PHYSIOLOGICAL APPROACH TO UNDERSTAND THE MECHANISMS OF HYDROGEN SULFIDE TOLERANCE USING A FISH STUDY SYSTEM

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PHYSIOLOGICAL APPROACH TO UNDERSTAND

THE MECHANISMS OF HYDROGEN SULFIDE

TOLERANCE USING A FISH STUDY SYSTEM

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Abstract: Hydrogen sulfide (H_2S) is well known as a toxic gas produced by the decomposition of organic matter and geothermal sources and also produced endogenously by cysteine catabolism. Exposure to H_2S drives hormetic effects including toxic inhibition of cytochrome c oxidase of the mitochondrial electron transport chain at high concentrations, and maintenance of normal vascular and neural functions at low concentrations. Abnormal elevation of cellular H_2S, due to either environmental exposure or defective detoxification, is correlated with vascular and metabolic dysfunction in most aerobic organisms, however *Poecilia mexicana* thrives in H_2S rich environments. The cellular mechanisms whereby organisms tolerate extreme H_2S are not fully understood. **Our central hypothesis is that sulfide tolerant fish have an enhanced H_2S detoxification capacity and/or resistance to H_2S toxicity following exposure, relative to non-tolerant fish.** Specifically, we hypothesized that sulfide tolerant fish differentially express genes involved in maintaining H_2S homeostasis. We found significant differences in gene expression patterns related to H_2S detoxification between lab-reared sulfide tolerant and non-tolerant populations originating from the Tacotalpa drainage. Since mitochondria are both the site of H_2S toxicity as well as enzymatic detoxification, we further hypothesized that tolerance is achieved by modifications to mitochondrial respiration. To test this, we compared mitochondrial function between 1) lab-reared and wild captured sulfide tolerant and non-tolerant populations originating from Tacotalpa drainage and 2) wild captured sulfide tolerant and non-tolerant populations originating from the Puyacatengo and Pichucalco drainages. We predicted that sulfide tolerant fish are able to maintain mitochondrial respiration in the presence of increasing concentrations of H_2S relative to non-tolerant fish and that the sulfide tolerant population captured from Pichucalco drainage, which has the highest concentration of environmental H_2S compared to other drainages tested, would exhibit the greatest degree of H_2S tolerance compared to the sulfide tolerant populations from drainages with lower environmental H_2S. We determined that mitochondria from sulfide tolerant fish have increased maximal and spare respiratory capacities following exposure to high concentrations of H_2S, relative to non-tolerant fish, and that the population captured from Pichucalco exhibits the greatest degree of tolerance compared to the other two drainages.

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CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Chemical properties of Hydrogen sulfide

Hydrogen sulfide (H_2S) is a toxic, colorless, and flammable gas with the characteristic odor of rotten eggs (Reiffenstein, Hulbert et al. 1992, Szabo 2007, Li, Hsu et al. 2009). H_2S exerts toxicity by inhibiting cellular respiration and can be lethal (Cooper and Brown 2008). H_2S solubility in lipophilic solvents is higher than in water, thus it can easily diffuse through the plasma membrane and reach intracellular compartments (Reiffenstein, Hulbert et al. 1992, Mancardi, Penna et al. 2009). H_2S, in its gaseous form, has many functions. However, it is very short lived due to various factors, such as oxidation with the presence of oxygen and pH (Kolluru, Shen et al. 2013). In aqueous solutions H_2S dissociates yielding a hydrosulfide (HS^-) ion (pKa = 7.04) and sulfide (S^{2-}) ion (pKa = 11.96) (Reiffenstein, Hulbert et al. 1992, Wang 2002) (equation 1). In the body, at a physiological pH of 7.4, approximately one-third of H_2S exists as the un-dissociated form (S^{2-}) and two-thirds as the dissociated form (HS^-) (Reiffenstein, Hulbert et al. 1992).

$$H_2S \rightarrow H^+ + HS^- \rightarrow H^+ + S^{2-}$$

Equation 1: H_2S dissociation in aqueous solution

1.2 Environmental H_2S

In nature, H_2S is produced primarily by the decomposition of organic matter and is found in sediments and grass marshes of the intertidal zone, natural gas, volcanic and sulfur-spring emissions as well as deep-sea hydrothermal vents (Dorman, Moulin et al. 2002, Hildebrandt and Grieshaber 2008, Li, Hsu et al. 2009, Hooper, Shane et al. 2010). In marine sediments, sulfur bacteria produce H_2S due to breakdown of organic matter (Bagarinao 1992). Once oxygen is depleted by aerobic bacteria in the sediments, anaerobic bacteria use H_2S as an alternate electron acceptor in decomposing the remaining organic matter (Bagarinao 1992). Due to their high temperature deep-sea hydrothermal vents are considered an extreme environment. High temperature interactions between seawater and rocks produce H_2S at these vents (Bagarinao 1992). In addition, several different genera of colonic bacteria present in the microenvironment of the gut are capable of producing H_2S by using the fermentation products in the colon (Gibson, Macfarlane et al. 1993, Levine, Ellis et al. 1998).

H_2S is also emitted during industrial processes, such as the manufacture of pulp in paper mills (Marttila, Jaakkola et al. 1994), from tanneries during the processing of leather (Boshoff, Duncan et al. 2004), and oil refineries (Li, Hsu et al. 2009). Therefore, H_2S is a well-known industrial pollutant. Environmental exposure to H_2S quickly affects the eyes and respiratory membranes, such as olfactory and nasal mucosa, while the major target affected is the nervous system (Reiffenstein, Hulbert et al. 1992). According to Oesterhelweg & Püschel, human volunteers exposed to concentrations of up to 30-ppm H_2S exhibited symptoms including headache, nausea, vomiting and breathlessness and under higher H_2S concentrations (150 – 250 ppm), respiratory tract irritation and pulmonary edema were observed (Oesterhelweg and Püschel 2008, Li, Hsu et al. 2009). Table 1 highlights the responses in humans to various concentrations of H_2S gas (Reiffenstein, Hulbert et al. 1992). At the cellular level,

H_2S reversibly inhibits cytochrome c oxidase (COX), the terminal electron acceptor of the mitochondrial electron transport chain, by binding with the heme a_3 group and Cu_B in COX (Cooper and Brown 2008). This inhibition of cellular respiration by H_2S can ultimately lead to death (Reiffenstein, Hulbert et al. 1992, Cooper and Brown 2008).

Table 1.1: Human physiologic responses to various concentrations of H_2S

Concentration, ppm	**Response**
0.003-0.02	Odor threshold
3-10	Obvious unpleasant odor
20-30	Strong offensive odor
30	Sickening sweet odor
50	Conjunctival irritation
50-100	Respiratory irritation
100-200	Olfactory paralysis
250-500	Pulmonary edema
500	Anxiety, headache, ataxia, dizziness, tachypnea, knockdown
500-1000	Respiratory paralysis, neural paralysis, cardiac arrhythmias, death

Inhibition of COX by H_2S occurs in other organisms other than humans. Aquatic vertebrates may be subjected to substantially elevated ambient H_2S through biogenic production and from a variety of industrial and agricultural processes (Bagarinao 1992). In freshwater fishes, exposure to high concentrations of H_2S can lead to reduced growth and survival, necrosis, lower survival of eggs, and higher incidence of offspring deformities (Bagarinao 1992).

1.3 Endogenous production of H_2S

Over the years, H_2S had only been recognized for its toxicity as an environmental hazard. Prior to Abe and Kimura's report in 1996, there were no known physiologic functions for H_2S (Abe and Kimura 1996). However, multiple studies have recently demonstrated that H_2S is produced endogenously at very low concentrations and functions as a physiologically relevant signaling molecule (Du, Hui et al. 2004, Li, Hsu et al. 2009). The best understood physiological response to H_2S is vasodilation. The K_{ATP} channel is the major molecular target of H_2S for its vasorelaxant effect. H_2S is able to open K_{ATP} channels (which hyperpolarize the cell) in vascular smooth muscle cells in a non-ATP associated manner, promoting vasodilation, which is critical for blood pressure regulation (Zhao, Zhang et al. 2001). Other than the vasodilatory effect, recent studies signify that H_2S is important in multiple physiological functions including anti-inflammatory, pro-angiogenic, anti-apoptotic, colonic smooth muscle relaxation, and anti-oxidant effects (Szabo 2007, Li, Rose et al. 2011, Olson 2011, Stein and Bailey 2013).

H_2S is produced in animal cells as a by-product of cysteine catabolism (Stipanuk and Ueki 2011) by enzymes in the reverse transsulfuration pathway. Cysteine is a sulfur-containing amino acid, which is important for protein synthesis, detoxification and diverse metabolic functions. Cystathionine γ-lyase (CSE) and cystathionine beta-synthase (CBS) are enzymes responsible for endogenous production of H_2S in tissues via utilization of L-cysteine as the main substrate (Wang 2002, Leffler, Parfenova et al. 2006, Cooper and Brown 2008, Qu, Lee et al. 2008). Thiocysteine and homocysteine are also substrates for CSE and CBS enzymatic production of H_2S (Qu, Lee et al. 2008). CBS and CSE have different tissue distributions (Qu, Lee et al. 2008) as demonstrated by studies reporting CBS-dependent H_2S production in the nervous system whereas CSE is the main H_2S forming enzyme in the cardiovascular system (Moore, Bhatia et al. 2003, Qu, Lee et al. 2008).

Another enzyme involved with endogenous H_2S production is 3-mercaptopyruvate sulfurtransferase (MST) which, along with cysteine aminotransferase (CAT) produces H_2S from cysteine and α-ketoglutarate (α-KG) (Shibuya, Mikami et al. 2009, Kimura 2011).

Other than enzymatic endogenous production of H_2S, non-enzymatic production involves glutathione (GSH) as the substrate (Wang 2012). Glutathione (GSH) is a water-soluble tripeptide composed of the amino acids glutamine, cysteine, and glycine and functions as an important antioxidant; GSH plays a role in the detoxification of a variety of electrophilic compounds and peroxides in cells (Townsend, Tew et al. 2003). Figure 1 summarizes the enzymatic and non-enzymatic endogenous production of H_2S (Wang 2012). Moreover, nonenzymatic reduction of elemental sulfur to H_2S using reducing equivalents obtained from the oxidation of glucose (equation 2) provides additional endogenous H_2S (Searcy and Lee 1998, Wang 2012).

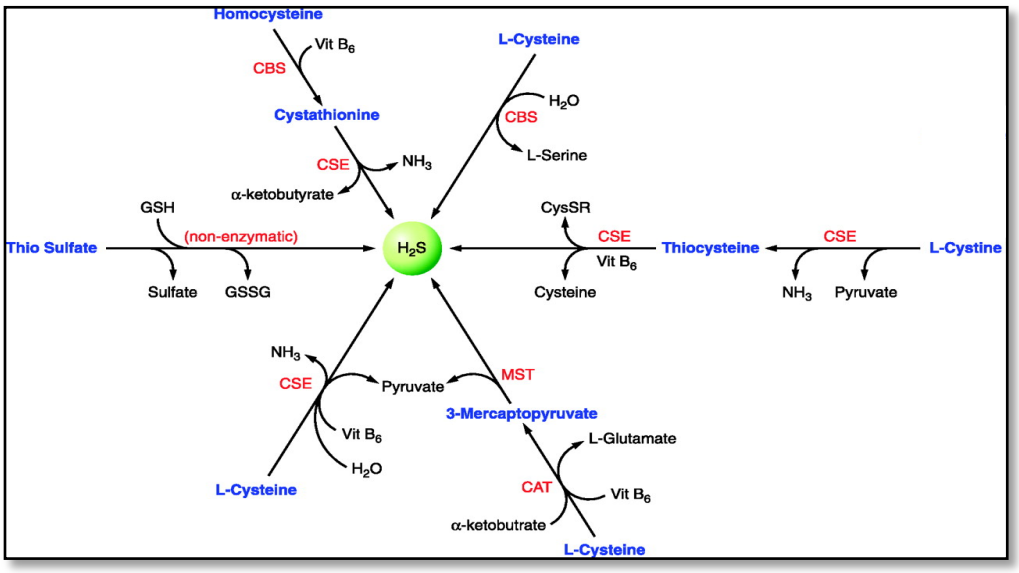


Figure 1.1: The enzymatic and non-enzymatic endogenous production of H_2S modified from Wang in 2012 (Wang 2012)

$$2C_6H_{12}O_6 + 6\ S_0 + 3H_2O \rightarrow 3C_3H_6O_3 + 6H_2S + 3CO_2$$

Equation 2: Endogenous nonenzymatic production of H_2S

Finally, it is important to discuss endogenous and exogenous production of H_2S in the GI tract. H_2S is endogenously produced in colonic smooth muscle cells and regulates gastrointestinal motility (Hosoki, Matsuki et al. 1997, Farrugia and Szurszewski 2014), whereas bacteria in the GI tract exogenously produce H_2S. The main substrates used by sulfate-reducing bacteria (SRB) for H_2S production are fermentation products formed by other bacteria, such as acetate, propionate, lactate, butyrate, succinate, ethanol, pyruvate and some amino acids (Gibson, Macfarlane et al. 1993). SRB generate H_2S in the lumen of the colon, therefore exposing epithelial colonocytes to "exogenous" H_2S that can readily permeate cells and enter the blood stream. However, normal colonic epithelial cells efficiently detoxify the vast majority of this "exogenous" H_2S generated by bacteria (Levitt, Furne et al. 1999, Lagoutte, Mimoun et al. 2010).

1.4 Detoxification of H_2S

Since endogenous H_2S is produced via normal metabolic processes and exogenous H_2S is produced within the gut by bacteria, a means to continuously detoxify H_2S is necessary to prevent the inevitable accumulation of lethal levels of H_2S within the body. Sulfide detoxification via oxidation occurs within the body in different tissues (Grieshaber and Volkel 1998). Detoxification occurs predominantly in the liver and colon mainly in mammals, and the metabolites are excreted in the urine and feces (Levine, Ellis et al. 1998, Levitt, Furne et al. 1999).

While H_2S at concentrations of 50 – 100 ppm (Reiffenstein, Hulbert et al. 1992) inhibits mitochondrial activity, mitochondria are the sites of detoxification and removal of H_2S (Levitt, Furne et al. 1999, Stein and Bailey 2013). Recently, sulfide:quinone oxidoreductase (SQR), a mitochondrial membrane flavoprotein, has been shown to be the first enzyme in the mitochondrial sulfide oxidation enzyme system (Hildebrandt and Grieshaber 2008). In mitochondria, two H_2S molecules are oxidized to elemental sulfur (S^o) via SQR, resulting in the formation of SQR-persulfide (SQR-SSH) and

electrons released from this process are fed into the ubiquinone (Q) pool further driving the generation of ATP from oxidative phosphorylation (Hildebrandt and Grieshaber 2008). One of the two persulfides on the oxidized SQR is further oxidized to hydrogen sulfite (H_2SO_3) by sulfur dioxygenase present in the mitochondrial matrix and then the other persulfide group joins with H_2SO_3 to produce thiosulfate ($H_2S_2O_3$) by sulfur transferase, and is excreted. Thiosulfate reductase may also convert $H_2S_2O_3$ to sulfate (H_2SO_4) (Hildebrandt and Grieshaber 2008, Blachier, Davila et al. 2010, Stein and Bailey 2013). Figure 2 shows the sulfide oxidation pathway modified from Hildebrandt and Grieshaber in 2008 (Hildebrandt and Grieshaber 2008, Lagoutte, Mimoun et al. 2010).

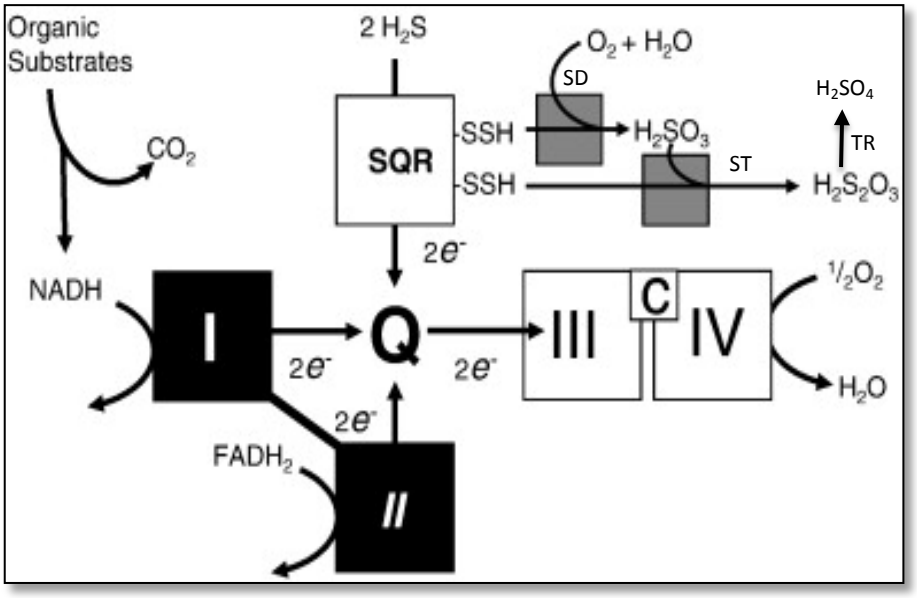


Figure 1.2: Sulfide oxidation pathway in the mitochondria modified from Hildebrandt and Grieshaber in 2008 (SD: sulfur dioxygenase, ST: sulfur transferase and TR: Thiosulfate reductase) (Hildebrandt and Grieshaber 2008)

In the colon, H_2S produced by SRB is processed by a reaction that converts this gas to a nonvolatile metabolite, thiosulfate, which is excreted in the urine and feces (Levine, Ellis et al. 1998, Levitt, Furne et al. 1999, Hildebrandt and Grieshaber 2008). These strict anaerobes within the gut use H_2S as a terminal electron acceptor during oxidative reactions (Gibson, Macfarlane et al. 1993). Since

H_2S is a potentially toxic compound for the intestinal epithelial cells when present in excess, SQR activity in the epithelia for sulfide detoxification is exceptionally robust and very important (Blachier, Davila et al. 2010).

1.5 Extremophile animals

Even though elevated exogenous H_2S levels in the environment are toxic to most terrestrial and aquatic organisms, there are some organisms that thrive in extreme environments with high H_2S concentrations (Reiffenstein, Hulbert et al. 1992), such as the lugworm (*Arenicola marina*) (Groenendaal 1980), tube worms (*Riftia pachyptila*) (Goffredi, Childress et al. 1997), echiuran worm (*Urechis caupo*) (Ma, Zhang et al. 2012), killifish (*Fundulus parvipinnis*) (Bagarinao and Vetter 1992), tambaqui fish (*Colossoma macropomum*) (Affonso, Polez et al. 2002) and Atlantic molly (*Poecilia mexicana*) (Plath, Hauswaldt et al. 2007). Proposed adaptive mechanisms for sulfide tolerance and protection include exclusion of sulfide at the body wall in the lugworm owing to its impermeability and the presence of a thick covering of sulfide-oxidizing bacteria (Groenendaal 1980). Mitochondrial sulfide oxidation to less toxic sulfur compounds via SQR activity is also an important adaptation shown by vertebrates and invertebrates (Grieshaber and Volkel 1998).

The lugworm, *A. marina*, is often used to study strategies of sulfide tolerance because it is highly abundant in intertidal flats where micromolar concentrations of sulfide regularly occur (Völkel and Grieshaber 1994, Grieshaber and Volkel 1998). *A. marina* mitochondria oxidize sulfide to thiosulfate via SQR (Völkel and Grieshaber 1994). This sulfide oxidation occurs in body wall tissue, which serves as the first defensive barrier against its harsh environment and thus may prevent sulfide from reaching the internal organs at a significant concentration (Grieshaber and Volkel 1998). The Echiuran worm, *U. caupo*, is another sulfide-adapted animal found along the coast of California, which is similar to the lugworm's ecological habitat (Ma, Zhang et al. 2012). Like the lugworm, echiurans worm can produce ATP from sulfide oxidation in the mitochondria (Ma, Zhang et al.

2012). The deep-sea hydrothermal vent tubeworm, *R. pachyptila*, relies upon internal chemolithoautotrophic bacterial symbionts to support its large size and high growth rates while supplying sulfide to the bacteria (Goffredi, Childress et al. 1997). H_2S accumulation is limited in these organisms because of their symbionts and any remaining sulfide is bound by hemoglobin (Goffredi, Childress et al. 1997).

The Atlantic molly (*P. mexicana*) is a live-bearing fish species that can survive in extreme sulfur habitats, such as sulfide freshwater springs and sulfur caves in Southern Mexico (Tobler, Schlupp et al. 2006). *P. mexicana* is found in two populations, colonized separately within a small area. These two populations are considered sulfide tolerant and non-tolerant populations, due to the presence or absence of H_2S in their habitat (Tobler, DeWitt et al. 2008, Tobler, Palacios et al. 2011). This patchy occurrence of H_2S in their habitat is considered a strong divergent selective pressure and an environmental stressor between the two populations. There are four sulfur spring drainages (Rio Tacotalpa, Rio Pichucalco, Rio Puyacatengo and Rio Ixtapangajoya) located in Southern Mexico that are the habitat to these populations (Figure 3). They have evolved in convergence of many phenotypic traits in behavior, morphology and physiology. Phenotypic variations have been observed in these two populations (Tobler, DeWitt et al. 2008). For example, in comparison to non-tolerant populations, sulfide tolerant populations exhibit increased head size and large gill surface area (Tobler and Hastings 2011). Larger gills provide more surface area for oxygen uptake in this extreme habitat (Bagarinao 1992, Tobler and Hastings 2011). Due to the hypoxic environment, the sulfide tolerant population shows behavioral changes such as performing aquatic surface respiration (ASR) to maximize of uptake oxygen (Plath, Tobler et al. 2007). Phenotypic adaptations are maintained in sulfide tolerant fish reared in the lab in generations without H_2S. Furthermore, recent evidence demonstrated that tolerant populations in two (Puyacatengo and Pichucalco) out of three sulfur spring drainages in Southern Mexico have evolved H_2S-resistant COX via amino acid substitutions causing conformational changes in COX that prevent

H_2S binding, suggesting that aerobic respiration may proceed in the presence of elevated H_2S (Pfenninger, Lerp et al. 2014).

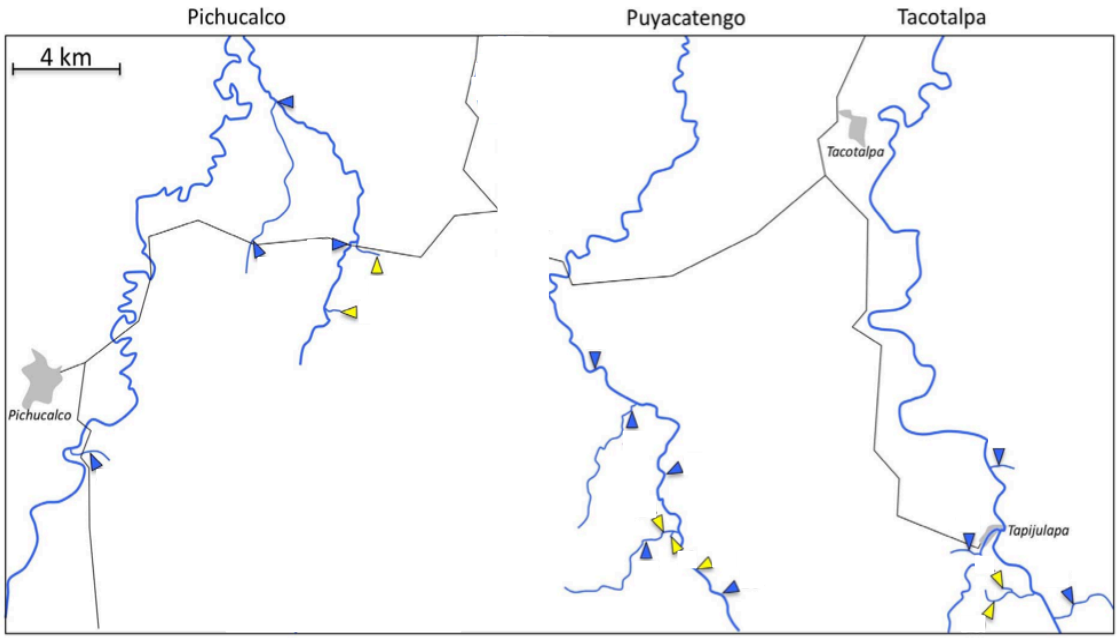


Figure 1.3: Three freshwater sulfur-springs located in southern Mexico modified from Palacios et al. (2013), Blue represent non-sulfidic sites and yellow represents sulfidic sites (Palacios, Arias-Rodriguez et al. 2013).

How *P. mexicana* sulfide tolerant fish can withstand extreme H_2S is an emerging area of research. Although phenotypic differences are known, the physiological or the cellular mechanisms of tolerance are not well defined. Pfenninger et al.(2014) revealed that H_2S tolerance displayed by the sulfide tolerant population from the Tacotalpa drainage is not due to an H_2S-resistant COX (Pfenninger, Lerp et al. 2014). Therefore, another yet to be identified mechanism enables the survival of these fish in their extreme H_2S environment. How can *P. mexicana* in Tacotalpa drainage tolerate this extreme H_2S? Are they better able to detoxify H_2S? Do they develop any resistance to withstand

H_2S toxicity? Is the tolerance capacity of this population due to one of the above reasons or a combination of several effects?

