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ORIGINAL PAPER

Pulsatile urea excretion in Gulf toadfish: the role of circulating serotonin and additional 5-HT receptor subtypes

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Abstract

The neurochemical serotonin (5-HT) is involved in stimulating pulsatile urea excretion in Gulf toadfish (*Opsanus beta*) through the 5-HT_{2A} receptor; however, it is not known if (1) the 5-HT signal originates from circulation or if (2) additional 5-HT receptor subtypes are involved. The first objective was to test whether 5-HT may be acting as a hormone in the control of pulsatile urea excretion by measuring potential fluctuations in circulating 5-HT corresponding with a urea pulse, which would suggest circulating 5-HT may be involved with urea pulse activation. We found that plasma 5-HT significantly decreased by 38% 1 h after pulse detection when branchial urea excretion was significantly elevated and then returned to baseline. This suggests that 5-HT is removed from the circulation, possibly through clearance or excretion, and may be involved in the termination of pulsatile urea excretion. There appeared to be no pulsatile release of 5-HT from peripheral tissues to trigger a urea pulse. The second objective was to determine if additional 5-HT receptor subtypes, such as an additional 5-HT_2 receptor (5-HT_{2C} receptor) or the 5-HT receptors that are linked to cAMP (5-$HT_{4/6/7}$ receptors), played a role in the stimulation of urea excretion. Intravenous injection of 5-HT_{2C}, 5-HT_4, 5-HT_6, and 5-HT_7 receptor agonists did not result in a urea pulse, suggesting that these receptors, and thus cAMP, are not involved in stimulating urea excretion. The involvement of circulating 5-HT and the 5-HT_{2A} receptor in the regulation of pulsatile urea excretion may provide insight into its adaptive significance.

Keywords 5-HT_{2c} · 5-HT_4 · 5-HT_6 · 5-HT_7 · cAMP · tUT

Introduction

The unique handling of nitrogenous waste, specifically the excretion of urea, by Gulf toadfish (*Opsanus beta*) has led to extensive research into the neuroendocrine mechanisms controlling urea release in this species (reviewed by McDonald et al. 2012a). This research has revealed that a chronic increase in circulating levels of the stress hormone cortisol (Hopkins et al. 1995) results in the switch from ammonia excretion to urea production due to an increase in urea production enzymes (Laberge et al. 2009) and an increase in toadfish urea transporter (tUT) mRNA expression in the gill (McDonald et al. 2009). After the switch to ureotely, a drop in cortisol occurs 2–4 h before a urea pulse and then returns to baseline levels immediately after the urea pulse (Wood et al. 1997, 2001). This drop in plasma cortisol is believed to be permissive to the activation of the urea transporters involved in the urea pulse, because intra-arterial infusion of cortisol causes a reduction in pulse size and total urea excretion (McDonald et al. 2004, 2009; Rodela et al. 2009a, b).

In addition to cortisol, pulsatile urea excretion is also regulated by the monoamine neurochemical serotonin (5-HT; 5-hydroxytryptamine) as injections of 5-HT into the bloodstream result in urea pulses of natural size in vivo (Wood et al. 2003) and in a significant increase in the rate of urea excretion of the in vitro perfused gill preparation (McDonald et al. 2012b). A urea pulse is also induced when toadfish

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are treated with fluoxetine, a selective serotonin reuptake inhibitor (SSRI) antidepressant that inhibits the 5-HT transporter (SERT) and results in an increase in circulating 5-HT concentrations (Amador et al. 2018; McDonald et al. 2011, 2012a; Morando et al. 2009). Thus, 5-HT stimulates a pulse of urea from the gill; however, it is not clear from where the 5-HT originates. One possibility is that 5-HT is released from internal sources within the gill (e.g., intrinsic serotonergic neurons or neuroepithelial cells), acting in a paracrine fashion (Bailly et al. 1992; Dunel-Erb et al. 1982; Jonz and Nurse 2003; Porteus et al. 2012). Alternatively, 5-HT may originate from peripheral sources, such as the liver and intestine (where 5-HT is synthesized), heart, or kidney, as these tissues contain high concentrations of 5-HT in other teleost species (Caamano-Tubio et al. 2007; Nagai et al. 1997; Piomelli and Tota 1983; Stoyek et al. 2017). These tissues are also responsible for uptake of 5-HT from circulation through the 5-HT transporter (SERT) in Gulf toadfish (Amador and McDonald 2018b). Release of 5-HT from these tissues would reflect 5-HT acting in a hormonal fashion—a role of 5-HT that is relatively understudied but for which there is increasing support (Cartolano et al. 2019a; reviewed by McDonald 2017)—and may be measured by changes in circulating 5-HT concentrations. Supporting this idea, a urea pulse is triggered immediately after injection of 5-HT into the bloodstream (McDonald et al. 2012b; Wood et al. 2003).

Whatever the source, the stimulation of pulsatile urea excretion by 5-HT appears to be mediated by the 5-HT_{2A} receptor as intra-arterial injections of the 5-HT_2 receptor agonist α-methyl-5-HT result in a urea pulse that is inhibited by the 5-HT_{2A} receptor antagonist ketanserin (Mager et al. 2012; McDonald and Walsh 2004). McDonald and Walsh (2004) proposed the 5-HT_{2A} receptor over the 5-HT_{2B} or the 5-HT_{2C} receptors because of the affinities of α-methyl-5-HT and ketanserin for each of the receptors and the sensitivity of the pulsatile urea excretion mechanism to the compounds (i.e., attenuation of pulsatile urea excretion occurred even at ketanserin concentrations that were 100-fold less than α-methyl-5-HT; McDonald and Walsh 2004). However, based on its somewhat similar affinity to α-methyl-5-HT and ketanserin (within an order of magnitude), the question of whether the 5-HT_{2C} receptor could also be involved has persisted. In any case, both the 5-HT_{2A} and 5-HT_{2C} receptors are G-protein-coupled receptors whose activation leads to an increase in intracellular inositol phosphate (IP_3) and Ca^{2+}. In contrast, mammalian urea transporters (UTs) are regulated by arginine vasopressin (AVP) and the V_2 vasopressin receptor, a G-protein-coupled receptor whose activation results in increases in cAMP and intracellular Ca^{2+} (Potter et al. 2006). Earlier work on toadfish suggested that arginine vasotocin (AVT), a homologue of the mammalian AVP, may be involved in regulating toadfish pulsatile urea excretion as injection of AVT leads to small urea pulses (Wood et al. 2001). Because of this potential role of AVT and its link with cAMP, we wondered if the 5-HT_{2A} receptor is solely responsible for the stimulation of urea pulses or if, perhaps, another 5-HT receptor that is linked to increases in cAMP (the 5-HT_4, 5-HT_6, or 5-HT_7 receptor) is also involved (McDonald et al. 2004, 2012a; McDonald and Walsh 2004).

