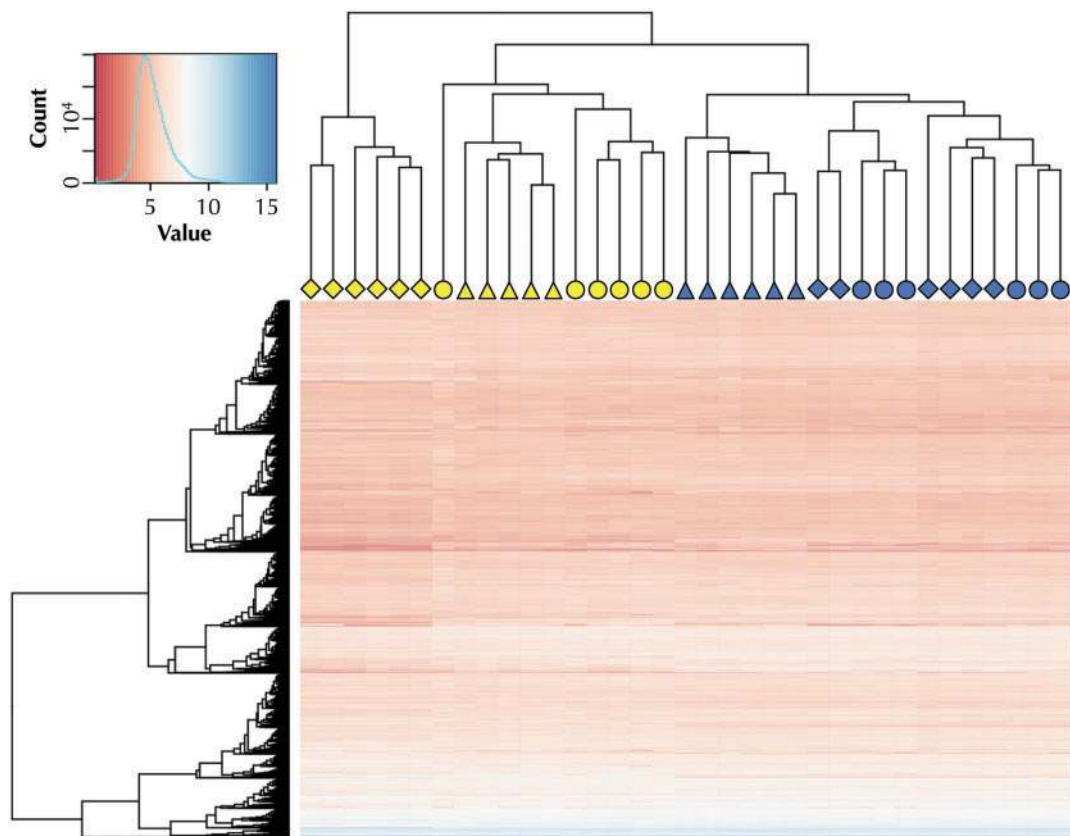
FIG. 2. Results of a hierarchical cluster analysis and heat map showing expression variation in the 10,000 top expressed genes. Symbol shapes designate the population of origin for each individual in the analysis (○: Tacotalpa drainage; △: Puyacatengo drainage; ◇: Pichucalco drainage). Yellow symbols represent individuals from sulfide springs, blue symbols those from nonsulfidic reference habitats.

structuring among nonsulfidic fish (Puyacatengo individuals were contained in a single subcluster), there was no apparent differentiation between fish from the Tacotalpa and Pichucalco drainages. This indicated that gene expression patterns in nonsulfidic habitats were relatively similar irrespective of river drainage, and considering that the Tacotalpa and Pichucalco drainages are most distant physically, geographic isolation alone likely played a minor role in driving population differences. In contrast, individuals from sulfide spring populations appeared in two separate clusters. One cluster only included individuals from the Pichucalco drainage (*P. sulphuraria*), which exhibited the most distinct gene expression patterns. The other cluster consisted of individuals from the Tacotalpa and Puyacatengo drainages, which—with the exception of an outlier individual from the Tacotalpa drainage—were arranged in distinct subclusters. General patterns recovered by the hierarchical cluster analysis were also evident in results from ordination of gene expression data (supplementary fig. S2, Supplementary Material online).

Weighted gene correlation network analysis (WGCNA) of the top 10,000 expressed genes revealed 15 modules of coexpressed genes (fig. 3A). Ten of the 15 modules were significantly correlated with habitat type (presence or absence of H$_2$S), with modules 5 and 10 exhibiting correlation coefficients >0.9 (fig. 3B). These modules primarily contained genes associated with enzymatic H$_2$S detoxification and processing of sulfur compounds (positively correlated with the presence

of H$_2$S, module 10), or with genes associated with immune function as well as cell migration and chemotaxis (negatively correlated with the presence of H$_2$S, module 5). In contrast, there were much fewer significant associations between any modules and specific river drainages or specific populations, and correlation coefficients were considerably <0.9. This again supports that geographic distance per se plays a minor role in shaping population differences in gene expression and indicates that the presence or absence of H$_2$S is a main driver of gene expression networks as well as connectivity among genes.

