PHYSIOLOGICAL AND TRANSCRIPTOMIC ASPECTS OF ADAPTATION TO EXTREME ENVIRONMENTS

by

COURTNEY NICOLE PASSOW

B.S., Texas A&M University, 2011

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Division of Biology
College of Arts and Sciences

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2016

Abstract

Extremophiles are organisms with the ability to survive in environments characterized by strong physicochemical stressors lethal to most other organisms, providing excellent models to further our understanding of life's capacities and limitations to deal with far-from-average conditions. I studied how physiological processes varied among fish residing in starkly different environmental conditions to understand how organisms cope with extreme environments and disentangle the roles of short-term plastic responses and evolved population differences in shaping physiological responses. I used the *Poecilia mexicana* model, a series of extremophile fish populations that has colonized toxic hydrogen sulfide (H_2S) rich springs and caves, to address three major objectives: (1) I investigated the energetic consequences of life in extreme environments and tested whether predicted reductions in organismal energy demands evolved repeatedly along replicated environmental gradients. (2) I characterized variation in gene expression among populations and organs to test for interactive effects between different stressors and identify potential physiological mechanisms underlying adaptation to H_2S and cave environments. (3) I conducted common garden and H_2S-exposure experiments to test how evolutionary change and plasticity interact to shape variation in gene expression observed in nature.

To address these objectives, I measured variation in metabolic physiology and quantified variation in physiological processes through genome-wide gene expression analyses. I found that adaptation to extreme environments directly impacts energy metabolism, with fish living in extreme environments consistently expending less energy overall. Reductions in energy demand have evolved in convergence and were primarily mediated through a life history shift (reduction in body mass). The quantification of gene expression across divergent habitats and organs revealed organ-specific physiological responses in H_2S-rich and cave habitats. Gene expression variation

in the relevant genes was primarily shaped by evolutionary change in gene regulation, and ancestral plastic responses play a minor role in causing the observed expression differences between replicated sulfidic and nonsulfidic populations in nature. Overall, my research has implications for understanding the capacities and constraints that shape life in extreme environments and aids in our understanding of modifications in physiological pathways mediating adaptation to elevated H_2S and perpetual darkness.

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Dedication

I would like to dedicate this work to my family and friends. I would especially like to dedicate this to my loving and extremely supportive grandmother, Grace Stark. Although she passed away before I was able to complete my dissertation, I would not have pursued or even completed this degree program and project without her love, guidance and support.

Preface

Introduction

Adaptation is an important component of responses to changing environments (Davis and Shaw 2001), as local adaptation can allow populations to persist in novel environments characterized by strong abiotic and biotic conditions (Franks and Hoffmann 2012; Savolainen et al. 2013). Local adaptation typically involves modification of morphological, behavioral, or physiological traits mediating an increase in fitness under specific environmental conditions (Howarth 1993; Kawecki and Ebert 2004). Hence, phenotypic and corresponding genetic differentiation along environmental gradients has frequently been used to infer local adaptation in natural systems (Hoffmann and Sgro 2011). Recent studies have made considerable progress towards identifying the genetic basis of morphological adaptations (Turner et al. 2010, Jones et al 2012), yet few have focused on mechanisms mediating physiological adaptation (Cheviron et al. 2012, Storz and Wheat 2010). However, phenotypic variation in the wild does not always have a simple genetic basis because of the interactive effects of phenotypic plasticity and heritable population differences (Ghalambor et al. 2007). This is particularly true for populations occurring along physiochemical gradients, as adaptation typically occurs at multiple hierarchical levels, including short-term acclimatization, developmental plasticity, and genetic variation (Whitehead 2012). Disentangling the mechanisms driving phenotypic variation in natural populations and determining the adaptive value of plasticity is critical in evolutionary biology (Via et al 1995, Schmitt et al. 1999), because plasticity can either be adaptive (incurring a fitness advantage; Badyaev 2005), non-adaptive (where it acts as a constraint; Miner et al. 2005), or selectively neutral (Ghalambor et al 2007). Since many species are currently experiencing increased environmental variation (Walther et al.

2002), understanding how selection acts on heritable and plastic components of phenotypes is imperative (Nussey et al. 2005).

Organisms living in extreme environments are ideal models to study physiological adaptation, because they can thrive in places that are lethal for most others through the modification of structural, physiological, and behavioral traits (Waterman 2001). Extreme environments exert relatively simple and strong selection; nonetheless, whole-organism responses to physiochemical stress appear to be complex, including traits at multiple levels of biological organization. Organisms that survive under these conditions have become a focal point in scientific research, particularly to understand the evolution of stress adaptations (Gostincar et al. 2010). Research on extremophiles has predominately focused on bacteria and archaea (Schönknecht et al. 2013), but there is a diversity of metazoan extremophiles (Garbarino et al. 2015; Riesch et al. 2015a). In particular, vertebrates residing in extreme environments provide unique systems, because many of them exhibit evolutionary replication across environmental gradients spanning extreme and benign habitats (Tobler et al. 2008a; Kavembre et al. 2016).

Two major aims have governed the study of physiological adaptation in harsh environments: (1) Research has focused on understanding the energetic consequences of life in extreme environments. Low energy availability and maintenance costs in extreme environments can exert selection for efficient energy use (Sibly and Calow 1989). While many studies have investigated immediate metabolic costs of exposure to stressors and energy limitation (McKenzie et al. 2007; McCue 2010), whether and how long-term exposure to diverse environmental conditions drives adaptive shifts in organismal energy demands remains an open question. Furthermore, it remains unclear whether potential adaptive reductions in energy demands evolve in convergence and through the same proximate mechanisms. Addressing these questions and

elucidating the selective forces that shape microevolutionary change in metabolic rate variation is critital for understanding patterns of metabolic scaling at macroevolutionary scales (Feder 2000). In addition, an understanding of energetic consequences of life in extreme environments is critical to predict evolutionary change in other traits that are sensitive to energy availability, including changes in life history characteristics (Hayes et al. 1992). (2) Research has focused on identifying the physiological mechanisms that allow organisms to tolerate extreme environmental conditions. Although recent efforts have made progress in identifying the genetic basis of adaptive traits in natural systems (Oleksiak et al. 2002; Savolainen et al. 2013), physiological adaptations are not expected to have a simple genetic basis because of the dynamic interactions between acclimation, developmental plasticity, and heritable population differences (Ghalambor et al. 2007; Ghalambor et al. 2015). Next generation sequencing tools such as RNA sequencing (RNA-seq; Wang et al. 2009) make it possible to quantify genetic changes in organisms along environmental gradients in nature (Cheviron et al. 2012; Smith et al. 2013). This in turn provides insight into the physiological pathways that may be linked to adaptation and, in combination with laboratory experiments, will allow for understanding how heritable and plastic responses interact to shape variation in nature (Ghalambor et al. 2015).

Model and objectives

Organisms residing in starkly different environments provide unique opportunities to address the problems outlined above. Hence, I focused on extremophile fish of the *Poecilia mexicana* complex, a group of livebearers that has repeatedly colonized hydrogen sulfide rich springs and caves (Tobler et al. 2008a; Tobler et al. 2011). Evolutionarily independent pairs of sulfidic and non-sulfidic populations occur in four replicated river drainages in southern Mexico (Pichucalco,

Ixtapangajoya, Puyacatengo and Tacotalpa; Tobler et al. 2008a; Palacios et al. 2013), whereas the two cave habitats, one sulfidic cave and one nonsulfidic cave, occur in one drainage, the Tacotalpa (Tobler et al. 2006; Tobler et al. 2008b). Sulfidic habitats are characterized by high concentrations of hydrogen sulfide (H_2S), a potent respiratory toxicant that is lethal for most metazoans because it inhibits cytochrome c oxidase (COX; Cooper and Brown 2008), interrupting the electron transport chain and effectively halting ATP production (Reiffenstein et al. 1992). Cave habitats are characterized by the absence of light, which has direct effects on organismal function and has shaped the evolution of phenotypic traits (e.g., sensory biology, circadian rhythms and loss of eyes and pigmentation; Koilraj et al. 2000; Protas et al. 2007; Tobler et al. 2010). The *P. mexicana* system is well characterized in terms of environmental and phenotypic variation, and morphological (Parzefall 2001; Tobler at al. 2008a) life history, (Riesch et al. 2010; Riesch et al. 2016), and behavioral traits (Plath et al. 2007b; Plath 2008) vary among the sulfidic and cave environments. In the sulfidic environments, there is also evidence of convergence across the drainages in regards to head and gill size and sulfur tolerance (Tobler et al. 2011a). There is also significant genetic differentiation and low rates of gene flow between the sulfidic and nonsulfidic, as well as cave and surface environments (Plath et al. 2007b; Plath et al. 2010b). Nonetheless, we are still lacking basic knowledge about physiological adaptations that mediate survival in this system, even though the environmental factors present in this system are expected to have profound effects on physiological processes. Consequently, my dissertation had four overarching objectives:

Chapter 1. Variation in energy availability in extreme environments can exert selection for efficient energy use through the reduction of body mass or metabolism. Hence, I focused on

quantifying the evolution of routine metabolism in extremophiles across four divergent habitats. To address this, I measured routine metabolic rates and mass to determine the major driver of reduced energetic demands in laboratory and common-garden reared extremophile fish adapted to the presence or absence of sulfide and light.

Chapter 2. Constraints on energy acquisition should precipitate in strong selection for reduced energy in extremophiles. Nonetheless, we know very little on whether the same mechanisms (reduced body size *vs* suppressed metabolism) shape energy demands in sulfidic fish. I tested whether convergent evolution shapes energy demands across evolutionarily independent lineages of extremophile fish. I measured routine metabolism and mass in paired sulfidic and nonsulfidic fish collected across four independent lineages in the wild and laboratory to determine whether there was evidence of convergent evolution shaping energy demands, and whether it was the same mechanisms driving this variation.

Chapter 3. Inferences about physiological mechanisms of adaptation based on gene expression analyses depend on strength of the abiotic stressor and organs being analyzed. Thus, I focused on determining variation in gene expression in fish adapted to sulfide or darkness as well as in different organs. I quantified patterns of expression, differential expression and functional variation within three extremophile populations (sulfidic surface, nonsulfidic cave and sulfidic cave) and three different organs (gill, liver and brain) to determine how expression varies among habitat types and organs.

Chapter 4. Differential gene expression is an important mechanism mediating local adaptation. However, gene expression is notoriously plastic, which complicates inferences about the adaptive value of expression. I tested how evolutionary changes and plasticity interact to shape variation in gene expression. To address this, I studied expression responses in wild-caught and laboratory populations of sulfide spring fish to determine whether common-garden reared fish exhibited similar expression changes compared to the wild-caught fish due to adaptation to H_2S and whether there was evidence of evolved constitutive differences and/or plastic responses to H_2S exposure.

Synopsis

The costs of maintaining homeostasis in the presence of physicochemical stressors can profoundly affect an organism's energy budget (Sibly and Calow 1989; Parson 1996). Likewise, convergent evolution should shape changes in energy demands across the evolutionarily independent lineages (Schluter 2000; Losos 2010). Results from chapter 1 revealed that fish residing in extreme environments were characterized by a reduction in body size, thus effectively reducing their overall energetic demands, despite elevated routine metabolism in cavefish observed in laboratory and wild-caught fish. Chapter 2 also provided evidence of convergent evolution through the consistent reduction of energetic demands in sulfidic fish across multiple river drainages and illuminated how fish use a combination of reduced body size and routine metabolism to adapt to extreme environments.

Changes in gene expression among environmental gradients with strong abiotic and biotic sources of selection have been instrumental for identifying genetic variation underlying adaptation (Cheviron et al. 2008; López-Maury et al. 2008). Nonetheless, we still lack knowledge about how different sources of selection affect different organs (Alvarez et al. 2015). We also know little

about whether plasticity or evolutionary changes are the major drivers in variation observed in expression. Overall, in chapter 3, I documented extensive variation in gene expression among closely related populations and within different organs, illuminating the importance of focusing on multiple stressors and organs when determining the genetic changes underlying adaption in nature. In chapter 4, I identified similar patterns of differential expression in wild-caught and laboratory fish that corresponded to H_2S adaptation and found evidence for both evolved expression differences as well as H_2S inducibility in sulfide fish. Thus, results from chapter 4 suggest that evolved changes in expression not ancestral plasticity are responsible for variation in gene expression across sulfidic and non-sulfidic populations.

My research has implications for understanding the capacities and constraints that shape life in extreme environments. Research focusing on metabolic consequences of life in extreme environments contributes to understanding the evolutionary mechanisms shaping organismal physiology and has broad implications for life history evolution. My research also aids in understanding the physiological pathways being modified in environments with strong sources of selection. Although studies have quantified shared gene expression responses in populations exposed to similar stressors, most have focused on invertebrates or plants when quantifying expression variation in different tissues (Bos et al. 2016; Dean et al. 2016). Hence, we know very little about changes in expression in vertebrates exposed to strong abiotic and biotic stressors. My research addresses critical question in ecology and evolution research by illuminating variation in natural systems at multiple biological scales and between different tissues (Alvarez et al. 2015). Likewise, my research highlights the importance of multifarious selection in shaping evolutionary change and phenotypic expression (Holmstrup et al. 2010) and illuminates expression variation in candidate genes associated with H_2S adaptation has likely been shaped by evolutionary change

rather than ancestral plasticity. This in turn reveals that molecular evolution (Pfenninger et al. 2014; Pfenninger et al. 2015) and regulatory changes (Kelley et al. 2016) both play critical roles in mediating adaptation to extreme environments.

Chapter 1 - Reduction of energetic demands through modification of body size and routine metabolic rates in extremophile fish[1]

Courtney N. Passow, Ryan Greenway, Lenin Arias-Rodriguez,

Punidan D. Jeyasingh and Michael Tobler

Abstract

Variation in energy availability or maintenance costs in extreme environments can exert selection for efficient energy use, and reductions in organismal energy demand can be achieved in two ways: reducing body mass or metabolic suppression. Whether long-term exposure to extreme environmental conditions drives adaptive shifts in body mass or metabolic rates remains an open question. We studied body size variation and variation in routine metabolic rates in locally adapted populations of extremophile fish (*Poecilia mexicana*) living in toxic, hydrogen sulfide-rich springs and caves. We quantified size distributions and routine metabolic rates in wild-caught individuals from four habitat types. Compared to ancestral populations in nonsulfidic surface habitats, extremophile populations were characterized by significant reductions in body size. Despite elevated metabolic rates in cavefish, the body size reduction precipitated in significantly reduced energy demands in all extremophile populations. Laboratory experiments on common garden-raised fish indicated that elevated routine metabolic rates in cavefish likely have a genetic basis. The results of this study indicate that adaptation to extreme environments directly impacts energy metabolism, with fish living in cave and sulfide spring environments overall expending less energy during routine metabolism.

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Introduction

Animals require energy for maintenance, growth, and reproduction, and since individuals' energy expenditure may be greater or less than the environmental energy availability, they can modulate a variety of physiological processes to balance energy supply and expenditure (Cho et al. 1982). Metabolic rate is a physiological measure of the rate at which organisms burn calories from assimilated food resources to produce energy for organismal functioning, and understanding metabolic rate variation is critical for investigating ecological processes at multiple levels of organization (Brown et al. 2004; Sibly et al. 2012). The majority of metabolic rate variation in animals coincides with variation in body mass and temperature (Peters 1983; Gillooly et al. 2001; Brown et al. 2004; Clarke and Fraser 2004; Cano and Nicieza 2006). Nonetheless, mass- and temperature-adjusted metabolic rates can vary substantially even among closely related taxa (McNab 1986; Clarke and Johnston 1999; Nagy et al. 1999; Lovegrove 2000; Schaefer and Walters 2010). Elucidating the selective forces that shape such residual metabolic rate variation in allometric plots and underlie macroevolutionary patterns in the diversification of metabolic rates is a critical challenge in physiology (Garland and Carter 1994; Feder et al. 2000).

Resource availability is a strong source of selection driving adaptive modification of metabolic rates within and among closely related species (Mueller and Diamond 2001; McCue 2010; Moiroux et al. 2012). From an energetic point of view, fitness can be described as the conversion rate of energy into offspring (Brown et al. 1993), which ultimately is limited first by the rate at which organisms can acquire energy from the environment and then by the rate at which

they can allocate energy to reproduction (as opposed to maintenance or growth). Consequently, reductions of environmental resource availability or increases in maintenance or growth costs are predicted to constrain the amount of energy for reproduction and exert selection for a reduced overall energy demand that allows for maximizing relative energy allocation to the production of offspring. Organisms can reduce their overall energy demand in two fundamental ways: (1) They can reduce their body size by reducing energy allocation to growth, which simultaneously reduces total energy expenditure for maintenance (Blanckenhorn 2000; Wikelski and Romero 2003; Pafilis et al. 2009; McNab 2010). (2) Organisms can reduce metabolic rate independently of body size, which changes the allometric relationship between metabolism and body size (Guppy and Withers 1999; Wang et al. 2006; Burton et al. 2011). It is important to note that these mechanisms are not mutually exclusive but may work in synchrony, such that focusing merely on body size or metabolic rates alone can lead to erroneous conclusions (McNab 1999, McNab 2002; Van Voorhies et al. 2004; McCue 2010).

Systems in which closely related populations occur in habitats with starkly different environmental conditions provide an excellent opportunity to study evolutionary change in organismal energy demand. This is especially true for species that have invaded extreme environments, and comparisons between populations in localized extreme environments and adjacent "benign" habitats allow for a powerful approach examining the effects of stressors on organismal physiology as well as the evolutionary trajectories of populations. Exposure to physicochemical stressors profoundly affects energy budgets of organisms, because the maintenance of homeostasis precipitates in considerable energetic costs through investments in physiological, morphological, or behavioral coping mechanisms (Calow 1989; Sibly and Calow 1989; Parsons 1996). As such, continuous exposure to environmental stressors should select for

increased metabolic rates (e.g., Knoblauch et al. 1999). However, the evolution of increased metabolic rates in extreme environments is typically constrained by a reduced supply of resources required for metabolic expenditure (Waterman 1999; Waterman 2001). While many studies have investigated immediate metabolic costs of exposure to physicochemical stressors and energy limitation (e.g., Haney and Nordlie 1997; Penttinen and Kukkonen 1998; Rose et al. 2006; Wang et al. 2006; McKenzie et al. 2007; McCue 2010), it remains unclear how metabolic rates and metabolic rate plasticity evolve when populations adapt to diverse environmental stressors. Here, we explicitly tested for differences in body size and routine metabolic rate in the context of resource limitation in extremophile fish. Such information will be important for a more general understanding of metabolic rate evolution (Naya et al. 2013).

Poecilia mexicana is a livebearing fish that has repeatedly colonized extreme environments characterized by the presence of naturally occurring, toxic hydrogen sulfide (H_2S) and the absence of light in caves (Tobler and Plath 2011; Tobler et al. 2011). In Mexico's Cueva del Azufre system, both environmental factors occur in a natural, factorial design forming four distinct habitat types: nonsulfidic surface streams, sulfidic surface streams, a nonsulfidic cave, and a sulfidic cave (Tobler et al. 2008a; Plath and Tobler 2010). Populations from different habitat types are characterized by adaptive trait divergence (Tobler et al. 2008a; Tobler and Plath 2011) and are reproductively isolated with low rates of gene flow among populations living under different environmental conditions (Plath et al. 2007a; Plath et al. 2010a). Reproductive isolation is mediated at least in part by natural selection against non-adapted, migrant individuals and by sexual selection through ecotype-assortative mating (Tobler 2009; Tobler et al. 2009b; Riesch et al. 2011a).

The strikingly different environmental conditions in this system are expected to profoundly affect organismal energy budgets. Caves have widely been considered as energy limited because of a lack of photosynthetic primary production (Poulson and White 1969; Langecker 2000), and some cave organisms accordingly have evolved lower metabolic rates (e.g., Hüppop 1985, Hüppop 1986; Hervant et al. 2000; Poulson 2001a; Wilhelm et al. 2006). Similarly, exposure to H_2S has been shown to constrain energy acquisition in *P. mexicana* (Tobler et al. 2009a). H_2S causes and aggravates hypoxia in natural environments (Bagarinao 1992), driving exposed fish to trade-off time between benthic foraging and aquatic surface respiration, which directly mediates survival in the toxic and hypoxic environment (Plath et al. 2007b; Tobler et al. 2009a). In addition, exposure to perpetually sulfidic environments requires active detoxification for survival. Sulfide spring fish exhibit a heritable and constitutive increase in H_2S detoxification ability through up-regulation of the sulfide:quinone oxidoreductase pathway (Tobler et al. 2014), a process that is energetically costly (Ip et al. 2004; Hildebrandt and Grieshaber 2008). Accordingly, some organisms adapted to sulfidic environments have been documented to increase energy consumption in the presence of H_2S (Gorodezky and Childress 1994; Schneider 1996). Clearly, exposure to extreme environmental conditions impacts organismal energy budgets through reduced energy availability, reduced ability for energy acquisition, and/or increases in organismal maintenance costs, which is reflected in *P. mexicana* from both sulfidic and cave environments consistently having a lower body condition than fish from nonsulfidic surface habitats (assessed through abdominal distension: Plath et al. 2005; body fat content: Tobler 2008; and mass-length relationships: Tobler et al. 2006). Consequently, adaptation to these environments should be linked to energy metabolism, and we hypothesized that extremophile populations should be selected for reductions in overall energy demand. To test this overarching hypothesis, we addressed the following objectives: (1) We

quantified size distributions of fish in different habitat types to test whether adaptation to extreme environments was associated with body size reduction. (2) We quantified routine metabolic rates in wild-caught individuals to test whether adaptation to extreme environments was associated with metabolic rate suppression. (3) We tested for a genetic basis of variation in metabolic rates and metabolic rate plasticity in response to energy availability by quantifying routine metabolic rates in common garden raised individuals subjected to different food treatments.

Material and Methods

Study Site

To disentangle potential effects of the presence of hydrogen sulfide and permanent darkness in caves on organismal energy demands, we focused on a set of habitats in the Cueva del Azufre system, where these environmental factors occur in a natural, factorial design: (1) a nonsulfidic surface habitat (Arroyo Bonita), (2) a sulfidic surface habitat (El Azufre), (3) a nonsulfidic cave (Cueva Luna Azufre), and (4) a sulfidic cave (Cueva del Azufre, chamber V). All sites were located within <4 km of each other and were situated near the village of Tapijulapa in the Mexican state of Tabasco (Tobler et al. 2008a). The sulfidic cave is segregated into different chambers with varying exposure of light and high densities of *P. mexicana* (Gordon and Rosen 1962; Parzefall 2001), while the nonsulfidic cave is considerably smaller than the sulfur cave, completely dark, and only maintains a small *P. mexicana* population (Tobler et al. 2008b). Fish from the sulfidic surface habitat were collected in the El Azufre, a stream that drains the Cueva del Azufre and eventually joins the Rio Oxolotan. The nonsulfidic habitat (Arroyo Bonita) also is a tributary of the Rio Oxolotan and similar in size and structure to that of the El Azufre (Tobler et al. 2008a). All procedures conducted for this study were approved by the Institutional Animal Care and Use Committee at Oklahoma State University (ACUP: AS10-15).

Size distribution in natural populations

To compare size distributions in the four habitat types, we assembled data from previous studies (Tobler et al. 2006; Tobler 2008; Tobler et al. 2008a; Tobler et al. 2008b; Tobler et al. 2008c) as well as several unpublished projects. In all cases, fish were collected using seines (4 m long, 4 mm mesh width), sexed, and weighed (blotted wet weight to the closest 0.01 gram). Mass-based size distributions were then analyzed using analysis of variance (ANOVA) with body mass as a dependent variable. We included sex, presence or absence of H_2S (i.e., sulfidic vs. nonsulfidic habitat), and presence or absence of light (i.e., cave vs. surface habitat) as independent variables.

Determining routine metabolic rates in natural populations

For the quantification of metabolic rates in wild-caught fish, specimens were collected in June 2012. Upon capture, fish were immediately transferred into insulated coolers with aerated water and transported to a nearby field station at the Centro de Investigación e Innovación para la Enseñanza y el Aprendizaje (CIIEA) in Teapa, Tabasco. Prior to metabolic rate trials, fish were allowed to acclimate to laboratory conditions for at least 48 hours. During that time, they were kept in 70-liter tanks with filtered and aerated water. The temperature was kept between 24 and 26°C. All fish were subjected to a 12:12 hour light:dark cycle.

We employed a closed chamber respirometry approach to quantify individual's routine metabolic rate, which is defined as the oxygen consumption of unconstrained, post-absorptive organisms capable of spontaneous motor activity (Fry 1957; Steffensen 1989). This approach has been widely used to quantify metabolic costs associated with a variety of factors, including exposure to environmental stressors (Haney and Nordlie 1997; Pirozzi and Booth 2009),

locomotion (Basolo and Alcaraz 2003; Seibel and Drazen 2007), elaborate morphological structures (Allen and Levinton 2007), mating behaviors (Hoback and Wagner 1997), and gestation (Timmerman and Chapman 2003). The protocol for measurements of oxygen consumption included the following steps for each individual: (1) As detritivores, *P. mexicana* have a relatively fast gut passage time of <6 hours (Tobler & Scharnweber, unpublished data). Hence, fish were not fed 24 hours prior to trials to assure that metabolic rate measurements were conducted on post-absorptive individuals (Timmerman and Chapman 2004b; Norin and Malte 2011). (2) Fish were then haphazardly chosen from stock tanks and placed into individual respirometry bottles filled ¾ with water for a 12-hour acclimation period under continuous aeration. Bottles had a total volume of 580 ml and were painted solid black to prevent light penetration. Four bottles were placed together in a black equipment box with a lid to further minimize light exposure and with water to minimize temperature fluctuations in the respirometry bottles. Realized mean temperatures in respirometry bottles ranges from 26.1 to 27.2 °C across all trials. (3) After the acclimation period, the respirometry chambers were flushed with fresh, aerated water to remove metabolic waste products (Timmerman and Chapman 2004a) and capped with a fitted lid that allowed for the insertion of an oxygen probe. Once capped, water was added into the chamber using a squirt bottle to remove any excess air, and a YSI ProDO optical dissolved oxygen probe (YSI Inc., Yellow Springs, OH) was inserted into each bottle (this probe monitors dissolved oxygen concentration in conjunction with temperature). Plumbers putty was fitted around the oxygen probe to prevent any diffusion of gases. Once all four respirometry bottles were set up, the lid of the water bath was closed, and the probes were set to measure the dissolved oxygen concentration at 10-second intervals. All experiments were run for at least 6 hours or until the oxygen saturation reached 10% to prevent mortality. Probes were recalibrated regularly according to the manufacturer's

recommendations to maintain accuracy. Note that all metabolic rate trials were conducted in absence of H_2S even for sulfidic populations, because the reactivity of H_2S with oxygen in aqueous solution (Chen and Morris 1972) affects estimates of organismal oxygen consumption. (4) After the termination of a trial, individuals were weighed (blotted wet weight to the closest 0.01 gram) and sexed. Descriptive statistics for the body mass of individuals used are given in Table 1.1.

Raw data obtained from all trials represented measurements of oxygen concentration and temperature through time. For each individual trial, we first removed any outliers that were likely caused by instrumental error (<0.1% of data points). We also removed any data points from the first 60 min of each trial, as the flushing of the respirometry bottle with fresh water and the installation of the oxygen probe may have caused erratic fish activity (Timmerman and Chapman 2004b). Because fish metabolic rates may be affected by reduced ambient oxygen concentrations (Haney and Nordlie 1997; Ultsch et al. 1978), we only included data points measured at dissolved oxygen saturations >70%. Metabolic rate (in mg O_2/hour) was then calculated for each individual as the slope of a regression (multiplied by the volume of water in the respiratory bottle) with oxygen concentration as a dependent variable and time as an independent variable. For all regressions, R^2 was >0.90.

Routine metabolic rate data were analyzed using analysis of covariance (ANCOVA). Log_{10}-transformed routine metabolic rate was used as a dependent variable. We included sex, presence or absence of H_2S in natural populations (i.e., sulfidic *vs*. nonsulfidic habitat), and presence or absence of light in natural populations (i.e., cave *vs*. surface habitat) as independent variables. Temperature and mass (log_{10}-transformed) were included as covariates in all models. All three-way ($F \leq 1.909$, $P \geq 0.173$) and two-way ($F \leq 2.780$, $P \geq 0.101$) interaction terms were non-significant and thus excluded from the final model.

Genetic basis of variation in routine metabolic rates and metabolic rate plasticity

Metabolic rate variation in wild-caught fish may merely reflect plastic responses to life under different environmental conditions. Hence, we tested whether differences in routine metabolic rates documented in wild-caught individuals have a genetic basis by using common garden-raised fish from the same populations investigated in the field. In addition, we used experimental manipulations of energy availability to test for population differences in metabolic rate plasticity in response to energy availability.

Common garden raised fish came from stocks at the University of Oklahoma and Oklahoma State University. All animals used in the laboratory portion of this study were born and raised in captivity, and fish were maintained under nonsulfidic conditions with a 12:12 hour light:dark cycle. Individuals for the metabolic rate experiment were haphazardly chosen from stock tanks. Each fish was sexed and weighed (blotted wet weight to the closest 0.01 gram; Table 1.1), and five fish from the same population were introduced into a 40-liter tank with filtered and aerated water (these are low stocking densities compared to regular stock tanks to minimize competition between individuals). Tanks were assigned to different food treatments in a balanced fashion (i.e., neighboring tanks alternated in population and food treatment assignment) for a total of 8 tanks per treatment. During the experiment, all fish were fed with Earthworm Fish Flake food (American Brine Shrimp Company, Ogden, UT). To standardize resource availability, we calculated the total fish mass for each tank. For the high food treatment, we calculated the amount of food (F, in grams) as F=0.0125*(total fish mass)$^{0.65}$. Low food treatment groups received half the amount of food provided to high food treatment groups. Fish were fed the calculated amount of food twice a day from Monday through Friday and once a day during the weekend. All fish were kept on their

respective food treatment for at least 21 days prior to testing. During this time, the temperature was kept constant at 25° C. Upon completion of a trial, fish were returned to regular stock tanks.

In general, the experimental protocol for metabolic rate measurements was identical for the wild-caught and common-garden raised fish. There were only two critical differences: (1) Instead of handheld oxygen probes, the oxygen consumption measurements were conducted using a Loligo Systems (Tjele, Denmark) 4-channel respirometry system with fiber optic probes. Oxygen concentrations and temperature were measured every second. (2) The temperature in the water bath was controlled by using an Ebo-Jäger 75-watt aquarium heater (EHEIM GmbH & Co.) in conjunction with a Mighty-Pro chiller (Aqua Euro USA). Fish were either tested at 20, 25, or 30°C to test for potential population differences in the temperature dependence of metabolic rates. The four habitat types investigated here vary both in mean temperatures and temperature variability (with extreme environments typically exhibiting higher averages and lower variability), and the chosen temperatures reflect the range *P. mexicana* typically encounter in natural habitats (Tobler et al. 2006; Tobler et al. 2008a).

Statistical analyses were conducted as outlined above for the wild-caught fish, except that the ANCOVA model for the laboratory-reared fish also included food treatment (high *vs*. low) as an independent variable. Three-way interactions ($F \leq 2.684$, $P \geq 0.103$), as well as interaction terms including temperature ($F \leq 0.379$, $P \geq 0.539$), were non-significant and hence excluded from the final model.

Results

Size distributions

Overall, we assembled mass data for 1,454 individuals. ANOVA indicated that males exhibited a significantly smaller body size than females in all populations (Table 1.2A), which is likely related

to the fact that male poeciliids – unlike females – have determinate growth (Constantz 1989; Reznick and Miles 1989). More importantly, we detected a significant cave by sulfide interaction (as well as significant cave and sulfide main effects), indicating significant body size differences among populations residing in different habitat types. Individuals from the ancestral population in the nonsulfidic surface habitat were by far the largest with a mass of 2.01 ± 1.20 g (mean ± standard deviation; Figure 1.1A). In contrast, individuals from extreme environments all exhibited a reduction in the overall body mass, with individuals >3.00 g being exceedingly rare (Figure 1.1B-D). Among the populations inhabiting extreme environments, sulfidic surface fish were the largest (0.92 ± 0.53 g), sulfidic cave individuals were intermediate (0.75 ± 0.48 g), and fish from the nonsulfidic cave were the smallest (0.48 ± 0.34 g).

Routine metabolic rate variation in natural populations

Analysis of the wild-caught fish revealed significant variation in routine metabolic rates among locally adapted populations. Body mass – as expected – explained most variation in routine metabolism (Table 1.2B). While there was no difference between sulfidic and nonsulfidic populations, cavefish exhibited significantly higher routine metabolic rates than surface fish (Figure 1.2A). Temperature did not significantly affect metabolic rates in wild-caught fish, likely because the temperature range was relatively narrow (26-27 °C).

Routine metabolic rates and metabolic rate plasticity in common garden raised fish

Temperature and mass explained the bulk of variation on routine metabolic rates of laboratory-reared individuals (Table 1.2C, Figure A.1). Furthermore, laboratory experiments confirmed the significantly higher routine metabolic rates in cave populations (Figure 1.2B),

as documented in wild-caught fish. Similarly, there was no significant difference between fish from sulfidic and nonsulfidic habitats. In addition, fish in the high food treatment exhibited higher routine metabolic rates than those in low food treatments, although these differences were dependent on body mass (see significant food by mass interaction term in Table 1.2C). Specifically, reductions in routine metabolic rates were more pronounced in larger individuals than smaller ones (Figure 1.3). Finally, there was no evidence for population differences in response to food treatments or in the temperature dependence of metabolic rates.

Discussion

Our study revealed significant variation in traits associated with energy metabolism among locally adapted populations of *Poecilia mexicana* inhabiting contrasting environments characterized by the presence or absence of light and toxic hydrogen sulfide. In particular, we found significant reductions of body size in extremophile populations and significant among population variation in routine metabolic rates of wild-caught individuals, although analyses of body size variation and metabolic rates yielded somewhat contradictory results. Laboratory experiments using common garden raised fish revealed genetic variation in routine metabolic rates, likely indicating evolved differences in energy metabolism among populations of the same species.

Reducing organismal energy demands: patterns and mechanisms

Reduced resource availability and increased maintenance costs often associated with extreme environments are predicted to exert selection for reduced energy demands, allowing organisms adapted to the extreme conditions to maximize the relative investment into reproduction (Brown et al. 1993; Parsons 1996). Our analyses indicated significant reductions in body mass for all populations from habitats with extreme environmental conditions; supporting the notion that

selection acts to reduce organismal energy demands. Contrary to predictions, however, cavefish exhibited significantly higher routine metabolic rates than fish from surface populations (irrespective of the presence of H_2S in natural waters). These contradictory results beg the question whether variation in body size and metabolic rate balance each other in a way that overall organismal energy demands do not vary among populations living under different environmental conditions, or whether reductions in body mass outweigh increases in routine metabolic rates. Simulating total energy expenditure of average individuals from the different populations under simultaneous consideration of population-specific size distributions and allometric metabolic rate functions (see Appendix A for details) indicated that body mass reductions outweigh differences in routine metabolic rate. In fact, estimates of total energy expenditure were significantly and substantially reduced (between 27 and 49%) in fish from extreme habitats compared to the ancestral nonsulfidic surface population (Figure A.2), which provides unequivocal evidence for a reduction of energy demands in extreme environments. Hence, the reduction in body size outweighed the increase in metabolic rates, highlighting that variation in body size and metabolic rates need to be considered simultaneously, because investigating one without the other may lead to spurious conclusions (e.g., McNab 1999).

Reductions in energy demands in extremophile populations were primarily driven by modification of body size, rather than metabolic rate suppression. This parallels results from selection experiments, in which mice selected for high locomotor activity maintained stable energy budgets despite of high costs, because they exhibited reductions in body size rather than reductions in mass-specific costs associated with running (Rezende et al. 2009). Consequently, modification of body size may face fewer evolutionary constraints than modification of metabolic rates, which would explain the tight correlation between mass and metabolic rate across a broad range of taxa

(Brown et al. 2004; Gillooly et al. 2001). While common garden experiments revealed a genetic component to variation in routine metabolic rates (cavefish retained elevated routine metabolic rates when raised in the laboratory for multiple generations), it remains unclear whether variation in body size among populations living in contrasting environments is driven by genetically based evolutionary change or phenotypic plasticity. Particularly in fishes that have indeterminate growth, there is substantial evidence for both heritable and plastic components underlying variation in body size (e.g., Campton 1992; Hughes et al. 2005; Hard et al. 2008). Overall, our study provides strong evidence that living in and adapting to extreme environments is linked to modification of energy metabolism, even though proximate mechanisms remain to be studied and ultimate mechanisms may differ between sulfidic and cave habitats.

Metabolic rate variation in cave environments

Caves are typically considered to have low resource availability due to the lack of photosynthetic primary production (Poulson and White 1969; Langecker 2000). Accordingly, a diversity of cave organisms have been reported to exhibit reduced metabolic rates compared to close relatives from surface habitats (see Hüppop 1985 for a review). To our knowledge, no previous studies have investigated to what degree body size reduction and metabolic rate suppression have contributed to decreases in energetic demands. However, it is important to note that inferences about energy metabolism in other cave organisms have primarily focused on mass-adjusted routine metabolic rates (e.g., Poulson 1963; Culver and Poulson 1971; Hüppop 1985; Hervant et al. 1997; Hervant et al. 2001), and – at least in some cases – there is evidence for selection for increased body size in cave populations (Culver et al. 1995; Christiansen 2012), presumably to increase starvation resistance in temperate caves with temporal periodicity of food (Hüppop 2000). Contrary to other

cave organisms, our data indicate that cavernicolous individuals of *Poecilia mexicana* have higher routine metabolic rates than relatives from surface habitats, such that overall reductions in energetic demands are primarily driven by reductions of body size. This discrepancy may be explained by the fact that resource availability in many tropical caves is comparatively stable over time due to reduced seasonality (Hüppop 2000). Hence, cave populations of *P. mexicana* may have adapted to perpetual rather than temporal shortages of energy. In the cave habitats investigated here, continuous supply of food is mediated by bat colonies depositing guano (in both caves) and by chemoautotrophic primary production by sulfide oxidizing bacteria (in the sulfidic cave; Roach et al. 2011). Interestingly, fish from the nonsulfidic cave lacking any sort of primary production exhibited a more pronounced reduction of simulated total energy expenditure than fish from the sulfidic cave; hence, overall energy availability is likely a key determinant of metabolic rate evolution in this system.

Cavefish having higher routine metabolism as compared to their surface counterparts also poses the question whether increased energy consumption was caused by behavioral differences. Poeciliids generally are diurnal (Coleman 2011). The darkness of the respirometry chambers could have reduced activity levels of surface fish, while cavefish remained active and maintained elevated routine metabolic rates. Indeed, cave populations of *P. mexicana* are characterized by sensory and behavioral adaptations to permanent darkness, which are absent in surface ancestors (Parzefall 2001; Plath et al. 2004; Rüschenbaum and Schlupp 2013). However, quantifying activity of fish in complete darkness indicated that cavefish did not have consistently higher activity than surface fish. Instead, fish from extreme habitats generally had a higher activity than those from the ancestral nonsulfidic surface population, and sex differences were idiosyncratic across all populations investigated (significant three-way interaction term including presence of light,

presence of H_2S, and sex; Figure A.3; Table A.3). Nonetheless, future studies should more rigorously tests how individual variation in behavior affects metabolic rates and *vice versa* (see Biro and Stamps 2010; Careau and Garland Jr 2012).