The adaptive significance of toadfish pulsatile urea excretion is not yet fully understood and the mechanisms by which 5-HT controls urea excretion may further elucidate its purpose. Our first objective was to test whether 5-HT may be acting as a hormone in the control of pulsatile urea excretion. This was addressed by measuring potential fluctuations in circulating 5-HT levels around the time of a urea pulse. We hypothesized that an increase in 5-HT concentrations in blood taken from the systemic circulation prior to or during a urea pulse would be a consequence of 5-HT release from a peripheral location to activate tUT. Alternatively, a decrease in circulating 5-HT during or after a urea pulse may be a result of 5-HT being removed from the bloodstream to terminate the urea pulse. The second objective was to determine if another 5-HT receptor, either from the 5-HT_2 receptor family, e.g., the 5-HT_{2C} receptor, or the 5-$HT_{4/6/7}$ receptors that are linked to increases in cAMP, plays a role in stimulation of urea excretion. We also tested whether these receptors were involved in stimulating changes in circulating cortisol concentrations, as some of these receptors have been implicated in stimulating cortisol secretion directly from the interrenal cells (Medeiros et al. 2010; Medeiros and McDonald 2012). We hypothesized that the 5-HT receptors that are linked to cAMP may also be involved in controlling pulsatile urea excretion, and that both the 5-HT_{2C} and 5-HT_4 receptors are involved in stimulating cortisol release.

Materials and methods

Experimental animals

Gulf toadfish (*O. beta*) were collected by commercial shrimpers in Biscayne Bay, Florida, USA (Florida Fish and Wildlife Conservation Commission Special Activity Licenses #SAL-12-0729-SR and #SAL-16-0729-SR) between August 2012–May 2013 and November 2016–November 2017. Toadfish were treated with a dose of malachite green (0.1 mg L^{-1}) and formalin (30 mg L^{-1}) upon arrival in the laboratory to treat for the parasite *Cryptocaryon irritans*. Fish were held in aerated 75-L flow-through stock tanks containing PVC shelters at temperatures that reflect seasonal changes in Biscayne Bay (18–28 °C) and were fed once a week with thawed shrimp. All procedures were approved by the University of Miami Institutional Animal Care and Use Committee (IACUC).

Experimental series

Series I. Correlating fluctuations in plasma 5-HT with pulsatile urea excretion

Coarse time series: plasma 5-HT measured every 2 h during urea pulsing and non-pulsing periods To determine whether 5-HT fluctuates on the same time scale as cortisol (Wood et al. 1997), serial blood samples were taken every 2 h for 72 h and compared with pulsatile urea excretion. During January 2017, six fish (95–285 g, average weight 167 ± 28 g) were held in crowded conditions to induce ureotelism in a 6-L plastic tub served with flow-through seawater (Hopkins et al. 1995; Walsh et al. 1994; Wood et al. 1995). After 1 week of crowding conditions during which the fish were not fed, the caudal artery was cannulated to take serial blood samples without disturbing the fish. Fish were placed in MS-222 (1 g L^{-1}), mass was recorded, and a Clay-Adams PE50 catheter filled with heparinized saline was placed in the caudal artery through an incision in the caudal muscle (Wood et al. 1997; Axelsson and Fritsche 1994). Fish recovered for 36 h in individual plastic 1.5-L containers with flow-through, aerated seawater. After recovery, flow-through seawater was turned off and water was collected continually in hourly samples (4 mL h^{-1}) for 72 h using a peristaltic pump (Gilson MINIPULS 3, Middleton, WI, USA) and fraction collectors (Bio-Rad Model 2110, Hercules, CA, USA). During this 72-h period, 150-µL blood samples were taken from the caudal artery catheter every 2 h. The blood was centrifuged at 16,100*g* for 10 min at 5 °C and 100 µL of plasma was flash-frozen in liquid nitrogen and stored at − 80 °C for later analysis of 5-HT. The red blood cells (RBCs) and any remaining plasma were mixed with 100-µL 150-mM NaCl and returned to the fish after each sample to replace the blood volume taken. Every 24 h, the containers were flushed with fresh seawater. After 72 h, fish were euthanized in an overdose of MS-222 (3 g L^{-1}) and water samples were stored at − 20 °C for later analysis of urea.

Fine time series: plasma 5-HT measured during urea pulses via real-time urea pulse detection To get a better resolution of circulating 5-HT around the time of a pulse, [^{14}C]-urea (America Radiolabeled Chemicals Inc. St. Louis, MO, USA) was used as a tracer (method of Wood et al. 1997) to monitor pulsatile urea excretion, because [^{14}C]-urea can be quantified within 5 min of taking a water sample. From November to December 2017, the caudal artery or vein was cannulated as described above in an uncrowded ($n = 6$) and crowded fish ($n = 7$) group, and fish (52–83 g, average weight 69 ± 3 g) were left to recover for at least 48 h in individual plastic 1.5 L containers with flow-through, aerated seawater. After recovery, fish were injected through the catheter with 5–15 µCi 100 g^{-1} of [^{14}C]-urea in 300 µL 100 g^{-1} of 150 mM NaCl. After a 1-h equilibration period, flow-through seawater was turned off and water sampling started. Water samples (4 mL) were collected by pipette every 30 min and immediately added to a scintillation vial with 10 mL of Ecolume Scintillation Cocktail (MP Biomedicals, Santa Ana, CA, USA) and [^{14}C]-urea was counted. When a pulse was suspected, as measured by an increase of about 100 counts per min (cpm) from the previous water sample, an immediate subsequent water sample was taken to confirm. Once the pulse was confirmed, blood sampling immediately began and 100 µL blood samples were taken at $t = 0$ h (when the pulse was confirmed) and then again approximately at $t = 15$ min, 30 min, 1 h, and 2 h. Blood sampling time points were then adjusted and grouped into different ranges ($t = -0.25$ to 0, 0–0.5, 0.5–1, 1–1.5, and 1.5–2.5 h) after the exact start time of a urea pulse > 100 µmol-N kg^{-1} was calculated. At the time of the blood sample, 100-µL 150-mM NaCl was injected into the fish via the catheter to replace the blood volume taken. The blood was centrifuged and plasma was stored as above for later analysis of 5-HT, total urea (radiolabeled + unlabeled urea), and [^{14}C]-urea (radiolabeled urea). Fish were euthanized at the end of sampling in an overdose of MS-222 (3 g L^{-1}).

