Mangrove Fishes Rely on Emersion Behavior and Physiological Tolerance to Persist in Sulfidic Environments

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Accepted 12/31/2018; Electronically Published 4/11/2019

Online enhancements: supplemental table and figures.

ABSTRACT

Hydrogen sulfide (H_2S) is a potent respiratory toxin that makes sulfidic environments tolerable to only a few organisms. We report the presence of fishes (*Kryptolebias marmoratus*, *Poecilia orri*, *Gambusia* sp., and *Dormitator maculatus*) in Belizean mangrove pools with extremely high H_2S concentrations (up to 1,166 μM) that would be lethal for most fishes. Thus, we asked whether the three most prevalent species (*Kryptolebias*, *Poecilia*, and *Gambusia*) persist in sulfidic pools because they are exceptionally H_2S tolerant and/or because they can leave water (emerse) and completely avoid H_2S. We show that both physiological tolerance and emersion behavior are important. *Kryptolebias* demonstrated high H_2S tolerance, as they lost equilibrium significantly later than *Poecilia* and *Gambusia* during H_2S exposure (1,188 $\pm$ 21 μM H_2S). However, the fact that all species lost equilibrium at an ecologically relevant [H_2S] suggests that physiological tolerance may suffice at moderate H_2S concentrations but that another strategy is required to endure higher concentrations. In support of the avoidance behavior hypothesis, H_2S elicited an emersion response in all species. *Kryptolebias* was most sensitive to H_2S and emersed at H_2S concentrations 52% and 34% lower than *Poecilia* and *Gambusia*, respectively. Moreover, H_2S exposure caused *Kryptolebias* to emerse more frequently and spend more time out of water compared to control conditions. We suggest that physiological H_2S tolerance and emersion behavior are complementary strategies. The superior H_2S tolerance and amphibious capability of *Kryptolebias* may explain why this species was more prevalent in H_2S-rich environments than other local fishes.

Keywords: ecological toxicant, H_2S, *Kryptolebias marmoratus*, *Poecilia orri*, *Gambusia* sp., amphibious fish, loss of equilibrium, hypoxia.

Introduction

Hydrogen sulfide (H_2S) is acutely toxic for most aquatic animals at micromolar concentrations, and thus it is an extreme environmental stressor (Smith et al. 1977; Bagarinao 1992). H_2S is naturally produced in many aquatic environments (e.g., coastal wetlands, mangrove forests, hydrothermal vents, freshwater springs) through biological or geochemical processes (Tobler et al. 2016). Such extreme environmental stressors can shape biological communities by acting as ecological filters (Tobler et al. 2016). H_2S toxicity, for example, imposes strong selection pressure against nontolerant species, allowing only a few tolerant species (mostly invertebrates) to pass through the "filter" and persist in H_2S-rich environments (Turnipseed et al. 2003; Tobler et al. 2006; Greenway et al. 2014). Consequently, species richness generally declines with increasing H_2S concentration (Tobler et al. 2006). However, some fishes have evolved remarkable tolerance to H_2S through physiological modifications.

The primary mechanism of H_2S toxicity is the inhibition of cytochrome *c* oxidase (COX), which impairs mitochondrial respiration and the aerobic production of ATP (Cooper and Brown 2008). In an H_2S-tolerant species (*Poecilia mexicana*), sequence modification of COX reduces H_2S binding and ensures continued mitochondrial function in the presence of H_2S (Kelley et al. 2016). Other fishes may compensate for the inhibition of direct toxicity targets (e.g., COX) by relying on anaerobic ATP production (i.e., glycolysis; Affonso et al. 2002). Tolerance can also be conferred through detoxification of H_2S in the body. Several fishes from sulfidic environments demonstrate a high capacity for oxidizing H_2S into a less toxic form (i.e., thiosulfate; Bagarinao and Vetter 1989, 1993).

Fishes may utilize behavioral strategies in addition to physiological approaches to minimize the flux of H_2S into the body. For instance, bearded gobies (*Sufflogobius bibarbatus*) make diel migrations between sulfidic and nonsulfidic microenvironments (Salvanes et al. 2011), whereas other species (e.g., *P. mexicana*) utilize

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Physiological and Biochemical Zoology 92(3):316–325. 2019. 1522-2152/2019/9203-8089$15.00. DOI: 10.1086/703117

alternative respiratory strategies, such as aquatic surface respiration (ASR), where they encounter well-oxygenated water with lower [H_2S] (Plath et al. 2007; Greenway et al. 2014). Perhaps the most complete avoidance strategy is demonstrated by amphibious fishes that can leave water for extended periods and avoid H_2S altogether. Amphibious fishes are known to emerse for biotic reasons (e.g., to avoid predators; Ord et al. 2017), and abiotic stressors in the aquatic environment are also strong emersion stimuli (Sayer and Davenport 1991; Graham 1997). This raises the question: Does emersion behavior provide a complementary or even alternative strategy to physiological tolerance, thereby allowing the utilization of periodically H_2S-rich environments from which fully aquatic species may be excluded?

