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ORIGINAL PAPER

Hydrogen sulphide toxicity and the importance of amphibious behaviour in a mangrove fish inhabiting sulphide-rich habitats

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Abstract

We investigated amphibious behaviour, hydrogen sulphide (H_2S) tolerance, and the mechanism of H_2S toxicity in the amphibious mangrove rivulus (*Kryptolebias marmoratus*). We found that fish emersed (left water) in response to acutely elevated [H_2S] (~ 130–200 µmol l^{-1}). The emersion response to H_2S may be influenced by prior acclimation history due to acclimation-induced alterations in gill morphology and/or the density and size of neuroepithelial cells (NECs) on the gills and skin. Thus, we acclimated fish to water (control), H_2S-rich water, or air and tested the hypotheses that acclimation history influences H_2S sensitivity due to acclimation-induced changes in (i) gill surface area and/or (ii) NEC density and/or size. Air-acclimated fish emersed at significantly lower [H_2S] relative to fish acclimated to control or H_2S-rich water, but exhibited no change in gill surface area or in NEC density or size in the gills or skin. Despite possessing exceptional H_2S tolerance, all fish lost equilibrium when unable to emerse from environments containing extremely elevated [H_2S] (2272 ± 46 µmol l^{-1}). Consequently, we tested the hypothesis that impaired blood oxygen transport (i.e., sulphemoglobin formation) causes H_2S toxicity in amphibious fishes. In vitro exposure of red blood cells to physiologically relevant [H_2S] did not cause a substantial increase in sulphemoglobin formation. We found evidence, however, for an alternative hypothesis that H_2S toxicity is caused by impaired oxidative phosphorylation (i.e., cytochrome c oxidase inhibition). Collectively, our results show that amphibious behaviour is critical for the survival of *K. marmoratus* in H_2S-rich environments as fish experience impaired oxidative phosphorylation when unable to emerse.

Keywords Hydrogen sulphide · Amphibious fish · Emersion · Sulphemoglobin · Cytochrome c oxidase

Introduction

Hydrogen sulphide (H_2S) is a lethal waterborne toxin that is naturally produced in a variety of aquatic habitats through geochemical and biological processes (Kelley et al. 2016). H_2S easily diffuses across biological membranes and causes acute toxicity in most exposed organisms at low micromolar concentrations (Völkel and Berenbrink 2000; Riesch et al. 2015; Tobler et al. 2018). The primary mechanism of H_2S toxicity is through the inhibition of cytochrome c oxidase (COX), the terminal enzyme in the electron transport chain. In mitochondria, H_2S reversibly binds to COX, thereby inhibiting oxidative phosphorylation (Bagarinao and Vetter 1989). As well, several authors have proposed that impaired blood oxygen transport may also contribute to H_2S toxicity through the complete, or partial, conversion of oxyhemoglobin (oxyHb) to sulphemoglobin (SHb)—a hemoglobin (Hb) derivative with a much lower oxygen affinity (Völkel and Berenbrink 2000; Pietri et al. 2011; Riesch et al. 2011). Consequently, cellular anoxia and the prevention of aerobic ATP production cause death in many H_2S exposed organisms. Nevertheless, various aquatic organisms, including some fishes, continue to survive, grow, and reproduce in H_2S-rich environments (Bagarinao and Vetter 1989; Riesch et al. 2010, 2011; Taylor 2012).

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Many of the fishes that inhabit H_2S-rich environments possess physiological modifications that promote H_2S tolerance. For example, populations of H_2S-tolerant *P. mexicana* and *P. sulphuraria* possess amino acid substitutions in the COX1 protein that result in reduced sensitivity to H_2S (Pfenninger et al. 2014). Furthermore, other species of H_2S-tolerant fishes (e.g., *Cyprinus carpio, Fundulus parvipinnis*, and *Gillichthys mirabilis*) possess Hb that has a low susceptibility to SHb formation (Bagarinao and Vetter 1992; Völkel and Berenbrink 2000). These physiological modifications improve H_2S tolerance of fishes by sustaining aerobic metabolism.

Many fishes also use alternative respiratory strategies to mediate survival in H_2S-rich environments. *P. mexicana* spend a substantial portion of time (~ 73%) performing aquatic surface respiration (ASR) in sulphidic water (Plath et al. 2007). As well, *Hoplosternum littorale* and *Megalops atlanticus* significantly increase air-breathing frequency when exposed to H_2S (Brauner et al. 1995; Geiger et al. 2000). Although these strategies enhance oxygen uptake, they do not inhibit the diffusion of H_2S across biological membranes. As a result, one approach—available only to amphibious fishes—is to avoid aquatic H_2S exposure altogether by leaving water (Abel et al. 1987; Rossi et al. 2019). Amphibious fishes leave water (emerse) in response to various unfavorable abiotic factors (Ebeling et al. 1970; Davenport and Woolmington 1981; Regan et al. 2011; Urbina et al. 2011; Robertson et al. 2015; Gibson et al. 2015), including H_2S (Abel et al. 1987; Rossi et al. 2019). Abel et al. (1987) found that ~ 4 µmol l^{-1} H_2S elicited emersion in laboratory-reared amphibious mangrove rivulus (*Kryptolebias marmoratus*) (Abel et al. 1987). However, we recently observed wild *K. marmoratus* immersed in stagnant pools that experience surges in H_2S up to 1166 µmol l^{-1} (Rossi et al. 2019), a concentration 4 orders of magnitude higher than previously shown to elicit emersion in laboratory-reared individuals (Abel et al. 1987). Thus, both the sensitivity and tolerance of the highly amphibious *K. marmoratus* to aquatic H_2S remain uncertain.

Recent acclimation history of amphibious fishes may impact H_2S sensitivity. One mechanism through which acclimation history may alter H_2S sensitivity is through changes in gill surface area. Acclimation-induced changes in gill surface area likely alter the diffusion of both H_2S and O_2 across the gills. In fishes, H_2S detoxification primarily occurs through the oxidation of H_2S to less toxic byproducts (Bagarinao 1992, Ip et al. 2004), and thus, enhanced O_2 uptake would be beneficial for fishes inhabiting H_2S-rich environments (Tobler et al. 2011). Consequently, acclimation-induced changes in gill surface likely impact the ability of fishes to detoxify H_2S and, therefore, may influence H_2S sensitivity and amphibious behaviour. Air-acclimated amphibious fishes reduce gill surface area through an increase in the height of the interlamellar cell mass (ILCM; Ong et al. 2007; Turko et al. 2011, 2019). In addition, exposure to aquatic H_2S may increase gill surface area, as previously noted in wild *P. Mexicana* collected from sulphidic environments (Tobler et al. 2008). Alternatively, acclimation history may impact H_2S sensitivity through alterations in the density and/or size of serotonergic neuroepithelial cells (NECs). Serotonergic NECs are chemoreceptive cells found on the gills of fishes (Milsom and Burleson 2007) as well as on the skin of the two species of amphibious fishes studied to date (*Kryptolebias marmoratus*, Regan et al. 2011; *Periophthalmodon schlosseri*; Zaccone et al. 2017). H_2S has been demonstrated to be a potent stimulator of serotonergic NECs (Olson et al. 2008; Porteus et al. 2014) and NEC stimulation appears to trigger emersion behaviour in *K. marmoratus* (Regan et al. 2011). Moreover, in *K. marmoratus*, the density and size of serotonergic NECs can be altered by acclimation to various environmental conditions (e.g., hypoxia, Regan et al. 2011; hypercapnia; Robertson et al. 2015). Therefore, we tested the hypotheses that acclimation history of amphibious fishes influences H_2S sensitivity due to acclimation-induced changes in (i) gill surface area and/or (ii) serotonergic NEC density and/or size.