Poecilia mexicana is a valuable vertebrate model to understand the mechanisms of H_2S tolerance mainly due to the presence of two populations within the same species, wherein one inhabits sulfide springs (tolerant) and the other (non-tolerant) in adjacent non-sulfidic habitats (Tobler, DeWitt et al. 2008, Tobler, Palacios et al. 2011, Palacios, Arias-Rodriguez et al. 2013). The main advantage of using this vertebrate model is that one population lives in naturally occurring H_2S while the other closely related population lives in H_2S-free freshwater providing a unique opportunity to compare the mechanisms that are essential for tolerance in the same vertebrate species. While other H_2S tolerant organisms such as lugworms, echiuran worms and tubeworms are used for H_2S studies, non-tolerant populations don't exist. Another advantage of using *P. mexicana* as the model species is the whole transcriptome, annotated by Kelley et al. in 2012 (Kelley, Passow et al. 2012), is available. In this study we use transcriptome data to design primers for genes to study the molecular basis for adaptation in the tolerant population of *P. mexicana* to its extreme environment. Collectively, these features of *P. mexicana* make it a unique model to study the mechanisms involved in H_2S detoxification and resistance in a vertebrate species.

1.6 Rationale of the study and Broad Aims

Even though there is information about phenotypic diversity (Tobler, DeWitt et al. 2008), whole transcriptome differences (Palacios, Arias-Rodriguez et al. 2013) and behavioral changes (Plath, Tobler et al. 2007) between the two populations of *P. mexicana*, the cellular mechanisms whereby organisms tolerate extreme H_2S are not currently understood (Tobler, Passow et al. 2016). There is a fundamental gap in our knowledge about the gene expression and mitochondrial functional differences between these two populations following exposure to H_2S. Therefore, our **central hypothesis is that sulfide tolerant fish differentially modify H_2S detoxification capacity and**

resistance to H_2S toxicity following exposure, relative to non-tolerant fish. To test this, we propose the following three aims.

Aim 1: Measure expression of a panel of genes relevant to H_2S physiology across tissue types in lab-reared sulfide tolerant versus non-tolerant fish populations (Tacotalpa lineage) following H_2S exposure. **Aim 2:** Compare mitochondrial function in tolerant versus non-tolerant fish livers upon increasing doses of H_2S in both lab-reared and wild-captured fish (Tacotalpa lineage). **Aim 3:** Identify mitochondrial adaptations in wild-captured tolerant versus non-tolerant populations of Puyacatengo and Pichucalco lineages, which both harbor a H_2S-resistant COX enzyme.

CHAPTER II

DEVELOPMENT OF A LABORATORY DESIGN FOR H_2S EXPOSURE:

GENE EXPRESSION ANALYSES

2.1 Introduction

Physiochemical stressors affect biological processes at all levels of organization and hydrogen sulfide (H_2S) is a stressor that occurs in a wide variety of aquatic environments (Bagarinao 1992, Grieshaber and Volkel 1998). In response to continuous environmental exposure of such physiological stressors, organisms often modulate physiological pathways (Feder and Hofmann 1999). *Poecilia mexicana* is a widespread live bearing fish occurring along the Atlantic versant of Mexico and northern Central America. In southern Mexico, multiple lineages have independently colonized freshwater springs rich in H_2S (Tobler, Palacios et al. 2011, Palacios, Arias-Rodriguez et al. 2013). Compared to populations in adjacent non-sulfidic habitats, sulfide spring fish are

characterized by a higher sulfide tolerance (Tobler, DeWitt et al. 2008, Tobler and Hastings 2011, Tobler, Palacios et al. 2011). Sulfide tolerant and non-tolerant populations have also diverged in morphology (Tobler and Hastings 2011, Tobler, Palacios et al. 2011), energy metabolism (Passow, Henpita et al. 2017), behavior (Plath, Tobler et al. 2007), and life history strategies (Riesch, Plath et al. 2010, Riesch, Plath et al. 2014). Despite the lack of major physical barriers preventing fish migration, sulfide tolerant and adjacent non-tolerant populations are genetically differentiated, and reproductive isolation is at least partially mediated by natural and sexual selection against migrants (Tobler, Riesch et al. 2009, Plath, Hermann et al. 2010, Plath, Pfenninger et al. 2013). Even though this system is well characterized in terms of environmental and phenotypic variation, the molecular basis of adaptation to perpetual H_2S exposure remains poorly understood.

To begin to understand the mechanism of H_2S tolerance, it is important to investigate the expression of genes relevant to H_2S physiology. Table 2.1 shows the panel of genes we selected and their relevance to H_2S biology.

As mentioned in the first chapter, SQR is responsible for normal H_2S detoxification in all tissues (Levitt, Furne et al. 1999, Hildebrandt and Grieshaber 2008). Most metazoans can detoxify H_2S to some extent through an evolutionarily conserved SQR detoxification pathway linked to the respiratory chain (Hildebrandt and Grieshaber 2008). SQR, which is associated with the inner mitochondrial membrane, catalyzes H_2S in the first step of mitochondrial metabolism of sulfide and paradoxically provides two electrons to the electron transport chain for oxidative phosphorylation (Hildebrandt and Grieshaber 2008). Thus, SQR detoxifies H_2S but also provides a means to utilize H_2S for energy production. Therefore, understanding differences in SQR gene expression by sulfide tolerant populations relative to non-tolerant populations (with and without exposure to H_2S) may provide insight into the potential mechanisms of H_2S tolerance.

Table 2.1: Genes of interest relevant to H_2S biology

Gene of Interest	Relevance to H_2S	References
SQR *(sulfide:quinone oxidoreductase)*	Mitochondrial H_2S detoxification	(Hildebrandt and Grieshaber 2008)
COX *(cytochrome c oxidase)*	Mitochondrial respiration and the primary target of H_2S toxicity	(Reiffenstein, Hulbert et al. 1992, Cooper and Brown 2008)
CSE *(cystathionine γ-lyase)*	Involved in endogenous production of H_2S in vascular tissues	(Wang 2002, Leffler, Parfenova et al. 2006, Cooper and Brown 2008, Qu, Lee et al. 2008)
VEGF *(vascular endothelial growth factor)*	Hypoxia responses that drives angiogenesis	(Liu, Pan et al. 2010, Qipshidze, Metreveli et al. 2012)
CYP *(cytochrome p450)*	H_2S metabolizing enzyme	(Caro, Thompson et al. 2011)

COX is the terminal electron acceptor of the mitochondrial electron transport chain and the primary target of H_2S toxicity (Cooper and Brown 2008). Thus, tissues with high oxygen demand (e.g., brain and heart) are especially sensitive to disruption of oxidative metabolism by H_2S due to its inhibition of COX. Measuring COX gene expression in response to H_2S in tolerant fish may suggest possible adaptations to inhibition of COX by H_2S.

Vascular endothelial growth factor (VEGF) is induced by hypoxia and is an important

driver of angiogenesis, which results in endothelial cell (EC) proliferation, migration and new vessel formation (Hoeben, Landuyt et al. 2004, Liu, Pan et al. 2010). ECs are both targets and sources of H_2S and H_2S promotes the growth of EC (Papapetropoulos, Pyriochou et al. 2009). Stimulation of ECs with VEGF upregulates CSE gene expression and enzymatic activity, thereby enhancing H_2S release (Papapetropoulos, Pyriochou et al. 2009). In the presence of cysteine or homocysteine, CSE catalyzes the production of H_2S (Stipanuk and Ueki 2011). CSE is expressed abundantly in the cardiovascular system (Hosoki, Matsuki et al. 1997, Moore, Bhatia et al. 2003). Recent biomedical research indicates that endogenous H_2S plays a critical role in cell signaling, and deficiency or excess of H_2S can lead to disease development (Li and Moore 2008, Li, Rose et al. 2011, Whiteman, Le Trionnaire et al. 2011). Consequently, organisms may not attempt to just eliminate H_2S from their system, but rather maintain optimal concentrations to assure proper physiological functioning. While we have an increasing understanding of sulfide metabolism in low tolerance species, it remains largely unknown how organisms like sulfide tolerant *P.mexicana* withstand continuous exposure to environmental H_2S and cope with its adverse effects. Therefore, it is important to investigate VEGF and CSE gene expression in the non-tolerant population following H_2S exposure in comparison to the sulfide tolerant population.

The CYP family of enzymes is mainly found in the liver and is critical for the metabolism of endogenous and exogenous substances (Wang, Chen et al. 2013) and a superfamily of heme-containing enzymes (Guengerich 1991). CYP enzymes are a significant source of reactive oxygen species (ROS) and oxidative stress in the liver (Caro, Thompson et al. 2011). CYP2J6 of *P. mexicana* (which is homologous to human CYP2J6) is explored in this study. The CYP2J family enzymes metabolize H_2S to a toxic metabolite that aggravates toxicity (Caro, Thompson et al. 2011). Therefore, CYP2J6 expression upon H_2S exposure may be down regulated in tolerant fish to reduce the toxicity of H_2S.

In this chapter, we investigated gene expression of the primary target of H_2S toxicity

(COX; (Cooper and Brown 2008)); the enzyme involved in the first step of sulfide oxidation (SQR; (Hildebrandt and Grieshaber 2008)); a key enzyme in endogenous H_2S production (Cystathionine γ lyase, CSE; (Stipanuk and Ueki 2011)); an enzyme which has been implicated in exacerbating H_2S toxicity (CYP; (Caro, Thompson et al. 2011)); and – considering the tight environmental and physiological links between H_2S and hypoxia (Bagarinao 1992, Fago, Jensen et al. 2012) – a key cytokine mediating organismal hypoxia responses (vascular endothelial growth factor, VEGF; (Ferrara and Davis-Smyth 1997). Considering the strong patterns of local adaptation in fish from sulfidic and non-sulfidic habitats, we expected population differences in either the constitutive expression of genes or in H_2S-induced gene expression responses. Specific predictions for each gene are highlighted in Table 2.2.

To measure the expression of our candidate genes, we collected gill, liver, heart and brain tissue from both populations as target tissues. We primarily focused on gill tissue for our study because the gills are in direct contact with environmental H_2S. The gill is a critical biological barrier that, due to its permeability and retention, determines the potential uptake of gases from the water. Thus, changes in gill gene expression of our candidate genes may be the most informative for our initial study. The liver is another tissue of interest in this study because it is the main organ where H_2S is detoxified (Hildebrandt and Grieshaber 2008). The heart and brain are the most energy demanding organs in the body (Cooper and Brown 2008). Therefore, measuring the expression of selected genes relevant to H_2S physiology in heart and brain is also crucial as H_2S is a potent inhibitor of ATP production.

Hypothesis and predictions

Here, we focused on a vertebrate that naturally inhabits sulfidic and non-sulfidic environments to quantify the expression of genes putatively involved in responses to deviations of H_2S homeostasis through exposure to environmental H_2S. We hypothesized that sulfide

tolerant fish differentially express SQR, COX, CSE, CBS, VEGF and CYP relative to non-sulfidic population under both control and H_2S exposure conditions, and we were particularly interested in elucidating potential effects of genetic variation among populations, and their interactions. Here, we focused on one evolutionary lineage from the Rio Tacotalpa drainage (Tobler, Schlupp et al. 2006, Tobler, DeWitt et al. 2008), including a sulfide tolerant and a non-tolerant population, and used common garden raised individuals that have never previously been exposed to H_2S to test for variation in candidate gene expression upon exposure to two different sulfide treatments (short and long term exposure).

SQR expression is predicted to increase in both populations in the presence of environmental H_2S. Specifically, we predicted that the sulfide tolerant population expresses more SQR with and without H_2S, in comparison to the non-tolerant population, to enhance H_2S detoxification as they adapted. Gene expression of COX was expected to be high in both populations under H_2S exposure because H_2S inhibits COX enzyme functionally, thus we predicted this inhibition would result in feedback to upregulate COX gene expression. We further predicted that VEGF would be upregulated in both non-tolerant and tolerant populations under short and long term H_2S exposure in response to hypoxic conditions, potentially initiating angiogenesis to increase capillary density for maximal delivery of oxygen to tissues. It was predicted that the expression of CSE (an enzyme responsible for endogenous H_2S production) would be low in the sulfide tolerant population under both conditions due to ancestral exposure to severely elevated H_2S. As previously mentioned, stimulation of ECs with VEGF upregulates CSE gene expression and enzymatic activity and increases H_2S release (Papapetropoulos, Pyriochou et al. 2009). Therefore, CSE may be transiently upregulated under H_2S exposure along with the upregulation of VEGF due to hypoxia in the non-tolerant population. CYP was predicted to be down regulated in the sulfide tolerant population under H_2S exposure, possibly as an adaptive means to reduce the formation of toxic metabolites. Furthermore, we predicted that gene

expression variation among individuals under H_2S exposure would be more pronounced in the gills as opposed to livers, heart and brain, because gills are in direct contact with environmental H_2S. These predictions regarding gene expression are summarized in table 2.2.

Table 2.2: Candidate genes investigated in this study, with predicted directions and relative strength of expression changes in sulfide tolerant fish in the presence or absence of environmental H_2S and non-tolerant fish in the presence of environmental H_2S, relative to non-tolerant fish in the absence of environmental H_2S

Gene of Interest	Absence of H_2S	Presence of H_2S	
	Sulfide tolerant	Non-tolerant	Sulfide tolerant
SQR	↑↑	↑	↑↑
COX	↓	↑	↑
CSE	↓	↑↑	↓
VEGF	↑	↑↑	↑
CYP	↓	↓↓	↓

2.2 Methods

2.2.1 Fish populations

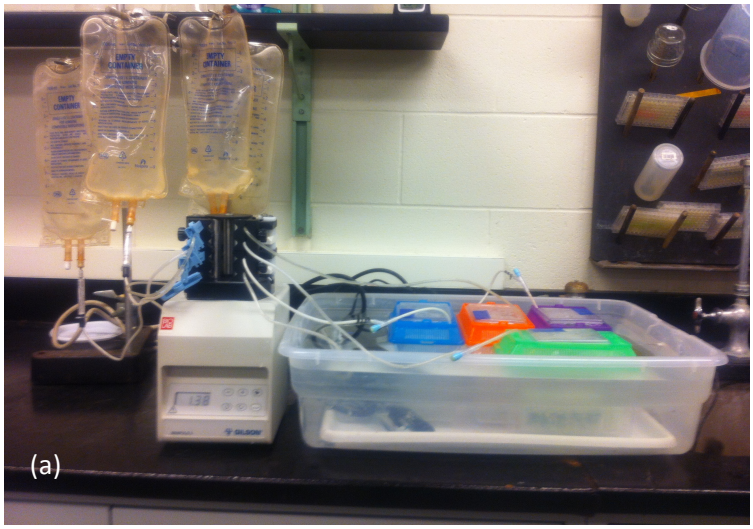
Two populations of sulfide tolerant and non-tolerant *P. mexicana*, were used for this study. Lab-reared fish were from stocks at Oklahoma State University that were established in 2010. Initially fish in those two populations were captured from the Rio Tacotalpa drainage in Mexico, one from a H_2S rich spring (El Azufre I) and one from an adjacent freshwater stream (Arroyo Bonita). Then the stocks were maintained under non-sulfidic conditions. All animals used in this study were born and raised in captivity; hence, even fish derived from the sulfide

spring population have not had any exposure to environmental H_2S during their lifetime. All procedures were approved by the Institutional Animal Care and Use Committee at Oklahoma State University (protocol #AS 1015).

2.2.2 H_2S exposure experimental setup

For sulfide exposure experiments, fish were individually placed in 1-L containers situated in a temperature controlled (25 °C) water bath (Fig. 2.1a, b). Peristaltic pumps were used to continuously supply fluids from reservoirs that either contained a H_2S solution or a non-sulfidic control at a flow rate of 84 mL/h. Experimental containers continuously overflowed into the water bath, such that the fluid volume in the experimental containers remained constant. There were two distinct H_2S exposure treatments (short and long term) and without H_2S as a control. (1) For the short-term exposure treatment, individuals from the sulfidic and the non-sulfidic population were exposed to sulfide from a 5.0 mM H_2S stock solution for 8 h. (2) For the long-term exposure treatment, individuals from the sulfidic and the non-sulfidic population were exposed to sulfide from a 0.5 mM H_2S stock solution for 48 h.

H_2S stock solutions were generated by dissolving an appropriate amount of sodium sulfide hydrate ($Na_2S \cdot 6H_2O$) in water. Prior to the sulfide dissolution the water was deoxygenated through purging with nitrogen to prevent sulfide oxidation in an aqueous solution (Cline and Richards 1969, Chen and Morris 1972, Butler, Schoonen et al. 1994). Solutions were stored in IV bags to prevent contact with oxygen throughout trials. For control treatments (n = 6 for each population), the same water was used without adding sodium sulfide.



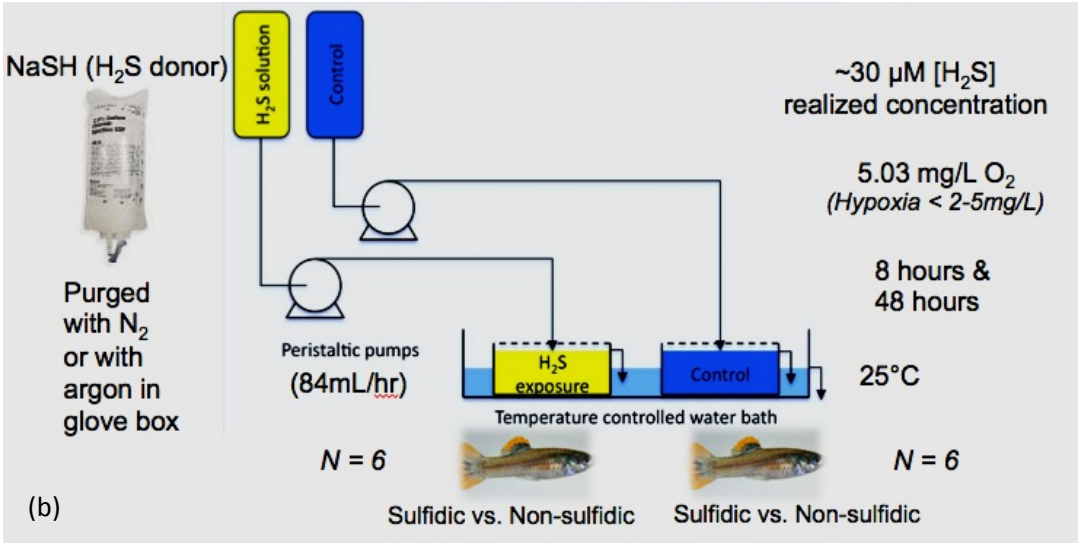


Figure 2.1: (a) Original experimental setup. (b) A diagram of the experimental setup for H_2S exposure of individual fish modified from Tobler, et al (2014). Sulfidic water (via addition of H_2S stock solutions) and non-sulfidic water (control) were continuously pumped into tanks by peristaltic pumps (Tobler, Henpita et al. 2014).

Even though we exposed fish to 5.0 mM and 0.5 mM H_2S concentrations for short term and long term exposure experiments respectively, the realized sulfide concentrations measured in

the short-term treatment averaged 38.05 ± 16.74 μM and 19.28 ± 10.15 μM (mean±SD) in the long-term treatment. Hence, despite the different stock solution concentrations used, realized sulfide concentrations in the experimental setup were relatively close between exposure types, which was likely caused by sulfide oxidation in an aqueous solution and volatilization (Cline and Richards 1969, Chen and Morris 1972, Bagarinao 1992). Oxygen concentrations averaged 5.03 mg/L and 2.82 mg/L in the short and long term exposure trials, respectively. Since lower than 2 mg/L of oxygen concentration is considered hypoxic, both type of experimental trials were not hypoxic to fish. Overall, experimental concentrations of H_2S both in the short and long term treatments were lower than in sulfide springs in nature, where long-term average concentrations are approximately 130 μM in springs of the Tacotalpa drainage (Tobler, Schlupp et al. 2006). However, reduced experimental concentrations were necessary to assure the survival of non-sulfidic fish for the duration of the experiment. For each population, exposure, and exposure type, the sample size was n = 6; the exception was the long-term exposure control group of the sulfidic population, for which we could not amplify any RNA in one individual (total N = 47).

2.2.3 RNA isolation and cDNA synthesis for gill, heart, liver and brain tissues

After the exposure experiments, individual fish were immediately euthanized by pithing (Nickum, Bart Jr et al. 2004), and then gills on both sides of the body, liver, heart and brain were removed. Tissues were immediately stored in the fixative RNALater (Life Technologies, Grand Island, NY, USA) at −20 °C (Wong, Wiley et al. 2012). For RNA extraction, gill, liver and heart tissues were transferred to tubes containing RNA lysis buffer (Qiagen, Germantown, MD, USA)) with β-mercaptoethanol (1:100) and homogenized (PRO Scientific Bio-Gen PRO200, Oxford, CT, USA). Lysates were centrifuged at 1000xg and RNA was isolated from the supernatant using the RNeasy Mini kit (Qiagen). For RNA extraction from brain, tissues were transferred to tubes containing 1mL TriReagent and homogenized (PRO Scientific Bio-Gen PRO200). The mixture

was allowed to sit for 2-5 min and transferred to tube containing 100uL Chloroform. Then it was vortexed for 30 seconds and allowed to sit for 10 min. Next, the samples were centrifuged for 15 min 14,000xg 4°C and the aqueous phase carefully collected and transferred into a micro centrifuge tube. For the extraction, 500uL isopropanol was added and vortexed for 10 seconds. The solution was incubated at room temperature for 10 min to precipitate RNA and centrifuged for 10 min 14,000xg 4°C.The supernatants were discarded carefully and 1mL of 75% ethanol was added to the RNA pellet. The tubes were centrifuged for 5 min 14,000xg at 4°C and then the ethanol was discarded and the RNA pellets were air dried. The RNA pellets were resuspended in 80uL DNAse/RNAse-free water and 10uL DNase enzyme + 10uL 10X DNase buffer was added per sample. The samples were incubated at 37°C water bath for 30 min to allow DNase to degrade any contaminating DNA and then 200uL water was added to each sample. Next, 300uL of low pH phenol (pH 4.3) was added and centrifuged for 6 min 15,000xg 4°C. The upper phase (275uL) was transferred to a fresh tube and 275uL of phenol/choloroform (neutral) was added followed by vortexing. The samples were centrifuged for 6 min 15,000xg 4°C, transferred 250uL of upper phase to fresh DNase/RNase-free tube and then 25uL of sodium acetate and 625uL of 100% ethanol were added per sample. The samples were inverted to mix and placed −80 °C overnight. On the next day, samples were centrifuged for 15 min 15,000xg 4°C and the supernatant was removed. 300uL of 70% cold ethanol was added to the RNA pellet and gently taped to mix/loosen/wash RNA pellet. The samples were then centrifuged for 15 min 15,000xg 4°C, ethanol was removed and pellets air dried. Finally, samples were resuspended in 20uL DNase/RNAse-free water.

After extraction, RNA concentrations were measured with a Nano Drop-1000 Spectrophotometer (Thermoscientific, Waltham, MA, USA). RNA (1 µg) was treated with Genomic Wipeout Buffer (Qiagen) to eliminate genomic DNA contamination, and cDNA was synthesized using the QuantiTect Reverse Transcription System (Qiagen).

2.2.4 Real time quantitative PCR (qPCR)

Primers (Table 2.3) for qPCR were developed for each candidate gene (COX, SQR, CSE, CYP, and VEGF-A) based on annotated sequences in the *P. mexicana* reference transcriptome (Kelley, Passow et al. 2012) using the Primer Quest qPCR primer design program (Integrated DNA Technologies). In addition, we quantified the expression levels of a reference gene (β-actin; (Nygard, Jørgensen et al. 2007). Prior to gene expression studies in experimental samples, all primers were tested across a serial dilution of gill cDNA. Experimental cDNA was used at a 1:4 dilution, and qPCR was performed on an Eppendorf Mastercycler (Eppendorf, Hauppauge, NY, USA) using Perfecta SYBR Fastmix (Quanta Biosciences, Gaithersburg, MD, USA). All samples were run in triplicate with primers designed to amplify fish SQR, COX, CSE, CBS, VEGF and CYP (Integrated DNA Technologies).

Table 2.3: Primer sets, including both forward (F) and reverse primers (R), used during qPCR for the quantification of expression levels in candidate and reference genes

Target gene	Direction Sequence
SQR	F 5′ CAGCTACCAGGACAACACTTT 3′ R 5′ ACTCCACAGCTTTAATGTCGAT 3′
COX	F 5′ GTAGAATCTCCCGTCCGTATTT 3′ R 5′ GTCCAGGGACTGCATCTATTT 3′
CSE	F 5′ GATGAAGGTGGTGGACATCAA 3′ R 5′ GGGCGCTGGAAATAAGAAGA 3′
VEGF	F 5′ GCTCTAGCTGGCGTTCTTTA 3′ R 5′ GAGAGGAGAAAGCGTTTGTTTG 3′
CYP	F 5′ CCTGCTGACAGGATTTCTCTAC 3′ R 5′ CCTCCAGAACCTGGTCTATCT 3′
β-actin (Reference gene)	F 5′ GTCTCCTTCATCGTTCCAGTTT 3′ R 5′ CTGTGCAGAACAACCACATTTC 3′

2.2.5 Data analysis

Gene expression was quantitated relative to the housekeeping gene, β-actin, (Nygard, Jørgensen et al. 2007) and expressed as a fold change of gene expression using the delta-delta Ct method (Livak and Schmittgen 2001). Due to the fact that certain gene expression changes were greater than 100 fold, we expressed the data using log10-transformed values of fold-change and Ct is an exponential and not a linear term (Livak and Schmittgen 2001). Population (sulfide tolerant vs. non- tolerant origin), exposure (control vs. H_2S exposure), and type of exposure (short vs.

long term) were used as independent variables. Gene expression patterns between fish populations were compared using three-way analysis of variance (ANOVA) in SPSS. Differences in expression with a $p<0.05$ was considered as statistically significant.

