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A waterborne chemical cue from Gulf toadfish, *Opsanus beta*, prompts pulsatile urea excretion in conspecifics

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HIGHLIGHTS

- We hypothesized that pulsatile urea excretion in naïve toadfish could be triggered by waterborne cues from a conspecific
- Naïve fish pulse urea within 7 h when exposed to seawater that held a conspecific and 20 h in response to clean seawater
- Ammonia but not urea, cortisol of 5-HT could be one component of a multi-component cue
- Further studies are needed to fully identify the chemical cue as well as determine the adaptive significance

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ABSTRACT

The Gulf toadfish (*Opsanus beta*) has a fully functional ornithine urea cycle (O-UC) that allows it to excrete nitrogenous waste in the form of urea. Interestingly, urea is excreted in a pulse across the gill that lasts 1–3 h and occurs once or twice a day. Both the stress hormone, cortisol, and the neurotransmitter, serotonin (5-HT) are involved in the control of pulsatile urea excretion. This and other evidence suggests that urea pulsing may be linked to toadfish social behavior. The hypothesis of the present study was that toadfish urea pulses can be triggered by waterborne chemical cues from conspecifics. Our findings indicate that exposure to seawater that held a donor conspecific for up to 48 h (pre-conditioned seawater; PC-SW) induced a urea pulse within 7 h in naïve conspecifics compared to a pulse latency of 20 h when exposed to seawater alone. Factors such as PC-SW intensity and donor body mass influenced the pulse latency response of naïve conspecifics. Fractionation and heat treatment of PC-SW to narrow possible signal candidates revealed that the active chemical was both water-soluble and heat-stable. Fish exposed to urea, cortisol or 5-HT in seawater did not have a pulse latency that was significantly different than seawater alone; however, ammonia, perhaps in the form of NH_4Cl, was found to be a factor in the pulse latency response of toadfish to PC-SW and could be one component of a multi-component cue used for chemical communication in toadfish. Further studies are needed to fully identify the chemical cue as well as determine its adaptive significance in this marine teleost fish.

1. Introduction

The Gulf toadfish (*Opsanus beta*) has a fully functional ornithine-urea cycle (O-UC) and is one of the few species of teleost fish that as mature adults can excrete their nitrogenous waste primarily in the form of urea (ureotely; [7,72]). In nature, toadfish excrete 50% urea and 50% ammonia; the excretion of urea acting as a 'chemical cloak' by partially masking the scent of ammonia from predators [9]. In the laboratory, nearly 100% ureotely can be induced in toadfish by means of crowding and/or confinement and the associated stress results in an increase in circulating levels of the stress hormone, cortisol [25,83,86], an upregulation in O-UC enzymes and urea production [25,31,74], and an increase in toadfish facilitated diffusion urea transporter (tUT) transcript in the gill [40,52,73]. Urea is then excreted in a distinct pulse across the gill, once or twice a day, for up to 3 h *via* tUT [80–83]. Cortisol plays an additional role in the control of pulsatile urea excretion; namely, two to 2-4 h before a urea pulse plasma cortisol levels decrease, a urea pulse occurs, and plasma cortisol levels rise rapidly thereafter [83,86]. The decrease in cortisol does not directly induce a urea pulse event [43,53,83,84,86] but instead, the drop in cortisol is believed to allow

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the monoamine neurotransmitter, serotonin (5-HT; 5-hydroxytryptamine), to directly stimulate urea excretion *via* a specific 5-HT receptor-mediated pathway [23,34,41,42,84].

Interestingly, in addition to the control of pulsatile urea excretion, both cortisol and 5-HT are well-documented for their role in the social behavior of fish. Namely, within a social hierarchy, subordinate fish are characterized by elevated circulating levels of cortisol and brain 5-HT turnover, elevated levels of the 5-HT metabolite, 5-hydroxyindoleacetic acid (5-HIAA), and elevated 5-HIAA/5-HT ratios (reviewed by [62]). The neurochemical link between toadfish pulsatile urea excretion and social behavior has led to the consideration that toadfish urea pulsing may be a means of chemical communication between conspecifics. Chemical cues are defined as stimuli that elicit a sensory response in a conspecific that, unlike chemical signals, is not necessarily associated with specialized or evolved neural mechanisms (reviewed by [80]). They can be passed between aquatic organisms for a variety of reasons: to indicate alarm or disturbance ([15,21,30,46,47,66,79]; reviewed by [78]), reproductive or social/territorial status [2,4–6,19,35,36,45,48,76], and for aggregation and migration [22,63–65,69]. Chemical cues can be derived from bile acids, steroids and prostaglandins, amino acids or peptides, nucleotides, released alone or in combination and are dispersed using a wide variety of routes amongst individuals or to entire populations of conspecifics (reviewed by [70]; reviewed by [80]).