We studied mangrove fishes occupying ephemeral pools in a mangrove forest on Long Caye, Belize, where H_2S had previously been detected but not extensively quantified. In this habitat, H_2S is naturally produced from the decomposition of organic matter under low oxygen conditions and the activity of anaerobic sulfate-reducing bacteria in the sediment (Nedwell 1982; Taylor 2012). In preliminary observations, the amphibious mangrove rivulus (*Kryptolebias marmoratus*) appeared more prevalent in aquatic environments containing elevated H_2S relative to other local fish species (*Poecilia orri* [mangrove molly], *Gambusia* sp. [species unknown as not morphologically distinct], *Dormitator maculatus* [fat sleeper]). *Kryptolebias* is a cyprinodontiform fish that is extremely tolerant of poor water quality but emerses when aquatic conditions fall beyond thresholds (e.g., extreme hypoxia; Regan et al. 2011) and can survive on land for at least 2 mo (Taylor 1990). H_2S has been shown to drive emersion in *Kryptolebias* in one previous study (Abel et al. 1987). Relatively little is known about the natural history of *Poecilia*, *Gambusia*, or *Dormitator*, but to the best of our knowledge, there are no reports of amphibious behavior in these fishes. In the present study, we first tested the hypothesis that *Kryptolebias*, *Poecilia*, and *Gambusia* persist in H_2S-rich mangrove pools because they are highly H_2S tolerant. This tolerance hypothesis predicts that fishes should not lose equilibrium even when exposed to the highest [H_2S] measured in mangrove pools inhabited by fish. Furthermore, due to the suspected primarily aquatic lifestyle of *Poecilia* and *Gambusia*, these species should be more physiologically tolerant of high [H_2S] than amphibious *Kryptolebias*. We also tested the complementary hypothesis that emersion behavior allows fishes to persist in H_2S-rich mangrove pools by leaving water to avoid H_2S. This avoidance hypothesis predicts that H_2S should be a driver of emersion in all species and particularly in the highly amphibious *Kryptolebias*.

Methods

Field Sampling

We collected four fish species: *Kryptolebias marmoratus*, *Poecilia orri*, *Dormitator maculatus*, and an unknown species of *Gambusia* (*Gambusia* sp.) from 11 geographically proximate study sites (fig. 1; maximum intersite distance: 750 m) on Long Caye, Lighthouse Reef Atoll, Belize, between April 18 and April 30, 2018. Going forward, all study species will be referred to by genus name only for brevity. All fish ($N = 628$) were captured from mangrove pools using dip nets, Taylor cup traps, or Gee minnow traps (Ritchie and Johnson 1986; Taylor 1990). Standard length (mm) and mass (g) were measured for calculation of condition factor (Fulton's K), as described by Froese (2006). We selected a subset of fish: *Kryptolebias* (hermaphrodites; $n = 28$), *Poecilia* (mixed sex; $n = 28$), and *Gambusia* (mixed sex; $n = 25$) for experimental use, and they were not weighed and measured until after experimentation to minimize handling stress. These experimental fish ($N = 81$) were individually maintained in 120-mL plastic holding cups (~60 mL seawater, 36.3‰ ± 0.2‰ [mean ± SEM]; pH = 8.1) for 24 h prior to use in experiments. No *Dormitator* were used in experiments as relatively few were captured. We performed all experiments in seawater of pH 6.4, adjusted using HCl (a pH measured in the field; see table 1), to target the toxic sulfide species (H_2S) of interest. In all experiments, water dissolved oxygen levels were between 3.3 and 4.5 mg L^{-1}, and temperature ranged between 27.5° and 30.0°C. Within 24 h of taking length and mass measurements or following experimentation, we returned fish to their capture site. All experimental procedures were approved by the University of Guelph Animal Care Committee (protocol 3983) according to guidelines established by the Canadian Council on Animal Care.

Our study period coincided with the end of the dry season, which typically runs from December to May. Water chemistry parameters were measured at each study site several times in April 2018 (table 1). We measured dissolved oxygen (mg L^{-1}), pH, temperature (°C), and salinity (‰) using an electronic field probe (Multiparameter Meter, Hanna Instruments, RI). We assayed total sulfide (H_2S, HS^-, S^{2-}) concentration (μM) at each site colorimetrically using the methylene blue method (Cline 1969). Water samples were diluted to fall within the linear range of the chosen methylene dye (40–250 μM total sulfide). Subsequently, we calculated H_2S concentration (μM) from [total sulfide] as described by Broderius and Smith (1977) using pH and the pK_1 value (Tumanova et al. 1957) that encompassed the range of water temperatures measured in the field.

Loss of Equilibrium

To measure H_2S tolerance in *Kryptolebias* (0.23 ± 0.03 g, $n = 8$), *Poecilia* (0.23 ± 0.04 g, $n = 8$), and *Gambusia* (0.19 ± 0.03 g, $n = 8$), we exposed fish to seawater (pH = 6.4) containing 1,188 ± 21 μM H_2S, made using $NaS \cdot 9H_2O$ (Sigma Aldrich). We recorded the time until loss of equilibrium (LOE). This concentration was chosen because it was the highest-recorded field measurement at a site where fish were captured (table 1, site 10). Fish were considered to have lost equilibrium when they could no longer maintain an upright position in the water column and were unresponsive to three consecutive caudal peduncle prods (Regan et al. 2017). LOE experiments were carried out in 350-mL plastic containers with a mesh barrier 1 cm below the water line to prevent emersion and aquatic surface respiration. Three of the eight *Gambusia* used in this experiment were previously used in the time control emersion behavior experiment detailed below.

