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RESEARCH ARTICLE

Mild hypoxia exposure impacts peripheral serotonin uptake and degradation in Gulf toadfish (*Opsanus beta*)

John Sebastiani*, Allyson Sabatelli and M. Danielle McDonald

ABSTRACT

Plasma serotonin (5-hydroxytryptamine, 5-HT) homeostasis is maintained through the combined processes of uptake (via the 5-HT transporter SERT, and others), degradation (via monoamine oxidase, MAO) and excretion. Previous studies have shown that inhibiting SERT, which would inhibit 5-HT uptake and degradation, attenuates parts of the cardiovascular hypoxia reflex in gulf toadfish (*Opsanus beta*), suggesting that these 5-HT clearance processes may be important during hypoxia exposure. Therefore, the goal of this experiment was to determine the effects of mild hypoxia on 5-HT uptake and degradation in the peripheral tissues of toadfish. We hypothesized that 5-HT uptake and degradation would be upregulated during hypoxia, resulting in lower plasma 5-HT, with uptake occurring in the gill, heart, liver and kidney. Fish were exposed to normoxia (97.6% O_2 saturation, 155.6 Torr) or 2 min, 40 min or 24 h mild hypoxia (50% O_2 saturation, ∼80 Torr), then injected with radiolabeled [^{3}H]5-HT before blood, urine, bile and tissues were sampled. Plasma 5-HT levels were reduced by 40% after 40 min of hypoxia exposure and persisted through 24 h. 5-HT uptake by the gill was upregulated following 2 min of hypoxia exposure, and degradation in the gill was upregulated at 40 min and 24 h. Interestingly, there was no change in 5-HT uptake by the heart and degradation in the heart decreased by 58% within 2 min of hypoxia exposure and by 85% at 24 h. These results suggest that 5-HT clearance is upregulated during hypoxia and is likely driven, in part, by mechanisms within the gill and not the heart.

KEY WORDS: 5-hydroxytryptamine, MAO, Monoamine oxidase, SERT, Serotonin transporter, Hypoxia

INTRODUCTION

Serotonin (5-hydroxytryptamine, 5-HT) is a signaling molecule used in dozens of pathways within the vertebrate body. Arguably best known for its role in the central nervous system, 5-HT was first described based on its role as a vasoconstrictor circulating in the plasma of mammals (Rapport et al., 1948). Circulating 5-HT is found at concentrations of 10^{-8} to10^{-9} mmol l^{-1} in the plasma, a range that appears to be consistent across vertebrate clades (Maurer-Spurej, 2005), and is a reflection of the balance between 5-HT production and clearance from the blood. Clearance occurs when 5-HT removed from the blood and moved into cells to be stored, degraded into metabolite (waste) or removed from the body (Sebastiani and McDonald, 2021). The vasoactive properties of 5-HT in the plasma make clearance an important factor to consider in cardiovascular and respiratory physiology, particularly within teleost fish during exposure to low oxygen (hypoxia). The typical teleost hypoxia response involves a suite of physiological reflexes, many of which directly or indirectly involve 5-HT (McDonald et al., 2010; Lillesaar, 2011; Shakarchi et al., 2013; Jonz et al., 2016; Amador et al., 2018). In response to hypoxia exposure, Gulf toadfish (*Opsanus beta*) exhibit decreased caudal arterial pressure, bradycardia, increased ventilation amplitude and decreased ventilation frequency (Panlilio et al., 2016), which collectively are believed to result in a greater gill lamellar perfusion and increased gas exchange. However, injection of exogenous 5-HT into rainbow trout (*Oncorhynchus mykiss*) results in the opposite: tachycardia and localized increases to vascular resistance within the branchial circuit, which impairs gas exchange (Sundin et al., 1995; Sundin and Nilsson, 2000). Furthermore, there is also evidence that circulating 5-HT exerts tonic vasoconstriction on blood vessels under normal conditions, and that improper regulation of circulating 5-HT may lead to hypertension and death in humans (Brenner et al., 2007). Combined, this suggests that homeostasis of circulating 5-HT is critical for maintaining physiologically optimal vascular resistance and blood flow at rest, and that removal of circulating 5-HT may help teleosts persist during hypoxic conditions.

Uptake into cells primarily occurs through the 5-HT transporter SERT, although 5-HT can also move through other promiscuous transporters such as the norepinephrine and dopamine transporters (Ni and Watts, 2006; Gershon and Tack, 2007; Daws, 2009). In gulf toadfish, the heart has the highest SERT mRNA expression and performs the highest 5-HT uptake of all measured organs (Amador and McDonald, 2018a). SERT mRNA expression has also been measured in gill, liver and, to a lesser extent, kidney, with high mRNA expression in the gill translating to significant transport-mediated 5-HT uptake (Amador and McDonald, 2018b). It is not known whether tissue 5-HT uptake changes when fish are exposed to hypoxia. Degradation of 5-HT is mediated by the rate-limiting enzyme monoamine oxidase (MAO) (Bortolato et al., 2010), which is bound to the mitochondria. Thus, degradation occurs intracellularly and is dependent on uptake. Degradation of 5-HT is important for the regulation of circulating 5-HT in teleost fish under normoxic conditions. For example, treating gulf toadfish with the MAO inhibitor clorgyline results in a significant increase in plasma 5-HT concentrations (Sebastiani and McDonald, 2021). Illustrating the potential role of the gill in 5-HT degradation, Olson (1998) reported that, after perfusing rainbow trout gills with [^{3}H]5-HT, two-thirds of remaining radiolabel in the effluent was bound to metabolite (Olson, 1998). It is not known whether degradation of 5-HT is upregulated during hypoxia.

Finally, there are two main excretion pathways for 5-HT: hepatic (via bile) and renal (via urine) (Amador and McDonald, 2018b). 5-HT concentrations are higher in bile than in urine and both fluids have 3.3- to 6.2-fold higher 5-HT concentration than the plasma

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(Amador and McDonald, 2022). Furthermore, high concentrations of 5-HIAA in rainbow trout urine (200-fold higher than plasma 5-HIAA) suggest that metabolite may dominate over unmetabolized 5-HT in the urine (Some and Helander, 2002). Combined, these data suggest that 5-HT and its metabolite are actively excreted into these fluids and that excretion is an important mechanism to rid the body of excess 5-HT and 5-HIAA under normoxic conditions. However, the extent to which excretory processes contribute to 5-HT homeostasis during hypoxia is not known.