Although various amphibious fishes are remarkably H_2S tolerant, prolonged exposure to environmentally relevant levels of aquatic H_2S causes acute toxicity in some species (i.e., *Boleophthalmus boddaerti, K. marmoratus*) (Ip et al. 2004; Rossi et al. 2019). Thus, we also examined the mechanism of H_2S toxicity by testing the hypotheses that impaired blood oxygen transport causes H_2S toxicity in amphibious fishes and/or that H_2S toxicity in amphibious fishes is caused by impaired oxidative phosphorylation. These hypotheses predict that (i) fish exposed to a physiologically relevant [H_2S] in vitro would contain significantly higher levels of SHb relative to H_2S-naïve fish and (ii) fish exposed to H_2S until loss of equilibrium (LOE) would have significantly lower COX activity relative to H_2S-naïve fish. The amphibious mangrove rivulus (*K. marmoratus*) is the ideal model organism to study the behavioural and physiological responses of amphibious fishes to H_2S, because they naturally inhabit crab burrows and ephemeral pools containing high levels of H_2S (Taylor 2012; Rossi et al. 2019) and can survive for weeks out of water (Taylor 1990). We acclimated fish to three ecologically relevant treatments: water (control), H_2S-rich water, and air for 3 days and examined emersion behaviour in response to acutely elevated aquatic H_2S and gill surface area. We then examined NEC density and size in the gills and skin, activity and LOE in elevated [H_2S], whole-body [total sulphides] at LOE, in vitro SHb formation, and COX activity following H_2S-induced LOE in water- and air-acclimated fish.

Materials and methods

Experimental animals

We obtained laboratory adult *K. marmoratus* hermaphrodites (strain 50.91, 0.047–0.150 g; Tatarenkov et al. 2010) from a breeding colony housed at the Hagen Aqualab at the University of Guelph, Guelph, ON, Canada. Fish were raised and maintained in individual 120 ml semi-transparent plastic containers (FisherBrand Collection Containers, Fisher Scientific) under constant conditions (pH 8.0, 25 °C, 12:12 photoperiod; Frick and Wright 2002a). Fish were maintained in artificial brackish water (15‰) prepared using Instant Ocean Sea Salt (Spectrum Brand Inc., Atlanta, GA, USA). We performed water changes once per week and fed fish *Artemia* sp. nauplii three times per week. Wild fish (0.121–0.356 g) were obtained and maintained as described by Rossi et al. (2019). Briefly, wild fish were captured from mangrove pools on Long Caye, Lighthouse Reef Atoll, Belize in May 2018 using dip nets, Taylor cup traps, and Gee minnow traps (Ritchie and Johnson 1986; Taylor 1990). Following capture, fish were maintained in 120 ml semi-transparent plastic containers (pH 8.1, 36.3 ± 0.2‰) (Rossi et al. 2019). All experiments in this study were approved by the University of Guelph Animal Care Committee (Animal Utilization Protocol 3891 and 3983).

Experimental protocol

Three series of experiments were conducted. In Series I, we acclimated intact fish to water (control), H_2S-rich water, or air (3 days) and examined emersion behaviour in response to increasing [H_2S], as well as gill surface area. In Series II, we acclimated intact fish to water (control) or air (3 days) and assessed NEC density and size in the gills and skin, LOE and activity level in elevated [H_2S] as well as SHb formation and COX activity. Acclimation to H_2S-rich water (3 days) was not included in Series II experiments, as preliminary results revealed no effect of H_2S acclimation on emersion behaviour or gill morphology. In Series III, we examined emersion behaviour in response to increasing [H_2S] and LOE in elevated [H_2S] in wild *K. marmoratus* to determine if these parameters are altered in laboratory fish as a result of generations of de-acclimation to aquatic H_2S.

Series I

To determine the effects of acclimation history on the H_2S emersion response, we acclimated fish to one of three treatments: water (control), H_2S-rich water, or air. We acclimated all experimental fish to room temperature (21.4 ± 0.2 °C) in 120 ml plastic containers for 7 days prior to experimental use. Fish were randomly assigned to treatment groups and were acclimated to respective treatments for the final 3 days of the 7 day room temperature acclimation. We achieved H_2S acclimation by placing fish in water (pH 6.7, 15‰) initially containing 92.7 ± 2.5 µmol l^{-1} H_2S prepared from $Na_2S{\cdot}9H_2O$ (Sigma-Aldrich Canada, Oakville, Canada) dissolved in reverse osmosis water. However, H_2S is an unstable compound and unavoidable oxidation and volatilization of H_2S results in decreases in [H_2S] over time. We performed water changes every 8 h (pH 6.7, 15‰, 92.7 ± 2.5 µmol l^{-1} H_2S) via siphoning to maintain elevated H_2S levels (Fig. S1). Overall, the mean concentration that fish were exposed to during the acclimation period was 51.6 ± 7.2 µmol l^{-1} H_2S (Fig. S1). To mimic conditions in their natural habitat, we acclimated fish to water at pH 6.7 (Rossi et al. 2019). Conducting experiments at pH 6.7 also allowed us to better target the toxic H_2S species of interest, as it constitutes a higher proportion of total sulphides (H_2S, HS^-, S^{2-}) at low pH. We acclimated control fish to water (pH 6.7, 15‰) and performed water changes every 8 h to mimic those in H_2S-acclimated fish. Air acclimation was achieved as described by Ong et al. (2007). Fish in all treatment groups were fasted for the final 3 days of the acclimation period, as emersed fish are unable to feed.

Following acclimation, fish were transferred to individual experimental containers and allowed to acclimate for 30 min. Preliminary experiments verified that a 30-min acclimation period was sufficient for fish to recover from handling stress (L. Tigert, A. Turko, and P. Wright, unpublished data). Experimental containers were surrounded by a moist foam-lined platform (~ 4 cm wide) (Rossi et al. 2019) that provided an area for fish to jump out of water (i.e., emerse) onto. Water (pH 6.7, 15‰) in the experimental container was continuously mixed with a stir bar separated from fish with a mesh divider. To determine the [H_2S] at which fish emersed, we increased the [total sulphide] of experimental water by ~ 50 µmol l^{-1} min^{-1} via the addition of boluses (1 ml) of sulphide stock solution until emersion occurred. Sulphide stock solution causes water pH to increase (Geiger et al. 2000); thus, we simultaneously added HCl (2 µl, 2.5 mol l^{-1}) with each sulphide bolus to maintain pH within a narrow range (pH 6.8 ± 0.03, range 6.6–7.2). We performed additions of sulphide stock solution and HCl behind a visual barrier to ensure that fish behaviour was not influenced by these additions. Emersion was considered to have occurred when 50% of the fish's body was out of water (Robertson et al. 2015). Immediately following emersion, fish were euthanized and fixed in 10% buffered formalin for gill histology.