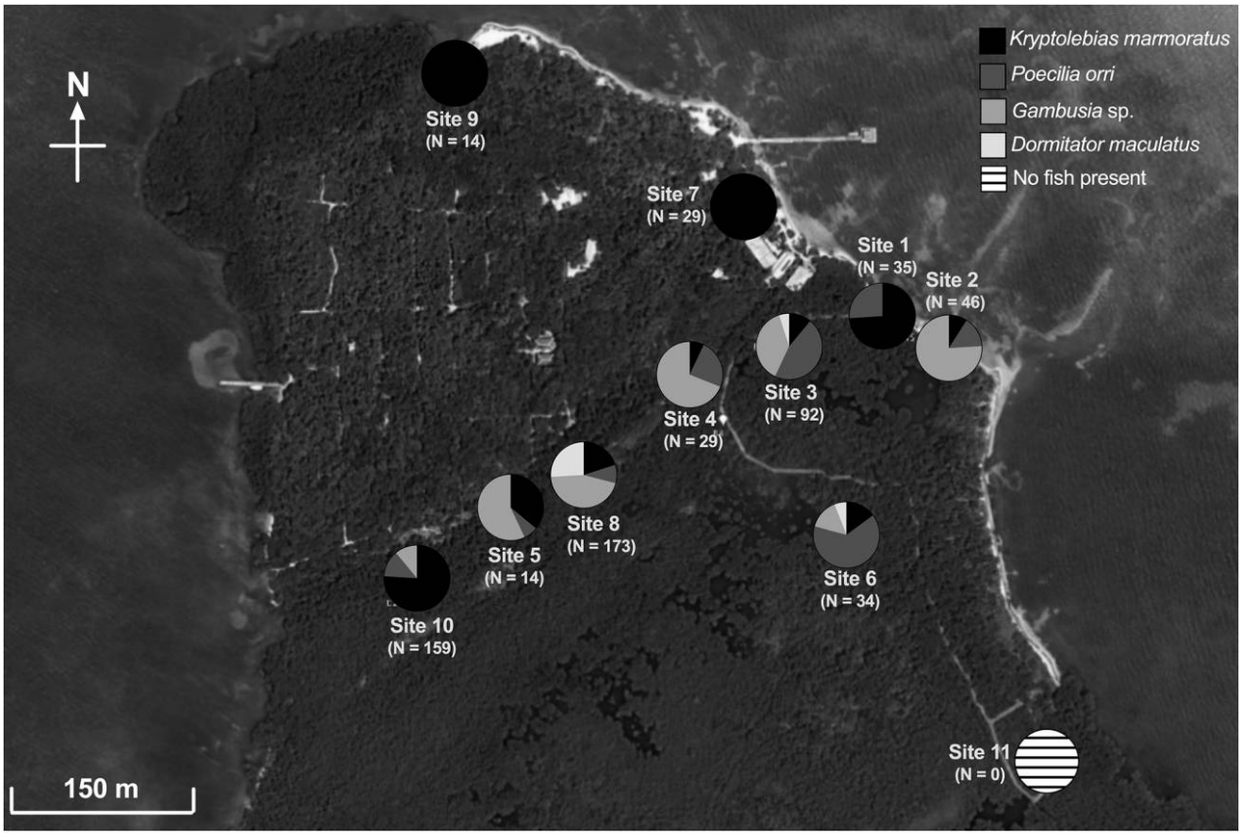

Figure 1. Map showing the northern tip of Long Caye, Lighthouse Reef Atoll, Belize, where field sampling occurred. Eleven study sites (1–11) are depicted at their GPS coordinates with the total number of fish of all species (*N*) listed for each site. Pie charts depict the percentage of each species captured at each site (see also table A1): *Kryptolebias marmoratus* (black), *Poecilia orri* (dark gray), *Gambusia* sp. (medium gray), *Dormitator maculatus* (light gray), and sites where no fish were collected (striped). Scale bar = 150 m. Image captured from Google Earth Pro, 2018, version 7.3.1.4507.

Assessment of H_2S as a Driver for Emersion

To assess whether increasing concentrations of H_2S would result in emersion by *Kryptolebias* (0.27 ± 0.03 g, $n = 8$), *Poecilia* (0.25 ± 0.05 g, $n = 8$), and *Gambusia* (0.30 ± 0.07 g, $n = 9$), we individually acclimated fish to an experimental chamber containing 350 mL seawater (pH = 6.4) for 1 h. The experimental chamber was surrounded at the water line by an oval platform (~4 cm wide) lined with a moist foam substrate onto which the fish could emerse. The seawater in the experimental chamber was continuously mixed with a stir bar (separated from fish by a mesh divider). Following the 1-h acclimation period, we increased the $[H_2S]$ in the experimental chamber by ~35 μM min^{-1} via the repeated addition of a bolus (150 μL) of 100 mM total sulfide stock solution. The addition of sulfide stock caused pH to increase; thus, pH was continuously recorded with a pH meter (Accumet AB15, Fisher Scientific) and maintained at ~6.4 (range 6.3–6.6) using small (μL) simultaneous additions of concentrated HCl. We added sulfide and HCl behind a visual barrier to minimize fish disturbance. At the time of emersion, we recorded water temperature and pH and used a water sample to empirically determine $[H_2S]$ as above.

Assessment of Emersion Behavior in Response to H_2S

To assess the degree of terrestriality in *Kryptolebias* (0.34 ± 0.07 g, $n = 8$), *Poecilia* (0.39 ± 0.11 g, $n = 8$), and *Gambusia* (0.32 ± 0.09 g, $n = 8$), we individually acclimated fish to the same experimental chamber as above (pH = 6.4) for 1 h without a stir bar or divider. Following the acclimation period, fish were video recorded (Logitech Quickcam Pro, Fremont, CA) for 1 h in seawater (pH = 6.4) to establish individual baseline emersion behavior. At the end of this control period, we drained the experimental chamber via siphoning to minimize disturbance and immediately refilled with 350 mL of seawater containing H_2S (545 ± 66 μM H_2S, pH = 6.4). This concentration was chosen to ensure that H_2S could trigger emersion behavior as it is slightly above the mean concentration at which the least sensitive species (*Poecilia*) emersed in the experiment above. Fish were then video recorded for another 1 h. We analyzed videos to compare the number of emersions and the total time spent out of water (min) between the control hour and H_2S exposure hour. H_2S volatilizes and oxidizes and thus is lost from solution over time. Over the 1-h experimental period, an average of 33.5% ± 0.04% of the H_2S was unavoidably lost; however, final concentrations remained high (396 ± 12 μM).