Previous attempts to investigate how uptake, degradation and excretion contribute to 5-HT homeostasis during hypoxia were complicated by a likely metabolic downregulation that occurred in toadfish exposed to severe hypoxia (20 Torr) (Amador and McDonald, 2022). This is because an exposure to 20 Torr was likely below their critical oxygen tension (P_{crit}) estimated to be <40 Torr (Amador et al., 2018; Ultsch et al., 1981). Below P_{crit}, any aerobic process such as 5-HT transport via SERT and degradation via MAO are also likely to be downregulated. Therefore, the goal of this study was to measure uptake, degradation and excretion of 5-HT following exposure to mild hypoxia (~79.5 Torr, above toadfish P_{crit}). We hypothesized that, in response to mild hypoxia exposure, 5-HT uptake and degradation would be upregulated in the heart, gill, liver and kidney, and biliary and urinary excretion of 5-HT would also be upregulated. Combined, the upregulation of 5-HT clearance in response to hypoxia would lead to a decrease in circulating 5-HT concentrations, which may help relieve tonic peripheral vasoconstriction and allow for greater blood perfusion of critical organs.

MATERIALS AND METHODS

Experimental animals

Gulf toadfish [*Opsanus beta* (Goode and Bean 1880)] were caught as roller trawl bycatch by local shrimpers in Biscayne Bay, FL, USA. Upon arrival at the lab, toadfish were exposed to freshwater for 15 min and returned to seawater. For three consecutive days upon arrival, fish were treated with a final concentration 0.1 mg l^{-1} malachite green in 30 mg l^{-1} formalin to treat and prevent infection by ectoparasites. Two and a half weeks later, fish were treated with the same regimen (day one freshwater and malachite, days two and three malachite only). Afterwards, fish were treated every 2.5 weeks with 1 day of freshwater and malachite. Fish were housed in 20 gallon (91 liter) aquaria supplied with aerated, flow-through, filtered seawater taken from Biscayne Bay and were fed raw (previously frozen) shrimp weekly until satiation. Previous work done with toadfish suggests that 5-HT clearance is sensitive to temperature (Sebastiani and McDonald, 2021), therefore sampling only occurred when ambient flow-through temperatures fell to 20–23°C. Series i experiments were conducted between 9 March and 27 April 2021 with an average temperature of 19.9±0.6°C. Series ii experiments were carried out between 28 October and 24 November 2020, with an average temperature of 21.6±1.0°C. Series iii experiments were conducted between 21 and 28 September 2020, with an average temperature of 22.7±0.3°C. All protocols were carried out with the approval of the University of Miami Institutional Animal Care and Use Committee (IACUC).

Experimental procedures

Hypoxia exposure

Two flow-through header tanks were set up using concrete blocks and wire frame shelving. Each header tank received aerated water from a large seawater reservoir that allowed the water to be kept at room temperature. A bulkhead valve was installed on the bottom of each tank, allowing water to flow from the tank into a gang valve which split the flow from each header tank into four individual 1.5 liter plastic chambers. Each individual chamber received water at the same rate and had its own air stone bubbling air. One header tank was fitted with an air stone and bubbled with air (control). The second tank (hypoxia) was fitted with an air stone hooked to a N_2 cylinder. Nitrogen flow was controlled by a solenoid valve (Burkert Fluid Control Systems, Ingelfingen, Germany) hooked up to a custom-built automatic relay system. An O_2 probe (Vernier, Beaverton, OR, USA) was placed in the hypoxia header tank to continuously read O_2 levels in real time. The O_2 probe was plugged into a LabQuest Stream interface (Vernier), which was connected to a personal computer running LoggerLite software (Vernier). LoggerLite was configured so that when O_2 saturation dropped below a certain threshold, a signal was sent out through the interface to a digital control unit (Vernier), which would trip a relay. The relay would cause the solenoid valve on the N_2 tank to open or close, adjusting the flow of N_2. With the valve open, N_2 would flow into the header tank, decreasing the P_{O_2} of the water and drive the O_2 saturation down. Once the desired P_{O_2} was reached, the software caused the relay to immediately turn off the solenoid valve. Once the valve shut off, P_{O_2} would continue to decline by several Torr (usually 3–6 Torr), eventually plateauing as new flow-through water entered the header. P_{O_2} levels in the tank oscillated at the programmed level This system ensured O_2 levels were maintained over 24 h (Fig. 1A).

Series i: The metabolic effects of mild hypoxia

Adult toadfish (0.048±0.005 kg; 0.041–0.056 kg; n=15) were weighed and placed in a 1 liter closed system respirometry chamber (Loligo Systems) sitting inside one of two shielded, 20 gallon, flow-through aquaria as previously described (Amador et al., 2018). The control normoxia tank was continuously bubbled with air (average 97.6% O_2 saturation, 155.6 Torr), whereas the hypoxia exposure tank was continuously bubbled with air and intermittently bubbled with N_2 to maintain a target O_2 content of 50% O_2 saturation (~79.5 Torr). The tanks housing the hypoxia-exposed fish alternated for every exposure. Flow of N_2 to the hypoxia exposure tank was turned on or off by a relay based on real-time O_2 saturation data (see below). Temperature of each tank was recorded with the normoxia tank averaging 19.8±0.7°C and the hypoxia tank averaging 20.0±0.5°C. Each fish was acclimated in the respirometry chamber at the desired O_2 regime for 24 h. One hour prior to experimentation, a digital O_2 probe (Control Company, Galveston, TX) was calibrated and inserted into the flow-through probe vessel associated with the respirometer for baseline measurements. At the beginning of the experiment, the pump bringing fresh normoxic or hypoxic seawater into the respirometry chamber was unplugged and water lines leading to the respirometer were clamped shut. The pump recirculating the water within the closed respirometer system was left on. Percentage O_2 saturation measurements were recorded once every 60 s for a period of 20 min. When the experiment was concluded, the chambers were unclamped and flow of fresh normoxic or hypoxic seawater was restored. Fish were allowed to recover for 20 min. They were then returned to holding tanks and monitored for 24 h. We observed 100% survivorship.

Series ii: The effects of mild hypoxia on 5-HT uptake

Adult toadfish (0.103±0.045 kg; 0.027–0.198 kg; n=57) were anesthetized in 1 g l^{-1} buffered MS-222 for 10 min and then