Metabolic rate variation in sulfidic environments

Similar to fish in caves, fish in sulfidic habitats exhibited a body size driven reduction in simulated total energy expenditure (Appendix A; Fig A.2). In contrast, we did not find differences in routine metabolic rates between sulfidic and nonsulfidic populations (irrespective of whether they were located in cave or surface habitats). This contradicted with previous hypotheses that either predicted lower metabolic rates (in response to energy shortage or the rampant hypoxia in sulfidic environments) or higher metabolic rate in sulfidic fish (in response to increased metabolic costs of sulfide detoxification; Riesch et al. 2011b). It is important to note, however, that it remains unclear how routine metabolic rates measured in our experimental setup compare to routine metabolic rates *in situ*, because all oxygen consumption measurements were conducted in absence of H_2S. In general, the presence of physiochemical stressors and toxicants can increase metabolic rates, because coping strategies and detoxification pathways are energetically costly (Penttinen and Kukkonen 1998; Rose et al. 2006; McKenzie et al. 2007). In metazoans, H_2S detoxification is primarily linked to the sulfide:quinone oxidoreductase pathway (Griesbeck et al. 2000; Shahak and Hauska 2008), which oxidizes sulfide to less toxic forms of sulfur while consuming energy (Ip et al. 2004; Hildebrandt and Grieshaber 2008). *Poecilia* from sulfidic habitats have consistently up-regulated genes associated with H_2S detoxification both in the natural habitats (Kelley et al., unpublished data) and upon experimental sulfide exposure in the laboratory (Tobler et al. 2014). However, it remains to be tested whether exposure to H_2S increases metabolic rates in *P. mexicana*

in a similar fashion as in some sulfide adapted invertebrates (Gorodezky and Childress 1994; Schneider 1996), in which case the present study would have overestimated differences in overall energy consumption between sulfidic and nonsulfidic populations. Because H_2S also blocks cytochrome c oxidase (COX) in the mitochondrial respiratory chain (Cooper and Brown 2008), sulfide exposure can also cause metabolic rate depression (Brauner et al. 1995; Blackstone et al. 2005; Volpato et al. 2008), and this may be particularly relevant for the populations investigated here. Unlike other evolutionarily lineages of sulfide spring *Poecilia* in southern Mexico that have evolved H_2S-resistant COXs, sulfide spring populations in the Tacotalpa drainage used in the current study exhibit an H_2S-susceptible COX similar to those found in ancestral nonsulfidic populations (Pfenninger et al. 2014). Consequently, there is also a possibility that our study actually underestimated differences in energy consumption between sulfidic and nonsulfidic populations in their natural habitats, and future experiments will need to isolate the potential effects of H_2S exposure in driving metabolic rate variation in natural populations.

Conclusions

Variation in metabolic rates is central to several physiological and ecological theories (e.g., Kooijman 2000; Brown et al. 2004), but we know comparatively little about the microevolutionary mechanisms that drive macroevolutionary patterns of metabolic rate variation. Our study indicates that adaptation to extreme environmental conditions is manifested in changes in energy metabolism (Parsons 1996), leading to striking intraspecific variation in energetic demands at small spatial scales. Notably, extremophiles have consistent reductions of body size in natural habitats that drive an overall reduction of energy demands. Hence, environmentally induced changes in energy supply and demand may be a major diving force in metabolic rate.

Figures

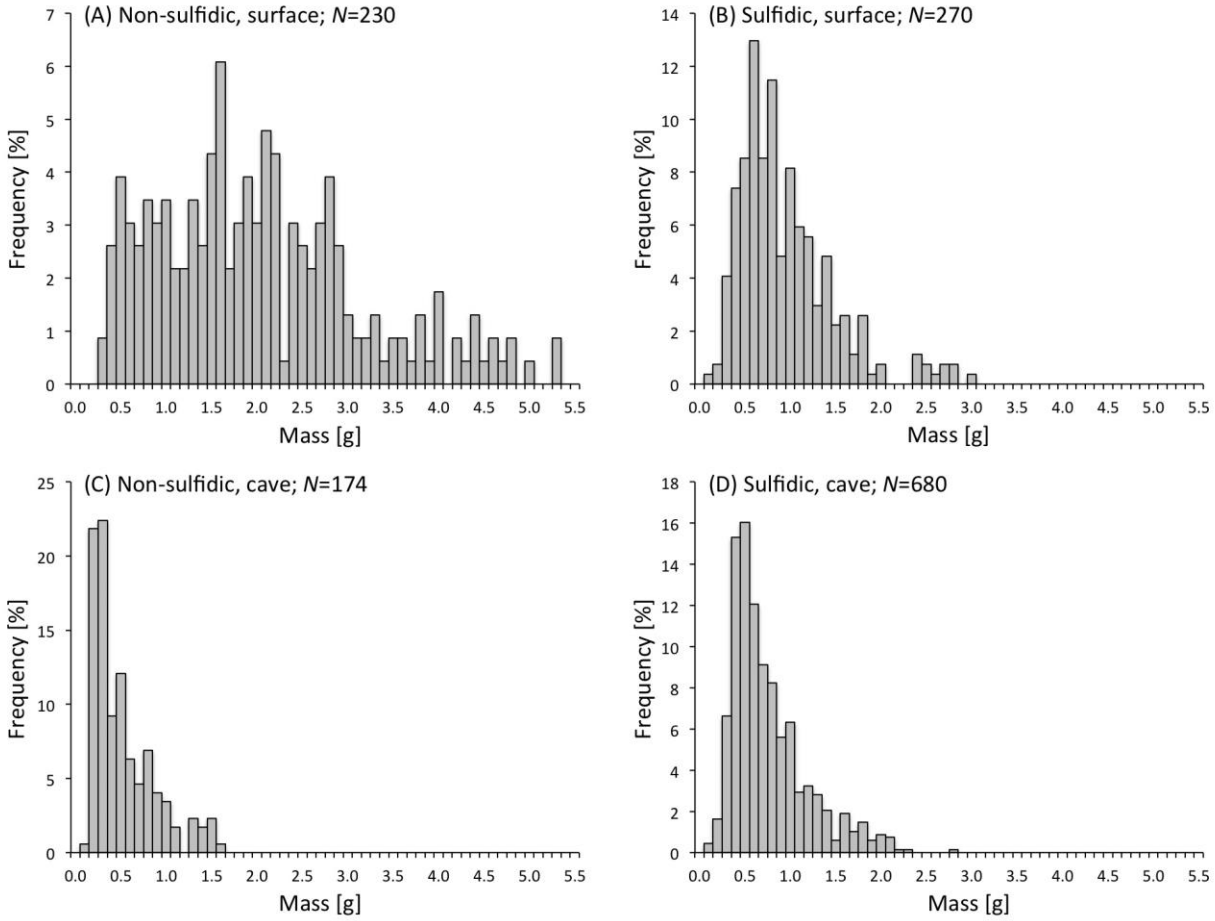

Figure 1.1 Frequency distribution based on body size

Frequency distributions based on body mass in natural populations (A: Arroyo Bonita; B: El Azufre; C: Cueva Luna Azufre; D: Cueva del Azufre). Frequencies are expressed as the percentage of individuals within a size class in each population.

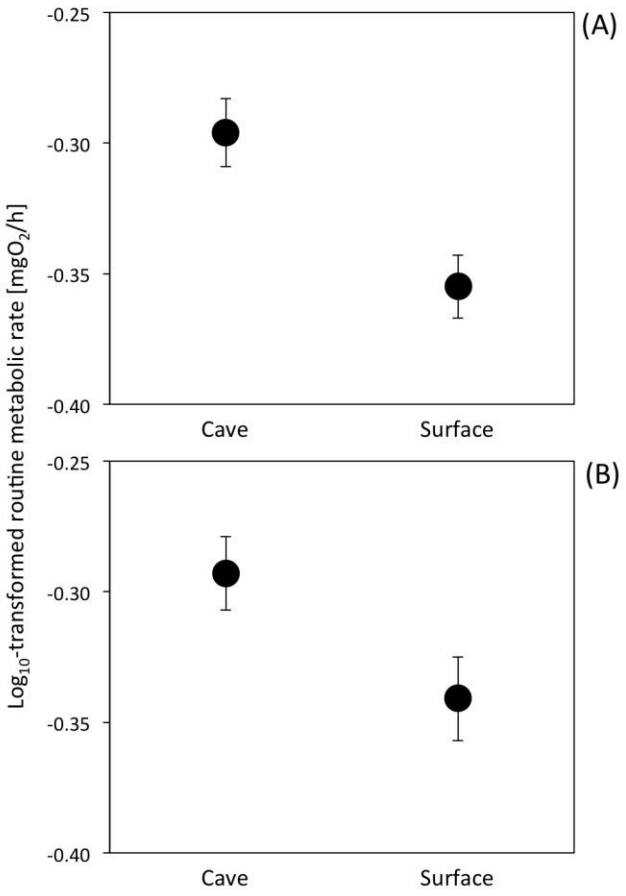

Figure 1.2 Routine metabolism

Differences in routine metabolic rates between cave and surface populations for wild-caught individuals. Depicted are estimated marginal means (± standard deviation) of routine metabolic rates derived from the analytical model in Table 1.2B. Mean values of covariates used for the calculation of estimated marginal means were as follows: mass (log_{10}-transformed) = -0.134 g; temperature = 26.7 °C. **B.** Differences in routine metabolic rates between cave and surface populations for laboratory-reared individuals. Depicted are estimated marginal means (± standard deviation) of routine metabolic rates derived from the analytical model in Table 1.2C. Mean values of covariates used for the calculation of estimated marginal means were as follows: mass (log_{10}-transformed) = -0.125 g; temperature = 25.9 °C.

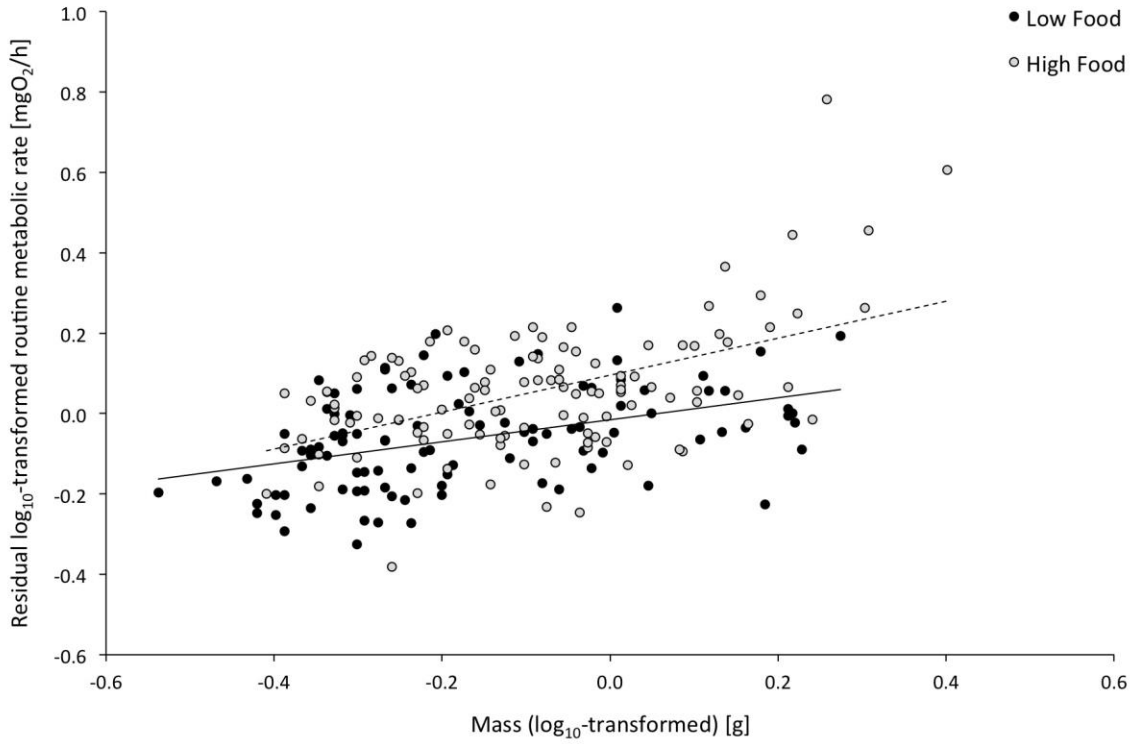

Figure 1.3 Relationship between body mass and routine metabolism

Relationship between body mass and routine metabolic rates for laboratory-raised fish in the high and low food treatments. Depicted are residuals from the analytical model (without mass and food treatment) presented in Table 1.2C.

Tables

Table 1.1 Descriptive statistics

Descriptive statistics for body masses as well as sample sizes of fish used in metabolic rate trials with wild-caught and common garden raised individuals of *Poecilia mexicana*.

	Wild-caught females	Wild-caught males	Laboratory-raised females		Laboratory-raised males	
			High food	Low food	High food	Low food
Nonsulfidic surface	1.07 ± 0.47	1.07 ± 0.80	1.01 ± 0.33	1.09 ± 0.37	0.70 ± 0.17	0.52 ± 0.04
	0.23 – 1.88	0.26 – 2.32	0.39 - 1.67	0.61 - 1.69	0.52 – 0.91	0.48 – 0.58
	(*N*=15)	(*N*=5)	(*N*=23)	(*N*=24)	(*N*=6)	(*N*=4)
Sulfidic surface	1.04 ± 0.45	0.74 ± 0.21	1.17 ± 0.53	0.49 ± 0.10	0.54 ± 0.09	0.48 ± 0.08
	0.43 – 2.04	0.51 – 0.98	0.56 – 2.52	0.34 – 0.66	0.41 – 0.71	0.36 – 0.67
	(*N*=16)	(*N*=4)	(*N*=11)	(*N*=10)	(*N*=17)	(*N*=19)
Nonsulfidic cave	0.58 ± 0.21	0.24 ± 0.08	1.08 ± 0.27	0.94 ± 0.35	0.53 ± 0.11	0.54 ± 0.14
	0.21 – 0.81	0.16 – 0.41	0.53 – 1.88	0.73 – 1.81	0.41 – 0.74	0.38 – 0.82
	(*N* = 7)	(*N*=8)	(*N*=21)	(*N*=15)	(*N*=8)	(*N*=13)
Sulfidic cave	1.15 ± 0.36	0.69 ± 0.55	1.02 ± 0.50	0.91 ± 0.42	0.69 ± 0.13	0.58 ± 0.16

	0.36 – 1.71 (*N*=12)	0.40 – 2.00 (*N*=7)	0.55 – 2.03 (*N*=15)	0.50 – 1.66 (*N*=11)	0.45 – 0.82 (*N*=12)	0.29 – 0.45 (*N*=13)

Note that all measurements of body mass are provided as mean ± standard deviation as well as the range (minimum – maximum) in grams. The sample size in each experimental group is provided in parentheses. Overall sample size for the field experiment was *N*=74 and for the laboratory experiment *N*=222.

Table 1.2 ANOVA and ANCOVA of body size variation and routine metabolism

Results of analyses of (co)variance explaining variation in body size and metabolic rates. (A) Comparison of size distributions of fish residing in different habitat types. (B) Comparison of routine metabolic rates in wild-caught individuals. (C) Comparison of routine metabolic rates in common garden-raised individuals subjected to different resource availability treatments.

Variable	*df*	*F*	*P*	η_p^2
(A) Body mass (Log_{10} – transformed)				
Sex	1	149.682	<0.001	0.100
Cave	1	398.866	<0.001	0.228
Sulfide	1	100.839	<0.001	0.070
Sex × Cave	1	0.660	0.417	<0.001
Sex × Sulfide	1	1.632	0.202	0.001
Cave × Sulfide	1	292.731	<0.001	0.178
(B) Routine metabolic rate of wild-caught fish (Log_{10}-transformed)				
Sex	1	0.001	0.971	<0.001
Cave	1	9.458	0.003	0.122
Sulfide	1	3.322	0.073	0.047
LogMass	1	210.822	<0.001	0.756
Temperature	1	0.884	0.351	0.013

(C) Routine metabolic rate of laboratory-reared fish (Log_{10}-transformed)				
Sex	1	0.019	0.889	<0.001
Food	1	31.87	<0.001	0.137
Cave	1	7.616	0.006	0.037
Sulfide	1	0.294	0.589	0.001
LogMass	1	76.603	<0.001	0.277
Temperature	1	265.84	<0.001	0.571
Food*Cave	1	1.484	0.225	0.007
Food*LogMass	1	10.438	0.001	0.05
Food*Sex	1	3.859	0.051	0.019
Food*Sulfide	1	3.34	0.069	0.016
Cave*LogMass	1	1.315	0.253	0.007
Cave*Sex	1	0.575	0.449	0.003
Cave*Sulfide	1	0.007	0.933	<0.001
Sex*LogMass	1	0.257	0.613	0.001
Sulfide*LogMass	1	0.161	0.689	0.001
Sulfide*Sex	1	0.082	0.775	<0.001

Note that the effect size for each of the terms in a model was estimated by use of partial Eta squared (η_p^2).

Chapter 2 - Convergent evolution of reduced energy demands in extremophile fish

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Abstract

Convergent evolution in organismal function can arise from nonconvergent changes in traits that contribute to that function. Theory predicts that low resource availability and high maintenance costs in extreme environments select for reductions in organismal energy demands, which could be attained through modifications of body size or metabolic rate. We tested for convergence in energy demands and underlying traits by investigating livebearing fish (genus *Poecilia*) that have repeatedly colonized toxic, hydrogen sulfide-rich springs. We quantified variation in body size and routine metabolism across replicated sulfidic and nonsulfidic populations in nature, modelled organismal energy demands, and conducted a common garden experiment to test whether population differences had a genetic basis. Sulfidic populations generally exhibited smaller body sizes and lower routine metabolic rates compared to nonsulfidic populations, which together caused significant reductions in energy demands in extremophile populations. Maintenance of population differences in routine metabolism of common garden reared individuals indicated evolved differences. In combination with other studies, these results suggest that reductions in energy demands represent a common theme in adaptation to physiochemical stressors, which has broad implications for life history evolution.

Introduction

Convergent evolution, where disparate lineages exposed to similar environmental conditions independently evolve similar phenotypes, is a central theme in evolutionary diversification (Endler 1986; Schluter 2000; Losos 2010). Convergence has been documented in a wide variety of traits and in response to different sources of selection (e.g., Schluter and McPhail 1992; Reznick et al. 1996; Landry and Bernatchez 2010). Although convergent evolution is frequently interpreted as evidence for the deterministic nature of natural selection, adaptation to similar environmental conditions does not consistently lead to identical evolutionary outcomes, with individual lineages sometimes diverging in unique, nonconvergent ways (Langerhans and DeWitt 2004; Kaeuffer et al. 2011; Losos 2011). One reason for nonconvergent trait evolution is that natural selection optimizes overall organismal function rather than specific traits that contribute to function (Kaeuffer et al. 2011; Losos 2011). Hence, there may be alternative phenotypic modifications that result in similar fitness (Arnold 1983), and convergence at one level of organization can arise from nonconvergent changes at lower hierarchical levels (Manceau et al. 2010).

The evolution of organismal energy demands is a primary example of how similar functional changes can arise through different mechanisms. Reductions in energy demands can be achieved through two mutually non-exclusive mechanisms: Organisms can evolve a smaller body mass, which decreases costs associated with growth and maintenance (Blanckenhorn 2000). Alternatively, they can evolve lower metabolic rates independent of body size, thus reducing their overall metabolic expenditure (Burton et al. 2011). We were interested in testing whether colonization of and adaptation to extreme environments leads to convergent shifts in organismal energy demand, and whether the same underlying mechanisms are driving variation in energetic demands (i.e., relative contributions of body mass *vs*. metabolic rate reduction) across replicate

populations. Life in extreme environments is associated with elevated energetic costs, including low availability or quality of food, coping strategies that affect rates of resource acquisition, and increased maintenance costs in the presence of environmental stressors (Parson 2005). Life history theory predicts that all of these factors constrain the amount of energy available for reproduction and exert selection for a reduction of organismal energy demands, ultimately allowing for the maximization of relative energy allocation to the production of offspring (Brown et al. 1993). Accordingly, there is circumstantial evidence supporting the hypothesis that extreme environmental conditions favor the reduction of energy demand (e.g., Hüppop 2000; Tieleman and Williams 2000; Passow et al. 2015).

Our study focused on the *Poecilia mexicana* species complex (Poeciliidae), in which multiple lineages have colonized toxic, hydrogen sulfide (H_2S) rich springs across four river drainages in southern Mexico (Tobler et al. 2011; Palacios et al. 2013). Sulfide spring populations exhibit adaptive trait divergence and are reproductively isolated from adjacent, ancestral populations residing in nonsulfidic environments (Tobler et al. 2011; Plath et al. 2013). Based on theoretical and empirical considerations, the presence of H_2S should affect organismal energy budgets in multiple ways. H_2S causes and aggravates hypoxia in natural environments (Bagarinao 1992), forcing fish to trade-off benthic foraging with aquatic surface respiration, which mediates short-term survival (Tobler et al. 2009a). In addition, H_2S constraints aerobic energy production in the mitochondria (Cooper & Brown 2008), and fish have to rely on less efficient anaerobic metabolism for the generation of ATP (Kelley et al. 2016). Finally, tolerating exposure to H_2S requires active detoxification (Tobler et al. 2014; Kelley et al. 2016), and enzymatic sulfide oxidation to a less toxic form requires energy (Ip et al. 2004).

Constraints in energy acquisition and production, as well as increased maintenance costs should precipitate in strong selection for a reduction in energy demands in sulfidic spring populations. Indeed, a recent study of *Poecilia mexicana* in sulfidic and nonsulfidic caves uncovered evidence for lower energetic demands in extremophile populations compared to ancestral populations in nonsulfidic surface habitats (Passow et al. 2015). However, that study was conducted on a small number of focal populations, and all extremophile populations (including the sulfidic and nonsulfidic cave) were derived from a common ancestor (Tobler et al. 2008a). Consequently, it remains to be tested whether convergent evolution shapes energy demands across evolutionarily independent lineages of extremophile fish, and if does, how reductions in body size and mass-specific metabolic rates contribute to such reductions. Here, we investigated multiple sulfidic and nonsulfidic populations to address the following objectives: (1) We quantified size distributions of fish populations in sulfidic and nonsulfidic habitats across four river drainages to test whether adaptation to extreme environmental conditions is consistently associated with the reduction of body size. (2) We quantified routine metabolic rates (RMR) of wild-caught fish to test whether extremophile population exhibited consistent reductions in metabolic rates. (3) We modelled total energy demand based on empirical data collected on body size and metabolic rate allometry to quantify the combined effects of body mass and metabolic rate reductions in different populations. (4) We quantified RMR in common garden reared individuals to test whether differences between sulfidic and nonsulfidic populations are caused by genetic variation.

Materials and methods

Study sites and mass-based size distribution

We analysed body size distributions and RMR in wild-caught *Poecilia* from five sulfidic springs

and six adjacent, nonsulfidic habitats in different tributaries of the Rio Grijalva (Pichucalco, Ixtapangajoya, Puyacatengo, and Tacotalpa river drainages; Figure 2.1, Table 2.1). To quantify body size distributions, fish were collected using seines (4 m long, 4 mm mesh width), and blotted wet weight was measured for each individual to the closest 0.001g. Mass-based size distributions were analysed using general linear models (GLM) with body mass (log_{10}-transformed) as the dependent variable. Sex, presence or absence of H_2S, drainage, and population (nested in the drainage by sulfide interaction) were included as independent variables. The three-way interaction term was not significant ($F_{3,2458} = 1.230$, $P = 0.297$) and was excluded from the final model.

Quantification of routine metabolic rates

To quantify RMR, which is defined as the oxygen consumption of unconstrained, post-absorptive organisms capable of spontaneous movement (Steffensen 1989), specimens were collected from focal populations and transported to a nearby field station. Fish were acclimated to standardized laboratory conditions for at least 48 hours. We used a closed chamber respirometry system to measure oxygen consumption. This approach has been widely used to quantify metabolic costs associated with a variety of traits and environmental conditions (Haney and Nordlie 1997; Seibel and Drazen 2007; Pirozzi and Booth 2009; Culumber 2015). Methods followed protocols implemented in a previous study (Passow et al. 2015): (1) Fish were not fed 24 hours prior to trials to assure measurements were conducted on post-absorptive individuals (Timmerman and Chapman 2004b). (2) Fish were haphazardly chosen and placed into 500mL Nalgene respirometry bottles that were painted black on the outside to reduce light penetration. Bottles were then placed together in a water bath to minimize temperature fluctuations (average ± SD: 25.13 ± 1.92°C). Fish were left undisturbed in the bottles with aerated water to acclimate to the ambient conditions.

(3) After acclimation, respirometry bottles were flushed with fresh aerated water to remove metabolic waste products that could affect metabolism and capped with a lid that had a hole drilled in the top to allow for the insertion of a YSI ProODO optical dissolved oxygen probe (YSI Inc., Yellow Springs, OH, USA). Plumbers putty was put around the probe to prevent gas exchange during the trial. Probes were set to measure the dissolved oxygen concentration at 10-second intervals. Note that all trials were conducted in absence of H_2S even for sulfidic populations, because the reactivity of H_2S with oxygen in aqueous solution affects the measurement of oxygen consumption rates (Chen and Morris 1972). (4) After the termination of a trial, individuals were weighed and sexed (see Table 2.1 for descriptive statistics). For each trial, we removed outliers (random readings of zero oxygen) that were likely caused by instrumental error. We also removed data points from the first 60 min of each trial, as the flushing of the bottle with fresh water and the installation of the probe may have caused erratic fish activity (Timmerman and Chapman 2004b). Because fish metabolic rates may be affected by reduced ambient oxygen concentrations (Haney and Nordlie 1997), we only included data points measured at dissolved oxygen saturations ≥70%. Metabolic rate (in mgO_2/hour) was then calculated for each individual as the slope of a regression (multiplied by the volume of water in the bottle) with oxygen concentration as a dependent variable and time as an independent variable (mean $R^2 = 98.9\%$). Routine metabolic rate data were analysed using GLM. Log_{10}-transformed routine metabolic rate was used as the dependent variable. We included sex, presence or absence of H_2S, drainage, and population (nested in the drainage by sulfide interaction) as independent variables. Temperature and mass (log_{10}-transformed) were included as covariates in all models. Three-way interaction terms and interactions with covariates were not significant ($F \leq 1.616$, $P \geq 0.186$) and were excluded from the final model.

Simulated total metabolism based on body size and routine metabolic rates

To determine how variation in body size and RMR interact to shape organismal energy demands, we modelled total routine metabolic rates for all populations based on the empirical data on size distributions and allometric metabolic rate functions (see Passow et al. 2015 for details). For each habitat type, we first resampled size distributions based on the field data collected 1000 times. For each resampled individual, total metabolic rate was calculated as $\log_{10}(MR_{tot})=b*\log_{10}(\text{mass})+a$, where b was the slope and a the intercept of a regression describing the relationship between mass and metabolic rate for each population. To account for uncertainty associated with the estimation of slopes and intercepts, values for b and a were randomly chosen from within the 95% confidence interval of each parameter. The simulated values of total routine metabolic rate consequently represent estimates of the energy demand of average individuals in each population, taking into account among-population variation in both body mass and metabolic rate allometry. Simulated total metabolic rates were analysed using GLM with presence or absence of H_2S in natural populations, drainage, and populations (nested in the drainage by sulfide interaction) as independent variables.

Routine metabolic rates and metabolic rate plasticity in common-garden raised fish

Metabolic rate variation in wild-caught fish may merely reflect plastic responses to exposure to different environments. Hence, we conducted a common garden experiment to test whether variation in mass-adjusted RMR between sulfidic and nonsulfidic populations has a genetic basis. Fish were collected from a subset of sites (Table 2.1) and transported to the laboratory at Kansas State University. Juveniles born to wild-caught mothers were isolated in family groups and raised to adulthood (standard length >30 mm). All fish were kept under nonsulfidic conditions with a

12:12 hour light:dark cycle and a constant temperature of 25°C. The experimental protocol for metabolic rate measurements was identical to the one for wild-caught fish (average temperature during trials ± SD: 24.17 ± 1.06°C). Data on metabolic rates (log_{10}-transformed) from wild-caught and common-garden-reared fish were combined and used as dependent variable in a GLM. We included sex, presence or absence of H_2S, drainage, and rearing environment (i.e. wild-caught *vs.* laboratory-reared) as independent variables. Temperature and mass (log_{10}-transformed) were included as covariates. Three-way and four-way interactions, as well as interactions with the covariates were not significant ($F < 2.125$, $P > 0.146$) and were excluded from the final model. All procedures were approved by the Institutional Animal Care and Use Committee at Kansas State University (Protocol #3418). Statistical analyses were conducted with SPSS 17 (SPSS Inc., Chicago, IL, USA).

Results

Reduced body size in extreme environments

We measured body mass for *N*=2,747 individuals collected across all sites (see Table 2.1 for descriptive statistics) and found significant differences between sulfidic and nonsulfidic populations (Table 2.2A). Individuals from sulfidic populations were consistently smaller than those from nonsulfidic populations of the same drainage (Figure 2.2A). We found that the magnitude of difference varied among the different drainages, and that there was significant variation among the specific populations analysed. Note that males were consistently smaller than females. In addition, sample sizes were generally lower for males than females, which is reflective of the highly female biased sex ratio in natural populations (Plath et al. 2007b).

Reduced routine metabolism in extreme environments

We measured RMR in *N*=347 wild-caught individuals from 5 sulfidic and 6 nonsulfidic populations. Body mass and temperature explained most of the variation in RMR, but RMR also varied among populations and drainages (Table 2.2B). Most importantly, individuals from sulfidic habitats generally exhibited lower mass-adjusted RMR than those from nonsulfidic habitats. We found that this pattern was more pronounced in the Ixtapangajoya and Pichucalco river drainages (Figure 2.2B), which explains the significant drainage by habitat type interaction in the model. Simulations of total energy demands indicated that the presence of H_2S explained the bulk of variation in the dataset (Table 2.2C), and fish from sulfidic populations exhibited substantially lower energetic demands (Figure 2.2C). The magnitude of differences between sulfidic and nonsulfidic populations among drainages also varied (Figure 2.2C).

Heritable and plastic variation in the reduction of routine metabolic rates

Comparison of RMR between wild-caught and common garden-reared individuals from a subset of populations indicated that energy consumption rates generally were higher in the field than in the laboratory (Table 2.2D). Nonetheless, differences in energy consumption rates between sulfidic and nonsulfidic sites were maintained – or even amplified – in the laboratory, with sulfidic fish having lower mass-adjusted RMR than nonsulfidic fish (Figure 2.2D).

Discussion

We investigated the evolution of energy demands in extreme environments by comparing body size and metabolic rate variation among locally adapted fish populations inhabiting replicated sulfidic and nonsulfidic habitats. We found that fish from sulfide springs were both smaller and

had lower mass-adjusted RMRs. Nonetheless, differences were not pronounced in all river drainages, indicating some variation in the reduction of energetic expenditure. Simulating total metabolic rates based on variation in body mass and RMR allometry, however, indicated pronounced reductions in overall energy demands of extremophile populations, revealing a pattern of convergent evolution in extremophile populations. The maintenance of population differences in mass-adjusted RMR of laboratory-reared fish further suggests evolved reductions in energy demands of extremophile populations.

Reduction in energy demands common theme in adaptation to extreme environments

These results are consistent with theoretical considerations that predict reductions in energy demands of extremophiles in response to selection mediated by high maintenance costs and/or low resource availability (Brown et al. 1993; Parson 2005). They are also consistent with empirical evidence from other extremophile organisms (Hüppop 1985; Koslow 1996; Timmerman and Chapman 2004; Passow et al. 2015), perhaps suggesting that reductions in energy demands are a common theme in adaptation to extreme environments. While most inferences about energy demands of extremophiles have solely been drawn from analyses of metabolic rates, our study suggests that variation in body size and metabolic rates should be considered jointly. We found no or small differences in mass-adjusted RMR between sulfidic and nonsulfidic populations in some drainages, such that reductions in energy demand were primarily driven by reductions in body size. In others, modifications of both body size and metabolic rates jointly contributed to reductions in energy demand. Hence, even if overall energy demands have evolved in convergence across the different sulfide spring populations, the relative contributions of underlying mechanisms driving this change varied among lineages.

Plastic and genetic contributions to routine metabolic rates

Variation in routine energy consumption rates among organisms is primarily explained by body mass and temperature (Gillooly et al. 2001). Nonetheless, both plastic and genetic factors can cause deviations from mass and temperature-dependent metabolic scaling relationships (Burton et al. 2011), and analysis of common-garden reared individuals uncovered evidence for both. Effects of plasticity were evident, as common-garden raised fish exhibited lower mass-adjusted RMR compared to wild-caught individuals. At the same time, differences in mass-adjusted RMR between sulfidic and nonsulfidic populations are partially driven by genetic variation among populations, because sulfide spring fish retained lower oxygen consumption rates even when raised under standardized, nonsulfidic conditions. Consequently, reductions in overall energy demands documented in the field may at least in part be driven by evolutionary differentiation among proximate populations that are exposed to contrasting environmental conditions, although it remains unclear how epigenetic effects may have influenced metabolic rate variation in our experiment (Burggren 2014). Interestingly, reductions of energy demands also parallel divergence in reproductive life history traits (Riesch et al. 2009; Riesch et al. 2014) and the expression of costly morphological (Eifert et al. 2015; Schulz-Mirbach et al. 2016) and behavioural traits (Plath 2008; Bierbach et al. 2012; Doumas and Tobler submitted), bolstering the notion that changes in energy supply and demand represent important drivers of evolution in extreme environments.

Non-mutually exclusive mechanisms driving reduction in energetic demands across replicated gradients

Future studies will need to investigate what proximate mechanisms are involved in reducing mass-adjusted metabolic rates in sulfide spring environments, because several non-mutually exclusive mechanisms could be at work. (1) Variation in metabolic rates may simply reflect differences in activity rates or other aspects of behaviour (Careau et al. 2008; Careau and Garland 2012). Since metabolic rates were quantified using closed chamber respirometry, individual fish were capable of spontaneous movements. Population differences in general activity patterns or the expression of costly behaviours could consequently shape variation in metabolic rates. While a previous study found no correlation between activity and metabolic rates (Passow et al. 2015), there is evidence that fish from sulfidic environments have reduced costly behaviours associated with aggregation and mating (Plath 2008; Bierbach et al. 2012; Doumas and Tobler submitted). (2) Reduced metabolic rates in sulfide spring fishes may be a consequence of physiological modifications that have occurred in response to selection from the presence of H_2S. H_2S is potent respiratory toxicant that directly interferes with mitochondrial function and ATP production (Cooper and Brown 2008). At least in some sulfide spring populations investigated here (Pichucalco and Puyacatengo drainages), there is evidence for adaptive modification of cytochrome c oxidase, which represents the primary toxicity target of H_2S and the enzyme responsible for oxygen consumption by mitochondrial oxidative phosphorylation (Pfenninger et al. 2014). Modified cytochrome oxidase in sulfide spring populations of *P. mexicana* allows for the maintenance of aerobic ATP production in presence of H_2S (Pfenninger et al. 2014), but it remains unclear whether sulfidic and nonsulfidic populations differ in mitochondrial oxygen consumption rates in the presence of H_2S. (3) Reduced metabolic rates may be a consequence of physiological modifications in response to variation in oxygen or energy availability among habitat types, as oxygen limitation (Hochacka et al. 1996; Richards 2009) as well as quantitative (Wang et al. 2006) and qualitative (Jeyasingh 2007)

differences in diets can affect metabolic expenditure. Indeed, sulfide springs are extremely hypoxic (Tobler et al. 2006), and genes associated with anaerobic metabolism are up-regulated in natural populations (Kelley et al. 2016). In addition, sulfidic and nonsulfidic populations differ in both resource acquisition rates (Tobler et al. 2009a) and dietary resource use (Tobler et al. 2015a; Tobler et al. 2016a).

Conclusions

In summary, our study adds to a growing body of literature suggesting that adaptation to extreme environments is accompanied by changes in energy metabolism that likely underlie selection on life history traits. Reductions in in overall energy demands exhibited strong patterns of convergent evolution, but convergence was less evident in body size distributions and mass-specific routine metabolic rates that underlie organismal energy demand. Disentangling convergent and nonconvergent responses to physiochemical stressors that modulate the environmental supply of resources and organismal maintenance costs is critical for understanding organisms' resilience to environmental stress and predicting species responses in the face of global environmental change (Gardner et al. 2011).

Figures

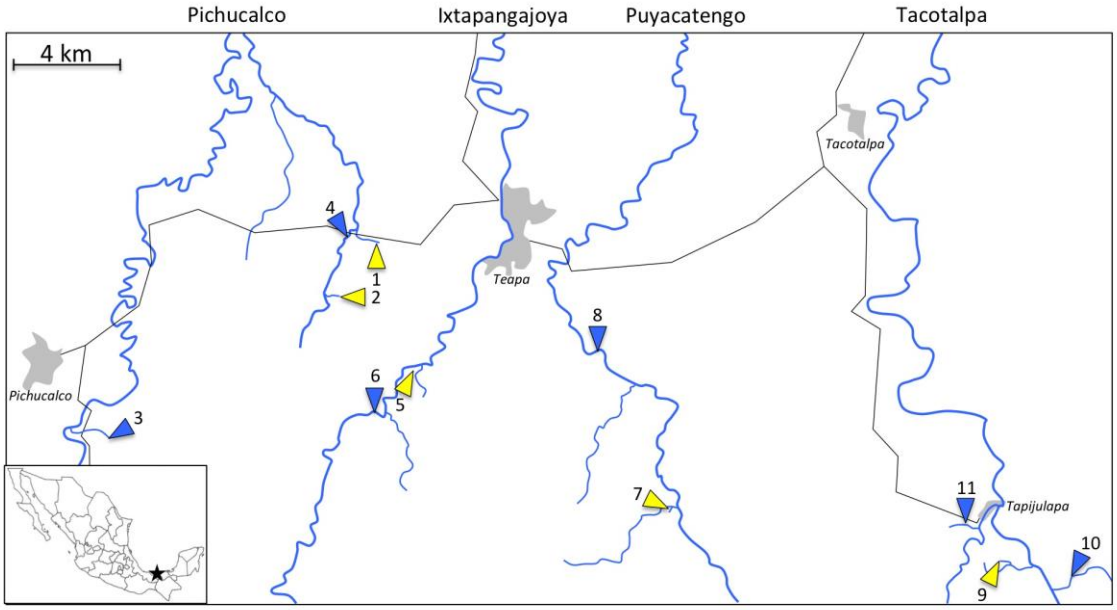

Figure 2.1 Map of study region

Map of the study region adapted from Palacios et al. (2013). Depicted are the localities of focal sulfidic and nonsulfidic sites across four river drainages in southern Mexico. Yellow arrows represent sulfidic sites and blue arrows represent nonsulfidic sites. The numbers correspond to sites as described in Table 2.1. Note that the location of major towns (shaded areas) and streets (black lines) has been added for orientation. The insert indicates the locality of the study area within Mexico.