The hypothesis that urea pulsing may be a means of chemical communication between toadfish was first tested by Sloman et al. [60] by examining the behavior and the pattern of pulsatile urea excretion in size-matched toadfish housed in the same aquarium. In that study, it appeared that social status did not affect pulsatile urea excretion, that is, neither the fish that eventually was designated as the dominant nor the ultimate subordinate consistently pulsed first nor was there any difference in urea pulse size based on ultimate social status. However, independent of social position, the timing of the urea pulse of the second fish in a pair was dependent on the pulsing of the first fish; in seven out of eight pairs, the second fish pulsed within 2 h of the first, compared to <10% of pulses occurring within any two hour period when toadfish were held apart [60,86]. That pulsatile urea excretion influences urea excretion in another fish suggests that a chemical cue is present within the urea pulse.

The goal of the present study was to further investigate the potential role of pulsatile urea excretion as a means of chemical communication in toadfish by testing the hypothesis that waterborne substances from one ureotelic toadfish could induce pulsatile excretion in another toadfish isolated from other sensory cues. Naïve toadfish were exposed to seawater that previously held a conspecific (pre-conditioned seawater; PC-SW) and the time it took for the naïve toadfish to pulse (pulse latency) was compared to toadfish held in clean seawater. The potential for heat-treated or solid-phase extracted PC-SW as well as certain individual substances, specifically urea, ammonia, cortisol and 5-HT, to induce pulsatile urea excretion in naïve toadfish was also tested.

2. Materials and methods

2.1. Experimental animals

For experiments conducted at the University of Ottawa, Gulf toadfish, *Opsanus beta* (Goode and Bean), were purchased from Gulf Specimen Marine Laboratory (Panacea, FL) and were express delivered in individual 5 L plastic bags housed within an insulated shipping container to Ottawa, ON, Canada. For experiments conducted at the University of Miami Rosenstiel School of Marine and Atmospheric Science (RSMAS), toadfish were collected from Biscayne Bay by commercial shrimp trawlers (Florida Fish and Wildlife Conservation Commission Special Activity License #SAL-12-0729-SR). Upon arrival at either location, fish were treated with a freshwater dip (10 L distilled water and 500 mL seawater, for 3 min), followed by a treatment of formalin (15 $mg \cdot L^{-1}$) for 1 h to prevent infection by the ciliate *Cryptocaryon irritans* as described previously [71]. In the laboratory, toadfish were transferred to glass aquaria (80 L) with re-circulated, aerated seawater (32 ppt) maintained at 23 °C with a 12/12 light-dark photoperiod. Toadfish were acclimated in these aquaria for at least seven days prior to the start of experimentation. Fish were fed raw squid weekly, and food was withheld two days prior to the start of the experiment and throughout the remainder of the experiment.

Fish used in the following experiments ranged in mass from 0.055 kg to 0.345 kg (average mass 0.166 kg ± 0.014 kg; $N = 117$). In our experience, toadfish of this size range are sexually mature and exhibit dominance behaviors. All experiments were carried out at 23 ± 0.5 °C and were in accordance with the Canadian Council of Animal Care (CCAC) guidelines and with the approval of the University of Ottawa Animal Care Committee (Protocol BL-255) and the University of Miami Animal Care and Use Committee (#A-3224-01).