Series II. Determining if additional 5-HT receptors subtypes are involved in stimulating a urea pulse via agonist injections

To determine if other 5-HT receptors subtypes are involved in stimulating pulsatile urea excretion, 5-HT receptor agonists were injected and urea excretion was monitored. Fish (50–125 g) were crowded for up to 1 week and the caudal artery or vein was cannulated as described above. Fish recovered for 48 h in individual 1.5-L containers with a flow-through of aerated seawater. About 0.5–1 h prior to injections with 5-HT receptor agonists, flow-through seawater was turned off and a 2-mL water sample was taken (pre-sample). Immediately before injections, another water sample was taken ($t = 0$) and an initial blood sample was taken and centrifuged and the plasma was flash-frozen in liquid nitrogen. Fish were then injected intravenously via the catheter with 100 µL 100 g^{-1} of 150 mM NaCl (controls) or 0.34 mg CP809101 (5-HT_{2C} receptor agonist; Tocris, Minneapolis, MN, USA), 0.45 mg RS67506 (partial 5-HT_4 receptor agonist; Tocris), 0.4 mg BIMU8 (full 5-HT_4 receptor agonist; Tocris), 0.33 mg EMDT oxalate (5-HT_6 receptor agonist; Tocris), or 0.52 mg LP12 hydrochloride (5-HT_7 receptor agonist; Tocris), each in 100 µL 100 g^{-1} of 150 mM NaCl. These doses yielded an estimated circulating concentration of 3.3×10^{-5} M for each agonist, which is an order of magnitude greater than 5-HT and α-methyl-5-HT used in the previous studies (McDonald and Walsh 2004; Wood et al.

2003) and three orders of magnitude higher than circulating concentrations. We used these high levels to ensure receptor stimulation, since it was predicted that these receptors play a smaller role in pulsatile urea excretion, likely due to low receptor density of lower affinity for 5-HT. Water samples were collected by pipette at $t = 0.5$, 1, and 1.5 h post-injection and a final blood sample was taken immediately after the last water sample at $t = 1.5$ h. Water samples were frozen at − 20 °C and later analyzed for urea and ammonia concentrations. Fish that pulsed before injection were not included in nitrogenous waste excretion analysis. Plasma samples were stored at − 80 °C and later analyzed for urea and cortisol. Each agonist was tested at a separate time, so water and plasma variables were compared to the respective controls for each agonist.

Analytical techniques and calculations

Urea concentrations in water and plasma were measured using the diacetyl monoxime method (Rahmatullah and Boyde 1980). Radiolabeled [^{14}C]-urea in the water and plasma was quantified on a PerkinElmer Tri-Carb 2910TR liquid scintillation analyzer (Waltham, MA, USA) using QuantaSmart software (PerkinElmer). Tests showed that quench was constant for these colorless samples, so a quench correction was not performed. Plasma [^{14}C]-urea was counted after adding 25 µL plasma and 4 mL of filtered seawater to 10 mL Ecolume Scintillation Cocktail. To then quantify the total concentration of urea excreted by fish from [^{14}C]-urea measurements, counts per minute (cpm) in water samples were converted to urea concentrations using plasma specific activity (SA) and these equations (Wood et al. 1997):

$$\text{SA}\ (\mu\text{mol-N cpm}^{-1}) = \frac{\text{total plasma urea concentration}\ (\mu\text{mol-N mL}^{-1})}{\text{plasma [}^{14}\text{C]-urea counts}\ (\text{cpm mL}^{-1})}$$

$$\text{Water urea}\ (\mu\text{mol-N kg}^{-1}) = \frac{\text{water [}^{14}\text{C]-urea}\ (\text{cpm mL}^{-1}) \times \text{SA}\ (\mu\text{mol-N cpm}^{-1}) \times \text{container volume (mL)}}{\text{fish mass (kg)}}.$$

An ELISA kit (ALPCO Diagnostics, Salem, NH, USA) was used to quantify plasma 5-HT concentrations. For *Series I.i.*, plasma 5-HT was quantified in blood samples selected on the criterion that they were collected within 6 h before and after the time of a large, singular urea pulse (> 500 µmol-N kg^{-1}) that did not occur within 15 h of another pulse > 100 µmol-N kg^{-1} ($n = 11$ pulses). To compare these 5-HT fluctuations during pulsing periods to non-pulsing periods, plasma 5-HT was also quantified in samples selected on the criterion that they were taken within 10-h time periods that did not occur within 10 h of a pulse > 100 µmol-N kg^{-1} ($n = 9$ non-pulsing periods). Plasma samples were collected every 2 h on the even hour and water samples were collected hourly. Due to the unpredictable nature of urea pulses, 5 of the 11 urea pulses measured occurred on an even hour, with a plasma sample taken at the time of the start of the pulse, and six urea pulses occurred on an odd hour, with no plasma sample taken at the time of pulse. To account for these discrepancies, 5-HT concentrations were pooled for every 2 h as no significant difference was found between pulses that occurred on even and odd hours. For *Series I.ii.*, plasma 5-HT concentration and plasma [^{14}C]-urea counts from five blood samples taken during 2.5 h of a pulse were converted to a percent change from the value in the first blood sample taken at the beginning of the urea pulse. These values were then separated into six time periods in reference to the start of the pulse.

Water ammonia concentration was determined using the indophenol blue method (Ivancic and Degobbis 1984). The proportion of urea-N (i.e., 2 mol N per mol urea) excreted out of total nitrogen excreted is the percent ureotely. To measure plasma cortisol, a ^{125}I-cortisol radioimmunoassay kit (MP Biomedicals) was used.

Statistics

GraphPad Prism software (Version 7.0a, La Jolla, CA, USA) was used to conduct statistical tests. Data were tested for normality using the D'Agostino–Pearson test or the Shapiro–Wilk test if sample size was too small. For *Series I.i.* coarse time series sampling of 5-HT fluctuation every 2 h, a one-way ANOVA was used with time as the main treatment for pulses and non-pulsing periods. All values were then combined for pulses and non-pulsing periods and compared using a Student's t test. For *Series I.ii.* fine time series sampling plasma variables over the course of a pulse, percent change for 5-HT was log transformed and one-way ANOVAs were used to compare water urea concentration, plasma 5-HT concentration, and plasma [^{14}C]-urea among time periods. For *Series II*, a Student's t test was used to compare agonists and respective controls for total urea, ammonia, and nitrogen, and % ureotely. A two-way ANOVA was used to compare urea excretion over time with time and treatment as the main factors, and if the data could not be transformed to meet the assumptions of normality, a non-parametric

Kruskal–Wallis test was used for each treatment with time as the main factor. A two-way ANOVA with repeated measures was used to compare plasma urea and cortisol between treatments and time. Values are presented as mean ± SEM (*n*).

Results

Series I. Correlating fluctuations in plasma 5-HT with pulsatile urea excretion

Coarse time series: plasma 5-HT measured every 2 h during urea pulsing and non-pulsing periods

There was no significant fluctuation in plasma 5-HT during the 6 h before or 6 h after large pulses (> 500 µmol-N kg^{-1}; $n = 11$ pulses) that occurred in 6 fish ($p > 0.05$; Fig. 1a). All pulses lasted between 1 and 3 h with an average length of 2.2 ± 0.2 h. During non-pulsing periods, no significant differences in plasma 5-HT were measured among time points ($p > 0.05$; Fig. 1b). However, plasma 5-HT concentrations during non-pulsing periods were 1.6 times greater than plasma 5-HT measured within 6 h of a pulse, a significant difference ($p < 0.0001$).