Table 1: Minimum and maximum water chemistry parameters measured at each sampling site

Site	Temp (°C)	Salinity (‰)	DO (mg L^{-1})	pH	Total sulfide (μM)	H_2S (μM)
1	32.4–35.5 (2)	22.8–26.3 (2)	.5–3.5 (2)	7.5–7.6 (2)	0–0 (2)	0–0 (2)
2	34.7–35.1 (2)	23.3–23.7 (2)	1.7–4.3 (2)	7.5–7.6 (2)	0–0 (2)	0–0 (2)
3	27.6–33.1 (4)	21.6–27.3 (3)	1.2–5.8 (4)	7.2–7.5 (4)	0–0 (2)	0–0 (2)
4	29.0–32.0 (3)	22.9–26.1 (3)	.1–7.9 (3)	6.9–7.4 (3)	0 (1)	0 (1)
5	30.6–31.2 (3)	32.1–35.6 (3)	3.0–5.2 (3)	7.0–7.2 (3)	0–0 (2)	0–0 (2)
6	32.9–33.8 (2)	31.7–34.7 (2)	5.5–5.9 (2)	7.8–7.9 (2)	0–0 (2)	0–0 (2)
7	27.8–32.6 (2)	1.1–2.8 (2)	.0–1.9 (2)	7.3–7.7 (2)	0–10 (2)	0–5 (2)
8	28.0–32.7 (12)	32.4–34.5 (7)	.4–3.6 (12)	6.8–7.4 (12)	0–102 (9)	0–45 (9)
9	27.9–32.0 (12)	36.3–40.3 (7)	.2–2.3 (10)	6.3–7.5 (12)	61–641 (7)	17–492 (7)
10	27.8–32.6 (9)	34.8–42.9 (8)	.0–3.4 (9)	6.3–7.8 (7)	0–2,881 (6)	0–1,166 (6)
11	26.5–30.0 (2)	39.5–41.4 (2)	.0–1.3 (2)	7.2–7.6 (2)	107–2,402 (2)	18–1,077 (2)

Note. Zero values indicate undetectable measures (i.e., <40 μM total sulfides). Values in parentheses indicate the number of readings included in the range. No statistical analyses were performed.

We also conducted a separate time control experiment to ensure that differences in the number of emersions and total time spent out of water (min) between hour 1 and hour 2 were the result of H_2S exposure and not disturbance, low pH, or chamber familiarity. *Kryptolebias* (0.43 ± 0.06 g, $n = 4$), *Poecilia* (0.32 ± 0.03 g, $n = 4$), and *Gambusia* (0.21 ± 0.04 g, $n = 4$) were used in this experiment. After the 1-h control period in this experiment, we drained the experimental chamber as above and replaced with 350 mL of H_2S-free seawater (pH = 6.4).

Statistical Analysis

We assessed all data for normality and homogeneity of variance using Shapiro-Wilk and Bartlett's tests, respectively. A nonparametric Kruskal-Wallis test, followed by a Dunn's post hoc test using a Bonferroni correction factor, was used to compare the Fulton's K of the three species across study sites, as normality and homogeneity of variance assumptions were violated and could not be corrected by data transformation. To compare Fulton's K of *Dormitator* at the two sites at which it was found, an unpaired t-test was used. When comparisons between species were required, we tested for a phylogenetic signal using an Abouheif's C_{mean} test. Of the many phylogenetic signal tests available, Abouheif's C_{mean} test is thought to be most appropriate when small numbers of species are compared (Abouheif 1999), although all phylogenetic signal tests have a high rate of type II error when there are fewer than 20 species (Münkemüller et al. 2012). Abouheif's C_{mean} tests revealed no phylogenetic signal in either the time to LOE ($P = 0.49$) or [H_2S] at emersion data ($P = 0.50$); therefore, incorporating phylogeny into statistical methods was unnecessary (Björklund 1997; Abouheif 1999). Thus, we performed a Kruskal-Wallis test, followed by a Dunn's multiple-comparison test using a Bonferroni correction factor, to compare the time to LOE between species and a one-way analysis of variance (ANOVA), followed by a Tukey's multiple-comparison test, to compare the [H_2S] at which species emersed; data were log transformed to meet parametric assumptions. We used paired t-tests to determine the effect of constant H_2S exposure on emersion behavior (number of emersions, % time spend out of water) in *Kryptolebias* and *Poecilia*. We did not perform statistical tests on the emersion behavior of *Gambusia* to constant H_2S as fish did not emerse in response to control or H_2S-rich water. We designated significance at $\alpha = 0.05$ and performed all statistical analyses in RStudio (ver. 1.1.447).

Results

Water Chemistry

Water parameters varied considerably on spatial and temporal scales, both across and within mangrove pools (table 1; fig. A1; figs. A1, A2 are available online). While H_2S is prevalent in the sediments across the mangrove ecosystem (Harbison 1986; Kryger and Lee 1995), we detected it in the water at only five of the 11 study sites (sites 7–11). We measured extremely high [H_2S] at sites 10 and 11 (>1 mM). At site 11, we found high [H_2S] in conjunction with white microbial mats coating the surface of the water; however, we never observed or captured fish there.

Species Proportion and Condition Factor across Sites

Kryptolebias was the predominant species at three of the four sites where we detected H_2S and captured fish (sites 7–10). *Kryptolebias* constituted 100% of fish captured at sites 7 and 9 and 76% of fish captured at site 10 (fig. 1; table A1, available online). Condition factor in *Kryptolebias* did not differ across study sites (Kruskal-Wallis; $P = 0.66$; table 2). In comparison, at sites where we did not detect H_2S (sites 1–6), species richness was higher (up to four fish species colocated at a single site), with either *Poecilia* or *Gambusia* being the dominant species at five of these six H_2S-free study sites (fig. 1; table A1). *Poecilia* and *Gambusia* collected at site 3 were in better condition than those at other sites. *Poecilia* at site 3 had a significantly higher Fulton's K than those collected at sites 4, 6, and 8 (Dunn's; $P < 0.05$), while *Gambusia* collected at site 3 had a significantly higher Fulton's K than those collected at site 8 (Dunn's; $P < 0.05$; table 2). We captured *Dormitator* at only three study sites (sites 3, 6, and 8) in low proportions (≤26%; table A1), and condition factor of this species