2.2. Experimental manipulations

2.2.1. Series i: response to PC-SW

Ureotely was induced in toadfish *via* a crowding/confinement protocol (6 toadfish in one 8 L aquaria, density = ~90 $g \cdot L^{-1}$; [74]) following a 48 h fasting period. Crowded ureotelic toadfish were then transferred into individual aquaria (3 L) for 48 h to collect all excretions (*i.e.*, to pre-condition the seawater). To determine whether a naïve toadfish can recognize and respond to PC-SW from an ureotelic toadfish, naïve toadfish (*i.e.*, fish that had previously only been housed in the 80 L aquaria at low density [6 toadfish in 80 L aquaria], had not been previously exposed to PC-SW and were not ureotelic as ureotelism is induced by crowding and/or confinement; [25,31,74]) were placed in 3 L of PC-SW and water samples (2 mL) were collected at one-hour intervals over a 48 h treatment period *via* PE60 tubing attached to fraction collectors (100/120 V-2110 fraction collector, BioRad, Mississauga, ON). Water samples were transferred to the fraction collectors by peristaltic pump (Minipuls 3 peristaltic pump, Gilson Inc. Middleton, WI). All experimental fish were held in the same aquaria for the duration of the experiment and visual barricades (opaque foam blocks) were put in place to remove any possibility that visual cues may be used to signal pulsing events. In parallel, control experiments were carried out by transferring naïve fish into 3 L aquaria containing unaltered fresh seawater. Following water collection, samples were placed at 4 °C and analyzed for ammonia and urea concentrations no later than one week after collection and pulse latency (time-to-first-pulse in h) in the naïve fish was calculated.

2.2.2. Series ii: dilution of PC-SW

To identify whether there is a specific threshold of chemical cue needed to trigger a pulsing event, a dilution experiment was carried out. Two toadfish were placed in a 6 L aquarium supplied with aerated seawater (replenished every 24 h) for 72 h to pre-condition the seawater. Naïve fish were individually placed in the aerated exposure tanks (3 L), of varying PC-SW concentrations (0, 0.17, 1.7, 3.3, 8.3, 16.7, 33.3, 100%), and were housed for a 48 h exposure period. Water samples (2 mL) were collected hourly over the entire 48 h experiment *via* fraction collectors, stored and analyzed as described above.

2.2.3. Series iii: manipulation of PC-SW

Collection and pooling of PC-SW was carried out as described in *Series ii* using two large toadfish with an average weight of 0.118 kg. The PC-SW was divided into two aliquots; the first was unaltered and stored at 4 °C for approximately 2 h and the second was heat-treated for 60 min in a 90 °C water bath (Isotemp 220, Fisher Scientific, Toronto, ON). Naïve fish were then separated into three groups; one group was exposed to clean seawater (negative control), another to unaltered PC-SW (positive control), and a third to heat-treated PC-SW. A second collection of PC-SW was carried out using two large toadfish with an average weight of 0.168 kg. The PC-SW was divided into two aliquots; the

first was unaltered and stored and the second was passed through an octadecylsilane solid phase extraction cartridge (Sep-Pak C18; Waters, Milford, MA) to remove non-polar compounds. Non-polar compounds from the PC-SW extracted by the C18 column were collected by elution with (0.25 L) methanol and the methanol fraction was then dried under vacuum and the residue was reconstituted in 8 L of seawater. Naïve fish were separated into three groups; one group was exposed to clean seawater (negative control), another to the non-polar fraction (the methanol eluate from the C18 column) of PC-SW, a third to the polar fraction (the aqueous eluate from the C18 column) of the PC-SW and a fourth to unaltered PC-SW (positive control). Water samples (2 mL) were collected hourly over the entire 48 h experiment *via* fraction collectors, stored and analyzed as described above.

2.2.4. Series iv: neurochemical or metabolite exposure

Since cortisol and 5-HT are highly involved in controlling the pulsatile urea excretion mechanism of toadfish and PC-SW contains urea and a certain amount of ammonia ([42]; reviewed by [84]), the ability of these compounds to elicit a urea pulse on their own when placed in seawater was tested. The cortisol concentration (10 $mg \cdot L^{-1}$) used in this experiment was predicted to be the amount of cortisol one toadfish may excrete into its environment based on average plasma cortisol levels [25,40,43,53,83,86]. The 5-HT concentration (3 $\mu mol \cdot L^{-1}$) was based on average plasma 5-HT levels as well as the concentration needed to elicit a response *via* intra-arterial injection [10,84]. The concentration of urea (100 $\mu mol\text{-}N \cdot L^{-1}$) was chosen from previous studies reporting the typical size of a urea pulse and the ammonia concentration (50 $\mu mol\text{-}N \cdot L^{-1}$, added as NH_4Cl) was chosen from previous studies reporting ammonia excretion rates [25,81]. Naïve toadfish were then exposed to a one-time dose of cortisol, 5-HT, urea or ammonia, after 2 h of background water sampling. Water samples were collected on a 30 min time interval for the initial 6 h post injection after which water collection was carried out on an hourly basis. Samples were then stored at 4 °C and analyzed no longer than one week post experimentation for urea and ammonia concentrations.