Fine time series: plasma 5-HT measured during urea pulses via real-time urea pulse detection

Using the more sensitive [^{14}C]-urea approach, a total of 6 large pulses were analyzed from six different fish. Similar to *Series i*, all pulses lasted between 1.5 and 3 h with an average length of 1.9 ± 0.2 h. A representative graph of one individual shows plasma 5-HT concentration and [^{14}C]-urea fluctuation during a urea pulse (Fig. 2). On average, as a urea pulse occurred, urea excreted into the surrounding water was significantly elevated at 1 h into the urea pulse ($p < 0.05$; Fig. 3a). Plasma 5-HT concentrations significantly decreased by 38% 1–1.5 h into a urea pulse and then returned to baseline levels after the next half hour ($p < 0.05$; Fig. 3b). Plasma [^{14}C]-urea was significantly reduced by 10% 1.5–2.5 h after a urea pulse ($p < 0.05$; Fig. 3c).

Series II. Determining if additional 5-HT receptors subtypes are involved in stimulating a urea pulse via agonist injections

None of the receptor agonists stimulated a urea pulse as there were no significant differences in urea excretion over time compared to respective controls ($p > 0.05$; Fig. 4). There were also no effects of agonists on urea, ammonia, total nitrogen excretion, or % ureotely ($p > 0.05$), except that fish injected with CP809101 (5-HT_{2C} receptor agonist) excreted about 65% less total nitrogen than controls ($p = 0.037$; Table 1). The receptor agonists also had no effect on plasma urea concentration ($p > 0.05$; Fig. 5). Fish in the EMDT oxalate (5-HT_6 receptor agonist) injection group had overall 1.3-fold higher plasma urea compared to their respective control fish ($p = 0.041$), but this was not due to EMDT oxalate injection as pre- and post-injection plasma urea values were not significantly different ($p > 0.05$; Fig. 5d). Finally, fish injected with RS67506 (partial 5-HT_4 receptor

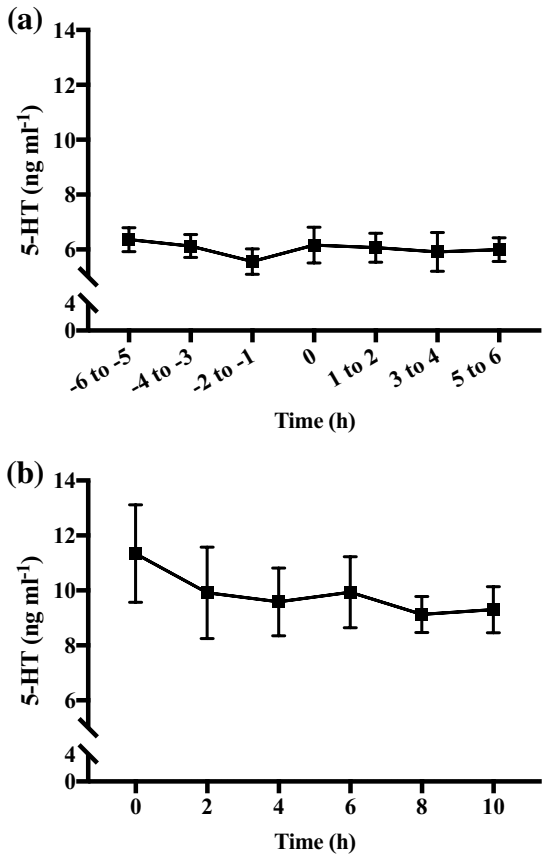

Fig. 1 Plasma 5-HT concentrations (ng mL^{-1}) (**a**) within 6 h of a urea pulse ($t = 0$) ($n = 11$) and **b** during non-pulsing periods ($n = 9$). Values are mean ± SEM

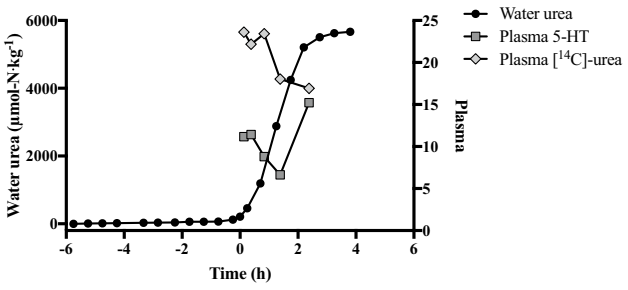

Fig. 2 Representative graph of a urea pulse (left axis) at $t = 0$ and the corresponding plasma values (right axis) for 5-HT concentration (ng mL^{-1}), and [^{14}C]-urea (× 10 cpm $µL^{-1}$)

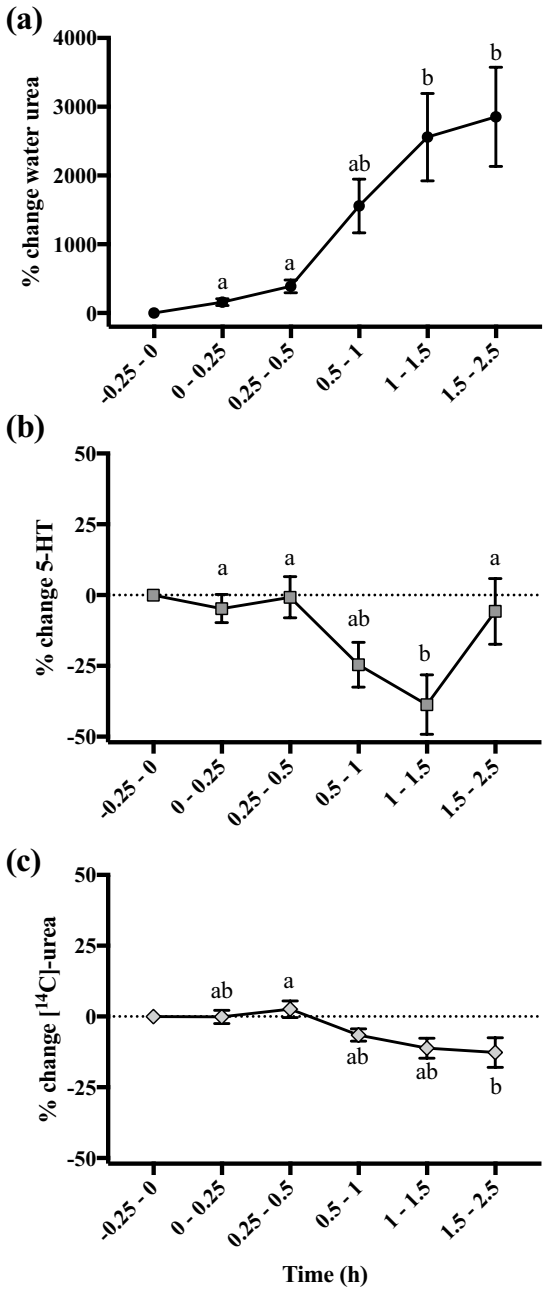

Fig. 3 **a** Water urea concentration, **b** plasma 5-HT concentration, and **c** plasma [^{14}C]-urea from all six pulses are presented as percent change from the initial water or blood sample taken for each fish and are separated into different time periods where $t=0$ is the beginning of a pulse. The sample size for each time period is − 0.25 to 0 h (water $n=6$; plasma $n=3$), 0–0.25 h (water $n=3$; plasma $n=6$), 0.25–0.5 h (water $n=6$; plasma $n=5$), 0.5–1 h (water $n=6$; plasma $n=4$), 1–1.5 h (water $n=6$; plasma $n=6$), and 1.5–2.5 h (water $n=6$; plasma $n=6$). Values are mean ± SEM. Different letters denote significance, $p<0.05$