2.3 Results

This study provided potential mechanisms of H_2S tolerance that will lead us in important directions for selecting functional assays to employ. The data that we gathered during this study is shown in figure 2.2-6 and the statistical analysis results are presented in Table 2.4-8.

For SQR, there was a significant difference between the two populations in liver ($F_{1,40}$ = 19.12, P = <0.01), heart ($F_{1,40}$ = 58.36, P = <0.01) and brain ($F_{1,40}$ = 59.56, P = <0.01). Short term and long term exposure experiments (type of exposure) were significantly different regarding SQR expression in brain ($F_{1,40}$ = 8.36, P = 0.01) while H_2S exposure (control vs. H_2S exposure) was a significantly different in gills ($F_{1,39}$ = 8.51, P = 0.01), heart ($F_{1,40}$ = 5.57, P = 0.02) and brain ($F_{1,40}$ = 24.77, P = <0.01). When considering the interaction between population and type of exposure for SQR, there was a significant difference observed in gills ($F_{1,39}$ = 10.97, P = <0.01), heart ($F_{1,40}$ = 4.42, P = 0.04) and brain ($F_{1,40}$ = 8.90, P = 0.01). For SQR, a significant difference was noted for the interaction between H_2S exposure (control vs. H_2S exposure) and type of exposure in gills ($F_{1,39}$ = 11.06, P = <0.01) and brain ($F_{1,40}$ = 5.54, P = 0.02) and for the interaction between population and H_2S exposure in gills ($F_{1,39}$ = 11.51, P = <0.01) There was a significant three-way interaction between populations, H_2S exposure and type of exposure in gills ($F_{1,39}$ = 11.96, P = <0.01). In long term exposure experiments in the presence or the absence of H_2S, the sulfide tolerant population upregulated SQR compared to the non-tolerant population in all four tissues (Figure 2.2). In short term exposure experiments in the absence of H_2S (under control condition), the sulfide tolerant population upregulated SQR compared to the non-tolerant population in all four tissues. In the gill tissue, with the presence of H_2S, non-tolerant population

upregulated SQR while sulfide tolerant population down regulates SQR compare to control condition. As predicted, we observed that SQR exhibited the greatest degree of upregulation in gill tissues relative to the other three target tissues.

Similar to SQR, there was a significant difference in COX gene expression between population and H_2S exposure in brain tissue ($F_{1,40} = 6.91$, $P = 0.01$) and gill tissue ($F_{1,39} = 13.40$, $P = <0.01$) respectively. Likewise, considering the interaction of population and type of exposure, there was a significant difference in COX at gill ($F_{1,39} = 5.72$, $P = 0.02$) and brain tissue ($F_{1,40} = 8.18$, $P = 0.01$). There was a minimal upregulation in the sulfide tolerant population in the absence of H_2S compared to non-tolerant population in gills, liver and brain tissues in both exposure experiments (Figure 2.3A,B & D). The sulfide tolerant population, however, displayed moderate down regulation in the presence of H_2S compared to control experiment in gill and brain tissue in the short term exposure experiments (Figure 2.3A & D).

There were significant differences in CSE expression between populations in gill tissue ($F_{1,39} = 5.09$, $P = 0.03$), H_2S exposure in gill ($F_{1,39} = 14.95$, $P = <0.01$) and liver tissue ($F_{1,40} = 9.60$, $P = <0.01$), type of exposure in gill ($F_{1,39} = 14.63$, $P = <0.01$), population vs. type of exposure interaction in gill tissue ($F_{1,39} = 4.92$, $P = 0.03$) and brain tissue ($F_{1,40} = 6.12$, $P = 0.02$) as well as H_2S exposure vs. type of exposure in liver tissue ($F_{1,40} = 4.12$, $P = 0.05$). Following short term exposure to H_2S, there was upregulation of CSE gene expression by non-tolerant population compared to the control in gill, liver and brain tissues (Figure 2.4A, B & D). CSE gene expression was down regulated in both populations in the presence of H_2S compared to the control in heart tissue while CSE upregulation was observed in liver tissues (Figure 2.4B & C). In the long term exposure experiments CSE was upregulated in non-tolerant compared to the control in gill and liver tissues while CSE gene expression by heart tissue from the sulfide tolerant population was upregulated in both the presence and absence of H_2S compared to non-tolerant

population (Figure 2.4C).

Analyses of VEGF gene expression revealed significant differences between populations in brain tissue ($F_{1,40}$ = 4.62, P = 0.04), H_2S exposure in liver tissue ($F_{1,40}$ = 4.44, P = 0.04) and brain tissue ($F_{1,40}$ = 4.16, P = 0.05), type of exposure in liver tissue ($F_{1,40}$ = 7.13, P = 0.01) and heart tissue ($F_{1,40}$ = 7.04, P = 0.01), interaction between populations vs. H_2S exposure in brain ($F_{1,40}$ = 8.63, P = 0.01), interaction between populations vs. type of exposure in gill tissues ($F_{1,39}$ = 15.18, P = <0.01) and in brain tissue ($F_{1,40}$ = 22.03, P = <0.01) and the interaction between H_2S exposure vs. type of exposure in liver ($F_{1,40}$ = 4.24, P = 0.05). In the presence of H_2S, sulfide tolerant population down regulated VEGF expression in gill tissue following short term exposure experiment with compare to its control (Figure 2.5A). In the presence of H_2S, both sulfide tolerant and non-tolerant populations upregulated VEGF expression in heart in both exposure experiments than the control condition (Figure 2.5C).

Our final target gene was CYP and there was a significant difference of its expression between populations ($F_{1,39}$ = 9.86, P = <0.01) in gill tissue and type of exposure in gill tissues ($F_{1,39}$ = 8.65, P = 0.01), in heart tissue ($F_{1,40}$ = 5.79, P = 0.02) and in brain tissue ($F_{1,40}$ = 4.80, P = 0.03). In both exposure experiments, both populations down regulated CYP in the presence of H_2S compared to control in gill, heart and brain tissues (Figure 2.6A, C & D).

Overall, gene expression differences were much more pronounced and variable in gills as opposed to other targeted tissues, and predictions of expression responses were partially fulfilled.

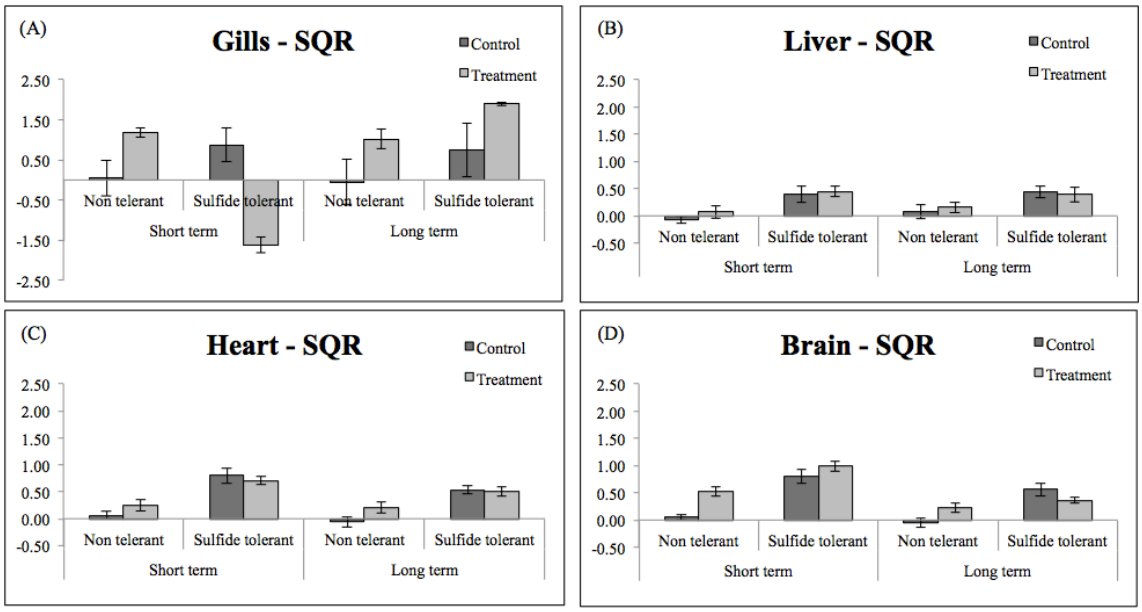


Figure 2.2: Log 10-fold change in gene expression relative to the reference (β-actin) for SQR in (A) gills, (B) liver, (C) heart and (D) brain. The dark gray bars represent control (no H_2S exposure) with standard error while the light gray bars represent H_2S exposed treatment with standard error.

Table 2.4: Results of three-way ANOVA examining variation in gene expression of SQR between populations (sulfide tolerant vs. non-tolerant origin), H_2S exposure (control vs. H_2S exposure), and type of exposure (short term vs. long term) in gills, liver, heart and brain. Only the effects that significant ($\alpha' = 0.05$) are presented here.

Target Tissue	Effect	df	F	p value
Gills	H_2S exposure	1	8.51	0.01
	Population x Type of exposure	1	10.97	<0.01
	Population x H_2S exposure	1	11.51	<0.01
	Type of exposure x H_2S exposure	1	11.06	<0.01
	Population x Type of exposure x H_2S exposure	1	11.96	<0.01
	Error	39		
Liver	Population	1	19.12	<0.01
	Error	40		
Heart	Population	1	58.36	<0.01
	H_2S exposure	1	5.57	0.02
	Population x Type of exposure	1	4.42	0.04
	Error	40		
Brain	Population	1	59.56	<0.01
	Type of exposure	1	8.36	0.01
	H_2S exposure	1	24.77	<0.01
	Population x Type of exposure	1	8.90	0.01
	Type of exposure x H_2S exposure	1	5.54	0.02
	Error	40		

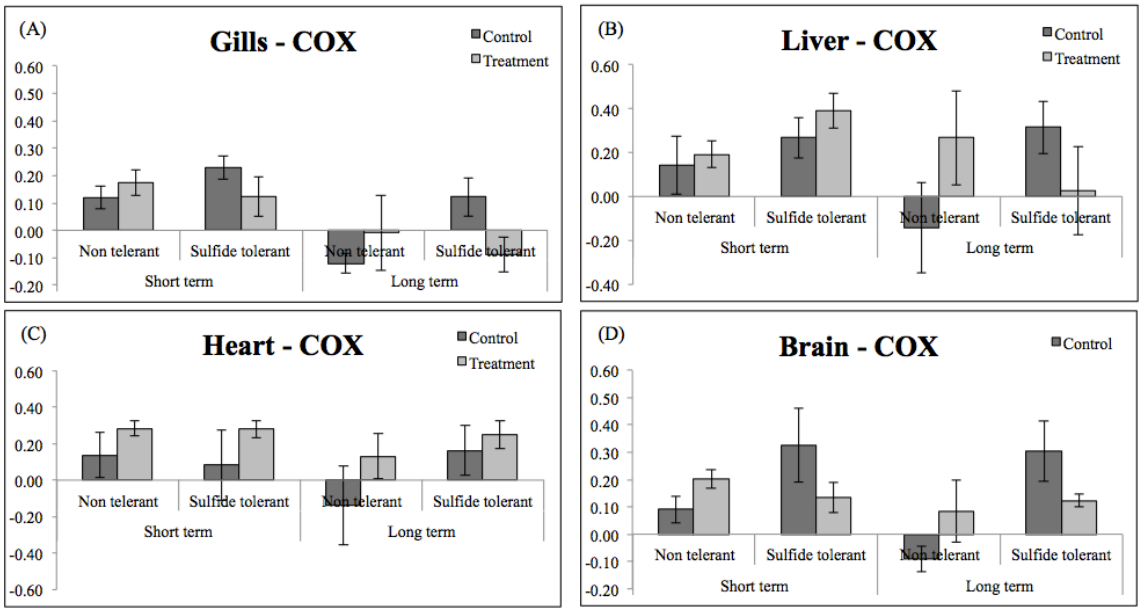


Figure 2.3: Log 10-fold change in gene expression relative to the reference (β-actin) for COX in (A) gills, (B) liver, (C) heart and (D) brain. The dark gray bars represent control (no H_2S exposure) with standard error while the light gray bars represent H_2S exposed treatment with standard error.

Table 2.5: Results of three-way ANOVA examining variation in gene expression of COX between populations (sulfide tolerant vs. non-tolerant origin), H_2S exposure (control vs. H_2S exposure), and type of exposure (short term vs. long term) in gills and brain. Only the effects that significant ($\alpha' = 0.05$) are presented here.

Target Tissue	Effect	df	F	p value
Gills	H_2S exposure	1	13.40	<0.01
	Population x Type of exposure	1	5.72	0.02
	Error	39		
Brain	Population	1	6.91	0.01
	Population x Type of exposure	1	8.18	0.01
	Error	40		

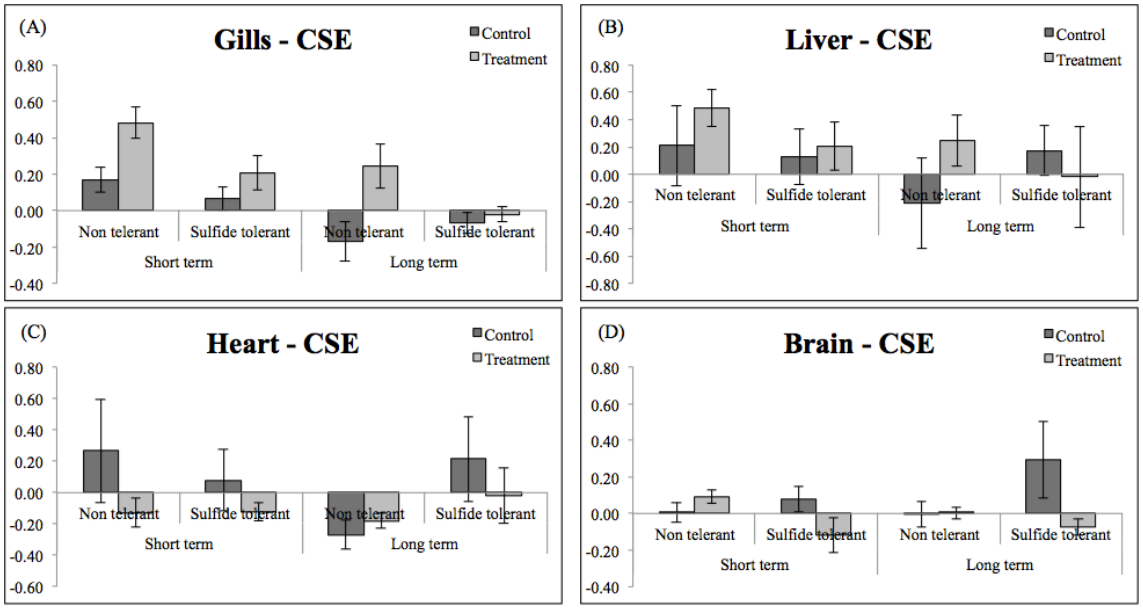


Figure 2.4: Log 10-fold change in gene expression relative to the reference (β-actin) for CSE in (A) gills, (B) liver, (C) heart and (D) brain. The dark gray bars represent control (no H_2S exposure) with standard error while the light gray bars represent H_2S exposed treatment with standard error.

Table 2.6: Results of three-way ANOVA examining variation in gene expression of CSE between populations (sulfide tolerant vs. non-tolerant origin), H_2S exposure (control vs. H_2S exposure), and type of exposure (short term vs. long term) in gills, liver and brain. Only the effects that significant ($\alpha' = 0.05$) are presented here.

Target Tissue	Effect	df	F	p value
Gills	Population	1	5.09	0.03
	Type of exposure	1	14.63	<0.01
	H_2S exposure	1	14.95	<0.01
	Population x Type of exposure	1	4.92	0.03
	Error	39		
Liver	H_2S exposure	1	9.60	<0.01
	Type of exposure x H_2S exposure	1	4.12	0.05
	Error	40		
Brain	Population x Type of exposure	1	6.12	0.02
	Error	40		

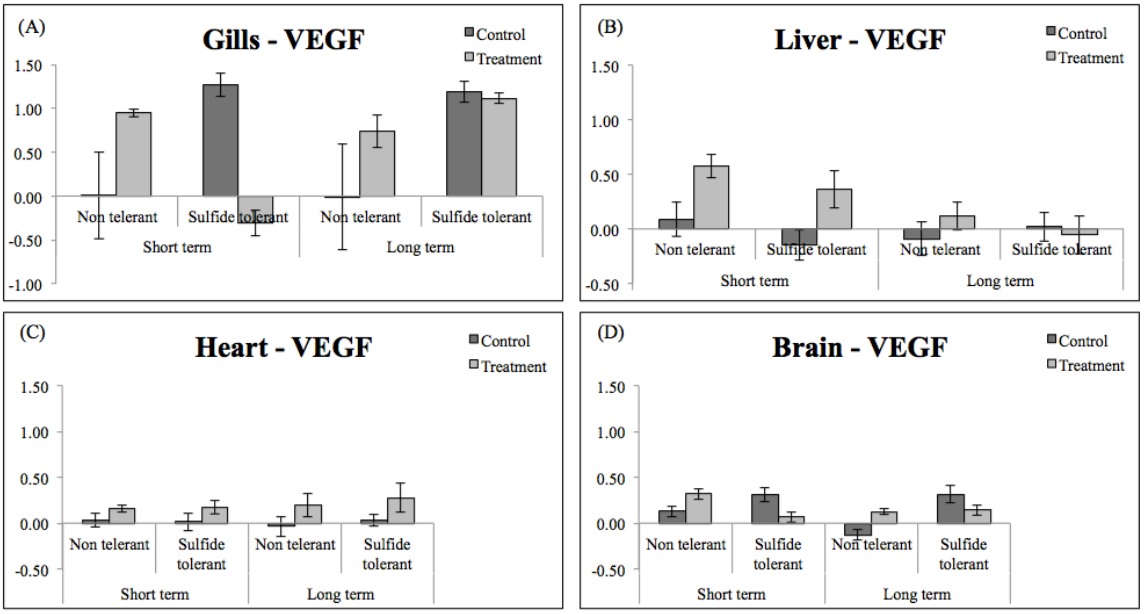


Figure 2.5: Log 10-fold change in gene expression relative to the reference (β-actin) for VEGF in (A) gills, (B) liver, (C) heart and (D) brain. The dark gray bars represent control (no H_2S exposure) with standard error while the light gray bars represent H_2S exposed treatment with standard error.

Table 2.7: Results of three-way ANOVA examining variation in gene expression of VEGF between populations (sulfide tolerant vs. non-tolerant origin), H_2S exposure (control vs. H_2S exposure), and type of exposure (short term vs. long term) in gills, liver, heart and brain. Only the effects that significant ($\alpha' = 0.05$) are presented here.

Target Tissue	Effect	df	F	p value
Gills	Population x Type of exposure	1	15.18	<0.01
	Error	39		
Liver	Type of exposure	1	7.13	0.01
	H_2S exposure	1	4.44	0.04
	Type of exposure x H_2S exposure	1	4.24	0.05
	Error	40		
Heart	Type of exposure	1	7.04	0.01
	Error	40		
Brain	Population	1	4.62	0.04
	H_2S exposure	1	4.16	0.05
	Population x Type of exposure	1	22.03	<0.01
	Population x H_2S exposure	1	8.63	0.01
	Error	40		

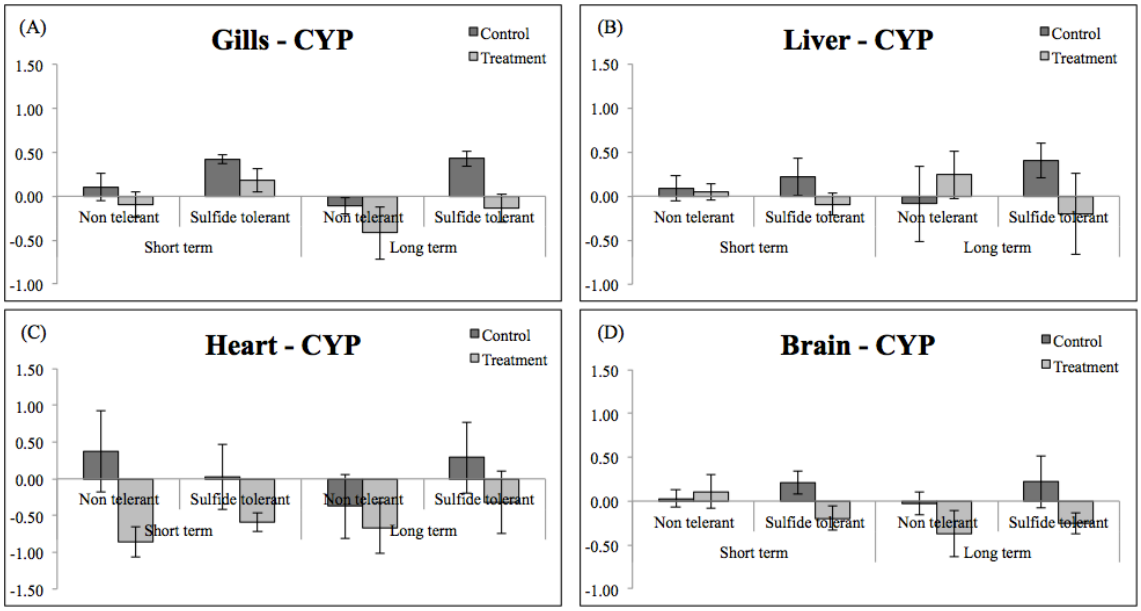


Figure 2.6: Log 10-fold change in gene expression relative to the reference (β-actin) for CYP in (A) gills, (B) liver, (C) heart and (D) brain. The dark gray bars represent control (no H_2S exposure) with standard error while the light gray bars represent H_2S exposed treatment with standard error.

Table 2.8: Results of three-way ANOVA examining variation in gene expression of CYP between populations (sulfide tolerant vs. non-tolerant origin) and type of exposure (short term vs. long term) in gills, heart and brain. Only the effects that significant ($\alpha' = 0.05$) are presented here.

Target Tissue	Effect	df	F	p value
Gills	Population	1	9.86	<0.01
	Type of exposure	1	8.65	0.01
	Error	39		
Heart	Type of exposure	1	5.79	0.02
	Error	40		
Brain	Type of exposure	1	4.80	0.03
	Error	40		

2.4 Discussion

Our study documented complex changes in the expression of candidate genes associated with H_2S toxicity, detoxification, and endogenous production. According to the results, gene expression differences were the most prominent in gill tissue relative to the other tissues. We can speculate that this is because gills are in direct contact with environmental H_2S and serve as the main entry point of H_2S into the body. Depending on the gene, expression levels not only varied between sulfide tolerant and non-tolerant populations, but whether and how fish were exposed to H_2S also played a critical role.

In both exposure experiments (short term and long term) in the presence or the absence of H_2S, the sulfide tolerant population upregulated SQR compared to the non-tolerant population in all four tissues (Figure 2.2). As predicted, these results provide an indication that, even without H_2S exposure during their lifetime, sulfide tolerant fish may have a higher detoxification capacity compared to non-tolerant fish. Even though H_2S negatively affects energy production in aerobic organisms through the interruption of oxidative phosphorylation (Cooper and Brown 2008), enzymatic detoxification of H_2S through SQR contributes electrons to oxidative phosphorylation for ATP production (Völkel and Grieshaber 1994, Grieshaber and Volkel 1998). For example, the hypoxic core of a colon cancerous tumor overproduces H_2S and is able to utilize the H_2S as a metabolic fuel to further cancer progression (Szabo, Coletta et al. 2013). Therefore, we speculate that sulfide tolerant fish may able to use H_2S as an energy fuel for their bioenergetic process.

Furthermore, SQR was highly upregulated in gill tissue compared to the other three target tissues. In the gills, individuals from the sulfide tolerant population displayed higher expression under control treatments, but H_2S exposure elicited complex, population-specific changes in SQR expression. In both populations, SQR expression increased in long term exposure experiments while, only in response to short term exposure for the non-tolerant population. These results are

consistent with the findings of other studies conducted with Echiuran worm from H_2S rich environments and with the human colonocytes (Lagoutte, Mimoun et al. 2010, Ma, Zhang et al. 2012, Mimoun, Andriamihaja et al. 2012). Also genomic patterns of enzymatic pathways suggest that some *P. mexicana* tolerant populations enhance H_2S detoxification (Kelley, Arias-Rodriguez et al. 2016).

The reason for SQR down regulation in sulfide tolerant population following short term exposure is uncertain. But we can speculate these fish rely on alternative mechanisms such as reductions of H_2S uptake that helps to reduce toxicity (Vismann 1991) or alternative enzymatic detoxification pathways (Hildebrandt and Grieshaber 2008). Enzymes of the SQR pathway are upregulated or under positive selection in fish with high H_2S tolerance (Kelley, Arias-Rodriguez et al. 2016). It remains to be investigated whether sulfide tolerant and non-tolerant populations of *P. mexicana* also exhibit structural differences in SQR that may affect detoxification abilities. Overall, our results add to the growing body of evidence that SQR is critically involved in sulfide tolerance observed across a wide variety of organisms living amidst environmental H_2S.