Series II

To determine if changes in H_2S sensitivity were related to NEC density and/or size in the gills and skin, we used immunohistochemistry. Immediately following acclimation, fish were euthanized and whole gill baskets and skin samples (~2–5 mm regions) were collected. Dorsal and ventral skin samples were collected directly anterior to the anal fin. All skins were separated from underlying connective tissue following fixation.

To determine the effect of air acclimation on H_2S tolerance, we individually exposed water- and air-acclimated fish to water (pH 6.7, 15‰) containing 2272 ± 46 µmol l^{-1} H_2S and recorded the time until loss of dorsal–ventral equilibrium (LOE) occurred. Sulphide exposure was achieved in modified 120 ml containers that did not permit emersion or ASR. We considered LOE to have occurred when fish were unable to maintain an upright position in the water column and were unresponsive to 3 tail prods (Regan et al. 2017). We also exposed additional laboratory fish from each treatment group ($n = 3$) to water (50 ml, pH 6.7, 15‰) for 30 min to ensure that acidic water alone did not induce LOE. No fish lost equilibrium in response to pH 6.7 water. To determine [total sulphides] in individual fish at LOE, fish were euthanized via immersion in an ice bath (Wilson et al. 2009) and analyzed immediately.

To determine if acute H_2S exposure impacted whole-body movement, we performed LOE experiments as described above in a separate group of fish and recorded activity of fish from below using a digital video camera (Microsoft LifeCam VX-2000, Redmond, WA). We terminated experiments upon the first occurrence of LOE as prodding caused alterations in natural activity. Almost all of the fish (13 of 14) recovered following LOE.

Following acclimation, in a separate group of fish, we collected blood to test for SHb formation. Fish were euthanized and blood was collected via caudal severance using heparinized microhematocrit tubes (Bianchini and Wright 2013). Due to the small size of *K. marmoratus*, we combined blood from 9 fishes (~1 µl $fish^{-1}$) for examination (total 9–10 µl). Blood samples were rinsed with saline (0.9% NaCl, 1 mg ml^{-1} EDTA; Miwa and Iunui 1991) and centrifuged at 1500 *g* to pellet, but not lyse, red blood cells (RBCs). Following removal of the supernatant, RBCs (0.9 µl) were exposed to one of three in vitro treatments: control (0 mmol l^{-1} total sulphides), a concentration of total sulphides representative of that measured in the body at LOE (0.67 mmol l^{-1} and 0.96 mmol l^{-1} for water- and air-acclimated fish, respectively), or a supraphysiological concentration of total sulphides (10 mmol l^{-1}) prepared in phosphate buffered saline (PBS; mmol l^{-1}: NaCl, 137; Na_2HPO_4, 15.2; KCl, 2.7; KH_2PO_4, 1.5) at neutral blood pH (pH 7.8; Patrick et al. 1997). As a positive control, we also exposed RBCs of water-acclimated fish to 10 mmol l^{-1} total sulphides at a lower pH (pH 7.0) to maximize SHb formation. We exposed RBCs of water- and air-acclimated fish to H_2S treatment for 14.7 min and 20.3 min, respectively. Exposure periods were determined based on the average time required for fish from each treatment group to lose equilibrium when exposed to water (pH 6.7, 15‰) containing 2272 ± 46 µmol l^{-1} H_2S. Following incubation, samples were centrifuged for 4 min at 20,000*g* to lyse RBCs. The hemolysate was collected to test for the presence of two Hb derivatives, oxyHb and SHb.

Following acclimation, in a separate group of fish, we measured whole-body COX activity. We compared control fish COX activity to fish that were exposed to water (pH 6.7, 15‰) containing elevated [H_2S] until LOE, as described previously. All fish were then euthanized, weighed, and homogenized as described by Borowiec et al. (2015) and enzyme activity was measured.

Series III

To examine the H_2S emersion threshold and H_2S tolerance of wild fish in the field, we performed emersion experiments and LOE experiments, as described in Series I and Series II, respectively. Experiments were performed at a field site on Long Caye, Lighthouse Reef Atoll, Belize. Captured fish were maintained in 120 ml plastic containers for 24 h prior to experimental use, as described by Rossi et al. (2019). All parameters aside from temperature were consistent between laboratory and field experiments, as the temperature of experimental water could not be modified in the field. Experiments were performed between 27.5 and 28 °C. We terminated all experiments after 1 h of H_2S exposure.

Analyses

Water analysis

We determined [total sulphides] in water samples colorimetrically using the methylene blue method (Cline 1969). We calculated aquatic [H_2S] using water pH, room temperature, and [total sulphides] as described by Broderius and Smith (1977).

Gill morphology

We fixed, decalcified, embedded, sectioned, stained, and analyzed gills as described by Turko et al. (2018). Briefly, fixed fish were decalcified, paraffin embedded, sectioned in 5 µm increments, and stained with haematoxylin and eosin. Photomicrographs of the gills were taken using a Nikon Eclipse 90i™ microscope. Five gill lamellae from each arch were randomly selected for analysis. The height of each lamella and size of adjacent ILCM were measured

using ImageJ software (National Institutes of Health, Bethesda, MD, USA; Ong et al. 2007).

Immunohistochemistry

Immunohistochemical procedures were modified from Regan et al. (2011). Fixed gill baskets and skin samples were permeabilized for 48 h at 4 °C (1% Triton X-100 in PBS, pH 7.8). Following permeabilization, we incubated tissues in a combination of antibodies raised in rabbit against serotonin (5-HT; Sigma-Aldrich) and a zebrafish neuron-specific antibody raised in mouse (zn-12; Developmental Studies Hybridoma Bank, University of Iowa, Iowa, USA) at dilution factors of 1:250 and 1:100, respectively, at 4 °C overnight. We then removed excess primary antibodies with PBS and incubated tissues in secondary antibodies for 1 h at room temperature in darkness. Goat anti-rabbit fluorescein isothiocyanate (FITC; 1:50) and goat anti-mouse Alexa Fluor 594 (1:100) were used to indirectly label 5-HT and zn-12 immunoreactivity of NECs and nerve fibers, respectively. Following incubation, we immediately removed excess secondary antibodies with PBS and mounted tissues on glass microscope slides in PermaFluor (Thermo Fisher Scientific, Massachusetts, USA). Tissues were viewed using epifluorescence (Nikon Eclipse 90i™, Tokyo, Japan) and confocal microscopes (Nikon A1R MP™, Tokyo, Japan).

