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RESEARCH ARTICLE

Mitochondrial physiology and responses to elevated hydrogen sulphide in two isogenic lineages of an amphibious mangrove fish

Keri E. Martin[1], Suzanne Currie[2] and Nicolas Pichaud[3,*]

ABSTRACT

Hydrogen sulphide (H_2S) is toxic and can act as a selective pressure on aquatic organisms, facilitating a wide range of adaptations for life in sulphidic environments. Mangrove rivulus (*Kryptolebias marmoratus*) inhabit mangrove swamps and have developed high tolerance to environmental H_2S. They are hermaphroditic and can self-fertilize, producing distinct isogenic lineages with different sensitivity to H_2S. Here, we tested the hypothesis that observed differences in responses to H_2S are the result of differences in mitochondrial functions. For this purpose, we performed two experimental series, testing (1) the overall mitochondrial oxidizing capacities and (2) the kinetics of apparent H_2S mitochondrial oxidation and inhibition in two distinct lineages of mangrove rivulus, originally collected from Belize and Honduras. We used permeabilized livers from both lineages, measured mitochondrial oxidation, and monitored changes during gradual increases of sulphide. Ultimately, we determined that each lineage has a distinct strategy for coping with elevated H_2S, indicating divergences in mitochondrial function and metabolism. The Honduras lineage has higher anaerobic capacity substantiated by higher lactate dehydrogenase activity and higher apparent H_2S oxidation rates, likely enabling them to tolerate H_2S by escaping aquatic H_2S in a terrestrial environment. However, Belize fish have increased cytochrome *c* oxidase and citrate synthase activities as well as increased succinate contribution to mitochondrial respiration, allowing them to tolerate higher levels of aquatic H_2S without inhibition of mitochondrial oxygen consumption. Our study reveals distinct physiological strategies in genetic lineages of a single species, indicating possible genetic and/or functional adaptations to sulphidic environments at the mitochondrial level.

KEY WORDS: Mitochondrial respiration, Cytochrome *c* oxidase, Oxidative capacity, Liver, H_2S tolerance, Metabolism

INTRODUCTION

Hydrogen sulphide (H_2S) is produced naturally in aquatic ecosystems by bacterial metabolism and geothermal activity (Beauchamp et al., 1984) and is present in a wide variety of aquatic ecosystems. H_2S can both stimulate and depress metabolic functions (Nicholls and Kim, 1982); when external sources increase internal levels above a threshold, it can quickly become toxic (Reiffenstein et al., 1992). Owing to its toxicity, H_2S can act as a selective pressure influencing behavioural, morphological and/or physiological adaptations that confer tolerance to elevated levels of H_2S (Tobler et al., 2008). Generally, adaptations to H_2S tolerance can be separated into three distinct strategies: (1) limiting H_2S from entering the body, (2) mutualistic relationships with sulphur-oxidizing bacteria and/or (3) internal detoxification (Kelley et al., 2016). Variations in avoidance behaviours are key for limiting H_2S exposure. For example, mobile animals (e.g. teleost fishes) leave areas with elevated H_2S (Abel et al., 1987; Rossi et al., 2019; Martin and Currie, 2020) whereas sessile organisms such as bivalves can close exposed biological membranes and rely on anaerobic metabolism (Vismann, 1991). Alternately, other H_2S-tolerant invertebrates host colonies of sulphur-oxidizing bacteria that change H_2S into less toxic forms [i.e. elemental sulphur (S_2), sulphite (SO_3^{2-}), sulphate (SO_4^{2-}) or thiosulphate ($S_2O_3^{2-}$)] (*Soleyma velum*, Cavanaugh, 1983; *Olavius algarvensis*, Dubilier et al., 2001). Lastly, H_2S can be detoxified internally through sulphur oxidation into less toxic byproducts (i.e. SO_4^{2-}, $S_2O_3^{2-}$) that are stored/excreted (Bagarinao, 1992). Clearly, adaptations to H_2S are varied and are determined by environmental factors of an organism's habitat (Tobler et al., 2008). Often H_2S-tolerant animals have multiple adaptations at different levels of organization (i.e. whole body, tissue, organ or cellular), and each adaptation contributes to overall tolerance (Vismann, 1991).

Marine invertebrates are arguably the most well-studied group of H_2S-tolerant animals. In contrast, we know relatively less about H_2S tolerance in aquatic vertebrates even though several fish species inhabit sulphidic-rich environments. Their strategies for H_2S tolerance provide fascinating models with which to study adaptations to challenging environments. For example, in Mexican caves, distinct populations of guppies (*Poecilia mexicana*) have independently evolved multiple strategies of H_2S tolerance, separating them from closely related non-sulphidic populations (Tobler et al., 2018). Similar to *P. mexicana*, the amphibious mangrove rivulus (*Kryptolebias marmoratus*) lives in a sulphidic environment (native mangrove forests), resulting in constant exposure to fluctuating levels of H_2S ranging from 0 to 1116 µmol l^{-1} (Rossi et al., 2019). Mangrove rivulus show a remarkable tolerance for H_2S, surviving both short-term (>20 min) exposure to ∼2500–3500 µmol l^{-1} H_2S and long-term (3 days) exposure to 200 µmol l^{-1} H_2S (Cochrane et al., 2019; Martin and Currie, 2020). Importantly, mangrove rivulus are hermaphroditic, and capable of self-fertilization, allowing for the production of isogenic lineages from a single parental genotype to study genetic differences of phenotypic traits (Tatarenkov et al., 2010). As in *P. mexicana* (Tobler et al., 2008; Greenway et al., 2020), there are differences in responses to H_2S among isogenic lineages of amphibious rivulus. Mangrove rivulus from the Honduras lineage are more sensitive to H_2S, emerging from water at lower H_2S concentrations compared with the Belize lineage (Martin and Currie, 2020). We are only beginning to understand the behavioural and physiological strategies used by mangrove rivulus to tolerate its

[1]Department of Biology, Mount Allison University, Sackville, NB, Canada, E4L 1E4. [2]Department of Biology, Acadia University, Wolfville, NS, Canada, B4P 2R6. [3]Department of Chemistry and Biochemistry, University of Moncton, Moncton, NB, Canada, E1A 3E9.

*Author for correspondence (nicolas.pichaud@umoncton.ca)

S.C., 0000-0002-7734-7200; N.P., 0000-0002-2820-8124

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sulphide-rich habitat. To date, these strategies include avoidance/limited body exposure (Abel et al., 1987; Rossi et al., 2019; Martin and Currie, 2020) and modified H_2S-resistant haemoglobin (Cochrane et al., 2019), but these cannot fully explain their H_2S tolerance. Genetically distinct sub-populations of fish serve as critical tools for studying the adaptation of organisms to extreme environments (Tobler et al., 2018), but we do not know what physiological mechanisms may determine the observed differences in isogenic lineages in mangrove rivulus. We require a better understanding of internal detoxification mechanisms of H_2S, which could help to identify underlying physiological differences between lineages to improve our knowledge of how H_2S can act as a selective pressure on fishes inhabiting challenging environments.