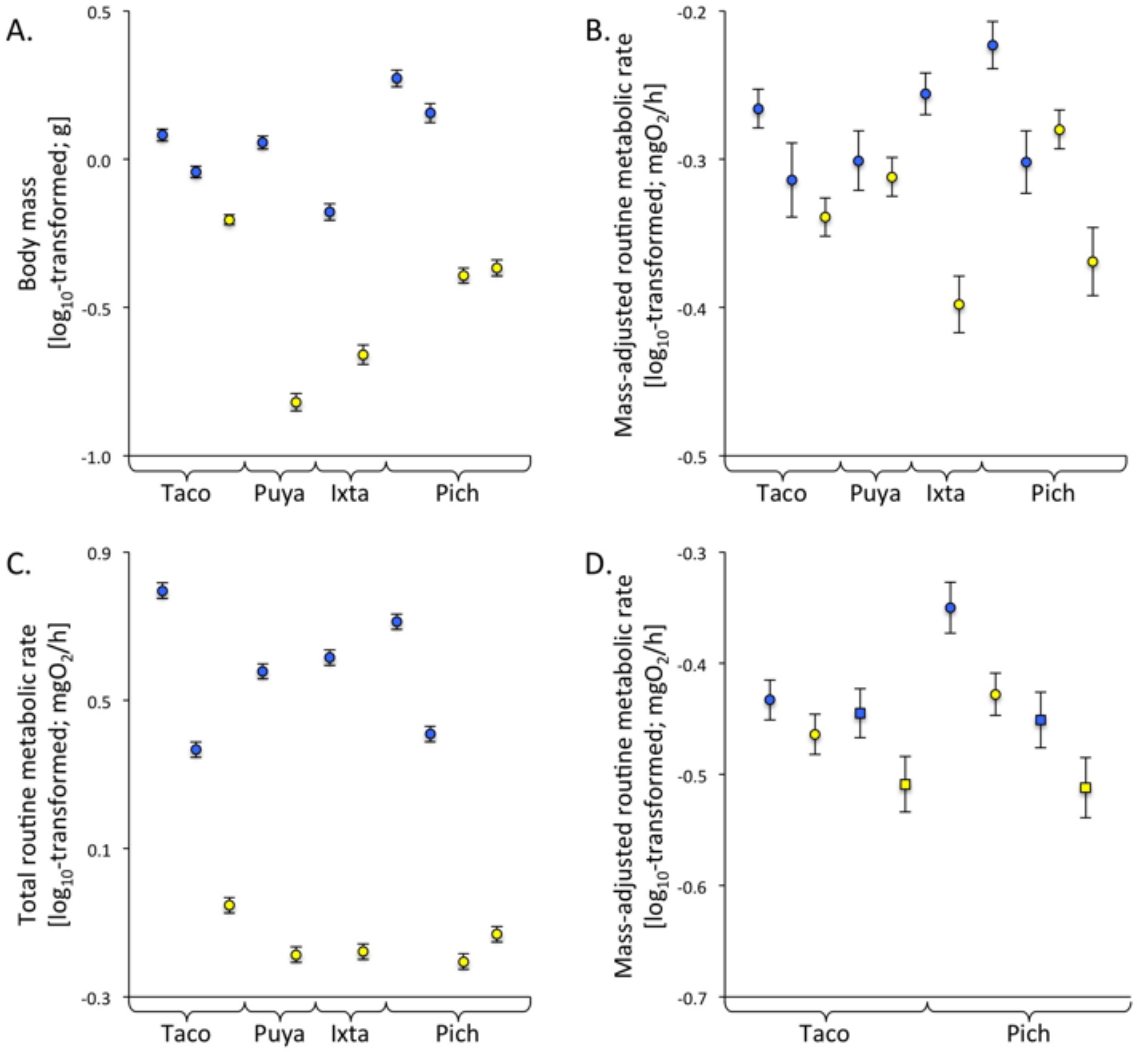

Figure 2.2 Population differences in body size and routine metabolism

Population differences in A. body size, B. mass-adjusted routine metabolic rate, and C. total routine metabolic rate. Depicted are estimated marginal means (EMM ± standard error) based on analytical models presented in Table 2.1. Populations are organized by river drainage; blue symbols represent nonsulfidic populations, yellow symbols sulfidic ones. Mean values of covariates used for the calculation of EMM of mass-adjusted routine metabolic rate were as follows: mass (log_{10}-transformed) = -0.083 g; temperature = 25.13 °C. D. Differences in mass-adjusted routine metabolic rate between wild-caught (circles) and laboratory-reared (squares) fish from a subset of populations. Mean values of covariates used for the calculation of EMM were as follows: mass (log_{10}-transformed) = -0.141 g; temperature = 24.17 °C.

Tables

Table 2.1 Descriptive statistics of populations investigated

List of population investigated for this study, including latitude and longitude of collection localities. The table also provides descriptive statistics of body masses from fish used to characterize size distributions in natural populations as well as to quantify routine metabolic rates (RMR) in wild-caught and laboratory-reared specimens. We report body masses [g] in means (± standard deviation) and ranges (in parentheses), as well as sample sizes separately for males and females of each population. Note that ID numbers correspond to the numbers in Figure 2.1.

ID	Site	Lat/Long	H_2S	Size distribution		MR wild-caught		MR laboratory-reared	
				Females	Males	Females	Males	Females	Males
Rio Pichucalco drainage									
1	Baños del Azufre	17.552, -92.999	+	0.49±0.35 (0.04-1.84) N=141	0.58±0.29 (0.04-1.73) N=43	0.69±0.28 (0.34-1.31) N=34	0.47±0.26 (0.20-0.87) N=9	0.52±0.15 (0.29-0.78) N=9	0.41±0.13 (0.22-0.60) N=6
2	La Gloria	17.532, -93.015	+	0.67±0.99 (0.12-5.26) N=115	0.45±0.27 (0.16-1.25) N=43	0.96±0.61 (0.16-2.46) N=12	0.85±0.52 (0.49-1.22) N=2	-	-
3	Arroyo Rosita	17.485, -93.104	-	2.35±1.65 (0.21-9.04) N=119	2.03±1.36 (0.51-6.10) N=27	2.19±1.07 (0.57-5.52) N=26	2.31±1.54 (0.51-6.00) N=9	0.74±0.25 (0.48-1.13) N=11	0.70±0.25 (0.34-1.09) N=8
4	Rio El Azufre, west branch	17.556, -93.008	-	1.57±0.91 (0.30-4.80) N=113	1.88±1.01 (0.59-3.92) N=17	1.78±0.65 (0.84-3.37) N=14	1.36±0.85 (0.46-2.79) N=6	-	-
Rio Ixtapangajoya drainage									
5	La Esperanza, large spring	17.511, -92.983	+	0.35±0.20 (0.06-0.89) N=126	0.18±0.07 (0.10-0.32) N=19	0.79±0.25 (0.40-1.22) N=17	0.19±0.05 (0.14-0.23) N=3	-	-
6	Rio Ixtapangajoya	17.495, -92.998	-	1.52±1.60 (0.07-8.54) N=153	0.60±0.48 (0.13-1.94) N=38	1.48±0.57 (0.40-2.62) N=26	1.06±0.77 (0.24-2.99) N=13	-	-
Rio Puyacatengo drainage									
7	La Lluvia, small spring	17.464, -92.895	+	1.83±0.80 (0.21-2.89)	0.34±0.15 (0.13-0.74)	1.83±0.80 (0.21-2.89)	0.34±0.15 (0.13-0.74)	-	-

				N=123	N=107	N=28	N=16		
8	Rio Puyacatengo at Vicente Guerrero	17.510, -92.914	-	1.79±0.83 (0.75-3.42) N=167	1.44±0.83 (0.55-2.72) N=62	1.79±0.83 (0.75-3.42) N=15	1.44±0.83 (0.55-2.72) N=5	-	-
Rio Tacotalpa drainage									
9	El Azufre I	17.442, -92.775	+	0.85±0.56 (0.16-3.00) N=306	0.63±0.27 (0.07-1.56) N=112	0.65±0.45 (0.16-1.58) N=36	0.73±0.40 (0.30-2.04) N=10	0.61±0.27 (0.30-1.17) N=17	0.37±0.25 (0.19-0.95) N=8
10	Arroyo Bonita	17.427, -92.752	-	1.68±1.16 (0.17-7.80) N=251	1.59±1.23 (0.25-4.69) N=71	1.34±0.81 (0.23-3.73) N=31	1.06±0.50 (0.25-2.32) N=13	0.47±0.12 (0.30-0.59) N=9	0.73±0.69 (0.27-2.54) N=10
11	Arroyo Tacubaya	17.454, -92.785	-	1.23±0.72 (0.15-5.23) N=265	0.87±0.54 (0.20-2.23) N=55	0.37±0.21 (0.14-0.79) N=19	0.60±0.30 (0.60-1.18) N=3	-	-

Table 2.2 Results of general linear model

Results of general linear models analysing variation in body size and metabolic rates. A. Comparison of body mass among populations. B. Comparison of routine metabolic rates in wild-caught individuals. C. Comparison of simulated total metabolic rates. D. Comparison of routine metabolic rates in wild-caught and common-garden raised individuals for a subset of populations. Effects with relative variance > 0.5 are highlighted in bold.

Variable	*df*	*F*	*P*	η_p^2	Relative variance
A. Body mass					
Sex	1	24.02	<0.001	0.010	0.056
Drainage	3	54.961	<0.001	0.063	0.356
H_2S	1	506.565	<0.001	0.171	0.966
Population (Drainage × H_2S)	4	132.192	<0.001	0.177	1.000
Sex × Drainage	3	12.018	<0.001	0.014	0.079
Sex × H_2S	1	0.005	0.942	<0.001	<0.001
Drainage × H_2S	3	55.620	<0.001	0.063	0.356
B. Routine metabolic rate (wild-caught fish)					
Mass (log_{10}-transformed)	1	637.391	<0.001	0.670	1.000
Temperature	1	185.013	<0.001	0.371	0.554
Sex	1	0.585	0.445	0.002	0.003
Drainage	3	2.800	0.040	0.026	0.039
H_2S	1	22.439	<0.001	0.067	0.100
Population (Drainage × H_2S)	3	7.870	<0.001	0.070	0.104
Sex × Drainage	3	2.080	0.103	0.019	0.028
Sex × H_2S	1	0.383	0.536	0.001	0.001
Drainage × H_2S	3	5.862	0.001	0.053	0.079
C. Simulated energy demand					
Drainage	3	5.305	0.001	0.001	0.005
H_2S	1	3038.772	<0.001	0.217	1.000
Population (Drainage × H_2S)	3	103.974	<0.001	0.028	0.129
Drainage × H_2S	3	6.352	<0.001	0.002	0.009
D. Routine metabolic rate (laboratory-reared fish)					
Mass (log_{10}-transformed)	1	294.558	<0.001	0.557	1.000
Temperature	1	6.405	0.012	0.027	0.048
Sex	1	1.817	0.179	0.008	0.014
Drainage	1	2.754	0.098	0.012	0.022
H_2S	1	10.109	0.002	0.041	0.074
Field/Laboratory	1	12.221	0.001	0.050	0.090
Sex × Drainage	1	1.312	0.253	0.006	0.011
Sex × H_2S	1	<0.001	0.993	<0.001	<0.001

Sex × Field/Laboratory	1	0.112	0.739	<0.001	<0.001
Drainage × H_2S	1	1.207	0.273	0.005	0.009
Drainage × Field/Laboratory	1	4.430	0.036	0.019	0.034
H_2S × Field/Laboratory	1	0.151	0.698	0.001	0.002

Chapter 3 - Tissue-specific responses to toxic hydrogen sulfide and permanent darkness in livebearing fishes

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Abstract

Adaptation is a key process in the evolution of biodiversity. Nonetheless, elucidating the physiological pathways that are modified under different environmental condition remains a major task for the evolutionary biology, especially in non-model organisms. Next-generation sequencing technologies have transformed the quantification of genome-wide gene expression patterns in non-models and natural systems through the use of RNA sequencing. Here, we performed an RNA-sequencing study on three different organs and fish adapted to hydrogen sulfide (H_2S)-rich environments and the absence of light to quantify patterns of gene expression associated with adaptation to extreme environments. We specifically asked: (1) How does gene expression and functional responses change among populations living in different habitats and organs?, (2) Is there evidence of shared expression responses in populations exposed to the same environmental conditions and how do shared responses vary among levels of organization? and (3) what inferences can analyses of differential expression provide about potential molecular mechanisms underlying adaptation? We assembled and annotated transcriptomes of *Poecilia mexicana* from 16 wild-caught individuals with four individuals and four tissue types per population (nonsulfidic surface, sulfidic surface, nonsulfidic cave, and sulfidic cave). We mapped all reads to the *Poecilia mexicana* genome and identified variation in gene expression patterns between ecotypes and tissues by using the EdgeR package to identify genes that are differentially expressed. We

uncovered rampant variation in gene expression. Organ type was the major driver of expression among samples illuminating the importance of choosing focal organs when studying gene expression in nature. We also identified variation in the amount of shared differentially expressed genes, where shared responses increased with level of biological organization. Nonetheless, shared responses were more common in the sulfidic habitats, highlighting the effects of H_2S impact on gene regulation. Overall, our analyses provided insights into transcriptional variation in a unique system that coincides with adaptation to H_2S and darkness. Likewise, functional annotations of differentially expressed genes provide a springboard for investigating molecular mechanisms putatively underlying adaptation to extreme environments.

Introduction

Next-generation sequencing technologies (NGS; Metzker 2010) have transformed the quantification of genome-wide gene expression patterns in natural systems through the use of RNA sequencing (RNA-seq; Wang et al. 2009). Analyses of gene expression variation along environmental gradients have been instrumental for identifying molecular mechanisms underlying adaptation and characterizing organismal responses to novel environments with strong abiotic and biotic sources of selection (Cheviron et al. 2008; López-Maury et al. 2008, Whitehead et al. 2011a; Morris et al. 2014). Studies often focus on replicated gradients in which multiple populations are exposed to similar stressors allowing for the identification of shared and unique expression responses (Rifkin et al. 2003; Whitehead et al. 2011b; Leder et al. 2015). Nonetheless, environments are more complex than one simple stressor (Holmstrump et al. 2010). Thus, it is imperative to focus on expression responses in natural systems where closely related populations are exposed to multiple sources of selection that occur in different combinations. This is

particularly important because gene expression changes to multiple stressors may not be additive, and interactive effects and trade-offs may hamper our ability to predict organismal responses in natural systems when multiple sources of selection coincide (Suh et al. 2003; Kopec et al. 2011).

Inferences about potential mechanisms of adaptation based on gene expression analyses may also depend on the tissues that are being analyzed. Stressors with broad, systemic effects (e.g., thermal stress) might elicit relatively consistent expression responses across different tissue types (Akashi et al. 2016). Alternatively, patterns of gene expression responses might be idiosyncratic and vary substantially among organs, tissues, or even cell types (Birnbaum et al. 2003; Bailey et al. 2013; Bos et al. 2016). For example, expression responses to hypoxia exposure vary due to differences in the metabolic requirements among organs (Gracey et al. 2000, Whitehead and Crawford 2005). While the analysis of gene expression responses in multiple organs is becoming more common (Whitehead and Crawford 2005; Chan et al. 2009 Alvarez et al. 2015; Akashi et al. 2016; Uebbing et al. 2016), it often remains unclear how organ-specific expression changes relate to potential functional consequences and patterns of local adaptation (Oleksiak et al. 2002).

Here, we characterized gene expression variation in a system of extremophile fishes that are exposed to different combinations of environmental stressors and asked whether the analysis of multiple organs affects inferences about the putative functional consequences of expression differences. *Poecilia mexicana* (Poeciliidae) is a small livebearing fish that has colonized sulfide springs and caves in Mexico's Cueva del Azufre system, giving rise to a unique group of closely related populations living in geographically proximate habitat types with vastly different environmental conditions (nonsulfidic surface streams, sulfidic surface streams, a nonsulfidic cave, and a sulfidic cave; see Figure. 3.1; Parzefall et al. 2001; Tobler et al. 2008a). Sulfidic habitats are characterized by high concentrations of hydrogen sulfide (H_2S), a potent respiratory

toxicant that binds to cytochrome c oxidase in the respiratory chain, effectively halting ATP production (Bagarinao 1992; Cooper and Brown 2008; Tobler et al. 2016b). Caves are characterized by the absence of light, which has direct effects on organismal function (e.g., sensory biology and circadian rhythms; Poulson and White 1969; Langecker 2000; Niemiller and Soares 2015). Both H_2S and permanent darkness also shape the biotic environment, affecting resource availability (Hüppop 2000; Roach et al. 2011) as well as competition and predation regimes (Riesch et al. 2010). Previous studies have indicated that populations exposed to specific combinations of H_2S and darkness are locally adapted (Figure 3.1D), exhibiting phenotypic differences in behavioral (Parzefall et al. 2001; Plath 2008), sensory (Plath et al. 2004; Tobler et al. 2010), physiological (Passow et al. 2015), morphological (Tobler et al. 2008a), and life-history characteristics (Riesch et al. 2014). In addition, microsatellite analyses have indicated significant genetic differentiation and low rates of gene flow among populations in different habitat types (Plath et al. 2007a; Plath et al. 2010b), and reproductive isolation is in part mediated by natural and sexual selection against non-adapted migrants (Tobler 2009; Tobler et al. 2009b).

Previous transcriptome studies on organisms living in H_2S-rich environments have documented differential expression of genes associated with H_2S detoxification and the processing of sulfur compounds, as well as aerobic and anaerobic ATP production and energy metabolism (Wong et al. 2015; Kelley et al. 2016; Liu et al. 2016). Analysis of transcriptomes of cave-dwellers have revealed differential expression of genes associated with eye development and function, as well as energy metabolism (Gross et al. 2013; Meng et al. 2013). Here we performed an RNA-seq study on three different organs and fish from nonsulfidic surface, sulfidic surface, sulfidic cave and nonsulfidic cave populations to quantify patterns of gene expression. We specifically asked the following questions: (1) How does gene expression vary among populations living in different

habitat types, and are the functional responses similar among organs? (2) Is there evidence of shared expression responses in populations exposed to the same environmental conditions (presence of H_2S or absence of light), and how do shared responses vary among levels of organization? (3) What inferences can analyses of differential expression provide about potential molecular mechanisms underlying adaptation to specific environmental conditions?

Methods

Sample collections

Samples for transcriptome analyses were collected in the Cueva del Azufre system near the village of Tapijulapa, Tabasco, Mexico (Figure 3.1A; Tobler et al. 2008a). Fish were caught at four sites: Arroyo Bonita (nonsulfidic surface stream), El Azufre II (sulfidic surface stream), Cueva Luna Azufre (nonsulfidic cave), and Cueva del Azufre (sulfidic cave). Upon capture, four adult females per site were immediately euthanized, weighed, and measured (Table B.1). Gills, livers, brains, and eyes were then extracted using sterilized scissors and forceps and separately preserved and stored in RNAlater (Ambion, Inc). Procedures for all experiments were approved by the Institutional Animal Care and Use Committee at Kansas State University (Protocol #3418).

We focused on the gill, liver, brain and eyes because of *a priori* expectations about their roles in adaptation to the environmental conditions encountered in this system. Gills are an important organ involved in the maintenance of homeostasis (Evans et al. 2011; Evans et al. 2013), and since they exhibit a high surface area directly exposed to the water, they provide an important contact point for H_2S exposure (Tobler et al. 2016b). The liver plays important roles in detoxification and modulating energy metabolism (Dorman et al. 2002; Green et al. 2008), and both processes are relevant in the context of H_2S and cave adaptation (Bagarinao and Vetter 1990;

Aspiras et al. 2015). The brain has previously been shown to play an important role in adaptation to perpetual darkness (Langecker 2000; Poulson 2001b), and brain morphology varies among populations in the Cueva del Azufre system (Eifert et al. 2015). Furthermore, H_2S can have neurotoxic effects (Kombian et al. 1998). Finally, eyes are frequently modified or lost during evolution in caves (Jeffery and Martasian 1998), and eyes are significantly smaller – albeit still functional – in *P. mexicana* from cave compared to surface populations (Fontanier and Tobler 2009).

RNA extraction, cDNA library preparation, and sequencing

The protocol for transcriptomic analyses was previously employed for *P. mexicana* (Kelley et al. 2012). We collected 10-30 mg of tissue, placed it into TTXT bags (Covaris), froze it using liquid nitrogen, and then pulverized. Total RNA was then extracted using Qiagen's RNeasy Plus mini kit. mRNA was then purified using the Ribo-Zero Magnetic Gold Kit (Epicentre) and cleaned twice using Agencourt RNAclean XP beads (Beckman Coulter Inc.). mRNA was fragmented to 400 nt using NEB's RNA Fragmentation Buffer and incubating at 94 °C for 4 minutes. First-strand cDNA synthesis was performed using 12 µl of eluted mRNA, 1 µl of random Hexamers:Olido dT primers (3 µg :1 µg), 4 µl of first strand reaction buffer (Invitrogen), 2 µl of 0.1 M DTT (Invitrogen), and 1 µl of 10 mM dNTP mix (Biolabs). We then added 1 µl SuperScript III RT to the mixture. The whole solution was incubated at 25°C for 50 minutes and immediately placed on ice to terminate the reaction. We then added 5 µl of 5X first-strand buffer, 1 µl of DTT, 2 µl of 10 mM dNTP mix with dUTP (ABI), 15 µl of 5X second-strand reaction buffer (Invitrogen), and 3.75 µl of NEB second strand enzyme mix. The reaction mix was incubated at 16°C for 2 hours. After second strand synthesis, the reaction was cleaned up using Agencourt RNAclean XP beads and

eluted into 50 µl of nuclease-free H_2O. The double stranded cDNA was used as an input for the KAPA HTP Library Preparation Kit (KAPA Biosystems) for end-repair, A-tailing and adapter ligation with Trueseq barcoded adapters, and library amplification. For the library amplification reaction, we ran the initial denaturation at 98 °C for 45 sec, followed by 12 cycles of denaturation at 98 °C for 15 sec, annealing at 60 °C for 30 sec, and extension at 72 °C for 30 sec, and finishing with a final extension at 72 °C for 60 sec. RNA-sequencing libraries were quantified using Qubit and Agilent 2100 Bioanalyzer High Sensitivity DNA chip. We then pooled libraries based on nM concentrations. Libraries were sequenced on an Illumina HiSeq 2000 across two lanes with paired-end 101 bp reads. Due to low coverage, some samples were re-run on a single HiSeq lane and reads were then concatenated together for each sample. Read counts and quality for eye samples were not sufficient for data analyses; thus, all following analyses are restricted to gill, liver, and brain samples.

Reference transcriptome assembly and annotation

All raw RNA-seq reads were sorted by barcode and trimmed twice (quality 0 and then quality 24) using the program Trimgalore! (Krueger 2015; Table B.2). Trimmed reads were then mapped to the *Poecilia mexicana* reference genome (Version 1.0, Genbank accession number: LMXC01000000; plus corresponding mitochondrial sequences, GenBank Accession number: KC992991) using BWA (Li and Dubin 2009). We then used the cufflinks package (version 2.2.1) to extract expressed regions, merge the regions for all individuals, and create a multifasta file representing the reference transcriptome for further analyses (Trapnell et al. 2010). Transcripts were annotated using a BLASTx search against the Human database (critical E-value = 0.001),

and we retained the top BLAST hit for further analyses. Sequences with a match were also annotated with Gene Ontology (GO) IDs (Gene Ontology Consortium 2004).

Quantifying gene expression variation

To analyze variation in gene expression among populations and organs, we mapped all trimmed reads to the multifasta file using BWA (Li and Dubin 2009) and then estimated transcript abundance with eXpress (Roberts and Pachter 2013). Transcripts that did not have at least 2 counts per million in at least 3 samples were removed from further analyses. To describe multivariate variation in gene expression, we conducted a weighted co-expression network analysis on the top 10,000 expressed genes in the package WGCNA (Langfelder and Horvath 2008). Before construction the networks, we used the variance-stabilizing transformation function in the DEseq2 package in R (Love et al. 2014) and set the soft threshold (i.e., power) to 3, which was the lowest value that optimized topology (Langfelder and Horvarth 2008). We then constructed a consensus dendrogram based on the most significant modules and tested for associations between individual modules and predictor variables (presence of H_2S, absence of light, organ, individual, population) by using Pearson correlations.

To quantify differential gene expression among populations, we treated the ancestral, nonsulfidic surface population as a reference and conducted pairwise comparisons with each of the populations inhabiting an extreme environment (sulfidic surface, nonsulfidic cave, and sulfidic cave). Pairwise comparisons were conducted separately for each organ by using the bioconductor package edgeR (Robinson et al. 2010, Robinson and Oshlack 2010). The genome-wide false discovery rate was set to 0.05, and the *P*-value cut-off to 0.05. These analyses provided a list of up and downregulated transcripts for each population and organ. We tested whether the number of

differentially expressed transcripts varied among populations and organs using analysis of variance (ANOVA). We included expression direction (i.e., up or downregulated), population, and organ as independent variables. Furthermore, we tested whether the putative biological processes associated with differentially expressed genes in each habitat was consistent among different organs. To do so, we used the *enrichPathway* function in the ReactomePA package (Yu and He 2016) to identify the enriched pathways in each group and then used the *compareCluster* function in the Clusterprofiler package (Yu et al. 2012) to compare the results among organs. Correlations in enrichment between organs were assessed using Pearson correlation within each population.

Identifying shared organismal responses in H_2S and cave environments

We tested for shared organismal responses to H_2S and perpetual darkness by comparing differentially expressed genes between the sulfidic surface and cave populations (shared responses to H_2S), as well as between the nonsulfidic and the sulfidic cave populations (shared responses to darkness). Because theory predicts that the degree of shared responses should be a function of the level of biological organization that is being analyzed as selection acts on the highest level (Elmer and Meyer 2011), we quantified shared responses at the level of the transcript (transcript ID based on our reference transcriptome), gene (same BLAST hit in SwissProt), and function (same GO terms associated with BLAST hit). Shared responses were then quantified by calculating Jaccard's index based on shared transcripts, shared genes, and shared GO terms for each of the comparisons (Jaccard 1901). Jaccard's indices (log_{10}-square-root-transformed) were then analyzed using ANOVA. We included expression direction, environmental factor (i.e., H_2S and darkness), level organization, and organ as independent variables.

Biological functions associated with adaptation to H_2S and cave environments

We assessed the biological functions of differentially expressed genes to test for functional changes potentially associated with adaptation to H_2S and perpetual darkness. To do so, we conducted a GO term enrichment analysis (*P*-value cutoff 0.0001), a technique that assigns genes into their functional characteristics, using the program GOrilla (Eden et al. 2009) for each population and organ separately. Transcripts with evidence for differential expression served as the target set that was tested against all transcripts in the reference transcriptome.

Results

Reference transcriptome

Sequencing transcriptomes for different organs yielded a total of 133,785,284 reads for gill tissues (14 individuals in final dataset), 95,374,496 reads for brain tissues (11 individuals in final dataset), and 136,779,442 reads for liver tissues (16 individuals in final data set; see Table B.2 for details). Due to low read counts and quality we excluded 5 brain and 2 gill libraries. Mapping against the reference genome resulted in a total of 63,590 transcripts from 11,966 unique loci. The total length of the merged transcriptome was 225,876,000bp, with an N50 of 5,290bp. BLAST against the Human database using SwissProt resulted in matches for 48,501 transcripts (76.43 %; see Table B.3), 13,504 of which were associated with Gene Ontology (GO) terms.

Gene expression variation

Weighted co-expression network analysis revealed five modules of co-expressed transcripts (Figure 3.2A-B). Organ identity was significantly correlated with all modules, while none of the other predictor variables (presence or absence of H_2S, presence or absence of light, population,

and individual) exhibited any significant associations. Gene expression consequently varied substantially among organs, while effects of environmental conditions were comparatively minor. Hence, we analysed among-population variation in gene expression separately for each organ.

Comparing the number of differentially expressed genes among populations from the different extreme habitats (relative to the ancestral, nonsulfidic surface population) indicated that organ and the interaction between organ and habitat type explained the majority of variation (Table 3.1A). In the two sulfidic habitats, the number of differentially expressed genes was the highest in the gills (Figure 3.3). In the nonsulfidic cave, however, the number of differentially expressed genes was greatest in the liver and lower in the brains and gills respectively (Figure 3.3).

Differentially expressed genes also varied in their function (Figure 3.4). Overall, positive correlation in the enrichment of functional classes was relatively rare, indicating that the nature of differentially expressed genes depended on organ. Among few exceptions were similar functional responses in upregulated transcripts between brain and liver in the nonsulfidic cave, upregulated transcripts between brain and gill in the sulfidic surface habitat, upregulated transcripts between liver and gills in the sulfidic cave, and downregulated transcripts between brain and liver in the sulfidic cave.

Shared organismal responses in H_2S and cave environments

By comparing differentially expressed transcripts between populations, we identified shared responses to H_2S and cave environments. The extent of shared responses was a function of interactive effects between environmental factors, organs, and the level of biological organization (Table 3.1B). As predicted by theory, the amount of shared responses increases with level of organization (Figure 3.5A). However, the amount of shared responses at each level of organization

and the magnitude of increase from one level to the next significantly differed between environmental factors. In cave environments, there were no shared differentially expressed transcripts and only about 20 % of GO terms associated with differentially expressed genes were shared among populations (Figure B.1). In contrast, shared responses were much higher between sulfidic environments at each level of organization, with about 40% of GO terms associated with differentially expressed genes appearing in both populations (Figure B.2). Population differences were also evident among organs (Figure 3.5B). While there were no significant differences among organs in the two cave populations, shared responses were significantly more prevalent in gills between the two sulfidic populations.

Biological functions potentially associated with adaptation to H_2S and cave environments

To identify functional changes potentially associated with adaptation to the different environmental conditions, we conducted a GO term enrichment analysis for up and downregulated genes for each tissue and population (Table B.5). For brevity, we only discuss enriched terms that are shared between both sulfidic or both cave habitats.

No GO terms were significantly enriched across all organs in the two sulfidic habitats (Table B.4A-B.4B). However, several genes associated with H_2S detoxification and sulfur metabolism were consistently upregulated in the gills (Table B.4A). This was reflected in significant enrichment in terms associated with sulfur (GO:0044272, GO:0006790) and glutathione metabolism (GO:0006749). In addition, there was enrichment in terms associated with oxidative stress responses (GO:0006979). Downregulated genes in the gills were predominately associated with the transport of ions and other molecules (GO:0030001, GO:0006814, GO:0006811, and associated terms; Table B.4B). In the liver, significant enrichment was only

uncovered for downregulated genes, many of which were associated with metabolism of lipids and fatty acids (GO:0035337, GO:0006631,GO:003583, and associated terms; Table B.4B). In the brain, upregulated genes were enriched for processes associated with energy metabolism and ATP production (GO:0006096, GO:00016051, GO:00006757; Table B.4A). Downregulated genes in the brain were associated with the organization of the extracellular matrix and DNA integration (GO:0030198, GO:0015074).

No GO terms were significantly enriched in all organs of the two cave populations. In the gills, up and downregulated genes were primarily involved in ion transport and the regulation of pH (Table B.4C-B.4D). In the liver we found no evidence for shared enrichment in upregulated genes, and downregulated genes were associated with responses to chemicals and the regulation of biological quality and protein secretion (GO:0042221, GO:0065008, and associated terms; Table B.4D). Surprisingly, there was no consistent enrichment in any GO term in the brain of the two cave populations (Table B.4C-B.4D).

Discussion

Transcriptome analyses among closely related fish populations in proximate but environmentally distinct habitat types uncovered rampant variation in gene expression. Organ type was the major driver of expression differences among samples, and organ type also affected the number and function of differentially expressed genes in each habitat type. Consequently, choosing focal organs can significantly impact the conclusions of gene expression studies in nature. As predicted by theory, identification of shared differentially expressed genes between the two sulfidic and the two cave habitats suggested that the proportion of shared responses increases with the level of biological organization. However, shared responses at any level of organization were much more

common in the sulfidic habitats than the cave habitats, highlighting that the effects of H_2S impact gene regulation differently than the absence of light. Our analyses provided insights into transcriptional variation in a unique system with coinciding sources of selection, and functional annotation of differentially expressed genes provide a springboard for investigating molecular mechanisms putatively underlying adaptation to extreme environments.

Expression variation among organs and environments

Considering different organs are fulfilling vastly different functions despite being composed of cells with the same genome (Whitehead and Crawford 2005), it was not surprising that the majority of variation on gene expression was observed among organ types. However, the gene expression responses to the presence of H_2S and the absence of light in caves also significantly varied among organs, both in terms of the number of genes that were differentially expressed and their function. So even though H_2S-rich and cave environments are often assumed to exert strong selection, the consequences of exposure are not systemic and uniform but specific to particular organs. In the nonsulfidic cave for example, the majority of differentially expressed genes occurred in the liver, and the enrichment in reactome pathways differed among organs (especially gills *vs*. liver and brain). In both sulfidic habitats, differentially expressed genes were mostly concentrated in the gills, which are directly exposed to environmental H_2S. Furthermore, genes associated with H_2S detoxification and the metabolic processing of sulfur are primarily differentially expressed in the gills but not the brain and the liver. This may suggest that the bulk of H_2S is detoxified peripherally in the gills (rather than the liver as in other vertebrates; Dorman et al. 2002), thus shielding other organ systems from the toxic effects. Consequently, the choice of tissue substantially affects inferences about gene expression variation. Many studies still focus on the analysis of single

organs (Wang et al. 2014; Narum et al. 2015), whole organisms (Gross et al. 2013) or lack formal analyses of how expression variation among organs affect the results (Hinaux et al. 2013; Uyhelji et al. 2016). This is not necessarily problematic when *a priori* hypotheses are being tested, but a focus on single organs may also lead to skewed or misleading results that affect our inferences about organismal responses to environmental variation.

Our results also indicate that gene expression responses in populations exposed to multiple stressors are not the mere sum of the responses to individual stressors. Although the sulfidic cave population (exposed to H_2S and darkness) had the highest total number of differentially expressed genes, the number of differentially expressed genes in the gills was higher in the sulfidic surface population, and the number of differentially expressed genes in the liver was higher in the nonsulfidic cave population. In addition, only a subset of responses in the sulfidic cave were actually shared with the sulfidic surface and the nonsulfidic cave population (see below), such that the functional overlap of responses was lower than what a comparison of simple numbers insinuated. This result highlights that there are likely interactive, nonadditive effects between H_2S exposure and living in a cave environment. Such interactions have also been documented in experimental studies that manipulated exposure to multiple physiochemical stressors (Yang et al. 2007; Garcia-Reyero et al. 2012; Maes et al. 2013) and will complicate our ability to predict organismal responses along complex environmental gradients.

Shared responses and the predictability of change in different environments

Shared responses between the two sulfidic and the two cave populations increased with levels of biological organization (from transcript to gene and function). This was particularly evident between the two cave populations that did not share a single differentially expressed transcript in

some organs, yet differentially expressed genes shared about 20% of functional attributes (GO terms; Figure 3.5A). The increase in shared responses with level of organization is predicted by theory (Manceau et al. 2010; Elmer and Meyer 2011) and has been documented in other systems (Shapiro et al. 2004; Linnen et al. 2009; Rosenblum et al. 2010; Pfenninger et al. 2015). It likely arises as a consequence of selection optimizing overall organismal performance rather than traits contributing to performance (see Arnold 1983; Wainwright et al. 2005), and modulation of different transcripts may mediate similar adaptive modifications of relevant physiological pathways.

More importantly, the proportion of shared responses was significantly higher between the two sulfidic than the two cave habitats. This was particularly evident at the levels of transcripts and genes (>20 % of responses shared between sulfidic habitats, <3% of responses shared between cave habitats), but substantial differences were also observed at a functional level (20 % *vs*. 37 %). There are several nonmutually exclusive hypotheses about the mechanisms that could be driving this pattern. (1) The number of shared differentially expressed genes may be a function of phylogenetic relatedness; i.e., shared responses may have been higher between the two sulfidic populations, because they are more closely related. To test this, we called single nucleotide polymorphisms (SNPs) in our dataset and analyzed the relationship among populations (see supplementary material for details). The results indicated that the three-extremophile populations were closely related, and unlike previous microsatellite analyses (Tobler et al. 2008a; Plath et al. 2010a), there was no evidence for significant population structure. Nonetheless, inference of population splits indicated that the nonsulfidic and sulfidic cave populations were more closely related to each other than to the sulfidic surface population (Figure B.4). Hence, phylogenetic history alone is unlikely to explain the low level of shared responses between the two cave

populations. (2) Our classification of habitats into discrete types may be an oversimplification of complex ecological gradients (see Kaeuffer et al. 2012). Besides the presence of H_2S and the absence of light, there may be additional strong sources of selection in the nonsulfidic cave that we have not considered and may shape the distinct pattern of gene expression. For example, despite their proximity, the two caves differ in ambient temperature, the ionic composition of the water (other than the presence of H_2S), and the availability of trophic resources (Tobler 2008; Tobler et al. 2008b; Rosales Lagarde 2012). (3) Some sources of selection may elicit more predictable evolutionary responses than others. At least in the organs investigated here, the presence of H_2S – unlike the absence of light – has clear-cut molecular targets (*e.g.*, cytochrome c oxidase in the respiratory chain and hemoglobin) that affect specific physiological functions (*e.g.*, energy metabolism, oxygen transport, and oxidative stress) (Li et al. 2011; Olson 2011; Tobler et al. 2016b). H_2S's direct interaction with specific proteins could constrain the diversity of adaptive solutions that mitigate the toxic effects, ultimately leading to more predictable outcomes in gene expression variation in replicated populations. Indeed, previous studies have found that sulfide spring populations exhibit positive selection on and differential expression of genes associated with highly conserved pathways involved in H_2S toxicity and detoxification (Pfenninger et al. 2014, Pfenninger et al. 2015; Kelley et al. 2016). Furthermore, the notion that H_2S elicits more predictable organismal responses is supported by the level of shared responses between populations being disproportionally high in the gills, which are directly exposed to environmental H_2S. Whether physiochemical stressors with clear molecular targets generally elicit more predictable patterns of genetic evolution than other sources of selection remains to be tested in a broader set of systems (Pfenninger et al. 2015).

Potential mechanisms underlying adaptation to extreme environments

Functional annotations of differentially expressed genes provided insights about potential mechanisms underlying adaptation to sulfidic and cave environments. Populations in sulfidic environments upregulated key enzymes involved in H_2S detoxification, including multiple components of the sulfide:quinone oxidoreductase and the glutathione pathways (Hildebrandt and Grieshaber 2008; Jackson et al. 2012). In addition, we found evidence for upregulation of genes associated with the toxic effects of H_2S. Specifically, H_2S negatively affects aerobic ATP production and creates oxidative stress through the interruption of the mitochondrial respiratory chain (Eghbal et al. 2004; Cooper and Brown 2008), and genes associated with energy metabolism and anaerobic ATP production as well as oxidative stress responses were upregulated. Enrichment analysis also indicated downregulation of ion transporters in the gills; genes associated with lipid and fatty acid metabolism in the liver, and extracellular matrix components in the brain. Future studies will need to address the functional consequences of these expression changes and test whether they are adaptive in sulfidic environments. The sulfidic surface and cave habitats also differ from normal surface streams in a variety other aspects of the environment (e.g., salinity, pH, and oxygen concentrations), hence some of the documented gene expression changes may have arisen in response to these correlated environmental variables.

There were only a few shared enriched GO terms significant in both cave populations. Changes in the expression of ion transporters and the regulation of pH were likely driven by variation in water chemistry and not sources of selection that are typically associated with cave environments, although some ion channels have been associated with the development of pigmentation (Bellono et al. 2014), and both cave populations exhibit significant reductions of pigmentation in the skin (Tobler et al. 2008b). Despite the lack of shared responses, it is important

to note that there was evidence for significant enrichment in each cave population for several functions typically associated with cave adaptation. This includes genes associated with energy and lipid metabolism that could be related to resource scarcity in caves (see Hüppop 2000), regulation of circadian rhythms that are likely related to the absence of light (Koilraj et al. 2000), and genes associated with neuronal development and axon guidance that may be related to changes in the brain anatomy of cave fish (Eifert et al. 2015). While the high proportion of shared responses between the two sulfidic habitats facilitated the identification of potential mechanisms contributing to adaptation to H_2S, the largely unique expression patterns in the two caves makes inferences about adaptation to perpetually dark habitats much less straightforward. This is somewhat surprising because evolution in caves is typically associated with strong patterns of convergent phenotypic evolution (Dowling et al. 2002; Jeffery 2009). Furthermore, regressive evolution of pigmentation in cavefish is even caused by convergent molecular modifications (Protas et al. 2006).

Conclusion

Overall, this study has documented extensive variation in gene expression among spatially proximate and genetically closely related populations. Laboratory experiments using one of the extremophile populations (sulfidic surface) have indicated that a substantial part of expression variation observed in nature is due to evolutionary change in gene regulation, and shared ancestral plastic responses to H_2S exposure play a negligible role in explaining population differences (Passow unpublished data). Considering the rampant variation in gene expression and functional traits among closely related populations, the fish of the Cueva del Azufre system provide a unique opportunity to study the genomic basis of complex phenotypic variation in nature.