2.2.5. Series v: the impact of PC-SW intensity and donor body mass

To determine the effect of body mass on pulse latency, a "small donor" (0.085 kg) and a "large donor" (0.393 kg) were held individually in 3 L aquaria to pre-condition the seawater. Urea was used as a proxy measurement to quantify the 'intensity' of the pulse because the actual chemical cue(s) is (are) currently unknown. The first treatment was PC-SW collected from the small donor 72 h after individual confinement that contained 10 $\mu mol\text{-}N \cdot L^{-1}$ urea, the second treatment was PC-SW collected from the large donor 12 h after individual confinement that contained 200 $\mu mol\text{-}N \cdot L^{-1}$ urea and the third treatment was PC-SW collected from the large donor 72 h after individual confinement that contained 3000 $\mu mol\text{-}N \cdot L^{-1}$ urea. Naïve fish of various sizes (0.084 to 0.360 kg) were exposed to PC-SW for 48 h and water sampled as described above. With this design it was possible to elucidate whether a large donor elicits a different response from conspecifics compared to a small donor and to also determine whether large naïve fish react differently to the chemical cue compared to small naïve fish.

2.3. Analytical techniques and calculations

Water urea concentrations were quantified using a diacetyl monoxime method adapted from Rahmatullah and Boyd [50]. Water ammonia concentrations were quantified by the indophenol blue method adapted from Ivancic and Degobbis [29]. Samples were assayed in duplicate then analyzed on a spectrometer (SpectraMax plus384 absorbance microplate reader, Molecular Devices Corporation, Sunnyvale, CA) using SoftMax Pro V5 analysis software (Molecular Devices Corporation).

Urea and ammonia excretion values ($\mu mol\text{-}N \cdot kg^{-1}$) were calculated using the equation:

$$\text{Excretion} = (\Delta C \cdot V_f) \cdot M^{-1}$$

in which ΔC represents the change in water urea or ammonia concentration ($\mu mol\text{-}N \cdot L^{-1}$), V_f is the volume (L) of the experimental container and M is the body mass (kg). To obtain urea-N values urea concentrations were multiplied by two as urea contains two N. The percent ureotely was determined as the amount of urea excreted ($\mu mol\text{-}N \cdot kg^{-1}$) divided by the total nitrogen excreted. A sudden appearance of urea in the sampled water that exceeded a threshold of 100 $\mu mol\text{-}N \cdot kg^{-1}$ was defined as a urea pulse as outlined by McDonald et al. [43]. Pulse latency was calculated as the time (h) to pulse following exposure to experimental water.

2.4. Statistical analysis

Data are reported as means ± 1 standard error of the mean (S.E.M.) (N = number of fish). Figures are presented as cumulative nitrogen excreted ($\mu mol\text{-}N \cdot kg^{-1}$) over a 48 h period as well as means ± 1 S.E.M. of first pulsing event (pulse latency) and % ureotely. Unpaired two-tailed Student's *t*-test or a one-way ANOVA with a Holm-Sidak multiple comparisons test was performed using commercial software (SigmaPlot 10.1, SPSS) to detect significant differences between toadfish exposed to varying experimental conditions compared to fresh seawater (control). The Mann-Whitney test was used to detect differences between means when groups did not exhibit equality of variance. Alpha = 0.05 was used as the limit of significance.

3. Results

Naïve toadfish exposed to PC-SW had a significantly lower pulse latency, pulsing almost 3-fold faster when placed in PC-SW (6.7 ± 1.7 h, N = 6) compared to individuals placed in seawater alone (19.7 ± 3.5 h, N = 12; Fig. 1). Toadfish that were exposed to increasing intensities of PC-SW demonstrated a decrease in pulse latency (Fig. 2). A significant decrease in pulse latency compared to 0% PC-SW (*i.e.*, seawater control) was first measured when toadfish were exposed to 8.3% PC-SW with a further decrease in pulse latency when exposed to 16.7% PC-SW or greater (Fig. 2A). Similarly, a significant increase in % ureotely was measured in fish that were exposed to concentrations of PC-SW that were at least 33.3% of the original chemical cue or higher (Fig. 2B).