Comparison of gene expression between individuals from the three paired sulfidic and nonsulfidic sites revealed significant evidence for differential expression in each of the river drainages (fig. 4). Overall, 1,626 transcripts were significantly upregulated and 1,827 downregulated in sulfide spring fish in at least one of the river drainages (supplementary table S4, Supplementary Material online). Nonetheless, the number of differentially expressed genes was not equally distributed across different river drainages. Gene expression differences were least pronounced in the Puyacatengo (303 upregulated and 336 downregulated genes) and the Tacotalpa drainages (494 upregulated and 493 downregulated), and most pronounced in the Pichucalco drainage (1,215 upregulated and 1,420 downregulated). The vast majority of gene expression differences between sulfidic and nonsulfidic populations was unique to a particular drainage; 82% of differentially expressed
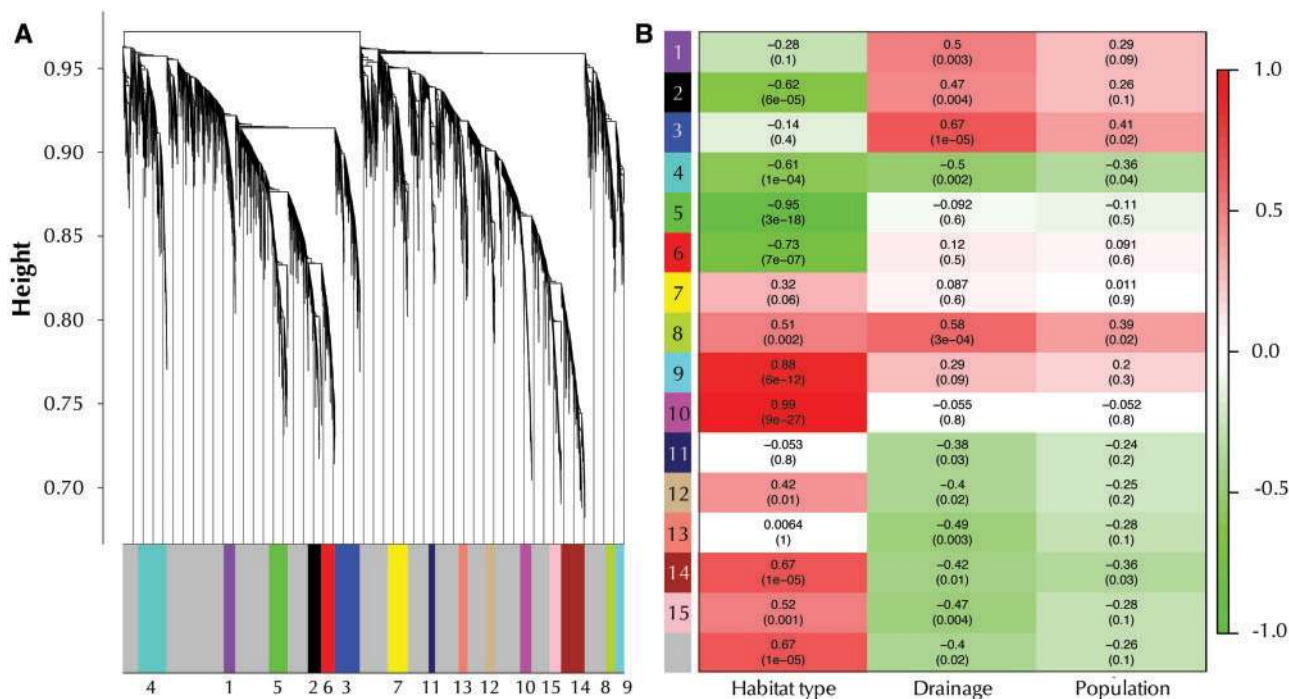
FIG. 3. Results of WGCNA of the top 10,000 expressed genes. (A) Average linkage clustering tree based on topological overlap distances in gene expression patterns of sulfidic and nonsulfidic fish. Branches of the dendrogram correspond to modules, as shown in the color bar below. Note branches associated with the gray color represent genes that are not associated with any module. (B) Correlation between module eigenvalues and habitat type (presence or absence of H_2S), river drainage, and population (collection site). Each row represents a module corresponding to color and identification number. Reported are Pearson correlation coefficients (on the top of each cell) and P values (on the bottom in parentheses). Cell coloration represents the correlation value according to the scale bar on the right.

transcripts were upregulated and 81% downregulated in only one of the three population pairs (fig. 5). Populations in the Pichucalco drainage exhibited the highest number of unique gene expression differences (951 unique upregulated and 1,139 unique downregulated transcripts), followed by the populations in the Tacotalpa (305 upregulated and 241 downregulated) and the Puyacatengo (81 upregulated and 97 downregulated) drainages.

The differences in the number of unique differentially expressed genes in each of the drainages largely corresponded to previously documented differences in the divergence times between different pairs of sulfidic and nonsulfidic populations, which is consistent with neutral drift driving a large portion of gene expression variation among populations (Whitehead and Crawford 2006). The sulfide spring population in the Pichucalco drainage represents the oldest (~300,000 years) and most distinct lineage and shares little gene flow with populations from adjacent nonsulfidic habitats (Plath et al. 2013; Pfenninger et al. 2014). Unlike sulfide spring populations in the other river drainages, they are not closely related to *P. mexicana* in adjacent nonsulfidic habitats, but rather to populations in Northern Mexico (Tobler et al. 2011; Palacios et al. 2013). Hence, distinctness of gene expression patterns in the sulfide spring population of the Pichucalco river drainage likely reflects phylogenetic divergence. In contrast, populations in the Tacotalpa and Puyacatengo drainages have colonized sulfide springs more recently (<50,000 years ago; Pfenninger et al. 2014). Sulfide

springs in these drainages have been colonized independently (Tobler et al. 2011; Palacios et al. 2013), and sulfidic populations remain connected to adjacent nonsulfidic populations by low rates of gene flow (Plath et al. 2013).

Overall, these results suggest that the presence of H_2S and historical patterns of population connectivity have interacted to shape population differences in gene expression patterns across small spatial scales. Accordingly, there was little evidence that convergent evolution has shaped genome-wide gene expression patterns in different sulfide spring populations. The absence of convergence at the transcriptome level maybe due to several, nonmutually exclusive reasons (Kaeuffer et al. 2012). First, the documented variation in gene expression may be selectively neutral and not affect fitness along the environmental gradient from sulfide springs to regular freshwater habitats. Variation in gene expression hence could reflect the effects of genetic drift that causes random differences among populations (Whitehead and Crawford 2006). Second, variation in gene expression may be related to population attributes and sources of selection that are unrelated to the presence or absence of H_2S per se. For example, variation in age structure, reproductive status, parasitization, and the presence or absence of other abiotic and biotic environmental factors could all affect transcription of genes (Naumova et al. 2012; Choi et al. 2014; Yu et al. 2014; McTaggart et al. 2015; Whittington et al. 2015), potentially causing unique patterns of gene expression in individual populations. Finally, absence of convergence could be a
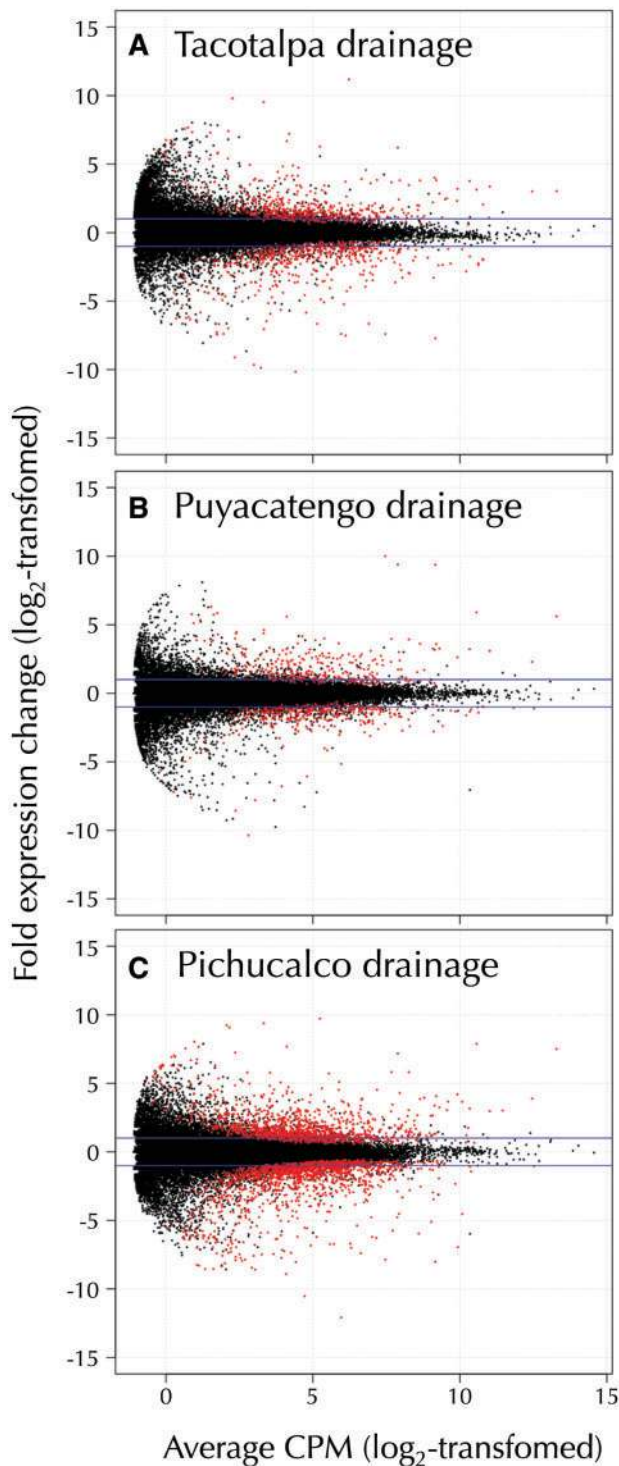
Fig. 4. Volcano plots depicting the fold change in gene expression (log_2-transformed) between the sulfidic and nonsulfidic populations as a function of the concentration of each transcript (in counts per million [CPM], log_2-transformed). Transcripts with evidence for significant differential expression (FDR $\leq$ 0.001) are colored in red. The horizontal blue lines indicate a 4-fold difference in transcript abundance. Results are shown separately for the Tacotalpa (A), Puyacatengo (B), and Pichucalco (C) river drainages each of the river drainages investigated (panels A–C).

