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Physiology

Seeing in the swamp: hydrogen sulfide inhibits eye metabolism and visual acuity in a sulfide-tolerant fish

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In fish, vision may be impaired when eye tissue is in direct contact with environmental conditions that limit aerobic ATP production. We hypothesized that the visual acuity of fishes exposed to hydrogen sulfide (H_2S)-rich water would be altered owing to changes in cytochrome c oxidase (COX) activity. Using the H_2S-tolerant mangrove rivulus (*Kryptolebias marmoratus*), we showed that a 10 min exposure to greater than or equal to 200 µM of H_2S impaired visual acuity and COX activity in the eye. Visual acuity and COX activity were restored in fish allowed to recover in H_2S-free water for up to 1 h. Since *K. marmoratus* are found in mangrove pools with H_2S concentrations exceeding 1000 µM, visual impairment may impact predator avoidance, navigation and foraging behaviour in the wild.

1. Introduction

Vision in most animals is important for interpreting the surrounding environment. The response to visual stimuli directs many critical behaviours (e.g. navigation, foraging and predator avoidance) that ultimately affect survival. Eye tissue is energetically demanding because rod and cone photoreceptors in the retina need ATP to generate membrane potentials, as well as synthesize, and transport proteins [1–4]. In aquatic animals, environmental perturbations that impact aerobic metabolism (e.g. hypoxia) may harm vision [5].

One abiotic factor that severely limits aerobic metabolism in aquatic animals is hydrogen sulfide (H_2S). H_2S reversibly binds to cytochrome c oxidase (COX) within the mitochondrial electron transport chain, inhibiting oxidative phosphorylation and decreasing aerobic ATP production [6–8], among other toxic effects [9]. Not surprisingly, most fishes are intolerant of H_2S [6], but a few species have survived and even speciated in H_2S-rich water [10,11]. Is vision jeopardized in fishes exposed to H_2S?

We tested the hypothesis that the visual acuity of fishes exposed to H_2S-rich water would be impaired owing to reduced COX activity in the eye tissue. We studied the H_2S-tolerant mangrove rivulus (*Kryptolebias marmoratus*) that inhabits O_2-poor, H_2S-rich pools (up to 1166 µM of H_2S) in mangrove forests [12]. We measured both visual acuity and COX activity in the eye of fish at various H_2S concentrations (100–400 µM) and followed recovery after exposure.

2. Methods

(a) Animals

We obtained adult hermaphrodites of the self-fertilizing *Kryptolebias marmoratus* ($n = 107$; 6–12 months old) from a breeding colony in the Hagen Aqualab at the University of Guelph, Guelph, Ontario, Canada. Our experiments were performed on fish from the isogenic HON11 strain, which originated from the Bay Islands,

Utila, Honduras [13]. Although the HON11 strain has been maintained in the laboratory for approximately 25 years [13], adult hermaphrodites produce genetically identical offspring and the resulting isogenic strains remain stable for several generations [14]. We held fish in 120 ml containers under constant conditions (approx. 60 ml, 15‰, 25°C, pH approx. 8, 12 : 12 h light : dark cycle) and fed fish brine shrimp (*Artemia* sp. nauplii) three times per week. We acclimated fish for 7 days to water of pH 6.7 (approx. 60 ml, 15‰, 22°C) before the start of the experiment because of the lower pH found in natural mangrove swamps [12]. Moreover, conducting experiments at lower pH values ensures a higher proportion of H_2S is present, compared to less-toxic sulfide species (HS^- and S^{2-}) [15].

(b) Experimental protocol

(i) Hydrogen sulfide and visual acuity

To determine visual acuity, we used an optokinetic response (OKR) machine modified from existing designs [16,17]. We transferred fish into a cuvette plugged with mesh, which was then placed in a stationary water-filled beaker within the rotating drum of the OKR machine (10.5 r.p.m.). We gradually decreased the size of black and white gratings on the rotating drum to identify the smallest grating that elicited a visual response [16].

We tested visual acuity in fish exposed to one of three H_2S concentrations: approximately 100, 200 or 400 µM of H_2S (pH 6.7, 15‰; $n = 9$–12 per [H_2S]), prepared using $Na_2S \cdot 9H_2O$. Since *K. marmoratus* leap out of the water (emerse) to escape H_2S when concentrations approach 200 µM [8,12], we chose H_2S concentrations at, above and below this threshold. We first tested the visual acuity of fish in H_2S-free water after a 10 min acclimation to the OKR machine (control). We then replaced the water in the beaker with H_2S-rich water and waited for 10 min before repeating the OKR test. Finally, we allowed fish to recover in H_2S-free water for 10 min, before assessing visual acuity a third time. A 10 min acclimation was used to provide enough time for fish to adjust to their surroundings while minimizing the time for H_2S oxidation/volatilization. We repeated the experiment at 400 µM of H_2S in a separate subset of fish but used a 1 h recovery period ($n = 13$). We recorded all responses through a dissection microscope (WILD Heerbrugg C.B.S. 155) with a cell phone camera and adaptor (Carson Universal Optical Smartphone Adaptor). Water samples were taken from each treatment before and after the visual acuity test to verify H_2S concentrations [15,18] (electronic supplementary material, table S1).