The density of NECs in the gills was determined as described by Robertson et al. (2015). Briefly, images were taken of six gill filaments randomly selected from the first gill arch on the left side of each fish. We then totaled the number of NECs in the selected arches and divided by six to obtain an average number of NECs per filament. To quantify NEC density in the skin, we counted the number of NECs in a 0.14 mm^2 sampling area in dorsal and ventral skin samples. NEC density in the skin was standardized to body size (number of NECs per mm^2 divided by standard length), as preliminary analysis revealed that cutaneous NEC density was significantly and positively correlated with standard length. To estimate NEC size, we manually measured the area of the asymmetrical NECs in all samples using ImageJ (National Institutes of Health, Bethesda, MD, USA). All clearly visible cells that were not overlapping were measured.

Activity

We determined activity of fish using video footage analyzed using ImageJ (National Institutes of Health, Bethesda, MD, USA). Fish were considered to be inactive when body and fin movements were undetectable.

Whole-body [total sulphides]

Following LOE experiments, fish were euthanized via immersion in an ice bath, rinsed in H_2S-free water, and blotted dry. Fish were then homogenized in PBS. Immediately following homogenization, we assayed [total sulphides] in homogenates using the methylene blue method (Cline 1969). The [total sulphides] was interpolated from a standard curve that was prepared using excess tissue homogenate instead of dH_2O.

In vitro SHb formation

Using the hemolysate, the presence of two Hb derivatives, oxyHb (bands visible at 540 and 576 nm) and ferrous SHb (band visible between 613 and 623 nm; Bagarinao and Vetter 1992), were examined by performing a spectral scan (Spectramax Plus Spectrophotometer, Molecular Devices, San Jose, CA) from 450 to 750 nm in 1 nm steps.

COX activity

To determine COX activity, homogenates were centrifuged at 20,000*g* for 4 min to remove cellular debris. Immediately following centrifugation, we assayed COX activity in the supernatant (28 °C) as described by Borowiec et al. (2015). We determined protein concentration of extracts with bovine serum albumin as a standard (Bradford 1976). Preliminary experiments verified that enzyme activity was linear with time, as well as proportional to the quantity of tissue homogenate.

Statistical analyses

We initially assessed all data for normality using Shapiro–Wilk tests and homogeneity of variance using Bartlett's test. We used one-way analysis of variance (ANOVA), followed by Tukey's post hoc test, to determine whether acclimation treatment had a significant effect on the H_2S emersion response. We used a Kruskal–Wallis test to determine whether acclimation treatment had a significant effect on gill surface area, as normality and homogeneity of variance assumptions were violated and could not be corrected by data transformation. We used *t* tests (two-tailed) to determine whether treatment had a significant effect on NEC density and size in the gills, NEC size in the ventral skin and dorsal skin, time to LOE, the [total sulphides] in whole-body homogenates following LOE, and activity during H_2S exposure. Data were log transformed when assumptions of normality and homogeneity of variance were violated. We used a two-way ANOVA to determine the effects of air exposure and body region (i.e., dorsal vs. ventral) on cutaneous NEC density. We used a two-way ANOVA with Tukey's post

hoc test to determine the effects of acclimation treatment and H_2S exposure on COX activity. We did not statistically analyze the effect of in vitro H_2S exposure on globin subtype, as blood from multiple fish was pooled for examination ($n=2$). Results were considered significant at $\alpha<0.05$. Throughout the text, values are presented as mean ± SEM. Values represent [H_2S] unless otherwise stated.

Results

Emersion behaviour

All fish emersed in response to acutely elevated levels of H_2S (Fig. 1). Air-acclimated fish emersed at significantly lower [H_2S] relative to water-acclimated (control), H_2S-acclimated, and wild fish ($p<0.01$; Fig. 1). The H_2S emersion threshold for wild fish was not statistically different relative to the water- or H_2S-acclimated fish ($p>0.05$; Fig. 1).

Gill morphology

Acclimation history did not have a significant effect on gill coverage by the ILCM ($p>0.05$; Fig. 2).

Neuroepithelial cells (NECs)

Serotonergic NECs were present on the efferent aspect of the gill filaments and on the skin of *K. marmoratus*

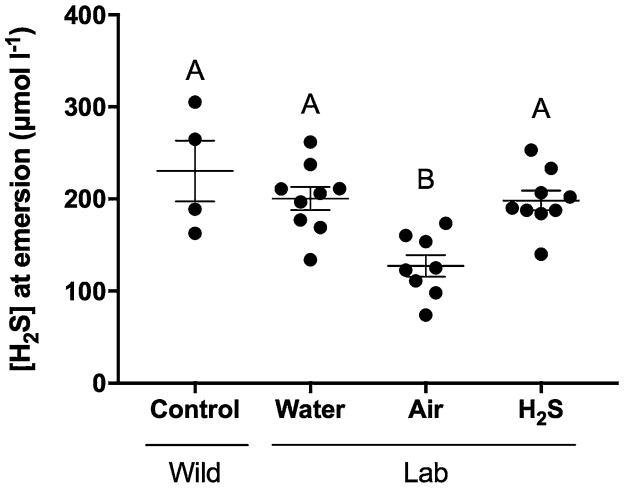

Fig. 1 Concentration of H_2S that induced *K. marmoratus* to leave water (emerse). Wild fish (control, $n=4$) were captured at Long Caye, Belize. Laboratory fish were acclimated to water (control; $n=9$), air ($n=8$), or H_2S-rich water (51.6 ± 7.2 µmol l^{-1}; $n=9$) for 3 days. Different letters indicate significant differences between treatment groups ($p<0.01$). Error bars represent SEM

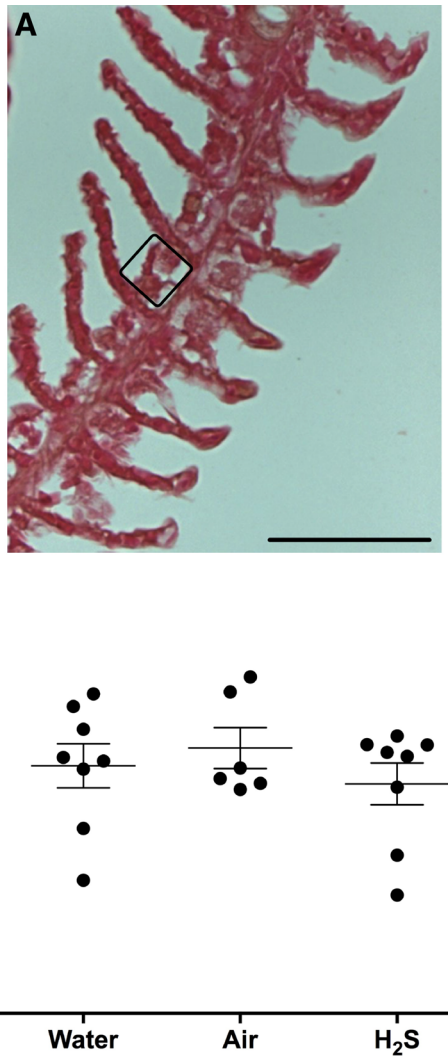

Fig. 2 Gill morphology of *K. marmoratus*. **a** Representative image depicting gills of fish following 3 day exposure to air. An example of an interlamellar cells mass (ILCM) is present in a black box. Scale bar = 50 µm. **b** Lamellar ILCM coverage (%) of gill lamellae in fish acclimated to water (control; $n=8$), air ($n=6$), or H_2S-rich water (51.6 ± 7.2 µmol l^{-1}; $n=8$) for 3 days. Error bars represent SEM