agonist) and LP12 HCl (5-HT$_7$ receptor agonist) and control fish in the BIMU8 (full 5-HT$_4$ receptor agonist) trial had significantly lower cortisol in the final blood sample compared to the initial sample pre-injection ($p=0.025$, $p=0.004$, $p=0.03$; Fig. 6b, c, e, respectively), but there were no effects of BIMU8, CP809101, and EMDT oxalate on plasma cortisol ($p>0.05$; Fig. 6a, c, d).

Discussion

We hypothesized that an increase in blood 5-HT concentrations prior to or during a urea pulse would be a consequence of pulsatile 5-HT release to activate the pulse, whereas a decrease in circulating 5-HT during or after a urea pulse may be a result of 5-HT clearance from the circulation to terminate the pulse. We were originally unsure if we would be able to detect changes in 5-HT concentrations related to the urea pulse in the blood from the caudal artery or vein given that it was so far downstream from the gill and there could be possible uptake, metabolism, and excretion from other tissues between the caudal vessel and the gill (Amador and McDonald 2018b), which might confound the effect of the urea pulse. For example, the intestine and liver regulate peripheral 5-HT by deaminating circulating 5-HT and releasing 5-HIAA into the blood which is then cleared by the kidney (Caamano-Tubio et al. 2007). We did not find any differences in 5-HT concentrations between the caudal artery and caudal vein, which was expected, because the majority of uptake and clearance do not occur between these vessels (Amador and McDonald 2018b; Caamano-Tubio et al. 2007), and, as discussed below, we were able to detect circulating 5-HT fluctuations that provided information about how a urea pulse is elicited.

With the coarse time series sampling protocol, we investigated whether there would be changes in circulating 5-HT concentrations on a similar timescale as circulating cortisol concentrations. The previous studies have found that 2–4 h before a urea pulse circulating cortisol levels, which are elevated in ureotelic toadfish, decrease by 60%; cortisol concentrations then return to their original, elevated levels after a urea pulse (Wood et al. 1997, 2001). However, as no clear pattern of circulating plasma 5-HT on this time scale could be ascertained in the present study, we suspected that sampling every 2 h lacked the resolution to pinpoint the changes in circulating 5-HT—a conclusion that is supported by our results in the fine time series sampling protocol (below). Plasma 5-HT concentrations measured within 6 h of a large urea pulse were significantly lower compared to non-pulsing periods, which we do not believe are responsible for causing the urea pulse (instead, shorter, transient changes are responsible), but support the idea that 5-HT is maintained at elevated concentrations in between pulses as evidenced below. Furthermore, the reduced 5-HT within 6 h of a pulse with less inter-individual variation may, instead, suggest that 5-HT is under tight control around the time of a urea pulse.

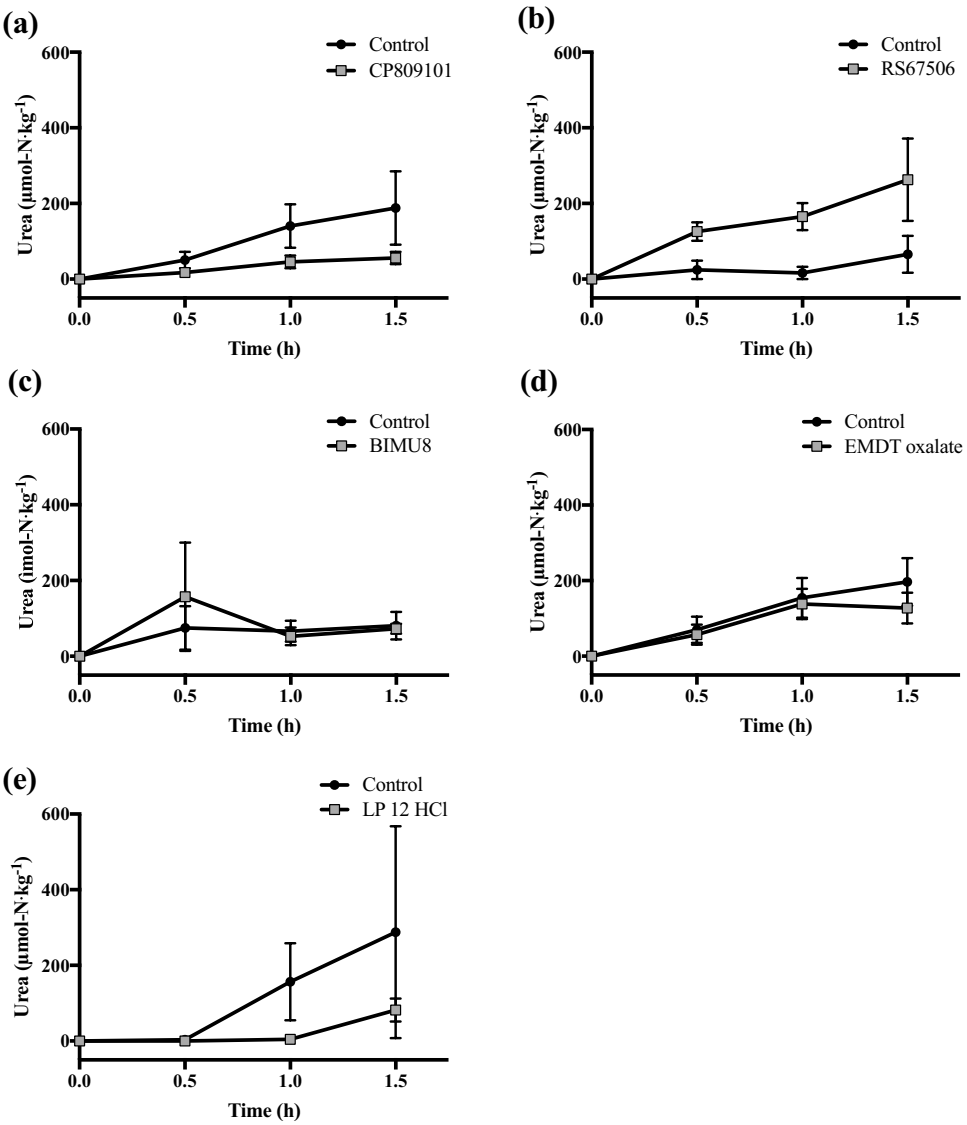

Fig. 4 Total urea excretion after injection at $t = 0$ h for **a** control $n = 6$ and CP809101, a 5-HT_{2C} receptor agonist, $n = 8$; **b** control $n = 2$ and RS67506 (partial 5-HT_4 receptor agonist) $n = 5$; **c** control $n = 4$ and BIMU8 (full 5-HT_4 receptor agonist) $n = 4$; **d** control $n = 8$ and EMDT oxalate (5-HT_6 receptor agonist) $n = 8$; and **e** control $n = 6$ and LP12 HCl (5-HT_7 receptor agonist) $n = 5$. Values are mean ± SEM