Journal of Experimental Biology (2022) 225, jeb244064. doi:10.1242/jeb.244064

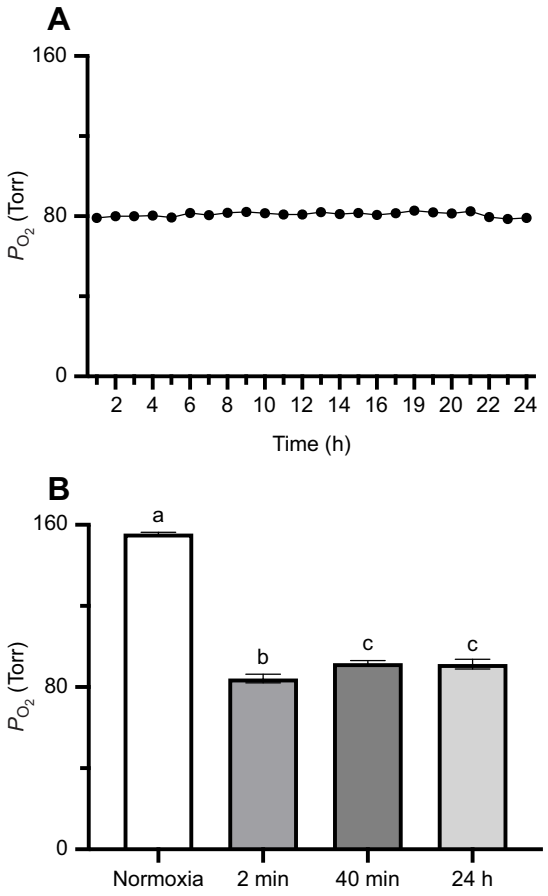

Fig. 1. Mean P_{O_2} for the hypoxia header tank over 24 h and in the individual chamber at the end of each exposure 2 min, 40 min and 24 h. (A) P_{O_2} (Torr) of the hypoxia header tank over 24 h. (B) P_{O_2} of the individual chamber at the end of each exposure for series ii and series iii (n=25, 16, 15, 14 for normoxia, 2 min, 40 min, and 24 h mild hypoxia, respectively). Values are means±1 s.e.m. Bars not sharing a letter are significantly different (P<0.05).

surgically implanted with a caudal vessel catheter (Intramedic PE 50 tubing; Becton Dickinson, Franklin Lakes, NJ, USA) filled with heparinized saline (150 mmol l^{-1} NaCl with 50 IU ml^{-1} sodium heparin; Sigma-Aldrich) and sealed by heat flare as described previously (Wood et al., 1997). Fish were allowed to recover in individual isolated 1.5 liter plastic chambers with aerated, flow-through seawater for 36–48 h before experimentation, with temperature recorded every 24 h. After recovery, fish were treated according to four different oxygen schemes. (1) Normoxia: fish received aerated flow-through water from the control header tank in addition to aeration from individual air stones (97.9%±3.6 O_2 saturation, ∼155.6 Torr). (2) 2 min hypoxia exposure: on the morning of the experiment, the hypoxia header tank was set to a lower %O_2 saturation (20%, 32 Torr) to achieve target O_2 saturation (50%=79.5 Torr) after 2 min (as measured by O_2 probes). Water flow to the individual chambers was disconnected and fish continued to receive air bubbled into their chambers. At t=0, a large hose was attached directly to the header outflow and hypoxic water was delivered directly into a chamber for 2 min. Controls were run alongside 2 min exposures that also had water delivered through the large hose to control for the change in flow. (3) 40 min hypoxia exposure: on the morning of the experiment, the hypoxia relay was turned on and the header tank was set to the target O_2 saturation, but individual air stones were kept on so that the fish would still be held in normoxic water. At t=0, air flow through the air stones was turned off. The chamber then went through a 20 min ramp period, where O_2 saturation in the chamber slowly dropped as it received hypoxic water from the header tank. Once the ramping period had been completed and the target O_2 saturation of 50% (79.5 Torr) reached, fish were held for 20 min for a total hypoxia exposure time of 40 min. (4) 24 h exposure: the hypoxia header was set to target O_2 saturation of 50% (79.5 Torr) and flow to air stones were turned off 24 h prior to takedown. While the P_{O_2} of all hypoxia treatments was significantly lower than normoxia, the P_{O_2} of the 2 min hypoxia exposure was significantly lower than the 40 min and 24 h exposure, which were not different from each other. All fish were injected with 0.2 µCi [^{3}H]5-HT, 2 µl saline^{-1} g^{-1} fish (40 Ci mmol^{-1}; American Radiolabeled Chemicals, St Louis, MO, USA) 2 min before the end of the normoxia/hypoxia exposure. Then, at the end of the exposure, a 200 µl blood sample was taken from fish and the fish was euthanized in 3 g l^{-1} MS-222. A 25 µl aliquot of whole blood was set aside, the rest was centrifuged for 5 min at 14,000 ***g*** to isolate plasma. Temperature and O_2 saturation in the chamber were recorded at the time of takedown. Tissues were removed, beginning with the heart to prevent circulation of [^{3}H], followed by brain, gills, liver and kidney. Bile and urine were collected by carefully puncturing the gall bladder and urinary bladder, respectively, with scissors and draining the contents into labeled bullet tubes. Volume of liquid samples was measured using a pipette; if the sample contained less than 50 µl of liquid it was not considered usable. Interestingly, many fish did not have any urine in their bladder at time of takedown, resulting in low sample size. It is unclear if this is due to a biological response by the fish to hypoxia, or possibly a reflection of some type of bias in our collection methods. Tissues and fluids were immediately processed for the analysis of [3H]5-HT and remaining plasma, bile and urine samples were frozen in liquid N_2 for later analysis of 5-HT concentration. Metabolite concentrations were not measured in this study.

Series iii: The effects of mild hypoxia on 5-HT degradation

Adult toadfish (0.053±0.018 kg; 0.021–0.103 kg; n=24) were transferred to individual chambers and temperature was recorded. Fish were allowed to acclimate for 24 h. The same O_2 regimes outlined in series ii were used. Once hypoxia treatment had completed, fish were euthanized by overdose in 3 g l^{-1} buffered MS-222. Fish were dissected, with the brain, heart, gill and kidney collected in addition to bile. Prior to being flash frozen in liquid N_2, each tissue was cut in half and placed in separate tubes. All samples were flash-frozen and stored at −80°C.

Analytical techniques and calculations

For series i, O_2 consumption rate ($\dot{M}_{O_2}$) was determined by measuring the change in %O_2 saturation every 3 or 4 min while a fish was in a respirometer. The $\dot{M}_{O_2}$ was then calculated by converting the %O_2 saturation to concentration and normalizing by the mass of the fish. The multiple measurements of $\dot{M}_{O_2}$ for each individual fish was averaged together to get one $\dot{M}_{O_2}$ value per fish.

For series ii, for whole blood, plasma, urine and bile, 25 µl samples were pipetted into scintillation vials containing 4 ml UltimaGold liquid scintillation cocktail (PerkinElmer, Waltham, MA, USA). Organ samples were weighed and placed into glass scintillation vials. Organs were digested in 5 ml g^{-1} 1 mol l^{-1} nitric acid and placed in a water bath set to 60°C for 48 h. Digested tissues were then centrifuged at 2500 rpm for 5 min and 25 µl of homogenate was added to 4 ml of UltimaGold liquid scintillation cocktail. For both tissues and fluids, the amount of radioactivity was

Journal of Experimental Biology (2022) 225, jeb244064. doi:10.1242/jeb.244064

measured using an LS6500 liquid scintillation counter (Beckman Coulter, Fullerton, CA, USA) with on-board color quench correction. The amount of radioactivity (cpm 25 µl^{-1}) was multiplied by 40 to obtain cpm ml^{-1}. The amount of 5-HT taken up by each tissue was determined by finding the ratio of endogenous 5-HT (ng ml^{-1}, obtained using ELISA, ALPCO Diagnostics) to tritium activity (cpm ml^{-1}) in each plasma sample (ng cpm^{-1}). Uptake was then quantified as the radioactivity of each tissue homogenate multiplied by the specific activity of the plasma from the same fish. This was then divided by the dilution factor of nitric acid to obtain uptake (ng g^{-1}).