(Fig. 3). Air exposure did not significantly alter NEC density or size in the gills or skin of *K. marmoratus* ($p>0.05$; Table 1; Fig. 4). Cutaneous NECs were unevenly distributed both within and between the dorsal and ventral skin (Figs. 3d, g, 4), with the ventral skin containing significantly more NECs relative to the dorsal skin ($p<0.0001$; interaction, $p>0.05$, Fig. 4). Cutaneous NECs were often irregularly shaped and possessed membrane processes (Fig. 3d). Zn-12 immunopositive nerve fibers were observed in close proximity to gill and skin NECs (Fig. 3c, f, i). Interestingly, additional unidentified cells that were zn-12-immunopositive, but 5-HT-negative, were present within the afferent aspect of the gill filaments

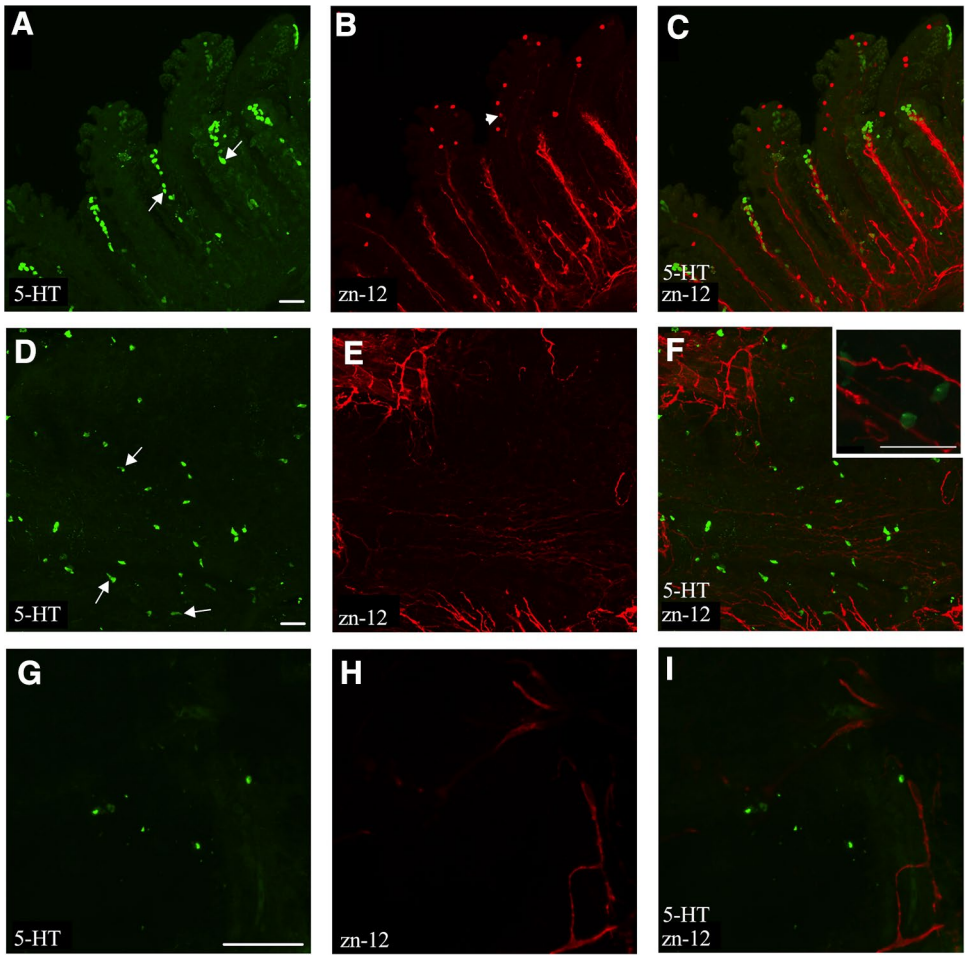

Fig. 3 Representative confocal images of 5-HT-immunoreactive neuroepithelial cells (NECs; green) and zn-12-immunoreactive nerve fibers (red) in the **a–c** gills, **d–f** ventral skin and **g–i** dorsal skin of *K. marmoratus*. **a–c** NECs in the gills were mainly present in the distal tips along the efferent aspect of gill filaments (arrows) and were located amongst nerve fibers. **b** Additional zn-12-immunoreactive cells that were 5-HT-negative (arrowhead) were also found in the gills along the afferent aspect. Note that filaments from both hemibranchs are visible. **d–i** Cutaneous NECs were unevenly distributed within and between the ventral and dorsal skin and were located in close proximity to zn-12 immunoreactive nerve fibers (inset). **d** Cutaneous NECs commonly possessed membrane processes (arrows). **c, f, i** Are composite images of (**a, b**), (**d, e**), and (**g, h**), respectively. Scale bars = 100 µm

Table 1 Neuroepithelial cell density and size in the gills and size in the skin of *K. marmoratus* after 3 days of water or air exposure

	Water	Air
Gills		
NEC density (filament^{-1})	8.2 ± 0.8	6.9 ± 0.8
NEC size (µm^2)	22.3 ± 1.9	20.2 ± 1.6
Ventral skin		
NEC size (µm^2)	37.8 ± 1.6	36.4 ± 1.6
Dorsal skin		
NEC size (µm^2)	40.3 ± 7.8	34.7 ± 4.8

Mean ± SEM. Sample size of $n = 6$ and $n = 5$ for NEC measurements in the gills of water- and air-acclimated fish, respectively. Sample size of $n = 10$ and $n = 9$ for NEC size in the skin of water- and air-acclimated fish, respectively

of water- and air-acclimated fish (Fig. 3b). These cells did not appear to be associated with zn-12-positive nerve fibers.

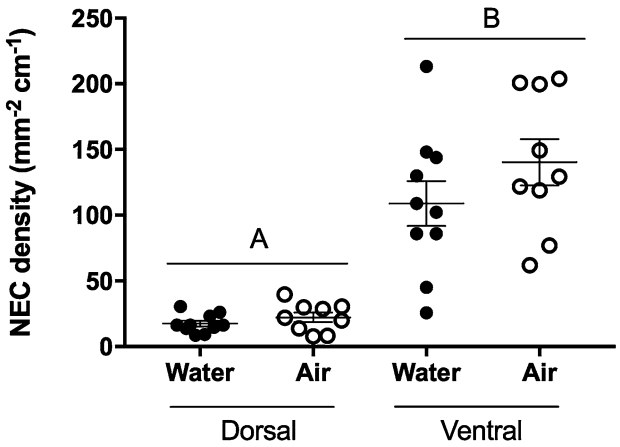

Fig. 4 Neuroepithelial cell density in the dorsal and ventral skin of *K. marmoratus*. Fish were acclimated to water (control; $n = 10$) or air ($n = 9$) for 3 days. Different letters indicate a significant difference between NEC density in the dorsal and ventral skin. Error bars represent SEM

Loss of equilibrium and whole-body [total sulphides]

Air-acclimated fish were more H_2S-tolerant relative to water-acclimated fish. Air-acclimated fish lasted 1.4 times longer than water-acclimated fish in 2.3 mmol l^{-1} H_2S before losing equilibrium ($p < 0.05$; Fig. 5a). At LOE, the concentration of total sulphides in air-acclimated fish was ~ 30% greater than that measured in water-acclimated fish ($p < 0.01$; Fig. 5b). Wild fish maintained equilibrium in 2.3 mmol l^{-1} H_2S until termination of the experiment (1 h; data not shown).