Table 2: Calculated condition factor (Fulton's *K*) for mangrove fish species (*Kryptolebias marmoratus*, *Poecilia orri*, *Gambusia* sp., and *Dormitator maculatus*) at each sampling site

	Fulton's *K*			
Site	*Kryptolebias*	*Poecilia*	*Gambusia*	*Dormitator*
1	1.56 ± .09 (22)	2.87 ± .20 (6)[ac]	…	…
2	1.56 ± .03 (4)	2.61 ± .25 (7)[ac]	1.86 ± .09 (18)[ab]	…
3	1.51 ± .06 (10)	2.90 ± .10 (41)[a]	2.08 ± .10 (25)[a]	2.04 ± .22 (5)
4	1.74, 1.82 (2)	2.14 ± .09 (7)[bc]	1.83, 1.82 (2)	…
5	1.46, 1.19 (2)	2.93 (1)	…	…
6	1.66 ± .26 (5)	2.31 ± .10 (21)[bc]	…	…
7	1.48 ± .04 (29)	…	…	…
8	1.50 ± .08 (31)	2.21 ± .07 (17)[bc]	1.75 ± .06 (64)[b]	2.06 ± .04 (45)
9	1.43 ± .06 (10)	…	…	…
10	1.50 ± .05 (113)	2.44 ± .07 (19)[ac]	1.09, 1.79 (2)	…
11	…	…	…	…

Note. Data are presented as mean ± SEM, except where the sample size is <3, where individual values are reported separated by a comma. Numbers in parentheses indicate sample size. Letters in superscript indicate statistically significant differences within a species across sites. Statistical comparisons were not made between species, nor were sites with a sample size <3 included in statistical analyses.

did not differ across sites (Kruskal-Wallis; $P = 0.43$; table 2). No other fish species were found at any of our study sites.

Loss of Equilibrium

All species, with the exception of one *Kryptolebias* individual, lost equilibrium within our a priori–determined 1-h experimental duration; thus, this single trial was terminated prior to LOE (fig. 2*A*, square). However, the time to LOE varied among species (Kruskal-Wallis; $P < 0.01$), indicating significant species differences in tolerance. *Kryptolebias* lost equilibrium significantly later than both *Poecilia* and *Gambusia* (Dunn's; $P < 0.05$), and *Poecilia* lost equilibrium significantly later than *Gambusia* (fig. 2*A*).

Emersion in Response to Increasing H_2S

All species emersed in response to increasing concentrations of H_2S. However, both the propensity to emerse and the concentration of H_2S that elicited emersion was species specific (ANOVA; $P < 0.01$). *Kryptolebias* emersed at a significantly lower [H_2S] than both *Poecilia* and *Gambusia* (Tukey; $P < 0.01$; fig. 2*B*). However, in four of nine experimental trials, *Gambusia* did not emerse, despite unimpeded access to an emersion platform, and instead lost equilibrium (fig. 2*B*, squares). Length, mass, and Fulton's *K* were not significantly different in fish that lost equilibrium compared to those that emersed (*t*-test; $P > 0.5$).

Emersion Behavior in Response to Constant H_2S

In contrast to rapidly increasing H_2S, exposure to a constant lower [H_2S] was only an emersion stimulus for *Kryptolebias* and some *Poecilia* individuals but not for *Gambusia*. Exposure to H_2S significantly increased the number of emersions (*t*-test; $P < 0.01$; fig. 3*A*) and the percent time spent out of water (*t*-test; $P < 0.01$; fig. 3*B*) in *Kryptolebias*. Although *Poecilia* exposed to H_2S did not significantly change the number of emersions (*t*-test; $P = 0.10$; fig. 3*D*) or the percent time spent out of water (*t*-test; $P = 0.08$; fig. 3*E*), the data were bimodal. Some *Poecilia* (5/8) were nonresponsive (i.e., did not emerse in response to H_2S; fig. 3*G*), while others (3/8) emersed and remained out of water for more than 90% of the H_2S-exposure hour (fig. 3*F*). H_2S caused one *Poecilia* individual to lose equilibrium after 41 min; this fish was excluded from the statistical analysis because the experimental period was incomplete. *Gambusia* did not emerse in either the control or H_2S-exposure hour (fig. 3*H*, 3*I*); thus, we did not perform statistical analysis. Three *Gambusia* individuals lost equilibrium within 7–34 min of H_2S exposure. These *Gambusia* did not differ significantly in length, mass, or Fulton's *K* (*t*-tests; $P > 0.1$) than fish that maintained equilibrium. We show representative traces outlining the emersion activity during the H_2S exposure hour for each species (fig. 3*C*, 3*F*, 3*G*, 3*J*). In the time control experiment, we detected neither statistical significance in the number of emersions (*t*-test, $P = 0.23$) nor the percent of time spent out of water (*t*-test, $P = 0.09$) between hours 1 and 2 for all species (data not shown).

Discussion

We asked whether *Kryptolebias*, *Poecilia*, and *Gambusia* can persist in H_2S-rich mangrove pools (up to 1,166 μM H_2S) because they are highly H_2S tolerant and/or because emersion behavior allows them to avoid H_2S. Our results suggest that both physiological H_2S tolerance and emersion behavior are important. *Kryptolebias* demonstrated high physiological H_2S tolerance, as fish lost equilibrium significantly later than both *Poecilia* and *Gambusia* during H_2S exposure (1,188 ± 21 μM H_2S). However, the fact that all species lost equilibrium at an ecologically relevant [H_2S] suggests that physiological tolerance may suffice at moderate [H_2S], while another strategy is required to endure higher [H_2S]. In support of the avoidance behavior hypothesis, increasing [H_2S]