(ii) Cytochrome c oxidase activity

To determine if COX activity was impacted by H_2S, we exposed new groups of fish to either approximately 100, 200 or 400 µM of H_2S for 10 min, and then euthanized fish in ice water before quickly dissecting the eyes ($n = 6$–9 per treatment group). We also assessed the COX activity of control fish ($n = 10$), as well as fish allowed to recover from approximately 100, approximately 200 µM of H_2S for 10 min and from approximately 400 µM of H_2S for either 10 min or 1 h ($n = 7$–8 per recovery group). We homogenized tissues on ice in 200 µl of homogenization buffer (20 mmol l^{-1} Hepes, 1 mmol l^{-1} sodium EDTA and 0.1% Triton X-100) using a hypodermic needle and then centrifuged tissue homogenates at 20 000*g* (4 min) to remove cell debris. We measured COX activity in the supernatant (µM g wet mass^{-1} min^{-1}) as previously described [8,19].

(iii) Calculations and statistics

Visual acuity (cycles degree^{-1}) was calculated for each fish using the following equation:

$$\text{visual acuity} = \frac{1}{2\tan^{-1}(h/2a)}$$

where a is the distance between the fish's eye and the grating (6.5 cm), and h is the length of the smallest cycle (the combined length of one black and one white line on the grating) to elicit a visual response.

We assessed data for normality and homogeneity of variance using Shapiro–Wilk and Bartlett's tests, respectively, and log-transformed when necessary. We used repeated-measures ANOVAs, followed by Tukey's *post hoc* tests, to compare the visual acuity of fish between their control, treatment and recovery tests. We analysed the COX data with a one-way ANOVA, followed by a Dunnett's many-to-one comparison test to determine which treatment and recovery groups differed from the control (significant at $\alpha < 0.05$). We used RStudio (v. 1.1.463 [20]) with R (v. 3.6.1 [21]) for all statistical analyses and Prism (v. 8.0.2) for graphing.

3. Results

(a) Visual acuity

Visual acuity was impaired at the higher H_2S concentrations. At approximately 100 µM H_2S, there were no significant changes in visual acuity between control, treatment and recovery tests (ANOVA, $p = 0.62$; figure 1*a*). Exposure to approximately 200 µM of H_2S significantly reduced visual acuity relative to control (ANOVA, $p = 0.002$; Tukey, $p = 0.006$), but visual acuity was restored to control levels following recovery in H_2S-free water (Tukey, $p = 0.99$; figure 1*b*). At approximately 400 µM of H_2S, visual acuity also declined relative to the control (ANOVA, $p = 0.003$; Tukey; $p = 0.002$), but vision did not fully recover after 10 min in H_2S-free water, as visual acuity during recovery was not significantly different from control (Tukey, $p = 0.15$) or treatment levels (Tukey; $p = 0.12$; figure 1*c*). A longer recovery (1 h), however, restored visual acuity to control levels (ANOVA, $p < 0.0001$; Tukey, $p < 0.67$; figure 1*d*).

(b) Cytochrome c oxidase activity

COX activity in the eye decreased in response to the higher H_2S concentrations (ANOVA, $p < 0.0001$). COX activity was significantly lower in fish exposed to approximately 200 µM of H_2S (Dunnett's, $p = 0.02$) and approximately 400 µM of H_2S (Dunnett's, $p = 0.005$; figure 2) relative to the control. At the highest concentration, COX activity did not recover after 10 min (Dunnett's; $p = 0.005$), but was fully recovered after 1 h (Dunnett's, $p = 0.53$; figure 2).