Activity

During LOE experiments, air-acclimated fish were significantly less active than water-acclimated fish ($p < 0.001$; Fig. 6), exhibiting prolonged periods of inactivity lasting up to ~ 12 min. The proportion of time that fish spent active was negatively and significantly related to the time to LOE in both water- ($R^2 = 0.83$, $p < 0.01$) and air-acclimated ($R^2 = 0.80$, $p < 0.05$; Fig. 6) fish.

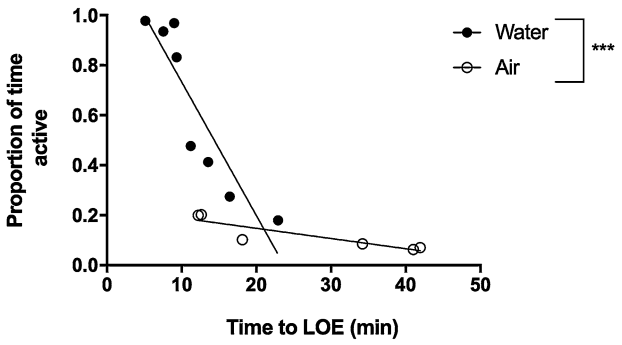

Fig. 6 Relationship between the proportion of time spent active and time to LOE in water- ($y = -0.05x + 1.3$, $R^2 = 0.83$, $p < 0.01$) and air-acclimated ($y = -0.004x + 0.2$, $R^2 = 0.80$, $p < 0.05$) fish exposed to elevated H_2S at pH 6.7 until LOE. Asterisks denote a significant difference between fish acclimated to water and air ($p < 0.001$)

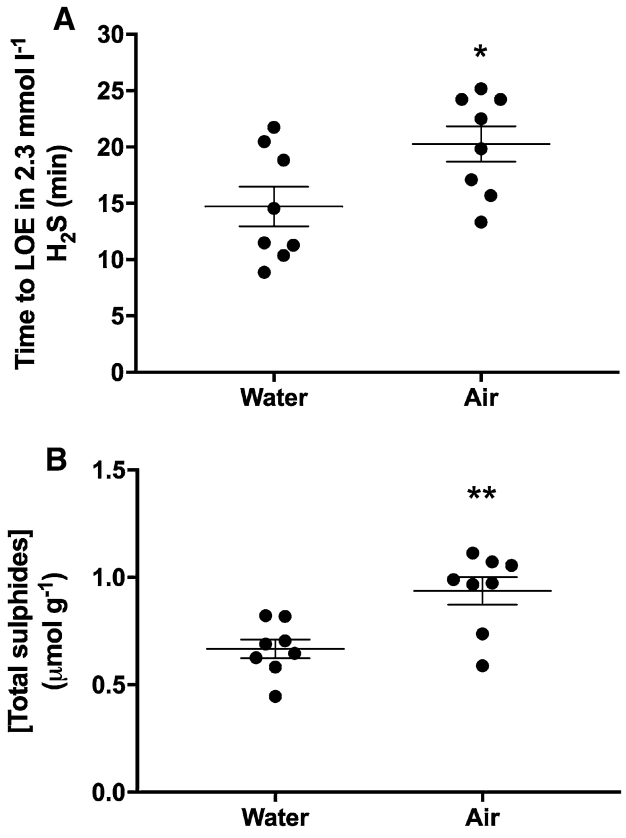

Fig. 5 Air acclimation increases the H_2S-tolerance of *K. marmoratus*. **a** Time required for *K. marmoratus* to lose dorsal–ventral equilibrium when exposed to 2272 ± 46 μmol l^{-1} H_2S and **b** [total sulphides] in whole-body homogenates at LOE. *K. marmoratus* were acclimated to water (control; $n = 8$) or air ($n = 8$) for 3 days. Asterisks denote a significant difference between fish acclimated to water and air (**a** $p < 0.05$, **b** $p < 0.01$). Error bars represent SEM

In vitro SHb formation

Exposure of RBCs to physiologically relevant concentrations of sulphide (matched to whole-body [total sulphides] at LOE) at neutral blood pH did not result in a substantial decrease in oxyHb or increase in SHb formation in water- (control; Fig. 7a) or air-acclimated fish (Fig. 7b). However, at supraphysiological concentrations of total sulphides (10 mmol l^{-1}), the proportion of oxyHb decreased, on average, by 9.1% and 16.9% in water- and air-acclimated fish, respectively, relative to the control exposure (Fig. 7a, b). Furthermore, SHb formation increased by 13.4% and 6.6% in water- and air-acclimated fish, respectively, relative to the control exposure (Fig. 7a, b). Exposure of RBCs to supraphysiological concentrations of sulphide at low pH caused a marked reduction (17.4%) in oxyHb and marked increase (41%) in SHb formation in water-acclimated fish (Fig. 7c).

COX activity

COX activity was significantly affected by H_2S exposure ($p < 0.0001$; interaction, $p > 0.05$; Fig. 8). In H_2S-exposed fish, COX activity was decreased by 45% in water-acclimated fish ($p < 0.0001$) and 29% in air-acclimated fish ($p < 0.01$) relative to fish from the same acclimation treatment exposed to control conditions (Fig. 8). Acclimation history did not significantly affect COX activity ($p > 0.05$; interaction, $p > 0.05$; Fig. 8).