H_2S directly inhibits cytochrome c oxidase (COX) of the mitochondrial respiratory chain by binding to COX and interrupting of aerobic ATP synthesis (Cooper and Brown 2008). We found significant evidence for population differences in brain tissue, H_2S exposure differences in gill tissue and their interactions in the expression of COX in gill and brain tissues. There was a minor upregulation by the sulfide tolerant population in the absence of H_2S compared to non-tolerant population in gills, liver and brain tissues in both exposure experiments (Figure 2.3A,B & D). The sulfide tolerant population, however, displayed moderate down regulation in the presence of H_2S compared to control experiment in gill and brain tissue in the short term exposure experiments (Figure 2.3A & D).

Though we predicted up regulation in COX expression in the sulfide tolerant population,

it may be that the upregulation of SQR gene expression (potentially indicating enhanced detoxification capability) reduces the H_2S toxicity levels such that COX is exposed to a lower than expected level of H_2S, thereby minimizing toxicity at COX. Sulfide tolerant populations in Puyacatengo and Pichucalco drainage exhibit H_2S tolerant COX enzyme by changing the tertiary protein structure of the enzyme while the sulfide tolerant population in Rio Tacotalpa drainage (population used in this study) exhibits H_2S-susceptible COX (Pfenninger, Lerp et al. 2014). This may also reflects that there is an alternative mechanism to reduce H_2S toxicity at COX enzyme. The lack of COX upregulation in the sulfide tolerant population in short term exposure is consistent with the findings of other studies (Roberts, Thomas et al. 2008, Tiranti and Zeviani 2013).

The source for the majority of H_2S biosynthesis in animal tissues is the cytosolic enzyme cystathionine γ lyase (CSE) (Leffler, Parfenova et al. 2006), which is primarily expressed in the cardiovascular system. The expression of CSE differed significantly across populations (in gill tissue), H_2S exposure (in gill and liver tissues), exposure type (in gill tissue), and the interactions between population and type of exposure (in gill and brain tissues), and H_2S exposure and type of exposure (in liver tissue). Following short term exposure, we observed upregulation of CSE, in the non-tolerant population in the presence of H_2S compared to the control experiments in gill, liver and brain tissues (Figure 2.4A, B and D). On the other hand, CSE expression was down regulated in both populations with H_2S relative to the control in heart tissue (Figure 2.4C). Following long term exposure, CSE was upregulated in non-tolerant in the presence of H_2S compared to the control in gill and liver tissues (Figure 2.4A and B).

Even though we predicted that the sulfide tolerant tissues would exhibit reduced expression of CSE to minimize the endogenous H_2S production (Stipanuk and Ueki 2011), the results revealed CSE was upregulated upon environmental exposure It is noteworthy that

upregulation of CSE in gills and liver upon H_2S exposure appeared to be primarily driven by individuals from the non-tolerant population, while there was difference in sulfide tolerant fish for CSE expression between the presence or absence of H_2S exposure. Hence, the additional studies to better understand the roll of CSE in response to H_2S exposure are needed.

Vascular endothelial growth factor (VEGF) is responsible for hypoxia responses that drive angiogenesis (Liu, Pan et al. 2010, Qipshidze, Metreveli et al. 2012). We found significant evidence that VEGF gene expression is affected by population difference (in brain tissue), H_2S exposure (in liver, heart and brain tissues), type of exposure (in liver and heart tissues) and the interactions of population differences vs. H_2S exposure (in brain tissue), the interactions of population differences vs. type of exposure (in gill and brain tissues) and H_2S exposure vs. type of exposure (in liver tissue). In the presence of H_2S, sulfide tolerant population down regulated VEGF expression in gill tissue in short term exposure experiment while upregulated expression in both populations in long term exposure experiment (Figure 2.5A). In liver and heart tissues VEGF expression upregulated in both populations in the presence of H_2S in both exposure experiments (Figure 2.5B and C).

We speculate this upregulation in long term H_2S exposure may be a means to promote angiogenesis to maximize oxygen uptake at the heart and liver; perhaps down regulation of VEGF at the gills is an approach to reduce capillary density thereby reducing uptake of environmental H_2S. Similar evidence for upregulation of VEGF upon H_2S exposure is supported in a variety of biomedical studies (Wang 2012). While our study provides strong evidence for population and tissue differences in VEGF regulation upon sulfide exposure, it remains unclear whether and how the observed differences in VEGF expression provide an adaptive advantage in H_2S rich environments.

Cytochrome P450 (CYP- CYP2J family enzymes) metabolize H_2S to a toxic metabolite

that aggravates toxicity (Caro, Thompson et al. 2011). From this study we found significant evidence for population differences and type of exposure differences in the gene expression of CYP2J in brain tissue and gill tissue respectively. In both exposure experiments, both populations down regulated CYP2J in the presence of H_2S compared to control in heart and brain tissues (Figure 2.6C and D). Since CYP2J has been implicated in exacerbating H_2S toxicity (Caro, Thompson et al. 2011), this trend of CYP2J down regulation in both populations studied herein is supported by previous findings (Caro, Thompson et al. 2011).

Overall, we detected some significant differences in candidate gene expression patterns related to H_2S biology between lab reared sulfide tolerant and non-tolerant populations originating from the Rio Tacotalpa drainage. All animals used in this study were born and raised in captivity; hence, even fish derived from the sulfide spring population have not had any exposure to environmental H_2S during their lifetime. Therefore, the significant differences in candidate gene expression patterns revealed that the sulfide tolerant population has maintained their tolerance for multiple generations even in the absence of environmental sulfide. More studies are needed to investigate the potential influences of maternal effects for this tolerance (Jaenisch and Bird 2003, Feil and Fraga 2012). This study utilized gene expression measurement to investigate the potential molecular mechanisms underlying H_2S toxicity and physiology. Overall, this study uncovered putative adaptive modifications at the molecular level to add to our current understanding of adaptations in behavioral, morphological, physiological, and life history traits that have previously been documented in sulfide spring fishes (Tobler, Palacios et al. 2011, Tobler and Plath 2011, Riesch, Plath et al. 2014).

A limitation of this study was the acute handling stress of fish while introducing them to the experimental setup, retaining them in a confined area during the experiment and during pithing. Due to these stressors fish undergo a series of neuroendocrine, biochemical,

physiological, and behavioral changes as coping mechanisms which may affect the results of the experiment (Davis 2006). Another limitation of this study was the H_2S oxidation in an aqueous solution and volatilization. Due to these factors it was challenging to maintain a constant H_2S concentration during H_2S solution preparation and exposure experiments. To normalize gene expression data we used beta-actin as the housekeeping gene (Nygard, Jørgensen et al. 2007), which was not expected to change expression levels across sample or treatment groups. Even though we did not expect to see any change in expression levels across sample or treatment, beta-actin expression changed upon H_2S exposure ($F_{1,39} = 4.055$, $P = 0.051$) (Tobler, Henpita et al. 2014). Its expression varies across the plates that could skew the end results of the targeted gene expressions. Therefore, it would be necessary to use another housekeeping gene (such as GAPDH) for future H_2S qPCR studies.

It will also be necessary to investigate whether sulfide tolerant populations of *P. mexicana* are able to disproportionally rely on anaerobic metabolism (Bagarinao 1992) or can increase detoxification capacity through reversed electron flow from coenzyme Q back to the mitochondrial complex I, which allows for H_2S detoxification by SQR even when COX is blocked (Lagoutte, Mimoun et al. 2010). Elucidating the mechanisms underlying the maintenance of H_2S homeostasis ultimately will shed light on the molecular adaptation to extreme environments (Grieshaber and Volkel 1998) and provide insights for biomedical applications attempting to use H_2S donating drugs and H_2S-inhibitors to treat a wide variety of diseases linked to disruptions of H_2S homeostasis (Szabo 2007, Zhao, Wang et al. 2010, Olson 2011). In order to rigorously address these questions, mitochondrial function assays are needed to further understand how tolerance is achieved in extremophile sulfide tolerant populations.

CHAPTER III

MITOCHONDRIAL ADAPTATIONS IN *Poecilia mexicana* TOLERANT POPULATIONS (TACOTALPA DRAINAGE) UPON HYDROGEN SULFIDE EXPOSURE

3.1 Introduction

An extreme environment is a setting wherein conditions are lethal to most organisms with respect to its physicochemical properties (Thiel 2011). However, extremophiles adapt to and even thrive under these conditions giving rise to unique ecological communities (Jaenicke and Böhm 1998). Investigation into the strategies utilized to exploit extreme conditions presents an opportunity to uncover biological processes across the molecular, cellular, organismal and population level to collectively demonstrate how environmental stressors drive evolution. Hydrogen sulfide (H_2S) is considered an environmental stressor for aquatic organisms (Kelley, Arias-Rodriguez et al. 2016), which inhibits cellular respiration thereby limiting survival and reproduction (Bagarinao 1992, Tobler, Schlupp et al. 2006). Extremophiles that inhabit H_2S rich environments display behavioral, morphological and physiological

adaptations to withstand this environmental stressor (Groenendaal 1980, Reiffenstein, Hulbert et al. 1992, Goffredi, Childress et al. 1997, Plath, Hauswaldt et al. 2007, Ma, Zhang et al. 2012).

As discussed in chapter one, H_2S is a toxic, flammable and lipid soluble gas with the characteristic odor of rotten eggs (Reiffenstein, Hulbert et al. 1992, Szabo 2007, Li, Hsu et al. 2009, Mancardi, Penna et al. 2009). H_2S exerts toxicity by inhibiting cytochrome c oxidase (COX), the terminal electron acceptor of the mitochondrial electron transport chain (ETC) and is lethal at micromolar concentrations (Cooper and Brown 2008). H_2S is exogenously produced by the decomposition of organic matter, during industrial processes, and is released by geothermal sources, such as sulfur springs as well as deep-sea hydrothermal vents (Bagarinao 1992, Marttila, Jaakkola et al. 1994, Dorman, Moulin et al. 2002, Boshoff, Duncan et al. 2004, Hildebrandt and Grieshaber 2008, Li, Hsu et al. 2009, Hooper, Shane et al. 2010). H_2S is also produced endogenously at very low concentrations as a by-product of cysteine catabolism and functions as a physiologically relevant signaling molecule, mainly in vasodilation (Zhao, Zhang et al. 2001, Wang 2002, Du, Hui et al. 2004, Leffler, Parfenova et al. 2006, Szabo 2007, Cooper and Brown 2008, Qu, Lee et al. 2008, Li, Hsu et al. 2009, Li, Rose et al. 2011, Olson 2011, Stipanuk and Ueki 2011, Stein and Bailey 2013). Maintenance of cellular H_2S homeostasis to circumvent toxic accumulation necessitates ongoing detoxification of H_2S, which is achieved via mitochondrial sulfide-quinone oxidoreductase (SQR) enzymatic activity (Grieshaber and Volkel 1998, Levine, Ellis et al. 1998, Levitt, Furne et al. 1999, Hildebrandt and Grieshaber 2008, Stein and Bailey 2013). Therefore, the mitochondria serve as both the target of H_2S toxicity as well as site of detoxification.

Abnormal elevation of cellular H_2S, due to environmental exposure or faulty detoxification, is correlated with vascular, metabolic and neurological dysfunction in most organisms (Bagarinao 1992, Reiffenstein, Hulbert et al. 1992, Cooper and Brown 2008). However, there are some organisms that thrive in H_2S-rich environments (Reiffenstein, Hulbert et

al. 1992), such as the lugworm (*Arenicola marina*) (Groenendaal 1980, Völkel and Grieshaber 1994, Grieshaber and Volkel 1998), tube worms (*Riftia pachyptila*) (Goffredi, Childress et al. 1997), echiuran worm (*Urechis caupo*) (Ma, Zhang et al. 2012), and Atlantic molly (*Poecilia mexicana*) (Plath, Hauswaldt et al. 2007). The Atlantic molly, which is the focus of this study, is a live-bearing fish species that survives and reproduces in extreme sulfur habitats, such as sulfide freshwater springs in Southern Mexico (Tobler, Schlupp et al. 2006).

Poecilia mexicana are found in two ecotypes, H_2S-tolerant and non-tolerant, depending on the presence or absence of H_2S in their habitat (Tobler, DeWitt et al. 2008, Tobler, Palacios et al. 2011). The presence of H_2S in the habitat produces a strong divergent selective pressure. H_2S-tolerant ecotypes are found in three sulfur spring drainages located in Southern Mexico and display convergent evolution of many phenotypic traits in behavior, morphology and physiology. In comparison to non-tolerant populations, sulfide tolerant populations exhibit increased head size, larger gill surface area, and perform aquatic surface respiration (ASR) to maximize the uptake of oxygen in this extreme habitat (Bagarinao 1992, Plath, Tobler et al. 2007, Tobler and Hastings 2011). Significant upregulation of SQR expression in sulfide tolerant *P. mexicana* suggests enhanced H_2S detoxification capability (Tobler, Henpita et al. 2014), as shown in other organisms (Völkel and Grieshaber 1994, Ma, Zhang et al. 2012) and mammalian colonocytes (Levine, Ellis et al. 1998, Levitt, Furne et al. 1999), which are exposed to elevated H_2S produced by sulfate reducing bacteria in the gut. Furthermore, recent evidence indicates that tolerant populations in two out of three sulfur spring drainages (Puyacatengo and Pichucalco drainages) in Southern Mexico have evolved H_2S-resistant COX via amino acid substitutions causing conformational changes in COX that prevent H_2S binding, suggesting aerobic respiration may proceed in the presence of elevated H_2S (Pfenninger, Lerp et al. 2014). However, this study revealed that H_2S tolerance displayed by the sulfide tolerant population from the Tacotalpa drainage is not due to an H_2S-resistant COX (Pfenninger, Lerp et al. 2014). Therefore, another yet

to be identified mechanism/strategy is enables the survival of these fish in their extreme H_2S environment.

There are multiple possibilities as to how H_2S tolerance may be achieved. *P. mexicana* may have adapted in a manner that doesn't require them to maintain aerobic ATP production (e.g., through anaerobic ATP production), or may have modified other components of the ETC that assures proper function (e.g., they may be able to avoid the generation of oxidative stress when COX is blocked). Furthermore, fish may have modified detoxification, enabling the ETC to function despite abnormal elevation of H_2S.

In this study, we utilized wild captured H_2S-tolerant and non-tolerant populations from the Tacotalpa drainage, along with lab-reared individuals from the same populations that have not been exposed to H_2S for several generations. Our study of both wild captured and lab reared *P. mexicana* provides not only a unique model to elucidate cellular mechanisms that produce tolerance, but also contributes to our understanding of the evolutionary process yielding tolerant and non-tolerant populations. Since mitochondria are both the site of H_2S toxicity and detoxification (Hildebrandt and Grieshaber 2008), our objective was to compare mitochondrial function upon H_2S exposure in *P. mexicana* fish derived from tolerant and non-tolerant Tacotalpa populations. In this study, we tested whether sulfide tolerant populations are able to maintain aerobic ATP production in presence of H_2S by using a coupling assay. We predicted that spare respiratory capacity would be maintained in sulfide tolerant populations both in wild-captured and lab-reared individuals, while non-tolerant populations would eventually cease to maintain their spare respiratory capacity. If there are modifications of ETC, without a H_2S-resistant COX (Complex IV of the ETC) in fish from the Tacotalpa drainage, we assumed that functional modifications exist elsewhere along the ETC in sulfide tolerant populations.

To test our hypothesis, we measured mitochondrial oxygen consumption rate (OCR), a reflection of ATP production, in liver mitochondria directly exposed to H_2S using ex vivo coupling flow assays (Rogers, Brand et al. 2011). The assays employed herein allow for measurement of spare respiration, which is the capacity of mitochondria to meet an increased demand for ATP production and is critical for coping with oxidative stressors. In addition, these assays enable us to identify functional differences at specific mitochondrial complexes. We found that mitochondria from sulfide tolerant fish maintain significant spare respiratory capacity following exposure to high concentrations of H_2S, relative to non-tolerant fish, and exhibit a greater degree of tolerance in the wild captured population, relative to lab reared.

3.2 Methods

3.2.1 Study organisms

This study focused on two populations of *Poecilia mexicana* from the Rio Tacotalpa drainage in Mexico: an H_2S-tolerant populations one from sulfide spring (El Azufre I) and an ancestral, non-tolerant population from an adjacent freshwater stream (Arroyo Bonita; see Tobler, Palacios et al. 2011 for details). For analyses of mitochondrial function in wild-caught individuals, fish were collected in their natural habitats using seines, transported to the laboratory at Oklahoma State University, and used for experiments within four to five weeks of their capture. To investigate the role of plasticity in mitochondrial function, we also reared fish from the same source populations in a common-garden setting for several generations. All stocks were maintained at 25 °C with a twelve-hour light-dark cycle. Laboratory stocks were maintained under non-sulfidic conditions. Therefore, laboratory-reared individuals irrespective of their habitat of origin had never been exposed to environmental H_2S in their lifetime. All procedures used were approved by the Institutional Animal Care and Use Committees at Kansas State University (protocol #3473) and Oklahoma State University (protocol #1015).

3.2.2 Preparation of reagents and solutions

All chemicals that were used to make mitochondrial isolation buffer (MSHE+BSA), mitochondrial assay solution (MAS, 3X) and substrates were purchased from Sigma-Aldrich (St. Louis, MO, USA). MSHE+BSA was prepared with 70 mM sucrose, 210 mM mannitol, 5 mM HEPES, 1 mM EGTA, 0.5% (w/v) fatty acid-free BSA, and doubled distilled water (DDW). MAS, 3X was prepared with 210 mM sucrose, 660 mM mannitol, 30 mM KH_2PO_4, 15 mM $MgCl_2$, 6 mM HEPES, 3 mM EGTA, 0.6% (w/v) fatty acid free BSA, and DDW. 3X MAS was then diluted to make 1X MAS, which was use for the preparation of substrates, ADP, and respiration reagents. As substrates, 0.5 M succinate, 0.5 M malate, 0.5 M pyruvate, and 40 mM ADP were prepared with DDW. As respiration reagents, 10 μM oligomycin, 10 μM Antimycin A/rotenone mix, and 3 μM FCCP [carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone] (Seahorse XF Cell Mito Stress Test Kit, Santa Clara, CA, USA) were prepared with 1X MAS. All reagents and solutions were adjusted to pH 7.2 with potassium hydroxide. Except for the respiration reagents, which were prepared fresh on the day of each experiment, all solutions were stored at 4 °C until used.

3.2.3 Isolation of mitochondria

To isolate mitochondria, fish were removed from stock tanks and immediately euthanized by pithing (Nickum, Bart Jr et al. 2004). Fish were then dissected to isolate livers, which were added to 500 μL MSHE-BSA and stored on ice. Due to the small size of the study species, livers from multiple individuals were pooled to obtain at least 60 mg of tissue, which provided a sufficient amount of mitochondrial to run one coupling assay (see below). Liver samples were homogenized on ice with a Bio-Gen PRO200 (PRO Scientific, Oxford, CT, USA) for 10 seconds at the lowest speed, and then 500 μL MSHE-BSA were added to each homogenate. Homogenates were centrifuged at 600 g for 5 minutes at 4° C, and the filtered supernatant of each sample was

again centrifuged at 5000 g for 5 minutes at 4 °C. The resulting pellets were then resuspended in 1 mL 1X MAS. 100 µL of the sample was taken to measure the total protein concentration (mg/ml) by using the BCA Assay reagent (Thermo Scientific Pierce, Rockford, IL, USA).

3.2.4 Measuring total protein concentration in samples

The protein concentration was determined by using bicinchoninic acid reagent (Thermo Scientific Pierce BCA Protein Assay Kit) with bovine serum albumin (BSA) as a standard. Series of eight standards (2000, 1500, 1000, 750, 500, 250, 125 and 0 µg/mL of BSA) and the working reagent (WR) were prepared as described in the manufacturer manual. 100 µL of the sample was centrifuged at 5000 g for 5 minutes at 4 °C. The resulting pellet was then resuspended in 1 mL 1X PBS (1:10 dilution). 25µL of each standard and unknown sample were pipetted into a microplate (Thermo Scientific Pierce 96-Well Plates, Thermo Scientific Pierce, Rockford, IL, USA) as to make three replicates. 200µL of the WR were added to each well and mixed plate thoroughly on a plate shaker for 30 seconds. Then the plate was covered and incubated at 37°C for 30 minute. The absorbance was measured at 562nm on the plate reader. The intensity of the colored reaction product is in proportion to the amount of protein that can be determined by comparing its absorbance value to a standard curve. The standard curve was prepared by plotting the average Blank-corrected 562nm measurement for each BSA standard vs. its concentration in µg/mL. Protein concentration in the sample was determined by reference to a standard curve consisting of known concentration of the standard protein.

3.2.5 Mitochondrial coupling assay

Mitochondrial function was assayed using a Seahorse XFe96 Extracellular Flux Analyzer (Agilent Technologies, Santa Clara, CA, USA), which allows for the quantification of oxygen consumption rates (OCR) of isolated mitochondria in 96-well plates (Ferrick, Neilson et al. 2008). Energy demand and substrate availability for mitochondria can be tightly controlled in this

setup through the sequential addition of compounds that either stimulate or inhibit components of the ETC: the addition of ADP enhances oxygen consumption, oligomycin blocks ATP synthase, FCCP uncouples oxygen consumption from ATP synthesis, and antimycin and rotenone block mitochondrial complexes I and III, respectively (figure 3.1).

Measuring mitochondrial OCR in presence of these different compounds during a coupling assay allows for the quantification of a variety of mitochondrial functions, including basal respiration, ATP-linked respiration, proton leak, spare respiration, maximal respiration and non-mitochondrial respiration (figure 3.2) (Rogers, Brand et al. 2011). Basal respiration indicates the energetic demand of the cell under baseline conditions after adding ADP. ATP-linked respiration can be used to calculate the decrease in oxygen consumption rate upon injection of the ATP synthase inhibitor oligomycin. This indicates ATP produced by the mitochondria that contributes to meeting the energetic needs of the cell along with the help of ATP synthase. Proton leak is the remaining basal respiration not coupled to ATP production. Proton leak can be a sign of mitochondrial damage or can be used as a mechanism to regulate the mitochondrial ATP production. Maximal respiration designates the maximal oxygen consumption rate attained by adding the uncoupler FCCP. FCCP mimics a physiological "energy demand" by stimulating the respiratory chain to operate at maximum capacity. This shows the maximum rate of respiration that the cell can achieve. Spare respiratory capacity indicates the capability of the cell to respond to an energetic demand as well as how closely the cell is to respiring to its theoretical maximum. The cell's ability to respond to demand can be an indicator of cell fitness or flexibility. Non-mitochondrial respiration is the oxygen consumption that persists due to a subset of cellular enzymes that continue to consume oxygen after rotenone and antimycin A addition. This is important for getting an accurate measure of mitochondrial respiration (XF Cell Mito Stress Test Kit; Agilent Technologies user manual).

Prior to a coupling assay, the XF^e 96 sensor cartridge was pre-hydrated with calibrant solution (200 μL per well) overnight at 37 ^{0}C.The 96 wells in the utility plate of the XF^e96 Flux Assay kit was filled with 0.2 mL of Seahorse XF Calibrant and the sensor cartridge was lowered onto the utility plate submerging the sensors in XF Calibrant (figure 3.3a). This setup was placed inside the kit cover and sealed during the overnight incubation at 37^{0}C. ADP, oligomycin, FCCP, and antimycin/rotenone solutions (XF Cell Mito Stress Test Kit; Agilent Technologies) were then loaded into the four injection ports (A, B, C and D at 96 wells), respectively for calibration (figure 3.3.b). These injected drugs enhance oxygen consumption, block ATP synthase, uncouple the oxygen consumption from ATP synthesis, and block mitochondrial complex I and complex III, respectively, enabling the measurement of basal respiration, coupled respiration (respiration linked to ATP production), maximal respiration, and uncoupled respiration (proton leak). Spare respiration and non-mitochondrial respiration were calculated using the resulting values. Once all the compounds were loaded, the cartridge was transferred carefully to the XF Analyzer for calibration immediately prior to the assay. Meanwhile, mitochondrial solution was added to each well of a 96-well plate (containing 4 μg mitochondria per well) along with substrates (succinate, malate, and pyruvate) and 1X MAS (figure 3.3d). The mitochondrial sample volume/protein content was calculated by using the results from the BCA Assay. After calibration, the 96-well plate containing equal amounts of isolated mitochondria was placed in the analyzer. To assess mitochondrial function/dysfunction in the sulfide tolerant fish relative to the non-tolerant fish under basal and H_2S conditions, sodium hydrosulfide (NaSH) was added directly into each well as a H_2S donor, immediately prior to placement in the analyzer. Overall, OCRs were measured (figure 3.3c) at seven different doses of H_2S (5, 15, 30, 50, 60, 80 and 90 μM) along with a non-sulfidic control (0 μM). After the addition of H_2S, coupling assays were conducted following the manufacturer's protocols. Each isolate was measured across a range of H_2S concentrations, but tissue limitations prevented measurements of all isolates across all concentrations. Each isolate

and concentration pairing was conducted in triplicate, and OCR measurements were normalized by protein content.