consequence of different mechanisms underlying adaptation in independent lineages of sulfide spring fishes (Wilkens and Strecker 2003; Hoekstra et al. 2006; Losos 2011). Although
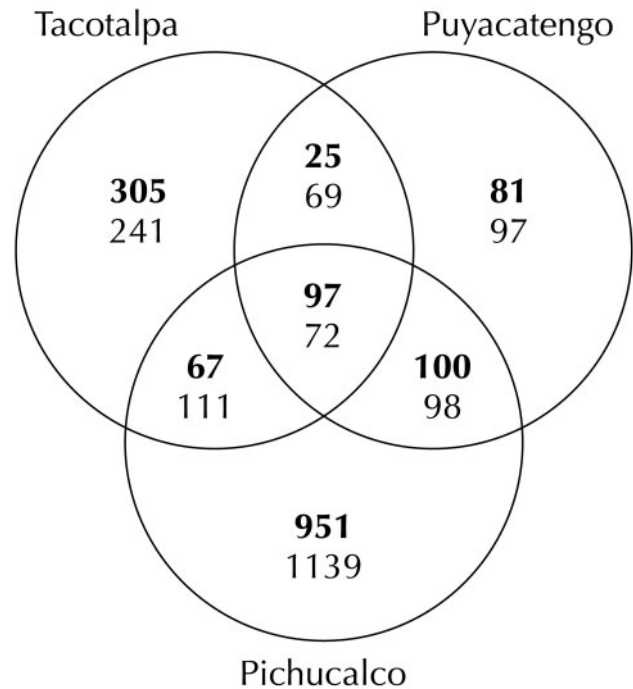

Fig. 5. Venn diagram depicting shared and unique variation in gene expression between sulfidic and nonsulfidic populations across three river drainages. Numbers in each section correspond to the number of differentially expressed transcripts derived from estimations of gene expression in eXpress. The number of upregulated transcripts are listed on top (in bold font) and the number of downregulated transcript below.

field-based studies of broad gene expression patterns are inadequate to differentiate how these factors may shape convergent evolution, identification of shared differentially expressed genes and vetting of functional annotations against a priori predictions can nonetheless provide insights about mechanisms of adaptation.

Shared Differentially Expressed Genes and Transcripts

Even though genome-wide variation in gene expression patterns showed little evidence for convergence, there was a small, but significant number of transcripts that were consistently differentially expressed in the same direction across two or more sulfidic and nonsulfidic population pairs. Of the 1,626 transcripts that showed evidence for upregulation in at least one of the drainages, 289 (18%) were shared between two drainages and 97 (6%) among all three drainages (table 1). Of the 1,827 transcripts that showed evidence for downregulation in at least one of the drainages, 350 (19%) were shared between two drainages and 71 (4%) among all drainages (table 2). Transcripts that are consistently differentially expressed among all three population pairs represent primary candidates for playing a role in adaptation to sulfide spring environments (Ghalambor et al. 2015), and the following discussion of the potential functional consequences of variation in gene expression—unless noted otherwise—refers only to differentially expressed genes shared among all population pairs.

Table 1. List of Genes that Were Consistently Upregulated in Sulfide Spring Fishes of All Drainages.