For series iii, to analyze monoamine oxidase (MAO), mitochondria from each tissue was isolated. Gill, heart, liver and kidney were thawed, weighed and mechanically homogenized in 9 volumes per weight 0.25 mol l^{-1} sucrose buffered to a pH of 7.4 with 0.02 mol l^{-1} sodium phosphate buffer (equal parts monobasic and dibasic sodium phosphate) as previously described (Tipton and Dawson, 1968). Samples were centrifuged at 1800 ***g*** for 20 min at 4°C. The supernatant was moved to a new tube and centrifuged again at 18,000 ***g*** for 20 min at 4°C. The supernatant was again removed, the pellet resuspended in half the original volume of phosphate-buffered sucrose, and centrifuged one more time at 18,000 ***g*** for 20 min at 4°C. The final supernatant was then discarded and the pellet was resuspended in 1 ml sodium phosphate buffer. Total protein concentration was measured using Bradford reagent (Sigma-Aldrich) and bovine serum albumin protein standards. Each sample was then diluted to a total protein concentration between 1–10 µg ml^{-1} to be analyzed for MAO activity using an assay kit (Sigma-Aldrich).

MAO activity (µmol g^{-1} h^{-1}) was calculated using the following:

$$\text{MAO activity} = \left(\frac{ab}{c}\right) \times 60,$$

where a is the observed activity of the assay (µmol l^{-1} min^{-1}), b is the dilution factor used to get the sample within total protein range and c is original mass of the tissue multiplied by resuspension volume (g l^{-1}). The resulting units, µmol g^{-1} min^{-1} were converted to µmol g^{-1} h^{-1} by multiplying by 60 (min h^{-1}). Assay activity values that fell within range of the standard curve but were negative were considered to have zero enzyme activity. Clearance ratios for each liquid tissue were calculated by dividing the 5-HT concentrations of the bile or urine by the 5-HT concentrations of the plasma in each fish.

Statistics

Statistics were preformed using Prism (GraphPad v.8). Data were checked for outliers using ROUT (Q=1%), and the data sets were analyzed for normality using the Shapiro–Wilk test. Normal data or log-transformed data that were not normally distributed were analyzed using a one-way ANOVA with Tukey's multiple comparisons test or unpaired Student's *t*-test. A *P*-value below 0.05 was considered significant for this study.

RESULTS

All hypoxia exposures were at or near the desired P_{O_2} of 79.5 Torr (Fig. 1A). Although P_{O_2} of all hypoxia treatments were significantly different from normoxic controls, the 2 min treatment group had a 9.1% lower P_{O_2} than the 40 min and 24 h groups (Fig. 1B, P<0.001). Average P_{O_2} in the respirometry chamber was significantly lower in water from hypoxia exposed tanks than from normoxia tanks (Fig. 2A, P<0.001). Fish held in normoxia had an $\dot{M}_{O_2}$ that was not significantly different from fish held in in hypoxia for 24 h (Fig. 2B). There was no difference in plasma 5-HT concentrations between fish exposed to 2 min of hypoxia (8.08±0.87 ng ml^{-1}; n=9) and their normoxic controls (7.96±0.95 ng ml^{-1}; n=9; Fig. 3A). Plasma 5-HT concentrations following 40 min (7.73±0.88 ng ml^{-1}; n=11) and 24 h (6.80±0.90 ng ml^{-1}; n=10) of hypoxia were 34% and 59% lower than normoxic controls (11.72±2.03 ng ml^{-1}; n=14), respectively (Fig. 3B, P=0.033). After 2 min of hypoxia exposure, there was a significant 1.56-fold increase in 5-HT uptake by the gill compared with controls (Fig. 4A). The heart, kidney and liver showed no change in 5-HT uptake following 2 min of hypoxia exposure (Fig. 4A). None of the tissues taken from 40 min or 24 h hypoxia-exposed fish showed any significant difference when compared with fish exposed to normoxia (Fig. 4B).

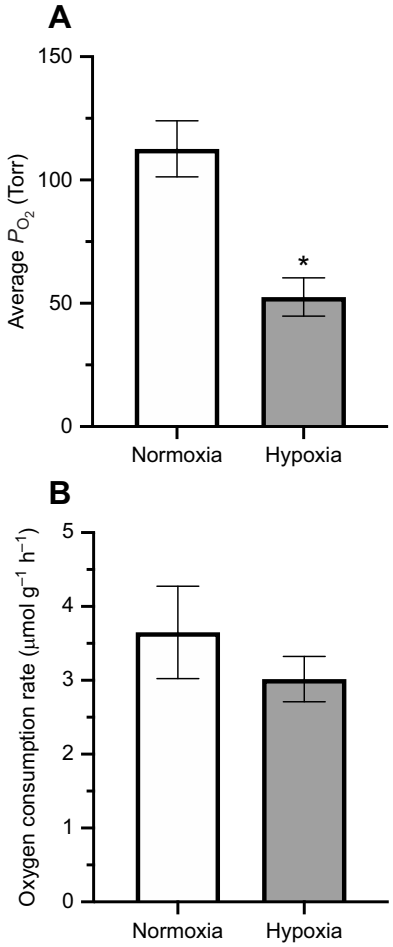

Fig. 2. Mean P_{O_2} and oxygen consumption rates of Gulf toadfish incubated in normoxia or mild hypoxia for 24 h. (A) P_{O_2} (Torr) and (B) oxygen consumption rates (µmol g^{-1} h^{-1}) of fish incubated in normoxia (P_{O_2}=160 Torr; n=8) and fish incubated in mild hypoxia (P_{O_2}=79.5 Torr; n=7). Values are means±1 s.e.m. Asterisk denotes a statistically significant difference (*P<0.05).