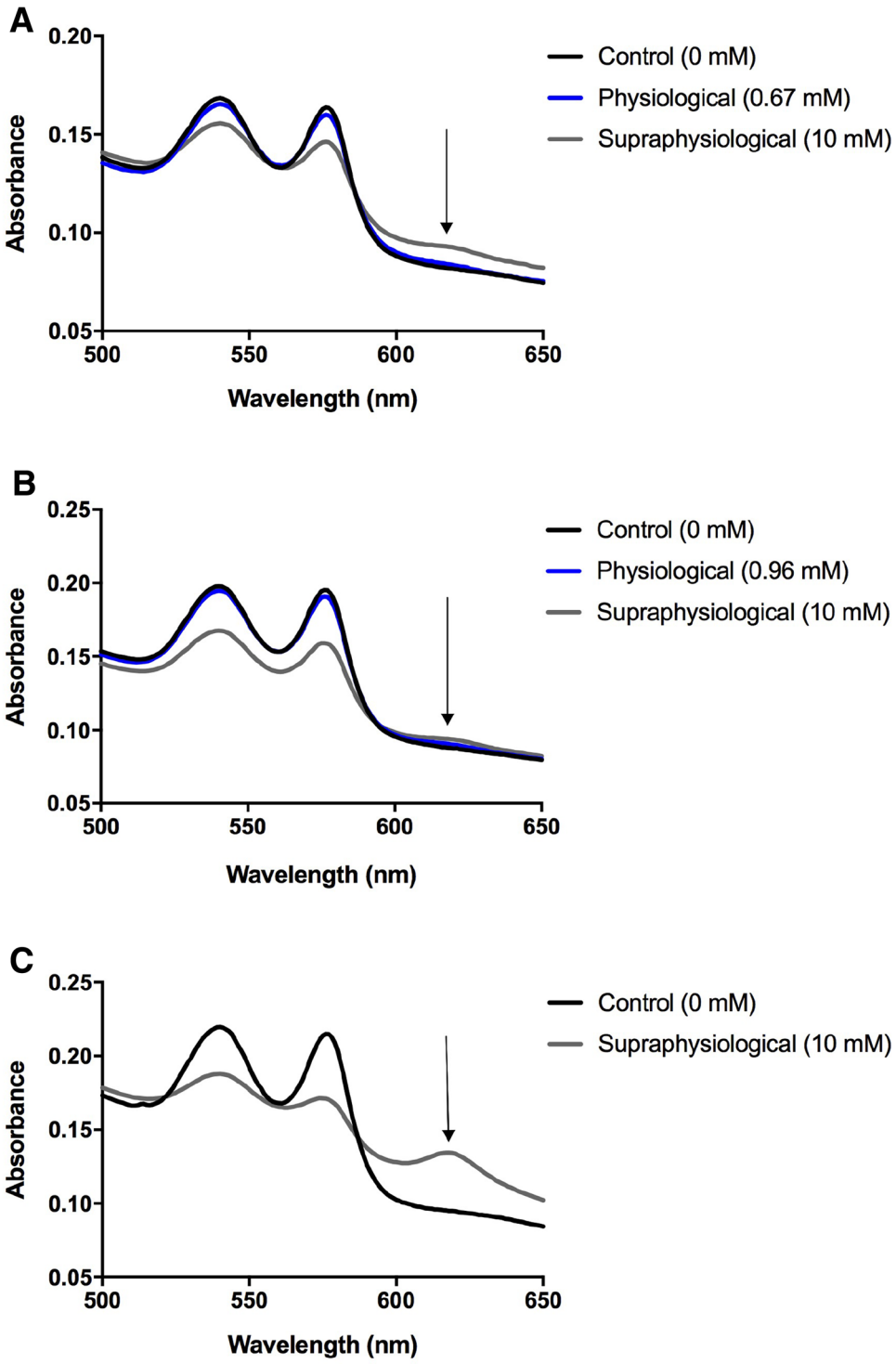

Fig. 7 Representative spectral scans of hemoglobin derivatives in *K. marmoratus* blood. **a, c** Hemoglobin isoforms in RBCs of *K. marmoratus* acclimated to water, and **b** hemoglobin isoforms in RBCs of *K. marmoratus* acclimated to air. RBCs of **a** and **b** were incubated with sulphide at neutral blood pH (pH 7.8). RBCs of **c** were incubated with sulphide at pH 7.0. Peaks at 540 nm and 576 nm are characteristic of oxyhemoglobin. The peak between 613 and 623 nm, as indicated by an arrow, is characteristic of sulphemoglobin

Discussion

Our results show that *K. marmoratus* is remarkably H_2S tolerant. All fish remained immersed in water containing > 70 µmol l^{-1} H_2S and survived for three days at a mean [H_2S] of ~ 50 µmol l^{-1} without access to air. These findings suggest that the H_2S tolerance of *K. marmoratus* is substantially greater than most species of fresh water fishes (96-h LC_{50} < 3 µmol l^{-1} H_2S; Bagarinao and Vetter 1989) and is likely also greater than that of other species of H_2S-tolerant fishes, including *F. parvipinnis* (96-h LC_{50} = 24 µmol l^{-1} H_2S) and *G. mirabilis* (96-h LC_{50} = 18 µmol l^{-1} H_2S; Bagarinao and Vetter 1989). This ability to persist in H_2S-rich aquatic environments appears

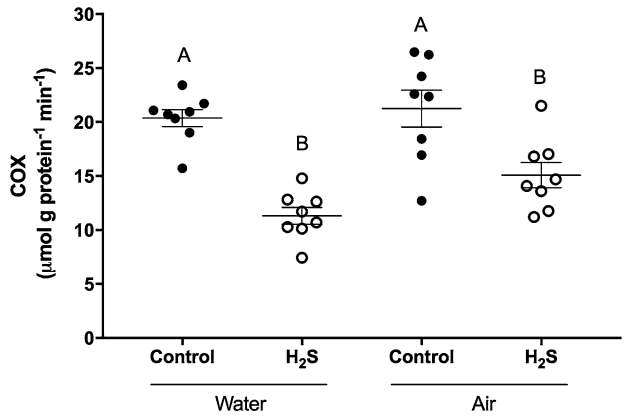

Fig. 8 Hydrogen sulphide (H_2S) exposure reduces cytochrome c oxidase (COX) activity in *K. marmoratus*. *K. marmoratus* were acclimated to water ($n=16$) or air ($n=16$) for 3 days. Following acclimation, half of the fish were exposed to elevated aquatic H_2S at pH 6.7 until LOE. Different uppercase letters indicate significant differences between groups ($p<0.01$). Error bars represent SEM

to be the result of both physiological modifications and behavioural strategies. We demonstrated that *K. marmoratus* possess Hb that is insensitive to physiologically relevant [H_2S]. In addition, we showed that *K. marmoratus* respond to acutely elevated levels of aquatic H_2S using behavioural avoidance (emersion). We suspect that the use of emersion behaviour is crucial for the survival of *K. marmoratus* inhabiting H_2S-rich environments as fish experience impaired oxidative phosphorylation when unable to emerse.

H_2S sensitivity

We found that both laboratory-reared and wild fish were equally sensitive to aquatic H_2S. However, the [H_2S] that elicited emersion behaviour in laboratory fish was two orders of magnitude greater than previously reported in the same species (Abel et al. 1987). Methodological differences may account for the discrepancy between studies. In particular, H_2S is highly labile and pH sensitive, and therefore, careful attention is required for sample processing.

H_2S sensitivity in *K. marmoratus* was influenced by prior acclimation to air, but not by acclimation to aquatic H_2S. We found that air-acclimated fish emersed at [H_2S] ~ 36–45% lower than that demonstrated to elicit emersion in fish from the remaining treatment groups. In *K. marmoratus*, air exposure (7 days) has been shown to cause a significant reduction in gill surface area (increase height of ILCM; Ong et al. 2007) that consequently impairs aquatic respiration (Turko et al. 2012, 2018). However, analysis of gill morphology revealed no change in gill surface area following 3 day air acclimation, suggesting that gill remodeling requires more than 3 days to occur. Consequently, we did not find evidence to support the hypothesis that acclimation-induced alterations in gill surface cause alterations in the H_2S emersion response.