Gene ID	Accession Number	Gene Name	Xmac Annotation	Number of Transcripts
TCONS_00000049	O75452	Retinol dehydrogenase 16	rdh1	1
TCONS_00002627	Q16878	Cysteine dioxygenase type 1	CDO1	1
TCONS_00002704	Q00610	Clathrin heavy chain 1	CLTCL1	2
TCONS_00002706	P53007	Tricarboxylate transport protein	si:dkey-178e17.1	1
TCONS_00002887	n/a		XLOC_002625	1
TCONS_00004013	Q9Y6N5	Sulfide:quinone oxidoreductase	sqrdl	1
TCONS_00004833	Q5BKX6	Solute carrier family 45 member 4	SLC45A4 (2 of 2)	1
TCONS_00005215	Q9BRT3	Migration and invasion enhancer 1	XLOC_004459	1
TCONS_00006194	P02144	Myoglobin	Mb	1
TCONS_00006595	Q8NHV1	GTPase IMAP family member 7	zgc:152658	1
TCONS_00006596	P10620	Microsomal glutathione S-transferase 1	mgst1.2	1
TCONS_00006640	P52294	Importin subunit alpha-5	kpna1	1
TCONS_00006715	Q5T6X4	Protein FAM162B	fam162a	3
TCONS_00007401	B7ZAP0	RAB GTPase activating protein 1-like	rabgap1l2	1
TCONS_00008470	P15121	Aldose reductase	akr1b1	1
TCONS_00010008	O00483	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex	ndufa4l2	1
TCONS_00010278	P41236	Protein phosphatase inhibitor 2	ppp1r2	1
TCONS_00010396	Q7KZN9	Cytochrome *c* oxidase assembly protein COX15	cox15	1
TCONS_00011484	Q96PR1	Potassium voltage-gated channel subfamily C member 2	kcnc2	1
TCONS_00011526	P58743	Prestin	slc26a5	1
TCONS_00011778	P21266	Glutathione S-transferase Mu 3	ENSXMAG00000009961	1
TCONS_00012045	Q12805	EGF-containing fibulin-like extracellular matrix protein 1	EFEMP1	1
TCONS_00012336	Q9H628	Ras-related and estrogen-regulated growth inhibitor-like protein	zgc:171704	2
TCONS_00012508	P53985	Monocarboxylate transporter 1	slc16a1	1
TCONS_00012770	P00390	Glutathione reductase	Gsr	1
TCONS_00013706	P99999	Cytochrome *c*	XLOC_011171	1
TCONS_00014743	Q6ZQY3	Acidic amino acid decarboxylase GADL1	GADL1 (1 of 2)	1
TCONS_00014769	Q6ZQY3	Acidic amino acid decarboxylase GADL1	GADL1 (2 of 2)	1
TCONS_00014831	Q96P48	Arf-GAP with Rho-GAP domain, ANK repeat, and PH domain-containing protein 1	ENSXMAG00000006436	1
TCONS_00014991	Q16822	Phosphoenolpyruvate carboxykinase	pck2	1
TCONS_00015284	P27144	Adenylate kinase 4	ak4	2
TCONS_00015294	Q9BYD5	Cornifelin	ponzr10 (4 of 5)	1
TCONS_00015352	Q8TDN7	Alkaline ceramidase 1	acer1	1
TCONS_00015381	Q96B33	Claudin-23	cldn23	2
TCONS_00016368	Q16831	Uridine phosphorylase 1	upp1	1
TCONS_00016470	P60174	Triosephosphate isomerase	tpi1a	1
TCONS_00017022	Q9UBX3	Mitochondrial dicarboxylate carrier	SLC25A10	2
TCONS_00017053	Q86WA9	Sodium-independent sulfate anion transporter	slc26a11	1
TCONS_00017057	O15525	Transcription factor MafG	MAFG (2 of 2)	1
TCONS_00017983	P09972	Fructose-bisphosphate aldolase C	aldocb	2
TCONS_00018345	P25325	3-mercaptopyruvate sulfurtransferase	zgc:162544 (1 of 2)	1
TCONS_00018346	P25325	3-mercaptopyruvate sulfurtransferase	zgc:162544 (2 of 2)	1
TCONS_00018398	Q9UQK1	Protein phosphatase 1 regulatory subunit 3C	ppp1r3ca	2
TCONS_00019672	Q9UPQ4	Tripartite motif-containing protein 35	TRIM35 (2 of 15)	1
TCONS_00020083	P10599	Thioredoxin	ENSXMAG00000001739	1
TCONS_00020502	P35558	Phosphoenolpyruvate carboxykinase	pck1	1
TCONS_00021089	Q684P5	Rap1 GTPase-activating protein 2	rap1gap2a	1
TCONS_00021347	P07339	Cathepsin D	ctsd	1
TCONS_00021997	O95571	Persulfide dioxygenase ETHE1	ETHE1	2
TCONS_00022001	O95571	Persulfide dioxygenase ETHE1	ETHE1	3
TCONS_00022084	Q86YN6	Peroxisome proliferator-activated receptor gamma coactivator 1-beta	ENSXMAG00000019179	1
TCONS_00022105	Q5VV67	Peroxisome proliferator-activated receptor gamma coactivator-related protein 1	XLOC_017890	1
TCONS_00022352	n/a		XLOC_018095	1
TCONS_00022678	P09105	Hemoglobin subunit theta-1	ENSXMAG00000000181	2
TCONS_00022913	Q7L5N7	Lysophosphatidylcholine acyltransferase 2	LPCAT4 (1 of 2)	1
TCONS_00022971	P00918	Carbonic anhydrase	cahz	2
TCONS_00023196	Q53RY4	Keratinocyte-associated protein 3	tmem54	1
TCONS_00023347	Q9Y241	HIG1 domain family member 1A	hig1	1
TCONS_00023958	Q15120	Pyruvate dehydrogenase kinase, isozyme 3	pdk3a	1
TCONS_00024060	Q56VL3	OCIA domain-containing protein 2	ociad2	1

Table 1. Continued

Gene ID	Accession Number	Gene Name	Xmac Annotation	Number of Transcripts
TCONS_00025513	P25325	3-mercaptopyruvate sulfurtransferase	ENSXMAG00000008042	1
TCONS_00025981	P06733	Alpha-enolase	eno1a	1
TCONS_00026107	n/a		abch1	1
TCONS_00026499	O60675	Transcription factor MafK	maff	1
TCONS_00026767	P0CG29	Glutathione S-transferase theta-2	ENSXMAG00000006919	4
TCONS_00026929	P48506	Glutamate-cysteine ligase catalytic subunit	gclc	1
TCONS_00028224	n/a		XLOC_022849	1
TCONS_00028623	n/a		XLOC_023166	1
TCONS_00028908	n/a		XLOC_023394	1
TCONS_00030449	Q9Y6M7	Sodium bicarbonate cotransporter 3	SLC4A7 (1 of 2)	2
TCONS_00030815	Q96FC7	Phytanoyl-CoA hydroxylase-interacting protein-like	ENSXMAG00000007363	1
TCONS_00033175	Q06830	Peroxiredoxin 1	prdx1	3
TCONS_00034020	Q8N4X5	Actin filament-associated protein 1-like 2	si:dkey-220o5.5	1
TCONS_00034143	O15394	Neural cell adhesion molecule 2	ENSXMAG00000017303	1
TCONS_00034516	n/a		XLOC_028149	1
TCONS_00034517	P07911	Uromodulin	XLOC_028150	1

NOTE.—Provided are the GenBank accession number for the top BLAST hit, the gene name, the corresponding identifier in the *Xiphophorus maculatus* (Xmac) reference genome, and the number of transcripts with evidence for upregulation for each gene. For additional information, see supplementary table S4, Supplementary Material online. n/a, not applicable.

GO enrichment analysis indicated significant overrepresentation of certain GO terms associated with shared differentially expressed genes relative to the background set (all 13,127 transcripts associated with a GO term). Specifically, shared upregulated genes were enriched for 60 GO terms associated with biological processes, 16 associated with molecular function, and 12 associated with cellular components (supplementary table S5 and figs. S3–S5, Supplementary Material online); shared downregulated genes were enriched for 13 terms associated with biological processes and 3 associated with cellular components (supplementary table S6 and figs. S6 and S7, Supplementary Material online). As predicted, the mitochondrial respiratory chain and other parts of mitochondria (GO:0070469, as well as GO:0044429 and associated terms) belonged to the cellular components with evidence for significant enrichment in shared differentially expressed genes, reflecting the fact that these components represent a nexus in H_2S toxicity and detoxification (Cooper and Brown 2008; Hildebrandt and Grieshaber 2008). In the following sections, we focus on the discussion of enriched GO terms associated with biological processes in the context of the three general mechanisms potentially driving elevated tolerance to environmental H_2S.

Evidence for Differential Expression of Genes that Could Mediate H_2S Exclusion

Increased tolerance to high environmental concentrations of H_2S may be mediated by the formation of barriers that minimize diffusion into the body (Vismann 1991). Our analyses have provided no evidence suggesting that sulfide spring fishes differentially express genes that could be associated with decreasing the flux of H_2S. In fact, genes encoding for claudins and keratins (table 2), which are associated with the establishment of skin barriers (Fuchs and Weber 1994; Günzel and Yu 2013) and enriched relative to the background set (GO:0061436), were consistently downregulated and not upregulated in sulfide spring populations. In addition, shared differentially expressed genes associated with the function of cellular membranes (basolateral and apical plasma membranes; GO:0016323 and GO:0016324) were primarily involved in ion transport during osmoregulatory processes (see below).