4. Discussion

Our results indicate that H_2S concentrations greater than or equal to 200 µM impaired visual acuity and COX activity in the eye, consistent with our hypothesis. Fish appeared to have a graded response to increasing H_2S concentrations, with no impairment of visual acuity or COX activity at lower H_2S concentrations, but impairment at higher concentrations, requiring longer recovery times at the highest concentrations (approx. 400 µM of H_2S). It is possible that the laboratory-reared fish used in our study may respond differently to H_2S than wild fish, which are routinely exposed to H_2S in their natural environment. However, we previously demonstrated that both laboratory-reared and wild *K. marmoratus* emerse at similar H_2S concentrations (approx. 200 µM [8,12]), suggesting that H_2S responses may not change considerably even after years in captivity. Emersion at approximately 200 µM by

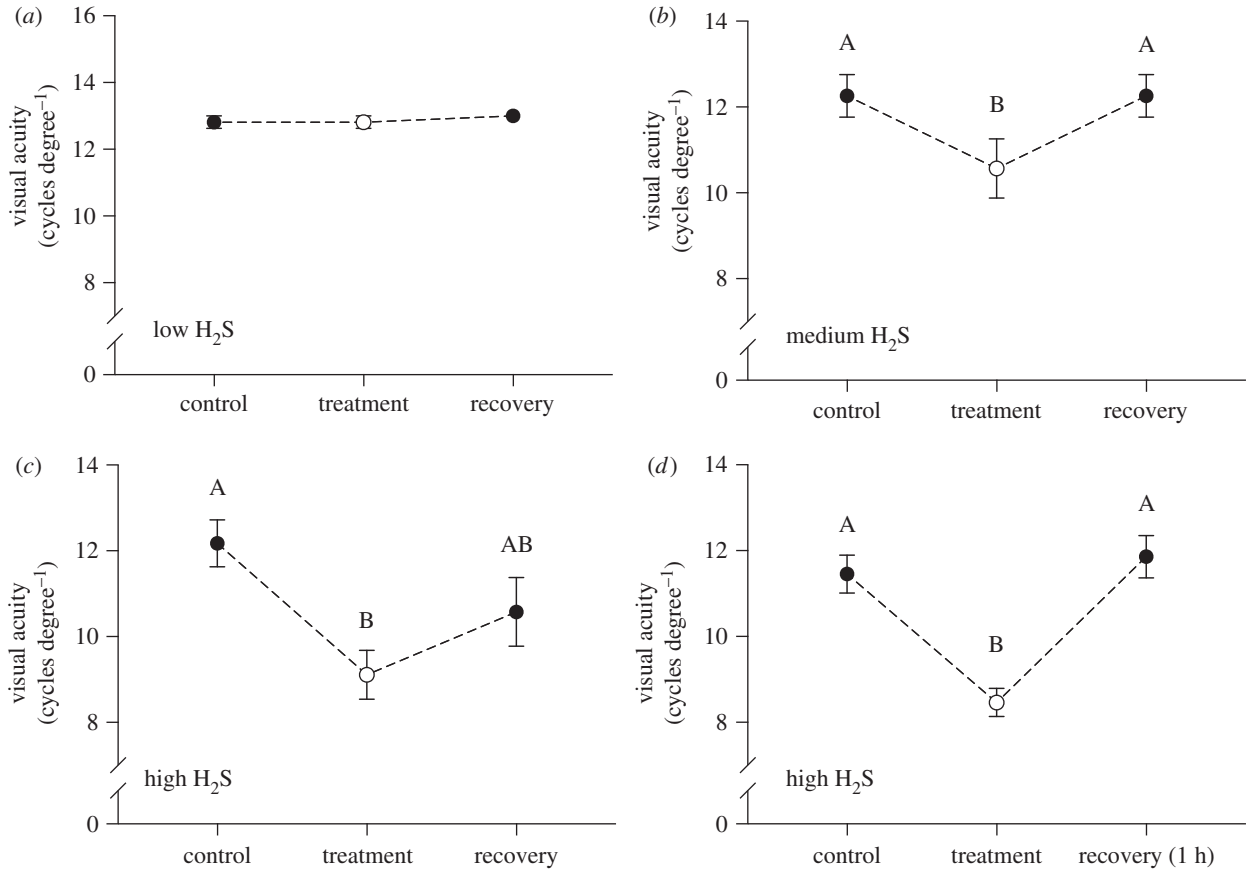

Figure 1. The visual acuity (cycles degree^{-1}) of adult mangrove rivulus (*Kryptolebias marmoratus*) during consecutive control, treatment and recovery OKR tests. Fish were tested at either (*a*) low (approx. 100 µM), (*b*) medium (approx. 200 µM) or (*c*,*d*) high (approx. 400 µM) H_2S concentrations. Different letters indicate significance ($\alpha < 0.05$). Means ± s.e.m. ($n = 9$–13 per group).