Figures

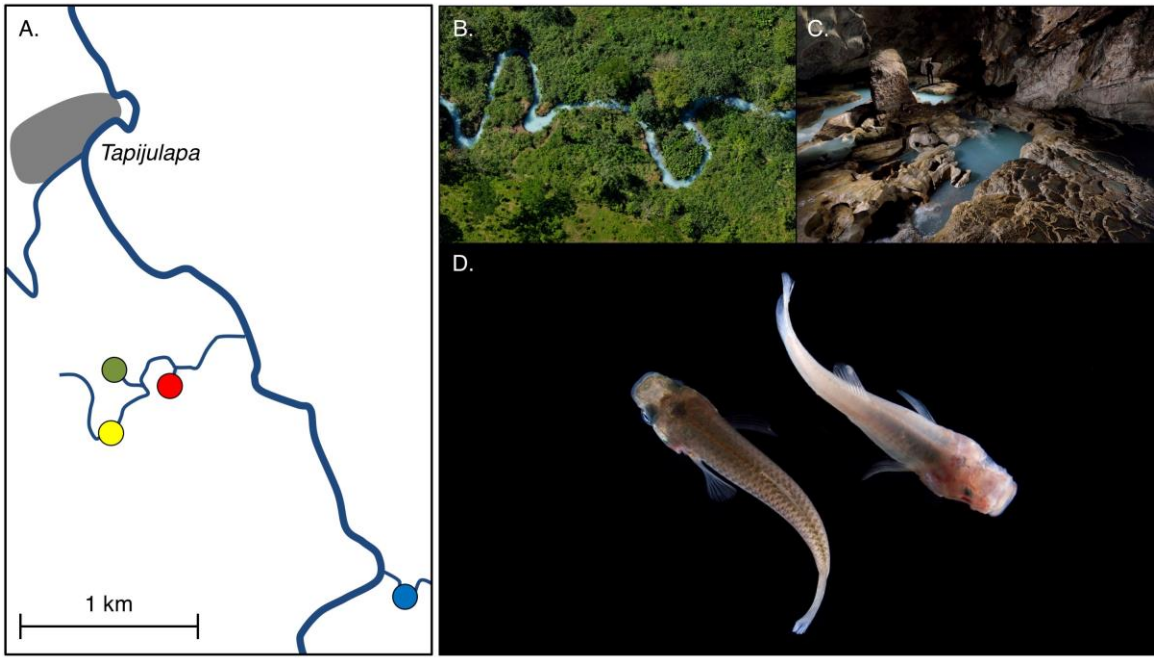

Figure 3.1 Overview of the study area and fish

(A) Overview of the study area near the village of Tapijuapa. Depicted are the four focal populations: nonsulfidic surface (blue), sulfidic surface (yellow), nonsulfidic cave (red), and sulfidic cave (green). (B) The sulfidic surface stream and (C) the sulfidic cave in which fish were collected for this study. (D) Phenotypic differences are evident between fish from the sulfidic surface (left) and the sulfidic cave habitat (right). Photos are courtesy of Robbie Shone.

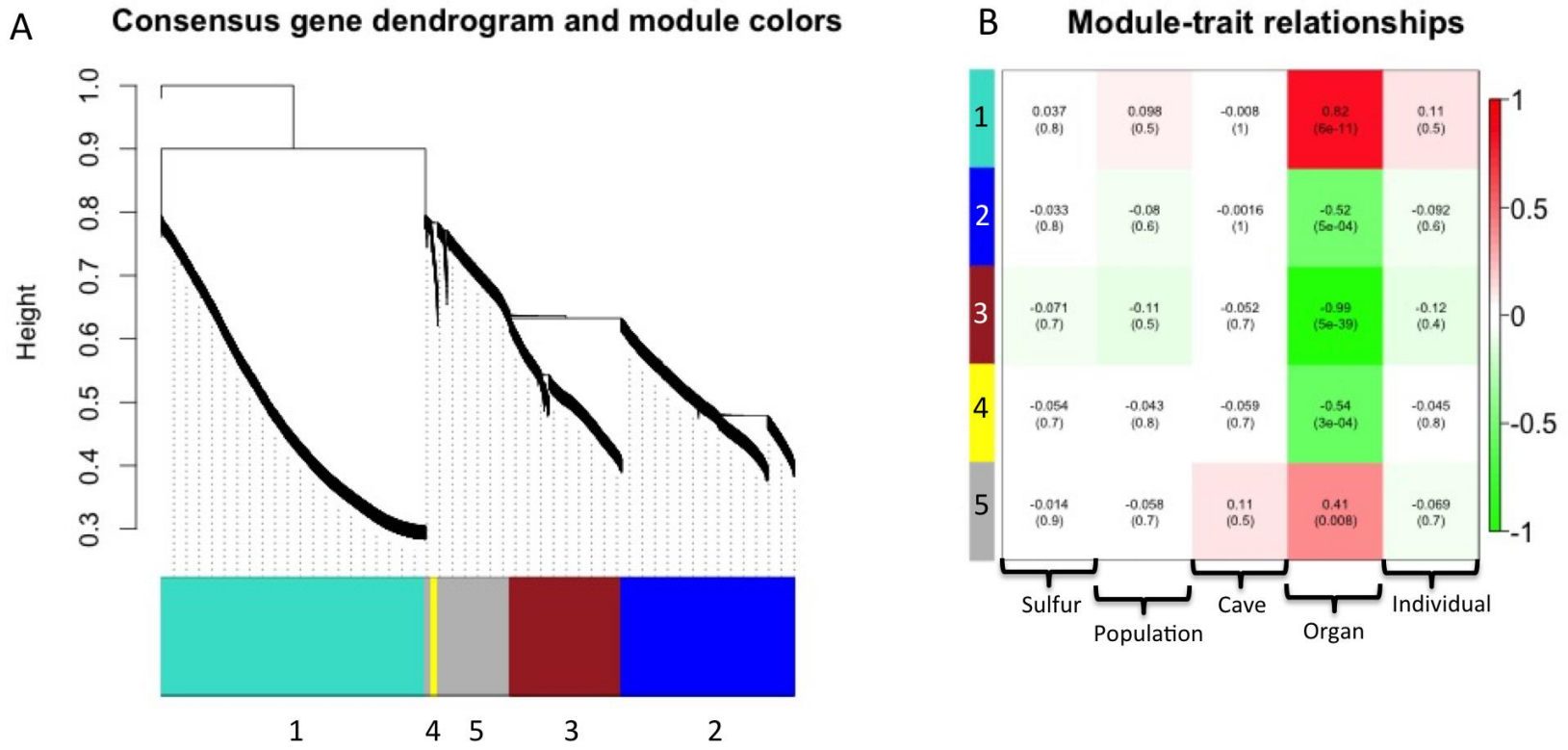

Figure 3.2 Weighted co-expression network analysis

Results of the weighted co-expression network analysis. (A) Linkage clustering dendrogram depicting modules of coexpressed genes as indicated by the color bar below. (B) Correlation between module eigenvalues and predictor variables. Rows represent modules of coexpressed genes. Reported are the Pearson coefficients (top) and p-values (bottom). Cell color corresponds to the degree of correlation according to the scale bar.

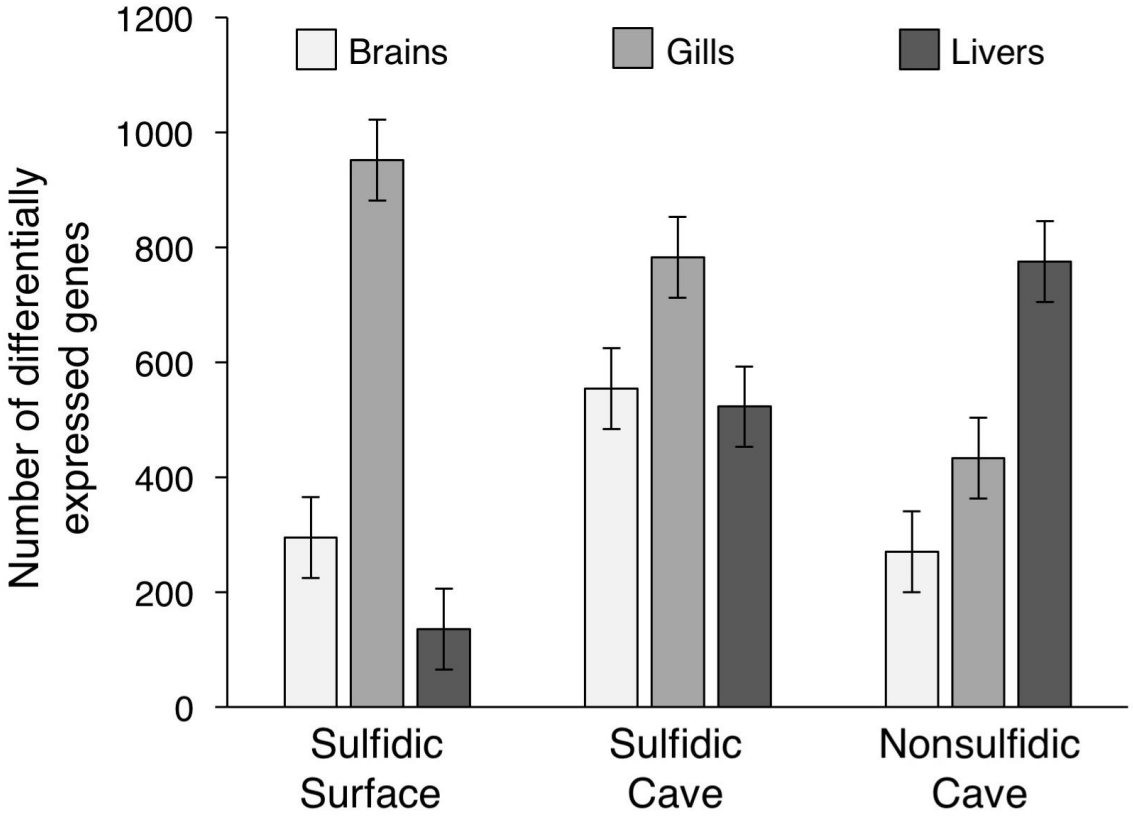

Figure 3.3 Number of differentially expressed genes

The number (estimated marginal means ± standard error) of differentially expressed genes (up and downregulated) among populations and organs. Estimated marginal means were derived from the ANOVA model in Table 3.1A.

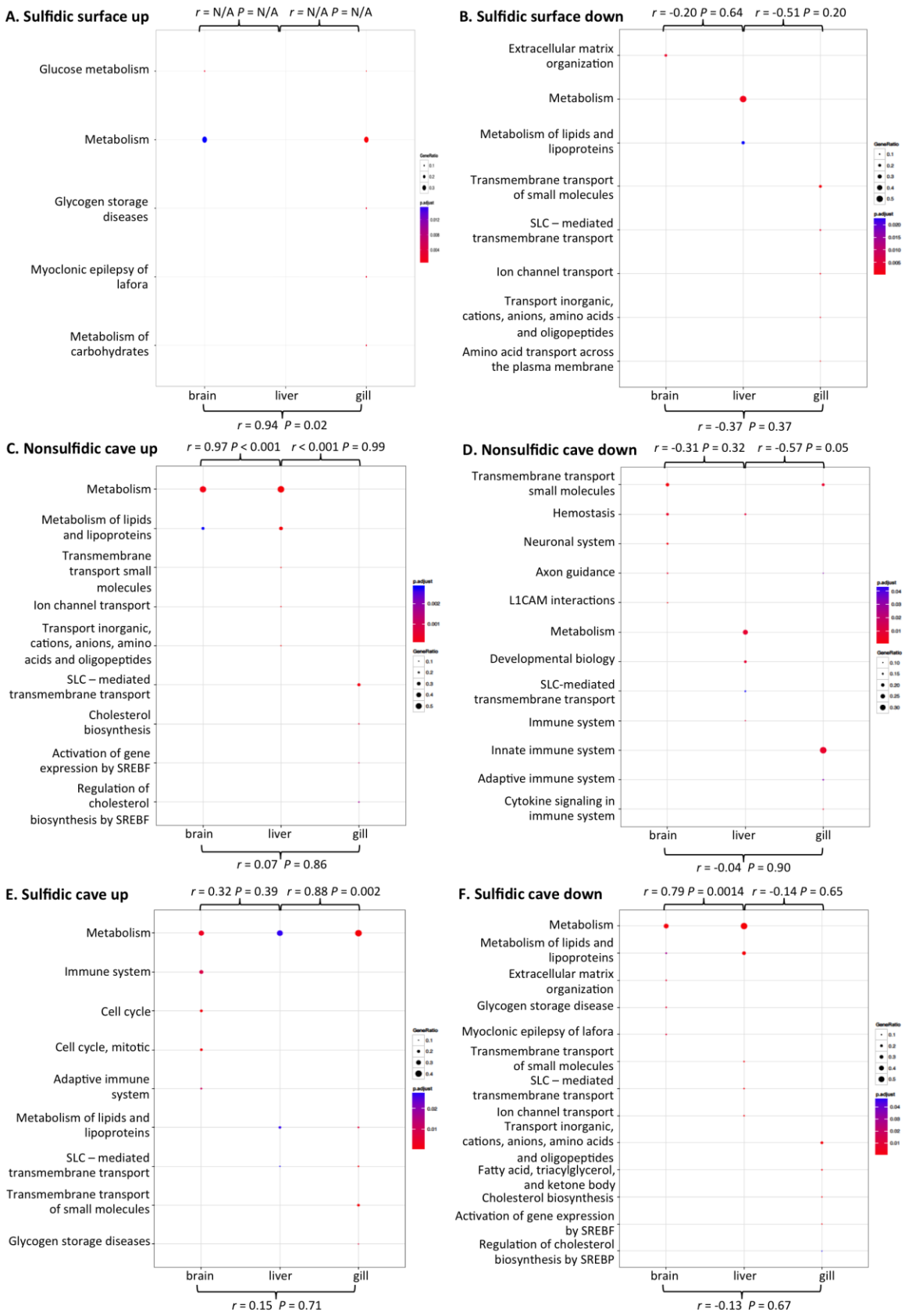

Figure 3.4 Comparison of the functional annotations

Comparison of the functional annotations of up and downregulated genes among organs. Depicted are the enriched reactome pathways of for each of the three populations: (A-B) sulfidic surface, (C-D) nonsulfidic cave, (E-F) sulfidic cave. The size of each dot corresponds to the number of genes in enriched each pathway (i.e., gene ratio). The color corresponds to theadjusted *P*-value for each category as indicated by the scale bar. Pearson correlation coefficients (*r*) and *P*-values indicate similarities in enrichment among organs.

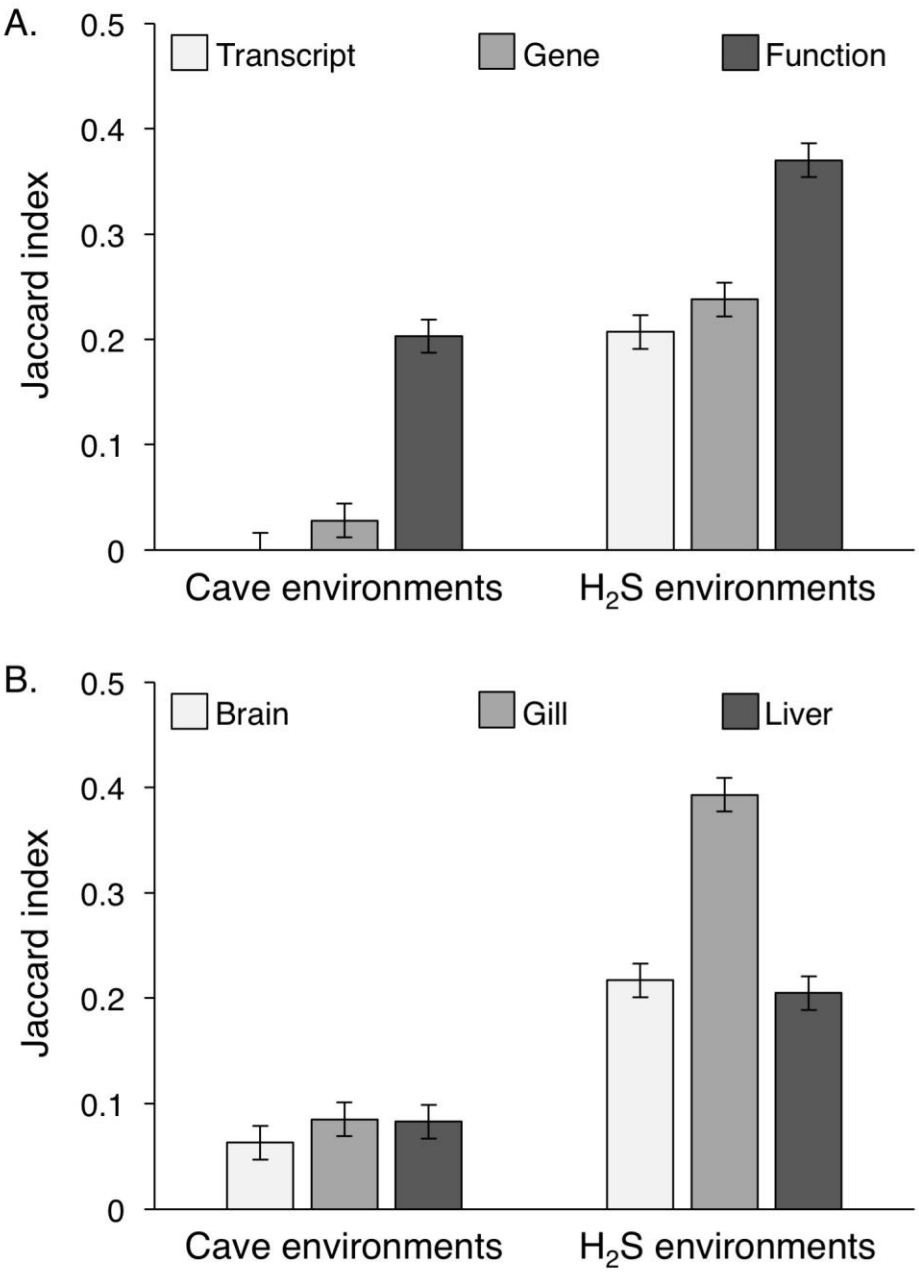

Figure 3.5 Jaccard index based on biological level of organization and organ

The proportion (estimated marginal mean of Jaccard index ± standard error) of shared (A) across populations and levels of biological organization, and (B) across populations and organs. Estimated marginal means were derived from the ANOVA model in Table 3.1B.

Tables

Table 3.1 Results of ANOVA

Results of analyses of variance (ANOVA) comparing (A) the number of differentially expressed genes and (B) the proportions of share responses (Jaccard index) among populations and organs.

Variables	***df***	***F***	***P***	η_p^2
A. Number of differentially expressed genes				
Habitat type	2	4.3	0.101	0.682
Organ	2	19.5	0.009	0.907
Expression direction	1	0.1	0.724	0.035
Habitat × Expression direction	2	0.8	0.501	0.292
Organ × Expression direction	2	1.6	0.313	0.441
Habitat × Organ	4	18.0	0.008	0.947
B. Proportion of shared responses				
Expression direction	1	0.3	0.619	0.016
Environmental factor	1	450.4	<0.001	0.966
Level of organization	2	152.7	<0.001	0.950
Organ	2	24.4	<0.001	0.753
Expression direction × Environmental factor	1	2.0	0.178	0.111
Expression direction × Level of organization	2	0.7	0.489	0.086
Expression direction × Organ	2	2.3	0.128	0.227
Environmental factor × Level of organization	2	27.7	<0.001	0.776
Environmental factor × Organ	2	15.2	<0.001	0.655
Level of organization × Organ	4	0.5	0.711	0.118

Chapter 4 - Evolutionary change shapes gene expression variation in locally adapted, extremophile fishes

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Abstract

Differential gene expression is an important mechanism mediating local adaptation. However, gene expression is also notoriously plastic, which can complicate inferences about the adaptive value of expression variation in natural systems. Here, we studied genome-wide expression responses in wild-caught and laboratory-reared populations of an extremophile fish (*Poecilia mexicana)* living in toxic, hydrogen sulfide (H_2S)-rich springs. In nature, there is evidence for differential expression between sulfidic and nonsulfidic populations for a wide variety of genes, but the mechanisms underlying variation in gene expression remain unexplored. In this study, we asked: (1) Do fish raised in a common-garden experiment and exposed to H_2S exhibit similar changes in transcription compared to sulfidic and nonsulfidic populations in the wild. (2) Is there evidence for evolved, constitutive differences in gene expression among populations that are maintained in the laboratory irrespective of H_2S exposure? (3) Is there evidence for evolutionary gain or loss of plastic responses upon exposure to H_2S. To address this, we collected 87 gills from common-garden raised individuals from four populations (two sulfidic and two nonsulfidic) across two independently colonized drainages exposed to a control and three varying doses of hydrogen sulfide. We also collected gills from 22 individuals in wild-

caught fish from the same populations. We generated cDNA libraries and then mapped transcripts against the *P. mexicana* genome to estimate gene expression variation. We recovered similar patterns of differential expression in wild-caught and laboratory populations and found evidence for both evolved constitutive expression differences as well as gain and loss of H_2S inducibility in sulfidic fish. Overall, this study suggests that evolution – and not ancestral plasticity - is responsible in generating variation in gene expression across replicated pairs of sulfidic and nonsulfidic populations.

Introduction

The modulation of gene expression is an important mechanism by which organisms respond to changes in environmental conditions. Variation in gene expression can be highly plastic over short periods of time and induced in response to specific environmental cues (Dietz and Somero 1992; Buckley et al. 2001; Cheviron et al. 2008). At the same time, regulatory changes governing the expression of genes have also been hypothesized to play an important role in adaptive evolution (King and Wilson 1975; Townsend et al. 2003; Wray et al. 2003; Nuzhdin et al. 2004). It is therefore not surprising that there is evidence for variation in gene expression among natural populations that live in different environments (Oleksiak et al. 2002; Savolainen et al. 2013). In fact, the study of gene expression variation in natural populations has been revolutionized by the advent of next generation sequencing techniques (RNA-seq; Wang et al. 2009) which facilitates genome wide analyses of gene expression even in non-model organisms (Oleksiak et al. 2002; Whitehead and Crawford 2005; Cheviron et al. 2012). Nonetheless, the presence of differentially expressed genes among populations is not sufficient to infer adaptation to

specific environmental conditions, and the environmental cues and the evolutionary mechanisms shaping expression variation are often unknown.

The study of gene expression variation in natural populations faces two primary challenges. First, environmental gradients are complex, even if different habitats seemingly differ in only one or a few environmental factors (Grether et al. 2001; Holmstrup et al. 2010; Tobler and Plath 2011). For example, the presence of a primary physiochemical stressor typically coincides with superimposed gradients of suites of abiotic and biotic environmental factors (Tobler et al. 2015b). Such co-variation of multiple variables among natural populations makes it impossible to establish the cause and effect relationships between sources of selection and the patterns of expression.

Second, adaptive variation in gene expression requires the documentation of evolved differences in gene regulation, but multiple mechanisms can underlie gene expression variation among natural populations: (1) Gene expression differences in nature could be the consequence of evolution in gene regulation. In this case, population differences documented in natural populations should be constitutive and maintained in the laboratory-reared individuals irrespective of exposure to different environmental conditions (Ferea et al. 1999). (2) Gene expression differences could be a consequence of the phenotypically plastic induction of gene expression in response to specific environmental conditions. If such plastic responses are shared between ancestral and derived populations, expression differences in natural populations are a mere consequence of habitat-dependent exposure histories. Hence, expression differences observed in natural populations should disappear when individuals are raised under standardized environmental conditions in the laboratory (Shaw et al. 2007). (3) Gene expression

differences could be a result of evolved population differences in the inducibility of expression responses (evolution of plasticity). Such plastic responses in expression may have evolved in derived populations. In this case, individuals from derived – but not ancestral – populations should modulate gene expression depending on the exposure to different environmental cues in the laboratory (Lande 2009). Alternatively, plasticity in expression patterns present in ancestral populations may have been lost in derived ones (canalization; Shaw et al. 2014). In this case, individuals from ancestral – but not derived – populations should modulate gene expression depending on the exposure to different environmental cues. It is important to note that these alternative scenarios are non-mutually exclusive and could occur simultaneously. For example, populations may differ in base-line expression levels in absence of an environmental cue (mechanism 1), they may up-regulate the expression levels upon exposure (mechanism 2), and they may differ in the magnitude of up-regulation (mechanism 3).

Inferring adaptive changes in gene expression consequently hinges on linking sources of selection to expression variation at particular loci and on excluding the possibility that expression differences are a mere product of shared ancestral plastic responses. This requires the simultaneous investigation of expression variation in natural populations and in laboratory experiments that standardize rearing environments and manipulate exposure to specific sources of selection in a factorial design (Ghalambor et al 2015). Here, we investigated the environmental sources of selection and the mechanisms underlying expression variation in replicated extremophile fish populations and their ancestors. *Poecilia mexicana* (Poeciliidae) is a small livebearing fish that is common in streams and rivers of Mexico and parts of Central America (Alda et al. 2013). In addition,

members of this species have independently colonized toxic, hydrogen sulfide (H_2S)-rich springs in multiple river drainages of southern Mexico (Tobler et al. 2011; Palacios et al. 2013). Hydrogen sulfide is a potent respiratory toxicant and creates extreme environmental conditions that are lethal for most metazoans (Bagarinao et al. 1992). It inhibits cytochrome c oxidase (COX) in the respiratory chain of mitochondria, thus halting ATP production (Reiffenstein et al. 1992; Cooper and Brown 2008). Sulfide spring populations of *P. mexicana* are locally adapted and exhibit convergent evolution in physiological, morphological, and life-history traits (Tobler et al. 2011; Riesch et al. 2014). In addition, they are undergoing ecological speciation and exhibit low gene flow with populations in adjacent nonsulfidic environments despite the lack of physical barriers (Plath et al. 2013).

Recent studies have provided insights into the molecular underpinnings of adaptation to sulfide spring environments. There is evidence for positive selection both on the molecular targets of H_2S toxicity (COX; Pfenninger et al. 2014) and genes associated with H_2S detoxification (sulfide:quinone oxidoreductase pathway; Pfenninger et al. 2015). There is also substantial variation in gene expression between sulfidic and nonsulfidic population pairs in different river drainages (Kelley et al. 2016). Functional annotation indicated that differentially expressed genes shared among replicated population pairs include loci involved in H_2S detoxification as well as the maintenance of ATP production in presence of H_2S (Kelley et al. 2016). Nonetheless, the functional links between differential expression at specific loci and the presence of H_2S remains elusive. Many differentially expressed genes have never been associated with H_2S toxicity, and sulfide springs differ from adjacent nonsulfidic habitats in a variety of abiotic and biotic conditions including temperature, pH, oxygen concentrations, salinity, resource availability, and

predation (see Riesch et al. 2015b for a review). In this study, we investigated variation in gene expression between sulfidic and nonsulfidic populations in the wild and in common garden raised individuals exposed to sulfidic and nonsulfidic conditions to address two primary questions: (1) What differentially expressed genes in the wild are differentially expressed because of the presence of H_2S? (2) Is differential expression at loci associated with H_2S exposure a product of evolution in gene regulation or a consequence of shared plastic responses induced by H_2S exposure?

Materials and methods

Sample collections and experimental H_2S exposures

Focal sulfidic and adjacent nonsulfidic populations of *Poecilia mexicana* were located in two major tributaries of the Rio Grijalva (Ríos Tacotalpa and Puyacatengo) in the Mexican states of Tabasco and Chiapas (Palacios et al. 2013). Sulfide springs in these drainages were colonized less than 100,000 years ago (Pfenninger et al. 2014). To quantify variation in gene expression in nature, a previous study analyzed gill tissues from freshly collected fish (Kelley et al. 2016; GenBank Sequence Read Archive study accession ID: PRJNA290391). We collected gravid females from the same sites and transported them to Kansas State University. Females were housed under standardized conditions in filtered and aerated 70-L tanks with nonsulfidic water. Once females dropped fry, they were removed from the tank, and family groups were raised to adulthood (>30 mm standard length).

Adult, laboratory-reared individuals were then used for an H_2S exposure experiment following a previously published protocol (Tobler et al. 2014). In brief,

peristaltic pumps were used to administer stock solutions into 1-L containers holding experimental animals over a 22-hour period (flow rate: 90 mL/hour). Since sulfide concentrations are variable within and among sulfide springs (Tobler et al. 2011), we exposed the fish to multiple H_2S concentrations with the goal to capture a broad diversity of gene expression responses for comparative analyses. Besides a control (0 mM of total sulfide), we prepared stock solutions at 0.5, 3.0, and 6.0 mM total sulfide. From each of the populations, we exposed up to six unrelated individuals per treatment (Table C.1). Immediately after the 22-hour exposure, individuals were euthanized, and gill tissues were removed and stored in RNAlater (Ambion). All procedures were approved by the Institutional Animal Care and Use Committee at Kansas State University (Protocol #3418).

RNA isolation and RNA-sequence library preparations

For all samples from the laboratory experiment, we pulverized 10-30 mg of sample per individual and extracted RNA using the Nucleospin RNA kit (Machery-Nagel). mRNA isolation and cDNA library preparation was conducted with diluted total RNA (between 1ng – 100μg) using the NEBNext Ultra Directional RNA Library Prep Kit with NEBNext Poly(A) mRNA Magnetic Isolation (New England Biolabs). We followed the manufacturer's instructions with minor modifications. When isolating, fragmenting, and priming the mRNA from the total RNA, the incubation period was 10 minutes at 94°C to obtain a fragment size of about 400 bp. The second interval of the incubation of the first strand cDNA synthesis was 50 minutes, and we then incubated samples for 10 minutes at 25 °C, 50 minutes at 42 °C, and 15 minutes at 70 °C. For adaptor ligation, we used a 1:50 dilution of the NEBNext adaptor. For PCR amplification, samples were incubated for 1

cycle at 37°C for 15 minutes; 1 cycle at 98°C for 30 seconds; 10 cycles at 98°C for 10 seconds, 65°C for 30 seconds, and 72°C for 30 seconds; 1 cycle at 72°C for 5 minutes. We purified the PCR reactions using AMPure XP beads (Beckman Coulter) and 80% ethanol for washing. Samples were washed a total of three times. After purification, 20 µl of the supernatant were transferred to clean PCR tubes and stored at 4 °C. Library quality was assessed using Bioanalyzer High Sensitivity DNA chip (Agilent). Libraries were then pooled in sets of 14 to 15 samples and sequenced on six lanes of an Illumina HiSeq 2500 with paired-end 101 bp reads at the University of Kansas Genome Center. Nine of the samples were subsequently resequenced at the Washington State University Spokane Genomics Core due to low read counts in the initial dataset. Preliminary analyses of gene expression variation revealed no consistent differences among sequencing lanes or providers (Figure C.3).

Transcriptome assembly and annotation

Raw RNA-seq reads from the field and the laboratory datasets were sorted by barcode, primer dimer was removed, and then reads were trimmed to quality 24 using TrimGalore! (Krueger 2015). Trimmed reads were then mapped to the reference genome of *Poecilia mexicana* (Version 1.0; Genbank accession number: LMXC01000000) and mitochondrial reference sequence, which included 13 coding and two rRNA (GenBank Accession number: KC992991). Mapping was conducted using BWA-mem (Li and Dubin 2009; Li 2013), which yielded the highest alignment percentage and quality for our data (as compared to the programs Bowtie, STAR, and Stampy; results not shown). We then used cufflinks (version 2.2.1) to extract mapped regions, cuffmerge to merge the extracted

regions for all individuals (Trapnell et al. 2010), and cd-hit-est with default values in the program cd-hit (Li and Godzik 2006; Fu et al. 2012) to remove any duplicate transcripts resulting from merging the laboratory and field datasets. Finally, we generated a multifasta file of all expressed loci using gffread in the cufflinks package, providing a reference transcriptome used for subsequent analyses.

Transcripts were annotated by using a BLASTx search against the SwissProt human database (critical E-value: 0.001; access date 10/17/2015). We also ran a BLASTn search on the Rfam database to potentially annotate any transcripts without a match in SwissProt (Nawrocki et al. 2015). There were only 328 hits to the Rfam database, indicating that there were few non-coding RNAs in our dataset. For all BLAST searches, we only retained the top BLAST hit per transcript. Sequences with a match were also annotated with Gene Ontology (GO) IDs (Gene Ontology Consortium 2004).

Quantifying gene expression variation

To estimate gene expression variation, we re-mapped the trimmed RNA-seq reads to the reference transcriptome and then quantified transcript abundance using eXpress (Roberts and Pachter 2013). We removed any transcripts that did not have at least 2 counts per million in at least 3 of the samples. To evaluate the major drivers of expression variation, we conducted a principle component analysis (PCA) using all 109 libraries. PC1 explained 15.1 % variance and was associated with differences between laboratory and field populations (Figure C.3). This may be caused by technical differences in library preparation (KAPA Library Preparation Kit *vs*. NEBNext Ultra Directional RNA Library Prep Kit) and sequencing (Illumina HiSeq 2000 at Stanford Center for Genomics and

Personalized Medicine *vs*. Illumina HiSeq 2500 at University of Kansas Genome Center and Washington State University Genomics Core), and we consequently removed variation associated with PC1 using the bioconductor package sva (Leek et al. 2012; Figure C.3).

To test the whether differentially expressed genes in the wild are differentially expressed because of H_2S and whether the expression was associated with evolution in gene regulation or plasticity, we identified differentially expressed genes between sulfidic and nonsulfidic populations for multiple subsets of our data. For all comparisons, differential expression was quantified based on normalized read counts using the bioconductor package edgeR (Robinson et al. 2010; Robinson and Oshlack 2010). We used *exactTest* with genome-wide false discovery rate (FDR) set to 0.05. Comparisons were conducted as described in Table 4.3, and potential overlaps in responses between drainages or populations were evaluated in R (version 3.1.0).

Weighted co-expression gene networks (WGCNA)

To complement our comparisons of gene expression patterns in nature and the laboratory, we conducted a co-expression gene network analysis with the R package WGCNA (Langfelder and Horvath 2008) using the top 10,000 expressed genes. We used the variance-stabilizing transformation function in the DEseq2 package in R (Love et al. 2014), which transforms library size normalized data on the $\log_2$ scale (Anders and Huber 2010). Before constructing the networks, we set the soft threshold (i.e., power) to 9, which was the lowest value that optimized the topology (Langfelder and Horvarth 2008).

Functional assessment of candidate genes

To assess the potential functional roles of candidate genes that were differentially expressed both in field and laboratory comparisons, we conducted an enrichment analysis of corresponding GO terms in GOrilla (Eden et al. 2009). We used default settings, with a *P*-value cut-off of 0.001. Enrichment analysis was conducted both for up and downregulated genes that were shared between the two drainages and between the rearing environment (Table C.4).

Results and discussion

Experimental design, transcriptome assembly, and annotation

Individuals for this study were collected in two pairs of sulfidic and nonsulfidic populations from two river drainages (Ríos Tacotalpa and Puyacatengo; Palacios et al. 2013). Gill transcriptomes from wild-caught fish (*N*=22) were taken from a previously published study (Kelley et al. 2016; GenBank Sequence Read Archive study accession ID: PRJNA290391). In the same populations, individuals were bred and offspring raised to adulthood in a common-garden setting. Laboratory-reared fish were used for an H_2S exposure experiment upon reaching maturity (*N*=87). Because gene expression responses may be dose-dependent and thresholds may be variable among populations, fish from each population were exposed to a range of H_2S concentrations (0.0 (control), 0.5, 3.0, and 6.0 mM) with the goal to induce expression variation in a breadth of sulfide sensitive genes.

Sequencing the gill transcriptomes yielded a total of 320,187,084 reads for the individuals collected in the field (*N*=22*)* and 2,046,844,024 reads for the individuals from the laboratory experiment (N=*87*) (Table C.1). Reads were mapped against the *Poecilia mexicana* reference genome (Version 1.0; Genbank accession number: LMXC01000000).

The transcriptome was generated in cufflinks (version 2.2.1) by extracting the mapped regions, merging the extracted regions for all individuals and then generating a FASTA file using *gffread utility* (Trapnell et al. 2010). This resulted in a transcriptome composed of 63,590 transcripts from 11,966 unique loci. The total length of the merged transcriptome was 225,876,000 bp with an N50 of 5,290 bp (see Table C.2 for additional summary statistics). A local BLAST search identified 48,601 loci (76.43 %) with matches in the SwissProt database (see supplementary Table C.3 for complete annotations of all individual transcripts). Of these, 13,499 were associated with Gene Ontology (GO) terms (Gene Ontology Consortium 2004).

Identification of differentially expressed genes in fish from the laboratory experiment through pairwise comparisons between control (0.0 mM total sulfide) and different treatment conditions (0.5, 3 and 6 mM total sulfide) did not reveal consistent dose-dependent responses among populations, both in terms of the number and identity of differentially expressed transcripts. Hence, all subsequent analyses rely on the comparison of expression levels between control and exposed individuals, where transcripts were considered differentially expressed if a significant difference was detected in at least one comparison (control *vs*. 0.5 mM, control *vs*. 3 mM, or control *vs*. 6 mM). This approach allowed for the retention of the highest diversity of genes responding to H_2S exposure, even if responses were not consistent across all treatments.

H_2S-induced variation in gene expression

A previous study documented rampant differences in gene expression between adjacent sulfidic and nonsulfidic populations (Kelley et al. 2016), but due to the complexity of

environmental differences between habitat types (Tobler and Plath 2011), it remains unclear which expression differences are tied to the presence of absence of H_2S. Addressing this problem requires the comparison of transcripts that are differentially expressed in natural populations with those that are differentially expressed in common-garden reared fish, where population differences in environmental exposure are limited to the presence or absence of H_2S. Hence, for each drainage, we quantified differential expression between sulfidic and nonsulfidic populations in nature and in the laboratory experiment (i.e., sulfidic population exposed to H_2S *vs*. nonsulfidic population under control conditions). Differential expression was quantified using edgeR (Robinson et al. 2010), and transcripts showing the same pattern in both comparisons are key candidates for adaptive variation in gene expression in direct response to H_2S.

In total, we identified 5,861 upregulated and 5,448 downregulated transcripts (Figure 4.1), most of which were differentially expressed only in the wild or only in the laboratory experiment. 603 upregulated and 552 downregulated transcripts showed identical patterns in the wild and the laboratory in at least one of the drainages, indicating that about 10.5 % of the documented expression variation likely represent adaptive variation in gene expression in response to H_2S (Table 4.1-4.2). Expression differences unique to wild populations (~40.4 %) are likely driven by other environmental factors that coincide with the presence of H_2S in sulfide springs, including differences in temperature, pH, salinity, oxygen concentrations, and biotic factors (Riesch et al. 2015b), which have been shown to induce gene expression variation in other systems (e.g., Gracey et al. 2000; Podrabsky and Somero 2004; Zippay and Hofmann 2010; Narum et al. 2013; Ghalambor et al. 2015). Expression differences unique to the laboratory experiment (~38.0 %) likely

arose as a byproduct of the experimental design that relied on raising fish under nonsulfidic conditions and only exposing them to H_2S for short periods of time as adults (as opposed to a continuous exposure in nature). The nature and duration of exposure to physiochemical stressors can significantly impact gene expression (Tobler et al. 2014; Narum and Campbell 2015). In addition, it is important to recognize that technical differences during the preparation of the field and laboratory datasets in library preparation and sequencing could contribute to the high number of differentially expressed transcripts unique to each dataset, even though we attempted to control for these statistically (see Materials and Methods; Figure C.3). Functional interpretations of differentially expressed genes that are unique to the field or laboratory dataset should therefore be made with caution.