The apparent lack of modifications to the integumentary system and respiratory surfaces is not entirely unexpected, because there is likely a trade-off between H_2S exclusion and oxygen acquisition. Barriers that reduce diffusion of H_2S likely reduce the diffusion of oxygen across gill epithelia. This trade-off is also reflected in the expression of a morphological trait, where sulfide spring fishes exhibit higher gill surface areas per unit body mass than fish from nonsulfidic environments (Tobler et al. 2011). Hence, effective exclusion of H_2S may be constrained by organisms' need for oxygen acquisition in the hypoxic waters of sulfide springs. Evidence for structural modifications that mediate H_2S exclusion from the body has also been scarce in other systems (see Bagarinao 1992 for a review), and behavioral adaptations, including alternative respiratory strategies that minimize the contact of respiratory surfaces with H_2S-rich water, may be more important to minimize the influx of H_2S into the body (Abel et al. 1987; Brauner et al. 1995; Greenway et al. 2014).

Evidence for Differential Expression of Genes that Could Mediate Maintenance of H_2S Homeostasis

Increased tolerance to high environmental concentrations of H_2S may be mediated by an increased ability of sulfide spring fishes to maintain endogenous H_2S homeostasis despite its continuous influx from the environment. Indeed, we found consistent upregulation of genes involved in enzymatic H_2S oxidation (GO:0019418 and associated terms), glutathione metabolism (GO:0006749 and associated terms), as well the transport of oxidized sulfur species (GO:0008272 and associated terms). Genes associated

Table 2. List of Genes that Were Consistently Downregulated in Sulfide Spring Fishes of All Drainages.

Gene ID	Accession Number	Gene Name	Xmac Annotation	Number of Transcripts
TCONS_00000141	Q9BQR3	Serine protease 27	zgc:165423	3
TCONS_00000307	P05787	Keratin, type II cytoskeletal 8	wu:fd11e11	1
TCONS_00000716	O43865	Adenosylhomocysteinase 2	ahcyl1 (2 of 3)	2
TCONS_00001614	n/a		XLOC_001505	1
TCONS_00003728	O43520	Phospholipid-transporting ATPase IC	atp8b3	1
TCONS_00003846	P51589	Cytochrome P450 2J2	ENSXMAG00000005929	1
TCONS_00004097	n/a		XLOC_003586	1
TCONS_00004170	Q13823	Nucleolar GTP-binding protein 2	gnl2	1
TCONS_00004688	n/a		XLOC_004052	1
TCONS_00005037	O00483	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4	NDUFA4 (1 of 2)	2
TCONS_00005679	O94900	Thymocyte selection-associated high mobility group box protein TOX	Tox	1
TCONS_00005785	P51857	3-oxo-5-beta-steroid 4-dehydrogenase	AKR1D1 (1 of 2)	1
TCONS_00006859	P05787	Keratin, type II cytoskeletal 8	krt4	1
TCONS_00007568	Q96IP4	Protein FAM46A	FAM46B (2 of 2)	1
TCONS_00007657	Q92539	Phosphatidate phosphatase LPIN2	LPIN2 (1 of 3)	1
TCONS_00008606	n/a		XLOC_007101	1
TCONS_00010147	P62508	Estrogen-related receptor gamma	Esrrgb	1
TCONS_00010450	O43490	Prominin-1	prom2	2
TCONS_00010764	Q8IXH8	Cadherin-like protein 26	CDH26 (2 of 4)	1
TCONS_00011212	Q5FVE4	Long-chain-fatty-acid-CoA ligase ACSBG2	acsbg1	1
TCONS_00011532	Q9HBU6	Ethanolamine kinase 1	etnk1	1
TCONS_00011703	Q96HN2	Adenosylhomocysteinase 3	ahcyl1 (3 of 3)	1
TCONS_00011898	Q9UPQ4	Tripartite motif-containing protein 35	trim35-12	1
TCONS_00012317	O60825	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 2	pfkfb2a	1
TCONS_00013668	Q9NZW5	MAGUK p55 subfamily member 6	mpp6b	1
TCONS_00014144	P08779	Keratin, type I cytoskeletal 16	krt97	3
TCONS_00014168	Q9GZV3	High affinity choline transporter 1	ENSXMAG00000017837	1
TCONS_00015517	Q9HBA0	Transient receptor potential cation channel subfamily V member 4	trpv4	1
TCONS_00015752	Q96QD5	DEP domain-containing protein 7	depdc7	1
TCONS_00016045	Q9NXI6	RING finger protein 186	im:7152348	1
TCONS_00016260	Q99259	Glutamate decarboxylase 1	gad1b	1
TCONS_00017126	n/a		XLOC_013895	1
TCONS_00018061	Q6UXB0	Protein FAM131A	FAM131A	1
TCONS_00018951	P52943	Cysteine-rich protein 2	CRIP3	1
TCONS_00019566	Q02218	2-oxoglutarate dehydrogenase	OGDH (2 of 2)	2
TCONS_00019615	Q8WVX9	Fatty acyl-CoA reductase 1	FAR2	1
TCONS_00019789	O14745	Na(+)/H(+) exchange regulatory cofactor NHE-RF1	SLC9A3R1 (2 of 2)	1
TCONS_00019956	Q86UD5	Mitochondrial sodium/hydrogen exchanger 9B2	si:dkey-162b23.4	2
TCONS_00020193	Q9NSE2	Cytokine-inducible SH2-containing protein	Cishb	1
TCONS_00020392	Q96S86	Hyaluronan and proteoglycan link protein 3	hapln3	1
TCONS_00020515	Q8N5B7	Ceramide synthase 5	cers5	1
TCONS_00021208	Q02779	Mitogen-activated protein kinase 10	map3k10	1
TCONS_00021855	Q04912	Macrophage-stimulating protein receptor	mst1rb	1
TCONS_00022897	P55017	Solute carrier family 12 member 3	ENSXMAG00000000951	2
TCONS_00023777	Q9NUV9	GTPase IMAP family member 4	ENSXMAG00000019889	2
TCONS_00024008	Q0VF96	Cingulin-like protein 1	CGNL1	1
TCONS_00024654	O76013	Keratin, type I cuticular Ha6	zgc:171226 (2 of 2)	1
TCONS_00024675	P08727	Keratin, type I cytoskeletal 19	zgc:171226 (1 of 2)	2
TCONS_00024983	O95267	RAS guanyl-releasing protein 1	rasgrp4	2
TCONS_00025014	Q9P0J1	[Pyruvate dehydrogenase [acetyl-transferring]]-phosphatase 1	pdp1	1
TCONS_00025959	Q8TD43	Transient receptor potential cation channel subfamily M member 4	trpm4a	1
TCONS_00027843	Q6NUK1	Calcium-binding mitochondrial carrier protein SCaMC-1	SLC25A24 (2 of 2)	1
TCONS_00028646	O14493	Claudin-4	Cldne	2
TCONS_00028684	Q9Y5Z4	Heme-binding protein 2	soul5	2
TCONS_00030067	Q8N1W1	Rho guanine nucleotide exchange factor 28	arhgef28	1
TCONS_00031911	P58107	Epiplakin	XLOC_025876	1
TCONS_00032188	P36269	Gamma-glutamyltransferase 5	GGT5 (3 of 3)	1