Table 1 Water urea excretion, ammonia excretion, total nitrogen excretion, and % ureotely for each 5-HT receptor agonist-treated fish and their respective controls

Treatment (*n*)	Urea (µmol-N kg^{-1})	Ammonia (µmol-N kg^{-1})	Total nitrogen (µmol-N kg^{-1})	% Ureotely
Control (2)	65.8 ± 48.8	38.4 ± 38.4	104.2 ± 10.4	59.1 ± 40.9
RS67506 (5)	267.1 ± 107.5	107.9 ± 30.5	375.0 ± 105.3	65.5 ± 9.8
Control (4)	81.0 ± 36.4	65.0 ± 7.9	146.0 ± 34.1	47.1 ± 16.1
BIMU8 (4)	75.7 ± 14.4	23.9 ± 18.7	99.6 ± 24.7	83.6 ± 12.2
Control (6)	206.3 ± 96.7	71.7 ± 34.1	277.9 ± 99.2	70.3 ± 9.9
CP909101 (8)	57.7 ± 17.0	37.8 ± 23.1	95.5 ± 33.0*	58.0 ± 15.1
Control (8)	200.2 ± 62.2	160.5 ± 65.5	360.7 ± 89.6	62.3 ± 9.1
EMDT oxalate (8)	147.0 ± 40.7	240.7 ± 83.3	387.7 ± 118.2	36.9 ± 10.5
Control (6)	287.8 ± 280.2	21.9 ± 10.0	309.6 ± 282.6	27.0 ± 19.2
LP12 HCl (5)	82.0 ± 30.2	18.0 ± 10.1	99.9 ± 38.4	86.9 ± 6.0

Values are mean ± SEM. Asterisk denotes significant difference from respective control ($p < 0.05$)

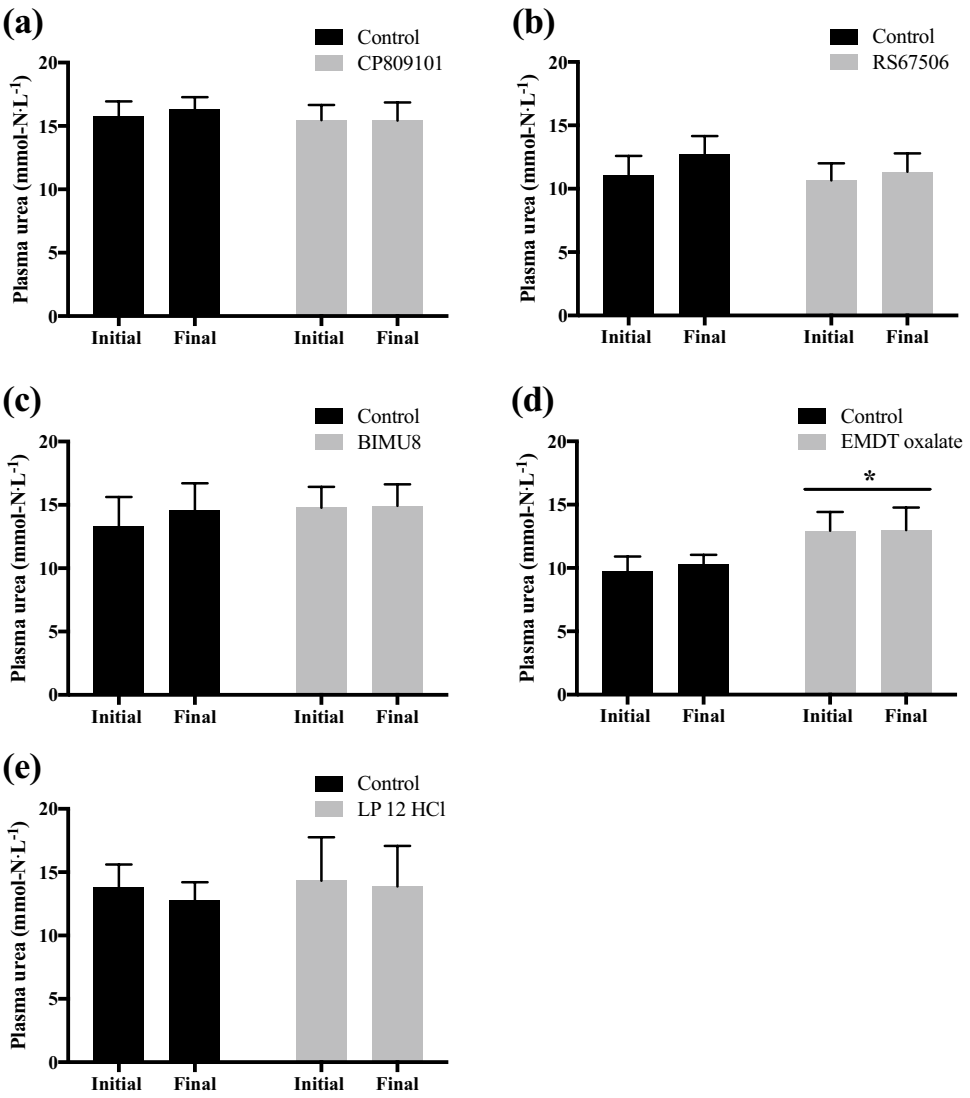

Fig. 5 Plasma urea concentrations in the initial ($t = 0$ h) and final ($t = 1.5$ h) blood samples from fish treated with **a** control $n = 6$ and CP809101 (5-HT_{2C} receptor agonist) $n = 9$; **b** control $n = 6$ and RS67506 (partial 5-HT_4 receptor agonist) $n = 6$; **c** control $n = 6$ and BIMU8 (full 5-HT_4 receptor agonist) $n = 6$; **d** control $n = 9$ and EMDT oxalate (5-HT_6 receptor agonist) $n = 10$; and **e** control $n = 8$ and LP12 HCl (5-HT_7 receptor agonist) $n = 7$. Values are mean ± SEM; asterisk denotes significant differences between treatments; $p < 0.05$