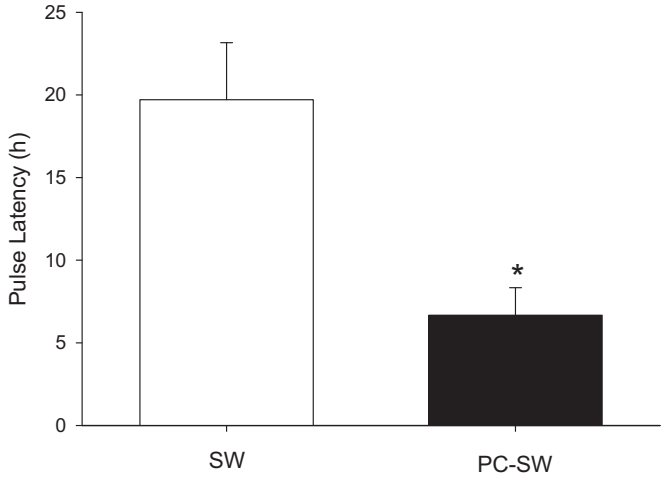

Fig. 1. Urea pulse latency of toadfish exposed individually to seawater control (SW) or PC-SW from a conspecific. Data are means ± 1 SEM; *indicates a statistically significantly difference from SW values.

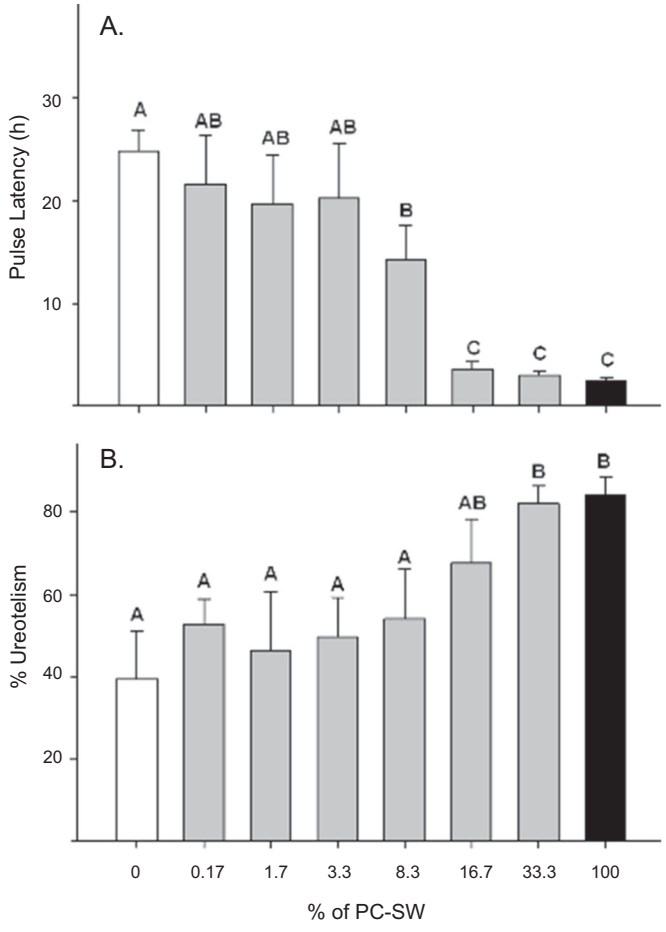

Fig. 2. (A) Urea pulse latency of toadfish exposed individually to varying dilutions of PC-SW. (B) % ureotely of toadfish exposed individually to varying dilutions of PC-SW. Data are shown as means ± 1 SEM; different letter indicates statistically significant differences amongst treatments.