MAO activity during normoxia was highest in the heart (Fig. 5A), over 18-fold higher than the next closest organ, the gill (Fig. 5B). MAO activity was very low in the kidney (Fig. 5C) and was not detectable in the liver (data not shown). MAO activity in the gill did not change after the 2 min hypoxia exposure, but there was a significant 3-fold increase after both the 40 min and 24 h hypoxia exposure compared with controls (Fig. 5B). In contrast, MAO

Journal of Experimental Biology (2022) 225, jeb244064. doi:10.1242/jeb.244064

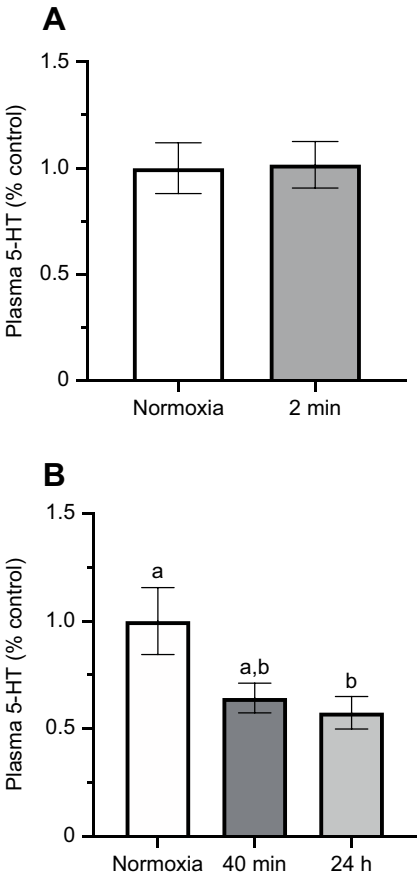

Fig. 3. Normalized plasma 5-HT concentrations of Gulf toadfish exposed to normoxia or to 2 min, 40 min or 24 h of mild hypoxia. (A) Fish exposed to 2 min of mild hypoxia (n=9) and their concurrent controls (n=9). (B) Fish exposed to 40 min (n=11) or 24 h (n=10) of mild hypoxia and their concurrent controls (n=14). Values are means±1 s.e.m. Bars not sharing a letter are significantly different (P<0.05).

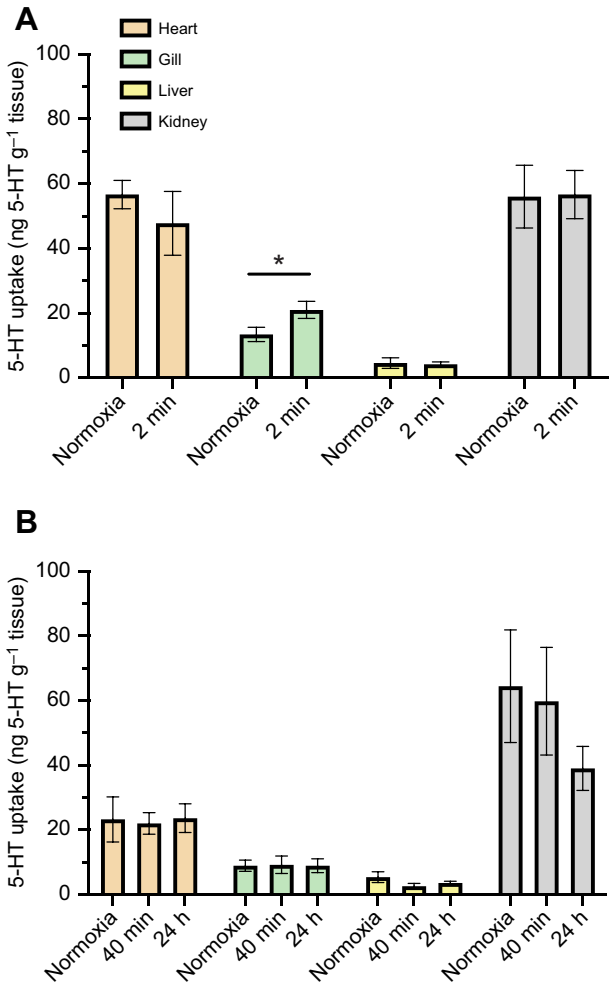

Fig. 4. 5-HT uptake in the heart, gill, liver and kidney of Gulf toadfish exposed to normoxia or to 2 min, 40 min or 24 h of mild hypoxia. (A) Fish exposed to 2 min of mild hypoxia and their concurrent controls. (B) Fish exposed to 40 min or 24 h of mild hypoxia and their concurrent controls. Values are means±1 s.e.m. (n=8–11). Bars connected by an asterisk indicate a significant effect of hypoxia exposure (*P<0.05).

activity in the heart decreased during hypoxia, with MAO activity being 43.1%, 22.7% and 15.5% of control values after the 2 min, 40 min and 24 h hypoxia exposure, respectively. Interestingly, there was no difference between MAO activity in the heart and gill at the 24 h timepoint. MAO activity in the kidney showed a similar increasing trend as the gill, but this result was not statistically significant (Fig. 5C).

There was no effect of any hypoxia treatment on either bile 5-HT or [^{3}H] concentrations when compared with normoxic controls (Fig. 6). However, clearance ratios were >>1 for bile for all fish and were unaffected by hypoxia exposure (Table 1). Many fish did not have urine in their bladder at time of takedown, resulting in low sample size for the urine data. Thus, the urine data should be considered preliminary findings. Similarly to the bile, hypoxia did not have any effect on 5-HT or [^{3}H] concentrations in the urine (Table 2). Clearance ratios were >1 in urine for all fish and were unaffected by hypoxia exposure (Table 1). While endogenous 5-HT concentrations were 38% higher in the bile than in the urine of normoxia fish (P=0.048), total counts of [^{3}H] were not different (see Fig. 6). Hypoxia had no significant effect on 5-HT or [^{3}H] concentrations in either bile or urine (Fig. 6, Table 2).

DISCUSSION

The goal of this study was to determine how 5-HT clearance in the periphery changes in response to mild hypoxia, with the idea that circulating 5-HT in the extracellular pool is exerting considerable tonic vasoconstriction on arteries under normal conditions, similarly to its effects reported in mammals (Brenner et al., 2007). The reduction in plasma 5-HT concentrations measured in the present study clearly shows 5-HT clearance from the plasma in response to prolonged mild hypoxia. It should be noted that the P_{O_2} was significantly higher in 40 min and 24 h hypoxia exposures than in 2 min exposures. This was likely due to the length of exposure, as the longer incubation times may allow more time for hypoxic water to equalize with the thin layer of air trapped above the water line, bringing the P_{O_2} up slightly. The difference in P_{O_2} is relatively small and unlikely to impact results, as all treatments were well above an estimated toadfish P_{crit} of about 30–40 Torr (our unpublished observations; Amador et al., 2018) and the P_{crit} established for the closely related *Opsanus tau* of 29.0±3.5 Torr (Ultsch et al., 1981). In addition, the average P_{O_2} was lower during the respirometry experiment than the P_{O_2} to which fish were exposed during the hypoxia exposures because closed system respirometry was used during which the P_{O_2} was continuously driven down as the fish consumed the available oxygen in the chamber. As such, the measured P_{O_2} is an average of the initial (target) P_{O_2} in addition to