Internal regulation of H_2S is a common strategy of H_2S tolerance as all animals have a limited ability to detoxify H_2S internally in mitochondria (Hildebrandt and Grieshaber, 2008). H_2S acts by reversibly binding to cytochrome *c* oxidase (Complex IV – COX) (Cooper and Brown, 2008), a key enzyme in the electron transport system (ETS) reducing O_2 to H_2O upon electron transfer from the other components of the ETS. All animals produce H_2S internally. Endogenous H_2S concentrations are controlled by mitochondrial oxidation, initiated by the enzyme sulphide:quinone oxidoreductase (SQR). This enzyme transfers electrons from the oxidation of H_2S to the ubiquinone (Q) in the inner mitochondrial membrane, enabling the sequential transport of electrons to complex III, cytochrome *c* and COX until the final acceptor of the ETS, oxygen (Hildebrandt and Griehaber, 2008; Greenway et al., 2020). However, if levels within the cell rise above the tolerable threshold, H_2S will inhibit COX, severely limiting mitochondrial ATP production (Grieshaber and Völkel, 1998). In isolated tissue, 5–15 µmol l^{-1} H_2S stimulates mitochondrial sulphide oxidation, and levels over 15 µmol l^{-1} begin to cause inhibition (e.g. marine clam, *Silemya reidi*, Powell and Somero, 1986; soil amoeba, *Acanthamoeba castellannii*, Lloyd et al., 1981; rat, Bartholomew et al., 1980). Key tissues involved in sulphur oxidation vary between species and include blood, outer body wall, kidney, liver, spleen and hepatopancreas (Bagarinao and Vetter, 1989), but the main site of H_2S enzymatic oxidation is always mitochondria. To our knowledge, there are two main strategies for H_2S tolerance in mitochondria: (1) H_2S-resistant enzymes and/or (2) increased oxidation capacity (Tobler et al., 2016). We know the H_2S-tolerant mangrove rivulus fish (*K. marmoratus*) does not have H_2S-resistant COX (Cochrane et al., 2019), suggesting a reliance on alternate mechanisms of H_2S tolerance, such as increased oxidation capacity, but this has yet to be investigated. In fact, we have no baseline information on the mitochondrial physiology of this emerging model organism.

In this study, we used two isogenic lineages of mangrove rivulus and measured mitochondrial physiology and responses to H_2S to test the overarching hypothesis that functional differences at the level of the mitochondria underpin physiological and behavioural responses to sulphidic environments. To this end, we conducted two experimental series on permeabilized liver tissue, allowing us to investigate the different components of the ETS involved in H_2S oxidation and its inhibitory effects. Given that we have no information on basic mitochondrial physiology in this species, our first experimental series was aimed at characterizing the overall mitochondrial oxidizing capacities of liver in the two lineages. Because of recently observed differences in H_2S tolerance between the Belize and the Honduras lineages (Martin and Currie, 2020), we predicted divergent oxidizing capacities resulting in metabolic differences in aerobic and anaerobic capacities. In the second experimental series, we measured the kinetics of apparent H_2S mitochondrial oxidation and its inhibitory effects on respiration in the two lineages. If independent adaptations to H_2S have evolved in the two isogenic lineages because of their differences in H_2S sensitivity (Martin and Currie, 2020), then we should observe mitochondrial functional differences. Specifically, we predicted that the two distinct isogenic lineages would have different strategies to cope with H_2S. These differences would be reflected by their capacities to resist increasing H_2S concentrations and to oxidize H_2S, suggesting potential independent adaptations to sulphidic environments.

MATERIALS AND METHODS

Animals

All experiments were performed using adult mangrove rivulus [*Kryptolebias marmoratus* (Poey 1880)] housed in a Conviron environmental chamber (CMP 3244) at Mount Allison University in New Brunswick, Canada. We housed fish individually in 120 ml containers with ∼80 ml brackish water (15 ppt; Instant Ocean, Aquarium Systems Inc.). Mangrove rivulus are hermaphrodites capable of self-fertilization (Taylor, 2012) and our fish have been bred in a laboratory for over 40 generations, creating distinct lineages with differentiation at multiple loci (Tatarenkov et al., 2010). We performed experiments with two distinct lineages: mangrove rivulus from the 50.91 lineage native to Twin Cayes, Belize (hereafter referred to as Belize), collected in 1991, and the Hon11 lineage from the Bay Islands, Honduras (hereafter referred to as Honduras), collected in 2006 (Tatarenkov et al., 2010). We maintained fish on a diel thermal cycle of 25°C (04:00 h) to 30°C (14:00 h), similar to mean temperature cycles experienced in mangrove tide pools in Belize (Ellison et al., 2012; Rossi et al., 2019). We maintained a photoperiod of 12 h:12 h light:dark and humidity of 60%. We fed fish live *Artemia* nauplii 4 days per week and bloodworms 1× per week, with bi-weekly water changes. All protocols were approved by the Mount Allison University Animal Care Committee in accordance with the Canadian Council on Animal Care (protocol no. 101840).

Series I: determination of metabolic capacity

To evaluate the metabolic capacity in our two isogenic lineages, we measured mitochondrial respiration rates in permeabilized livers and activities of key metabolic enzymes in liver homogenates.

Liver permeabilization

We netted fish (means±s.e.m.; mass: 0.047±0.001 g, length: 15.370±0.014 mm) from containers, sedated them on ice and then killed them with a cranial blow. We quickly dissected livers and transferred them to an ice-cold relaxing solution (2.77 mmol l^{-1} CaK_2EGTA, 7.23 mmol l^{-1} K_2EGTA, 5.77 mmol l^{-1} Na_2ATP, 6.56 mmol l^{-1} $MgCl_2$, 20 mmol l^{-1} taurine, 15 mmol l^{-1} Na_2 phosphocreatine, 20 mmol l^{-1} imidazole, 50 mmol l^{-1} MES,0.5 mmol l^{-1} dithiothreitol, pH 7.1). For permeabilization, we used two pairs of sharp forceps that were inserted centrally into the sample, and we repeatedly tore apart the tissue in different directions until 0.1 mm sized fragments were obtained in the form of a loosely connected network. For each mitochondrial oxygen consumption experiment, permeabilized livers were pooled (Honduras: two livers for a mean±s.e.m. of 1.56±0.15 mg; Belize: three livers due to smaller body size for a mean of 1.48±0.16 mg). We then incubated the permeabilized livers in respiration medium (140 mmol l^{-1} KCl, 5 mmol l^{-1} KH_2PO_4, 20 mmol l^{-1} HEPES, 3 mmol l^{-1} $MgCl_2$, 1% BSA w/v, pH 7.2) and put them on an orbital shaker at 4°C for 5 min. We then gently dry-blotted the permeabilized livers and weighed them on a

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Secura 225D-1S semi-micro balance (0.01 mg readability, Sartorius, Göttingen, Germany) before transferring them into the 2 ml chamber of an Oxygraph-O2K (Oroboros Instruments, Innsbruck, Austria) filled with air-saturated respiration medium with pyruvate (10 mmol l^{-1}) and malate (2 mmol l^{-1}). For the Belize lineage, we performed eight different experiments (N=8), and for the Honduras lineage, we performed six different experiments (N=6).