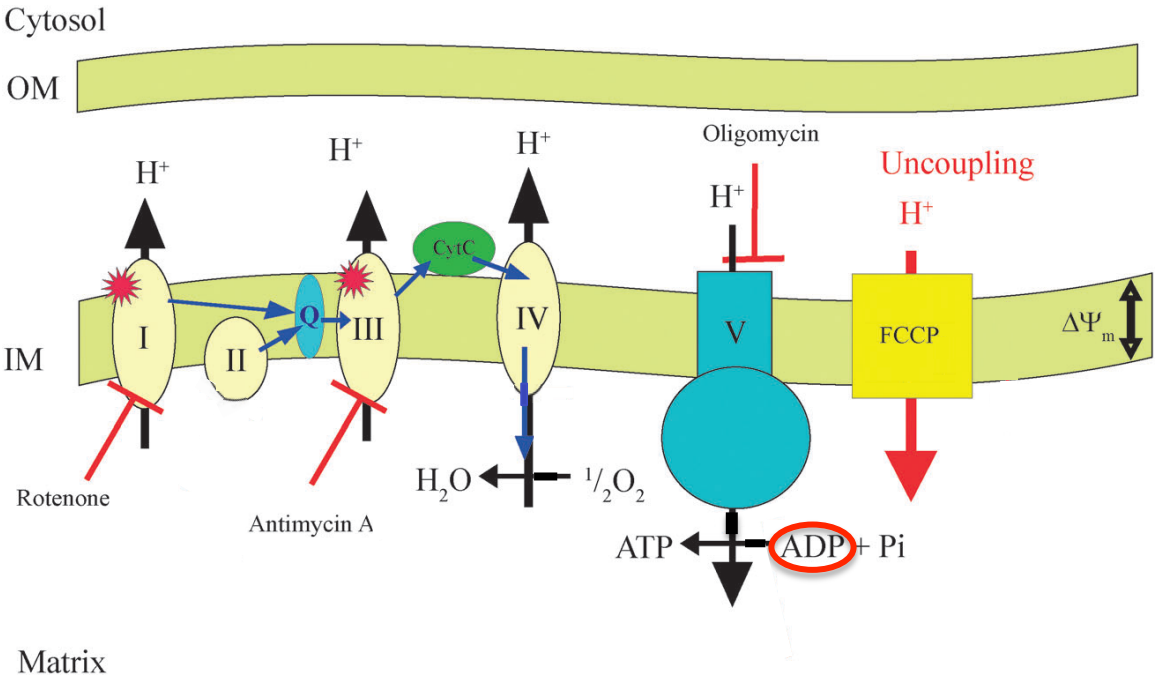


Figure 3.1: General representation of the coupling assay: drug targets and the effects on ETC complexes (modified from Papkovsky and Dmitriev, 2013). The ETC consists of four enzymes: complexes I (NADH-ubiquinol oxidoreductase), II (succinate-ubiquinol oxidoreductase), III (ubiquinol-cytochrome c oxidoreductase) and IV (cytochrome c oxidase) located in the inner mitochondrial membrane (IM). Transfer of electrons (blue arrows) is mediated by coenzyme Q10 (Q) and cytochrome c (cyt c) and results in O_2 consumption at complex IV. The proton gradient is used to produce ATP by complex V (ATP-synthase). The addition of ADP enhances oxygen consumption, oligomycin blocks ATP synthase, FCCP uncouples oxygen consumption from ATP synthesis, and antimycin and rotenone block mitochondrial complexes I and III, respectively (Papkovsky and Dmitriev 2013).

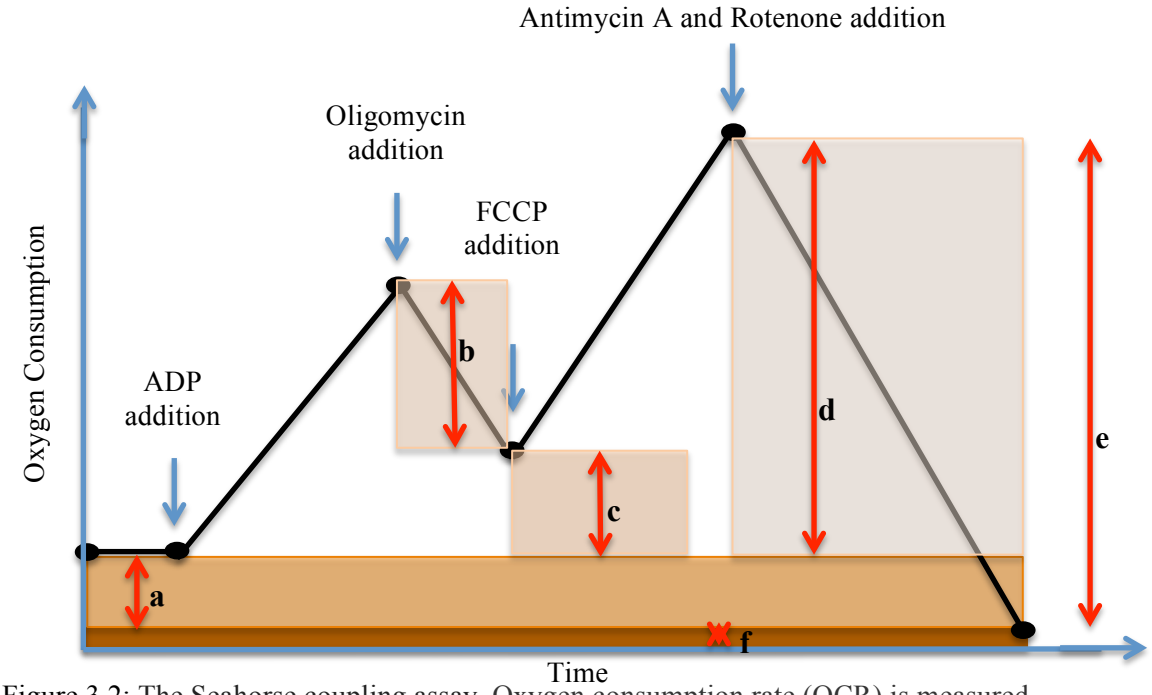


Figure 3.2: The Seahorse coupling assay. Oxygen consumption rate (OCR) is measured initially and after the addition of drugs to derive several parameters of mitochondrial respiration. Before adding ADP, (a) basal respiration, (derived by subtracting non-mitochondrial respiration from the initially resulting OCR) is calculated. Next oligomycin is added and (b) ATP-linked respiration (derived by subtracting the resulting OCR after addition of ADP from the resulting OCR after the addition of oligomycin) and (c) proton leak (derived by subtracting basal respiration from the resulting OCR after the addition of oligomycin) is calculated. Next FCCP is added and (e) maximal respiration (derived by subtracting non- mitochondrial respiration from the resulting OCR after the addition of FCCP) is calculated. Lastly, antimycin A and rotenone are added to shut down ETC function, revealing the non-mitochondrial respiration, f. (d) spare respiration is calculated by subtracting basal respiration from maximal respiration.

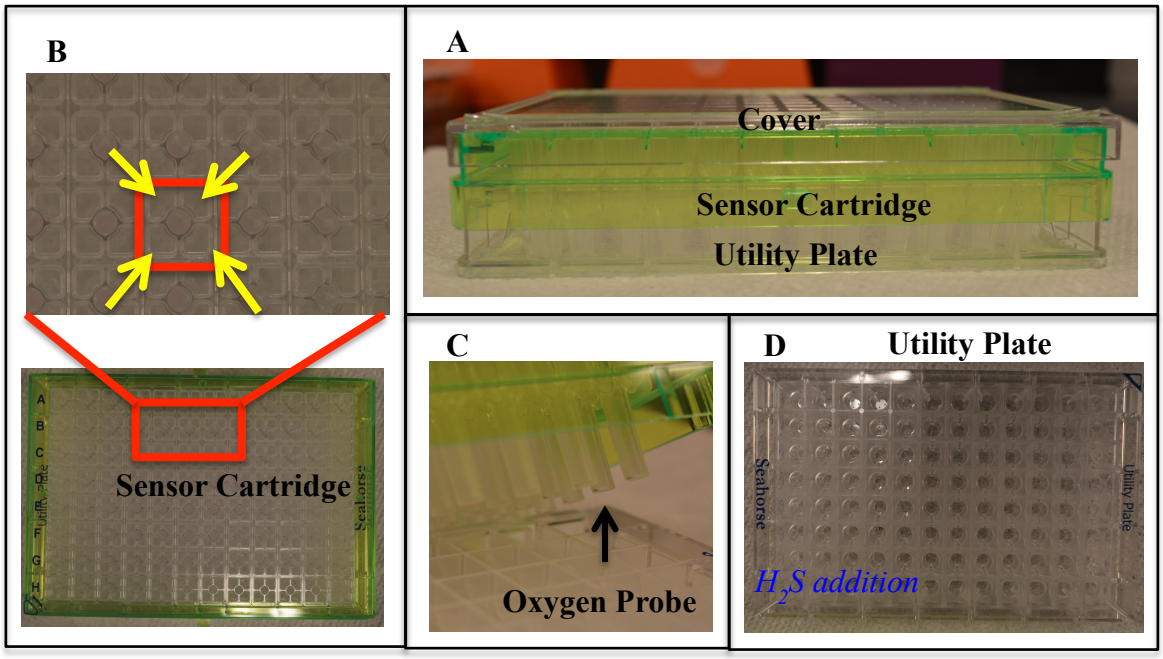


Figure 3.3: Mitochondrial coupling assay kit A). XF^{e}96 Flux Assay kit contains utility plate, sensor cartridge and the cover; B). Sensor cartridge and the enlarge view of the four drug injection ports at each well. ADP, oligomycin, FCCP, and antimycin/rotenone solutions were loaded into these four injection ports (A, B, C and D at 96 wells) respectively at the sensor cartridge for calibration; C). Oxygen probe that located in the sensor cartridge, measures oxygen consumption in mitochondrial solution in the utility plate; D). Utility plate. Mitochondrial solution was added to each well of the 96-well plate (4 ug mitochondria per well) along with substrates (succinate, malate, and pyruvate) and 1X MAS for coupling assay. According to the experimental design, sodium hydrosulfide (NaSH) was added directly each well as H_2S donor, just before a plate was placed in the analyzer.

3.2.6 Statistical analyses

Separate n-parameter logistic regressions were fit for each mitochondrial isolate with metrics of mitochondrial function (basal respiration, maximal respiration and spare respiratory capacity) as dependent variables, and H_2S concentration as the independent variable, using the

nplr package in R (Commo and Bot 2016). Based on regression models, area under the curve (AUC) was estimated based on Simpson's rule. AUC values were then compared between mitochondrial isolates from the sulfide tolerant and non-tolerant populations separately for wild-captured and lab-reared fish using ANOVA (two-way and three way).

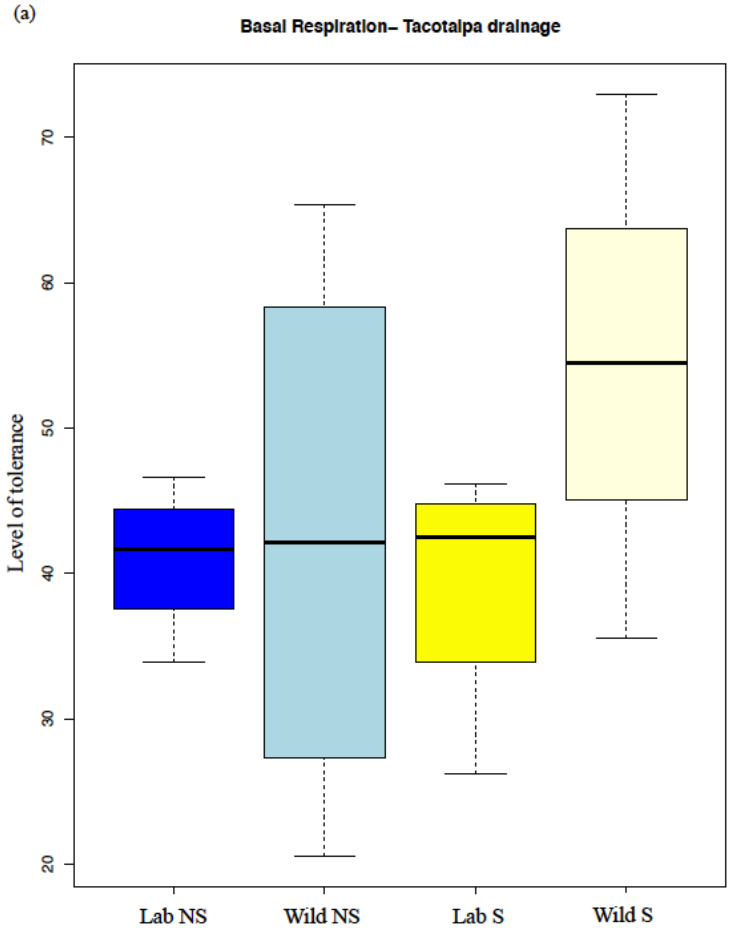
3.3 Results

To investigate mitochondrial function of H_2S-tolerant and non-tolerant *P. mexicana* populations from the Tacotalpa drainage, we extracted mitochondria from livers dissected from wild-caught and lab-reared fish. We then used the coupling assay on a Seahorse Extracellular Flux Analyzer to quantify different aspects of liver mitochondrial function across multiple H_2S concentrations, including basal and maximal oxygen consumption rates, and spare respiratory capacity.

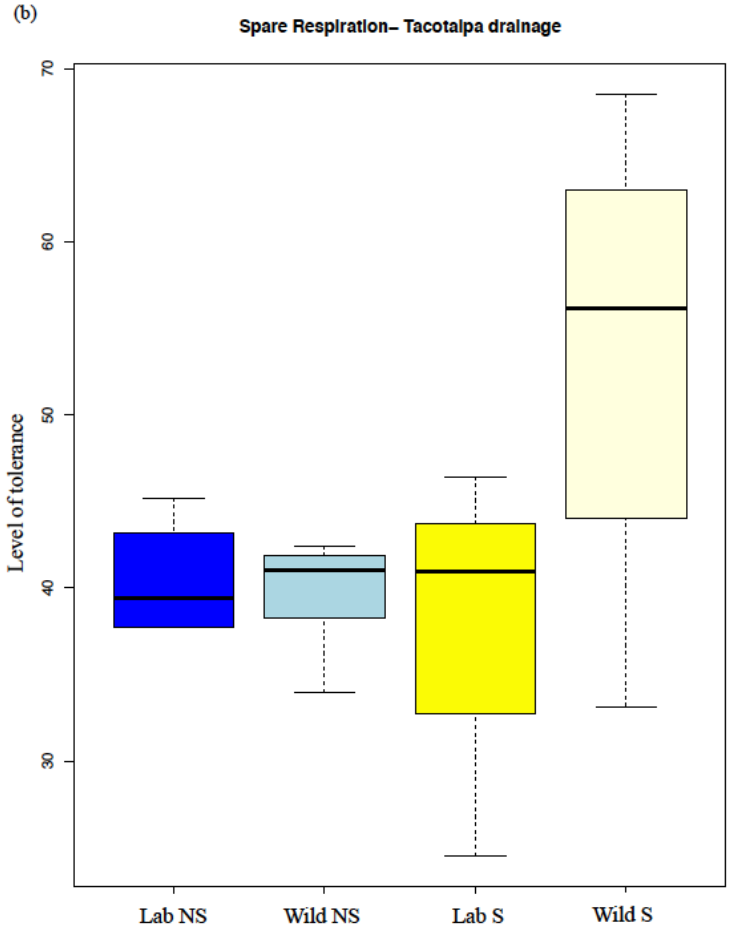
To quantitatively compare responses to H_2S exposure across mitochondrial isolates from different populations (sulfide tolerant *vs*. non-tolerant) and different origin (wild-captured *vs*. lab-reared), we calculated area under the curve (AUC, a metric of the overall functional response to H_2S) for basal, spare and maximal respiration (figure 3.4). Two-way ANOVA results are shown in table 3.1. Comparison of AUC for basal and spare respiration revealed no significant differences between the sulfide tolerant and non-tolerant population, either in wild-caught or lab-reared individuals (figure 3.4 a and b). In contrast, there were significant differences in maximal respiration between populations ($F_{1,13}$ = 8.93, P = 0.01), between their origin ($F_{1,13}$ = 13.19, P = <0.01) and also between population origin interaction ($F_{1,13}$ = 12.67, P = <0.01) (figure 3.4c). Mitochondria isolated from wild-captured, sulfide tolerant fish exhibited a higher AUC for maximal respiration compared to those from lab-reared sulfide tolerant and both lab-reared and wild captured non-tolerant fish (figure 3.4c).

As expected based on H_2S's toxic effects, all metrics of mitochondrial function generally declined with increasing H_2S concentrations (basal respiration - $F_{1,112}$ = 165.83, P = <2e-16; spare respiration - $F_{1,121}$ = 268.68, P = < 2.2e-16; and maximal respiration - $F_{1,121}$ = 361.19, P = < 2.2e-16) (figure 3.5). For spare respiration, there were significant interactions between concentration and population ($F_{1,121}$ = 19.42, P = 2.28e-05) and population and origin ($F_{1,121}$ = 4.64, P = 0.03). Similar to AUC results, there were significant differences in maximal respiration between populations ($F_{1,121}$ = 8.90, P = <0.01), origin ($F_{1,121}$ = 7.72, P = <0.01), interactions between concentration and population ($F_{1,121}$ = 19.34, P = 2.37e-05) and population and origin ($F_{1,121}$ = 11.59, P = <0.01). Mitochondria isolated from wild-captured, sulfide tolerant fish exhibited a higher AUC for maximal respiration compared to other populations, indicating that overall mitochondrial function across the different H_2S treatments was higher and started to deteriorate at higher concentrations in tolerant relative to non-tolerant fish. The same trends were evident for mitochondria derived from lab-reared individuals, although direct comparisons were not statistically significant. In part, the lack of significance was likely driven by low statistical power that arose from the limited availability of samples from lab-reared stocks.

(a)


Basal Respiration– Tacotalpa drainage
Level of tolerance
20
30
40
50
60
70
Lab NS
Wild NS
Lab S
Wild S

(b)


Spare Respiration– Tacotalpa drainage
Level of tolerance
70
60
50
40
30
Lab NS
Wild NS
Lab S
Wild S

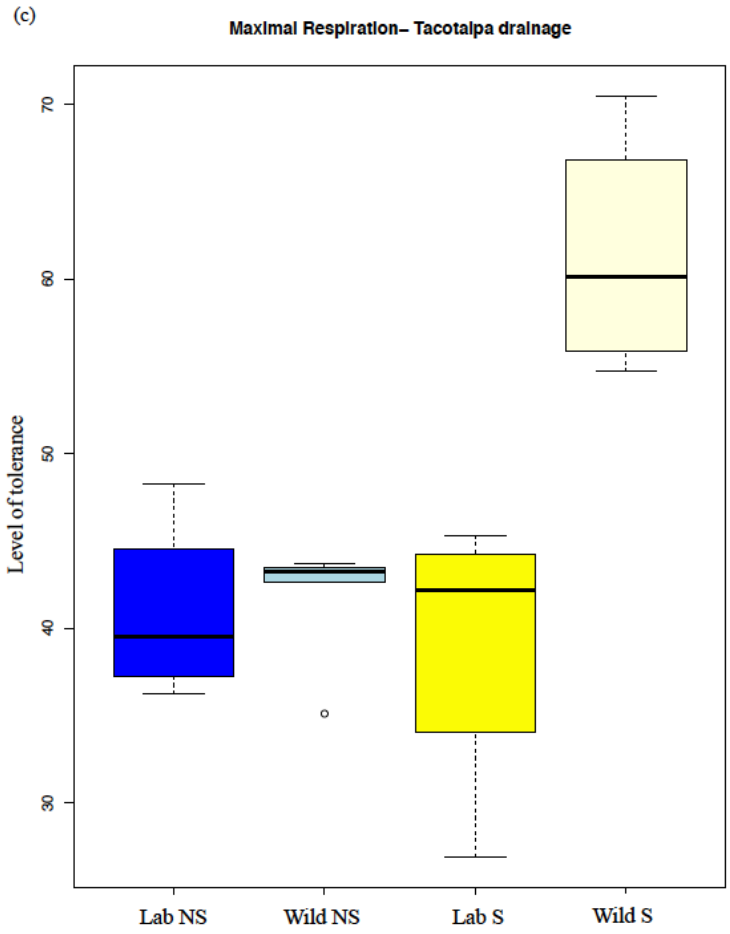
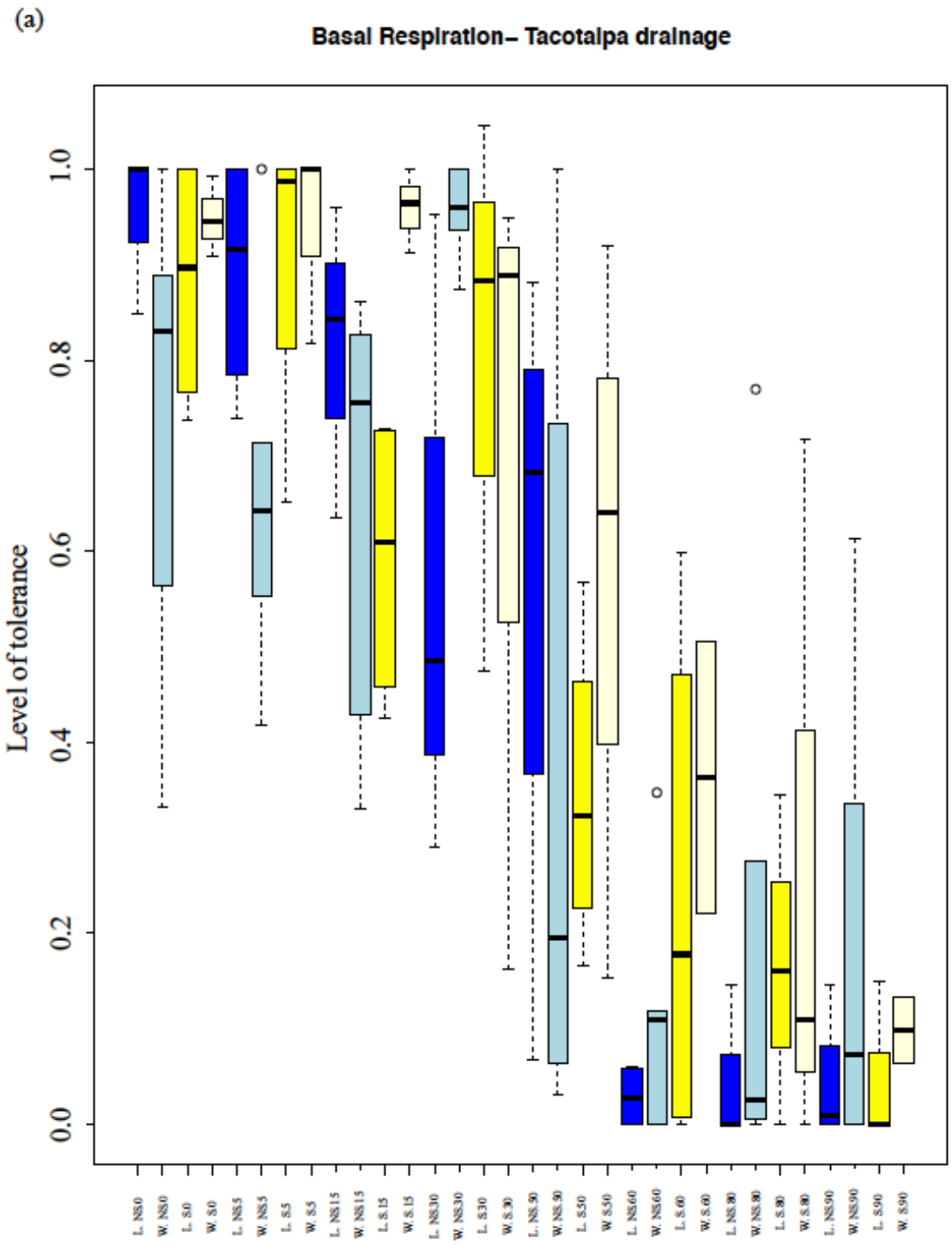


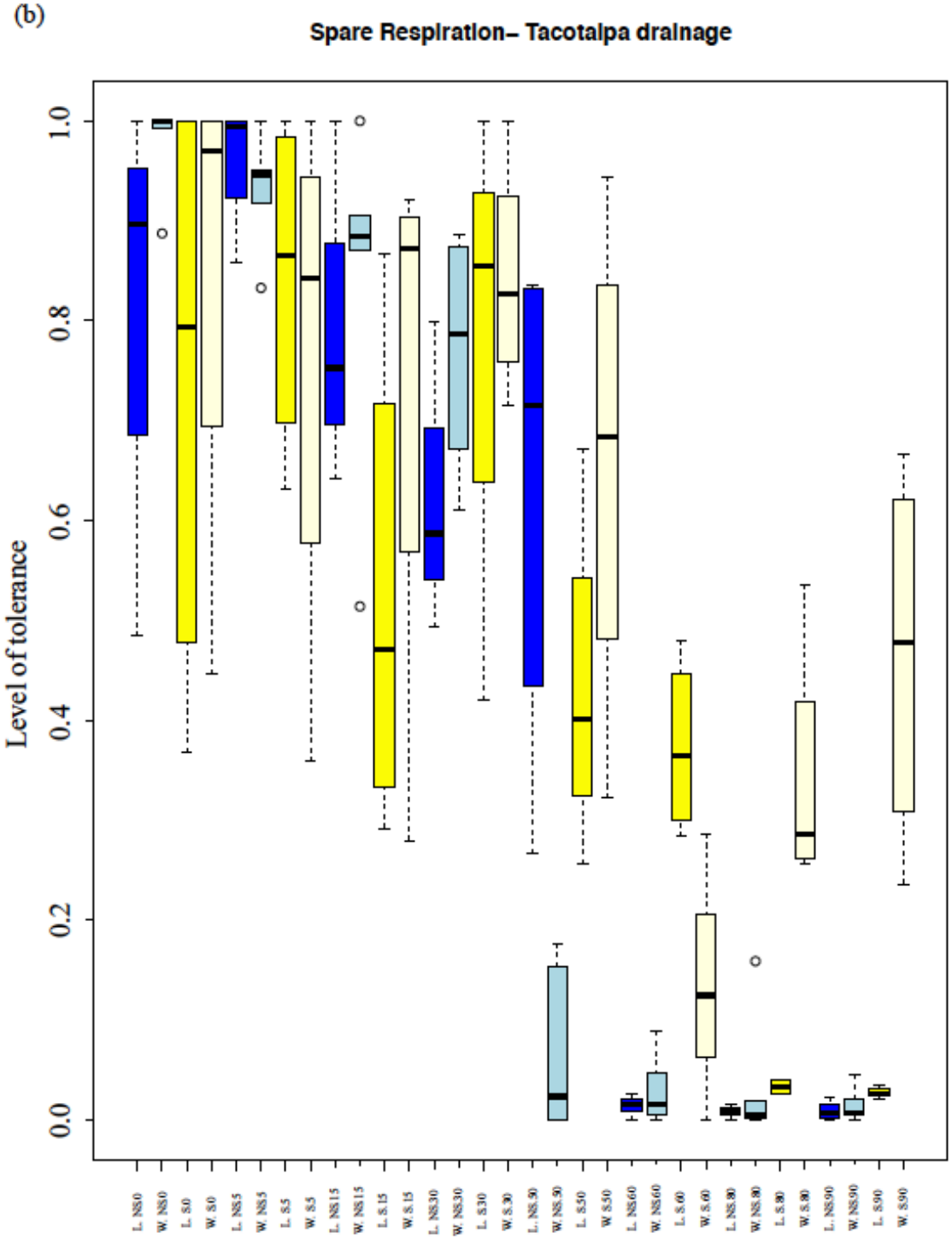
Figure 3.4: The level of tolerance (area under the curve, AUC) expressed by the four populations, lab-reared non-tolerant (Lab NS), wild captured non-tolerant (Wild NS), lab-reared sulfide tolerant (Lab S), and wild captured sulfide tolerant (Wild S). a). Basal respiration b). Spare respiration c). Maximal respiration. Blue boxes represent individuals from L.NS population, light blue boxes represent individuals from W.NS population, yellow boxes represent individuals from L.S population and plain yellow boxes represent individuals from W.S population. Within each box plot, the central rectangle length represents the first quartile to the third quartile of the data distribution. A line inside the rectangle shows the median and the lines above and below the box show the locations of the minimum and maximum of the data set. The dot outside the box represents the outlier.