Journal of Experimental Biology (2022) 225, jeb244064. doi:10.1242/jeb.244064

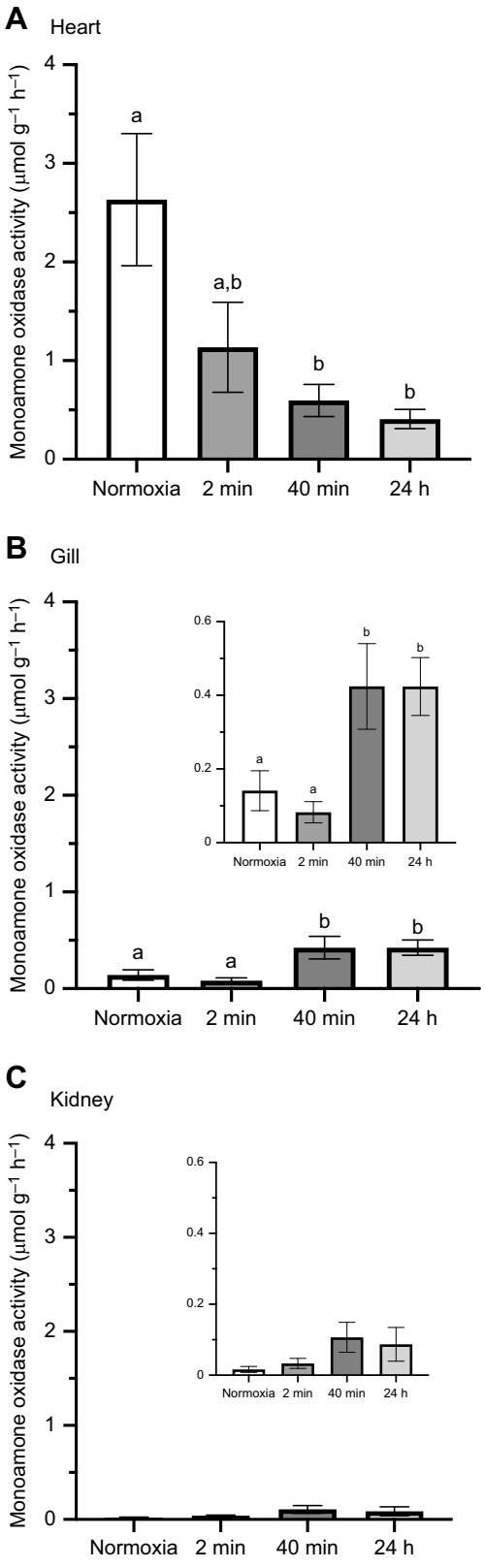

Fig. 5. Monoamine oxidase (MAO) activity in the heart, gill and kidney of toadfish exposed to normoxia or mild hypoxia for 2 min, 40 min or 24 h. MAO activity in (A) heart, (B) gill and (C) kidney of fish exposed to normoxia (n=8) or mild hypoxia for 2 min (n=8), 40 min (n=8) or 24 h (n=8). Inset in B depicts gill data scaled to the highest values obtained in that gill for clarity. Inset in C depicts kidney data scaled the same as inset in B. Values are means±1 s.e.m. Bars not sharing a letter are significantly different (P<0.05).

the P_{O_2} as oxygen was depleted. Therefore, we are confident that shifts in uptake and degradation activity are the result of a time-dependent process in the organs; however, effects due to changes in P_{O_2} cannot be ruled out.

There is evidence that circulating 5-HT levels in toadfish are maintained under resting conditions via homeostatic mechanisms (Sebastiani and McDonald, 2021), possibly in an effort to help keep blood pressure at physiologically safe levels, as in humans (Watts et al., 2012). The heart was hypothesized to be the primary location involved in 5-HT homeostasis in teleosts because of both high SERT mRNA expression and corresponding high SERT-mediated 5-HT uptake (Amador and McDonald, 2018b). Supporting this idea, 5-HT has been found in cells within the endocardial layer of the zebrafish (*Danio rerio*) atrium and the sinoatrial valve, and within intracardiac neurons (Stoyek et al., 2017). None of these cell populations colocalized with tryptophan hydroxylase, suggesting that the 5-HT in these cells was not produced but taken up (Stoyek et al., 2017). Additionally, the location of the heart within the cardiovascular circuit makes it uniquely suited for this homeostatic task as it is just downstream of the liver, which shows high activity of the 5-HT synthesizing enzyme tryptophan hydroxylase in several other teleost species (Nagai et al., 1997), and upstream of the gill, for which excess 5-HT in the bloodstream might be problematic and particularly detrimental during hypoxia (Sundin et al., 1995; Nilsson and Sundin, 1998). In further support of the heart playing a major role in 5-HT regulation during normoxia, at least in toadfish, the present study has found MAO activity in the heart is the highest of all peripheral tissues tested. MAO activity and distribution appears to vary considerably across teleost tissues and has been reported in the brain, heart, liver, kidney and intestine (Hall and Uruena, 1982; Edwards et al., 1986; Setini et al., 2005). Our findings in toadfish are contrary to a study in pacu (*Piaractus mesopotamicus*), which found that V_{max} of MAO in the kidney was 7-fold higher than in the heart (Salles et al., 2007).

In response to mild hypoxia, heart 5-HT uptake stayed consistent whereas heart MAO activity sharply decreased, which suggests that, if nothing else were to have changed, more 5-HT would have entered the gill circulation during hypoxia rather than less, as originally hypothesized. Plasma 5-HT concentrations did decrease over the hypoxia time-course, indicating first and foremost that 5-HT uptake and degradation rates by the heart did not drive changes in plasma 5-HT concentrations. As neither 5-HT production nor mRNA expression of tryptophan hydroxylase were measured in the present study, it cannot be ruled out that 5-HT synthesis in the liver or elsewhere was downregulated during hypoxia and the blood entering the heart had lower 5-HT concentrations. An earlier study demonstrated that plasma 5-HT concentrations sampled from the hepatic vein did not differ in toadfish exposed to normoxia compared with 25 min or 24 h hypoxia (Amador and McDonald, 2022); however, fish were exposed to severe hypoxia (4 Torr) making it inappropriate to make direct comparisons to the present study. Additionally, tryptophan hydroxylase, the rate-limiting enzyme in the 5-HT synthesis pathway, is oxygen dependent, and evidence has shown that 5-HT synthesis in the teleost brain is significantly reduced during hypoxia (Davis et al., 1973; Rahman and Thomas, 2009). MAO is also oxygen dependent and therefore the reduced MAO activity in response to prolonged hypoxia exposure could be due to limited oxygen (Gaweska and Fitzpatrick, 2011). The oxygen budget of the heart may allow for degradation of 5-HT during normoxia but not hypoxia, since most teleost fish myocardium obtain oxygen from returning venous blood passing through the lumen (Tota et al.,