Measurement of respiration rates

In our first experimental series, we were interested in describing and comparing mitochondrial functions of the two mangrove rivulus isogenic lineages. Mitochondrial oxygen consumption was measured at 27.5°C in permeabilized livers of fish using a SUIT protocol (Pesta and Gnaiger, 2012; Simard et al., 2018). After the signal was stabilized, we monitored the CI-LEAK respiration rate (non-phosphorylating state) in the presence of pyruvate and malate. Addition of ADP (5 mmol l^{-1}) allowed us to reach the CI-OXPHOS respiration rate, when electron transport in the ETS is coupled to the phosphorylation of ADP. Injection of cytochrome *c* (10 µmol l^{-1}, CIc-OXPHOS) allowed us to estimate the integrity of the mitochondrial outer membrane (Kuznetsov et al., 2008). We then added succinate (10 mmol l^{-1}) to evaluate the mitochondrial oxygen consumption with the contributions of complexes I and II (CI+CII-OXPHOS). We subsequently performed injections of rotenone (0.5 µmol l^{-1}, inhibitor of complex I) and malonate (5 mmol l^{-1}, inhibitor of complex II) before evaluating apparent mitochondrial H_2S oxidation (see Series II below). Mitochondrial respiration rates are expressed as pmol O_2 s^{-1} mg^{-1} tissue.

Mitochondrial ratios

We used the different respiration rates to calculate several mitochondrial ratios. First, we used CI-LEAK and CI-OXPHOS to calculate the P_I/L_I ratio at the level of complex I (CI-OXPHOS/CI-LEAK), which is an indicator of mitochondrial quality and of mitochondrial coupling (Gnaiger, 2009; Iftikar et al., 2014). We also evaluated the cytochrome *c* effect by dividing the CI-OXPHOS rate by the mitochondrial oxygen consumption obtained after injection of cytochrome *c* (CIc-OXPHOS): an injection of cytochrome *c* that results in a 15% increased oxygen consumption indicates a compromised outer mitochondrial membrane, likely owing to the permeabilization (Kuznetsov et al., 2008). These two ratios served as a quality control for our permeabilized preparations. Finally, we calculated the succinate contribution to the oxygen consumption as (CI+CII-OXPHOS–CIc-OXPHOS)/CI+CII-OXPHOS, which represents the percentage increase of oxygen consumption after addition of succinate; a ratio close to 0.0 indicates that the added substrate did not increase the oxygen consumption markedly, while a ratio of 0.5 indicates a 50% increase, a ratio of 1.0 indicates a 100% increase (doubling), and so forth.

Enzymatic activities and complex IV maximal capacity

We measured citrate synthase (CS) and lactate dehydrogenase (LDH) enzymatic activities in liver homogenates (N=8 and N=6 for Belize and Honduras, respectively) as proxies of aerobic and anaerobic metabolism, respectively. For this experiment, we used a BioTek Synergy H1 microplate reader (BioTek®, Montreal, QC, Canada) set at 27.5°C. Livers were homogenized in 100 mmol l^{-1} potassium phosphate buffer, pH 7.0. CS activity was measured at 412 nm for 4 min by measuring the reduction of 5,5-dithiobis-2-nitrobenzoic acid (DTNB, ε=13.6 ml cm^{-1} $µmol^{-1}$) using a 100 mmol l^{-1} imidazole-HCl buffer containing 0.1 mmol l^{-1} DTNB, 0.1 mmol l^{-1} acetyl-CoA and 0.15 mmol l^{-1} oxaloacetic acid, pH 8.0 (Ekström et al., 2017). LDH activity was determined at 340 nm for 4 min by following the disappearance of NADH (ε=6.22 ml cm^{-1} $µmol^{-1}$) using a 100 mmol l^{-1} potassium phosphate buffer complemented with 0.16 mmol l^{-1} NADH and 0.4 mmol l^{-1} pyruvate, pH 7.0 (Ekström et al., 2017). Enzymatic activities were normalized by total protein content measured by the bicinchoninic acid method (Smith et al., 1985) and are expressed as U mg^{-1} protein where U represents 1 µmol of substrate transformed to product in 1 min.

In separate experiments, we evaluated the complex IV maximal capacity in liver homogenates (N=8 and N=6 for Belize and Honduras, respectively). Livers were homogenized on ice in an imidazole buffer (50 mmol l^{-1} imidazole, 2 mmol l^{-1} $MgCl_2$, 5 mmol l^{-1} EDTA, 0.9% Triton X-100, pH 7.4) and the resulting homogenates were centrifuged at 750 ***g*** for 5 min at 4°C to remove cellular debris. We collected the supernatant and directly transferred it to the Oxygraph-O2K chambers with respiration medium complemented with 20 mmol l^{-1} glucose, 10 mmol l^{-1} ascorbic acid and 150 µmol l^{-1} cytochrome *c* (Blier and Lemieux, 2001; Pichaud et al., 2010). After signal stabilization, we monitored the complex IV maximal capacity at 27.5°C.

Series II: H_2S mitochondrial kinetics

Given the known effects of H_2S on mitochondrial dynamics in other H_2S-tolerant organisms, our second experimental series was designed to determine the effects of H_2S on mitochondrial functions in our two mangrove rivulus lineages. It is worth noting that the H_2S oxidation measured here reflects the incremental oxygen consumption rate observed upon H_2S addition and, thus, cannot be entirely attributed to SQR activity but also to other ETS components (such as complex III). Hence, the oxidation rates measured are hereafter referred to apparent H_2S oxidation.

Apparent H_2S mitochondrial oxidation and mitochondrial oxygen consumption inhibition

After inhibition of complexes I and II (described in Series I), we added increasing concentrations of sulphide to the chambers to evaluate apparent mitochondrial H_2S oxidation. Stock solutions of sulphide (20 mmol l^{-1}, 2 mmol l^{-1} and 0.2 mmol l^{-1}) were freshly prepared just before the experiment using $Na_2S{\cdot}9H_2O$ (Sigma-Aldrich Canada, Oakville, Canada) dissolved in deoxygenated reverse osmosis water. We used 6 mol l^{-1} hydrochloric acid (HCl) to maintain pH within environmentally relevant levels, to avoid precipitation of $Na_2S{\cdot}9H_2O$ (~6.7; Rossi et al., 2019) and to ensure that the primary sulphur species present was H_2S. For each experiment, one Oxygraph-O2K chamber contained the respiration medium and the experimental substrates with the permeabilized livers. The second of the paired chambers did not contain any tissue in order to account for chemical background owing to oxygen consumption in the presence of H_2S, which was subtracted from the respective values in the presence of tissue (Völkel and Grieshaber, 1997). Specifically, injections of 1, 2, 5, 10, 4×20 and 5×50 µmol l^{-1} of sulphide were performed until we observed complete inhibition of mitochondrial oxygen consumption. At the end of each experiment, we added antimycin A (inhibitor of complex III) to completely inhibit the electron transport in the ETS and ensure that the sulphide concentrations used were inhibiting mitochondrial respiration to the same level. As H_2S is both an electron donor to the mitochondrial sulphide oxidase and an inhibitor of complex IV, these concentrations allowed us to sequentially evaluate apparent mitochondrial H_2S oxidation at low sulphide concentrations (0 to 98 µmol l^{-1}) and mitochondrial

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oxygen consumption inhibition at high sulphide concentrations (98 to 348 µmol l^{-1}). For apparent H_2S mitochondrial oxidation, we used a two-parameter Michaelis–Menten curve to estimate the maximum rate of apparent H_2S oxidation (V_{max}) and the affinity of sulphide oxidase for H_2S (K_m) for each mangrove rivulus lineage according to:

$$y = \frac{V_{max} \times x}{K_m \times x}. \quad (1)$$

For H_2S mitochondrial oxygen consumption inhibition, we converted the data to percentage inhibition of mitochondrial respiration and used a four-parameter log-logistic function to estimate the concentration of H_2S causing 50% of maximal oxygen consumption inhibition (50% effective concentration, EC_{50}) according to:

$$y = a + \frac{d - a}{[1 + (x/c)^b]}, \quad (2)$$

where a is the theoretical response at zero concentration, b is the Hill's slope, c is the inflection point and d is the theoretical response at infinite concentration.