Table 3.1: Results of two-way analysis of variance (ANOVA) examining variation in basal, spare and maximal respiration in Tacotalpa drainage. Effects that are significant after accounting for multiple testing ($\alpha' = 0.05$) are highlighted in bold font.

Effect	df	F value	p
Basal respiration			
Population	1	0.27	0.61
Origin	1	1.07	0.32
Population x Origin	1	0.81	0.39
Error	12		
Spare respiration			
Population	1	1.91	0.19
Origin	1	2.46	0.14
Population x Origin	1	3.55	0.08
Error	13		
Maximal respiration			
Population	1	8.93	**0.01**
Origin	1	13.19	**<0.01**
Population x Origin	1	12.67	**<0.01**
Error	13		


(a)
Basal Respiration– Tacotalpa drainage
Level of tolerance
0.0
0.2
0.4
0.6
0.8
1.0
L. NS.0
W. NS.0
L. S.0
W. S.0
L. NS.5
W. NS.5
L. S.5
W. S.5
L. NS.15
W. NS.15
L. S.15
W. S.15
L. NS.30
W. NS.30
L. S.30
W. S.30
L. NS.50
W. NS.50
L. S.50
W. S.50
L. NS.60
W. NS.60
L. S.60
W. S.60
L. NS.80
W. NS.80
L. S.80
W. S.80
L. NS.90
W. NS.90
L. S.90
W. S.90

(b)


Spare Respiration– Tacotalpa drainage
Level of tolerance
0.0
0.2
0.4
0.6
0.8
1.0
L. NS0
W. NS0
L. S0
W. S0
L. NS5
W. NS5
L. S5
W. S5
L. NS15
W. NS15
L. S15
W. S15
L. NS30
W. NS30
L. S30
W. S30
L. NS90
W. NS90
L. S50
W. S50
L. NS60
W. NS60
L. S60
W. S60
L. NS80
W. NS80
L. S80
W. S80
L. NS90
W. NS90
L. S90
W. S90

(c)

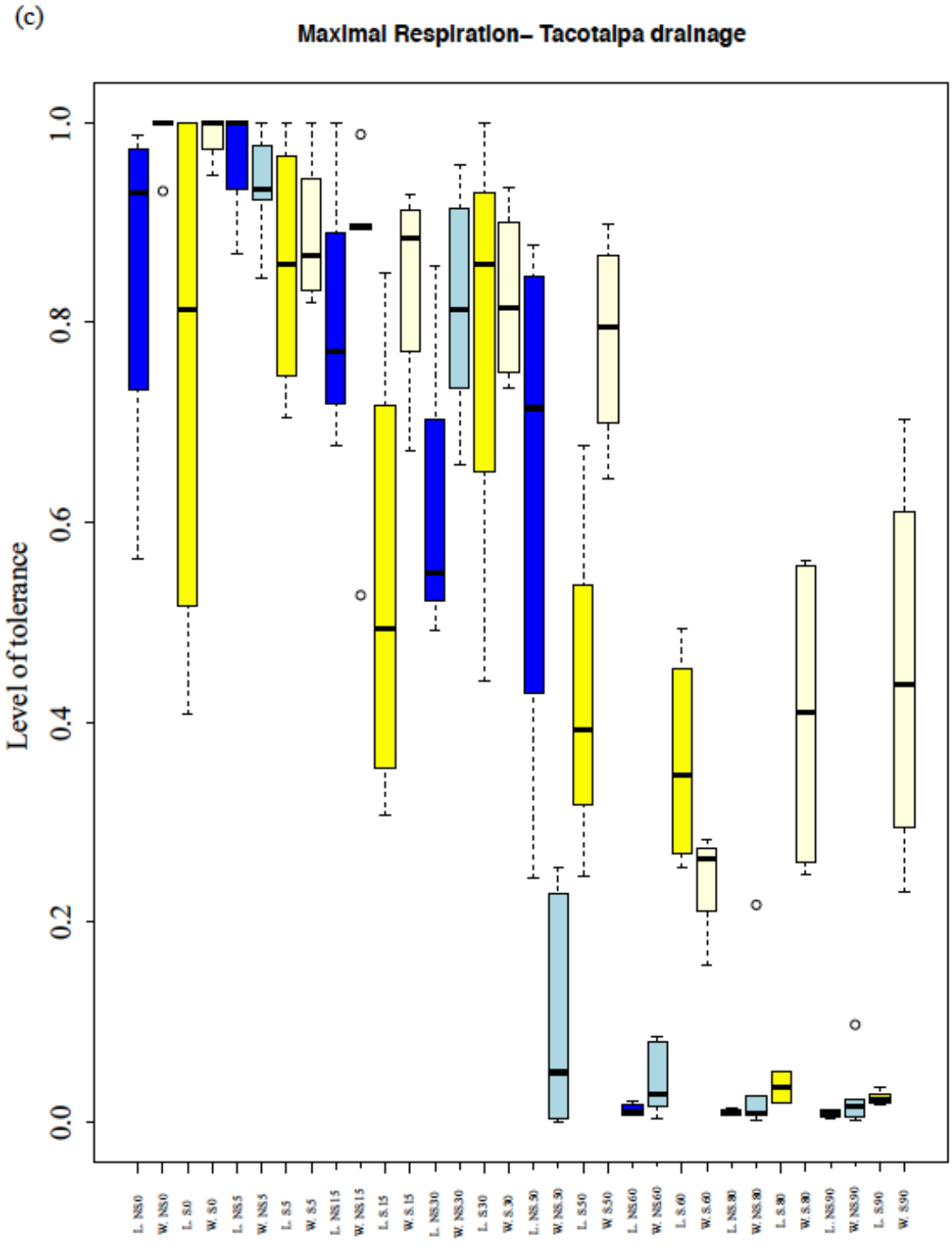

Maximal Respiration– Tacotalpa drainage
Level of tolerance
0.0
0.2
0.4
0.6
0.8
1.0
L. NS.0
W. NS.0
L. S.0
W. S.0
L. NS.5
W. NS.5
L. S.5
W. S.5
L. NS.15
W. NS.15
L. S.15
W. S.15
L. NS.30
W. NS.30
L. S.30
W. S.30
L. NS.50
W. NS.50
L. S.50
W. S.50
L. NS.60
W. NS.60
L. S.60
W. S.60
L. NS.80
W. NS.80
L. S.80
W. S.80
L. NS.90
W. NS.90
L. S.90
W. S.90

Figure 3.5: Basal (a), spare (b) and maximal (c) respiration in four populations, lab-reared non-tolerant (L.NS, blue boxes), wild captured non-tolerant (W.NS, light blue boxes), lab-reared sulfide tolerant (L.S, yellow boxes), and wild captured sulfide tolerant (W.S, plain yellow boxes) in Tacotalpa drainage. In each box plot, the central rectangle length represents the first quartile to the third quartile in data distribution. A line inside the rectangle shows the median and the lines above and below the box show the locations of the minimum and maximum of the data set. The dots outside the box represent the outliers.

Table 3.2: Results of three-way analysis of variance (ANOVA) examining variation in basal, spare and maximal respiration in the Tacotalpa drainage. Only the effects that are significant after accounting for multiple testing ($\alpha' = 0.05$) are presented here.

Effect	df	F value	p
Basal respiration			
Concentration	1	165.83	<2e-16
Error	112		
Spare respiration			
Concentration	1	268.6821	< 2.2e-16
Concentration x Population	1	19.4201	2.28e-05
Population x Origin	1	4.644	0.033
Error	121		
Maximal respiration			
Concentration	1	361.1847	< 2.2e-16
Population	1	8.8968	<0.01
Origin	1	7.722	<0.01
Concentration x Population	1	19.3392	2.37e-05
Population x Origin	1	11.5887	<0.01
Error	121		

3.4 Discussion

Overall, the results suggest that Tacotalpa sulfide tolerant fish population maintained aerobic ATP production in presence of H_2S, even without an H_2S-resistant COX. This ability may be lost – or at least reduced – after multiple generations under non-sulfidic conditions in the laboratory, suggesting that phenotypic plasticity plays a role in the ability to maintain mitochondrial function in this population.

This work elucidates the bioenergetic mechanisms that are responsible for sustaining life and shaping evolutionary processes in extreme sulfidic habitats, wherein the environmental stressor functions as both an inhibitor of and contributor to oxidative phosphorylation for ATP production. Basal respiration is a reflection of ATP production by oxidative phosphorylation under normal/basal conditions and only requires a part of total bioenergetic capacity (Desler, Hansen et al. 2012). Maximal respiration is the total bioenergetic capability while spare respiration is the capacity to increase OCR upon increased metabolic demand (Desler, Hansen et al. 2012).

Our study of sulfide tolerant population of *P. mexicana* in the Tacotalpa drainage reveals the mechanism whereby mitochondrial function is maintained under toxic levels of H_2S. This feat occurs via adaptations in mitochondrial function that enable significant increases, relative to non-tolerant populations, in spare and maximal respiration with increasing concentrations of H_2S. This adaptation is exhibited to a lesser extent in the tolerant populations reared under laboratory conditions lacking continuous environmental H_2S for multiple generations; thus, adaptation occurred in response to ancestral exposure to H_2S, is inherited in the absence of H_2S exposure and presumably requires ongoing selective pressure to produce maximal tolerance.

The overarching question is how do these tolerant ecotypes adapt to withstand environmental stressors while most others cannot? Specifically, how does toxic H_2S exposure

affect the biological processes in tolerant populations at the cellular level? What are the bioenergetic costs of life in sulfidic environments? Are these species able to minimize the flux of H_2S from the environment into their bodies? Or better able to detoxify H_2S? Do these organisms develop resistance to withstand H_2S toxicity? Finally, is H_2S tolerance due to one of the above reasons or a combination of several mechanisms? By addressing these basic questions, we will increase our understanding of adaptations to extreme environmental conditions.

P. mexicana exhibit behavioral adaptations, such as performing aquatic surface respiration (ASR) to maximize uptake of oxygen while in the presence of extreme H_2S (Plath, Tobler et al. 2007). Morphological adaptations include increased head size and gills (Tobler and Hastings 2011) to provide additional surface area for oxygen uptake (Bagarinao 1992, Tobler and Hastings 2011). Furthermore, it has been shown that tolerant populations of *P. mexicana* in two out of three sulfur spring drainages in Southern Mexico have evolved H_2S-resistant COX via amino acid substitutions causing conformational changes in COX that prevent H_2S binding (Pfenninger, Lerp et al. 2014). Taken together, these findings suggest that *P. mexicana* has evolved means to maintain aerobic respiration and mitigate H_2S toxicity. In addition, the wild captured sulfide tolerant population maintains a higher level of tolerance compared to lab-reared population presumably due to the ongoing selective pressure during their lifetime.

The sulfide tolerant population from the Tacotalpa drainage does not exhibit the modified H_2S-resistant COX as the sulfide tolerant populations in Puyacatengo and Pichucalco drainages do (Pfenninger, Lerp et al. 2014). Therefore, the higher H_2S tolerance driven by an enhanced spare respiratory capacity in the Tacotalpa sulfide tolerant population could be due to functional modifications elsewhere along the electron transport chain or a result of an accelerated detoxification. During the H_2S detoxification process in mitochondria, two H_2S are oxidized to elemental sulfur (S^o) via SQR and two electrons released from this process are fed into the ubiquinone (Q) pool contributing to the generation of ATP by oxidative phosphorylation

(Hildebrandt and Grieshaber 2008). Therefore, H_2S serves as a non-carbon source capable of fueling metabolism. As such, this gas has been proposed to play a key role in primordial forms of life in euxinic oceans of the Proterozoic eon (Olson and Straub 2015), microbial chemoautotrophs, and extremophiles inhabiting caves (Engel, Lichtenberg et al. 2007). Furthermore, overproduction of H_2S by human colon cancer is utilized to drive bioenergetics and angiogenesis providing metabolic and vascular support, respectively, for cellular proliferation (Szabo, Coletta et al. 2013). Moreover, chapter two results demonstrating up regulation of SQR expression in lab-reared sulfide tolerant fish (Tacotalpa ancestral origin) using real time quantitative PCR (Tobler, Henpita et al. 2014), which was confirmed in another study demonstrating upregulation of SQR protein expression in tolerant relative to non-tolerant gill tissue (Kelley, Arias-Rodriguez et al. 2016), supports the possibility that enhanced respiratory capacity is achieved by increased detoxification.

Taken together, our data suggest that the SQR pathway may function to minimize toxicity at COX by reducing the H_2S levels in the mitochondria while excess H_2S may be harnessed as a metabolic fuel by the H_2S-tolerant populations to drive the amplified spare respiratory capacity observed in this study (Nicholls and Kim 1982, Kelley, Arias-Rodriguez et al. 2016). Although enhanced detoxification has been shown in multiple systems, it is likely that overlapping mechanisms of tolerance exist. Genome-wide expression patterns in tolerant populations of *P. mexicana* inhabiting drainages adjacent to Tacotalpa also display upregulation of detoxification pathways (Kelley, Arias-Rodriguez et al. 2016).

Limitations of this experimental design will be discussed in chapter four. Future studies on adjacent populations will be necessary to determine whether greater maximal respiration may be achieved compared to the Tacotalpa populations, which lack a H_2S-resistant COX. Thus, a dual strategy of H_2S detoxification working synergistically with the H_2S-resistant COX expressed

by these neighboring tolerant populations of *P. mexicana* in Puyacatengo and Pichucalco drainages (Pfenninger, Lerp et al. 2014) may produce a greater degree of tolerance.

CHAPTER IV

MITOCHONDRIAL ADAPTATIONS IN *Poecilia mexicana* TOLERANT POPULATIONS (PUYACATENGO AND PICHUCALCO DRAINAGE) UPON HYDROGEN SULFIDE EXPOSURE

4.1 Introduction

Physiochemical stressors are abundant in natural environments and can profoundly impact organismal function, ecological dynamics, and long-term evolutionary outcomes. The effects of physiochemical stressors are particularly pronounced in extreme environments that exhibit stressful environmental conditions that few organisms can tolerate, leading to simple ecological communities dominated by specialized extremophiles (Waterman 1999, Bell 2012). Such extremophiles provide unique opportunities to study evolutionary innovations that facilitate organismal function in the face of adverse environmental conditions. Environmental variation is a key factor that affects phenotypic variation in the ecosystem and results in the emergence of locally adapted populations (Kawecki and Ebert 2004). Hydrogen sulfide (H_2S) is considered an environmental physiological stressor for aquatic organisms (Vismann 1991, Waterman 1999, Kelley, Arias-Rodriguez et al. 2016).

Extremophiles that inhabit H_2S rich environments display behavioral, morphological and physiological adaptations to withstand this environmental stressor, which leads to locally adapted phenotypic evolution in the populations (Groenendaal 1980, Reiffenstein, Hulbert et al. 1992, Goffredi, Childress et al. 1997, Plath, Hauswaldt et al. 2007, Ma, Zhang et al. 2012, Palacios, Arias-Rodriguez et al. 2013). *Poecilia mexicana* is a freshwater fish that is common in streams and rivers of Mexico and parts of Central America (Palacios, Arias-Rodriguez et al. 2013, Tobler, Passow et al. 2016). Members of this species have independently colonized H_2S rich springs in multiple drainages of southern Mexico (Tobler, Palacios et al. 2011, Palacios, Arias-Rodriguez et al. 2013). These organisms have adapted to H_2S rich environments, and therefore provide formidable systems to investigate mechanisms of tolerance to this physiochemical stressor. H_2S's toxic effects are primarily mediated by its ability to inhibit cytochrome c oxidase (COX), the terminal electron acceptor of the mitochondrial electron transport chain (ETC), which effectively halts aerobic ATP production (Cooper and Brown 2008) and causes high mortality over short periods of time even at low ambient concentrations (Beauchamp, Bus et al. 1984, Reiffenstein, Hulbert et al. 1992). However, all metazoans can eliminate H_2S enzymatically at low concentrations through the mitochondrial sulfide:quinone oxidoreductase (SQR) pathway (Hildebrandt and Grieshaber 2008, Jackson, Melideo et al. 2012).

There are four mechanisms that could potentially mediate tolerance observed in organisms inhabiting sulfide rich environments (Kelley, Arias-Rodriguez et al. 2016, Tobler, Passow et al. 2016). (a) Sulfide tolerant organisms may be able to minimize the flux of H_2S from the environment into the body via behavioral changes that minimize exposure to H_2S or through structural modifications in the integument and respiratory surfaces that exclude H_2S from the body (Grieshaber and Volkel 1998, Tobler, Passow et al. 2016). (b) Sulfide tolerant organisms could mitigate the direct molecular targets whose functions are impaired by the physiochemical stressor (Pfenninger, Lerp et al. 2014). (c) Detoxification pathways, which involve neutralizing

noxious compounds to non-toxic forms through enzymatic activity, could exhibit increased activity in sulfide tolerant organisms (Grieshaber and Volkel 1998, Hildebrandt and Grieshaber 2008). (d) Regulation of endogenous H_2S concentration may be mediated by symbiosis with sulfur oxidizing bacteria (Tobler, Passow et al. 2016). It is possible that these mechanisms overlap, working synergistically to maintain organismal function in the presence of H_2S. It is known that mitochondria are both the target of H_2S toxicity and the site of detoxification. Thus adaptation to environmental H_2S is likely accomplished at this level of cellular function. Specifically, tolerance in organisms adapted to H_2S-rich environments could be mediated by a modification of COX rendering the enzyme resistant to H_2S such that aerobic ATP production continues in the presence of H_2S and/or a modification of enzymes in the SQR pathways to enhance the rate of H_2S elimination beyond that required for endogenous production rates. A shift toward anaerobic ATP production to allow for the maintenance of organismal function when aerobic metabolism is inhibited by H2S is another possibility.

In the previous chapter, we focused on the mechanisms of tolerance in sulfide tolerant fish from one of the drainages (Tacotalpa) in southern Mexico. The genus *Poecilia* also colonized adjacent drainages, Puyacatengo and Pichucalco. Considering three drainages, *P. mexicana* is only found in Tacotalpa and Puyacatengo, while *P. sulphuraria* is the species in the Pichucalco drainage (Tobler, DeWitt et al. 2008, Palacios, Arias-Rodriguez et al. 2013). Sulfide tolerant populations in the Tacotalpa and Puyacatengo drainages colonized sulfide springs most recently (<50,000 years ago) compared to the sulfide tolerant population in the Pichucalco drainage (~300,000 years ago) (Pfenninger, Lerp et al. 2014, Kelley, Arias-Rodriguez et al. 2016). Sulfide tolerant populations in these three drainages are reproductively isolated, genetically differentiated and phenotypically distinct from the closely related non-tolerant populations within the same drainage and are characterized by morphological, physiological, and behavioral H_2S tolerance and life history adaptations that show strong signals of convergent evolution across drainages

(Tobler, Palacios et al. 2011, Palacios, Arias-Rodriguez et al. 2013). It is important to acknowledge that other environmental factors besides H_2S vary within and across drainages (Table 4.1) (Greenway, Arias-Rodriguez et al. 2014, Kelley, Arias-Rodriguez et al. 2016).

Table 4.1: Environmental variation in water chemistry within and among three drainages. Modified from Kelly et al. 2016 (NS indicates non-tolerant population while S indicates sulfide tolerant population) (Kelley, Arias-Rodriguez et al. 2016).

Site	Temperature [°C]	Specific conductivity [mS/cm]	pH	O_2 [mg/l]	H_2S [µM]
Tacotalpa drainage					
Arroyo Bonita (NS)	26.8±6.2	0.3±0.02	8.0±0.3	5.8±1.4	-0.8±1.9
El Azufre (S)	28.2±0.3	4.2±0.3	7.1±0.1	1.1±0.1	23.7±18.2
Puyacatengo drainage					
Río Puyacatengo (NS)	24.2±2.2	0.2±0.01	8.5±0.4	7.2±1.6	-0.1±0.4
La Lluvia springs (S)	25.7±0.2	2.3±0.2	7.2±0.3	1.7±0.4	26.2±18.3
Pichucalco drainage					
Arroyo Rosita (NS)	29.2±1.3	0.3±0.1	7.9±0.1	8.3±1.2	-0.1±0.5
Baños del Azufre (S)	31.9±0.7	2.7±0.2	6.9±0.1	1.1±0.9	190.4±119.7

Mitochondrial functional assays conducted in our previous study (chapter three) revealed that even without modification of COX, sulfide tolerant fish from the Tacotalpa drainage maintain aerobic ATP production in the presence of H_2S. In this study, we utilized wild captured sulfide tolerant and non-tolerant populations from the Puyacatengo and Pichucalco drainages, which are both known to display COX-resistance to H_2S (Pfenninger, Lerp et al. 2014). We hypothesized that in the presence of H_2S spare respiratory capacity is increased in sulfide tolerant populations while non-tolerant populations cease to maintain their spare respiratory capacity. To test our hypothesis, we measured mitochondrial oxygen consumption rate (OCR), a reflection of

ATP production, in liver mitochondria directly exposed to H_2S using ex vivo coupling flow assays (Rogers, Brand et al. 2011). The assays employed herein allow for measurement of spare respiration, which is the capacity of mitochondria to meet an increased demand for ATP production and is critical for coping with oxidative stressors. In addition, these assays enable us to identify functional differences at specific mitochondrial complexes. We found that mitochondria from sulfide tolerant fish maintain spare and maximal respiratory capacity following exposure to high concentrations of H_2S, relative to non-tolerant, and that sulfide tolerant fish from Pichucalco exhibit a greater degree of tolerance relative to the Puyacatengo sulfide tolerant population.

4.2 Methods

4.2.1 Study organisms

This study focused on two populations of *Poecilia mexicana* from the Puyacatengo drainage in Mexico: 1) H_2S-tolerant population from a small sulfide spring (La Lluvia) and, 2) an ancestral, non-tolerant population from an adjacent freshwater stream (Rio Puyacatengo). We also examined two populations from the Rio Pichucalco drainage in Mexico: 1) H_2S-tolerant population (*Poecilia sulphuraria*) from a sulfide spring (Banos del Azfufre) and, 2) an ancestral, non-tolerant population (*Poecilia mexicana*) from an adjacent freshwater stream (Rio El Azure). For analyses of mitochondrial function in wild-caught individuals, fish were collected in their natural habitats using seines, transported to the laboratory at Oklahoma State University, and used for experiments within a few weeks of their capture. All stocks were maintained at 25 °C with a twelve-hour light-dark cycle. All procedures used were approved by the Institutional Animal Care and Use Committees at Kansas State University (protocol #3473) and Oklahoma State University (protocol #1015).

4.2.2 Preparation of reagents and solutions

All chemical reagents and solutions were prepared as described in the methods section in chapter three.

4.2.3 Isolation of mitochondria and measuring total protein concentration in samples

Mitochondrial isolation and the total protein concentration measurements in the samples were conducted as described in the methods section in chapter three.

4.2.4 Mitochondrial coupling assay

Mitochondrial coupling assays were also performed as described in the methods section in chapter three.