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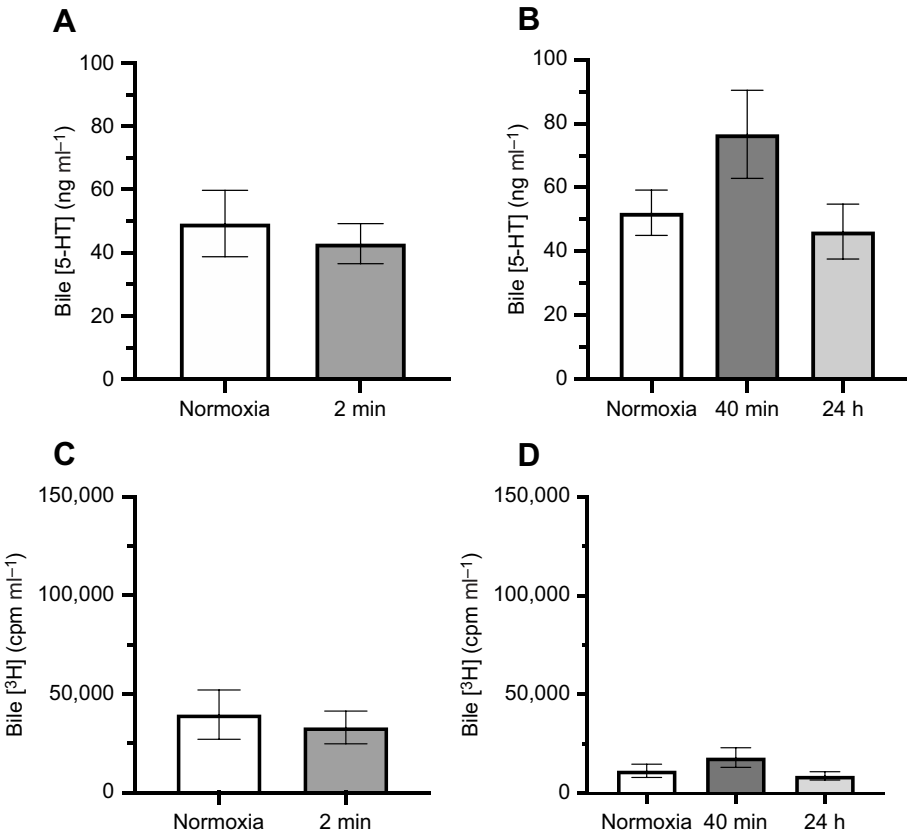

Fig. 6. Bile 5-HT concentration and [^{3}H] counts in fish exposed to normoxia or to mild hypoxia for 2 min, 40 min or 24 h. Bile 5-HT concentrations of fish exposed to (A) 2 min of mild hypoxia (*n*=7) and their concurrent controls (*n*=6), and (B) 40 min (*n*=8) or 24 h (*n*=8) of mild hypoxia and their concurrent controls (*n*=14). Bile [^{3}H] concentrations of fish exposed to (C) 2 min (*n*=11) of mild hypoxia and their controls (*n*=9), and (D) 40 min (*n*=9) and 24 h (*n*=8) of mild hypoxia and their controls (*n*=10). Values are means±1 s.e.m.

1983). It is also possible that changes in SERT-mediated 5-HT uptake and degradation in response to hypoxia may have nothing to do with whole body 5-HT homeostasis, but it is not clear that it would be related to the regulation of heart function during hypoxia. This is because, in contrast to the bradycardia and increased stroke volume experienced during hypoxia, intravenous injection of exogenous 5-HT in teleosts results in tachycardia and a decreased stroke volume (Sundin et al., 1995; Sundin and Nilsson, 2000; Pelster and Schwerte, 2012) by increasing the contractility of the heart (Burleson and Milsom, 1995; Côté et al., 2004; Kermorgant et al., 2014). By reducing 5-HT degradation, the heart is increasing the amount of 5-HT available to lumen-facing 5-HT receptors in the endocardium in the cardiac pool, which would oppose hypoxia reflexes. Further research on cardiovascular physiology of the gulf toadfish during hypoxia is needed.

While gill SERT mRNA expression and 5-HT uptake have been reported previously (Amador and McDonald, 2018a,b), to our knowledge this is the first study to quantify MAO activity in the gill during normoxia and the time course of 5-HT uptake and degradation within the gill in response to hypoxia exposure. However, isolated gill perfusions in trout have shown that approximately 80% of 5-HT is removed from the arterio-arterial pathway during a single pass through a combination of uptake and degradation (Olson, 1998), indicating that the gill is a major site for 5-HT homeostasis. In the present study, a significant increase in 5-HT uptake by the gill was only measured following 2 min of hypoxia exposure. However, we argue that 5-HT uptake continued to be upregulated after 40 min and 24 h of hypoxia exposure but was masked by the upregulation of MAO activity at these times, which resulted in the degradation of 5-HT into its metabolite 5-HIAA and prevented 5-HT accumulation in the tissue. Some 5-HIAA may be excreted across the gill (Amador and McDonald, 2018b), but more likely returns to the plasma for removal by the kidney via organic anion transporters (Dantzler, 1996). The gill is highly vasosensitive (Olson, 2002), and the removal of 5-HT from general circulation both at rest and during hypoxia may facilitate gas exchange depending on where in the branchial circuit removal is performed. Clearance by the gill may be a mechanism to modulate blood flow through the gill to maintain gas exchange during hypoxia, remove the blood of potentially harmful excess 5-HT before general body circulation, or both. If the gill is indeed responsible for driving down plasma 5-HT concentrations, the decrease in degradation observed in the heart may be a direct consequence. The finding that degradation rates in the gill and heart are matched after 24 h of

Table 1. 5-HT clearance ratios in Gulf toadfish during normoxia and after 2 min, 40 min and 24 h of hypoxia exposure

			Hypoxia		
		Normoxia	2 min	40 min	24 h
Experiment 1	Bile	7.4±2.2 (5)	5.6±1.3 (5)	–	–
	Urine	1.2±0.5 (2)	1.6±0.7 (3)	–	–
Experiment 2	Bile	7.8±2.2 (11)	–	12.3±4.3 (8)	7.2±1.3 (9)
	Urine	4.9±1.8 (8)	–	5.5±2.0 (3)	8.3±3.4 (3)

Values are means±s.e.m. (*n*).

Table 2. 5-HT concentrations and [^{3}H] counts in urine of Gulf toadfish during normoxia and after 2 min, 40 min and 24 h of hypoxia exposure

			Hypoxia		
		Normoxia	2 min	40 min	24 h
Experiment 1	5-HT (ng ml^{-1})	11.5±1.8 (2)	14.2±4.0 (4)	–	–
	[^{3}H] (cpm ml^{-1})	9918±7270 (2)	18,914±10,387 (5)	–	–
Experiment 2	5-HT (ng ml^{-1})	36.2±8.8 (9)	–	37.5±14.6 (3)	31.3±11.2 (3)
	[^{3}H] (cpm ml^{-1})	15,466±4632 (5)	–	92,665±45,319 (3)	24,362±17,162 (3)

Values are means±s.e.m. (*n*).

hypoxia exposure echoes the cardiorespiratory synchrony that occurs with heart rate and ventilation rate during hypoxia (Randall and Smith, 1967). Whether the control of circulating 5-HT is involved in those responses is yet to be investigated.