Complex IV maximal capacity and inhibition by H_2S

After evaluating the complex IV maximal capacity (see Series I), we injected sulphide step by step to inhibit this complex (0 to 498 µmol l^{-1}). Again, we evaluated the chemical background owing to oxygen consumption in the presence of H_2S in parallel without homogenates and subtracted this from the respective values in the presence of homogenates. The data were normalized by total protein content measured with the bicinchoninic acid method (Smith et al., 1985) for complex IV maximal capacity and converted to percentage inhibition of complex IV. We used a four-parameter log-logistic function to estimate the concentration of sulphide causing 50% of maximal complex IV inhibition (EC_{50}) according to Eqn 2.

Statistical analysis

Statistical data analyses were performed in R version 3.6.2 (https://www.r-project.org/). For mitochondrial respiration rates, mitochondrial ratios, complex IV, and CS and LDH activities, Student's *t*-tests were performed after verifying data normality (Shapiro–Wilk) and homogeneity of variances (*F* test), and data were transformed when required. For apparent H_2S mitochondrial oxidation, kinetic calculations of V_{max} and K_m were performed using the 'drc' package (Ritz et al., 2015) and comparison of kinetic parameters between Belize and Honduras lineages was performed using the means of differences ('compParm' function). For mitochondrial respiration and complex IV inhibition by H_2S, EC_{50} values for Belize and Honduras lineages were calculated with the 'drc' package and compared using the 'EDcomp' function (Ritz et al., 2015). Statistical significance was set at $P<0.05$.

RESULTS

Series I: determination of metabolic capacity in the two isogenic lineages

Mitochondrial respiration rates and mitochondrial ratios

Fish from the Belize lineage displayed statistically lower CI-LEAK and CI-OXPHOS respiration rates compared with fish from the Honduras lineage ($P<0.001$ and $P=0.009$, respectively; Fig. 1A). Moreover, the P_I/L_I ratio, an indicator of mitochondrial coupling at the level of complex I, was also lower in Belize compared with Honduras fish ($P=0.010$; Fig. 1B). For all liver permeabilizations, addition of cytochrome *c* did not increase respiration rates, attesting to the intactness of the outer mitochondrial membrane for both lineages (Fig. 1B). When measuring CI+CII-OXPHOS with addition of succinate as an oxidative substrate, fish from both lineages showed marked increases in mitochondrial oxygen consumption (135.5 and 93.7% increase for Belize and Honduras, respectively; Fig. 1A). However, no significant differences were detected between lineages for CI+CII-OXPHOS or for CII-OXPHOS (when rotenone was added to inhibit complex I). Interestingly, the succinate contribution to mitochondrial respiration was significantly higher for Belize than for Honduras fish ($P<0.001$; Fig. 1B). Altogether, these results suggest fundamental differences in mitochondrial physiology between lineages. Although the ratios calculated cannot be interpreted as definitive evidence for substrate oxidation efficiency at different steps of the ETS in the two lineages, they suggest that the Belize lineage relies more on succinate as an oxidative substrate for complex II than the Honduras lineage, and that the Honduras lineage has increased complex I oxidative capacity compared with the Belize lineage (Fig. 1).

Enzymatic activities and complex IV maximal capacity

To evaluate the general aerobic and anaerobic capacities of the Belize and Honduran lineages, we measured CS activity and complex IV maximal capacity (aerobic metabolism), as well as LDH activity (anaerobic metabolism). Interestingly, Belize fish had significantly lower LDH activity ($P<0.001$; Fig. 2A) than Honduras fish. Enzymatic activities of CS and complex IV maximal capacity were both significantly higher in Belize fish ($P=0.013$ and $P=0.004$, respectively; Fig. 2B,C). These results also demonstrate distinct lineage differences and indicate that Belize fish have an increased aerobic capacity and a decreased anaerobic capacity compared with fish from the Honduran lineage.

Series II: H_2S mitochondrial kinetics

Apparent H_2S mitochondrial oxidation and mitochondrial oxygen consumption inhibition

We used increasing concentrations of sulphide to stimulate entry of electrons into the ETS via sulphide oxidase. From 0 to approximately 100 µmol l^{-1} sulphide, we observed that the oxygen consumption was steadily increasing for both lineages. For both lineages, the apparent H_2S oxidation followed Michaelis–Menten kinetics, as denoted by good curve fits ($R^2=0.978$ and $R^2=0.971$ for Belize and Honduras, respectively; Fig. 3A). Interestingly, while K_m was not significantly different between lineages, V_{max} was significantly higher for Honduras fish ($P<0.001$; Fig. 3A). These data suggest that the Belize and Honduran lineages oxidized H_2S at different rates, but that both lineages have a sulphide oxidase with similar affinity for H_2S. Moreover, when higher sulphide concentrations were used to inhibit mitochondrial respiration, both lineages exhibited similar inhibition patterns. However, slightly but not significantly higher EC_{50} values were observed in Belize fish ($P=0.064$; Fig. 3B). These data suggest that mitochondrial respiration in our two lineages exhibits modest differences in sensitivity to H_2S.

Complex IV inhibition by H_2S

As complex IV is the mitochondrial complex sensitive to H_2S, we then measured the inhibition of complex IV maximal capacity by H_2S. For both lineages, complex IV maximal capacity was similarly inhibited by H_2S (Fig. 4). Specifically, both lineages showed maximal inhibition at approximately 300 µmol l^{-1} sulphide with