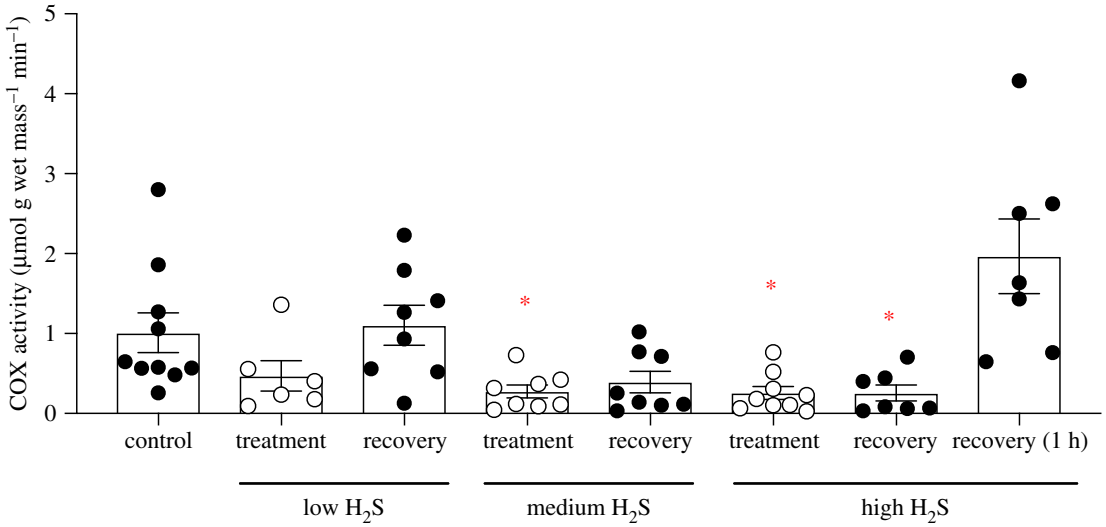

Figure 2. COX activity (µM g wet mass^{-1} min^{-1}) in the eye tissue of adult mangrove rivulus (*Kryptolebias marmoratus*). Fish were tested under control conditions (0 µM of H_2S; $n = 10$) and compared to treatment and recovery groups of low (approx. 100 µM; treatment $n = 6$, recovery $n = 8$), medium (approx. 200 µM; treatment $n = 8$, recovery $n = 8$) and high (approx. 400 µM; treatment $n = 9$, recovery $n = 7$, 1 h recovery $n = 7$) H_2S concentrations. Means ± s.e.m. Red asterisks indicate groups that are statistically different from control ($\alpha < 0.05$).

laboratory-reared and wild fish may be a strategy to avoid visual impairment. Interestingly, in a previous study, we found wild *K. marmoratus* occupying mangrove pools where H_2S concentrations exceeded 1000 µM [12]. Therefore, there may be circumstances when *K. marmoratus* remain in H_2S-rich water for prolonged periods of time (e.g. to avoid terrestrial predators), resulting in negative impacts on the visual system.

We found that *K. marmoratus* recover relatively quickly from moderate H_2S exposure. Recovery in H_2S-free water depends on both diffusive loss of H_2S [22], as well as detoxification mechanisms [6,23]. H_2S is detoxified in tissues (e.g. liver, spleen, kidney and gills) and blood by the oxidation of H_2S by thiosulfate, catalysed by quinone oxidoreductase [6,7]. At high H_2S concentrations, we found that recovery took up to 1 h,

presumably because a larger H_2S load required more time to diffuse from the tissues and/or be detoxified. Some H_2S-tolerant fishes express an H_2S-resistant COX1 isoform [10,23]; however, this does not appear to be the case in *K. marmoratus* [8], making them more susceptible to the toxic effects of H_2S.

Environmental H_2S may directly damage eye tissue. In humans (and other mammals), gaseous H_2S causes edema of the cornea, eye irritation and potentially blindness [24]. In a preliminary investigation, we did not observe any profound physical damage to the eye of *K. marmoratus* at approximately 400 µM of H_2S (electronic supplementary material, figure S1). Interestingly, the H_2S-tolerant cave-dwelling *Poecilia mexicana* has reduced eyes, possibly because they inhabit H_2S-rich and low light environments [25]. By contrast, *K. marmoratus* have relatively large eyes for their body size and possess a rete mirabile (electronic supplementary material, figure S1) which would facilitate O_2 delivery to the eye. Furthermore, *K. marmoratus* do not form sulphaemoglobin when exposed to elevated H_2S, which would prevent a reduction in the blood O_2 carrying capacity [8]. Thus, *K. marmoratus* may have evolved mechanisms to minimize visual impairment while living in a relatively toxic environment—an interesting avenue for future investigation.

Ethics. Experiments were approved by the University of Guelph Animal Care Committee (AUP 3891).

Data accessibility. The datasets supporting this article have been uploaded as part of the electronic supplementary material.

Authors' contributions. C.A.A., G.S.R. and P.A.W. designed the experiments. C.A.A. and G.S.R. carried out the work. C.A.A. and P.A.W. wrote the draft manuscript. C.A.A., G.S.R. and P.A.W. edited the manuscript. All authors approve the final version and agree to be held accountable for the work performed.

Competing interests. I/We declare I/we have no competing interests.

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