Across both drainages examined, 97 up and 80 downregulated transcripts showed identical patterns in the wild and the laboratory (Figure 4.1; Table 4.1-4.2). The majority of these transcripts were also recovered in a weighted gene co-expression network analysis (WGCNA; Langfelder and Hovarth 2008) of the top 10,000 expressed genes which revealed that the most supported modules corresponded to differences between sulfidic and nonsulfidic populations (Figure C.1-C.2). Thus, these transcripts are likely directly involved in facilitating tolerance to environmental H_2S as they show consistent responses to the presence and absence of H_2S across drainages both in the wild and the laboratory. Functional annotation of these transcripts can provide insights into the mechanisms underlying adaptation to H_2S, and we used GOrilla to test for enrichment of GO terms (Eden et al. 2009). Results indicated that upregulated transcripts were enriched for 63 terms associated with biological processes, while downregulated genes were enriched for 8 terms (Table C.4). Overall, enriched terms – and their underlying genes – closely aligned with

the results of a previous field study that inferred adaptive variation in gene expression in response to H_2S by identifying convergent patterns across three independent lineages (Kelley et al. 2016); hence, we only provide a brief overview of the putative functional links between the documented expression patterns and the evolution of H_2S tolerance in sulfidic populations.

The primary toxic effect of H_2S is its interference with oxidative phosphorylation, compromising the function of mitochondria (Reiffenstein et al. 1992). This occurs through the reversible binding of H_2S to cytochrome c oxidase (complex IV), which ultimately halts aerobic ATP production (Cooper and Brown 2008). It is therefore not surprising that differentially expressed genes were highly associated with mitochondrial cellular components (Table C.4). Furthermore, two primary groups of biological processes stand out among all the enriched GO terms and can directly be linked to H_2S toxicity and tolerance.

The first group broadly encompasses processes associated with ATP production and metabolic physiology. This includes genes involved in mitochondrial electron transport (GO:0006123 and GO:0006122), such as cytochrome c (*CYCS*), which can buffer the flow of electrons during blockage of complex IV, and genes associated with complex IV itself (*COX1* and *COX15*). In addition, several physiological pathways associated with anaerobic ATP production are also upregulated (e.g., gluconeogenesis, GO:0006094; pyruvate metabolism, GO:0006090). Together, these results indicate that gene expression differences between sulfidic and nonsulfidic population function to maintain aerobic ATP production in presence of H_2S and simultaneously increase anaerobic ATP production. Future studies will have to test this idea through physiological assays.

The second group of biological processes broadly encompasses pathways associated with enzymatic H_2S detoxification as well as sulfur processing and transport. H_2S detoxification in metazoans occurs through the sulfide:quinone oxidoreductase (SQR) pathway (Marcia et al. 2009), which oxidizes H_2S to nontoxic forms that can be excreted (Hildebrandt and Grieshaber 2008; Jackson et al. 2012). GO terms associated with H_2S oxidation were significantly enriched (GO:0019418, GO:0070221), including the upregulation of key genes associated with the SQR pathway (e.g., *SQRDL* and *ETHE1*). Likewise, we also saw significant enrichment in pathways associated with the physiological processing of sulfur, including metabolism of sulfur-containing amino acids and glutathione (GO:0006790, GO:0000096, GO:0006749), as well as the transport of oxidized sulfur species (GO:1902358, GO: 0015116, GO:0008272). H_2S detoxification through modulation of the SQR pathway has also been documented in other metazoans that live in sulfidic environments (Ma et al. 2012; Liu et al. 2016), perhaps indicating that modification of this highly conserved pathway represents a common theme in adaptation to environmental H_2S.

Overall, our results indicate that expression variation in a variety of genes associated with energy metabolism and ATP production as well with the physiological processing of sulfur are indeed a consequence of the presence of H_2S. These genes remained differentially expressed in laboratory fish that were reared under standardized conditions except for the exposure to H_2S, making it unlikely that hypoxia (Bagarinao 1992) and energy limitation (Plath et al. 2007b) – which are associated with the presence of H_2S in natural systems – are the primary drivers of changes in energy metabolism. Functional annotation of differentially expressed genes also suggests that adaptation to H_2S

likely involves mitigation of the toxic effects of H_2S through modification of pathways that contribute to ATP production and active detoxification, which is associated with maintenance of low endogenous concentrations (see Kelley et al. 2016). This is also supported by studies of molecular evolution that have detected signatures of positive selection both in genes encoding for toxicity targets (cytochrome c oxidase; Pfenninger et al. 2014) and in genes encoding for enzymes involved in H_2S detoxification (SQR; Pfenninger et al. 2015). Hence, both molecular evolution and changes in gene regulation appear to be associated with adaptation to H_2S.

Mechanisms underlying variation in gene expression

Comparison of gene expression variation between the sulfidic and nonsulfidic fish in the wild and laboratory indicated 1,155 differentially expressed transcripts (603 upregulated and 552 downregulated) that are likely candidates for adaptive differences in response to H_2S. Comparisons of gene expression patterns among different populations and exposure treatments allows for disentangling mechanisms that shape expression variation for each transcript. If expression differences of candidate transcripts are due to evolution of gene regulation that lead to constitutive population differences, expression differences should be maintained in laboratory-reared fish that have never been exposed to environmental H_2S. Comparing expression levels between sulfidic and nonsulfidic populations under control conditions indeed revealed that 20.8 % (Tacotalpa drainage) and 38.3 % (Puyacatengo drainage) of candidate transcripts remain differentially expressed irrespective of the presence of H_2S (Table 4.3). Transcripts with evidence for population

differences included transcripts with known functions in H_2S toxicity and detoxification (Table C.5).

Observed expression variation may also be a mere consequence of H_2S exposure. Quantifying expression differences between control and H_2S-exposed individuals for each population revealed that 24.1 % (Tacotalpa) and 53.0 % (Puyacatengo) of transcripts plastically responded to H_2S-exposure in at least one of the populations (Table 4.3), indicating that the number of transcripts with evolved constitutive expression differences and with H_2S-inducibility are roughly the same in each population, with the Puyacatengo having evidence of higher H_2S-inducibility. However, only 5.4 % (Tacotalpa) and 9.0 % (Puyacatengo) of transcripts actually exhibited the same plastic response in both the sulfidic and the nonsulfidic population of each drainage (ancestral plasticity; Table 4.3). This suggests that ancestral H_2S-induced plasticity in gene expression plays a relatively small role in shaping variation in natural populations, a notion that is further corroborated by the observation that only six transcripts (~0.5%) consistently exhibit H_2S-indicibility across all four populations examined here. These include two distinct transcripts each from key genes in enzymatic H_2S detoxification (*SQRDL* and *ETHE1*) as well as *UUP1* (encoding for Uridine phosphorylase 1) and *NMES1* (encoding for Normal mucosa of esophagus-specific gene 1 protein), whose functional links to H_2S exposure remain relatively elusive (Table C.6).

The majority of transcripts responded to H_2S exposure through either the evolution of plasticity or canalization (Table 4.3). Interestingly, the distribution of H_2S-responsive genes differs between the two river drainages. In the Tacotalpa, more transcripts were responsive to H_2S exposure in the derived, sulfidic population (15.4 *vs*. 3.3 %), indicating

that the colonization of sulfide springs was potentially accompanied with the evolution of plasticity in gene expression in response to H_2S. In contrast, more transcripts were responsive to H_2S exposure in the ancestral, nonsulfidic population of the Puyacatengo (40.7 *vs*. 3.4 %), which suggests that colonization of H_2S-rich habitats coincided with canalization of formerly plastic responses.

It is important to note that several transcripts showed evidence for multiple mechanisms; i.e., they were differentially expressed between sulfidic and nonsulfidic populations under control conditions, and they plastically responded to H_2S exposure in at least one of the populations. For example, *ETHE1* – a key enzyme in the SQR pathway – was constitutively higher expressed in sulfidic populations, and it was consistently upregulated upon exposure to H_2S, such that expression levels in sulfidic populations always exceeded those in nonsulfidic populations (Table C.5-C.6). Furthermore, a sizable number of transcripts could not be associated with any of the proposed mechanisms underlying expression variation in nature (56.9 % in the Tacotalpa, 26.7 % in the Puyacatengo). This is likely a consequence of our analytical approach that relied on the comparison of individuals in the control treatment against those exposed to H_2S irrespective of the actual concentration. So while a particular transcript may have occurred in our candidate list because there was evidence for differential expression in one comparison between controls from nonsulfidic populations and exposed individuals from sulfidic populations (see previous section), the signal may have not been strong enough to pass the significance threshold in subsequent analyses.

Despite these caveats, our analyses provide strong evidence that among population variation in the expression of genes likely associated with the presence of H_2S was

primarily shaped by evolutionary change. Less than 10% of transcripts exhibited shared responses between sulfidic and nonsulfidic populations (less that 1% if all populations were considered together), which clearly indicated that shared ancestral plasticity in response to H_2S does not drive the observed variation in gene expression. In contrast, there is broad evidence for evolution of constitutive expression differences between population as well as the gain and loss of H_2S-inducibility of gene expression. These results are consistent with recent studies that have documented interactions between evolution and plasticity that drive variation in expression in natural systems (McCairns and Bernatchez 2010; Leder et al. 2015; Narum and Campbell 2015).

Conclusions

Overall, our study made significant progress toward understanding factors shaping gene expression variation in a natural system of extremophile fishes. Even though H_2S is a potent respiratory toxicant and a strong source of selection, common garden and H_2S exposure experiments revealed that only about 10 % of genes that are differentially expressed among sulfidic and nonsulfidic population pairs in nature remain differentially expressed when fish are reared under standardized conditions. Other environmental factors that coincide with the presence of H_2S in sulfide springs (Grether et al. 2001; Holmstrup et al. 2010; Tobler and Plath 2011) as well as neutral processes (Whitehead and Crawford 2006) must also play a critical role in shaping gene expression variation among natural populations. This highlights the importance of multifarious selection in shaping evolutionary change and phenotypic expression, and understanding the mechanisms underlying adaptive evolution even along apparently simple environmental gradients will require that we

disentangle the interactive effects of co-varying sources of selection. Among the differentially expressed genes that are likely tied to the presence of H_2S, ancestral plasticity in response to H_2S exposure that is shared among populations appears to play a minor role in contributing to the observed expression variation in nature. Instead, the examination of the expression patterns of common garden raised fish revealed population differences in the constitutive expression levels and H_2S-inducibility, suggesting that expression variation in candidate genes has likely been shaped by evolutionary change. Hence, regulatory changes (Kelley et al. 2016) and molecular evolution affecting enzyme function (Pfenninger at al 2014; Pfenninger et al 2015) both play critical roles in mediating adaptation to sulfide springs.

The current study has also revealed three major, unaddressed questions relating to our understanding of adaptation to H_2S-rich environments: (1) Previous studies have identified a series of prominent candidate genes involved in energy metabolism and ATP production as well as H_2S detoxification and sulfur processing that are either under positive selection or differentially expressed in sulfide spring fish populations. Nonetheless, the effects of genetic variation and differences in gene regulation among populations on organismal performance in presence and absence of H_2S remain largely untested, even though such functional studies are critical to reveal molecular mechanisms of adaptation (Storz 2016). (2) Comparative analyses among lineages of *Poecilia* fishes that independently colonized sulfide springs have revealed stunning examples of convergent evolution at the molecular level (Pfenninger et al. 2014). Still, the majority of loci under positive selection and transcripts with evidence for differential expression are unique to specific lineages (Pfenninger et al. 2015, Kelley et al., 2016). In the current study, for

example, we found substantial differences both in the identity of differentially expressed transcripts and in the mechanisms that underlie expression variation. This raises questions about potential differences in strategies that may confer tolerance to H_2S. Sulfide spring populations in the Puyacatengo drainage have evolved a H_2S-resistent COX (Pfenninger et al. 2014) and exhibit more widespread differential expression of genes associated with oxidative phosphorylation than those in the Tacotalpa drainage (Table C.5-C.6). Hence, it remains to be tested whether such differences generally reflect different strategies to tolerate exposure to H_2S (e.g., mitigating of toxic effects during H_2S exposure *vs*. increased ability to detoxify H_2S; Tobler et al. 2016b). (3) While the present study revealed evidence for population differences in constitutive gene expression and H_2S-inducibility in gene expression, the regulatory mechanisms that underlie these population differences remain unknown. In biomedical models, H_2S has been shown to interact with a number of transcription factors (e.g., NF-κB, Nrf-2, Hif-1) and intracellular signaling molecules (mitogen-activated protein kinase pathway) (Li et al. 2011; Kabil et al. 2014). Whether these pathways are involved in H_2S adaptation remains to be tested, and future studies will have to illuminate the genetic mechanisms that give rise to the observed changes in gene regulation.

Figures

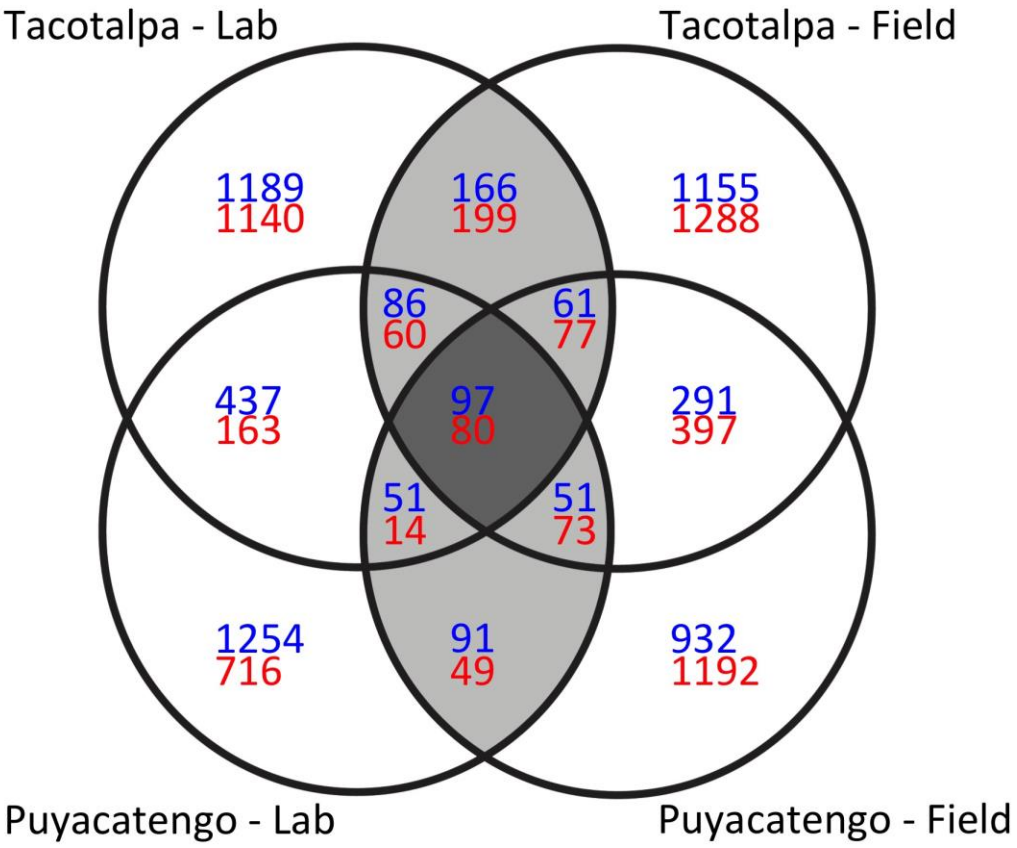

Figure 4.1 Venn diagram illustrating number of differentially expressed genes

Venn diagram illustrating the number of differentially expressed genes between sulfidic and nonsulfidic populations in the field and the laboratory for two river drainages (Puyacatengo and Tacotalpa). Values in blue are upregulated and those in red are downregulated in sulfidic populations. The shaded areas represent differentially expressed genes that show consistent patterns both in the field and the laboratory, providing candidates for adaptation to H_2S used on other analyses. The dark shaded area represents genes that showed identical patterns across drainages and are the focus for the interpretation of functional attributes.

Tables

Table 4.1 Candidate genes upregulated in laboratory and field populations

List of candidate genes that were consistently upregulated in both sulfidic populations in nature and the laboratory. Reported are the Transcript IDs, the accession number corresponding to the top BLAST hit, gene name, protein name, and the E-value.

Transcript ID	Accession Number	Gene	Protein	E-value
TCONS_00023606	O95571	ETHE1	Persulfide dioxygenase ETHE1, mitochondrial	2.00E-77
TCONS_00070357	Q9Y6N5	SQRDL	Sulfide:quinone oxidoreductase, mitochondrial	<1.00E-99
TCONS_00023604	O95571	ETHE1	Persulfide dioxygenase ETHE1, mitochondrial	8.00E-67
TCONS_00082242	Q96A26	FAM162A	Protein FAM162A	3.00E-30
TCONS_00040693	O43708	GSTZ1	Maleylacetoacetate isomerase	1.00E-09
TCONS_00012258	Q8IYE1	CCDC13	Coiled-coil domain-containing protein 13	4.00E-93
TCONS_00085696	Q56VL3	OCIAD2	OCIA domain-containing protein 2	2.00E-24
TCONS_00057314	Q9UBX3	SLC25A10	Mitochondrial dicarboxylate carrier	<1.00E-99
TCONS_00047163	Q643R3	LPCAT4	Lysophospholipid acyltransferase LPCAT4	<1.00E-99
TCONS_00061435	B7ZAP0	RABGAP1L	Rab GTPase-activating protein 1-like, isoform 10	7.00E-66
TCONS_00048669	O60675	MAFK	Transcription factor MafK	2.00E-40
TCONS_00023604	O95571	ETHE1	Persulfide dioxygenase ETHE1, mitochondrial	8.00E-67
TCONS_00061440	Q14145	KEAP1	Kelch-like ECH-associated protein 1	<1.00E-99
TCONS_00014089	P48506	GCLC	Glutamate--cysteine ligase catalytic subunit	<1.00E-99
TCONS_00006442	P58743	SLC26A5	Prestin	<1.00E-99
TCONS_00017095	Q15120	PDK3	[Pyruvate dehydrogenase (acetyl-transferring)] kinase isozyme 3, mitochondrial	<1.00E-99
TCONS_00084064	P13591	NCAM1	Neural cell adhesion molecule 1	3.00E-52
TCONS_00052515	P35558	PCK1	Phosphoenolpyruvate carboxykinase, cytosolic [GTP]	<1.00E-99
TCONS_00091432	P25325	MPST	3-mercaptopyruvate sulfurtransferase	<1.00E-99
TCONS_00071225	P53985	SLC16A1	Monocarboxylate transporter 1	<1.00E-99
TCONS_00010836	Q86UK0	ABCA12	ATP-binding cassette sub-family A member 12	3.00E-27

TCONS_00006391	O75439	PMPCB	Mitochondrial-processing peptidase subunit beta	<1.00E-99
TCONS_00033994	Q92597	NDRG1	Protein NDRG1	<1.00E-99
TCONS_00004173	O43175	PHGDH	D-3-phosphoglycerate dehydrogenase	<1.00E-99
TCONS_00044107	Q86YN6	PPARGC1B	Peroxisome proliferator-activated receptor gamma coactivator 1-beta	5.00E-16
TCONS_00004176	O43175	PHGDH	D-3-phosphoglycerate dehydrogenase	<1.00E-99
TCONS_00006390	O75439	PMPCB	Mitochondrial-processing peptidase subunit beta	<1.00E-99
TCONS_00033685	Q15849	SLC14A2	Urea transporter 2	<1.00E-99
TCONS_00094542	Q16822	PCK2	Phosphoenolpyruvate carboxykinase [GTP], mitochondrial	1.00E-63
TCONS_00023094	Q86WA9	SLC26A11	Sodium-independent sulfate anion transporter	<1.00E-99
TCONS_00094543	P35558	PCK1	Phosphoenolpyruvate carboxykinase, cytosolic [GTP]	<1.00E-99
TCONS_00094545	Q16822	PCK2	Phosphoenolpyruvate carboxykinase [GTP], mitochondrial	<1.00E-99
TCONS_00003330	P09972	ALDOC	Fructose-bisphosphate aldolase C	<1.00E-99
TCONS_00041978	P99999	CYCS	Cytochrome c	5.00E-50
TCONS_00066149	P48637	GSS	Glutathione synthetase	<1.00E-99
TCONS_00027992	Q8IWT1	SCN4B	Sodium channel subunit beta-4	2.00E-36
TCONS_00087011	P25325	MPST	3-mercaptopyruvate sulfurtransferase	3.00E-45
TCONS_00007928	Q96KS0	EGLN2	Egl nine homolog 2	<1.00E-99
TCONS_00041975	P99999	CYCS	Cytochrome c	7.00E-51
TCONS_00018972	Q5T1C6	THEM4	Acyl-coenzyme A thioesterase THEM4	1.00E-44
TCONS_00014090	P48506	GCLC	Glutamate--cysteine ligase catalytic subunit	<1.00E-99
TCONS_00093313	P48029	SLC6A8	Sodium- and chloride-dependent creatine transporter 1	<1.00E-99
TCONS_00023071	O15525	MAFG	Transcription factor MafG	5.00E-72
TCONS_00030199	P25325	MPST	3-mercaptopyruvate sulfurtransferase	<1.00E-99
TCONS_00050936	O75452	RDH16	Retinol dehydrogenase 16	<1.00E-99
TCONS_00034515	P27144	AK4	Adenylate kinase 4, mitochondrial	<1.00E-99
TCONS_00103938	N/A	N/A	N/A	N/A
TCONS_00027204	P10599	TXN	Thioredoxin	3.00E-33
TCONS_00065938	Q9UK39	NOCT	Nocturnin	<1.00E-99
TCONS_00101924	Q8TEY5	CREB3L4	Cyclic AMP-responsive element-binding protein 3-like protein 4	6.00E-42
TCONS_00023607	O95571	ETHE1	Persulfide dioxygenase ETHE1, mitochondrial	<1.00E-99
TCONS_00023605	O95571	ETHE1	Persulfide dioxygenase ETHE1, mitochondrial	<1.00E-99

TCONS_00070356	Q9Y6N5	SQRDL	Sulfide:quinone oxidoreductase, mitochondrial	<1.00E-99
TCONS_00085695	Q56VL3	OCIAD2	OCIA domain-containing protein 2	2.00E-24
TCONS_00012259	Q8IYE1	CCDC13	Coiled-coil domain-containing protein 13	4.00E-93
TCONS_00082240	Q96A26	FAM162A	Protein FAM162A	1.00E-27
TCONS_00079033	Q7KZN9	COX15	Cytochrome c oxidase assembly protein COX15 homolog	<1.00E-99
TCONS_00006440	P58743	SLC26A5	Prestin	<1.00E-99
TCONS_00034835	Q96B67	ARRDC3	Arrestin domain-containing protein 3	6.00E-41
TCONS_00048671	O60675	MAFK	Transcription factor MafK	2.00E-36
TCONS_00041984	P99999	CYCS	Cytochrome c	2.00E-54
TCONS_00045767	Q9C002	NMES1	Normal mucosa of esophagus-specific gene 1 protein	7.00E-27
TCONS_00027336	P00390	GSR	Glutathione reductase, mitochondrial	<1.00E-99
TCONS_00017098	Q15120	PDK3	[Pyruvate dehydrogenase (acetyl-transferring)] kinase isozyme 3, mitochondrial	<1.00E-99
TCONS_00017092	Q15120	PDK3	[Pyruvate dehydrogenase (acetyl-transferring)] kinase isozyme 3, mitochondrial	<1.00E-99
TCONS_00052517	Q16822	PCK2	Phosphoenolpyruvate carboxykinase [GTP], mitochondrial	<1.00E-99
TCONS_00006441	P58743	SLC26A5	Prestin	<1.00E-99
TCONS_00004630	Q969K7	TMEM54	Transmembrane protein 54	7.00E-24
TCONS_00051416	P53007	SLC25A1	Tricarboxylate transport protein, mitochondrial	6.00E-64
TCONS_00041977	P99999	CYCS	Cytochrome c	4.00E-24
TCONS_00103939	N/A	N/A	N/A	N/A
TCONS_00044106	Q86YN6	PPARGC1B	Peroxisome proliferator-activated receptor gamma coactivator 1-beta	9.00E-16
TCONS_00027832	Q06830	PRDX1	Peroxiredoxin-1	<1.00E-99
TCONS_00034015	O95388	WISP1	WNT1-inducible-signaling pathway protein 1	<1.00E-99
TCONS_00045227	P07339	CTSD	Cathepsin D	<1.00E-99
TCONS_00008835	P15121	AKR1B1	Aldose reductase	<1.00E-99
TCONS_00004172	O43175	PHGDH	D-3-phosphoglycerate dehydrogenase	<1.00E-99
TCONS_00041974	P99999	CYCS	Cytochrome c	6.00E-50
TCONS_00094540	Q16822	PCK2	Phosphoenolpyruvate carboxykinase [GTP], mitochondrial	<1.00E-99
TCONS_00094541	P35558	PCK1	Phosphoenolpyruvate carboxykinase, cytosolic [GTP]	<1.00E-99
TCONS_00003331	P09972	ALDOC	Fructose-bisphosphate aldolase C	<1.00E-99

TCONS_00033684	Q15849	SLC14A2	Urea transporter 2	8.00E-88
TCONS_00033686	Q15849	SLC14A2	Urea transporter 2	<1.00E-99
TCONS_00094544	P35558	PCK1	Phosphoenolpyruvate carboxykinase, cytosolic [GTP]	<1.00E-99
TCONS_00023072	O15525	MAFG	Transcription factor MafG	4.00E-72
TCONS_00002273	Q6ZQY3	GADL1	Acidic amino acid decarboxylase GADL1	<1.00E-99
TCONS_00086224	Q16831	UPP1	Uridine phosphorylase 1	<1.00E-99
TCONS_00086432	Q6ZNA5	FRRS1	Ferric-chelate reductase 1	3.00E-42
TCONS_00086225	Q16831	UPP1	Uridine phosphorylase 1	<1.00E-99
TCONS_00093314	P48029	SLC6A8	Sodium- and chloride-dependent creatine transporter 1	<1.00E-99
TCONS_00041979	P99999	CYCS	Cytochrome c	6.00E-50
TCONS_00030025	O95361	TRIM16	Tripartite motif-containing protein 16	1.00E-57
TCONS_00091203	Q9H628	RERGL	Ras-related and estrogen-regulated growth inhibitor-like protein	2.00E-19
TCONS_00086222	Q16831	UPP1	Uridine phosphorylase 1	<1.00E-99
TCONS_00034513	P27144	AK4	Adenylate kinase 4, mitochondrial	6.00E-74
lcl|KC992991.1_cds_AGW31980.1_11	P00395	MT-CO1	Cytochrome c oxidase subunit 1	<1.00E-99
TCONS_00050933	O75452	RDH16	Retinol dehydrogenase 16	<1.00E-99

Table 4.2 Candidate genes downregulated in laboratory and field populations

List of candidate genes that were consistently downregulated in both sulfidic populations in nature and the laboratory. Reported are the Transcript IDs, the accession number corresponding to the top BLAST hit, gene name, protein name, and the E-value.

Transcript ID	Accession Number	Gene	Protein	E-value
TCONS_00016699	Q04695	KRT17	Keratin, type I cytoskeletal 17	1.00E-74
TCONS_00026813	P10721	KIT	Mast/stem cell growth factor receptor Kit	<1.00E-99
TCONS_00103080	P11142	HSPA8	Heat shock cognate 71 kDa protein	<1.00E-99
TCONS_00086588	Q8NER1	TRPV1	Transient receptor potential cation channel subfamily V member 1	<1.00E-99
TCONS_00090425	Q8IZF2	ADGRF5	Adhesion G protein-coupled receptor F5	3.00E-79
TCONS_00001312	P60002	ELOF1	Transcription elongation factor 1 homolog	1.00E-38
TCONS_00038321	Q08209	PPP3CA	Serine/threonine-protein phosphatase 2B catalytic subunit alpha isoform	<1.00E-99
TCONS_00048728	Q96G23	CERS2	Ceramide synthase 2	<1.00E-99
TCONS_00095523	O00168	PXYD1	Phospholemman	1.00E-10
TCONS_00038324	Q08209	PPP3CA	Serine/threonine-protein phosphatase 2B catalytic subunit alpha isoform	<1.00E-99
TCONS_00083846	N/A	N/A	N/A	N/A
TCONS_00032764	Q9UBY9	HSPB7	Heat shock protein beta-7	3.00E-19
TCONS_00012141	Q96PQ7	KLHL5	Kelch-like protein 5	<1.00E-99
TCONS_00085220	Q8TB45	DEPTOR	DEP domain-containing mTOR-interacting protein	2.00E-53
TCONS_00028667	Q0VF96	CGNL1	Cingulin-like protein 1	<1.00E-99
TCONS_00073846	O75746	SLC25A12	Calcium-binding mitochondrial carrier protein Aralar1	<1.00E-99
TCONS_00026812	P10721	KIT	Mast/stem cell growth factor receptor Kit	<1.00E-99
TCONS_00098721	O60635	TSPAN1	Tetraspanin-1	1.00E-25
TCONS_00049661	Q92781	RDH5	11-cis retinol dehydrogenase	5.00E-14
TCONS_00038319	Q08209	PPP3CA	Serine/threonine-protein phosphatase 2B catalytic subunit alpha isoform	<1.00E-99
TCONS_00094906	Q96FJ2	DYNLL2	Dynein light chain 2, cytoplasmic	2.00E-25
TCONS_00042052	P12104	FABP2	Fatty acid-binding protein, intestinal	2.00E-56
TCONS_00013644	P41586	ADCYAP1R1	Pituitary adenylate cyclase-activating polypeptide type I receptor	4.00E-90
TCONS_00028430	N/A	N/A	N/A	N/A

TCONS_00033963	Q9H936	SLC25A22	Mitochondrial glutamate carrier 1	4.00E-68
TCONS_00037284	A8MVW5	HEPACAM2	HEPACAM family member 2	1.00E-77
TCONS_00028669	Q0VF96	CGNL1	Cingulin-like protein 1	2.00E-98
TCONS_00029376	Q9Y6R1	SLC4A4	Electrogenic sodium bicarbonate cotransporter 1	<1.00E-99
TCONS_00029371	Q9Y6R1	SLC4A4	Electrogenic sodium bicarbonate cotransporter 1	<1.00E-99
TCONS_00033964	Q9H936	SLC25A22	Mitochondrial glutamate carrier 1	4.00E-68
TCONS_00036290	Q00005	PPP2R2B	Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B beta isoform	<1.00E-99
TCONS_00050597	Q96HN2	AHCYL2	Adenosylhomocysteinase 3	<1.00E-99
TCONS_00032813	N/A	N/A	N/A	N/A
TCONS_00032970	N/A	N/A	N/A	N/A
TCONS_00048734	Q96G23	CERS2	Ceramide synthase 2	8.00E-79
TCONS_00094908	N/A	N/A	N/A	N/A
TCONS_00071139	O75746	SLC25A12	Calcium-binding mitochondrial carrier protein Aralar1	<1.00E-99
TCONS_00048735	Q96G23	CERS2	Ceramide synthase 2	3.00E-70
TCONS_00048737	Q96G23	CERS2	Ceramide synthase 2	7.00E-89
TCONS_00048101	P36269	GGT5	Gamma-glutamyltransferase 5	1.00E-96
TCONS_00091181	Q9UMS0	NFU1	NFU1 iron-sulfur cluster scaffold homolog, mitochondrial	1.00E-73
TCONS_00029374	Q9Y6R1	SLC4A4	Electrogenic sodium bicarbonate cotransporter 1	<1.00E-99
TCONS_00044131	O00483	NDUFA4	NADH-ubiquinone oxidoreductase MLRQ subunit	4.00E-31
TCONS_00013645	P41586	ADCYAP1R1	Pituitary adenylate cyclase-activating polypeptide type I receptor	1.00E-82
TCONS_00029375	Q9Y6R1	SLC4A4	Electrogenic sodium bicarbonate cotransporter 1	<1.00E-99
TCONS_00054294	Q9P2F6	ARHGAP20	Rho GTPase-activating protein 20	<1.00E-99
TCONS_00037282	A8MVW5	HEPACAM2	HEPACAM family member 2	7.00E-77
TCONS_00067010	Q99456	KRT12	Keratin, type I cytoskeletal 12	1.00E-58
TCONS_00074674	P21145	MAL	Myelin and lymphocyte protein	1.00E-40
TCONS_00042051	P12104	FABP2	Fatty acid-binding protein, intestinal	2.00E-44
TCONS_00090426	N/A	N/A	N/A	N/A
TCONS_00028429	N/A	N/A	N/A	N/A
TCONS_00028433	N/A	N/A	N/A	N/A
TCONS_00048100	P36269	GGT5	Gamma-glutamyltransferase 5	<1.00E-99

TCONS_00013643	P47872	SCTR	Secretin receptor	1.00E-73
TCONS_00037283	A8MVW5	HEPACAM2	HEPACAM family member 2	4.00E-78
TCONS_00078699	Q86X10	RALGAPB	Ral GTPase-activating protein subunit beta	<1.00E-99
TCONS_00068710	N/A	N/A	N/A	N/A
TCONS_00037285	A8MVW5	HEPACAM2	HEPACAM family member 2	3.00E-97
TCONS_00104440	P55017	SLC12A3	Solute carrier family 12 member 3	1.00E-81
TCONS_00050596	Q96HN2	AHCYL2	Adenosylhomocysteinase 3	<1.00E-99
TCONS_00013818	O60825	PFKFB2	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 2	<1.00E-99
TCONS_00081894	P51788	CLCN2	Chloride channel protein 2	<1.00E-99
TCONS_00062780	N/A	N/A	N/A	N/A
TCONS_00032765	P05186	ALPL	Alkaline phosphatase, tissue-nonspecific isozyme	<1.00E-99
TCONS_00094115	N/A	N/A	N/A	N/A
TCONS_00040976	Q8IXH8	CDH26	Cadherin-like protein 26	<1.00E-99
TCONS_00071140	O75746	SLC25A12	Calcium-binding mitochondrial carrier protein Aralar1	<1.00E-99
TCONS_00079909	Q99712	KCNJ15	ATP-sensitive inward rectifier potassium channel 15	<1.00E-99
TCONS_00048729	Q96G23	CERS2	Ceramide synthase 2	8.00E-71
TCONS_00005932	Q96CX2	KCTD12	BTB/POZ domain-containing protein KCTD12	6.00E-94
TCONS_00095524	O00168	FXYD1	Phospholemman	1.00E-10
TCONS_00047022	Q9GZV3	SLC5A7	High affinity choline transporter 1	<1.00E-99
TCONS_00029372	Q9Y6R1	SLC4A4	Electrogenic sodium bicarbonate cotransporter 1	<1.00E-99
TCONS_00029373	Q9Y6R1	SLC4A4	Electrogenic sodium bicarbonate cotransporter 1	<1.00E-99
TCONS_00086591	Q8NER1	TRPV1	Transient receptor potential cation channel subfamily V member 1	<1.00E-99
TCONS_00029377	Q9Y6R1	SLC4A4	Electrogenic sodium bicarbonate cotransporter 1	<1.00E-99
TCONS_00104439	P55017	SLC12A3	Solute carrier family 12 member 3	8.00E-41
TCONS_00012248	N/A	N/A	N/A	N/A
TCONS_00100574	O75746	SLC25A12	Calcium-binding mitochondrial carrier protein Aralar1	<1.00E-99

Table 4.3 Results of differential expression analysis

Results of differential expression analyses using different subsets of data. A. Comparisons between field and laboratory datasets were conducted to identify expression differences in nature that occur primarily as a consequence of the presence or absence of H_2S. For each drainage, we provide the numbers of up and downregulated transcripts, in which differential expression in nature is retained upon rearing fish under standardized laboratory conditions. B. Comparison of expression differences between sulfidic and nonsulfidic controls (representing transcripts with evidence for evolved population differences in constitutive expression) as well as between non-exposed (control) and exposed individuals from the same population (identifying transcripts that are plastically responding to H_2S exposure). Intersection of differentially expressed transcripts between sulfidic and nonsulfidic populations allowed for the categorization of individual transcripts as representing ancestral plasticity (H_2S-inducible in the ancestral, nonsulfidic and the derived, sulfidic population), evolution of plasticity (H_2S-inducible only in the derived, sulfidic population), and canalization (H_2S-inducible only in the ancestral, nonsulfidic population). Provided are the number of genes in each scenario and the percentage of representation based on the number of candidate transcripts. We also provide the number of transcripts that could not be assigned to a specific mechanism. Note that the percentages may exceed 100%, because individual transcripts may exhibit evidence for constitutive and plastic expression differences.

Mechanism	Comparison	Tacotalpa Up	Tacotalpa Down	Puyacatengo Up	Puyacatengo Down
A. Comparisons between field and laboratory data					
Candidate transcripts	$Sulfidic_{Field}$-$Nonsulfidic_{Field}$ ∩ $Sulfidic_{Exposed}$-$Nonsulfidic_{Control}$	410	416	290	216
B. Comparisons between different subsets of laboratory data					
Evolved differences in constitutive expression	$Sulfidic_{Control}$-$Nonsulfidic_{Control}$	68 (16.6%)	104 (25.0 %)	115 (39.7 %)	79 (36.6 %)
Ancestral plasticity	$Sulfidic_{Control}$-$Sulfidic_{Exposed}$ ∩ $Nonsulfidic_{Control}$-$Nonsulfidic_{Exposed}$	44 (10.7 %)	1 (0.2%)	41 (14.1 %)	4 (1.9 %)
Evolution of plasticity	($Sulfidic_{Control}$-$Sulfidic_{Exposed}$) – ($Sulfidic_{Control}$-$Sulfidic_{Exposed}$ ∩ $Nonsulfidic_{Control}$-$Nonsulfidic_{Exposed}$)	96 (23.4 %)	31 (7.5 %)	11 (3.8 %)	6 (2.8 %)

Canalization	$(Nonsulfidic_{Control}$-$Nonsulfidic_{Exposed}) - (Sulfidic_{Control}$-$Sulfidic_{Exposed} \cap Nonsulfidic_{Control}$-$Nonsulfidic_{Exposed})$	17 (4.1 %)	10 (2.4 %)	98 (33.8 %)	108 (50.0 %)
Unassigned transcripts		196 (47.8 %)	274 (65.9 %)	77 (26.6 %)	58 (26.9 %)

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Appendix A - Reduction of energetic demands through modification of body size and routine metabolic rates in extremophile fish

Materials and Methods

Modeling energy expenditure in idealized populations

Theory predicts that populations in extreme environments facing reduced resource availability, reduced acquisition, or elevated maintenance costs should be selected for reduced energetic demands. Overall energy demand is affected both by body size and metabolic rates, and inferences from either of these components in isolation can result in conflicting results (McNab 1999). Indeed, all populations in our study exhibited significant reductions in body mass as compared to the ancestral population in nonsulfidic surface habitats, but cavefish also exhibited higher routine metabolic rates than fish from surface habitats. Hence, testing the overarching hypothesis whether colonization and adaptation to extreme environments has lead to reductions in energy demand of *P. mexicana* requires directly contrasting total energy expenditure among populations. However, merely comparing empirically determined total routine metabolic rates (i.e., analyzing metabolic rates without body mass as a covariate) was not a tangible solution given our experimental design. We were striving to quantify metabolic rates for a broad size range of individuals in each population (to facilitate the determination of allometric relationships), and as a consequence, the size ranges of experimental animals used to quantify metabolic rates were not necessarily reflective of the size distributions in their respective natural populations. This was evident by the significant differences in body mass means and/or variances between fish used for oxygen consumption measurements and the actual size distribution in three of the four populations examined [e.g., laboratory-reared fish were smaller than wild-caught individuals for the nonsulfidic surface habitat

($F_{1,247}$=7.356, P=0.007); laboratory-reared fish were larger than wild-caught individuals for the nonsulfidic ($F_{1,185}$=30.080, P<0.001) and sulfidic cave habitats ($F_{1,696}$=4.064, P=0.044)], such that direct comparisons of empirically measured total routine metabolic rates (without mass as a covariate) would have over- or underestimated total energy demands depending on the population.

We therefore modelled total routine metabolic rates for individuals from each habitat type based on empirical data of size distributions as well as allometric metabolic rate functions. For each habitat type, we first resampled size distributions based on field data 1000 times. For each resampled individual, total metabolic rate was calculated as $\log_{10}(MR_{tot})=b*\log_{10}(\text{mass})+a$, where b is the slope and a the intercept of a regression describing the mass and metabolic rate relationship for each population. To account for uncertainty associated with the estimation of slopes and intercepts, values for b and a were randomly chosen from within the 95% confidence interval of each parameter (see Table A.1). The simulated values of total routine metabolic rate consequently represent estimates of the energy demand of average individuals in each population, taking into account variation in body mass and metabolic rate allometry. Simulated total metabolic rates were analyzed using ANOVA with presence or absence of H_2S in natural populations (i.e., sulfidic vs. nonsulfidic habitat) and presence or absence of light in natural populations (i.e., cave vs. surface habitat) as independent variables.