Using the finer time series sampling protocol, we determined that circulating 5-HT significantly decreased when a significant increase in urea excretion was measured 1 h after pulse detection (with urea pulses lasting an average of 2 h) and then returned to initial levels thereafter. In this experiment, blood samples were taken within a short time period and fish were re-injected with saline and RBCs to replace the blood volume after each blood sample; however, it is unlikely that the reduction in 5-HT concentration is due to dilution of 5-HT in the blood from the addition of saline, because an increase in 5-HT was measured at the end of the pulse. The decrease in [^{14}C]-urea measured in the present study supports the conclusion that urea is immediately cleared from the plasma when excreted at the gill (Wood et al. 1997). Since we did not measure an increase of plasma 5-HT at the beginning of the urea pulse, this could suggest that there is no pulsatile release of 5-HT from the gill or other peripheral tissues at the beginning of a urea pulse to activate pulsatile urea excretion. The constant 5-HT concentrations in the systemic blood plasma that were not significantly different up to 6 h before the urea pulse (using our coarse time series sampling protocol) could lend support that there is no pulsatile release of 5-HT from a peripheral source triggering pulsatile urea excretion. However, the hypothesis that circulating 5-HT may increase before a urea pulse due to release from peripheral tissues is difficult to test given that we could not predict the occurrence of a pulse earlier than 15 min beforehand using our fine sampling protocol; there could be a transient increase in plasma 5-HT within 2 h before a urea pulse, which would support the previous studies that found increases in circulating 5-HT cause a urea pulse (McDonald et al. 2012b; Wood et al. 2003). We believe a transient change in plasma 5-HT within 2 h before a urea pulse is possible based on our results that show 5-HT can significantly fluctuate within this time period. Our data also do not rule out the possibility of release of 5-HT

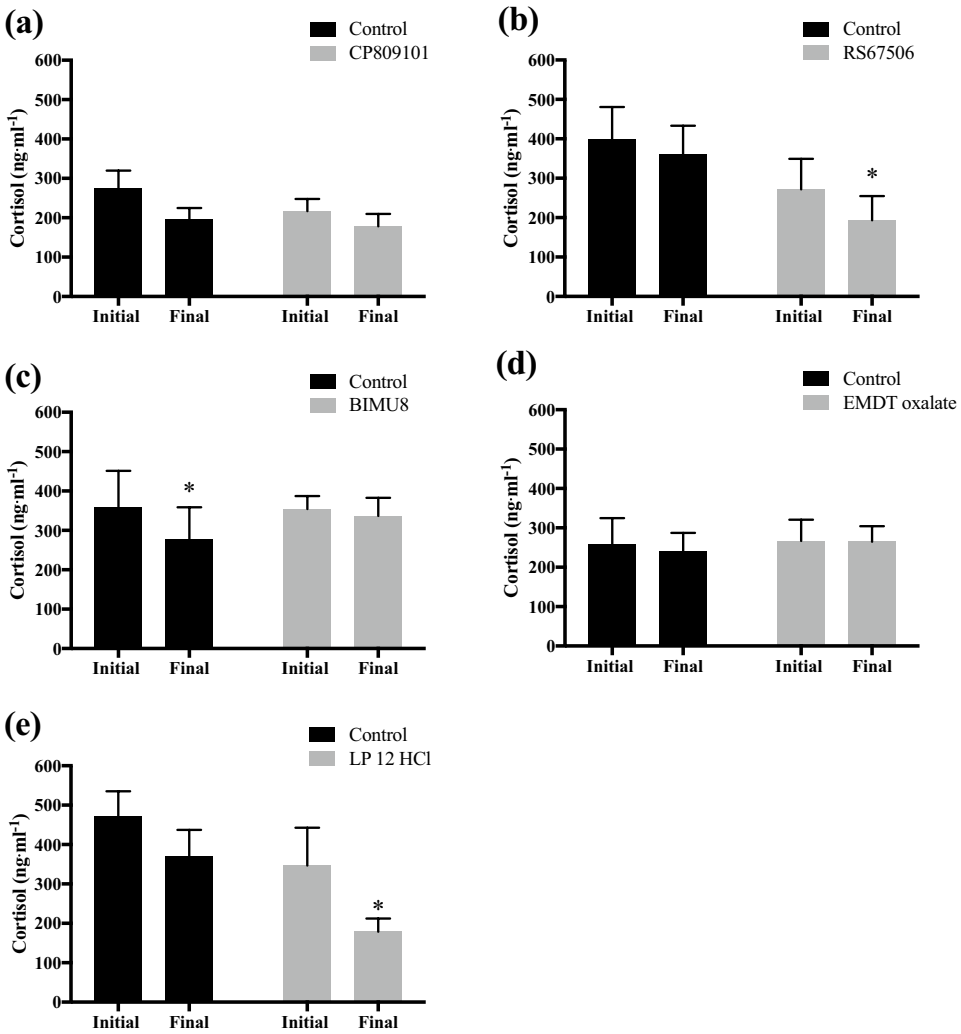

Fig. 6 Plasma cortisol concentrations in the initial ($t = 0$ h) and final ($t = 1.5$ h) blood samples from fish treated with **a** control $n = 6$ and CP809101 (5-HT_{2C} receptor agonist) $n = 9$; **b** control $n = 6$ and RS67506 (partial 5-HT_4 receptor agonist) $n = 6$; **c** control $n = 6$ and BIMU8 (full 5-HT_4 receptor agonist) $n = 6$; **d** control $n = 9$ and EMDT oxalate (5-HT_6 receptor agonist) $n = 10$; and **e** control $n = 8$ and LP12 HCl (5-HT_7 receptor agonist) $n = 7$. Values are mean ± SEM. Asterisk denotes significant differences between the initial and final time points; $p < 0.05$

from sources within the gill and the paracrine action of this release on 5-HT_{2A} receptors and changes in gill levels of 5-HT, its metabolite 5-hydroxyindoleacetic acid (5-HIAA), or 5-HT turnover during a urea pulse, as these have never been tested directly.

Instead, our data suggest that, during a urea pulse, 38% of 5-HT may be removed from circulation, perhaps by active uptake and/or excreted by the gill or another organ, and this removal may act to terminate the urea pulse. Because 5-HT triggers a urea pulse (Wood et al. 2003), our measured drop in plasma 5-HT may reflect a reduced availability of 5-HT leading to the end of a urea pulse. The gill is effective at removing ~ 80% of the 5-HT from arterial circulation through uptake (35%) and metabolism (40%; Olson 1998; Kullman 1994). Furthermore, significant uptake of 5-HT from the circulation by SERT and other non-selective 5-HT transporters has been measured in the toadfish gill, kidney, and heart (Amador and McDonald 2018a, b). The liver, kidney, and gill also play a role in excreting 5-HT and possibly 5-HT metabolites through mostly urine and bile (Amador and McDonald 2018b). During the urea pulse, circulating 5-HT may also be binding to the 5-HT_{2A} receptor, which is responsible for eliciting a urea pulse (Mager et al. 2012; McDonald and Walsh 2004), and, potentially, may be removed from the circulation. It may be possible that the circulating pool of 5-HT, which is in the nM range (reviewed by McDonald 2017), could stimulate the 5-HT_{2A} receptor which has a reported K_d in the nM range for mammals (Bonaventure et al. 2005; Bonhaus et al. 1995; Sleight et al. 1996); however, receptor binding may occur quickly and may not solely account for the reduction in circulating 5-HT measured during a pulse.