We subsequently hypothesized that alterations in the H_2S emersion threshold were caused by changes in serotonergic NEC density and/or size. We found that air exposure (3 days) did not cause significant hypertrophy or hyperplasia of serotonergic NECs on the gills or skin of *K. marmoratus*. Nevertheless, a transcriptomic analysis of the skin of *K. marmoratus* during air exposure (3 days) found significant increases in the expression of genes encoding for serotonin receptors (K. Dong, T. Blanchard, J. Schmitz, S. Kelly, P. Wright, A. Whitehead, in prep.). Therefore, increased H_2S sensitivity in air-acclimated *K. marmoratus* may instead be the result of an increase in the number of serotonin receptors. Further studies are required to confirm this idea. Interestingly, we found regional differences in NEC density in the skin. The ventral skin contained significantly more NECs relative to the dorsal skin, indicating that the skin of *K. marmoratus* may be regionally specialized with the ventral skin playing a greater sensory role. Surprisingly, we also found unidentified cells in the gills that were zn-12 immunopositive, but 5-HT-negative. Similar cells have previously been reported in the gill tufts of *Xenopus laevis* larvae and have been suggested to play a respiratory or osmoregulatory role within the gill (Saltys et al. 2006). However, the functional significance of these unusual cells remains unknown and further work is necessary to characterize their role in *K. marmoratus* gills.

H_2S tolerance

Air acclimation altered H_2S tolerance in *K. marmoratus*. Surprisingly, air-acclimated fish maintained equilibrium in high aquatic [H_2S] for a significantly longer duration relative to water-acclimated fish, despite exhibiting a significantly lower H_2S emersion threshold. We found that H_2S tolerance was mediated, in part, by a significant reduction in activity. Reduced activity levels likely enhanced H_2S tolerance in air-acclimated fish by conserving energy (ATP), thus allowing for sustained metabolic function and prolonged maintenance of equilibrium. We also demonstrated that COX inhibition in water- and air-acclimated fish was statistically similar despite significantly higher internal [total sulphides] in air-acclimated fishes at LOE, suggesting that air acclimation may reduce the sensitivity of COX to H_2S in *K. marmoratus*, thereby enhancing H_2S tolerance. However, additional research examining the structure and function of COX following air acclimation is required to confirm this speculation. Finally, air acclimation (3 days) increases cutaneous angiogenesis in *K. marmoratus* (Blanchard et al. 2019). Therefore, it is possible that enhanced cutaneous oxygen uptake also improves H_2S tolerance in air-acclimated fish by enhancing H_2S detoxification via enzymatic oxidation.

Despite being highly H_2S-tolerant, all fish lost equilibrium when unable to emerse from an environment containing elevated [H_2S]. Therefore, H_2S causes acute toxicity in *K. marmoratus*. SHb formation did not occur when hemolysates were exposed to physiologically relevant [total sulphides]. Consequently, we reject the hypothesis that impaired blood oxygen transport is the mechanism of H_2S toxicity in *K. marmoratus*. Previous in vitro studies reported significantly less SHb formation in hemolysates of H_2S-tolerant fishes (i.e., *F. parvipinnis, C. carpio*) relative to H_2S-intolerant species, such as *Oncorhynchus mykiss* (Bagarinao and Vetter 1992; Völkel and Berenbrink 2000). Moreover, in vitro SHb formation in H_2S-tolerant fishes (i.e., *F. parvipinnis, G. mirabilis*) only begins to occur within minutes following exposure to 1–5 mmol l^{-1} total sulphides (Bagarinao and Vetter 1992), concentrations that exceed levels found in the body of *K. marmoratus* at LOE. These findings suggest that various H_2S-tolerant fishes, including *K. marmoratus*, possess modified Hb that is insensitive to physiologically and ecologically relevant [H_2S]. Reduced sensitivity of Hb to H_2S likely contributes, in part, to the ability of *K. marmoratus* to remain immersed in H_2S-rich environments. However, the mechanism that causes *K. marmoratus* Hb to be insensitive to high [H_2S] remains unknown and worthy of future study.

We showed that H_2S-induced LOE in *K. marmoratus* is caused, in part, by impaired oxidative phosphorylation. COX activity in water- and air-acclimated *K. marmoratus* exposed to control conditions was similar to that previously reported by Frick and Wright (2002b) in the same species. Following LOE in response to elevated [H_2S], COX activity was significantly lower in water- and air-acclimated fish relative to the control. These findings demonstrate that impaired oxidative phosphorylation is an important mechanism of H_2S toxicity in *K. marmoratus*. Although *K. marmoratus* experience COX impairment when exposed to elevated H_2S, they may possess adaptations that facilitate sustained COX function in environments containing low to moderate [H_2S]. Pfenninger et al. (2014) demonstrated that H_2S tolerant *P. mexicana* and *P. sulphuraria* have evolved COX with reduced H_2S sensitivity. Consequently, we examined the amino acid sequence of COX1 in *K. marmoratus, P. mexicana*, and *P. sulphuraria* using BLAST and found that *K. marmoratus* does not possess the same amino acid substitution that is proposed to cause reduced H_2S sensitivity (Pfenninger et al. 2014). Alternatively, it is possible that the functional properties of COX are altered in *K. marmoratus* to sustain activity. For example, hypoxia-tolerant sculpins (Cottidae) possess COX with greater O_2 affinity relative to non-tolerant species (Lau et al. 2017). This adaptation may, therefore, also improve aerobic metabolism in H_2S-rich environments, a possibility that warrants further investigation.

Finally, we observed that fish spent increasing amounts of time near the air–water interface when exposed to elevated [H_2S]. Similar behavioural strategies have been reported in *P. mexicana* in response to high [H_2S], where fish significantly increase the use of ASR (Plath et al. 2007). The surface layer of the water contains both the highest levels of O_2 as well as the lowest levels of H_2S (Plath et al. 2007). Thus, these findings strongly suggest that behavioural strategies that allow for increased O_2 uptake and H_2S avoidance are important for mediating survival in H_2S-rich environments.

Conclusions

Overall, our findings indicate that *K. marmoratus* is highly H_2S tolerant. The findings in the present study are consistent with our field data, showing that *K. marmoratus* is significantly more H_2S tolerant than two co-located species, *Poecilia orri* and *Gambusia* sp. (Rossi et al. 2019). However, when exposed to extremely elevated levels of H_2S, *K. marmoratus* emerse. Video footage at the surface of crab burrows demonstrates that wild *K. marmoratus* frequently voluntarily emerse from H_2S-rich habitats for short periods of time each day (Turko and Wright 2015). This behaviour likely allows *K. marmoratus* to mitigate the toxic effects of H_2S by increasing O_2 uptake and preventing diffusion of H_2S into the body. However, when unfavorable conditions (e.g., terrestrial predators) prevent emersion, fish may succumb to elevated H_2S due to the impairment of oxidative phosphorylation. Consequently, our findings demonstrate that amphibiousness is a key adaptation that allows *K. marmoratus* to inhabit H_2S-rich environments that would otherwise cause acute toxicity.

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