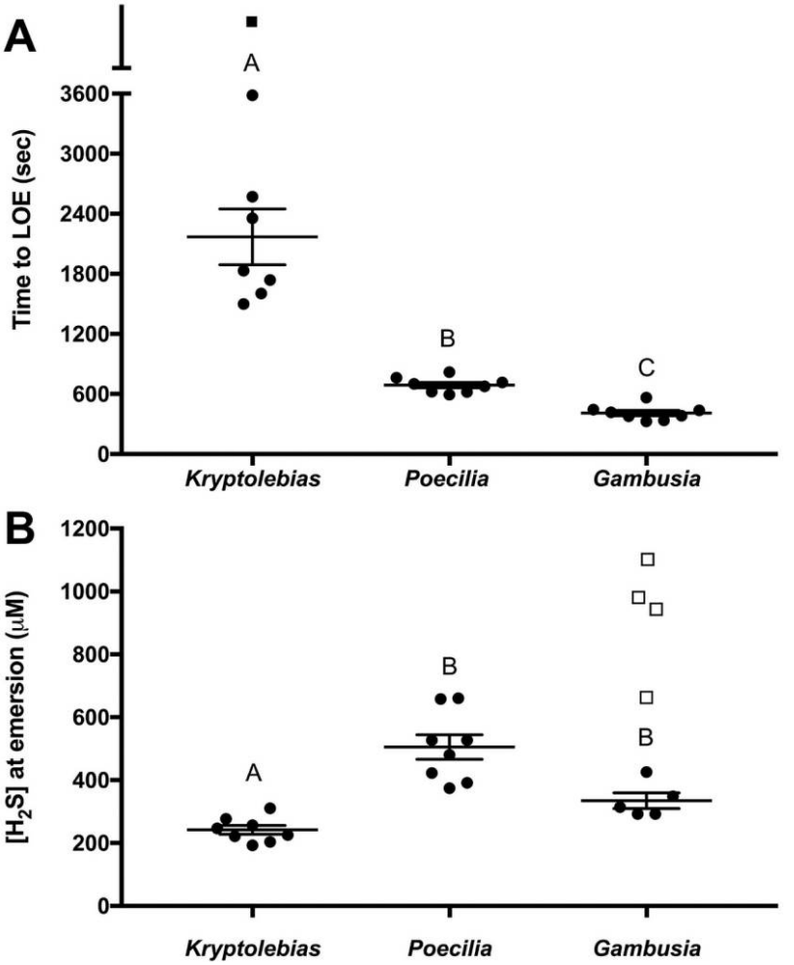

Figure 2. Physiological tolerance and behavioral sensitivity to H_2S. *A*, Filled circles indicate the time at which individual fish lost equilibrium. The broken *Y*-axis and filled square indicate a single individual who did not lose equilibrium within the 1-h experimental duration. *B*, Water [H_2S] at time of emersion. Circles indicate the [H_2S] at which fish emersed, and open squares indicate the [H_2S] at which *Gambusia* sp. lost equilibrium rather than emersing. The long horizontal bars denotes the mean of each group, while error bars indicate SEM. Different letters denote statistical significance. For all groups, $n = 8$, except for *Gambusia* sp. emersion data, where $n = 9$.

elicited an emersion response in all species. However, the amphibious *Kryptolebias* was highly sensitive to H_2S, as fish of this species emersed at lower [H_2S] than *Poecilia* and *Gambusia*. Moreover, H_2S exposure caused *Kryptolebias* to emerse more frequently and spend more time out of water than *Poecilia* and *Gambusia*. We suggest that physiological H_2S tolerance and emersion behavior are complementary strategies for survival in mangrove swamps. However, the superior ability of *Kryptolebias* to use both strategies may explain why this species was predominant at three of four study sites where H_2S was detected.

Our findings refute the hypothesis that mangrove fishes rely solely on physiological tolerance to persist in H_2S-rich pools. All fish lost equilibrium when exposed to an ecologically relevant [H_2S], except for one *Kryptolebias* individual. Loss of equilibrium is considered the point of ecological death, as fish are unable to escape lethal conditions in this immobilized state (Beitinger et al. 2000). While H_2S tolerance may be a valuable trait for fishes in pools with moderate [H_2S], fish must also employ alternative strategies to persist long-term. We expected that *Kryptolebias* would have lower physiological H_2S tolerance than *Poecilia* and *Gambusia* given their amphibious lifestyle and known aversion to H_2S (Abel et al. 1987). Instead, *Kryptolebias* was significantly more H_2S tolerant than both *Poecilia* and *Gambusia*. Tolerance to a stressor such as H_2S is often developed through prior or prolonged exposure (Bagarinao and Vetter 1993; Plath et al. 2007). It is possible that periodic H_2S exposure was sufficient to confer substantial H_2S tolerance in *Kryptolebias* over acclimatory, developmental, or evolutionary timescales. Alternatively, H_2S tolerance may not be dependent on the presence of H_2S in the environment (Tobler et al. 2016). Low O_2 availability constrains aerobic metabolism (Hochachka et al. 1996) in a similar manner to the inhibition of COX by H_2S exposure. Thus, physiological modifications to cope with aquatic hypoxia—which is pervasive in these mangrove pools (table 1; fig. A1)—may also confer H_2S tolerance in fishes. H_2S LC_{50} data are not available for *Kryptolebias*, and thus direct comparisons with other species are not possible. However, *Kryptolebias* appears