Our results from *Series II* indicate that, at the concentration tested, the 5-HT_{2C} receptor is not involved in the control of pulsatile urea excretion based on the ineffectiveness of the selective 5-HT_{2C} receptor (pk_i for 5-HT = 6.8–8.6) (Egan et al. 2000; Kimura et al. 2004; Knight et al. 2004; May et al. 2003) agonist, CP809101, to stimulate a urea pulse. The involvement of the 5-HT_{2B} receptor was not tested in the present study; however, its involvement was considered by

McDonald and Walsh (2004) to be less likely than either of the 5-HT_{2A} and 5-HT_{2C} receptors based on its low affinity to ketanserin, which is in direct contrast to the high sensitivity of the urea pulse to ketanserin in vivo (McDonald and Walsh 2004; Mager et al. 2012). The results of the present study also indicate that the 5-HT_4, 5-HT_6, and 5-HT_7 receptors, which are coupled to cAMP, are not involved in the control of pulsatile urea excretion. Thus, aside from the slight sensitivity of toadfish pulsatile urea excretion to AVT (Wood et al. 2001), there are no other similarities in the control of toadfish and mammalian UTs (Giulietti et al. 2014; McDonald et al. 2012a; Potter et al. 2006). The 5-HT_4, 5-HT_6, and 5-HT_7 receptors also did not mediate any changes in urea production, although fish treated with the 5-HT_6 receptor agonist (EMDT oxalate) had higher plasma urea both before and after injection compared to controls. The large variation in urea excretion, especially for the LP12 HCl control fish, is due to some fish releasing large urea pulses and some fish not pulsing during the 1.5-h monitoring period, which illustrates the unpredictability of urea pulses. While sex was not recorded in this study, this result could be explained if there were more females in the EMDT oxalate group, which have higher plasma urea compared to males (Cartolano et al. 2019a, b).

Based on the previous work, 5-HT can stimulate cortisol release in fish via the 5-HT_{1A}, 5-HT_2, and 5-HT_4 receptors (Lim et al. 2013; McDonald and Walsh 2004; Medeiros et al. 2010; Medeiros and McDonald 2012; Winberg et al. 1997). In the present study, we tested the involvement of 5-HT_{2C}, 5-HT_4, 5-HT_6, and 5-HT_7 receptors in stimulating cortisol secretion; however, none of these receptors appear to be involved in mediating an increase in plasma cortisol in vivo. Although the previous work showed cortisol secretion after injection with α-methyl-5-HT (5-HT_2 receptor agonist) in toadfish, the 5-HT_{2C} receptor agonist used in the present study (CP809101) did not stimulate cortisol release, suggesting that it might be the 5-HT_{2A} receptor and not the 5-HT_{2C} receptor responsible for cortisol release (Medeiros and McDonald 2012). After injection with the partial 5-HT_4 receptor agonist (RS67506) and 5-HT_7 receptor agonist (LP12 HCl), so that circulating levels were approximately 3.3×10^{-5} M, there was a surprising decrease in plasma cortisol within 1.5 h. Since urea excretion is tightly linked to plasma cortisol levels, it is possible that we might have missed an increase in urea excretion associated with this drop in plasma cortisol levels with our current sampling protocol (only up to 1.5-h post-injection and not longer). However, it has been shown in past studies that a drop in cortisol alone (stimulated by treatment with the cortisol production blocker, metyrapone) is not enough to stimulate a urea pulse (Wood et al. 1997, 2001), and so, for the drop in cortisol to result in a urea pulse in the present study, it would have to also be associated with the 5-HT_4 or 5-HT_7 receptor activation. In contrast to the present study, Medeiros and McDonald (2012) measured a stimulation in cortisol secretion when isolated toadfish kidneys were incubated for 2 h in RS670506 at a comparable dose of approximately 1×10^{-5} M (Medeiros and McDonald 2012). The opposing action of 5-HT_4 receptor stimulation in vivo compared to in vitro could be due to a one-time dose via injection versus continuous incubation or due to potential interfering compounds present in vivo versus an isolated system.

The previous research supports the idea that the sustained elevated cortisol before a urea pulse occurs inhibits the activation of the toadfish urea transporter (tUT) by 5-HT through downregulation of the 5-HT_{2A} receptor (Frere and McDonald 2013; Mager et al. 2012; McDonald and Walsh 2004; McDonald et al. 2004; Wood et al. 2003). Our present findings suggest that other likely candidate 5-HT receptors are not responsible for the regulation of pulsatile urea excretion. When cortisol is reduced before the pulse (Wood et al. 1997), the tonic inhibition on the 5-HT_{2A} receptor by cortisol is lifted, potentially allowing the insertion of the 5-HT_{2A} receptor into the basolateral membrane. This is supported by Frere and McDonald (2013) who showed that, when cortisol levels are reduced, the population of 5-HT receptors within the toadfish gill, as measured by binding of the 5-HT_{2A} receptor antagonist, [^{3}H] ketanserin, changes from a low number of high affinity receptors to a high number of low affinity receptors. Furthermore, that study also showed that a larger pulse size is elicited in response to α-methyl 5-HT when cortisol levels are reduced by metyrapone than in control fish with higher cortisol levels (Frere and McDonald 2013). Our present data suggest that circulating (hormonal action) or branchial (paracrine action) 5-HT can then bind to the receptor, triggering the urea pulse. As the urea pulse continues, our data show that 5-HT is removed from circulation during the first hour of a pulse—a decrease that can be detected as far downstream as the caudal vessel that may be a consequence of clearance from the circulation. We suggest that the low levels of 5-HT combined with the return of elevated cortisol levels may result in reduced 5-HT_{2A} receptor activation and/or receptor desensitization, and the termination of a urea pulse; however, any conclusions beyond this point in terms of the mechanisms of 5-HT or cortisol clearance are conjecture and further study is needed. Furthermore, the mechanism by which circulating 5-HT concentrations return to baseline values is not clear, but it could be either through increased production or reduced clearance.

Overall, the present study demonstrates that circulating levels of 5-HT fluctuate during the urea pulse, suggesting the potential hormonal role of 5-HT in the control of pulsatile urea excretion. Future research is needed to understand which mechanisms are responsible for the decreasing 5-HT during a urea pulse. Furthermore, our data indicate that the 5-HT_{2C}, 5-HT_4, 5-HT_6, and 5-HT_7 receptors are not involved

in mediating changes in urea excretion, urea production, or plasma cortisol concentrations. These data may provide insight into the adaptive significance of toadfish pulsatile urea excretion, currently hypothesized to be used for chemically communicating reproductive and social status (Sloman et al. 2005; Fulton et al. 2017; Cartolano et al. 2019a), since circulating 5-HT may also play a role in the control of reproduction (Prasad et al. 2015; Cartolano et al. 2019a) and social interactions (McDonald et al. 2011).

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