Note.—Provided are the GenBank accession number for the top BLAST hit, the gene name, the corresponding identifier in the *Xiphophorus maculatus* (Xmac) reference genome, and the number of transcripts with evidence for downregulation for each gene. For additional information, see supplementary table S4, Supplementary Material online.

with H$_2$S oxidation primarily belonged to the SQR pathway (including *SQR*, *persulfide dioxygenase* [*ETHE1*], and a *mitochondrial dicarboxylate carrier*; table 1), which represents the primary route of enzymatic H$_2$S detoxification in metazoans (Hildebrandt and Grieshaber 2008; Shahak and Hauska 2008). Upregulation of enzymes involved in glutathione metabolism (e.g., glutathione *s*-transferases and glutathione reductase) is also consistent with increased H$_2$S detoxification capability, because sulfur molecules sequestered by SQR may be transferred to glutathione (Jackson et al. 2012). Upregulation of genes associated with enzymatic H$_2$S oxidation has been documented in other animals (Ma et al. 2012; Liu et al. 2015) and human tissues (Lagoutte et al. 2010; Mimoun et al. 2012) exposed to elevated H$_2$S concentrations. A recent laboratory study also indicated that sulfide spring *P. mexicana* from one river drainage investigated here (Tacotalpa) retained higher constitutive SQR expression than reference populations even in the absence of H$_2$S (Tobler et al. 2014), and we verified the presence of differential amounts of protein of SQR using western blot (supplementary fig. S8, Supplementary Material online). Furthermore, a population genomic study has also uncovered significant signatures of positive selection on *SQR* in some sulfide spring populations of *P. mexixana* (Pfenninger et al. 2015), perhaps suggesting that there are both transcriptional and structural modifications to components of this pathway. Collectively, these results suggested that an increased expression of genes involved in H$_2$S detoxification likely contribute to the maintenance of low endogenous concentrations, and adaptation in sulfide spring fishes likely involves a higher regulatory capacity during environmental H$_2$S exposure.

In contrast, we found no evidence that an increased ability to maintain low endogenous H$_2$S concentration may be linked to lower rates of endogenous production, which is primarily mediated by the processing of sulfur-containing amino acids (Kabil et al. 2014). Several genes associated with the metabolic processing of sulfur amino acids (GO:0000096 and associated terms) were consistently upregulated in sulfide spring fishes. These included *cysteine dioxygenase* and *mercaptopyruvate sulfurtransferase* (table 1), the latter of which has been directly implicated in the endogenous production of H$_2$S (Stipanuk 2004; Stipanuk and Ueki 2011; Módis et al. 2012). Although the enzymatic processing of sulfur amino acids can result in the production of a wide variety of metabolites and may not precipitate in differences in endogenous H$_2$S production between sulfidic and nonsulfidic fish populations, these findings suggested that endogenous production of H$_2$S in sulfide spring fish is probably higher and certainly not lower than in close relatives from nonsulfidic habitats. This is consistent with a previous study that documented an increase in the expression of *cystathionine γ lyase*, a primary producer of endogenous H$_2$S (Stipanuk and Ueki 2011), upon short-term exposure to H$_2$S both in sulfidic and nonsulfidic populations (Tobler et al. 2014). It remains to be investigated whether upregulation of genes associated with the processing of sulfur-containing amino acids and endogenous H$_2$S production represents a maladaptive response to environmental H$_2$S exposure, or whether it contributes to H$_2$S processing and adaptation in ways not currently understood.

Evidence for Differential Expression of Genes that Could Reduce Side Effects of H$_2$S Toxicity

Increased tolerance to high environmental concentrations of H$_2$S may be mediated by modification of toxicity targets that allow for proper organismal function, even endogenous H$_2$S concentrations are elevated. H$_2$S toxicity is primarily mediated through its binding to COX, effectively halting oxidative phosphorylation (OXPHOS) in the mitochondrial respiratory chain. Our analysis indicated significant enrichment and upregulation of genes associated with the transfer of electrons from *cytochrome c* to oxygen (GO:0006123), which is mediated by COX. This included upregulation of *cytochrome c* (table 1), which receives electrons from coenzyme Q through complex III of the respiratory chain (Vedel et al. 1999). Although most proteins associated with the respiratory chain are organized in complexes, anchored in the inner membrane of mitochondria, and can only transfer electrons, cytochrome *c* is concentrated in the intermembrane space, only loosely associated with the inner membrane, and can permanently hold electrons until being processed by COX (Hatefi 1985; Saraste 1999). Upregulation of *cytochrome c* may be adaptive in sulfidic environments by buffering against deviations in the stoichiometric balance between different protein complexes of the respiratory chain (Lemos et al. 2004). Deviations in the stoichiometric balance are expected during exposure to environmental H$_2$S both because of COX blockage and because SQR—in addition to complexes I and II—transfers electrons to coenzyme Q (Lagoutte et al. 2010). Supplementary electron flow has been linked to oxidative stress (see below), and upregulation of *cytochrome c* could alleviate bottlenecks in electron flow. In addition, enrichment of genes associated with the transfer of electrons from cytochrome *c* to oxygen include proteins that are critical for the assembly of the COX enzyme (Glerum et al. 1997), although the adaptive significance of this transcriptional change remains unclear. A previous study already indicated that two lineages of sulfide spring fish in the *P. mexicana* complex (Puyacatengo and Pichucalco drainages) have evolved a COX that is resistant to the toxic effects of H$_2$S and allows for the retention of high enzyme activity in the presence of H$_2$S (Pfenninger et al. 2014). Consequently, there is evidence for both structural and transcriptional variation between sulfidic and nonsulfidic populations that are likely related to the maintenance of electron flow in the respiratory chain and the minimization of oxidative stress in the presence of H$_2$S.

Through its interference with COX function and OXPHOS, H$_2$S also has profound impacts on other aspects of energy metabolism. Even though there was no evidence for enrichment in other genes involved in the mitochondrial respiratory chain, it is important to note that there was evidence for consistent differential expression in other genes associated with OXPHOS and mitochondrial bioenergetics (TCONS_00005037 and TCONS_00005038, which are part of a subunit of NADH dehydrogenase [complex I in fig. 1A];

TCONS_00019566 and TCONS_00019567, which encode for oxoglutarate dehydrogenase, an enzyme of the citric acid cycle). Inspection of metabolic pathway maps generated with iPath indicated that components of complexes I and II of the respiratory chain (fig. 1A), which harvest electrons from organic substrates, were consistently downregulated in all sulfide spring populations (supplementary fig. S9, Supplementary Material online). This is consistent with a potential shift in the relative importance of H_2S and organic substrates as electron donors for OXPHOS (Völkel and Grieshaber 1997; Goubern et al. 2007). In addition, fish from sulfidic habitats upregulated and showed enrichment for a variety of genes involved in carbohydrate metabolism, particularly glycolysis (GO:0061621 and associated terms) and gluconeogenesis (GO:0006094 and associated terms). Glycolysis is a primary pathway producing ATP in an oxygen (OXPHOS)-independent manner through the processing of glucose, and gluconeogenesis generates glucose from a variety of carbon substrates (Richards 2009). Increases in glycolysis are commonly observed in organisms exposed to hypoxic conditions (Soengas and Aldegunde 2002; Richards 2009; Speers-Roesch et al. 2013). Potential upregulation of anaerobic metabolism in sulfide spring fish may be linked to hypoxia in sulfidic environments (reduced aerobic ATP production due to shortages on oxygen supply; see below) and/or to the interruption of OXPHOS by H_2S (reduced aerobic ATP production due to blocking of COX). Hence, future studies will need to unravel how COX blockage and oxygen availability interact to modulate anaerobic metabolism in sulfide spring fish.