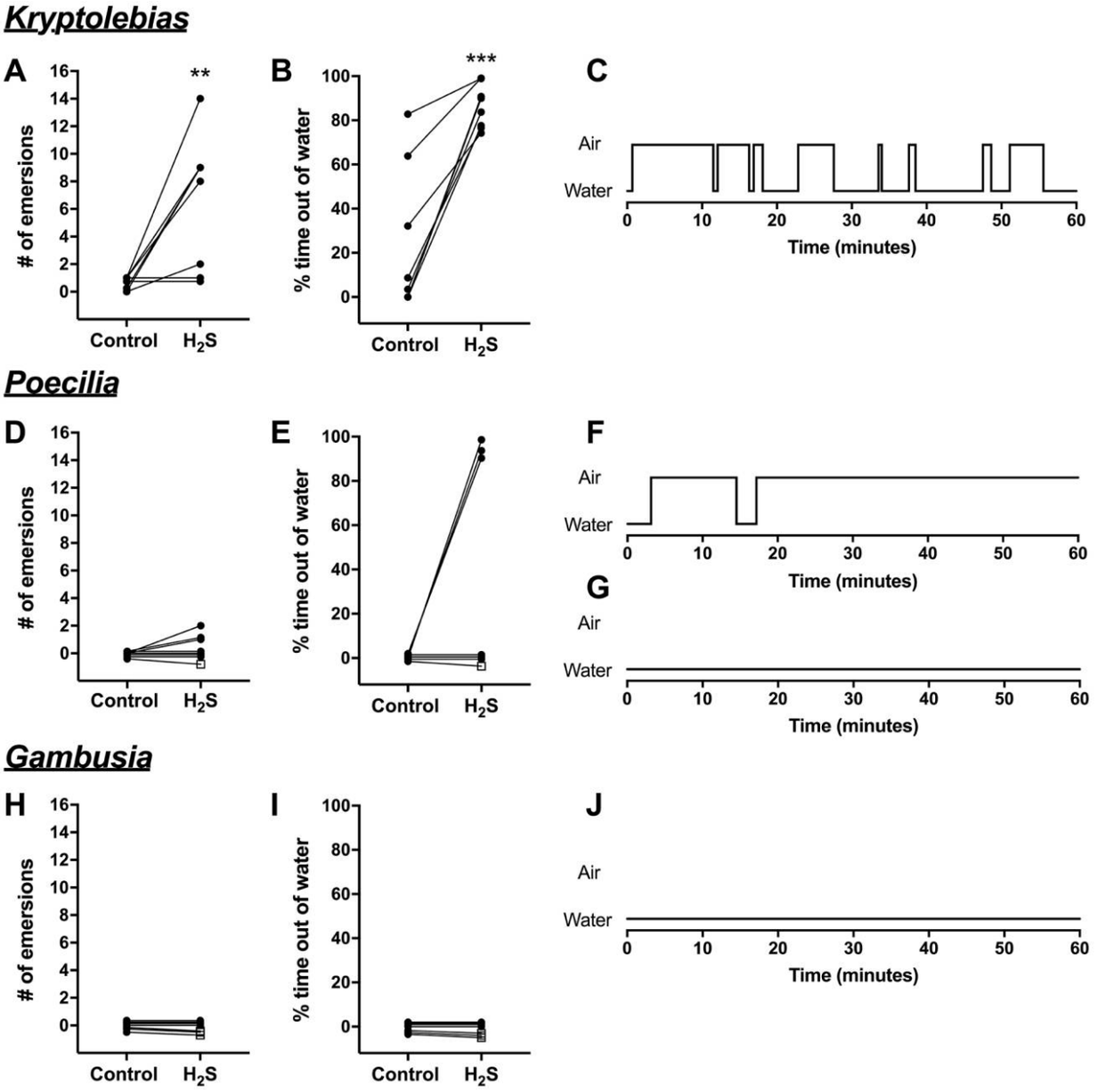

Figure 3. Emersion behavior—number of emersions (*A*, *D*, *H*), percentage of time spent out of water (*B*, *E*, *I*), and representative traces depicting an individual's emersion activity over time (*C*, *F*, *G*, *J*) for each species (*Kryptolebias marmoratus*, *Poecilia orri*, and *Gambusia* sp.) in response to constant H_2S exposure (545 μM) for 1 h. For each species, $n = 8$. Double asterisks indicate $P \leq 0.01$; triple asterisks indicate $P \leq 0.001$. Circles represent fish that emersed from water, while squares represent fish that did not emerse and instead lost equilibrium.

far more H_2S tolerant than several fully aquatic species that inhabit sulfidic environments. For instance, the 12-h LC_{50} for the flathead grey mullet (*Mugil cephalus*) was only 42 μM H_2S, and California killifish (*Fundulus parvipinnis*) could only tolerate 53 μM H_2S for ~20 h (Bagarinao and Vetter 1989). Overall, our findings suggest that physiological tolerance may be valuable at moderate H_2S concentrations, but other strategies are required to persist in mangrove pools when H_2S concentrations reach lethal levels. Currently, the physiological mechanisms behind H_2S tolerance in mangrove fishes remain unknown and worthy of study.

In support of the avoidance hypothesis, all species emersed in response to increasing [H_2S]. *Kryptolebias* are highly amphibious (Taylor 2000; Wright 2012; Turko and Wright 2015) and are known to voluntarily emerse in response to abiotic stressors (Regan et al. 2011; Gibson et al. 2015; Robertson et al. 2015), including H_2S (Abel et al. 1987). Surprisingly, *Poecilia* and *Gambusia* also emersed in response to H_2S, despite no previous literature reports of amphibious behavior in these species, although there are documented examples of other *Poecilia* and *Gambusia* species on land for brief sojourns. For example, *Gambusia affinis*