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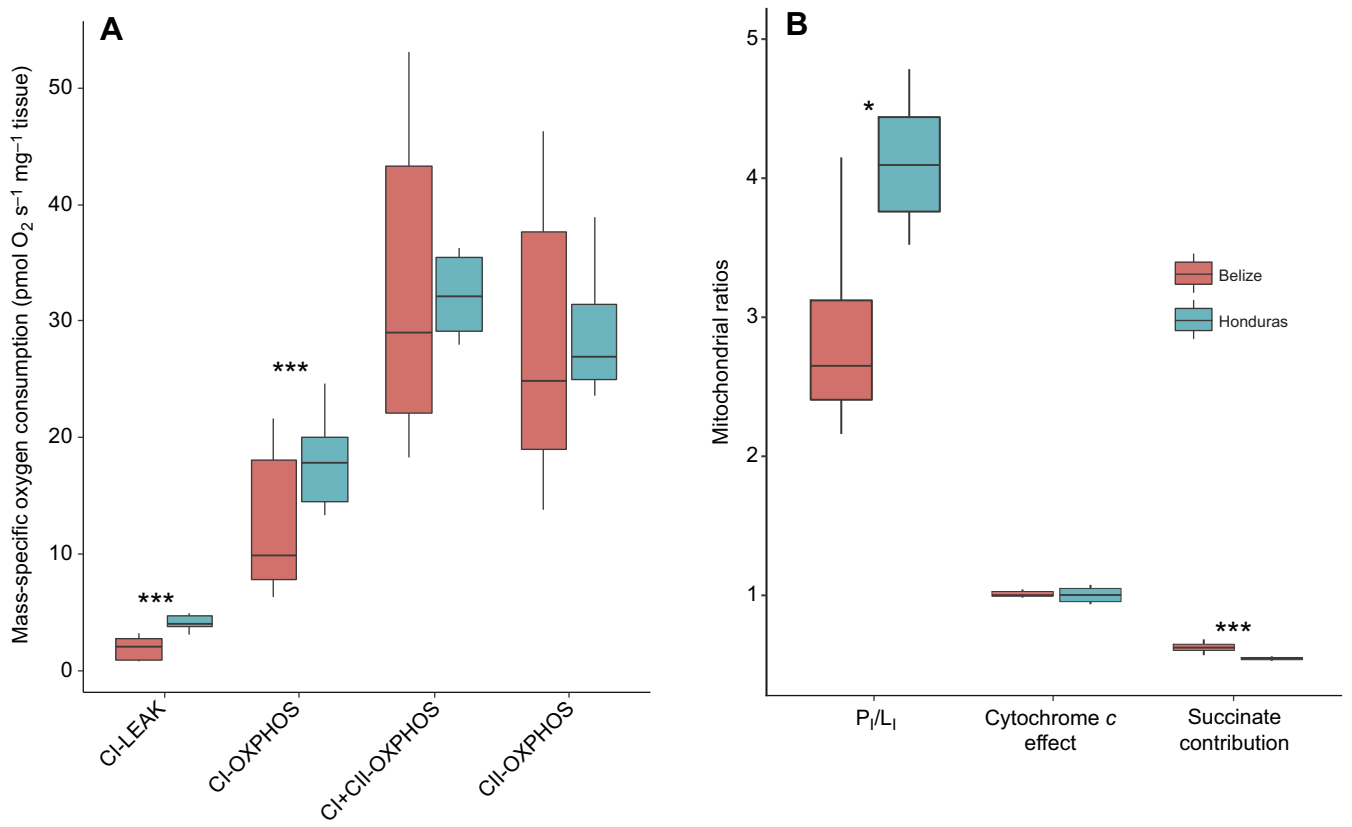

Fig. 1. Mass-specific oxygen consumption rates and mitochondrial ratios measured in permeabilized livers of mangrove rivulus fish, *Kryptolebias marmoratus*, from the Belize and Honduras lineages. (A) Mitochondrial oxygen consumption rates were measured in the presence of pyruvate+malate (CI-LEAK), +ADP (CI-OXPHOS), +succinate (CI+CII-OXPHOS) and +rotenone (CII-OXPHOS). (B) Calculated P_I/L_I ratio (CI-OXPHOS/CI-LEAK). The cytochrome *c* effect was calculated as CIc-OXPHOS/CI-OXPHOS, where CIc-OXPHOS represents the oxygen consumption rate measured after injection of cytochrome *c*, and succinate contribution=[(CI+CII-OXPHOS–CIc-OXPHOS)/CI+CII-OXPHOS]. Box plot values consist of the median (center line), the inter-quartile range (IQR, upper and lower edges of box), and the whiskers corresponding to maximum and minimum values no further than 1.5×IQR (Tukey style) for each lineage (*N*=8 for Belize and *N*=6 for Honduras), with asterisks denoting statistical differences (**P*<0.05, ****P*<0.001).

similar calculated EC_{50} (131.21±39.06 and 134.21±47.69 µmol l^{-1}, for Belize and Honduras, respectively; Fig. 4). Thus, despite having higher complex IV maximal capacity, complex IV of Belize lineage had the same sensitivity to H_2S as the Honduran lineage.

DISCUSSION

In this study, we present the first baseline mitochondrial metabolic function in two isogenic lineages of *K. marmoratus*, with different H_2S sensitivity. We tested the hypothesis that mitochondrial

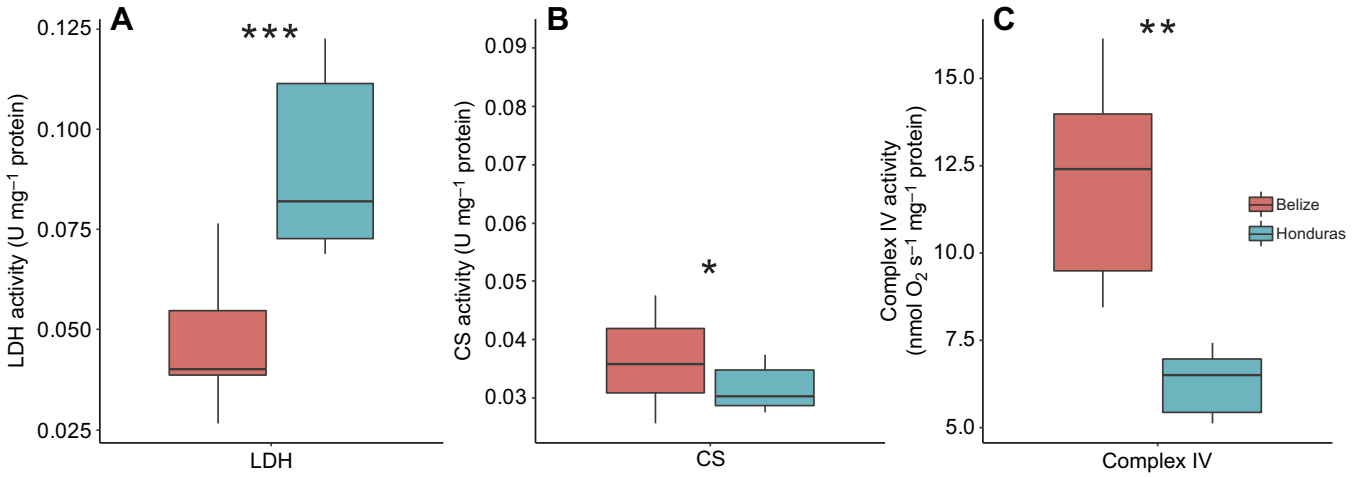

Fig. 2. Enzymatic activities measured in livers of mangrove rivulus fish, *Kryptolebias marmoratus*, from the Belize and Honduras lineages. (A) Lactate dehydrogenase (LDH), (B) citrate synthase (CS) and (C) complex IV maximal capacity. Box plot values consist of the median (center line), the IQR (upper and lower edges of box), and the whiskers corresponding to maximum and minimum values no further than 1.5×IQR (Tukey style) for each lineage (*N*=8 for Belize and *N*=6 for Honduras), with asterisks denoting statistical differences (**P*<0.05, ***P*<0.01, ****P*<0.001).