Fish activity in the darkness

Even though many cave organisms have been reported to have reduced activity levels compared to epigean ancestors (Hüppop 2000), elevated routine metabolic rates in cave populations of *Poecilia mexicana* may be caused by differences in activity. Specifically, the ancestral surface populations are diurnal and might rest in the darkness of respirometry chambers, but the cave

populations with derived adaptations to live in complete darkness (Parzefall 2001; Plath et al. 2004) could have maintained higher levels of activity. Hence, we conducted activity trials in darkness using common garden reared individuals from all four populations described in the main manuscript. If documented differences in routine metabolic rates were caused merely by behavioural variation, we predicted that cavefish should have higher activities than surface fish, and males should have higher activities than females.

To examine activity variation between the four populations in dark conditions, ten common garden raised fish were selected at random to perform dark activity trials. All fish used were born and raised in the laboratory, and were maintained under standard 12:12 hour light:dark cycles. Focal fish were introduced into 6-liter tanks that had a grid (2 cm x 2 cm) drawn on the front window. Fish were then allowed to acclimate to the experimental conditions and the complete darkness in the room for 10 minutes. After the acclimation period, individual fish were then filmed for 60 minutes using a Digital HD video camera recorder (HDR-SR11/SR12; Sony) with night-shot function. After trials, each individual was weighed, sexed, and returned to their respective stock tank.

Activity was determined by the number of grid quadrants an individual fish crossed per unit of time. Specifically, we counted the number of quadrants passed during 10-second time intervals every 10 minutes (from the start of a trial to its end one hour later). This resulted in 6 activity measurements per fish, which were averaged for data analysis.

Average activity (per 10-second interval; square-root-transformed) was analysed using analysis of variance (ANOVA) using SPSS 20 (IBM Inc.). The presence of H_2S (sulfidic or nonsulfidic population), the presence of light (cave or surface population), and sex were used as the independent variables. Note that body size (mass) did not have any significant effects (neither

as a main effect nor in any interaction term; $F \leq 1.090$, $P \geq 0.304$) and was thus excluded from the final model.

Results

Modeling energy expenditure in idealized populations

Taking into account habitat-specific natural size distributions and uncertainty associated with the estimation of allometric relationships between mass and metabolic rates in each population, we found that the presence or absence of light and sulfide, as well as the interaction term, had significant effects on the simulated total routine metabolic rates of individuals in each habitat type (Table A.2). Total energy consumption was estimated to be highest in the ancestral nonsulfidic surface population, but significantly reduced – to varying degrees – in populations inhabiting extreme environments (Figure A.2).

Fish activity in the darkness

Analysis of activity trials revealed complex differences among habitats and between sexes (see three-way interaction term including sulfide, cave, and sex; Table A.3). The presence of H_2S was the only significant main effect. Overall, fish from extreme environment tended to exhibit higher activity rates that fish from the nonsulfidic surface population (Figure A.3). In addition, sex differences in activity were highly idiosyncratic among populations. These analyses suggest than elevated routine metabolic rates in cave fish in general – and cave males in particular – were likely not driven by higher activity rates in these fish.

Appendix figures

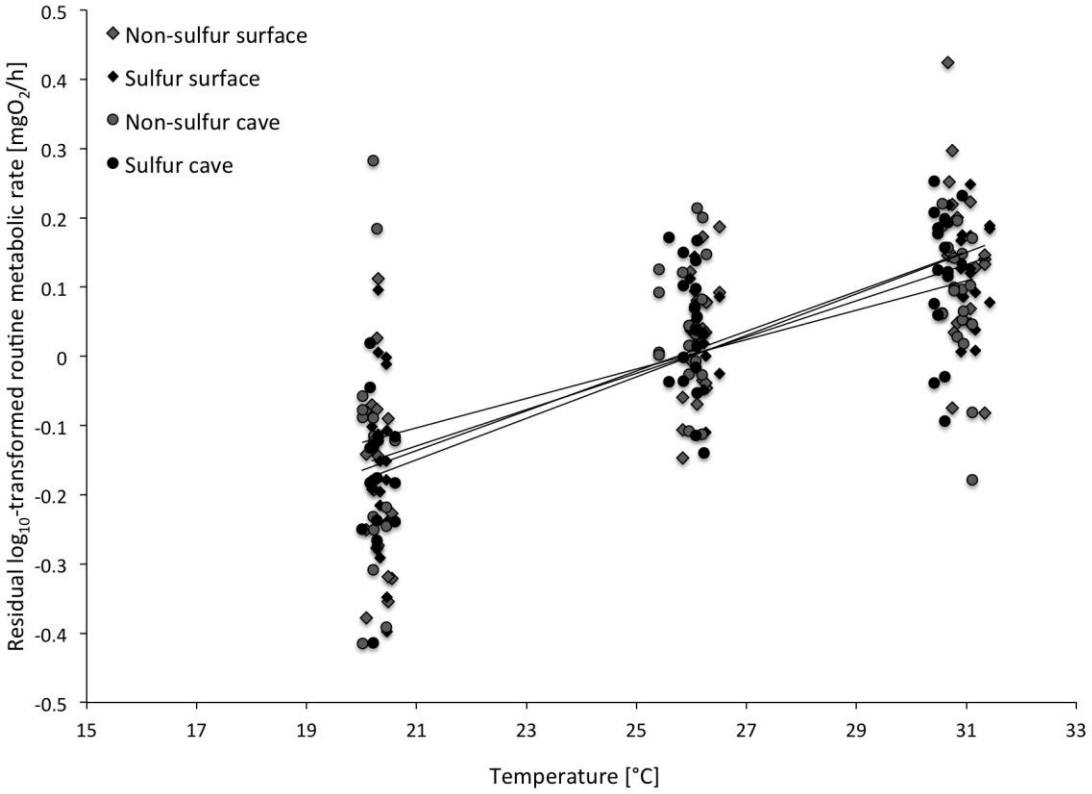

Figure A.1 Relationship between temperature and routine metabolism

Relationship between temperature and routine metabolic rate in trials with laboratory-reared fish. Depicted are residuals from the analytical model (without temperature) presented in Table A.2C for each population investigated. Overall, routine metabolic rate was significantly correlated with temperature (*post hoc* Pearson correlations: $r \geq 0.768$, $P \leq 0.001$), while the slopes did not differ significantly among populations (see main manuscript).

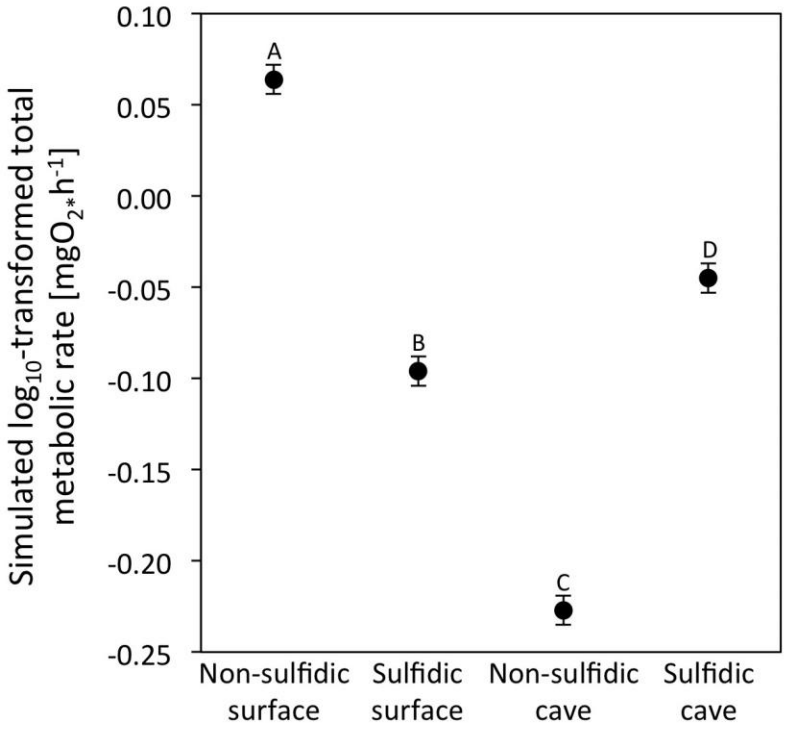

Figure A.2 Simulated total routine metabolism

Visualization of population differences in simulated total routine metabolic rates. Depicted are population-level means (± standard deviation) of total routine metabolic rates for both sexes combined. Means represent the energy demand of an average individual of each population, considering both variation in body mass and allometric metabolic rate functions. Pair-wise post-hoc tests (LSD, $P < 0.05$) revealed group differences as labeled by superscripts.

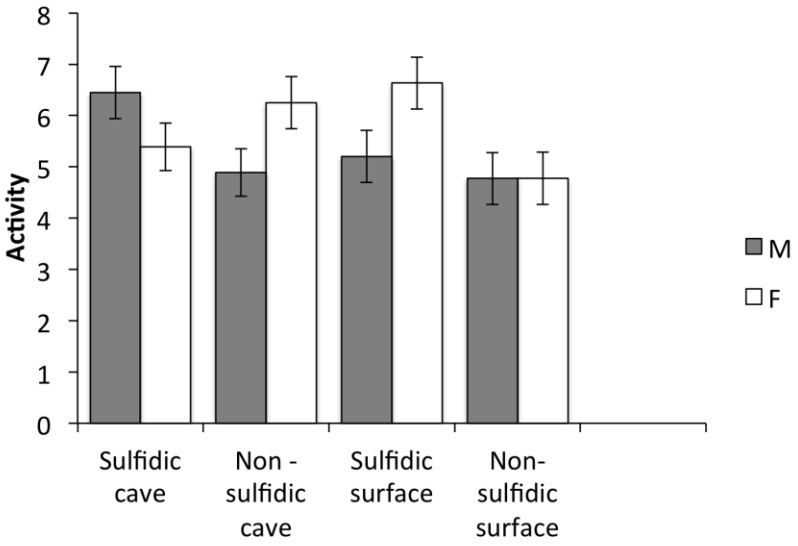

Figure A.3 Activity level of four divergent populations

Mean (±standard error) activity levels of four different *Poecilia mexicana* populations from contrasting environments. Data for males (M) are shown in grey, those for females (F) in white.

Appendix tables

Table A.1 Results of linear regression for total metabolic rate

Results of linear regressions with metabolic rate (log_{10}-transformed) as a dependent variable and body mass (log_{10}-transformed) as independent variable for each population investigated. Provides are basic statistical values (R^2, F, df, and P) as well as estimates for intercepts (a) and slopes (b). Values in brackets are the lower and upper bound limits of the 95% confidence intervals around estimates for intercepts and slopes, which were used for the modeling of total metabolic rates

	Nonsulfidic surface	**Sulfidic surface**	**Nonsulfidic cave**	**Sulfidic cave**
R^2	0.877	0.608	0.808	0.655
F	128.383	27.954	54.883	32.267
Df	1, 19	1, 19	1, 14	1, 18
P	<0.001	<0.001	<0.001	<0.001
Intercept (a)	-0.064 (-0.092, -0.035)	-0.056 (-0.086, -0.026)	-0.007 (-0.086, 0.073)	0.021 (-0.010, 0.052)
Slope (b)	0.595 (0.485, 0.706)	0.375 (0.226, 0.525)	0.524 (0.371, 0.677)	0.346 (0.218, 0.475)

Table A.2 Results of ANOVA for simulated total metabolic rate in natural conditions

ANOVA results of simulated total routine metabolic rates in idealized natural populations, considering both natural size distributions and error associated with population-specific estimates of the relationship between mass and metabolic rates.

Variable	*df*	*F*	*P*	η_p^2
Cave	1	745.387	<0.001	0.157
Sulfide	1	4.830	0.028	0.001
Cave × Sulfide	1	1481.383	<0.001	0.270

Table A.3 ANOVA for activity trials

ANOVA results from activity trials.

Source	*Df*	*F*	*P*
Corrected Model	7	2.41	0.041
Intercept	1	994.16	<0.001
Sulfide	1	4.49	0.041
Cave	1	1.28	0.265
Sex	1	1.52	0.227
Cave × Sex	1	0.65	0.426
Sulfide × Cave	1	1.29	0.264
Sulfide × Sex	1	0.50	0.483
Sulfide × Cave × Sex	1	7.49	0.010
Error	34		

Appendix A References

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Appendix B - Tissue-specific responses to toxic hydrogen sulfide and permanent darkness in livebearing fishes

Introduction

The number of shared differentially expressed genes at each level of organization may be a function of phylogenetic relatedness, as the shared responses may be higher in the sulfidic populations due to them being more closely related (Tobler et al. 2008a). To address this, using the same dataset we analyzed SNPs to look at relatedness across the cave and sulfidic populations. To gain a more comprehensive view on the demographics, we also included two more sulfidic and nonsulfidic pairs from two well-studied drainages (Pichucalco and Puyacatengo; Palacios et al. 2013) in the system.

Materials and methods

Single nucleotide polymorphism (SNP) calling

After read mapping, bam files from the same individual were combined into single bam file using the MergeSamFiles command in Picard Tools (v 1.138) (http://broadinstitute.github.io/picard/). In addition to data collected for this study, we also included sequences from Kelley et al. (2016) in the analysis to gain a comprehensive view on demographic patterns within the *Poecilia* system. With the additional data, we included two pairs of sulfidic and nonsulfidic populations from additional drainages (Pichucalco and Puyacatengo; Tobler et al. 2008a) and increased the sample size for the nonsulfidic and sulfidic surface populations from the Cueva del Azufre system.

Single nucleotide polymorphisms (SNP) were called on a per population basis using the UnifiedGenotyper tool in the Genome Analysis Toolkit (GATK) with EMIT ALL SITES (v. 3.5;

McKenna et al., 2010). Population vcf files were merged using the CombineVariants tool in GATK. The combined vcf was filtered following GATK recommended best practices (DePristo et al. 2011; Van der Auwera et al. 2013). The GATK filtered vcf was subsequently filtered using vcftools (v. 0.1.12b; Danecek et al. 2011) to include only biallelic sites that had at least 8-fold coverage per individual in 90% of the individuals. We also filtered the vcf such that no sites were within 5000 base pairs of one another. We excluded singletons for analyses that are sensitive to singletons (ADMIXTURE).

Population structure and relatedness analyses

We tested for evidence for population structure using the program ADMIXTURE (v. 1.23; Alexander et al. 2009). Vcf files (which included all populations) were converted into ped format using vcftools. We performed ten different runs for each independent value of K from 1-12 to check for convergence. We selected the best-supported K according to the cross-validation protocol implemented in ADMIXTURE.

To investigate population relatedness, we implemented TreeMix (v. 1.12; Pickrell and Pritchard 2012), which assembles a maximum likelihood bifurcating tree of population relatedness. Ped files were converted to TreeMix format using a python script that was included with the distribution of TreeMix. We rooted the tree using the sulfidic population from the Rio Pichucalco drainage lineage (*P. sulphuraria*; Pfenninger et al. 2014). The percentage of variance explained increased when we allowed one migration event (-m 1) (from 98.9% to 99.4%).

Results

Population structure and relatedness analyses

A total of 7616 SNPs passed our filters for the ADMIXTURE analyses, and 8368 SNPs passed our filters for the TreeMix analysis. In the ADMIXTURE analysis, the best-supported model for population structure was K = 4 when all populations were included (Figure B.3). Puyacatengo sulfidic and nonsulfidic populations clustered together as one population, as did the extremophile populations (sulfidic surface, nonsulfidic cave and sulfidic cave) in the Tacotalpa. The Pichucalco sulfidic population was an independent cluster, whereas the Pichucalco nonsulfidic population clustered most closely with the Tacotalpa nonsulfidic population.

In the bifurcating tree generated using TreeMix (Figure B.4), the two cave populations from the Tacotalpa appear most closely related to each other compared to the sulfidic populations from the Tacotalpa. Of the remaining populations, the nonsulfidic Tacotalpa population is most closely related to the other three populations in the Tacotalpa. Puyacatengo populations (sulfidic and nonsulfidic) were most closely related to one another. One migration event from the Pichucalco nonsulfidic population to the Tacotalpa nonsulfidic population was supported. Without migration, the tree explains 98.9% of the variance in the dataset, but with the same topology and one migration event, the tree explains 99.4% of the variance.

Appendix figures

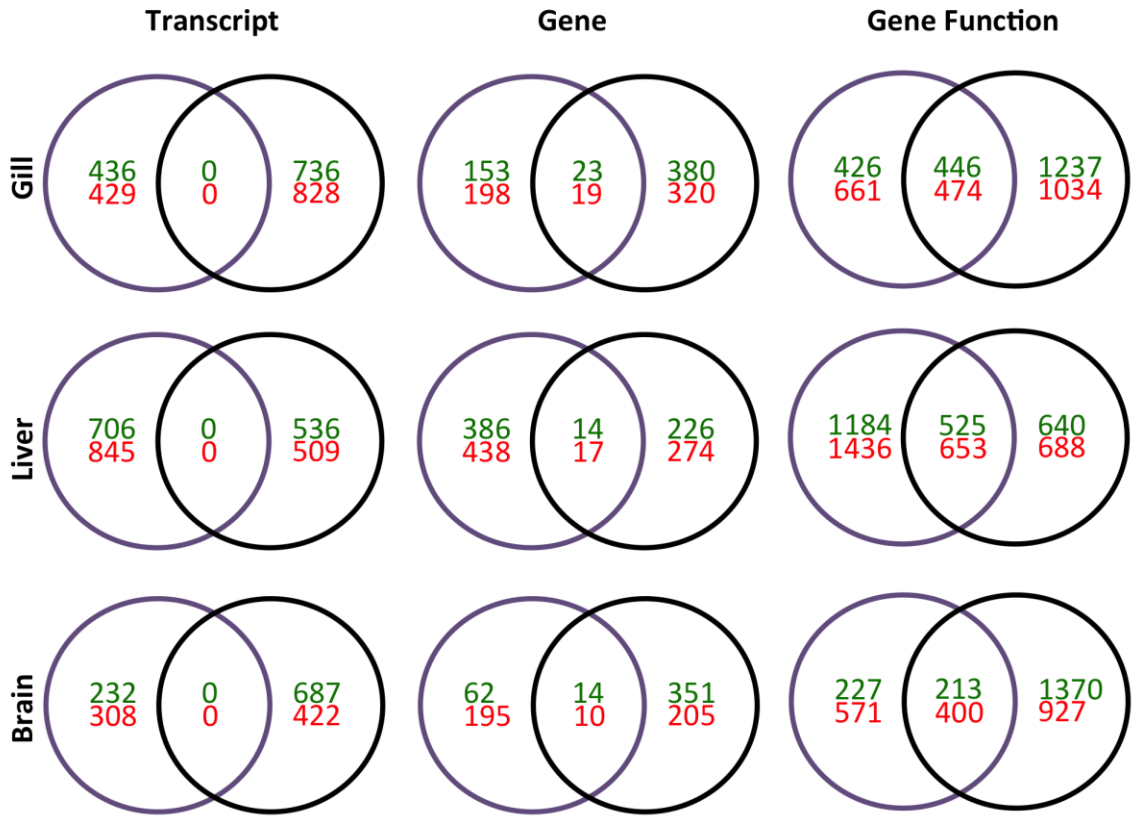

Figure B.1 Venn diagram of unique and shared expression in the cave

Venn diagrams depicting the number of unique and shared expression responses in the two cave habitats at the level of transcripts, genes, and functional annotation. These numbers were the basis for the calculation of the Jaccard index used to analyze shared responses among habitats, organs, and levels of biological organization. Purple circles are the nonsulfidic cave and black circles are the sulfidic cave population.

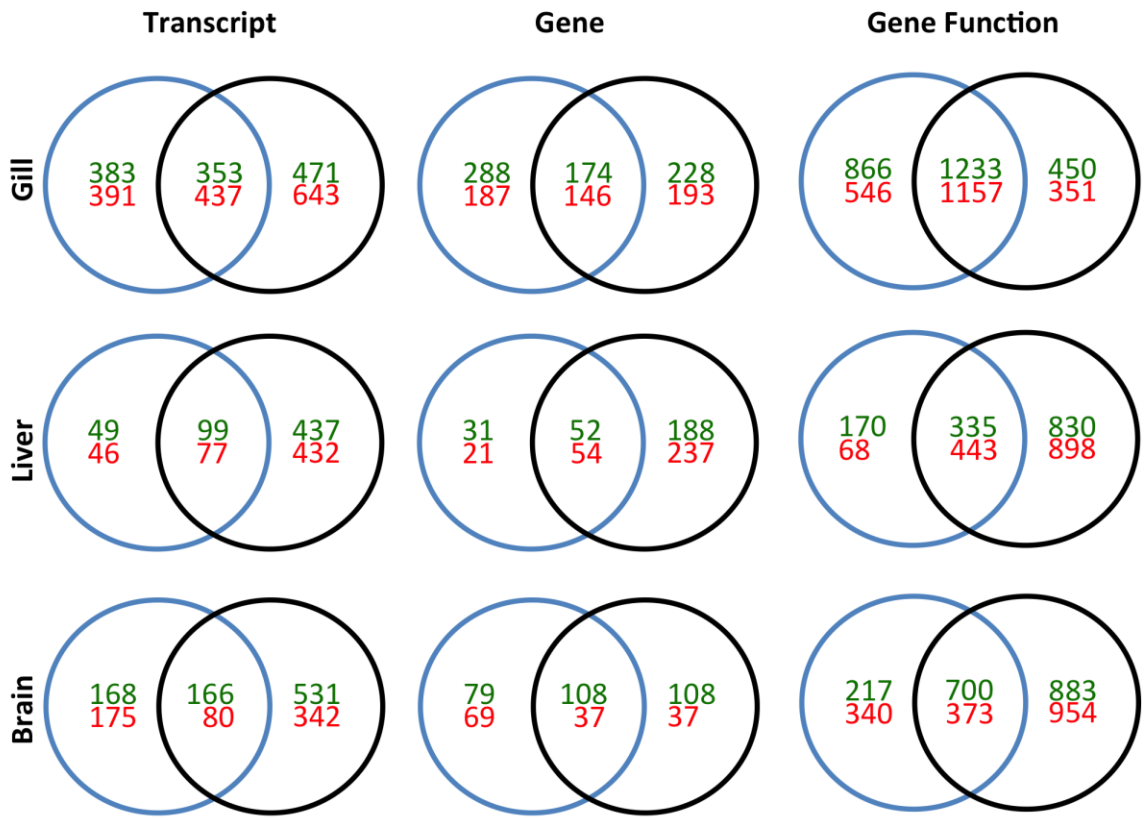

Figure B.2 Venn diagram of unique and shared expression in sulfur

Venn diagrams depicting the number of unique and shared expression responses in the two sulfidic habitats at the level of transcripts, genes, and functional annotation. These numbers were the basis for the calculation of the Jaccard index used to analyze shared responses among habitats, organs, and levels of biological organization. Blue circles are the sulfidic surface and black circles are the sulfidic cave population.

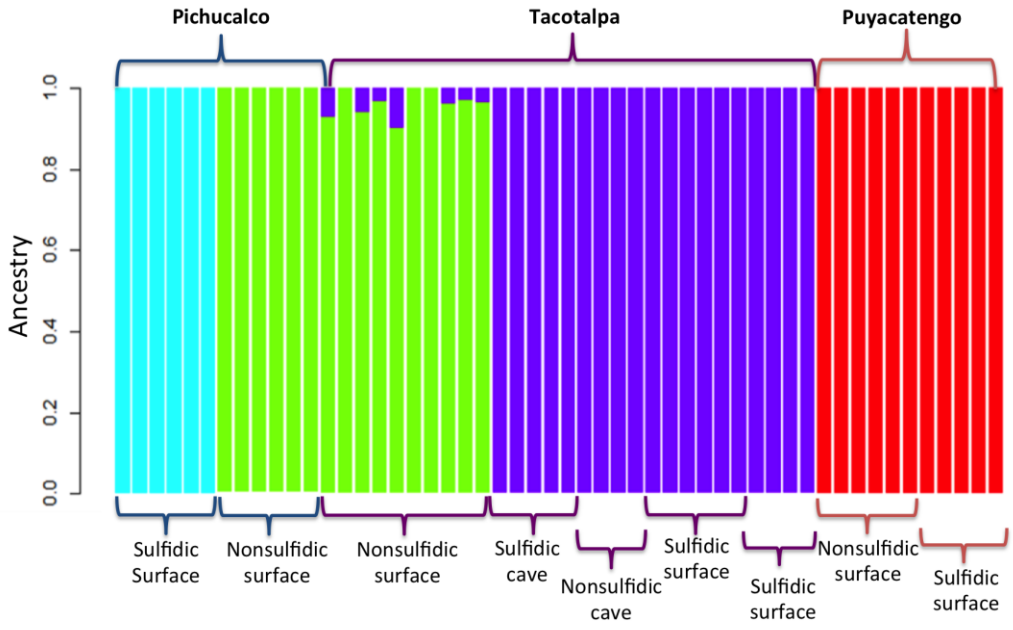

Figure B.3 Admixture plot of all three drainages

Output from the program ADMIXTURE based on the best-supported model (K = 4). Note that the focal extremophile populations in the Tacotalpa represent a single cluster (purple) without significant population structure. Populations in the Pichucalco drainage are designated in blue, populations in the Tacotalpa are designated in purple and populations in the Puyacatengo are designated in red.

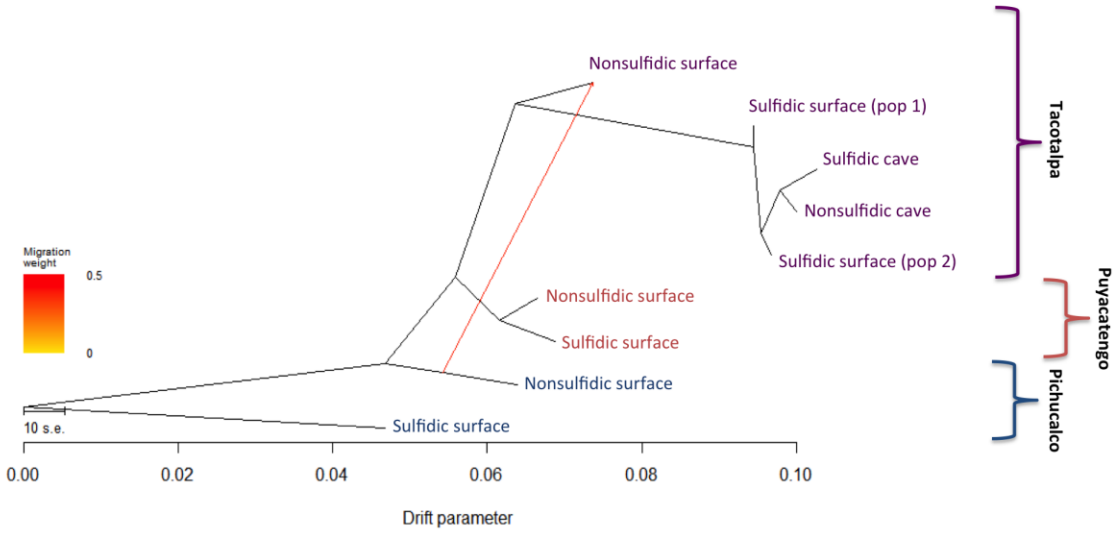

Figure B.4 Treemix plot of all three drainages

Treemix plot of the best model that includes one migration event from the nonsulfidic surface in the Pichucalco drainage, to the nonsulfidic surface in the Tacotalpa drainage. Populations in the Pichucalco drainage are designated in blue, populations in the Tacotalpa are designated in purple and populations in the Puyacatengo are designated in red.

Appendix tables

Table B.1 Basic statistics of sites and individuals used for study

Collection localities of samples used (A) in this study and (B) collected from a previously study (Kelley et al. 2016). For each site, we provided the drainage each population was collected from, GPS coordinates, average standard length (mm) and mass (g) (± standard deviation) of females used. Note, that mass data was not recorded for the individuals collected in Kelley et al. 2016 so we reported standard length only.

Site	Drainage	Coordinates	Standard length (mm)	Mass (g)
A. Populations from current study				
Nonsulfidic surface	Tacotalpa	17.427, −92.752	45.25 ± 5.50	1.93 ± 0.50
Sulfidic surface (population 2)	Tacotalpa	17.439, −92.775	32.75 ± 0.96	0.92 ± 0.11
Nonsulfidic cave	Tacotalpa	17.441, −92.773	39.00 ± 5.35	1.71 ± 0.64
Sulfidic cave	Tacotalpa	17.442, −92.775	43.25 ± 4.27	1.97 ± 0.37
B. Populations from Kelley et al. 2016				
Sulfidic surface (population 1)	Tacotalpa	17.442, −92.775	40.67 ± 4.08	
Nonsulfidic surface	Puyacatengo	17.504, −92.909	51.83 ± 5.19	
Sulfidic surface	Puyacatengo	17.464, −92.895	35.80 ± 5.72	
Nonsulfidic surface	Pichucalco	17.485, −93.104	40.33 ± 3.83	
Sulfidic surface	Pichucalco	17.552, −92.999	41.33 ± 7.89	

Table B.2 Descriptive sequencing statistics and sample size

Descriptive sequencing statistics for each population and organ before and after trimming. All numbers represent means (± standard deviation).

Samples	Sample Size	Raw read counts	Total read counts first trim	Average read counts second trim
Nonsulfidic surface gill	4	51,211,008 ± 3,066,659	50,494,010 ± 3,062,933	46,236,760 ± 2,694,877
Nonsulfidic surface liver	4	43,796,910 ± 1,468,678	40,962,508 ± 1,096,851	37,645,326 ± 1,019,676
Nonsulfidic surface brain	4	36,697,772 ± 3,592,346	35,880,998 ± 3,458,547	32,552,250 ± 2,950,330
Sulfidic surface Gill	3	29,414,736 ± 3,279,715	28,104,778 ± 3,172,097	25,884,956 ± 3,027,642
Sulfidic surface Liver	4	42,558,146 ± 4,628,663	41,840,232 ± 4,532,005	37,832,356 ± 3,769,800
Sulfidic surface Brain	2	15,295,696 ± 142,168	14,998,258 ± 135,061	13,544,718 ± 318,171
Nonsulfidic cave Gill	3	29,879,398 ± 947,819	29,344,370 ± 892,075	26,783,182 ± 487,324
Nonsulfidic cave Liver	4	33,927,432 ± 2,481,755	31,915,320 ± 2,427,735	29,008,504 ± 2,190,784
Nonsulfidic cave Brain	2	19,994,198 ± 5,192,999	18,891,230 ± 4,895,510	17,487,216 ± 4,497,547
Sulfidic cave Gill	4	39,945,364 ± 3,928,399	38,620,432 ± 3,981,537	34,880,386 ± 3,299,396
Sulfidic cave Liver	4	38,152,448 ± 2,765,622	35,404,608 ± 1,955,737	32,293,256 ± 1,633,588
Sulfidic cave Brain	3	35,553,494 ± 5,145,134	34,852,068 ± 5,095,666	31,790,312 ± 4,734,325

Table B.3 Transcript annotation results

Annotation results of the 48,601 transcripts with hits in SwissProt. For each transcript (specified by TCONS number), the table includes information on the sequence ID from SwissProt (including the accession number), percent of identical matches, alignment length, number of mismatches, number of gap openings, start and end of the alignment query, start and end of the alignment subject, E-value, and the bit score of the top BLAST hit for each transcript. Due to the large size, the table is provide in a separate excel spreadsheet (“Table BC.3 – Reference Transcriptome Annotations.xlsx”**)**

Table B.4 Gene ontology (GO) enrichment analysis

Results of the enrichment analysis of Gene Ontology (GO) terms of differentially transcripts that were shared between environments (sulfidic and cave) based on organ (gill, liver and brain). Provided are the GO term identifications, description of the enriched GO term, and gene names within each GO category obtained from the Gorilla enrichment analysis. Here we report the GO terms for differentially expressed genes that were (A) upregulated in the sulfur populations (B) downregulated in the sulfur populations (C) upregulated in the cave environments and (D) downregulated in the cave environments. Note, that we only report shared responses with terms associated with biological processes.

GO Identification	Description	Gene names
A. Sulfur upregulated		
Gill		
GO:0044272	Sulfur compound biosynthetic process	MLYCD, ACOT13, ACSS2, ACOT4, GSTM3, GSS, CDO1, ELOVL4, SLC25A1, GCLM, CHAC2, OGN, GSTZ1, ELOVL7, GCLC, MPST, MGST1, NCAN, ACSL1, SLC26A2, B3GNT7, GGT1, AKR1A1, MPC1, GSTO1, ELOVL6, CHAC1, GGT5
GO:0019752	Carboxylic acid metabolic process	PRKAG2, PRKAB1, TPI1, IL4I1, GADL1, SLC7A8, CNDP2, PSAT1, PGD, LPIN1, GLO1, BAAT, FABP6, ABCB11, CYP2J2, GSS, CDO1, IVD, SQRDL, PDK3, GSTZ1, GCLC, ELOVL7, RGN, PCK1, HNMT, ELOVL5, ABHD10, P4HB, GGH, PTGR1, SLC1A3, PLA2G10, ETHE1, PFKL, SRD5A2, SLC22A4, CCBL2, FADS2, CKB, MPST, NCAN, CRAT, SLC25A10, IDNK, HSD17B8, MLYCD, PGK1, PRKAB1, GADL1, PSAT1, PGD, ABCB11, GSS, MCEE, GCSH, GSTZ1, ELOVL7, ACADL, ACADS, ABHD10, GGH, P4HA1, PADI2, PARS2
GO:0016051	Carbohydrate biosynthetic process	PGK1, PCK2, PRKACB, PCK1, TPI1, AKR1B1, PGD, SLC25A1, PPP1R3C, GYS1, TALDO1, SLC25A10, G6PD, NR1D1, B3GNT3, B3GNT2, FBP1, RGN
GO:0044281	Small molecule metabolic process	ACOT13, GADL1, GSTM3, GSR, COX5B, HSD11B1, APOB, APOA4, GSS, LPCAT4, COX15, GSTZ1, ELOVL7, SLC16A9, LIPH, CYCS, CERS6, CD36, NT5C2, P4HB, B3GNT3, GGH, CA5B, PTGR1, ECSIT, TTPA, GMPR2, CRAT, AQP1, CMBL, PRKAG2, TPI1, ADCK3, GLO1, CYP2J2, CDO1, CA4, SQRDL,

		CA2, PDK3, MAN2B2, GCLC, PCK2, PCK1, SLC25A1, SLC22A4, FBP1, MGST1, IDNK, MTTP, PRKAB1, SLC9A1, FABP2, SLC7A8, CNDP2, PSAT1, PGD, FABP6, ABCB11, PNP, CHIT1, ABCB1, LDLRAP1, UPP1, RGN, ELOVL5, ABHD10, SLC1A3, PLA2G10, ETHE1, ALDH3B1, PFKL, SLC4A1, RDH11, PCSK9, SLC2A4, ALAS2, CFTR, SLC5A1, G6PD, ACER1, IL4I1, AKR1B1, B3GNT2, LPIN1, BAAT, IVD, TXN, HNMT, ERVK-6, UCP2, SRD5A2, CCBL2, CHAC2, MPP1, CKB, HMOX2, MPST, NCAN, SLC25A10, GLTPD1, COX6B1, COX4I1, GCSH, PC, ACADL, ACADS, P4HA1, PADI2, GYS1, OGN, COQ6, BLVRB, FGF7, GCLM, MMADHC, TALDO1, SMS, ACOT4, ACSS2, FTCD, PPAP2B, KEAP1, PARS2, PDSS2, HSD17B8, MLYCD, PGK1, PRKACB, RFK, MCEE, SURF1, CHIA, PYGM, ELOVL4, PPP1R3C, AKR1B10, GPX1, NARS2, ABHD14B, GBAS, RDH14, HS3ST3A1, CYP26C1
GO:1901700	Response to oxygen-containing compound	TIE1, PRKACB, CAPN2, GSR, SLC8A1, APLP1, SLC14A2, CXCL12, GSS, GCNT1, SPARC, IRG1, WT1, MMP2, CYCS, CD38, GGH, CLDN4, GJA3, GJB2, WNT5A, NLRP1, PYGM, TRIM16, FKBP1B, PRDX1, SLC2A4, PCSK9, PDXP, CXCL6, G6PD, CFTR, AQP1, NR1D1, PPARGC1B, AKR1B1, CMA1, ABCC2, LITAF, GPX1, PDGFRB, PLAU, CDO1, RARG, WFDC1, SLC26A5, PDK3, GRN, PPBP, TXN, DMTN, TGFB3, PCK1, TSPO, SORT1, UCP2, TGFBR2, KLF4, YES1, CXCL2, RAMP3, CCRN4L, THBD, SPON2, MGST1, GJD3, BNIP3, PRDX6, SLC9A1, APOB, APOA4, SLC26A3, P2RY6, CD36, P4HB, PFKL, KLF15, LPIN1, SLC22A6, MDM2, LYN, KRT8, EIF2AK2, HNMT, CDKN1A, HAVCR2, SRD5A2, ILDR2, IRF5
GO:0044283	Small molecule biosynthetic process	PRKAG2, PHOSPHO1, PRKAB1, ACER1, TPI1, GADL1, ADCK3, AKR1B1, LPIN1, PSAT1, PGD, BAAT, ABCB11, APOA4, CDO1, PNP, ELOVL7, RGN, PCK2, PCK1, ELOVL5, SLC1A3, SLC25A1, FADS2, FBP1, NCAN, SLC25A10, CFTR, G6PD, HSD17B8, PDSS2, MLYCD, PRKACB, RFK, FGF7, PC, TALDO1, ACSS2, PADI2, ELOVL4, COQ6

GO:1901564	Organonitrogen compound metabolic process	MLYCD, GLTPD1, PGK1, GADL1, GSTM3, RFK, GSR, PSAT1, PGD, GSS, LPCAT4, MCEE, MRPS18A, SURF1, GCSH, PC, COX15, GSTZ1, ACADL, TUFM, NT5C2, CTSZ, P4HA1, GGH, CHIA, SLC1A3, PADI2, PLA2G10, ETHE1, GMPR2, ALDH3B1, PFKL, ABCB6, PPP2CA, OGN, BLVRB, ALAS2, PDXP, CRAT, G6PD, AQP1, SLC25A35, MRPS16, ACER1, TPI1, IL4I1, AKR1B1, CMA1, PDGFRB, GPX1, NARS2, GLO1, FGF7, CDO1, IVD, SQRDL, GCLM, MRPS15, MMADHC, TALDO1, SLC25A51, ABHD14B, MRPL38, GCLC, SMS, GBAS, TSFM, TSPO, MRPL15, FTCD, HS3ST3A1, MRPS17, UCP2, SLC25A1, CCBL2, SCG5, CHAC2, PPAP2B, CKB, HMOX2, MPST, NCAN, SLC25A10, PARS2, MGST1, SLC7A8, SLC9A1, CNDP2, APOA4, PNP, CHIT1, SLC16A9, UPP1, CERS6, B3GNT3, P4HB, GFM1, NACA, PRKAG2, SLC25A38, PHOSPHO1, LPIN1, B3GNT2, BAAT, AMBP, ITIH3, EIF2AK2, HNMT, ERVK-6, SLC22A4, MPP1, ANPEP
GO:0009725	Response to hormone	PPARGC1B, GSTM3, AKR1B1, ABCC2, SLC9A1, LPIN1, CXCL12, APOB, MDM2, CDO1, CA2, SLC26A5, LYN, GRN, GCLC, TRIM25, CTSL, SLC34A2, HNMT, PCK1, P2RY6, GGH, CLDN4, CDKN1A, GJB2, UCP2, TGFBR2, RAMP3, ANXA2, SRD5A2, TRIM16, PCSK9, SLC2A4, LMO2, AQP1, PAQR8, NR1D1, PRKACB, PDGFRB, GCNT1, WFDC1, SPARC, ACADS, IRG1, WT1, LOX, S100B, CD38, TGFB3, TSPO, SORT1, MMP14, WNT5A, PAQR6, SMAD6, KEAP1
GO:0042592	Homeostatic process	PRKACB, GSR, SFXN5, FBXW7, SLC35G1, SLC8A1, CXCL12, TRPC4, ARRDC3, UMOD, TRPM2, SLC9A2, FTH1, ACADL, LDLRAP1, ACADS, FOXO3, PARP3, ATP6V1E1, CTSK, CD38, CXCR3, CLDN4, PLA2G10, GRHL3, PYGM, ABCB6, SLC4A1, FKBP1B, SLC26A11, PRDX1, PCSK9, SLC2A4, ALAS2, RAC1, CFTR, AQP1, NR1D1, AKR1B1, CMA1, ABCC2, GPX1, FGF7, PLAU, TMEM79, IVD, SCO1, CA2, PDK3, SLC26A5, GCLC, TXN, CYBRD1, GRIK2, PCK1, FADD, UCP2, MAFG, TXNRD3, EPOR, ABCA12, CKB, HMOX2, NR1D2, TRPV4, CNGB1, ALOXE3, GPR116, FN1, SLC9A3R1, ATP2A2, CRY1, WNK4, IRS1,