4.2.5 Statistical analyses

Separate n-parameter logistic regressions were fit for each mitochondrial isolate with metrics of mitochondrial function (basal respiration, maximal respiration and spare respiratory capacity) as dependent variable and H_2S concentration as independent variable, using the nplr package in R (Commo and Bot 2016). Based on regression models, AUC was estimated based on Simpson's rule (Commo and Bot 2016). AUC values were then compared between mitochondrial isolates from the sulfide tolerant and non-tolerant population using one-way and two-way ANOVA.

4.3 Results

To investigate mitochondrial function of H_2S-tolerant and non-tolerant fish populations from the Puyacatengo and Pichucalco drainages, we used the coupling assay on the Seahorse Extracellular Flux Analyzer to quantify different aspects of liver mitochondrial function across

multiple H_2S concentrations, including basal and maximal oxygen consumption rates, and spare respiratory capacity.

To quantitatively compare responses to H_2S exposure across mitochondrial isolates from different populations (sulfide tolerant *vs*. non-tolerant) in both drainages we calculated area under the curve (AUC, a metric of the overall functional response to H_2S) for basal (figure 4.1), spare (figure 4.2) and maximal respiration (figure 4.3). One-way ANOVA results are shown in table 4.2. Comparison of AUC for basal respiration revealed no significant difference between the sulfide tolerant and non-tolerant population in the Puyacatengo drainage, while there was a significant difference in AUC for basal respiration between the Pichucalco drainage sulfide tolerant and non-tolerant populations ($F_{1,6}$ = 3373.2 P = 1.12e-05). The AUC was significantly different for spare respiration between tolerant versus non-tolerant populations in the Puyacatengo drainage ($F_{1,6}$ = 113.38, P = 4.04e-05) and the Pichucalco drainage ($F_{1,6}$ = 34.50, P = <0.01). Likewise, the AUC for maximal respiration significantly differed between tolerant versus non-tolerant populations in the Puyacatengo drainage ($F_{1,6}$ = 59.61, P = <0.01) and the Pichucalco drainage ($F_{1,6}$ = 49.77, P = <0.01). Mitochondria isolated from sulfide tolerant fish in the Puyacatengo exhibited higher AUC for spare and maximal respirations compared to those from non-tolerant fish (figure 4.2a and 4.3a). Sulfide tolerant fish in the Pichucalco drainage exhibited higher AUC for basal, spare and maximal respirations compared to those from non-tolerant fish in same drainage and also to both populations in the Puyacatengo drainage (figure 4.1, 4.2 and 4.3).

For both sulfide tolerant and non-tolerant populations in the Puyacatengo and the Pichucalco drainages, all metrics of mitochondrial function generally declined with the increase of H_2S concentrations (Puyacatengo – basal respiration $F_{1,58}$ = 54.04, P = 7.54e-10; Pichucalco – basal respiration $F_{1,36}$ = 65.56, P = 1.27e-09; Puyacatengo – spare respiration $F_{1,60}$ = 91.07, P = 1.22e-13; Pichucalco – spare respiration $F_{1,36}$ = 39.40, P = 2.97e-07; Puyacatengo – maximal

respiration $F_{1,60}$ = 106.85, P = 6.01e-15 and Pichucalco – maximal respiration $F_{1,36}$ = 44.73, P = 8.45e-08) (figure 4.4, 4.5 and 4.6). In the Pichucalco drainage, there were significant differences between populations for basal respiration ($F_{1,36}$ = 25.82, P = 1.17e-05), spare respiration ($F_{1,36}$ = 20.80, P = 5.71e-05) and for maximal respiration ($F_{1,36}$ = 23.62, P = 2.30e-05) while in the Puyacatengo drainage we only observed significant differences between populations for spare respiration ($F_{1,60}$ = 6.66, P = 0.01) and for maximal respiration ($F_{1,60}$ = 8.08, P = <0.01). The interaction between populations and H_2S concentrations was only significant in the Puyacatengo drainage for maximal respiration ($F_{1,60}$ = 4.07, P = 0.05). Sulfide tolerant fish from the Pichucalco maintained a higher AUC in comparison to non-tolerant fish and they maintained a high basal, spare and maximal respiration at almost all the H_2S concentrations exposures tested. Estimated two-way ANOVA results were shown in table 4.3. Overall, these results show that sulfide tolerant fish populations in the both Puyacatengo and Pichucalco drainages maintained aerobic ATP production in the presence of H_2S.

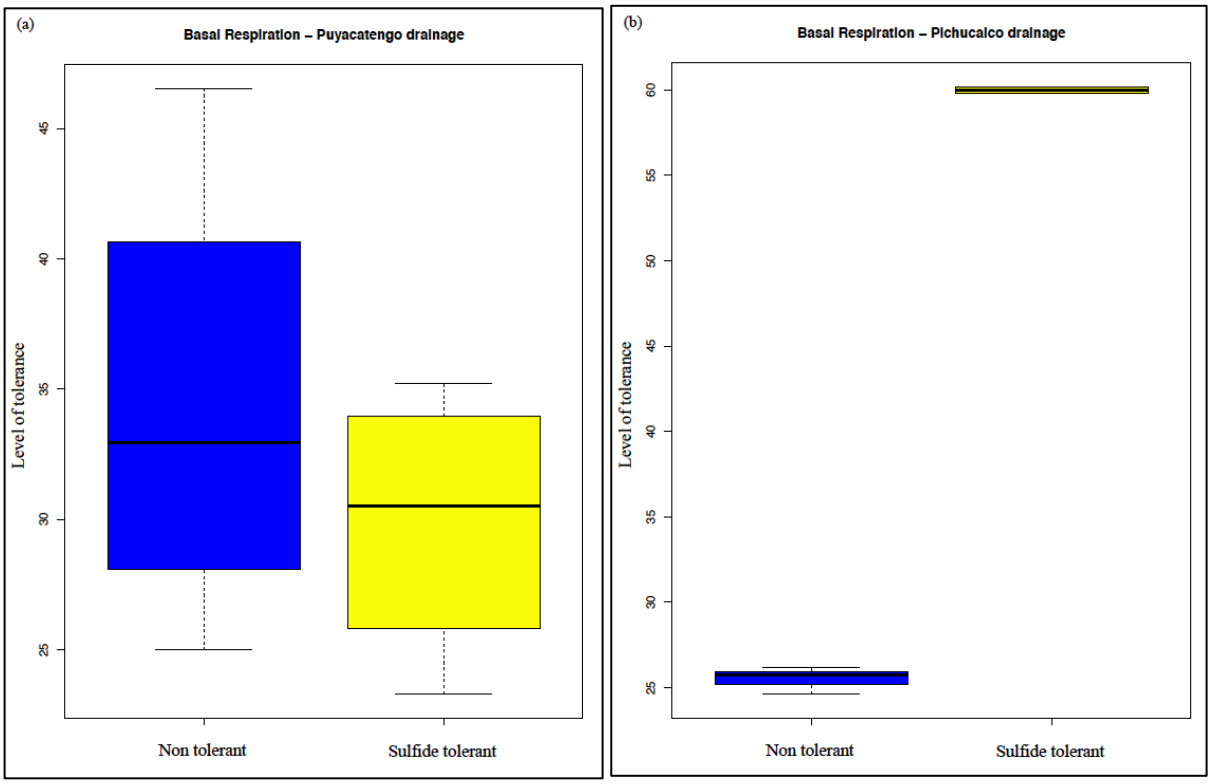


Figure 4.1: The level of tolerance (area under the curve, AUC) expressed by the two populations, non-tolerant and sulfide tolerant for basal respiration. a). Puyacatengo drainage b). Pichucalco drainage. Blue boxes represent individuals from non-tolerant population and yellow boxes represent individuals from sulfide tolerant population. In each box plot, the central rectangle length represents the first quartile to the third quartile in data distribution. A line inside the rectangle shows the median and the lines above and below the box show the locations of the minimum and maximum of the data set.

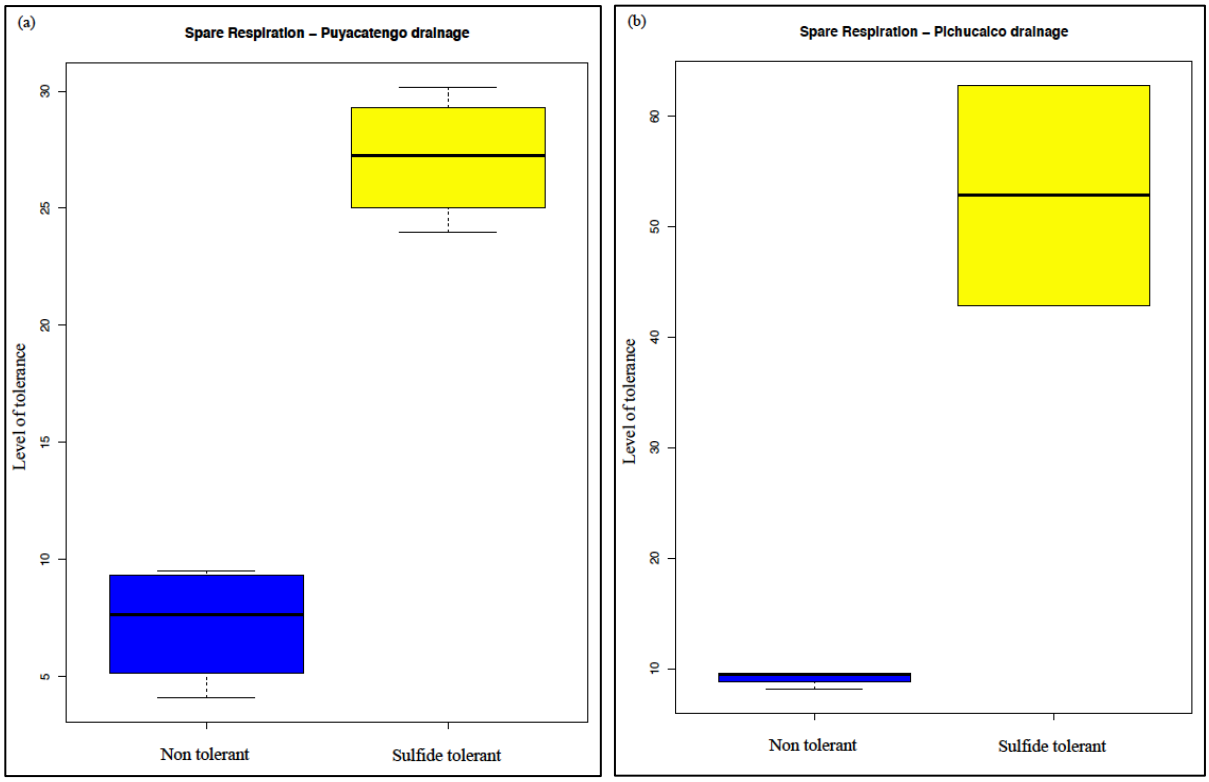


Figure 4.2: The level of tolerance (area under the curve, AUC) expressed by the two populations, non-tolerant and sulfide tolerant for spare respiration. a). Puyacatengo drainage b). Pichucalco drainage. Blue boxes represent individuals from non-tolerant population and yellow boxes represent individuals from sulfide tolerant population. In each box plot, the central rectangle length represents the first quartile to the third quartile in data distribution. A line inside the rectangle shows the median and the lines above and below the box show the locations of the minimum and maximum of the data set.

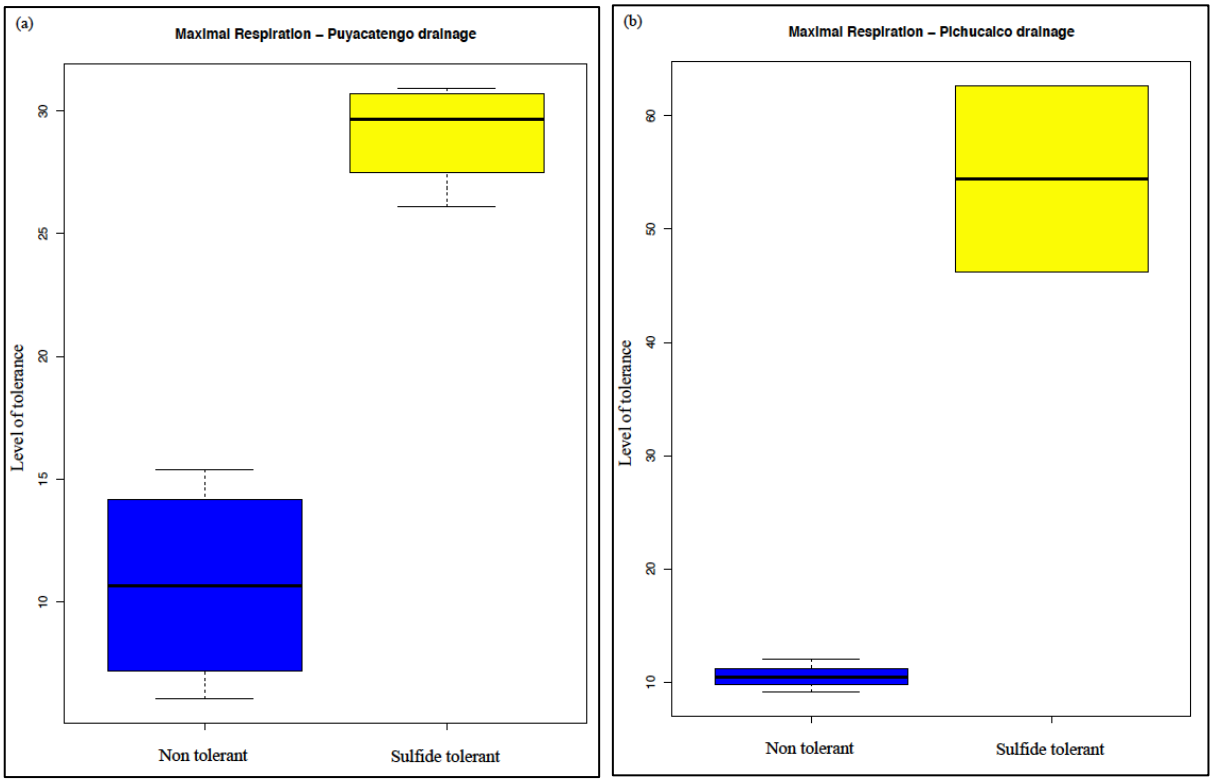
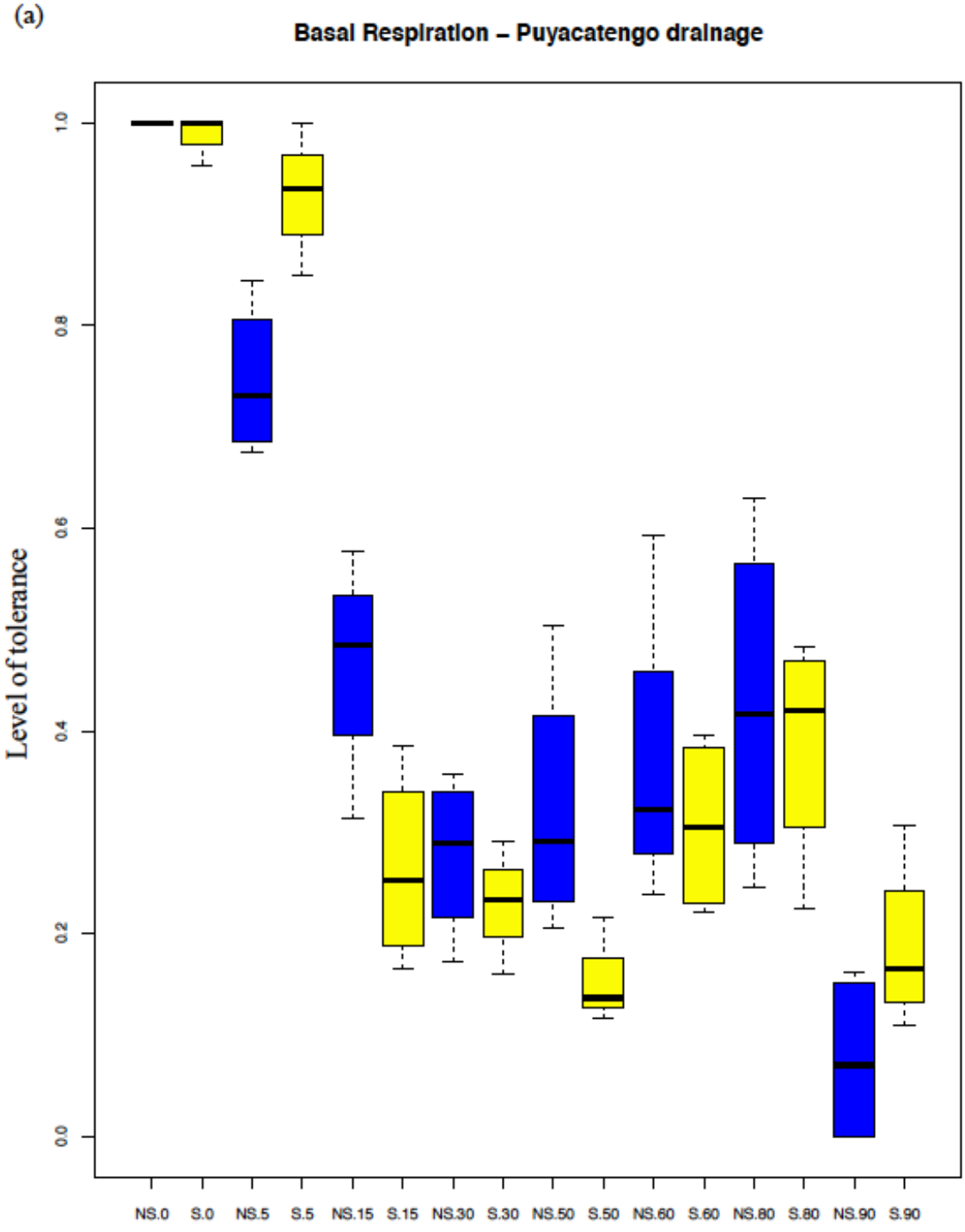


Figure 4.3: The level of tolerance (area under the curve, AUC) expressed by the two populations, non-tolerant and sulfide tolerant for maximal respiration. a). Puyacatengo drainage b). Pichucalco drainage. Blue boxes represent individuals from non-tolerant population and yellow boxes represent individuals from sulfide tolerant population. In each box plot, the central rectangle length represents the first quartile to the third quartile in data distribution. A line inside the rectangle shows the median and the lines above and below the box show the locations of the minimum and maximum of the data set.

Table 4.2: Results of one-way analysis of variance (ANOVA) examining variation in basal, spare and maximal respiration in the Puyacatengo and the Pichucalco drainages. Effects that are significant after accounting for multiple testing (α' = 0.05) are highlighted in bold font.

Effect	df	F value	p
A. Basal respiration			
a. Puyacatengo drainage			
Population	1	0.73	0.43
Error	6		
b. Pichucalco drainage			
Population	1	3373.2	**1.12e-05**
Error	3		
B. Spare respiration			
a. Puyacatengo drainage			
Population	1	113.38	**4.04e-05**
Error	6		
b. Pichucalco drainage			
Population	1	34.50	**<0.01**
Error	3		
C. Maximal respiration			
a. Puyacatengo drainage			
Population	1	59.61	**<0.01**
Error	6		
b. Pichucalco drainage			
Population	1	49.77	**<0.01**
Error	3		

(a)


Basal Respiration – Puyacatengo drainage
Level of tolerance
0.0
0.2
0.4
0.6
0.8
1.0
NS.0
S.0
NS.5
S.5
NS.15
S.15
NS.30
S.30
NS.50
S.50
NS.60
S.60
NS.80
S.80
NS.90
S.90

(b)

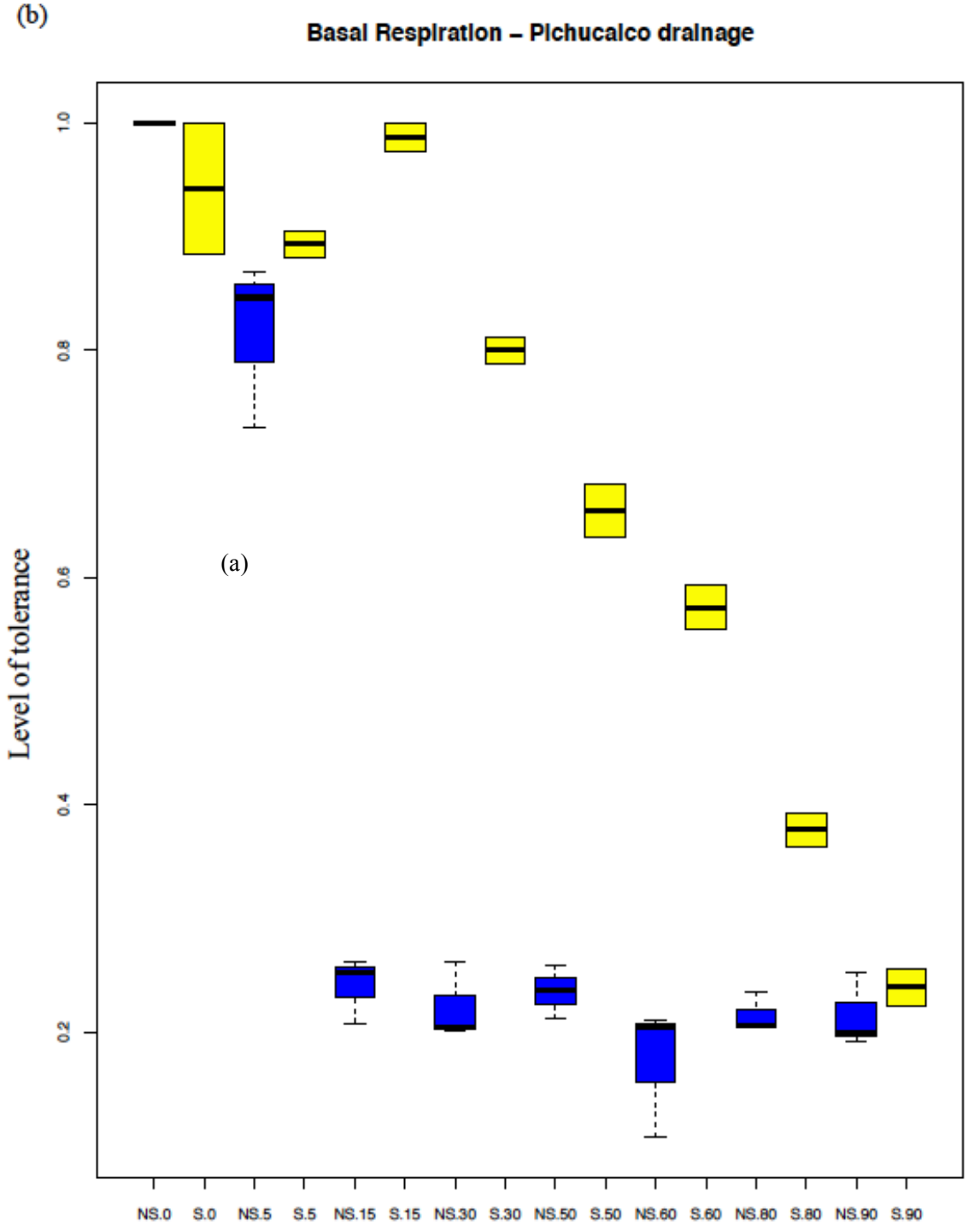
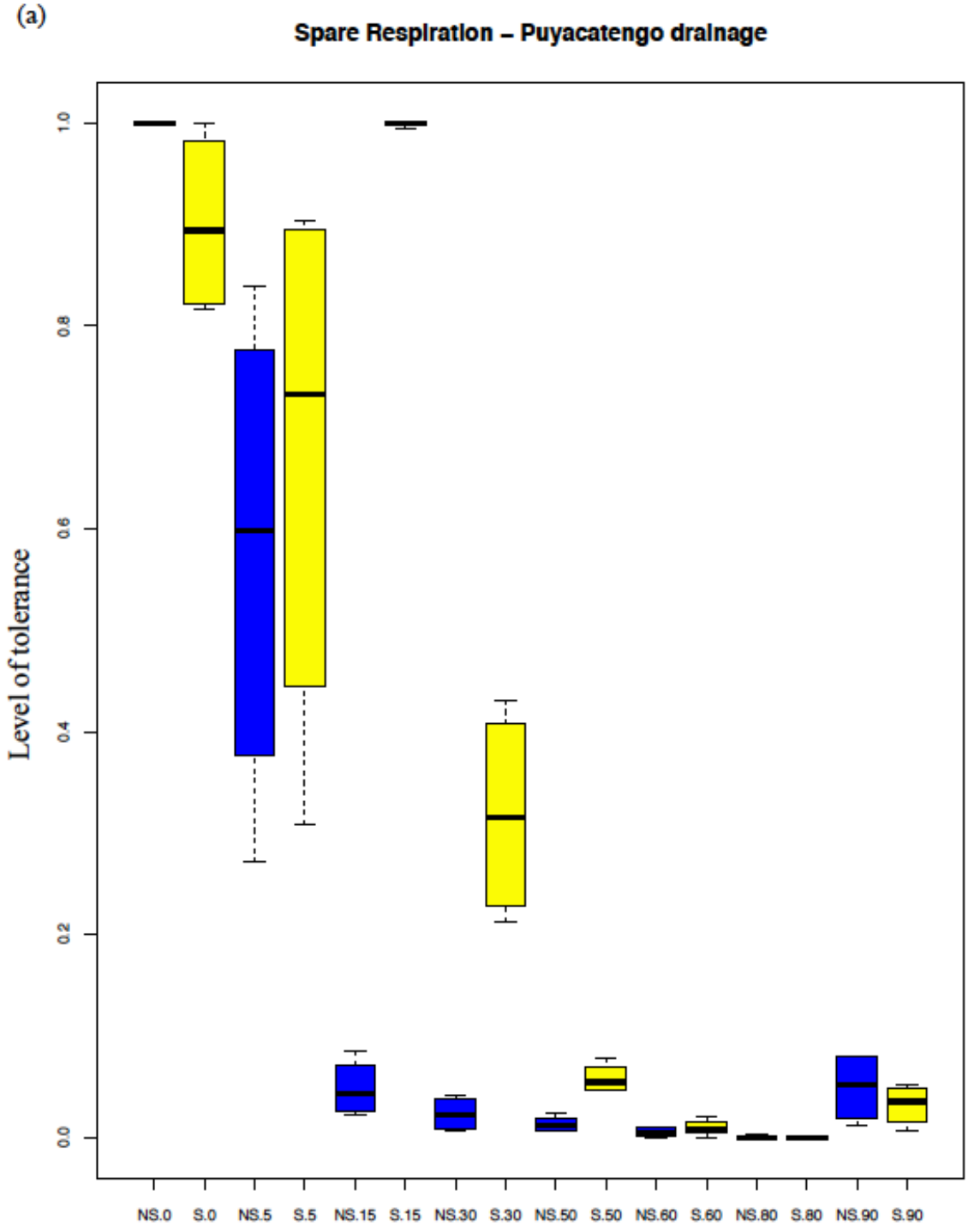

Basal Respiration – Pichucalco drainage
Level of tolerance
1.0
0.8
0.6
0.4
0.2
(a)
NS.0
S.0
NS.5
S.5
NS.15
S.15
NS.30
S.30
NS.50
S.50
NS.60
S.60
NS.80
S.80
NS.90
S.90

Figure 4.4: Basal respiration in two populations, non-tolerant (NS, blue boxes) and sulfide tolerant (S, yellow boxes) in the Puyacatengo drainage (a) and the Pichucalco drainage (b) across multiple H_2S concentrations. In each box plot, the central rectangle length represents the first quartile to the third quartile in data distribution. A line inside the rectangle shows the median and the lines above and below the box show the locations of the minimum and maximum of the data set.