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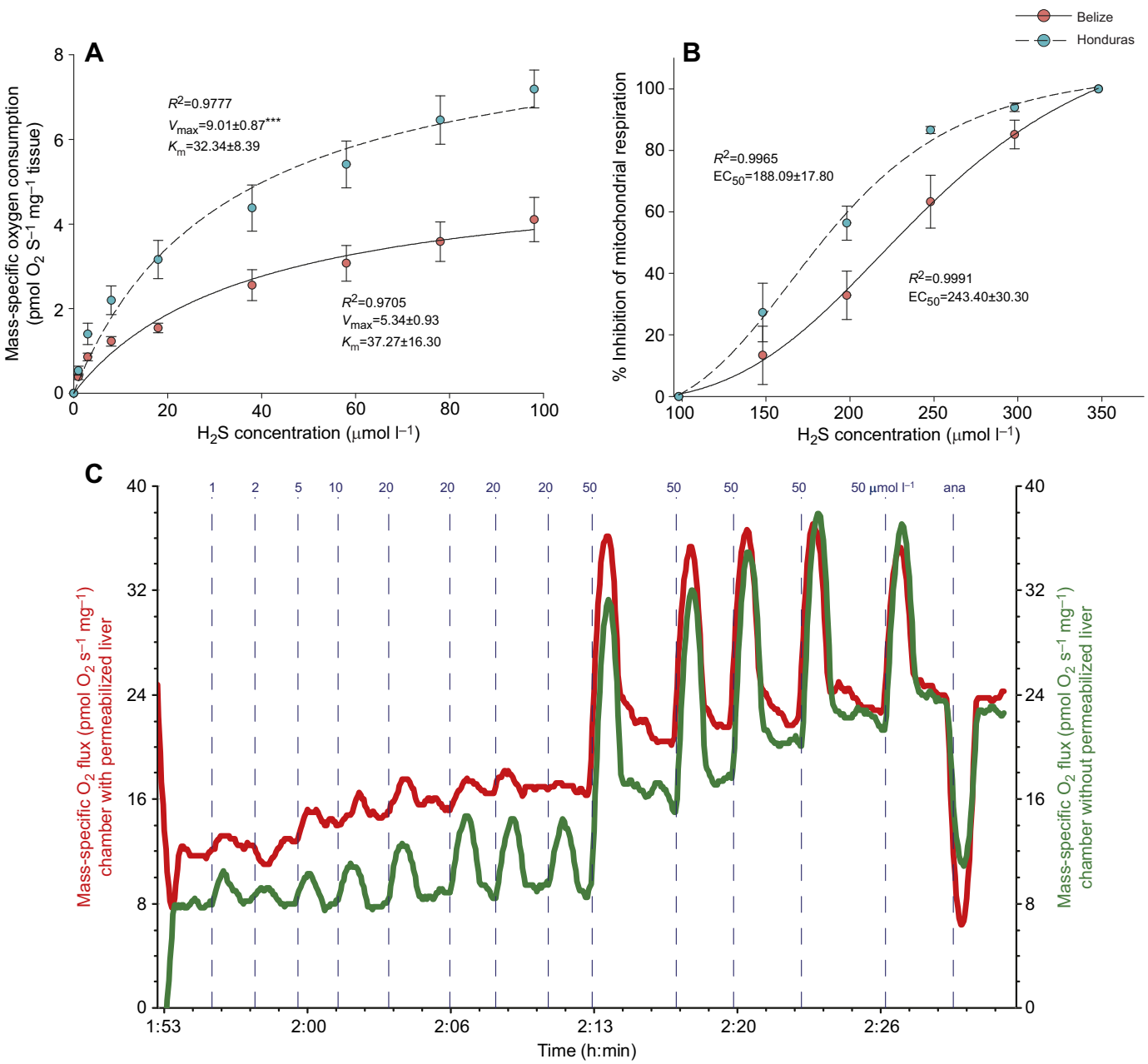

Fig. 3. Kinetics of apparent H_2S oxidation and inhibition of mitochondrial respiration at high H_2S measured in permeabilized livers of mangrove rivulus fish, *Kryptolebias marmoratus*, from the Belize and Honduras lineages. (A) Mitochondrial oxygen consumption was measured following experiments described in Fig. 1A, i.e. in the presence of pyruvate, malate, ADP, cytochrome *c* and succinate; after inhibition of complexes I and II by rotenone and malonate, respectively; and following successive injections of sulphide (0 to 98 µmol l^{-1}; *N*=8 for Belize and *N*=6 for Honduras). A Michaelis–Menten curve was fitted to estimate the maximum rate of apparent H_2S oxidation (V_{max}) and the affinity of sulphide oxidase for sulphide (K_m) for each mangrove rivulus lineage. (B) Mitochondrial oxygen consumption was measured following successive injections of sulphide (98 to 348 µmol l^{-1}) until complete inhibition was achieved (*N*=8 for Belize and *N*=6 for Honduras). The percentage of oxygen consumption inhibition was plotted against the H_2S concentration, and a four-parameter logistic curve was fitted to estimate the EC_{50} (half-maximal effective concentration) for both lineages. (C) Representative traces of apparent H_2S oxidation and mitochondrial respiration inhibition at high H_2S concentration. Each injection of sulphide (or antimycin A: ana, last injection) in the chambers is represented by a dashed blue line, and the green line representing the chemical background due to oxygen consumption in the presence of H_2S without sample was subtracted from the respective values in the presence of tissue (red line). H_2S concentrations on the *x*-axis refer to Na_2S concentrations used, and potential H_2S oxidation over time has been considered by measuring oxygen consumption in the presence of H_2S without tissue. Results are means±s.e.m. R^2 represents the coefficient of determination for the fitting curve. Asterisks denote significant differences between V_{max}, with ***P<0.001.

mechanisms underpin H_2S behavioural and physiological responses. To this end, we evaluated the apparent oxidation and inhibitory effects of H_2S on mitochondrial metabolism. Our results show that there are functional differences in mitochondrial physiology between lineages; the Honduran lineage has increased mitochondrial capacities at the level of complex I and higher anaerobic capacities compared with the Belize lineage, as demonstrated by LDH activity. However, the Belize lineage has a higher capacity to oxidize succinate, although mitochondrial respiratory rates are more variable than in the Honduran lineage.