Finally, H_2S has also been shown to be a potent generator of reactive oxygen species and oxidative stress (Eghbal et al. 2004). Sulfide spring fishes exhibited a consistent upregulation of genes involved in responses to reactive oxygen species (GO:0000302) and oxidative stress in general (GO:0006979). This does not only include the genes associated with the glutathione pathway discussed above, but also peroxiredoxin and thioredoxin (table 1), which act as antioxidants (Immenschuh and Baumgart-Vogt 2005; Koharyova and Kollarova 2008). Consequently, our analyses of gene expression variation across different population of sulfide spring fishes provided evidence for modification of genes that are directly or indirectly related to the toxic effects of H_2S.

Evidence for Differential Expression of Genes Associated with Environmental Factors that Correlate with the Presence of H_2S

Sulfide springs and adjacent nonsulfidic habitats not only differ in the presence and absence of H_2S, but they vary in a suite of abiotic and biotic environmental characteristics that may act as a source of selection and shape population differences in trait expression (Tobler and Plath 2011; Greenway et al. 2014). Sulfide springs are typically characterized by having lower dissolved oxygen concentrations, a lower pH, and higher concentrations of dissolved salts than adjacent nonsulfidic habitats (Tobler et al. 2006; Rosales 2012). Differences in gene expression both between

sulfidic and nonsulfidic populations as well as among different sulfidic populations could therefore be driven by a variety of environmental factors that were not measured in this study. For example, we found enrichment in genes that have been associated with responses to pH (GO:0009268), sodium transport (GO:0006814), water loss via skin (GO:0033561), and metabolic processes (GO:0061621, GO:0006094, and associated terms), which may be related to environmental factors correlated with the presence of H_2S. Furthermore, there was evidence for enrichment in a variety of other GO terms (supplementary tables S5 and 6, Supplementary Material online), for which the putative functional links to the presence of H_2S and other environmental factors remain elusive. Future studies will have to investigate how exposure to different environmental factors separately and jointly shapes patterns of gene expression in organisms that are exposed to multifarious selective regimes.

Conclusions

Transcriptome analyses in replicated lineages of sulfide spring fishes and corresponding reference populations provided evidence for substantial variation in gene expression across small spatial scales. Genes that were consistently differentially expressed across sulfide spring populations provide promising candidates for the identification of molecular mechanisms underlying adaptation to these extreme environments, although future work will also need to scrutinize how genes that were only differentially regulated in a subset of lineages may contribute to coping with H_2S toxicity. There was clear evidence for the upregulation of genes associated with the detoxification and subsequent excretion of H_2S. At the same time, differential expression was evident for genes associated with energy metabolism and oxidative stress, two primary physiological processes affected by H_2S. Hence, modification of processes associated with detoxification and toxicity likely complement each other to mediate elevated H_2S tolerance in sulfide spring fishes. Furthermore, the mitochondrial respiratory chain appears to represent a center of adaptation to the perpetual exposure to H_2S, which is consistent with its joint role in both H_2S toxicity and detoxification. Our analyses allow for the development of testable hypotheses about biochemical and physiological mechanisms of adaptation to H_2S-rich environments. Future studies will have to focus their efforts on three key problems: 1) Identifying the drivers underlying variation in gene regulation in natural populations will require controlled laboratory experiments. Rearing fish from replicated populations under standardized conditions and exposing individuals to varying H_2S concentrations will help disentangle plastic and genetic contributions to gene expression patterns observed in the wild (Whitehead 2012). 2) Quantifying genetic variation in protein-coding genes and identifying variants that have likely been shaped by natural selection will allow testing how variation in protein structure and protein regulation interact to mediate adaptation (Hoekstra and Coyne 2007; Wray 2007). 3) Quantifying the joint functional consequences of variation in gene expression and genetic variation will provide a mechanistic

understanding of the biochemical and physiological processes that govern differences in H_2S tolerance.

Our study contributes to a broader effort to understand molecular genetic mechanisms underlying life in extreme environment (Dassanayake et al. 2011; Teets et al. 2012; Kelley et al. 2014; Kavembe et al. 2015). The findings suggest that adaptation to physiochemical stressors involves the modification of multiple, complex physiological pathways that likely complement each other. Similar results have been found in other systems, where resistance to pesticides involves modifications to the integument that slows down diffusion of toxins into the body as well as alterations of toxicity targets that reduce their binding activity (Zhu et al. 2013). This illustrates the phenomenal challenges of relating variation in specific genes and their expression to physiological performance and ultimately organismal fitness (Storz and Wheat 2010). Acknowledging the complexity of organisms conceptually and empirically during investigations of adaptation will be paramount to understand the multifarious, layered strategies organisms use to cope with stressful environmental conditions. Although a wide variety of biochemical, physiological, structural, and behavioral coping strategies have been discovered for specific physicochemical stressors (including the one studied here; Riesch et al. 2015), disentangling the relative contributions of different traits to overall organismal performance remains a key challenge that should reveal potential synergies and redundancies among strategies. Ultimately, elucidating how organisms respond and adapt to physiochemical stressors that govern life in extreme environments will be instrumental for predicting the resilience and response of species to environmental stress in the face of global environmental change (Tobler et al. 2015).

Materials and Methods

Study Sites and Sample Collection

Samples of *Poecilia* for transcriptome analyses were collected in the area around the city of Teapa (Tabasco, Mexico). Sulfide spring complexes inhabited by *Poecilia* are located in the foothills of the Sierra Madre and are distributed across three major tributaries of the Río Grijalva (from east to west: Tacotalpa, Puyacatengo, and Pichucalco drainages; supplementary fig. S1, Supplementary Material online). Sulfide springs in each drainage were colonized independently by *P. mexicana*–like ancestors (Tobler et al. 2011; Palacios et al. 2013). All populations investigated here taxonomically belong to *P. mexicana*, except for the sulfide spring population in the Río Pichucalco drainage, which has been described as a distinct and highly endemic species (*P. sulphuraria*) (Alvarez del Villar 1948; Palacios et al. 2013). In each of the drainages, we collected individual fish from one sulfide spring complex and one proximate, nonsulfidic habitat of similar size and structure (*N* = 5–6 per site; see supplementary table S7, Supplementary Material online, for details). Fish were caught with a seine (2 × 4 meters). Immediately after capture, individual fish were sacrificed, measured and weighed, and gill tissues were extracted from both sides of the body using previously sterilized scissors and forceps. Tissues were preserved in 2 ml of RNAlater (Ambion, Inc.). All experiments were approved by the Institutional Animal Care and Use Committee of Kansas State University (ACUP #3473).