		ATP1A1, STAT5B, FGF23, CCR1, ATP1B1, LDLR, FLVCR1, SLC9A3, ATM, SSTR5, ITPKB, KCNMA1, KRT16, CHP1, MUC2, ANG, NGFR, CCDC109B, ATP6AP1, INSR, RHCG, SLC9A6, SOX4, SLC22A5, KCNJ2, SLC26A2, ERP44, HFE, TNFSF11, ATP2B3, PIGR
GO:0015701	Bicarbonate transport	CA4, SLC4A10, SLC4A1, CA2, SLC26A5, SLC26A11, SLC26A3, CA5B, CFTR, AQP1
GO:0048878	Chemical homeostasis	TRPV4, GPR116, ALOXE3, PVALB, MICU1, FN1, SLC9A3R1, ATP2A2, XPR1, CRY1, WNK4, IRS1, ATP1B1, LDLR, UMOD, TRPM2, KCNMA1, NGFR, ATP6AP1, INSR, ADCY9, CCL14, CLDN4, SLC39A6, KCNJ2, ATP6V1C1, PTPN11, SLC4A1, TRPV1, HFE, SLC25A23, RAB38, GPER1, ATP2B3, PTPRC, SLC4A10, CMA1, SLC7A8, ABCC2, SLC9A1, SLC35G1, CXCL12, APOB, APOA4, PLAU, TMEM79, TRPC4, IVD, CA2, SLC26A5, PDK3, SLC9A2, FTH1, SLC26A3, LDLRAP1, FOXO3, RGN, CYBRD1, ATP6V1E1, SLC34A2, PCK1, CD36, PLA2G10, UCP2, GRHL3, TTPA, MAFG, ABCA12, SLC31A1, FKBP1B, SLC26A11, SLC2A4, CLDN1, PCSK9, ALAS2, CKB, HMOX2, NR1D2, CFTR, AQP1
GO:0046364	Monosaccharide biosynthetic process	SLC25A1, PCK2, PCK1, TPI1, FBP1, AKR1B1, SLC25A10, PGD, G6PD, RGN, PGK1, PRKACB, PC, TALDO1
GO:0055114	Oxidation-reduction process	HSD17B8, PGK1, COX6B1, PRKACB, GSR, COX4I1, COX5B, PGD, MICAL1, BLOC1S2, MCEE, SURF1, COX15, FTH1, ACADL, ACADS, LOX, CYCS, ALKBH1, P4HA1, PIR, ETHE1, PYGM, GMPR2, ALDH3B1, PFKL, PPP1R3C, BMP2, GYS1, RDH11, AKR1B10, PRDX1, COQ6, BLVRB, CRAT, LEPREL1, PPP1R2, G6PD, NR1D1, PMPCB, TPI1, IL4I1, AKR1B1, GPX1, EPX, CDO1, IVD, PXDN, SQRDL, RFESD, TALDO1, SDHAF2, TXN, CYBRD1, SDR42E2, ACSS2, RDH14, SDR39U1, UCP2, CYP26C1, TXNRD3, RDH16, DUS3L, HMOX2, MTO1, SLC25A10, MGST1, DHRS13, PRKAG2, PRDX6, CYP2J2, HEPHL1, ALKBH3, PTGR1, ECSIT, PCDH12, SRD5A2, FADS2
GO:0006749	Glutathione metabolic process	CHAC2, GSTM3, GSR, GSTZ1, GCLC, CNDP2, G6PD, MGST1, GLO1, ETHE1, GSS, GCLM, GPX1

GO:0042221	Response to chemical	IL18, HAT1, GSTM3, GSR, COX4I1, APLP1, SLC14A2, CXCL12, CD274, GSS, GCNT1, SPARC, ACADS, FOXO3, IRG1, MMP2, LOX, CYCS, CD38, GGH, CLDN4, MMP14, GJA3, GJB2, BMP2, TRIM16, PRDX1, FKBP1B, LMO2, CXCL6, AQP1, NR1D1, PPARGC1B, LITAF, ABCC2, PDGFRB, CDO1, WFDC1, CA2, PDK3, GCLM, MEFV, PPBP, GCLC, CYBRD1, S100B, ADD3, PCK1, TSPO, SLC7A11, C1QA, SOX6, DNAJC4, SNAI2, RAMP3, PAQR6, KEAP1, MGST1, PAQR8, TIE1, PRKACB, CAPN2, SLC8A1, UMOD, WT1, CTSL, HLA-DPA1, SLC1A3, WNT5A, PYGM, NLRP1, ALDH3B1, PFKL, CLEC3B, PPP2CA, PCSK9, SLC2A4, PDXP, CFTR, G6PD, RALB, ACER1, CMA1, AKR1B1, TNMD, GPX1, RARG, PLAU, SLC26A5, NDRG1, GRN, CLU, RAP2A, TXN, TMEM100, DMTN, TGFB3, SERPINH1, SORT1, TRIM21, CHP2, UCP2, TGFBR2, KLF4, YES1, CXCL2, ANXA2, CCRN4L, FAM162A, THBD, SPON2, SMAD6, MPST, GJD3, GAS1, PRDX6, BNIP3, SLC7A8, SLC9A1, APOB, APOA4, XAF1, PNP, ABCB1, SLC26A3, TRIM25, CD36, P2RY6, P4HB, PTGR1, TTPA, KLF15, LPIN1, SLC22A6, MDM2, LYN, KRT8, EIF2AK2, SLC34A2, HNMT, CDKN1A, HAVCR2, SLC31A1, SRD5A2, ILDR2, IRF5, FBP1
GO:0006790	Sulfur compound metabolic process	MLYCD, ACOT13, GSTM3, GSR, GPX1, MICAL1, GLO1, GSS, MCEE, CDO1, SQRDL, GCLM, PC, ABHD14B, GSTZ1, GCLC, ELOVL7, TXN, SMS, ACOT4, ACSS2, ETHE1, ELOVL4, SLC25A1, CHAC2, OGN, MPST, NCAN, KEAP1, SLC25A10, MGST1, G6PD, ELOVL5, B3GNT3, CNDP2, B3GNT2, BAAT
GO:0006979	Response to oxidative stress	PPARGC1B, PRDX6, BNIP3, GSR, AKR1B1, ABCC2, MDM2, GSS, APOA4, PXDN, SEPP1, GCLC, FOXO3, TXN, CYCS, SLC7A11, CD36, P4HB, GJA3, GJB2, UCP2, KLF4, ALDH3B1, FKBP1B, PRDX1, MGST1, G6PD, AQP1, GPX1, SLC8A1, PDGFRB, EPX, GCLM, TRPM2, CD38, MMP14
GO:0009719	Response to endogenous stimulus	PRKACB, CAPN2, GSTM3, SLC8A1, APLP1, CXCL12, GSS, GCNT1, SPARC, ACADS, IRG1, WT1, MMP2, LOX, CTSL, CD38, GGH, CLDN4, MMP14, GJB2, WNT5A, NLRP1, PYGM, CLEC3B, BMP2, TRIM16, SLC2A4, PCSK9, LMO2, PDXP, CFTR, AQP1, NR1D1, PPARGC1B, AKR1B1, ABCC2, PDGFRB, TNMD, PLAU, CDO1, WFDC1, CA2, SLC26A5, GRN, GCLC, DMTN, TMEM100, S100B,

		TGFB3, PCK1, TSPO, SORT1, SLC7A11, SOX6, UCP2, TGFBR2, KLF4, SNAI2, YES1, ANXA2, RAMP3, PAQR6, THBD, SMAD6, KEAP1, MGST1, PAQR8, SLC9A1, APOB, SLC26A3, TRIM25, CD36, P2RY6, KLF15, LPIN1, SLC22A6, MDM2, LYN, SLC34A2, HNMT, CDKN1A, SRD5A2, IRF5
GO:0006575	Cellular modified amino acid metabolic process	GSTM3, FTCD, GSR, P4HA1, GGH, GPX1, PLA2G10, PADI2, GLO1, ETHE1, GSS, LPCAT4, GCLM, CCBL2, CHAC2, CKB, GSTZ1, GCLC, MPST, MGST1, G6PD, P4HB, CNDP2
GO:0010033	Response to organic substance	IL18, CAPN2, GSTM3, SLC9A1, CXCL12, CD274, APOB, APOA4, GSS, XAF1, UMOD, SLC26A3, TRIM25, CTSL, P2RY6, CD36, GGH, CLDN4, GJB2, NLRP1, PFKL, CLEC3B, TRIM16, FKBP1B, SLC2A4, PCSK9, LMO2, G6PD, CFTR, AQP1, NR1D1, PPARGC1B, KLF15, AKR1B1, CMA1, LITAF, ABCC2, LPIN1, SLC22A6, MDM2, PLAU, CDO1, CA2, LYN, SLC26A5, PDK3, GRN, CLU, MEFV, GCLC, EIF2AK2, TMEM100, SLC34A2, PCK1, HNMT, SERPINH1, SLC7A11, CDKN1A, UCP2, TGFBR2, KLF4, HAVCR2, CXCL2, SRD5A2, RAMP3, ANXA2, ILDR2, CCRN4L, IRF5, MGST1, PAQR8, TIE1, PRKACB, SLC8A1, APLP1, GCNT1, SPARC, ACADS, IRG1, WT1, MMP2, LOX, CD38, HLA-DPA1, MMP14, WNT5A, PYGM, BMP2, PPP2CA, PDXP, CXCL6, RALB, TNMD, PDGFRB, RARG, WFDC1, PPBP, DMTN, S100B, TGFB3, TSPO, SORT1, TRIM21, SOX6, DNAJC4, SNAI2, YES1, PAQR6, THBD, SPON2, SMAD6, KEAP1, GAS1, GJD3
Liver		
N/A	N/A	N/A
Brain		
GO:0006096	Glycolytic process	GAPDH, LDHA, PGM1, GPI, PFKFB1, PFKM, ALDOA, PGAM2
GO:0016051	Carbohydrate biosynthetic process	PCK1, PGM1, B3GNT3, AKR1B1, AKR1A1, ALDOA, PGAM2, UBB, AGL, MPDU1, CHST8, GAPDH, GPI, GPD1, PFKFB1, NR1D1
GO:0006757	ATP generation from ADP	GAPDH, LDHA, PGM1, GPI, PFKFB1, PFKM, ALDOA, PGAM2
B. Sulfur downregulated		

Gill		
GO:0055085	Transmembrane transport	TRPV4, MAL, SLC6A6, ATP2A2, NDUFA4, SLC6A13, ATP1B1, AHCYL1, TRPM2, SLC25A15, SLC4A4, KCNMA1, SLC12A3, ATP6AP1, SLC5A7, SLC44A4, TRPM5, ATP6V1C1, SLC3A2, CYB561, TRPV1, SLC7A5, SLC4A1, SLC25A23, ATP2B3, SLC38A2, ATP8B1, PRKAG2, CNGB1, MICU1, PKD1L2, CALHM3, SLC25A25, SLC38A4, GABRD, SLC9B2, SLC25A22, SLC38A3, CLCN2, ADCY9, KCNJ15, SLC30A3, RTN2, SLC30A2, TRPM4, KCNJ2, SLC39A6, KCNJ1, SLC25A5, PTPRC, SLC25A12, SLC7A3, HSPA8, AFG3L2, PRF1, ATP1A1, SLC9A3, SLC9A2, FXYD1, CPT1A, CPT1B, CCDC109B, NIPAL4, RHCG, SLC9A6, SLC26A2, TSC22D3, ABCB10, FLVCR1, MPC1, SLC22A5, CLCN1, SLC22A4
GO:0034220	Ion transmembrane transport	PRKAG2, TRPV4, CNGB1, SLC6A6, ATP2A2, PKD1L2, NDUFA4, ATP1A1, ATP1B1, SLC38A4, TRPM2, SLC9A3, GABRD, SLC9A2, SLC4A4, MPC1, SLC25A22, KCNMA1, SLC12A3, SLC38A3, FXYD1, CLCN2, CPT1A, CPT1B, CCDC109B, ATP6AP1, KCNJ15, RHCG, SLC9A6, SLC22A5, KCNJ2, CLCN1, SLC3A2, SLC4A1, SLC26A2, SLC22A4, TSC22D3, ATP2B3, SLC25A12, ATP8B1, MICU1, CALHM3, SLC25A25, SLC9B2, SLC25A15, SLC30A3, SLC30A2, TRPM4, SLC39A6, TRPM5, ATP6V1C1, TRPV1, SLC7A5, KCNJ1, PTPRC, SLC7A3, SLC38A2
GO:0030001	Metal ion transport	TRPV4, CNGB1, MICU1, SLC9A3R1, ATP2A2, PKD1L2, WNK4, SLC25A25, ATP1B1, SLC38A4, TRPM2, SLC9B2, SLC4A4, KCNMA1, SLC12A3, SLC38A3, ATP6AP1, SLC5A7, KCNJ15, DNM2, SLC30A3, SLC30A2, TRPM4, SLC39A6, KCNJ2, ATP6V1C1, TRPM5, SLC3A2, TRPV1, HFE, KCNJ1, ATP2B3, PTPRC, SLC38A2, ATP1A1, CCR1, SLC9A3, SLC9A2, CHP1, CCDC109B, NIPAL4, SLC9A6, SLC22A5, SLC22A4
GO:0006812	Cation transport	PRKAG2, TRPV4, CNGB1, SLC9A3R1, ATP2A2, PKD1L2, WNK4, NDUFA4, ATP1A1, CCR1, ATP1B1, SLC38A4, TRPM2, SLC9A3, SLC9A2, SLC4A4, KCNMA1, SLC12A3, CHP1, SLC38A3, CPT1A, CPT1B, CCDC109B, ATP6AP1, SLC5A7, KCNJ15, NIPAL4, RHCG, SLC9A6, SLC22A5, KCNJ2, SLC44A4, SLC3A2, SLC22A4, HFE, ATP2B3, MICU1, SEC14L1, CALHM3, SLC25A25,

		SLC9B2, SLC25A15, DNM2, SLC30A3, SLC30A2, TRPM4, SLC39A6, TRPM5, ATP6V1C1, TRPV1, KCNJ1, PTPRC, SLC7A3, SLC38A2
GO:0098655	Cation transmembrane transport	PRKAG2, TRPV4, CNGB1, MICU1, ATP2A2, PKD1L2, CALHM3, NDUFA4, SLC25A25, ATP1B1, TRPM2, SLC9B2, SLC25A15, SLC4A4, KCNMA1, SLC12A3, SLC38A3, ATP6AP1, KCNJ15, SLC30A3, SLC30A2, TRPM4, SLC39A6, KCNJ2, TRPM5, ATP6V1C1, SLC3A2, TRPV1, KCNJ1, ATP2B3, PTPRC, SLC7A3, ATP1A1, SLC9A3, SLC9A2, CPT1A, CPT1B, CCDC109B, RHCG, SLC9A6, SLC22A5, SLC22A4
GO:0006814	Sodium ion transport	PRKAG2, TRPV4, CNGB1, ATP2A2, PKD1L2, NDUFA4, ATP1A1, ATP1B1, SLC9A3, TRPM2, SLC9A2, SLC4A4, KCNMA1, SLC12A3, SLC38A3, CPT1A, CPT1B, ATP6AP1, CCDC109B, KCNJ15, RHCG, SLC9A6, SLC22A5, KCNJ2, SLC3A2, SLC22A4, ATP2B3, MICU1, CALHM3, SLC25A25, SLC9B2, SLC25A15, SLC30A3, SLC30A2, TRPM4, SLC39A6, TRPM5, ATP6V1C1, TRPV1, KCNJ1, PTPRC, SLC7A3
GO:0006811	Ion transport	TRPV4, SEC14L1, SLC6A6, ATP2A2, WNK4, NDUFA4, SLC6A13, ATP1B1, LDLR, TRPM2, SLC25A15, SLC4A4, KCNMA1, SLC12A3, ATP6AP1, SLC5A7, SLC44A4, TRPM5, ATP6V1C1, SLC3A2, TRPV1, SLC7A5, SLC4A1, HFE, ATP2B3, SLC38A2, ATP8B1, PRKAG2, ATP8B4, CNGB1, MICU1, SLC9A3R1, PKD1L2, CALHM3, SLC25A25, SLC38A4, GABRD, SLC9B2, SLC25A22, SLC38A3, CLCN2, KCNJ15, DNM2, SLC30A3, SLC30A2, PITPNC1, TRPM4, SLC39A6, KCNJ2, KCNJ1, PTPRC, SLC25A12, SLC7A3, ATP1A1, ACSL1, SLC9A3, SLC9A2, CHP1, FXYD1, CPT1A, CPT1B, CCDC109B, NIPAL4, RHCG, SLC9A6, SLC26A2, TSC22D3, CCR1, MPC1, SLC22A5, CLCN1, SLC22A4
GO:0009605	Response to external stimulus	MR1, AFG3L2, RALB, CNGB1, TRPV4, FN1, RNF152, PKD1L2, CRY1, PRF1, LITAF, ATP1A1, STAT5B, FGF23, HLA-DRB1, ACSL1, LDLR, MAPT, ARG1, CD4, SLC38A3, ANG, BHLHE40, NGFR, STRC, INSR, RHCG, DNMT3A, DNMT3B, KCNJ2, IFITM2, DEPDC5, DMBT1, FOS, MAG, MST1R, HFE, RDH5, MRC1, VCAM1, CYP1A1, PDE4D, IFI44, KIT, RBM4B, HSPA8, SBNO2, DDX3X,

		SIK1, CD63, BAIAP2, IGHV3-23, USP2, NLRP1, MOG, SLC38A2, MUC5B, CRP, PROX1, ALOX5, XPR1, SLC25A25, MX1, IGLC6, ALPL, SIPA1, ASNS, PTPRC
GO:0098662	Inorganic cation transmembrane transport	PRKAG2, TRPV4, CNGB1, MICU1, ATP2A2, PKD1L2, NDUFA4, ATP1B1, SLC25A25, TRPM2, SLC9B2, SLC4A4, KCNMA1, SLC12A3, ATP6AP1, KCNJ15, SLC30A3, SLC30A2, KCNJ2, SLC39A6, TRPM4, ATP6V1C1, TRPM5, SLC3A2, TRPV1, KCNJ1, ATP2B3, PTPRC, ATP1A1, SLC9A3, SLC9A2, CPT1A, CPT1B, CCDC109B, RHCG, SLC22A5, SLC9A6, SLC22A4
GO:0098660	Inorganic ion transmembrane transport	PRKAG2, TRPV4, CNGB1, ATP2A2, PKD1L2, NDUFA4, ATP1A1, ATP1B1, TRPM2, SLC9A3, GABRD, SLC9A2, SLC4A4, KCNMA1, SLC12A3, FXYD1, CLCN2, CPT1A, CPT1B, CCDC109B, ATP6AP1, KCNJ15, RHCG, SLC9A6, SLC22A5, KCNJ2, CLCN1, SLC3A2, SLC4A1, SLC26A2, SLC22A4, ATP2B3, MICU1, SLC25A25, SLC9B2, SLC30A3, SLC30A2, SLC39A6, TRPM4, ATP6V1C1, TRPM5, TRPV1, KCNJ1, PTPRC
GO:0015672	Monovalent inorganic cation transport	PRKAG2, SLC38A3, CNGB1, ATP6AP1, SEC14L1, SLC5A7, SLC9A3R1, KCNJ15, WNK4, NDUFA4, KCNJ2, SLC44A4, ATP6V1C1, ATP1B1, TRPM5, SLC3A2, SLC38A4, KCNJ1, TRPM2, SLC9B2, SLC4A4, KCNMA1, SLC12A3, SLC38A2, ATP1A1, SLC9A3, SLC9A2, CHP1, CPT1A, CPT1B, RHCG, SLC9A6, SLC22A5, SLC22A4
GO:0043269	Regulation of ion transport	PLA2R1, SLC9A3R1, ATP1A1, NOS1AP, FGF23, CCR1, ATP1B1, FCRL5, AHCYL1, ARG1, SLC9A3, GSTO1, KCNMA1, CHP1, CD4, SLC38A3, FXYD1, CLCN2, KCNJ15, KCNJ2, GNAO1, CLCN1, SEPT5, CRHR1, HFE, TNFSF11, PDE4D, SIK1, WNK3, CD63, S100A1, DNM2, TRPM5, PLCB4, PER2, KCNJ1, GPER1
GO:0048878	Chemical homeostasis	TRPV4, GPR116, ALOXE3, PVALB, MICU1, FN1, SLC9A3R1, ATP2A2, XPR1, CRY1, WNK4, IRS1, ATP1B1, LDLR, UMOD, TRPM2, KCNMA1, NGFR, ATP6AP1, INSR, ADCY9, CCL14, CLDN4, SLC39A6, KCNJ2, ATP6V1C1, PTPN11, SLC4A1, TRPV1, HFE, SLC25A23, RAB38, GPER1, ATP2B3, PTPRC, ATP1A1, CCR1, FGF23, FLVCR1, SLC9A3, SLC9A2, SSTR5, KRT16, CHP1, CCDC109B, RHCG, SLC9A6, SOX4, SLC26A2, TNFSF11

GO:1905039	Carboxylic acid transmembrane transport	SLC38A3, PRKAG2, CPT1A, CPT1B, SLC22A4, SLC38A4, SLC6A6, SLC22A5, SLC25A12, SLC25A22, MPC1, SLC7A5, SLC25A15, SLC7A3, SLC38A2
Liver		
GO:0046949	Fatty-acyl-CoA biosynthetic process	ACSL1, ACLY, ACSF2, ACACB, ELOVL6, TECR, ACLY, ELOVL5, ACSF2, ACACB, ELOVL6
GO:0035337	Fatty-acyl-CoA metabolic process	TECR, ACLY, DGAT1, ELOVL5, ACSF2, ACACB, ELOVL6, FAR1, ACSL1
GO:0071616	Acyl-CoA biosynthetic process	ACSL1, ACLY, ACSF2, ACACB, ELOVL6, TECR, ACSS2, ELOVL5
GO:0035384	Thioester biosynthetic process	TECR, ACLY, ELOVL5, ACSS2, ACSF2, ACACB, ELOVL6, ACSL1
GO:0032787	Monocarboxylic acid metabolic process	ACSL1, C3, PDP1, FA2H, ACOX3, HK1, ACLY, UGT2B17, ACSF2, ACACB, ELOVL6, GCK, CYP7A1, CYP8B1, SC5D, ABCB11, TECR, GRHPR, UGT8, CYP2D6, UGT2B15, NR5A2, ABCD3, AACS, PKLR, ACSS2, ELOVL5, NPC1, P4HB, HPGD, PER2, OGDH, HOGA1, FADS6, MSMO1, CYP27A1
GO:0008610	Lipid biosynthetic process	ACSL1, ANG, FA2H, PHOSPHO1, ACLY, ST3GAL2, ACSF2, ACACB, ELOVL6, P2RX1, PPM1L, APOA4, PTDSS1, CYP7A1, SPTLC3, LSS, CYP8B1, SC5D, EBP, CYP3A4, ABCB11, DHCR24, TECR, UGT8, NPC1L1, MVK, ABCD3, CYB5R3, AGPAT4, FAR1, TM7SF2, HSD17B7, FDPS, ACSS2, FDFT1, ELOVL5, PMVK, MVD, C14orf1, PISD, SQLE, CYP51A1, FADS6, HMGCR, HMGCS1, DGAT1, NSDHL, MSMO1, IDI1, CYP27A1
GO:0006631	Fatty acid metabolic process	ACOX3, ACLY, ACSS2, ELOVL5, HPGD, SC5D, PER2, TECR, C3, FA2H, FADS6, CYP2D6, MSMO1, ACSF2, ABCD3, ACACB, ELOVL6, AACS, ACSL1
GO:0006637	Acyl-CoA metabolic process	ACSL1, ACLY, ACSF2, ACACB, ELOVL6, ELOVL5, ACSS2, PMVK, MVD, OGDH, TECR, PIPOX, DGAT1, MVK, FAR1

GO:0035383	Thioester metabolic process	ACLY, ELOVL5, ACSS2, PMVK, MVD, OGDH, TECR, PIPOX, DGAT1, MVK, ACSF2, ACACB, ELOVL6, FAR1, ACSL1
GO:0035336	Long-chain fatty-acyl-CoA metabolic process	ACSL1, ACLY, ACSF2, ELOVL6, TECR, DGAT1, ELOVL5, FAR1
GO:0044255	Cellular lipid metabolic process	SPTLC3, PGM3, PEX11A, ABCA1, PPM1L, APOA4, FA2H, UGT8, NPAS2, ACACB, ABCD3, AGPAT4, AACS, FAR1, P2RX1, ACLY, ELOVL5, HPGD, PMVK, PISD, PER2, SDC2, RDH11, DGAT1, SOAT1, ALAS1, IDI1, ELOVL6, CYP7A1, PTDSS1, ACOX3, KDELC2, SC5D, CYP3A4, TECR, CYP2D6, MVK, ANG, FDPS, FDFT1, ACSS2, MVD, C3, HMGCR, FADS6, HMGCS1, MSMO1, ACSF2, PDE3A, PHOSPHO1, ACSL1, ST3GAL2
Brain		
GO:0015074	DNA integration	ERVK-11, GIN1, KRBA2, ERVK-10, NYNRIN
GO:0030198	Extracellular matrix organization	ADAMTS3, ACTN1, COL22A1, FN1, SULF1, MMP13, FOXF2, PXDN, BCAN, SDC3, ELF3, LAMA5, ECM2, VCAN, NCAN, FBLN1, ACAN, ITGB8, DCN, NOXO1, COL12A1, FBN2, COL5A1
GO:0043062	Extracellular structure organization	BCAN, ITGB8, DCN, ELF3, NOXO1, COL12A1, VCAN, FBN2, ACAN, COL5A1, ADAMTS3, ACTN1, COL22A1, FN1, SULF1, MMP13, FOXF2, PXDN, SDC3, LAMA5, ECM2, NCAN, FBLN1
C. Cave upregulated		
Gill		
GO:0006820	Anion transport	PRKAG2, ATP8B4, CLCN2, CPT1A, CPT1B, SLC6A6, SLC9A3R1, SLC3A2, ACSL1, LDLR, SLC4A1, SLC4A4, SLC25A12, SLC25A22, SLC12A3, ATP8B1, SLC4A10, MTTP, SLC13A2, SLC7A8, ABCC2, SLC22A6, BAAT, FABP6, ABCB11, SLC6A13, APOA4, SLC12A1, TRPC4, CA4, CA2, SLC26A5, SLC26A3, SLC16A9, SLC34A2, CD36, P2RY6, SLC7A11, CA5B, PLA2G10, SLC1A3, SLC43A2, SLC25A1, SLC22A4, ABCA12, SLC26A11, CLCC1, VDAC1, SLC25A10, CFTR, AQP1

GO:0055085	Transmembrane transport	SLC4A10, SLC7A8, SLC9A1, COX5B, SLC14A2, ABCB11, SLC6A13, SLC12A1, TRPC4, COX15, COX11, SLC9A2, FTH1, ABCB1, KCNN3, SLC26A3, SLC16A9, SLC12A3, ATP6V1E1, GJA3, GJB2, SLC1A3, KCNK10, SLC39A1, SLC4A1, FKBP1B, SLC26A11, SLC2A4, CLCC1, VDAC1, CFTR, SLC5A1, AQP1, PRKAG2, SLC13A2, ABCC2, SLC22A6, SLC26A5, SCN4B, CYBRD1, SLC34A2, SLC7A11, SLC43A2, SLC25A1, SLC22A4, SLC31A1, ABCA12, FAM26D, HMOX2, SLC25A10, SLC28A1, CNGB1, MAL, SLC6A6, PRF1, NDUFA4, ATP1B1, SLC25A25, AHCYL1, TRPM2, SLC9A3, SLC9B2, SLC4A4, SLC25A22, CLCN2, CPT1A, CPT1B, ATP6AP1, SLC30A2, SLC9A6, CACNB2, SLC39A6, KCNJ2, SLC44A4, SLC3A2, TSC22D3, ATP2B3, SLC25A12, HSPA8, ATP8B1
GO:0006811	Ion transport	PRKAG2, ATP8B4, CNGB1, SLC6A6, SLC9A3R1, NDUFA4, ATP1B1, SLC25A25, ACSL1, LDLR, TRPM2, SLC9A3, SLC9B2, SLC4A4, SLC25A22, SLC12A3, CLCN2, CPT1A, CPT1B, ATP6AP1, SLC30A2, SLC9A6, CACNB2, SLC39A6, KCNJ2, SLC44A4, SLC3A2, SLC4A1, HFE, TSC22D3, ATP2B3, SLC25A12, ATP8B1, SLC4A10, MTTP, SLC7A8, SLC9A1, COX5B, SLC35G1, FABP6, ABCB11, SLC6A13, APOA4, SLC12A1, TRPC4, COX15, COX11, SLC9A2, FTH1, KCNN3, SLC26A3, SLC16A9, HEPHL1, ATP6V1E1, CD36, P2RY6, CA5B, PLA2G10, SLC1A3, KCNK10, SLC39A1, FKBP1B, SLC26A11, CLCC1, VDAC1, CFTR, SLC5A1, AQP1, COX17, SLC13A2, ABCC2, SLC22A6, BAAT, CA4, CA2, SLC26A5, LYN, SCN4B, SLC34A2, SLC7A11, UCP2, SLC43A2, SLC25A1, RAMP3, SLC22A4, SLC31A1, ABCA12, FAM26D, SLC25A10
GO:0015711	Organic anion transport t	PRKAG2, SLC4A10, MTTP, SLC13A2, ABCC2, SLC7A8, SLC22A6, BAAT, FABP6, ABCB11, SLC6A13, APOA4, CA4, TRPC4, CA2, SLC26A5, SLC26A3, SLC16A9, SLC7A11, CD36, CA5B, PLA2G10, SLC1A3, SLC43A2, SLC25A1, SLC4A1, ABCA12, SLC22A4, SLC26A11, SLC25A10, CFTR, AQP1, ATP8B4, CPT1A, CPT1B, SLC6A6, SLC9A3R1, SLC3A2, ACSL1, LDLR, SLC4A4, SLC25A12, SLC25A22, ATP8B1
Liver		
N/A	N/A	N/A

Brain		
N/A	N/A	N/A
D. Cave downregulated		
Gill		
GO:0006885	Regulation of pH	MAFG, CA2, SLC26A5, SLC26A11, SLC26A3, CFTR, SLC26A2, ATP6AP1, SLC9A3, SLC9A2, RHCG, SLC9A6, CHP1
GO:0006820	Anion transport	PRKAG2, ATP8B4, CLCN2, CPT1A, CPT1B, SLC6A6, SLC9A3R1, SLC3A2, ACSL1, LDLR, SLC4A1, SLC4A4, SLC25A12, SLC25A22, SLC12A3, ATP8B1, ABCC2, CA5B, BAAT, SLC1A3, ABCB11, SLC6A13, CA4, TRPC4, SLC25A1, CA2, ABCA12, SLC26A5, SLC26A11, SLC26A3, CLIC2, SLC20A1, SLC25A10, CFTR, AQP1, SLC12A2
GO:0015711	Organic anion transport	PRKAG2, SLC38A3, ATP8B4, CPT1A, CPT1B, SLC6A6, SLC9A3R1, SLC22A5, SLC6A13, SLC3A2, ACSL1, LDLR, SLC26A2, SLC4A1, SLC22A4, SLC38A4, SLC4A4, SLC25A12, MPC1, SLC25A22, ATP8B1, ABCC2, CA5B, BAAT, SLC1A3, ABCB11, TRPC4, CA4, SLC25A1, CA2, ABCA12, SLC26A5, SLC26A11, SLC26A3, SLC25A10, CFTR, AQP1
GO:0042592	Homeostatic process	GSR, AKR1B1, ABCC2, SLC8A1, TRPC4, CA2, ARRDC3, PDK3, SLC26A5, FTH1, SLC26A3, GCLC, CYBRD1, GRIK2, PCK1, CXCR3, GATA2, UCP2, MUC6, MAFG, EPOR, SLC4A1, ABCA12, SLC26A11, PRDX1, FAM20A, CLDN1, SLC2A4, ALAS2, RAC1, NR1D2, CFTR, AQP1, NR1D1, TRPV4, CNGB1, ALOXE3, GPR116, FN1, SLC9A3R1, ATP2A2, CRY1, WNK4, IRS1, ATP1A1, STAT5B, FGF23, CCR1, ATP1B1, LDLR, FLVCR1, TRPM2, SLC9A3, SLC9A2, ATM, SSTR5, ITPKB, KCNMA1, KRT16, CHP1, MUC2, ANG, NGFR, CCDC109B, ATP6AP1, INSR, RHCG, SLC9A6, SOX4, CLDN4, SLC22A5, KCNJ2, SLC26A2, ERP44, HFE, TNFSF11, ATP2B3, PIGR
GO:0048878	Chemical homeostasis	TRPV4, GPR116, ALOXE3, FN1, SLC9A3R1, ATP2A2, CRY1, WNK4, IRS1, ATP1A1, CCR1, FGF23, ATP1B1, LDLR, FLVCR1, SLC9A3, TRPM2, SLC9A2, SSTR5, KCNMA1, KRT16, CHP1, NGFR, CCDC109B, ATP6AP1, INSR, RHCG, SLC9A6, SOX4, CLDN4, KCNJ2, SLC4A1, SLC26A2, HFE, TNFSF11, ATP2B3,

		ABCC2, SLC8A1, TRPC4, CA2, PDK3, SLC26A5, SLC26A3, FTH1, CYBRD1, PCK1, GRIK2, CXCR3, GATA2, UCP2, MAFG, EPOR, ABCA12, FAM20A, SLC26A11, SLC2A4, CLDN1, ALAS2, NR1D2, CFTR, AQP1
GO:0006811	Ion transport	ABCC2, SLC8A1, BAAT, ABCB11, SLC6A13, TRPC4, CA4, CA2, SLC26A5, SLC2A6, FTH1, KCNN3, SLC26A3, CLIC2, SCN4B, SLC12A3, SLC12A2, HEPHL1, ADD2, GRIK2, CA5B, UCP2, SLC1A3, SLC25A1, SLC4A1, ABCA12, RAMP3, SLC26A11, FAM26D, CLDN10, SLC20A1, SLC25A10, CFTR, AQP1, AFG3L2, TRPV4, MAL, SLC6A6, ATP2A2, PRF1, NDUFA4, ATP1A1, ATP1B1, AHCYL1, SLC9A3, TRPM2, SLC9A2, SLC4A4, KCNMA1, FXYD1, CPT1A, CPT1B, CCDC109B, ATP6AP1, SLC5A7, NIPAL4, RHCG, SLC9A6, SLC44A4, SLC3A2, CYB561, SLC26A2, TSC22D3, ATP2B3, ABCB10, ATP8B1, PRKAG2, CNGB1, PKD1L2, FLVCR1, SLC38A4, GABRD, SLC25A22, MPC1, SLC38A3, CLCN2, KCNJ15, SLC22A5, KCNJ2, CLCN1, SLC22A4, SLC25A5, SLC25A12, HSPA8
GO:0055085	Transmembrane transport	ABCC2, SLC8A1, SLC14A2, ABCB11, SLC6A13, TRPC4, SLC26A5, SLC2A6, FTH1, KCNN3, SLC26A3, PPBP, CLIC2, SCN4B, SLC12A3, SLC12A2, CYBRD1, DMTN, ADD2, GRIK2, GJA3, GJB2, SLC1A3, SLC25A1, SLC4A1, ABCA12, SLC26A11, SLC2A4, FAM26D, SLC20A1, SLC25A10, CFTR, AQP1, AFG3L2, TRPV4, MAL, SLC6A6, ATP2A2, PRF1, NDUFA4, ATP1A1, ATP1B1, AHCYL1, SLC9A3, TRPM2, SLC9A2, SLC4A4, KCNMA1, FXYD1, CPT1A, CPT1B, CCDC109B, ATP6AP1, SLC5A7, NIPAL4, RHCG, SLC9A6, SLC44A4, SLC3A2, CYB561, SLC26A2, TSC22D3, ATP2B3, ABCB10, ATP8B1, PRKAG2, CNGB1, PKD1L2, FLVCR1, SLC38A4, GABRD, SLC25A22, MPC1, SLC38A3, CLCN2, KCNJ15, SLC22A5, KCNJ2, CLCN1, SLC22A4, SLC25A5, SLC25A12, HSPA8
GO:0098656	Anion transmembrane transport	SLC4A1, SLC26A5, SLC26A11, SLC26A3, CLIC2, SLC20A1, CFTR, SLC1A3, SLC12A3, SLC12A2, SLC38A3, FXYD1, PRKAG2, CLCN2, CPT1A, CPT1B, SLC6A6, SLC22A5, CLCN1, SLC26A2, SLC38A4, SLC22A4, GABRD, SLC4A4, SLC25A12, MPC1, SLC25A22