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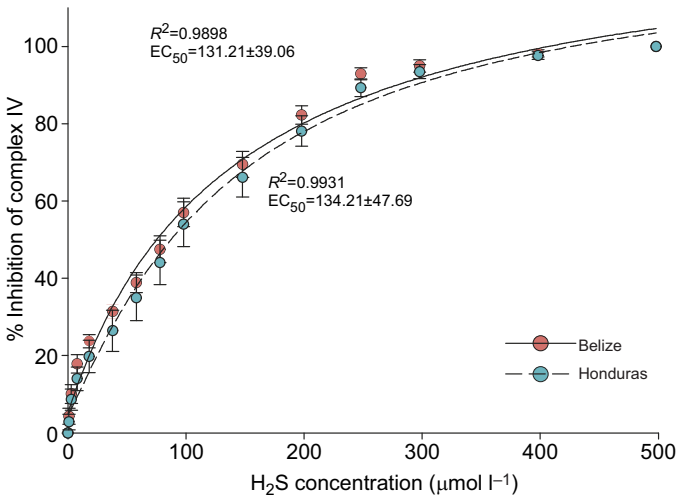

Fig. 4. Inhibition of complex IV maximal capacity by H_2S measured in livers of mangrove rivulus fish, *Kryptolebias marmoratus*, from the Belize and Honduras lineages. Oxygen consumption was measured with glucose, ascorbic acid and cytochrome *c* and following successive injections of sulphide (0 to 498 µmol l^{-1}) until complete inhibition was achieved (*N*=8 for Belize and *N*=6 for Honduras). The percentage of oxygen consumption inhibition was plotted against the H_2S concentration, and a four-parameter logistic curve was fitted to estimate the EC_{50} (half-maximal effective concentration) for both lineages. H_2S concentrations on the *x*-axis refer to Na_2S concentrations used, and potential H_2S oxidation over time has been considered by measuring oxygen consumption in the presence of H_2S without homogenate. Results are means±s.e.m. R^2 represents the coefficient of determination for the fitting curve.

The Belize lineage also shows higher activities of CS and COX. Furthermore, we present evidence of differences in H_2S mitochondrial kinetics in our two isogenic lineages. Apparent H_2S oxidation is higher in the Honduran lineage compared with the Belize lineage. Surprisingly, inhibitory effects of H_2S on mitochondrial oxygen consumption and COX activity were similar between lineages, although mitochondrial respiration is inhibited at slightly higher H_2S concentrations in the Belize lineage. These findings suggest that these two distinct genetic lineages have different strategies to cope with increasing H_2S concentrations in their environment, supporting our hypothesis and highlighting the importance of mitochondria in colonizing extreme environments.

Many distinctive differences in life history strategies, behaviour, metabolism and physiology have previously been found among isogenic lineages of mangrove rivulus (Grageda et al., 2005; Turko et al., 2018; Martin and Currie, 2020; Rossi and Wright, 2020). Notably, it has been shown that Honduran fish survive out of water for longer than Belize fish (Turko et al., 2018), which suggests differences in aerobic/anaerobic capacity between these lineages. Moreover, Honduras fish decrease their metabolic rate by 58% when emerged while Belize fish only reduce it by 31%, indicating that emersion tolerance is negatively associated with metabolic rate (Turko et al., 2019). Consistent with these previous studies, we show that Honduran fish have higher LDH activity than Belize fish, indicating a higher anaerobic capacity in the Honduran lineage (Fig. 2A). Similarly, we also observed increased activities of CS and complex IV in Belize fish, suggesting a higher aerobic capacity in this lineage (Fig. 2B,C). Collectively, these observed differences in aerobic/anaerobic metabolism between lineages may underpin the distinct emersion tolerance in isogenic lineages of mangrove rivulus.

For both lineages, permeabilized liver preparations were of good quality, markedly increasing oxygen consumption with the addition of ADP, but not changing with the addition of cytochrome *c* (Fig. 1B). Surprisingly, CI-LEAK and CI-OXPHOS were both significantly higher in the Honduran lineages, which translated to a higher calculated P_I/L_I ratio (Fig. 1). The higher CI-OXPHOS and P_I/L_I ratio both suggest increased oxidation capacity of complex I. In contrast, when succinate was added as substrate to measure CI+CII-OXPHOS (i.e. with complexes I and II substrates together), no differences between lineages were observed (Fig. 1A). However, owing to lower CI-OXPHOS in the Belize lineage, this suggests that Belize fish have a higher capacity to oxidize succinate, which is substantiated by the succinate contribution ratio (Fig. 1B). Although these ratios calculated cannot be interpreted as definitive evidence for substrate oxidation efficiency at different steps of the ETS in the two lineages, they suggest that the Belize lineage might rely more on succinate as an oxidative substrate for complex II than the Honduras lineage, which might have a greater complex I oxidative capacity than the Belize lineage (Fig. 1B). Succinate is a substrate that can accumulate in some fish upon exposure to hypoxia as an anaerobic end-product (Hochachka, 1980; Johnston et al., 1975; Johnston, 1975). Accumulation of succinate has been shown to occur in liver of North Sea eelpout (*Zoarces viviparus*) during heat stress as well as in marine invertebrates exposed to low oxygen during burrowing and has been suggested to be an indicator of mitochondrial anaerobiosis (Pörtner et al., 2004; Strahl et al., 2011; Van Dijk et al., 1999). In air-breathing fish such as the catfish (*Heteropneustes fossilis*), a consistent increase in complex II activity was observed in muscle tissue following air exposure for up to 18 h (Paital, 2014). It is now accepted that succinate accumulated during hypoxia can be rapidly oxidized by the mitochondria upon re-oxygenation in mammals and some aquatic vertebrates, a central process involved in ischemic/reperfusion injuries (Bundgaard et al., 2018, 2020; Chouchani et al., 2014; Cox and Gillis, 2020). Thus, our results in Belize fish might reflect increased capacity to re-oxidize succinate, accumulated during H_2S avoidance, upon return to a well-oxygenated environment. However, the calculated succinate contribution might not reflect real rates of succinate oxidation by complex II alone, as CII-OXPHOS is not different between lineages (Fig. 1A). Estimation of oxygen consumption with succinate and rotenone alone is thus needed for a proper evaluation of complex II contribution in both lineages and its implication in H_2S avoidance.

In addition to the differences in terms of respiration rates between lineages, Belize fish also displayed much more variable respiration rates than those from Honduras despite increased sampling size (*N*=8 and *N*=6 for Belize and Honduras lineages, respectively). This is mainly due to increased variation in body sizes in Belize fish. Interestingly, it has been recently shown that among-individual variation in mitochondrial functions could explain marked variation in growth performance independently of food intake (Salin et al., 2019). Thus, the increased variation in mitochondrial respiration rates observed here might be due to an increased growth performance in some individuals of the Belize lineage. It would be interesting to investigate whether (i) increased mitochondrial respiration in Belize fish is associated with higher growth rates, and (ii) both these parameters are linked to differences in H_2S avoidance.