RNA Isolation and RNAseq Library Construction

RNA was isolated from gills by pulverizing 50–100 mg of tissue frozen in liquid nitrogen with a Covaris Cryoprep at setting 3. RNA was then extracted with Qiagen's RNeasy Plus mini kit. PolyA+ mRNA was prepared from 50 μg total RNA using Invitrogen's Dynabeads mRNA purification kit. RNA was bound to and eluted twice from Dynabeads to minimize ribosomal RNA contamination. mRNA was fragmented to an average size of 400 nt using NEB's mRNA Fragmentation Module by incubation at 94 °C for 4 min. Fragmented mRNA was purified using Agencourt RNAClean XP beads and eluted in 12 μl ddH$_2$O. First-strand cDNA was synthesized in a 20 μl reaction using Invitrogen's double-stranded cDNA kit, primed with 1 μl of a mix of random hexamers:oligo dT primers (2 μg:1 μg), and incubated with Superscript II at 45 °C for 1 h. Double-stranded cDNA was synthesized directly from the first-strand cDNA using the NEBNext mRNA Second Strand Synthesis kit. After second-strand cDNA synthesis, the reaction was purified with Agencourt Ampure XP beads and eluted in 25 μl ddH$_2$O.

Double-stranded cDNA was used as input for Illumina sequencing library preparation with end-repair using the NEBNext end-repair kit, A-tailing with Taq polymerase, ligation with Truseq barcoded adapters, and amplification with Kapa Library Amplification Readymix. All steps were cleaned up with Ampure XP beads. RNAseq libraries were quantified on an Agilent 2100 Bioanalyzer High Sensitivity DNA chip and pooled in sets of 12 based on nanomolar (nM) concentration. Individuals were split into pools such that samples from different habitat types and drainages were sequenced together, and there was no evidence for significant lane effects. Libraries were sequenced on an Illumina HiSeq 2000 with paired-end 101 bp reads at the Stanford Center for Genomics and Personalized Medicine. Sequence data were deposited in the Sequence Read Archive on GenBank (study accession ID: PRJNA290391).

Transcriptome Assembly and Annotation

Raw RNA-seq reads from Illumina sequencing were sorted by barcode and dynamically trimmed to quality 20 using TrimGalore! (Krueger 2014). All reads shorter than 50 bases were removed, and only paired reads were used for the analysis, which resulted in removal of ~10% of sequenced bases (supplementary table S1, Supplementary Material online). Trimmed reads for each of the 35 individuals were mapped to the platyfish (*X. maculatus*) genome (version 4.4.2 release-75; Schartl et al. 2013). Mapping to the reference genome was accomplished using Stampy (version 1.0.23), which is designed for mapping reads to a divergent reference genome (Lunter and Goodson 2011). Cufflinks (version 2.2.1) was then used to extract putatively expressed regions for each individual, with the platyfish reference gene set as a guide (Trapnell et al. 2010). The number of unique loci to the *P. mexicana* data set was queried using cuffcompare. Putatively expressed

regions for each individual were merged with cuffmerge, and a FASTA file of all expressed loci was extracted using gffread, as implemented in Cufflinks. Platyfish mitochondrial transcripts were removed from the FASTA file and replaced with the appropriate *Poecilia* mitochondrial reference sequences, which included 13 protein-coding genes and the 16S ribosomal RNA sequence (GenBank: KC992995). Note that mapping reads to a de novo reference transcriptome assembly of *P. mexicana* using Trinity (see Kelley et al. 2012 for general approaches) yielded in qualitatively similar results. Mapping to the platyfish genome, however, led to fewer unannotated transcripts, which facilitated the functional interpretation of results.

To annotate transcripts, we first conducted a BLAST search of the longest transcript for each locus against the SwissProt database (http://ca.expasy.org/sprot/; BLASTX, critical *e*-value = 0.001; database accessed November 1, 2014). For each locus, we retained the top BLAST hit for subsequent analyses, and all transcripts for each locus were given the same SwissProt annotation. Sequences with a match in the SwissProt database were subsequently annotated with GO IDs (Gene Ontology Consortium 2004). GO IDs describe gene product characteristics and are hierarchically organized in terms of biological processes, molecular functions, and cellular components (Gene Ontology Consortium 2004).

Quantifying and Comparing Gene Expression Patterns

Transcript abundance was estimated by remapping the RNA-seq reads to the extracted transcriptome using Stampy (Lunter and Goodson 2011). Overall, transcript abundance (expression values) was estimated using eXpress (Roberts and Pachter 2013). We used the edgeR function calcNormFactors (Robinson et al. 2010; Robinson and Oshlack 2010) to estimate an effective library size, which accounts for differences in the number of total reads (total library size) among individuals. We modeled the common dispersion using the estimateCommonDisp function. We also modeled tagwise (gene-wise) dispersion using the estimateTagwiseDisp function to prevent outliers from driving any signal of differential expression between comparisons. We tested for differential expression between pairs of sulfidic and nonsulfidic ecotypes within the same drainage using an exact test implemented in edgeR. To control for multiple testing, we used a genome-wide false discovery rate (FDR) of 0.001 (Benjamini and Hochberg 1995).

To describe whether gene expression patterns in replicated sulfide spring populations changed in convergence, we conducted hierarchical cluster analysis. If gene expression patterns have changed in convergence, individuals from lineages living under the same environmental conditions (sulfidic vs. nonsulfidic) should cluster together. The hierarchical cluster analysis was performed on the top 10,000 expressed genes using the log-counts-per-million with prior count set to 5 to avoid taking the log of zero counts. We then used the heatmap.2 function from the GPLOTS package in R to generate hierarchical clusters. In addition, we conducted a WGCNA using the top 10,000 expressed genes. We used the variance-stabilizing transformation function in DESeq2

(Love et al. 2014) and constructed networks with the R package WGCNA (Langfelder and Horvath 2008). Clustering of gene expression profiles for the retained genes was consistent with hierarchical clustering based on the most expressed genes (fig. 2). The soft threshold (power) for the combined network was 6, which was the lowest value that optimized the scale free topology.

Identifying Potentially Adaptive Changes and Testing Hypotheses about the Function of Differentially Expressed Genes

The sets of differentially expressed genes with FDR < 0.001 for each pair of sulfidic and nonsulfidic ecotypes within the same drainage were intersected to identify genes with shared patterns of differential expression. GO annotations were then used in an enrichment analysis for biological processes, molecular function, and cellular components as implemented in GOrilla with a *P* value threshold of 0.001 (Eden et al. 2009). Furthermore, we visualized metabolic pathways associated with shared differentially expressed genes through annotation with KEGG orthologs (Kyoto Encyclopedia of Genes and Genomes; Kanehisa et al. 2012) as implemented in iPath v2 (Letunic et al. 2008).

Supplementary Material

Supplementary figures S1–S9 and tables S1–S7 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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