(a)


Spare Respiration – Puyacatengo drainage
Level of tolerance
0.0
0.2
0.4
0.6
0.8
1.0
NS.0
S.0
NS.5
S.5
NS.15
S.15
NS.30
S.30
NS.50
S.50
NS.60
S.60
NS.80
S.80
NS.90
S.90

(b)

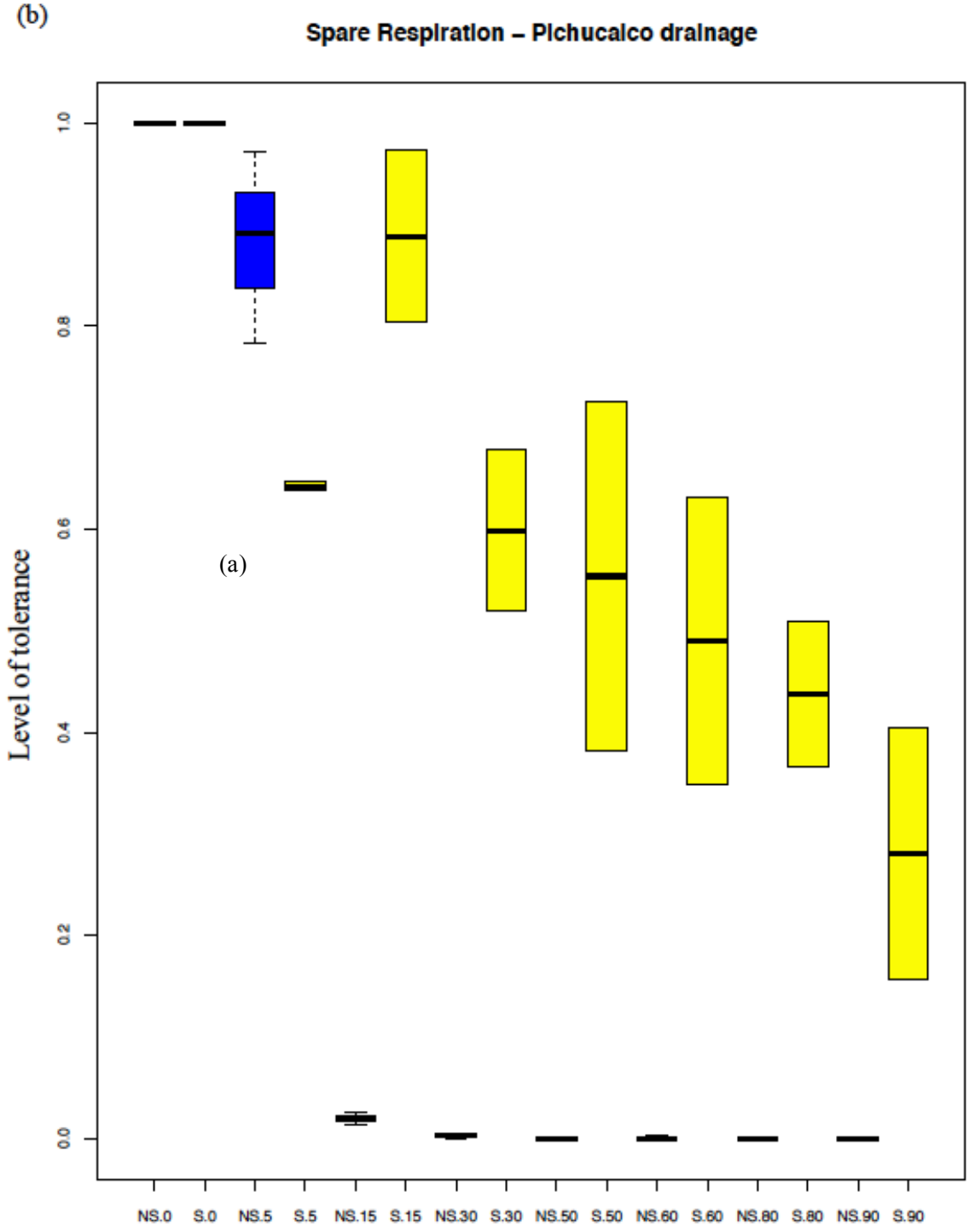
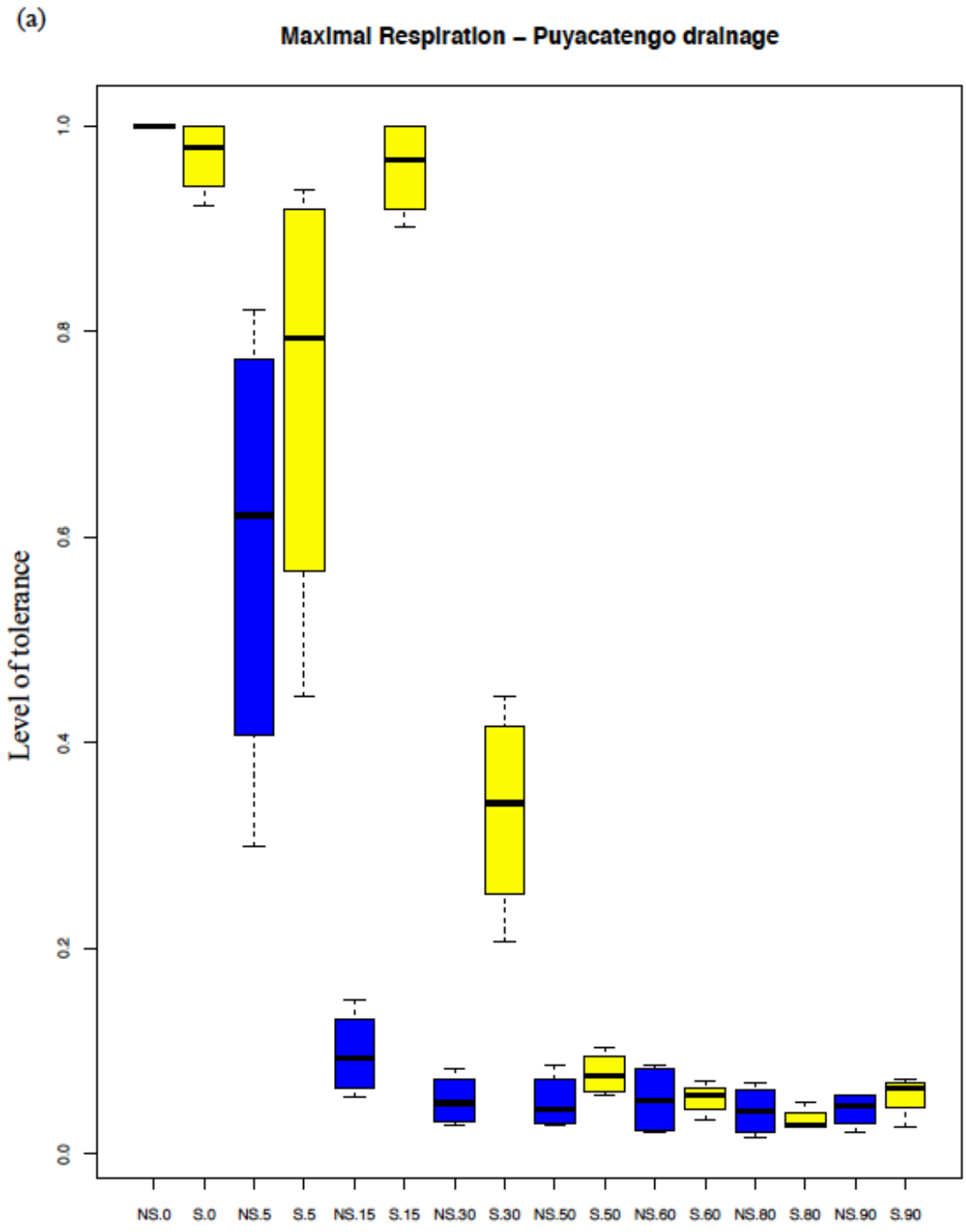

Spare Respiration – Pichucalco drainage
Level of tolerance
0.0
0.2
0.4
0.6
0.8
1.0
(a)
NS.0
S.0
NS.5
S.5
NS.15
S.15
NS.30
S.30
NS.50
S.50
NS.60
S.60
NS.80
S.80
NS.90
S.90

Figure 4.5: Spare respiration in two populations, non-tolerant (NS, blue boxes) and sulfide tolerant (S, yellow boxes) in the Puyacatengo drainage (a) and the Pichucalco drainage (b) across multiple H_2S concentrations. In each box plot, the central rectangle length represents the first quartile to the third quartile in data distribution. A line inside the rectangle shows the median and the lines above and below the box show the locations of the minimum and maximum of the data set.


(a)
Maximal Respiration – Puyacatengo drainage
Level of tolerance
0.0
0.2
0.4
0.6
0.8
1.0
NS.0
S.0
NS.5
S.5
NS.15
S.15
NS.30
S.30
NS.50
S.50
NS.60
S.60
NS.80
S.80
NS.90
S.90

(b)

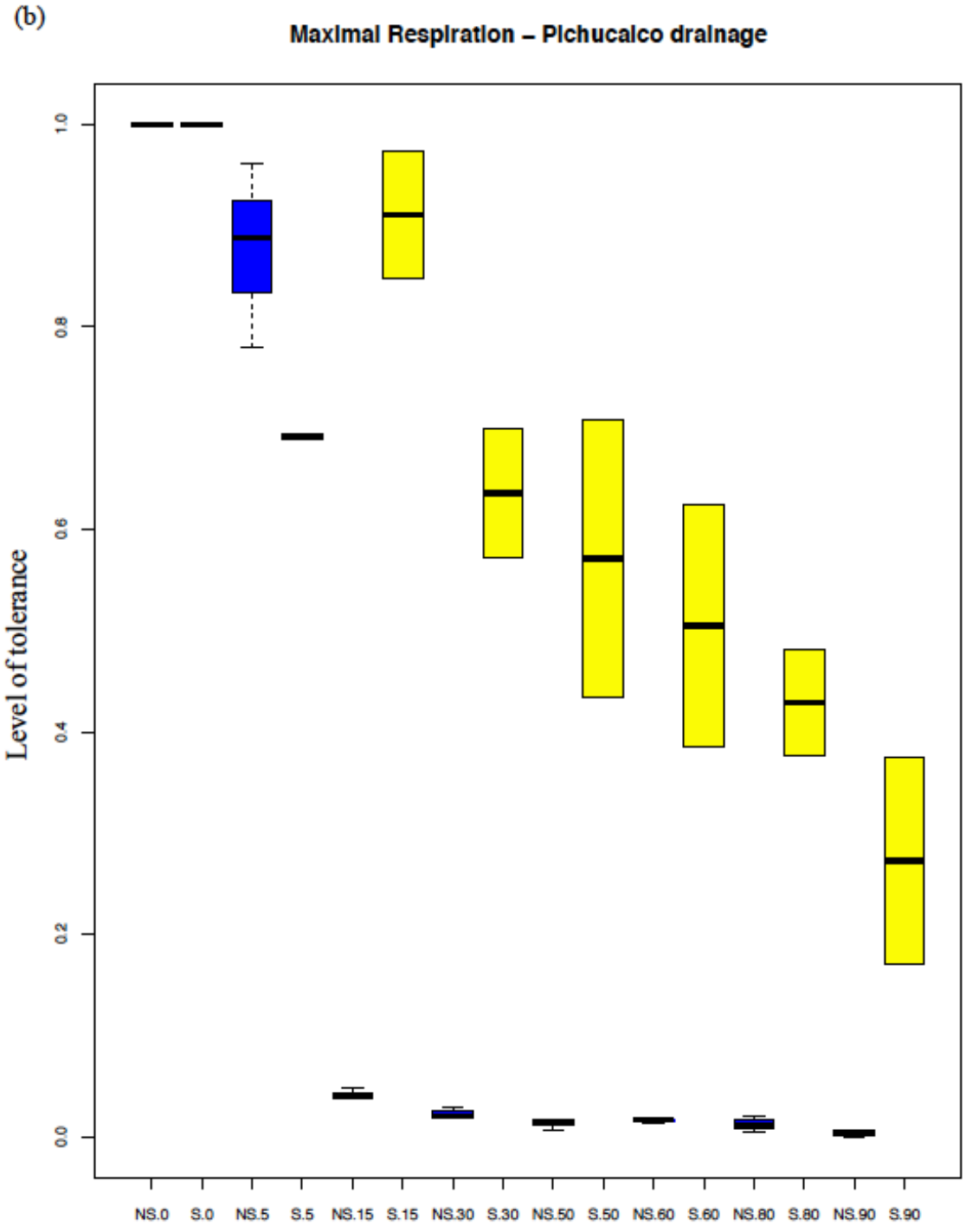

Maximal Respiration – Pichucalco drainage
Level of tolerance
0.0
0.2
0.4
0.6
0.8
1.0
NS.0
S.0
NS.5
S.5
NS.15
S.15
NS.30
S.30
NS.50
S.50
NS.60
S.60
NS.80
S.80
NS.90
S.90

Figure 4.6: Maximal respiration in two populations, non-tolerant (NS, blue boxes) and sulfide tolerant (S, yellow boxes) in the Puyacatengo drainage (a) and the Pichucalco drainage (b) across multiple H_2S concentrations. In each box plot, the central rectangle length represents the first quartile to the third quartile in data distribution. A line inside the rectangle shows the median and the lines above and below the box show the locations of the minimum and maximum of the data set.

Table 4.3: Results of two-way analysis of variance (ANOVA) examining variation in basal, spare and maximal respiration in the Puyacatengo and the Pichucalco drainages. Only the effects that are significant after accounting for multiple testing ($\alpha' = 0.05$) are presented here.

Effect	df	F value	p
Basal respiration – Puyacatengo drainage			
Concentration	1	54.04	7.54e-10
Error	58		
Basal respiration – Pichucalco drainage			
Population	1	25.82	1.17e-05
Concentration	1	65.56	1.27e-09
Error	36		
Spare respiration – Puyacatengo drainage			
Population	1	6.66	0.01
Concentration	1	91.07	1.22e-13
Error	60		
Spare respiration – Pichucalco drainage			
Population	1	20.80	5.71e-05
Concentration	1	39.40	2.97e-07
Error	36		
Maximal respiration – Puyacatengo drainage			
Population	1	8.08	<0.01
Concentration	1	106.85	6.01e-15
Population x concentration	1	4.07	0.048
Error	60		
Maximal respiration – Pichucalco drainage			
Population	1	23.62	2.30e-05
Concentration	1	44.73	8.45e-08
Error	36		

4.4 Discussion

Taken together with the chapter three studies, this body of work is the first to clarify the bioenergetic mechanisms that are responsible for sustaining life and shaping evolutionary processes in extreme sulfidic habitats wherein the environmental stressor is both an inhibitor of and contributor to oxidative phosphorylation for ATP production. This study reveals that sulfide tolerant fish populations in the Puyacatengo and the Pichucalco drainages maintain mitochondrial function during H_2S exposure. This achievement occurs via adaptations in mitochondria that enable significantly higher, relative to non-tolerant populations, maximal respiratory and spare respiratory capacities with increasing concentrations of H_2S.

The sulfide tolerant population from the Pichucalco drainage maintained higher tolerance relative to the Puyacatengo sulfide tolerant population. These findings concur with recent gene expression data in fish populations across drainages (Kelley, Arias-Rodriguez et al. 2016). Genome-wide expression patterns in tolerant populations from the Pichucalco drainage display significant upregulation of H_2S detoxification pathways (Kelley, Arias-Rodriguez et al. 2016). Each H_2S contributes two electrons to the electron transport chain upon detoxification, potentially serving as the source of energy to support the increased spare respiratory capacity we observed in the Pichucalco mitochondria in this study. Moreover, enhanced detoxification would maintain lower levels of H_2S further protecting COX activity, which also has reduced sensitivity in these populations.

Overall, gene expression differences in sulfide tolerant populations are least prominent in the Puyacatengo (303 up regulated and 336 down regulated genes) and the Tacotalpa drainages (494 up regulated and 493 down regulated), but most pronounced in the Pichucalco drainage (1,215 upregulated and 1,420 down regulated) (Kelley, Arias-Rodriguez et al. 2016). H_2S tolerance capacity is highest in the Pichucalco compared to other drainages, strengthening

previous studies that this population exhibits overlapping mechanisms of tolerance to exist in nature, such as modification of COX and enhancement of H_2S detoxification as suggested by genomic patterns of enzymatic pathways (Pfenninger, Lerp et al. 2014, Kelley, Arias-Rodriguez et al. 2016, Passow, Henpita et al. 2017). Furthermore, considering the age of colonization in these drainages, the Pichucalco sulfide tolerant population colonized significantly earlier (~300,000 years ago) than the sulfide population in the Puyacatengo (<50,000 years ago) (Pfenninger, Lerp et al. 2014, Kelley, Arias-Rodriguez et al. 2016) and shares little gene flow with populations in the adjacent non-tolerant population relative to other drainages (Pfenninger, Lerp et al. 2014). The Pichucalco drainage also has the highest H_2S concentration recorded than other drainages (Table 4.1), presumably providing the highest level of selective pressure. Furthermore, in addition to the presence of H_2S, sulfide springs also differ from non-sulfidic habitats in exhibiting higher specific conductivities, lower pH, and lower dissolved oxygen concentrations (Table 4.1) (Kelley, Arias-Rodriguez et al. 2016). These drainage specific environmental variations may serve as additional selective pressures that promote the utilization of one or more different strategies to survive these extreme habitats.

P. mexicana provides an ideal model system to study mechanisms involved in ecological speciation; H_2S appears to be key driver in the evolution of sulfide spring ecotypes (Tobler, Palacios et al. 2011, Riesch, Plath et al. 2014). Previous studies demonstrate that sulfide tolerant ecotypes in all drainages are reproductively isolated, genetically differentiated, and differ from conspecifics from adjacent non-sulfidic habitats in a series of phenotypic traits, including physiological, morphological, behavioral, and life history characteristics (Tobler, Palacios et al. 2011, Greenway, Arias-Rodriguez et al. 2014, Pfenninger, Lerp et al. 2014, Tobler, Passow et al. 2016, Passow, Henpita et al. 2017).

Pfenninger et al. (2014) revealed H_2S-resistant COX exist in sulfide tolerant fish in the Puyacatengo and the Pichucalco drainages, but not in Tacotalpa drainage (Pfenninger, Lerp et al. 2014). Our third chapter results indicate that, even without H_2S-resistant COX, sulfide tolerant fish in the Tacotalpa drainage maintain maximal respiration following H_2S exposure. Genetic patterns for H_2S detoxification pathways (Kelley, Arias-Rodriguez et al. 2016) and the SQR gene expression results in the second chapter considered together with findings in both the third and fourth chapters provide evidence that these extremophile populations use more than one strategy to achieve H_2S tolerance via mitochondrial modifications.

There were some limitations in this study. One major limitation was the small sample size available for experiments. The main reason for lower sample size was the high mortality of wild captured fish populations under laboratory conditions. One other major reason for lower biological replicates per population was the size of the fish. To obtain enough mitochondrial yield to run one coupling assay, multiple fish livers were pooled. Pooling liver mitochondria to obtain one biological replicate not only required higher fish numbers than expected, but increased variation to the results as well. Variation in the gender, age, size and the reproductive status of the used fish, also added variation to the results. Another limitation of this study was that mitochondrial membrane integrity may have been compromised during the isolation process. The centrifugation methods used to isolate mitochondria may have resulted in lower yield and decreased the mitochondrial integrity.

Future studies need to more rigorously address SQR enzymatic kinetics to identify whether SQR is modified such that the rate of detoxification is faster in tolerant fish. In addition to our direct contribution to evolution ecology, this body of work may also facilitate biomedical advances, such as the use of H_2S donating drugs to treat a variety of diseases related to H_2S

imbalance or in cancer biology wherein tumors overproduce H_2S (Szabo, Coletta et al. 2013, Módis, Bos et al. 2014).

CHAPTER V

CONCLUSIONS

Hydrogen sulfide (H_2S) is well known as a toxic gas that has hormetic effects including toxic inhibition of cytochrome c oxidase of the mitochondrial electron transport chain at high concentrations, and maintenance of normal vascular and neural functions at low concentrations. Even though elevated exogenous H_2S levels in the environment are toxic to most terrestrial and aquatic organisms, *Poecilia mexicana* thrives in H_2S rich environments. The cellular mechanisms whereby organisms tolerate extreme H_2S are not fully understood. Our central hypothesis was that sulfide tolerant fish have an enhanced H_2S detoxification capacity and/or resistance to H_2S toxicity following exposure, relative to non-tolerant fish. To test this, we proposed three aims. Aim 1: Measure expression of a panel of genes relevant to H_2S physiology across tissue types in lab-reared sulfide tolerant versus non-tolerant fish populations (the Tacotalpa lineage) following H_2S exposure. Aim 2: Compare mitochondrial function in tolerant versus non-tolerant fish livers upon increasing doses of H_2S in both lab-reared and wild-captured fish (the Tacotalpa lineage). Aim 3: Identify mitochondrial adaptations in wild-captured tolerant versus non-tolerant populations of the Puyacatengo and the Pichucalco lineages, which both harbor a H_2S-resistant COX enzyme.

First, we hypothesized that sulfide tolerant fish differentially express genes involved in maintaining H_2S homeostasis (chapter two). This study documented complex changes in the expression of candidate genes associated with H_2S toxicity, detoxification, and endogenous production. We found significant differences in gene expression patterns related to H_2S detoxification between lab-reared sulfide tolerant and non-tolerant populations originating from the Tacotalpa drainage.

Since mitochondria are both the site of H_2S toxicity as well as enzymatic detoxification, we further hypothesized that tolerance is achieved by modifications to mitochondrial respiration. To test this hypothesis we compared mitochondrial function between lab-reared and wild captured sulfide tolerant and non-tolerant populations originating from the Tacotalpa drainage (chapter three) and wild captured sulfide tolerant and non-tolerant populations originating from the Puyacatengo and the Pichucalco drainages (chapter four). We determined that sulfide tolerant fish are able to maintain mitochondrial respiration in the presence of increasing concentrations of H_2S relative to non-tolerant fish, and that the sulfide tolerant population captured from the Pichucalco drainage, which has the highest concentration of environmental H_2S compared to other drainages tested, exhibit the greatest degree of H_2S tolerance compared to the sulfide tolerant populations from drainages with lower environmental H_2S.

In summary, this dissertation documents a physiological approach to increase our understanding of H_2S tolerance in a fish (*Poecilia mexicana*) study system. This body of work is the first to identify the cellular mechanism that enables life in extreme sulfidic habitats. Our data show that these extremophile fish have adapted to an environmental stressor that is both an inhibitor of and contributor to oxidative phosphorylation for ATP production. These organisms not only survive in an extremely toxic environment by enhanced detoxification of H_2S at the mitochondria, but also utilize the stressor to enhance spare respiratory capacity. Lab-reared fish,

which had never been exposed to H_2S in their lifetime, exhibited a lesser degree of tolerance relative to wild-caught fish suggesting these adaptations are heritable and rely on continuous selective pressure to achieve maximal tolerance. While this directly contributes to the field of evolution ecology, these findings may also advance biomedical applications. It has become apparent that H_2S is able to protect the integrity of mitochondria and support mitochondrial function, thereby contributing to the preservation of cellular energetics which is relevant in treating inflammatory diseases. Likewise, understanding the exploitation of H2S as a metabolic fuel in certain cancers may aide in the identification of new drug targets to slow cancer growth.

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