GO:0055067	Monovalent inorganic cation homeostasis	SLC26A2, ATP6AP1, SLC9A3, SLC9A2, RHCG, SLC9A6, ATP1A1, KCNJ2, KCNMA1, CHP1, ATP1B1, MAFG, CA2, SLC26A5, SLC26A11, SLC26A3, SLC8A1, CFTR
Liver		
GO:0042221	Response to chemical	IL18, IL12B, LGALS1, TRIM25, NCOA3, MMP2, CCL16, CYCS, P2RY1, CD38, P2RX7, EIF4EBP1, SCARB1, CCL14, CLDN4, PTGR1, IL1R1, IL1B, TRIM16, GUCY2C, LMO2, CCL21, IL6R, PDE1B, GLUL, PLK3, TNC, ARSB, SLC12A5, SPHK1, PDGFRA, ACVR1, PDGFB, CA2, MX1, PTGS1, PTGS2, PPBP, FZD4, ADCY1, LRP8, ADD3, S100P, GNB3, CDKN1A, C3, GNAI2, IRF3, HID1, SIDT2, PDE4B, S100A16, IFIT1, SLC9A1, SLC8A1, CDK19, ERO1L, CHKA, KDM6B, NFKBIA, CHAT, LIPG, NLRP1, HGF, DSG2, NPNT, TESC, VCAM1, MSN, BAX, STAT1, ERN1, CYP26B1, UBB, CORO1B, IKBKE, JAK2, CD9, PLA2G1B, GRN, SSTR5, CLU, EIF2AK2, CD4, EPHA3, BDKRB2, SERPINH1, TNFRSF14, IGF1, RIPK2, TRIM21, CBX3, UCP2, TGFBR2, CXCL2, YES1, NLRP12, ACAP2, MB21D1, FAM162A, ERBB2, ABCG5, FLT3, PAQR9, ABCA1, CD274, APOA4, MTHFR, AGL, ABCD3, PPP5C, GCH1, GCK, AACS, P2RX1, ACO1, P4HB, ADNP2, EZR, PMVK, AHR, CCL3, CRHR1, CALU, CYB5A, SDC2, APRT, VCP, CYP7A1, H2AFZ, EEF2K, CRY1, NTRK3, DHCR24, NFIL3, NPC1L1, FZD7, NDRG1, BCAR3, BRSK2, SERPINF1, PKLR, ITPR2, ANG, CDK4, S100B, RORB, NPC1, SORT1, EPB41L5, COL4A2, SQLE, HMGCR, HMGCS1, MLXIPL, CCRN4L, PRKCA, SREBF2, SETD7, STT3B, PDE3A
GO:0010033	Response to organic substance	FLT3, PAQR9, ABCA1, CD274, APOA4, MTHFR, AGL, GCH1, PPP5C, ABCD3, AACS, GCK, TRIM25, P2RX1, CCL14, ADNP2, EZR, PMVK, AHR, CCL3, CRHR1, CALU, SDC2, APRT, VCP, CYP7A1, H2AFZ, EEF2K, CRY1, NTRK3, DHCR24, NFIL3, FZD7, SERPINF1, BRSK2, PKLR, ITPR2, ANG, CDK4, S100B, RORB, NPC1, SORT1, EPB41L5, COL4A2, C3, SQLE, HMGCR, HMGCS1, MLXIPL, CCRN4L, PRKCA, SREBF2, SETD7, STT3B, PDE3A, IFIT1, IL18, SLC9A1, SLC8A1, CDK19, IL12B, LGALS1, CHKA, NCOA3, MMP2, CCL16, P2RY1, P2RX7, CD38, EIF4EBP1, SCARB1, NFKBIA, CHAT, CLDN4, IL1R1, IL1B,

		NLRP1, HGF, DSG2, TRIM16, NPNT, TESC, VCAM1, LMO2, CCL21, MSN, IL6R, BAX, PDE1B, GLUL, STAT1, TNC, ARSB, SPHK1, ERN1, CYP26B1, ACVR1, PDGFB, CORO1B, IKBKE, CA2, JAK2, MX1, CD9, PLA2G1B, GRN, PTGS1, SSTR5, CLU, PTGS2, FZD4, PPBP, EIF2AK2, LRP8, ADCY1, CD4, EPHA3, S100P, GNB3, SERPINH1, IGF1, TNFRSF14, RIPK2, TRIM21, CBX3, CDKN1A, UCP2, TGFBR2, IRF3, HID1, CXCL2, YES1, ACAP2, NLRP12, MB21D1, SIDT2, ERBB2, PDE4B
GO:0014070	Response to organic cyclic compound	BAX, IFIT1, IL18, STAT1, SLC9A1, TNC, ARSB, SLC8A1, SPHK1, ACVR1, PDGFB, CA2, PTGS1, LGALS1, GRN, PTGS2, SSTR5, CHKA, EIF2AK2, ADCY1, LRP8, TRIM25, CD4, NCOA3, P2RY1, CD38, P2RX7, IGF1, NFKBIA, RIPK2, CLDN4, CDKN1A, CBX3, IL1B, TGFBR2, IRF3, C3, HID1, MB21D1, DSG2, VCAM1, MSN, PDE4B, CYP7A1, H2AFZ, EEF2K, FLT3, PAQR9, ABCA1, NTRK3, APOA4, MTHFR, AGL, ABCD3, SERPINF1, PPP5C, AACS, PKLR, P2RX1, ITPR2, CDK4, S100B, NPC1, EZR, PMVK, AHR, CCL3, CALU, SDC2, HMGCS1, PDE3A
GO:0065008	Regulation of biological quality	SLC4A10, EXOSC9, NCOA5, ACE, LGALS1, P2RY1, CD38, P2RX7, TENM1, SCARB1, HPS5, CCL14, BLK, CLEC4M, CLDN4, IL1R1, IL1B, STEAP4, CCL21, PDE1B, C9, GLUL, IL20RA, CDHR5, RHOU, LPCAT1, GLRX, SPHK1, SLC12A5, CYP3A4, PDGFRA, PDGFB, GZMB, CA2, RASSF2, USH1C, ITGAV, PTGS1, PTGS2, PPBP, FZD4, ADCY1, LRP8, ITGB3, PALM2, GNB3, CRTC1, C3, SWAP70, GNAI2, PTPN6, L1CAM, ITGA6, SIDT2, PDE4B, FHOD3, ALOXE3, F8, SLC9A1, SLC8A1, SFXN5, CAMK2G, ATP1B3, SLC6A13, ERO1L, AGTRAP, AGTR1, PNPLA2, TSPAN8, SLC1A1, HK1, GAA, CXCR3, NFKBIA, CHAT, EPB41L3, LIPG, HGF, NCF1, SLC4A1, RDH11, DSG2, CYSLTR1, TESC, MSN, CYBB, BAX, CRP, STAT1, ERN1, CCR1, CYP26B1, UBB, CORO1B, ATP8A1, JAK2, GNA13, CD9, PLA2G1B, CLU, SSTR5, CD2, CLIC2, EIF2AK2, ITPR1, TFPI, CD4, ANGPT1, BDKRB2, SERPINE2, RBFOX2, IGF1, TRIM21, TGM2, HCAR2, ANO9, UCP2, YES1, DOC2B, NLRP12, PLEKHO1, NR1D2, PIGR, SYT12, SMDT1, QRSL1, SEPT7, A2M, ABCG5, FLT3, PEX11A, ABCA1, SUN3, APOA4, HLA-DRB1, MTHFD1, TMEM97, FLNB, ZFP36L2, NR5A2, ACACB, GCH1, AACS,

		HPX, GCK, P2RX1, MYO10, XCR1, HSD17B7, ACO1, P4HB, EZR, LEPRE1, RAB20, SULT1A1, PYGL, CCL3, PER2, KCNK6, CRHR1, CALU, HEBP2, DGAT1, CREBL2, RAB11FIP5, ACTG1, SOAT1, APRT, DISC1, VCP, MYO5B, CLOCK, ARNTL, CYP7A1, LSS, ACOX3, GLUD1, MCU, ITGAM, DNAJC5, CRY1, SIPA1L1, NTRK3, IGSF9B, CNNM2, PPP1R3G, ISCU, ITGAX, BRSK2, SERPINF1, ITPR2, SULT1B1, ANG, CDK4, S100B, NPC1, HPSE, HMGCR, SPTBN1, MLXIPL, CCRN4L, PRKCA, SREBF2, PDE3A, EPS8
GO:0050708	Regulation of protein secretion	ARNTL, ANG, MCU, GLUD1, CRP, EZR, LEPRE1, CD274, CCL3, PER2, HLA-DRB1, SPTBN1, HMGCR, RAB11FIP5, PRKCA, BRSK2, GCK, CLOCK, AACS, BTN2A2, ITPR2, ANGPT1, RSAD2, GLUL, CD38, P2RX7, CLEC4E, IGF1, TNFRSF14, BLK, HCAR2, IL1B, UCP2, NLRP1, IRF3, DOC2B, NLRP12, TRIM16, JAK2, PLA2G1B, SSTR5, CD2, ITPR1, SIDT2
Brain		
N/A	N/A	N/A

Table B.5 Gene ontology enrichment unique to each organ analyzed

Results of the enrichment analysis of Gene Ontology (GO) terms of differentially transcripts that were unique within each organ analyzed. Provided are the GO term identification, description of the enriched GO term, the total number of genes in each reference set (N), the total number of genes with a specific GO term in each reference (B), the number of genes in the target set (n), and the number of genes in the intersections (b), P-value associated with the enrichment, false-discovery rate (q-value), and enrichment value obtained from Gorilla enrichment analysis. Due to the large size, the table is provide in a separate excel spreadsheet (Table B.5 – Unique GO annotations.xlsx). Each tab corresponds to the unique enriched GO terms for each organ and is labeled “Gill”, ”Liver” and “Brain.” Blue corresponds to upregulated GO enrichment, which red corresponds to downregulated enrichment.

Appendix B References

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Appendix C - Evolutionary change shapes gene expression variation in locally adapted, extremophile fishes

Appendix figures

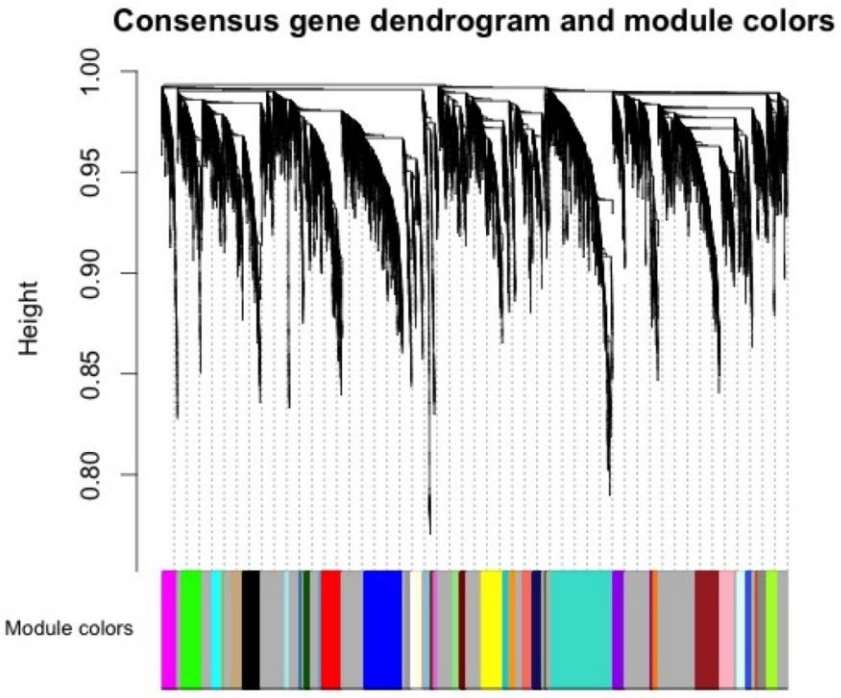

Figure C.1 Consensus gene dendrogram

Results of weighted gene co-expression network analysis of the top 10,000 expressed transcripts. Depicted is the average linkage clustering trees based on topological distance in gene expression patterns for comparisons across sulfidic and nonsulfidic fish in the field and the laboratory. Modules are defined according to colored bars. Anything in grey are transcripts that did not correspond to any module.

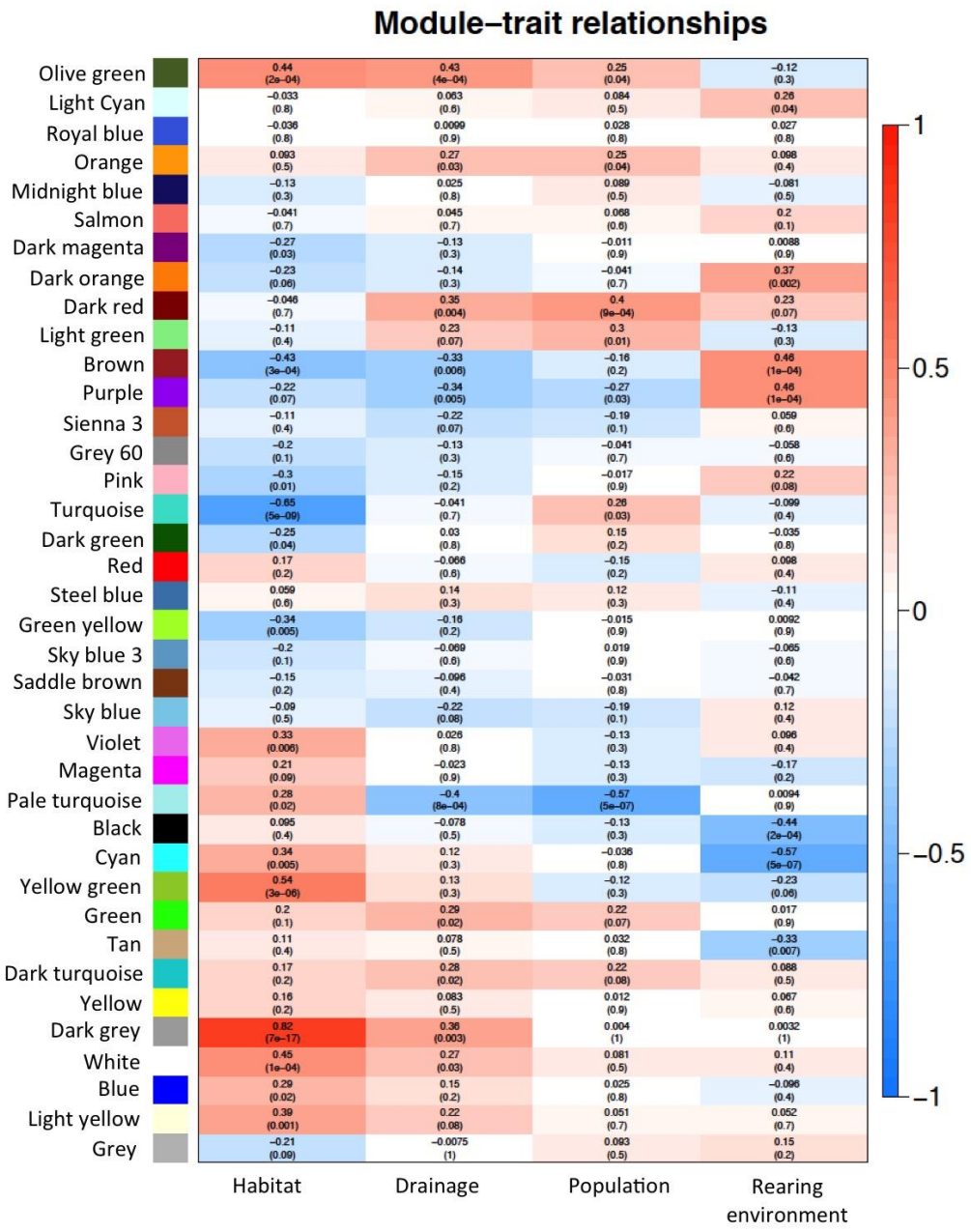
Module–trait relationships
Olive green
Light Cyan
Royal blue
Orange
Midnight blue
Salmon
Dark magenta
Dark orange
Dark red
Light green
Brown
Purple
Sienna 3
Grey 60
Pink
Turquoise
Dark green
Red
Steel blue
Green yellow
Sky blue 3
Saddle brown
Sky blue
Violet
Magenta
Pale turquoise
Black
Cyan
Yellow green
Green
Tan
Dark turquoise
Yellow
Dark grey
White
Blue
Light yellow
Grey
0.44 (2e−04) 0.43 (4e−04) 0.25 (0.04) −0.12 (0.3)
−0.033 (0.8) 0.063 (0.5) 0.084 (0.5) 0.26 (0.04)
−0.036 (0.8) 0.0099 (0.9) 0.028 (0.8) 0.027 (0.8)
0.093 (0.5) 0.27 (0.03) 0.25 (0.04) 0.098 (0.4)
−0.13 (0.3) 0.025 (0.8) 0.089 (0.5) −0.081 (0.5)
−0.041 (0.7) 0.045 (0.7) 0.068 (0.6) 0.2 (0.1)
−0.27 (0.03) −0.13 (0.3) −0.011 (0.9) 0.0088 (0.9)
−0.23 (0.06) −0.14 (0.3) −0.041 (0.7) 0.37 (0.002)
−0.046 (0.7) 0.35 (0.004) 0.4 (9e−04) 0.23 (0.07)
−0.11 (0.4) 0.23 (0.07) 0.3 (0.01) −0.13 (0.3)
−0.43 (3e−04) −0.33 (0.006) −0.16 (0.2) 0.46 (1e−04)
−0.22 (0.07) −0.34 (0.005) −0.27 (0.03) 0.46 (1e−04)
−0.11 (0.4) −0.22 (0.07) −0.19 (0.1) 0.059 (0.6)
−0.2 (0.1) −0.13 (0.3) −0.041 (0.7) −0.058 (0.6)
−0.3 (0.01) −0.15 (0.2) −0.017 (0.9) 0.22 (0.08)
−0.65 (5e−09) −0.041 (0.7) 0.26 (0.03) −0.099 (0.4)
−0.25 (0.04) 0.03 (0.8) 0.15 (0.2) −0.035 (0.8)
0.17 (0.2) −0.066 (0.6) −0.15 (0.2) 0.098 (0.4)
0.059 (0.6) 0.14 (0.3) 0.12 (0.3) −0.11 (0.4)
−0.34 (0.005) −0.16 (0.2) −0.015 (0.9) 0.0092 (0.9)
−0.2 (0.1) −0.069 (0.6) 0.019 (0.9) −0.065 (0.6)
−0.15 (0.2) −0.096 (0.4) −0.031 (0.8) −0.042 (0.7)
−0.09 (0.5) −0.22 (0.08) −0.19 (0.1) 0.12 (0.4)
0.33 (0.006) 0.026 (0.8) −0.13 (0.3) 0.096 (0.4)
0.21 (0.09) −0.023 (0.9) −0.13 (0.3) −0.17 (0.2)
0.28 (0.02) −0.4 (8e−04) −0.57 (5e−07) 0.0094 (0.9)
0.095 (0.4) −0.078 (0.5) −0.13 (0.3) −0.44 (2e−04)
0.34 (0.005) 0.12 (0.3) −0.036 (0.8) −0.57 (5e−07)
0.54 (3e−06) 0.13 (0.3) −0.12 (0.3) −0.23 (0.06)
0.2 (0.1) 0.29 (0.02) 0.22 (0.07) 0.017 (0.9)
0.11 (0.4) 0.078 (0.5) 0.032 (0.8) −0.33 (0.007)
0.17 (0.2) 0.28 (0.02) 0.22 (0.08) 0.088 (0.5)
0.16 (0.2) 0.083 (0.5) 0.012 (0.9) 0.067 (0.6)
0.82 (7e−17) 0.36 (0.003) 0.004 (1) 0.0032 (1)
0.45 (1e−04) 0.27 (0.03) 0.081 (0.5) 0.11 (0.4)
0.29 (0.02) 0.15 (0.2) 0.025 (0.8) −0.096 (0.4)
0.39 (0.001) 0.22 (0.08) 0.051 (0.7) 0.052 (0.7)
−0.21 (0.09) −0.0075 (1) 0.093 (0.5) 0.15 (0.2)
Habitat
Drainage
Population
Rearing environment
1
0.5
0
−0.5
−1

Figure C.2 Module-trait relationship

Depicted is the correlation between module eigenvalues and the habitat type (presence of absence of sulfide), river drainage (Tacotalpa or Puyacatengo), population (collection site for the four populations), and rearing environment (field or laboratory). Reported within each of the modules are the Pearson correlation coefficients on the top and the p-value on the bottom. Modules in red correspond to negative correlations while modules in blue correspond to positive correlations

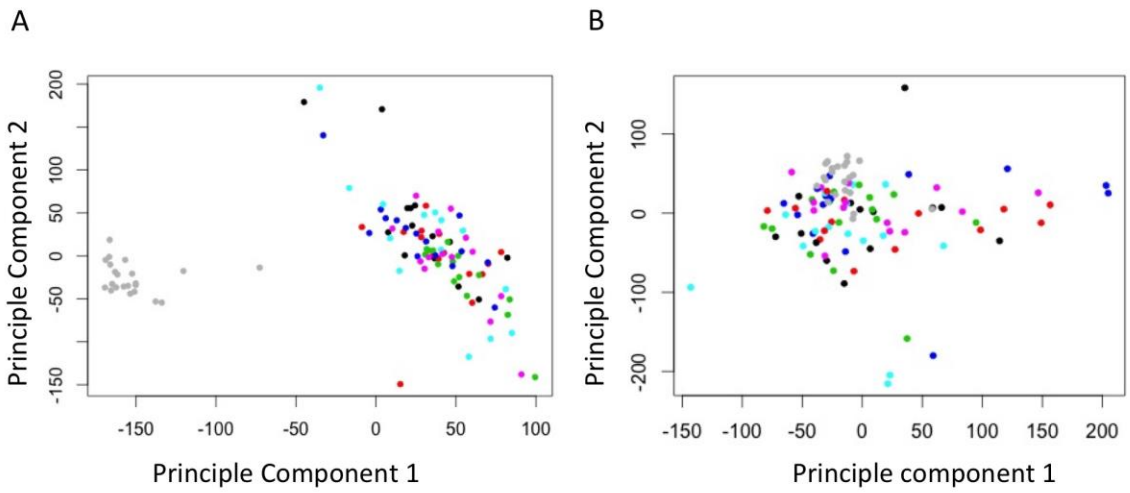

Figure C.3 Principle component analysis (PCA) based on the top 10,000 expressed genes

Principle component analysis (PCA) based on the top 10,000 expressed transcripts with counts per million at least > 0. We used the function prcomp in R and color-coded each cDNA library based on lane. Black is lane 1, red is lane 2, green is lane 3, blue is lane 4, teal is lane 5 pink is lane 6 and all in grey are the field data set. (A) Before PC1 was removed, PC1 explained 15.1% variance while PC2 explained 6.9% variance. (B) Once PC1 was removed with the program sva, PC1 explained 7.6 % variance whereas PC2 explained 6.5 % variance.

Appendix tables

Table C.1 Descriptive statistics of sequences and sample size

Average read counts and standard deviations for (A) all experimental groups in the laboratory as well as (B) the field dataset. Reported are the sample sizes for each group, initial average read counts for each group, average read counts after the first trim which was set at quality 0 in trimgalore to remove primer dimer, and average read counts after the second trim which was set at quality 24 in trim galore.

Samples	Sample size	Average read counts	Average read counts first trim	Average read counts second trim
A. Laboratory libraries				
Tacotalpa nonsulfidic control (0 mM)	6	25,924,417 ± 13,636,357	25,244,811 ± 13,310,933	24,244,383 ± 13,056,092
Tacotalpa nonsulfidic low (0.5 mM)	6	29,107,229 ± 6,320,881	28,371,810 ± 6,174,865	24,362,502 ± 5,790,764
Tacotalpa nonsulfidic medium (3.0 mM)	6	23,336,195 ± 10,057,855	22,724,253 ± 9,809,230	21,606,271 ± 9,788,479
Tacotalpa nonsulfidic high (6.0 mM)	5	30,567,106 ± 13,605,109	29,649,968 ± 13,049,987	27,966,482 ± 12,230,600
Tacotalpa sulfidic control (0 mM)	6	24,947,895 ± 8,975,276	24,373,533 ± 8,809,249	23,179,594 ± 8,504,193
Tacotalpa sulfidic low (0.5 mM)	6	23,257,544 ± 3,990,017	22,634,184 ± 3,875,480	21,392,520 ± 3,736,492
Puyacatengo nonsulfidic control (0 mM)	6	25,201,339 ± 8,635,045	24,550,960 ± 8,390,425	23,247,800 ± 7,894,734
Puyacatengo nonsulfidic low (0.5 mM)	6	20,626,282 ± 10,200,113	20,098,201 ± 9,979,353	19,087,913 ± 9,444,844
Puyacatengo nonsulfidic medium (3.0 mM)	5	26,504,144 ± 5,480,567	25,793,334 ± 5,361,733	24,299,024 ± 5,037,069
Puyacatengo nonsulfidic high (6.0 mM)	3	23,210,953 ± 9,353,835	22,657,868 ± 9,169,196	21,416,181 ± 8,846,355
Puyacatengo sulfidic control (0 mM)	6	25,550,198 ± 9,844,074	24,902,972 ± 9,626,721	23,592,980 ± 9,156,529
Puyacatengo sulfidic low (0.5 mM)	6	32,742,576 ± 16,118,726	31,894,185 ± 15,695,833	30,615,023 ± 15,363,220
Puyacatengo sulfidic medium (3.0 mM)	6	28,997,819 ± 11,706,512	28,235,659 ± 11,396,938	26,888,421 ± 11,178,422
Puyacatengosulfidic high (6.0 mM)	3	20,753,248 ± 7,506,904	20,275,093 ± 7,382,367	19,433,396 ± 7,385,660
Total	87	2,211,807,574	2,144,267,874	2,046,844,024
B. Field libraries				
Tacotalpa nonsulfidic	5	12,969,822 ± 2,040,643	12,852,499 ± 2,045,742	11,947,968 ± 1,764,465

Tacotalpa sulfidic	6	14,449,948 ± 6,657,938	14,288,309 ± 6,539,071	13,103,913 ± 5,448,064
Puyacatengo nonsulfidic	6	19,815,137 ± 4,656,133	19,499,851 ± 4,494,389	16,328,316 ± 3,937,914
Puyacatengo sulfidic	5	18,891,881 ± 4,952,350	19,928,181 ± 4,906,959	16,770,774 ± 4,226,391
Total	22	371,473,422	366,332,360	320,187,084

Table C.2 Basic statistics for the *Poecilia mexicana* transcriptome

Descriptive statistics for transcriptomic assembly and alignment to the *Poecilia mexicana* genome. Most metrics were determined from the perl script assemblathon_stats.pl written by Keith Bradnam (UC Davis) and licensed under a Creative Commons Attribution-NonCommercial-ShareAlike 3.0 Unported License.

***Poecilia mexicana* transcriptome basic statistics**	
Total number of unique transcripts	63,590
Total number of unique loci	11,966
Mean contig size	3,568
Median contig size	2,796
N50 (bp)	5,290
Longest contig	103,334
Total number of bases in transcriptome	225,876,000
GC %	46.82%

Table C.3 Annotations of unique loci generated in the reference transcriptome

Annotations of all 48,601 unique loci in the generated reference transcriptome of *Poecilia mexicana.* Table includes informaiton on the query sequence ID, subject sequence ID (which includes the accession number obtained from swissprot), percent of identical matches, alignment length, number of mismatches, number of gap openings, start and end of the alignment query, start and end of the alignment subject, E-value and the bit score of the top BLAST hit for each transcript. Due to the large size, the table is provide in a separate excel spreadsheet titled ("Table BC.3 – Reference Transcriptome Annotations.xlsx")

Table C.4 Shared enriched genes within wild-caught and common-garden reared fish

Results of the enrichment analysis of Gene Ontology (GO) terms of differentially transcripts that were shared between drainages) and rearing environments. Provided are the transcript identification number, a description of each GO term, false-discovery rate (q-value), P-value associated with the enrichment, the total number of genes in each reference set (N), the total number of genes with a specific GO term in each reference (B), the number of genes in the target set (n), and the number of genes in the intersections (b). We report terms associated with biological processes, molecular function, and cellular components for shared (**A**) upregulated and (**B**) downregulated transcripts.

GO Term	Description	P-value	FDR q-value	Enrichment	N	B	n	b
A. Up-regulated								
Biological Processes								
GO:0019752	carboxylic acid metabolic process	8.78E-11	1.22E-06	5.98	13499	794	54	19
GO:0006790	sulfur compound metabolic process	1.17E-10	8.09E-07	10.87	13499	299	54	13
GO:0043436	oxoacid metabolic process	6.99E-10	3.22E-06	5.3	13499	897	54	19
GO:0006082	organic acid metabolic process	8.27E-10	2.86E-06	5.24	13499	906	54	19
GO:0044281	small molecule metabolic process	9.62E-10	2.66E-06	3.4	13499	1986	54	27
GO:0006520	cellular amino acid metabolic process	3.54E-09	8.15E-06	8.23	13499	395	54	13
GO:0019319	hexose biosynthetic process	2.22E-08	4.40E-05	33.33	13499	45	54	6
GO:0046364	monosaccharide biosynthetic process	5.44E-08	9.41E-05	28.84	13499	52	54	6
GO:0044710	single-organism metabolic process	5.94E-08	9.13E-05	2.22	13499	3940	54	35
GO:0006749	glutathione metabolic process	2.91E-07	4.02E-04	34.72	13499	36	54	5
GO:0055114	oxidation-reduction process	4.63E-07	5.82E-04	4.57	13499	820	54	15
GO:0000096	sulfur amino acid metabolic process	5.69E-07	6.57E-04	30.49	13499	41	54	5
GO:0006575	cellular modified amino acid metabolic process	5.71E-07	6.07E-04	9	13499	250	54	9
GO:0019418	sulfide oxidation	6.02E-07	5.95E-04	149.99	13499	5	54	3
GO:0070221	sulfide oxidation, using sulfide:quinone oxidoreductase	6.02E-07	5.55E-04	149.99	13499	5	54	3
GO:0070813	hydrogen sulfide metabolic process	6.02E-07	5.20E-04	149.99	13499	5	54	3
GO:0006094	gluconeogenesis	6.44E-07	5.24E-04	29.76	13499	42	54	5
GO:0005996	monosaccharide metabolic process	7.34E-07	5.64E-04	10.64	13499	188	54	8
GO:0044711	single-organism biosynthetic process	1.43E-06	1.04E-03	3.67	13499	1157	54	17
GO:1901564	organonitrogen compound metabolic process	2.18E-06	1.51E-03	3.21	13499	1481	54	19
GO:0019318	hexose metabolic process	2.70E-06	1.78E-03	11.22	13499	156	54	7
GO:0016051	carbohydrate biosynthetic process	4.55E-06	2.86E-03	13.76	13499	109	54	6
GO:0006006	glucose metabolic process	1.10E-05	6.61E-03	11.81	13499	127	54	6

GO:0044283	small molecule biosynthetic process	1.39E-05	7.99E-03	6.1	13499	369	54	9
GO:0000098	sulfur amino acid catabolic process	1.68E-05	9.31E-03	57.69	13499	13	54	3
GO:0006979	response to oxidative stress	1.73E-05	9.20E-03	6.94	13499	288	54	8
GO:1901605	alpha-amino acid metabolic process	2.16E-05	1.11E-02	6.73	13499	297	54	8
GO:0042221	response to chemical	2.19E-05	1.08E-02	2.63	13499	1898	54	20
GO:0042592	homeostatic process	3.32E-05	1.58E-02	3.43	13499	1019	54	14
GO:0044272	sulfur compound biosynthetic process	4.37E-05	2.01E-02	9.26	13499	162	54	6
GO:0000302	response to reactive oxygen species	4.68E-05	2.09E-02	9.15	13499	164	54	6
GO:1901685	glutathione derivative metabolic process	5.60E-05	2.42E-02	39.47	13499	19	54	3
GO:1901687	glutathione derivative biosynthetic process	5.60E-05	2.35E-02	39.47	13499	19	54	3
GO:0044699	single-organism process	6.54E-05	2.66E-02	1.34	13499	9131	54	49
GO:0097066	response to thyroid hormone	7.65E-05	3.02E-02	35.71	13499	21	54	3
GO:0006564	L-serine biosynthetic process	9.38E-05	3.60E-02	124.99	13499	4	54	2
GO:0019401	alditol biosynthetic process	9.38E-05	3.51E-02	124.99	13499	4	54	2
GO:0006090	pyruvate metabolic process	1.01E-04	3.67E-02	16.39	13499	61	54	4
GO:0009058	biosynthetic process	1.27E-04	4.51E-02	1.93	13499	3502	54	27
GO:0046942	carboxylic acid transport	1.74E-04	6.01E-02	7.21	13499	208	54	6
GO:0015849	organic acid transport	1.78E-04	6.02E-02	7.18	13499	209	54	6
GO:0015711	organic anion transport	1.81E-04	5.95E-02	5.83	13499	300	54	7
GO:0045454	cell redox homeostasis	2.03E-04	6.53E-02	13.7	13499	73	54	4
GO:0006123	mitochondrial electron transport, cytochrome c to oxygen	2.33E-04	7.33E-02	83.33	13499	6	54	2
GO:1901700	response to oxygen-containing compound	2.38E-04	7.32E-02	3.03	13499	1072	54	13
GO:1901576	organic substance biosynthetic process	2.67E-04	8.04E-02	1.89	13499	3439	54	26
GO:0019725	cellular homeostasis	3.24E-04	9.54E-02	4.05	13499	556	54	9
GO:1902358	sulfate transmembrane transport	3.26E-04	9.39E-02	71.42	13499	7	54	2
GO:0019532	oxalate transport	3.26E-04	9.20E-02	71.42	13499	7	54	2
GO:0045604	regulation of epidermal cell differentiation	3.32E-04	9.18E-02	22.06	13499	34	54	3
GO:0044249	cellular biosynthetic process	4.23E-04	1.15E-01	1.88	13499	3324	54	25
GO:0006563	L-serine metabolic process	4.33E-04	1.15E-01	62.5	13499	8	54	2
GO:0045333	cellular respiration	5.38E-04	1.41E-01	18.75	13499	40	54	3
GO:0006534	cysteine metabolic process	5.55E-04	1.42E-01	55.55	13499	9	54	2
GO:0006122	mitochondrial electron transport, ubiquinol to cytochrome c	5.55E-04	1.40E-01	55.55	13499	9	54	2
GO:0008272	sulfate transport	5.55E-04	1.37E-01	55.55	13499	9	54	2
GO:0006950	response to stress	6.12E-04	1.49E-01	2.08	13499	2402	54	20
GO:0030641	regulation of cellular pH	6.22E-04	1.48E-01	17.86	13499	42	54	3
GO:0044273	sulfur compound catabolic process	6.22E-04	1.46E-01	17.86	13499	42	54	3

GO:0009414	response to water deprivation	6.92E-04	1.60E-01	50	13499	10	54	2
GO:0001101	response to acid chemical	6.99E-04	1.59E-01	5.56	13499	270	54	6
GO:0006820	anion transport	9.15E-04	2.04E-01	4.45	13499	393	54	7
GO:0009063	cellular amino acid catabolic process	9.33E-04	2.05E-01	9.17	13499	109	54	4
Molecular Function								
GO:0009055	electron carrier activity	1.91E-06	7.84E-03	15.96	13499	94	54	6
GO:0016491	oxidoreductase activity	2.04E-06	4.18E-03	5.24	13499	573	54	12
GO:0046943	carboxylic acid transmembrane transporter activity	2.92E-06	3.98E-03	14.85	13499	101	54	6
GO:0005342	organic acid transmembrane transporter activity	3.27E-06	3.35E-03	14.56	13499	103	54	6
GO:0008514	organic anion transmembrane transporter activity	1.10E-05	9.00E-03	11.81	13499	127	54	6
GO:0004611	phosphoenolpyruvate carboxykinase activity	1.57E-05	1.07E-02	249.98	13499	2	54	2
GO:0004613	phosphoenolpyruvate carboxykinase (GTP) activity	1.57E-05	9.18E-03	249.98	13499	2	54	2
GO:0016830	carbon-carbon lyase activity	3.29E-05	1.69E-02	21.74	13499	46	54	4
GO:0005310	dicarboxylic acid transmembrane transporter activity	1.15E-04	5.25E-02	31.25	13499	24	54	3
GO:0008509	anion transmembrane transporter activity	1.88E-04	7.68E-02	7.11	13499	211	54	6
GO:0015037	peptide disulfide oxidoreductase activity	2.33E-04	8.68E-02	83.33	13499	6	54	2
GO:0019531	oxalate transmembrane transporter activity	2.33E-04	7.95E-02	83.33	13499	6	54	2
GO:0016831	carboxy-lyase activity	3.03E-04	9.55E-02	22.73	13499	33	54	3
GO:0008271	secondary active sulfate transmembrane transporter activity	4.33E-04	1.27E-01	62.5	13499	8	54	2
GO:0015116	sulfate transmembrane transporter activity	4.33E-04	1.18E-01	62.5	13499	8	54	2
GO:0022857	transmembrane transporter activity	6.88E-04	1.76E-01	3.33	13499	751	54	10
GO:0016667	oxidoreductase activity, acting on a sulfur group of donors	7.62E-04	1.84E-01	16.67	13499	45	54	3
GO:0003824	catalytic activity	7.92E-04	1.80E-01	1.65	13499	4555	54	30
GO:0016209	antioxidant activity	9.22E-04	1.99E-01	15.62	13499	48	54	3
Cellular Component								
GO:0044429	mitochondrial part	2.29E-06	3.80E-03	4.34	13499	807	54	14
GO:0044444	cytoplasmic part	3.67E-06	3.05E-03	1.62	13499	6626	54	43
GO:0005743	mitochondrial inner membrane	1.48E-05	8.17E-03	6.05	13499	372	54	9
GO:0019866	organelle inner membrane	3.01E-05	1.25E-02	5.53	13499	407	54	9
GO:0005739	mitochondrion	5.62E-05	1.87E-02	3.27	13499	1069	54	14
GO:0031966	mitochondrial membrane	1.78E-04	4.92E-02	4.39	13499	513	54	9
B. Down-regulated								
Biological processes								
GO:0055085	transmembrane transport	1.05E-05	1.45E-01	4.33	13499	936	40	12
GO:0098656	anion transmembrane transport	1.05E-05	7.27E-02	11.77	13499	172	40	6
GO:0034220	ion transmembrane transport	6.69E-05	3.09E-01	4.88	13499	622	40	9

GO:0006811	ion transport	9.71E-05	3.36E-01	3.76	13499	987	40	11
GO:0006820	anion transport	1.37E-04	3.78E-01	6.01	13499	393	40	7
GO:0098660	inorganic ion transmembrane transport	3.18E-04	7.33E-01	5.24	13499	451	40	7
GO:0089711	L-glutamate transmembrane transport	4.63E-04	9.15E-01	61.36	13499	11	40	2
GO:0006835	dicarboxylic acid transport	8.46E-04	1.00E+00	16.07	13499	63	40	3
Molecular Function								
GO:0022857	transmembrane transporter activity	7.69E-06	3.15E-02	4.94	13499	751	40	11
GO:0015075	ion transmembrane transporter activity	1.18E-05	2.41E-02	5.31	13499	636	40	10
GO:0005215	transporter activity	1.38E-05	1.89E-02	4.21	13499	962	40	12
GO:0022891	substrate-specific transmembrane transporter activity	2.39E-05	2.45E-02	4.89	13499	690	40	10
GO:0008509	anion transmembrane transporter activity	3.35E-05	2.74E-02	9.6	13499	211	40	6
GO:0022892	substrate-specific transporter activity	1.08E-04	7.34E-02	4.1	13499	824	40	10
GO:0015103	inorganic anion transmembrane transporter activity	1.18E-04	6.89E-02	15.7	13499	86	40	4
GO:0015293	symporter activity	3.49E-04	1.78E-01	11.84	13499	114	40	4
GO:0005313	L-glutamate transmembrane transporter activity	4.63E-04	2.11E-01	61.36	13499	11	40	2
GO:0015172	acidic amino acid transmembrane transporter activity	5.55E-04	2.27E-01	56.25	13499	12	40	2
Cellular Components								
GO:0005886	plasma membrane	4.92E-04	8.16E-01	2.16	13499	2807	40	18
GO:0031226	intrinsic component of plasma membrane	5.15E-04	4.27E-01	3.38	13499	999	40	10
GO:0044425	membrane part	9.72E-04	5.37E-01	1.72	13499	4697	40	24

Table C.5 Up and downregulated transcripts with evidence of constitutive expression

Up and downregulated transcripts with evidence for population differences in constitutive expression. Note, that categories are separated by (1) shared across both drainages, (2) unique to the Tacotalpa, and (3) unique to the Puyacatengo. Reported are the Transcript IDs, accession number corresponding to the top BLAST hit, gene name, protein name, and the E-value. Values in blue are upregulated and values in red are downregulated transcripts. Due to the large size, the table is provide in a separate excel spreadsheet titled ("Table C.5 – Differential Expression Evolved Differences.xlsx")

Table C.6 Up and downregulated transcripts with evidence for changes in expression upon hydrogen sulfide exposure

Up and downregulated transcripts with evidence for expression changes upon H_2S exposure. Transcripts are organized based on evidence for (1) ancestral plasticity, (2) evolved plasticity in sulfidic populations, or (3) loss of plasticity in sulfidic populations. Reported are the Transcript IDs, accession number corresponding to the top BLAST hit, gene name, protein name, and the E-value. Values in blue are upregulated and values in red are downregulated transcripts. Due to the large size, the table is provide in a separate excel spreadsheet titled ("Table C.6 – Differential Expression Plastic Differences.xlsx")