Increased anaerobic capacity is a common strategy for surviving elevated H_2S levels and is a key feature of H_2S-tolerant organisms living in sediments that experience periodic H_2S exposure (Hand and Somero, 1983; Oeschger and Storey, 1990; Vismann, 1991). Moreover, it has been suggested that anaerobic capacity plays a key role in H_2S tolerance in fishes as a short-term coping mechanism prior to behavioural avoidance (Bagarinao and Vetter, 1989). Thus,

higher anaerobic capacity in the Honduran lineage might indicate increased sensitivity to H_2S, as demonstrated in a previous study (Martin and Currie, 2020). This suggestion is also in line with the lower COX activity detected in Honduran fish (Fig. 2C), as this complex is the primary inhibitory target of H_2S. It has been suggested that fishes with higher H_2S tolerance typically have higher sulphide oxidation abilities (Bagarinao and Vetter, 1989, 1990). In contrast, we show that despite a decreased COX activity and higher H_2S sensitivity (Martin and Currie, 2020), Honduran fish have a higher apparent mitochondrial capacity to oxidize H_2S than Belize fish (V_{max}=9.01±0.87 and V_{max}=5.34±0.93, respectively; Fig. 3A) combined with higher LDH activity. By way of comparison, amphibious mudskippers (*Boleophthalmus boddaerti*) demonstrate reliance on both aerobic and anaerobic mechanisms of H_2S detoxification, showing increased lactate levels and oxidation rates in the presence of H_2S (Ip et al., 2004). In the Mexican molly, *Poecilia mexicana*, individuals from geographically separated sub-populations respond differently to H_2S at the mitochondrial level, with certain populations demonstrating modified COX activity whereas other populations lack resistant COX and rely on other tolerance mechanisms (Greenway et al., 2020). Hence, a possible explanation for our results is that the Honduran and Belize lineages do not only rely on sulphide oxidation capacity to tolerate high H_2S, but have divergent physiological strategies (e.g. increased COX activity) to cope with high H_2S levels in their environment. Given that these lineages have been bred in the laboratory for many generations without environmental exposure to H_2S, we cannot rule out that the gene expression of the H_2S detoxifying enzyme, SQR, or other ETS components, may have altered over time and distinctly between the two lineages.

Despite possible genetic differences in coping with high H_2S, we demonstrate that mangrove rivulus from both the Honduran and Belize lineages have the capacity to detoxify H_2S in liver mitochondria. Both lineages are able to oxidize H_2S at concentrations up to 100 µmol l^{-1} before mitochondrial respiration starts to be inhibited (Fig. 3A and B). In contrast, the H_2S-tolerant California killifish (*Fundulus parvipinnis*) begins to decrease mitochondrial oxidation at approximately 20 µmol l^{-1} sulphide; in the less-tolerant speckled sanddab (*Citharichthys stigmaeus*), oxidation is inhibited at concentrations <10 µmol l^{-1} (Bagarinao and Vetter, 1990). Compared with other native mangrove fish such as molly (*Poecilia orri*) and gambusia (*Gambusia* sp.), mangrove rivulus are considerably more tolerant to elevated H_2S (Rossi et al., 2019). However, in the lugworm, *Arenicola marina*, sulphide oxidation is not inhibited by the presence of H_2S and continues to function up to ∼350 µmol l^{-1} (Völkel and Grieshaber, 1996), concentrations at which we observed total inhibition of mitochondrial respiration in both lineages (Fig. 3B). It is important to note that Martin and Currie (2020) reported that the Honduran and Belize lineages emerge from water at concentrations of ∼200 and ∼400 µmol l^{-1} H_2S, respectively. We found here that at a concentration of 200 µmol l^{-1} sulphide, mitochondrial respiration was decreased by approximately 50% and 30% in Honduran and Belize fish, respectively (Fig. 3B), suggesting that emersion concentration is related to apparent mitochondrial H_2S oxidation capacity in these fish. However, at ∼350 µmol l^{-1} sulphide, both lineages displayed complete inhibition of mitochondrial respiration (Fig. 3B). Hence, lineage differences in H_2S sensitivity observed by Martin and Currie (2020) cannot be fully explained by the apparent mitochondrial H_2S oxidation capacities in the liver noted here. Other, yet-to-be-determined mechanisms of sulphide detoxification are likely to play a role in lineage differences in H_2S sensitivity.

Surprisingly, the higher COX activity in Belize fish is not related to increased resistance of this complex to H_2S inhibition. Indeed, both lineages had very similar COX inhibition curves, with almost identical EC_{50} values for H_2S (131.21±39.06 and 134.21±47.69 µmol l^{-1} sulphide for Belize and Honduras, respectively; Fig. 4). To survive in sulphidic environments, different lineages of *P. mexicana* often modify the same physiological pathways in different ways (Tobler et al., 2018). Moreover in mangrove fishes, tolerance might be the result of selection of standing genetic variation and *de novo* mutations (Greenway et al., 2020; Pfenninger et al., 2014, 2015). COX is a mitochondrial complex encoded by both nuclear and mitochondrial DNA (mtDNA). mtDNA variations influence metabolic adjustments to environmental parameters such as temperature and diet (Baris et al., 2017; Healy et al., 2017; Pichaud et al., 2012, 2013). Thus, selection for specific mitochondrial genotypes and/or phenotypes might also have arisen from H_2S exposure in sulphidic environments. Indeed, in *P. mexicana*, it has been shown that two different lineages have shared amino acid substitutions in *cox1* mtDNA genes, which likely cause conformational changes in the COX1 subunit blocking the access to H_2S (Pfenninger et al., 2014). In our case, it is possible that mutations at the catalytic core of COX constituted by the three mtDNA encoded subunits (Kadenbach and Hüttemann, 2015) changed its activity in either the Belize or Honduran lineage, whereas the binding site of H_2S in COX1 (Cooper and Brown, 2008) remains unaffected. Future studies examining mtDNA divergences in COX subunits in different lineages of mangrove rivulus exposed to different H_2S concentrations could shed light on the role of potential mtDNA mutations in adaptations of this species to sulphidic environments.

In summary, we show that two distinct isogenic lineages of mangrove rivulus have differently adapted to sulphidic environments. These adaptations likely indicate divergences in mitochondrial function and metabolism that have allowed the lineages to develop different strategies to tolerate high H_2S levels in their environments. H_2S sensitivity and emersion time seem to be related to the aerobic/anaerobic capacities as well as to the sensitivity of COX to inhibition by H_2S of both lineages. Specifically, fish from the Honduran lineage have a higher anaerobic capacity, which allows them to emerge at lower H_2S concentrations (Martin and Currie, 2020) and thus be in terrestrial environments for an extended time (Turko et al., 2019). Honduran fish are also able to oxidize H_2S at higher rates, which allows rapid H_2S detoxification by their mitochondria. In contrast, fish from the Belize lineage can be exposed to higher H_2S concentrations in their environment because of increased COX activity and mitochondrial respiration, as their aerobic capacity is slightly more resistant to inhibition by H_2S. Moreover, the calculated increased capacity to oxidize succinate in Belize fish suggests that this metabolite might accumulate during anaerobic metabolism and is oxidized after oxygen concentration returns to normal in their aquatic environment. However, this has to be tested by specifically measuring succinate levels and oxygen consumption rates by complex II individually. Our study demonstrates the importance of mitochondrial H_2S oxidation in fish exposed to high H_2S concentrations and highlights possible genetic and/or functional mitochondrial adaptations to sulphidic environments. Mitochondrial functions might have been crucial for fish to adapt to these extreme environments and to develop avoidance mechanisms, allowing them to survive harsh, sulphidic conditions.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: K.M., S.C., N.P.; Methodology: K.M., N.P.; Validation: S.C., N.P.; Formal analysis: N.P.; Writing - original draft: K.M., S.C., N.P.; Writing - review & editing: K.M., S.C., N.P.; Funding acquisition: N.P., S.C.

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Data availability
All data collected are available from Mendeley at http://dx.doi.org/10.17632/x2c39mzr4t.1.

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