have been observed jumping onto land to avoid aquatic predators (Baylis 1982), and an unidentified *Poecilia* species was reported leaving water to reach an adjacent aquatic environment (Lefebvre and Spahn 1987). As increasing H_2S elicited an emersion response in all three species, the ability to sense H_2S is likely a common trait. However, the highly amphibious *Kryptolebias* was the most H_2S sensitive as they emersed at significantly lower H_2S concentrations than either *Poecilia* or *Gambusia*. The fact that the concentration of H_2S at emersion differed among species suggests that the mechanism(s) of H_2S detection differ, the threshold for detection differs, and/or the cost of air exposure is greater for *Poecilia* and *Gambusia*. The mechanism underlying the sensing of H_2S in *Kryptolebias*, *Poecilia*, and *Gambusia* is unknown but could be conferred by hypoxia-sensitive neuroepithelial cells (NECs) that also respond to H_2S in other fishes (Olson 2008; Porteus et al. 2014). NECs are typically localized on the gills of fishes (Dunel-Erb et al. 1982) but have also been reported in the skin of *Kryptolebias* (Jonz et al. 2004; Regan et al. 2011) and only one other species (*Periophthalmodon schlosseri*; Zaccone et al. 2017) to date. The greater H_2S sensitivity of *Kryptolebias* may be driven by a greater overall number/size of NECs across the body relative to *Poecilia* and *Gambusia*, but additional research is required to test this idea. Regardless, it is clear that *Kryptolebias* utilized emersion behavior in response to H_2S far more readily compared to the other two species. The low threshold for the emersion response in *Kryptolebias* is consistent with their well-documented highly amphibious natural history, where presumably the energetic and/or ecological costs on land are lower than for *Poecilia* and *Gambusia*. For example, H_2S-exposed *Gambusia* would often lose equilibrium rather than emerse, despite unimpeded access to the surface, suggesting that *Gambusia* may only use the emersion response as a last resort.

The amphibious *Kryptolebias* showed a high degree of terrestriality in response to constant H_2S, lending additional support to the avoidance behavior hypothesis. We observed *Kryptolebias* voluntarily leaving H_2S-free water on several occasions (figs. 2*A*–2*C*, A2). H_2S exacerbated this behavior as emersion frequency and time spent out of water increased. Interestingly, all *Poecilia* individuals emersed in response to increased H_2S, but only some *Poecilia* emersed in response to constant H_2S exposure. These interexperimental differences may be the result of methodological differences, such as the rate of H_2S change. The rate of change of an experimental variable (e.g., temperature in CT_{max} literature) can influence physiological and behavioral responses (Moyano et al. 2017). A subset of *Poecilia* emersed when exposed to constant H_2S for 1 h and remained on land for 90%–99% of the experimental duration with no apparent ill effects. Additional support for this amphibious-like phenotype in *Poecilia* is shown in figure A2, where a *Poecilia* individual is photographed emersed from H_2S-free water on the side of a bucket. *Gambusia* did not emerse when exposed to constant elevated H_2S and were never observed voluntarily emersed from H_2S-free water. Both *Poecilia* and *Gambusia* display the characteristic upturned mouth with a protruding bottom lip of aquatic fishes that perform ASR (Kramer 1987). While ASR was not quantified in this study, anecdotal observations suggest that *Poecilia* and *Gambusia* exposed to H_2S spent a large portion of time at the air-water interface. Therefore, ASR may be a key strategy used by nonamphibious *Poecilia* and *Gambusia* to tolerate elevated H_2S. Overall, the amphibious *Kryptolebias* demonstrated the highest degree of terrestriality in response to H_2S, followed by a subset of *Poecilia* individuals demonstrating some amphibious capability.

We found that mangrove pools exhibit considerable variation in [H_2S], pH, temperature, dissolved oxygen, and salinity on both spatial and temporal scales (table 1; fig. A1). The extremely high H_2S concentrations measured in this study are mong the highest reported in mangrove waters (Harbison 1986; Laurent et al. 2009; Satheeshkumar and Khan 2009). Remarkably, within a matter of hours, H_2S concentrations ranged from detectable to undetectable, and vice versa. At site 10, for instance, H_2S was 1,166 μM at noon and undetectable at 3 p.m. that same day (a rate of change of 387 $\mu M\ h^{-1}$). Low pH shifts the proportion of total sulfide (H_2S, HS^-, S^{2-}) toward the toxic form (H_2S; Broderius and Smith 1977). Interestingly, elevated H_2S concentrations were generally accompanied by low pH values. While O_2 levels varied across sites, most sites experienced intermittent hypoxia ($<2\ mg\ L^{-1}$; fig. A1). Low O_2 availability in mangrove pools supports the production of H_2S and can exacerbate H_2S toxicity in fishes by further constraining aerobic metabolism (Bagarinao and Lantin-Olaguer 1999). Taken together, these aquatic conditions and their fluctuating nature provide a challenging environment for fish respiration and survival. Further studies are required to assess the effects of multiple abiotic stressors on behavioral and physiological responses in mangrove fishes.

Conclusion

The H_2S concentrations measured in mangrove pools on Long Caye, Belize, are among the highest reported in mangrove waters. As these high concentrations exceed the limit of physiological tolerance for resident fishes, alternate coping strategies are necessary. The mangrove fishes studied here, particularly *Kryptolebias*, are highly H_2S tolerant but rely on the emersion response in their behavioral repertoire to survive when H_2S concentrations reach acutely toxic levels. We suggest that emersion behavior is an additional strategy fishes can exploit to pass through the ecological filter imposed by H_2S. Fishes that have successfully colonized H_2S-rich environments exemplify how both behavioral and physiological adaptations are often necessary to survive extreme environmental conditions.

Acknowledgments

We thank the staff at Itza Dive Lodge for logistical support. Emily Standen, Tamzin Blewett, and Glenn Tattersall are also thanked for assistance in the field and helpful commentary on the manuscript. Funding was provided by the National Sciences and Engineering Research Council of Canada (NSERC) Discovery grants to P.A.W. and S.C. (120513 and 061770, respectively), a University of Guelph Registrar's Research grant to G.S.R., an NSERC Canada Graduate Scholarships–Doctoral Program grant

to L.T., and a New Brunswick Innovation Foundation scholarship and Mount Allison Student Union academic enrichment grant to K.E.M. We declare no competing or financial interests. G.S.R, L.T., A.J.T., and P.A.W. conceived and designed the study. All authors collected field data and fish for experiments. G.S.R. and L.T. conducted all experiments and wrote the manuscript. All authors edited the manuscript.

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