The teleost gill processes a variety of other signaling molecules, including catecholamines (Olson, 1998). However, while isolated gill perfusions in trout have shown 80% of 5-HT to be removed during a single pass, only 15% of norepinephrine and 5% of epinephrine are removed from the same circuit (Olson, 1998), suggesting that the gill primarily controls 5-HT. The increase in gill MAO activity during hypoxia would therefore selectively degrade the 5-HT that opposes the hypoxia response by constricting arterial flow through the lamellae, while having little effect on the synergistic catecholamines which facilitate gas exchange and vasodilation within the gill.

The low amount of 5-HT uptake by the liver and the high amount of 5-HT uptake by the kidney was consistent with previous findings (Amador and McDonald, 2018b); and hypoxia had no effect on 5-HT uptake by either tissue. MAO activity within the kidney was low, but detectable, and showed some variability in response to hypoxia exposure, following trends similar to those seen in the gill. In contrast, MAO activity in the liver was below detectable levels in toadfish and was not sensitive to hypoxia exposure. MAO activity and distribution during normoxia has been reported in the liver and kidney of zebrafish, goldfish (*Carassius auratus*) and rainbow trout (Hall and Uruena, 1982; Edwards et al., 1986; Setini et al., 2005). While kidney MAO activity in toadfish was comparable to these other species (Hall and Uruena, 1982; Edwards et al., 1986), that there is undetectable hepatic MAO activity in toadfish is unlike other fish species measured to date (Hall et al., 1982; Edwards et al., 1986; Salles et al., 2007; Boyer, 2013). This is a surprising result considering that 5-HT is synthesized in the liver of many teleosts (Nagai et al., 1997) and the known role of the liver as a metabolic hub for many hormones, including cortisol and catecholamines (Mommsen et al., 1999; Semenova et al., 2017).

Clearance ratios (CRs) of 5-HT for the bile were >>1, and urine $CR_{5\text{-}HT}$ was more variable but always exceeded 1, indicating that 5-HT is concentrated in both fluids under normal conditions and during hypoxia, and concentrated in bile to a greater extent than in the urine. Thus, hepatic excretion of 5-HT appears to be a major clearance pathway, as well as a possible delivery route of 5-HT for the intestine. In mammals, intestinal 5-HT is typically sourced from intrinsic serotonergic nerves or synthesized within the enterochromaffin cells of the intestinal mucosa (Martin et al., 2017). 5-HT released in the intestinal lumen is associated with many digestive functions, including regulation of gut motility, water and fat absorption, and the secretion of bicarbonate and electrolytes via 5-HT receptors in the intestinal mucosa (Hansen, 2003; Tuo and Isenberg, 2003; Tuo et al., 2004; Gershon, 2013; Martin et al., 2017). Fish also release 5-HT into their intestinal lumen, even in species lacking enterochromaffin cells (Anderson et al., 1991) and 5-HT could be delivered to the intestine through the bile to participate in digestive functions as opposed to being excreted as waste. Furthermore, hypoxia exposure is known to decrease blood flow to the digestive system (Farrell and Richards, 2009), potentially limiting the local release of neuronal and endocrine 5-HT from within the gut.

A small amount of preliminary data were obtained from analyzing urine samples. Urine 5-HT concentrations were 61.9% of bile concentrations but total counts of [^{3}H] in the urine and bile, which includes both [^{3}H]5-HT and [^{3}H]5-HIAA (Sebastiani and McDonald, 2021), were not different. Since there was significant MAO activity measured in the kidney (and not in the liver), a significant proportion of [^{3}H] in the urine may be 5-HIAA; however, we were not able to discriminate between [^{3}H]5-HT and [^{3}H]5-HIAA in this study. This idea is supported by the work of Some and Helander (2002), who found 320 ng ml^{-1} 5-HIAA in the urine of rainbow trout (compared with 36 ng ml^{-1} 5-HT measured in this study) and is indicative of extremely efficient renal degradation and excretion of metabolite (Some and Helander, 2002; Caamaño-Tubío et al., 2007). The high 5-HIAA in the urine is likely accomplished *in vivo* through both renal degradation of 5-HT by MAO as well as highly efficient organic anion transporter-mediated secretion of 5-HIAA that was produced from other organs, such as the gill, (Hakim et al., 1970; Dantzler, 1996; Some and Helander, 2002; Villalobos et al., 2002; Aslamkhan et al., 2006). Interestingly, the number of hypoxia-treated fish that had urine in their urinary bladders at end of the experiment was low and decreased with the length of hypoxia exposure (73% for control cf. 33% after 40 min of hypoxia exposure and 22% after 24 h hypoxia exposure). This may indicate that toadfish urinate more frequently during hypoxia, which could be a mechanism to assist 5-HT clearance.

In conclusion, our data show that 5-HT is being removed from the plasma by the gill during exposure to mild hypoxia. The decrease in plasma 5-HT may facilitate the hypoxia response by removing a potent vasoconstrictor with antagonistic effects that would impair gas exchange and would also support the heart bradycardia response. This would be consistent with previous work, where inhibiting SERT (and therefore 5-HT uptake) with fluoxetine prevented the typical decrease in caudal arterial pressure and bradycardia measured in toadfish during hypoxia (Panlilio et al., 2016). 5-HT uptake may also be supplying the organs with functional 5-HT as tissue physiology shifts during hypoxia stress. Clearance of 5-HT by the gill is likely linked to the facilitation of gas exchange, while the physiological significance of the heart 5-HT dynamics during hypoxia remains unclear. Further research on the role 5-HT plays in cardiac function and how that role changes during exposure to mild hypoxia, is needed. Furthermore, understanding any differences between biliary 5-HT and locally synthesized intestinal 5-HT would be key to understanding why 5-HT appears at such high levels in the bile. Further research into the urinary and biliary excretion rates of both 5-HT and metabolite during hypoxia would help expand on this currently understudied area.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: J.S., M.D.M.; Methodology: J.S., M.D.M.; Validation: J.S., A.S.; Formal analysis: J.S.; Investigation: J.S., M.D.M.; Resources: M.D.M.; Data curation: J.S.; Writing - original draft: J.S., M.D.M.; Writing - review & editing: J.S., A.S., M.D.M.; Visualization: J.S., M.D.M.; Supervision: M.D.M.; Project administration: M.D.M.; Funding acquisition: M.D.M.

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