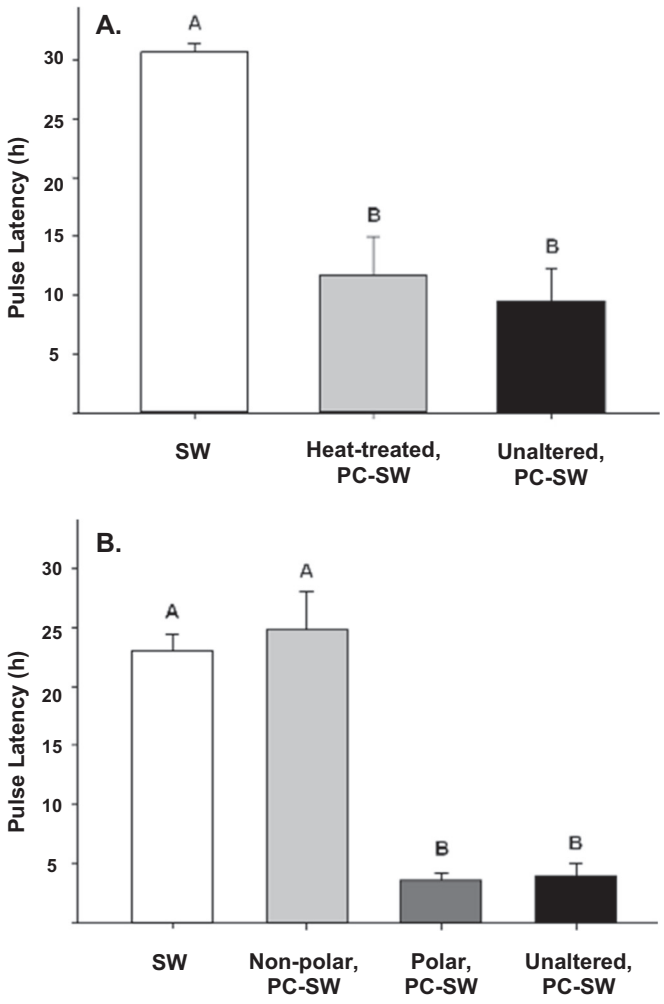

Fig. 3. (A) Pulse latency of toadfish exposed individually to seawater control (SW), heat-treated PC-SW, and unaltered PC-SW. (B) Pulse latency of toadfish exposed individually to SW, the non-polar fraction of PC-SW, the polar fraction of PC-SW or unaltered PC-SW. Data are shown as means ± 1 SEM; different letters indicates statistically significant differences amongst treatments.

The pulse latency of fish exposed to heat-treated PC-SW was not significantly different from the pulse latency response of fish to unaltered PC-SW and exposure to either resulted in fish pulsing approximately 2.5-times faster than fish place in seawater alone (Fig. 3A). Exposure to the polar fraction of PC-SW (3.8 ± 0.6 h, $N = 18$) resulted in a significantly lower pulse latency when compared to control seawater exposure (Fig. 3B). In contrast, pulse latency in toadfish exposed to the non-polar fraction of PC-SW was not different than control seawater alone (Fig. 3B). No significant differences in pulse latency were detected between fish exposed to waterborne cortisol or 5-HT compared to control individuals exposed to seawater alone (Fig. 4). Furthermore, no significant differences in pulse latency were detected between fish exposed to waterborne urea or control seawater (Fig. 5A). However, when exposed to ammonia, toadfish pulsed urea 1.4-times faster than control fish (Fig. 5B). Along these lines, when taking into account the ammonia concentration of all PC-SW trials, there was a significant correlation between ammonia concentration and pulse latency, with a higher ammonia concentration in the water prior to a urea pulsing event from naïve toadfish exposed to PC-SW resulting in a lower pulse latency (Fig. 5C).

Body mass as well as the intensity of the PC-SW influenced the pulse latency response of naïve toadfish (Fig. 6). There was no significant correlation between pulse latency and naïve fish body mass when naïve toadfish were exposed to clean seawater ($r^2 = 0.033$, $P = 0.5$, Fig. 6A). Furthermore, the pulse latency of small naïve fish (<0.115 kg; 14.0 ± 9.2 h, $N = 3$) exposed to clean seawater was not significantly different than that of large naïve fish (>0.115 kg; 21.8 ± 3.5 h, $N = 13$).

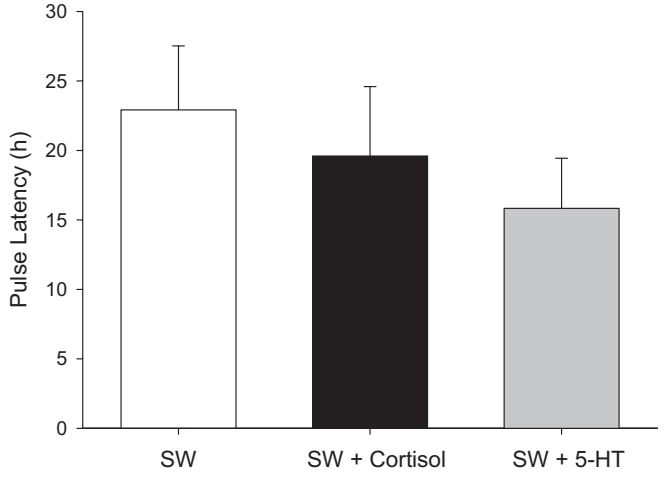

Fig. 4. Pulse latency of toadfish exposed individually to 10 mg·L^{-1} of waterborne cortisol or 3 μmol·L^{-1} 5-HT compared to seawater control (SW). Data are shown as means ± 1 SEM.

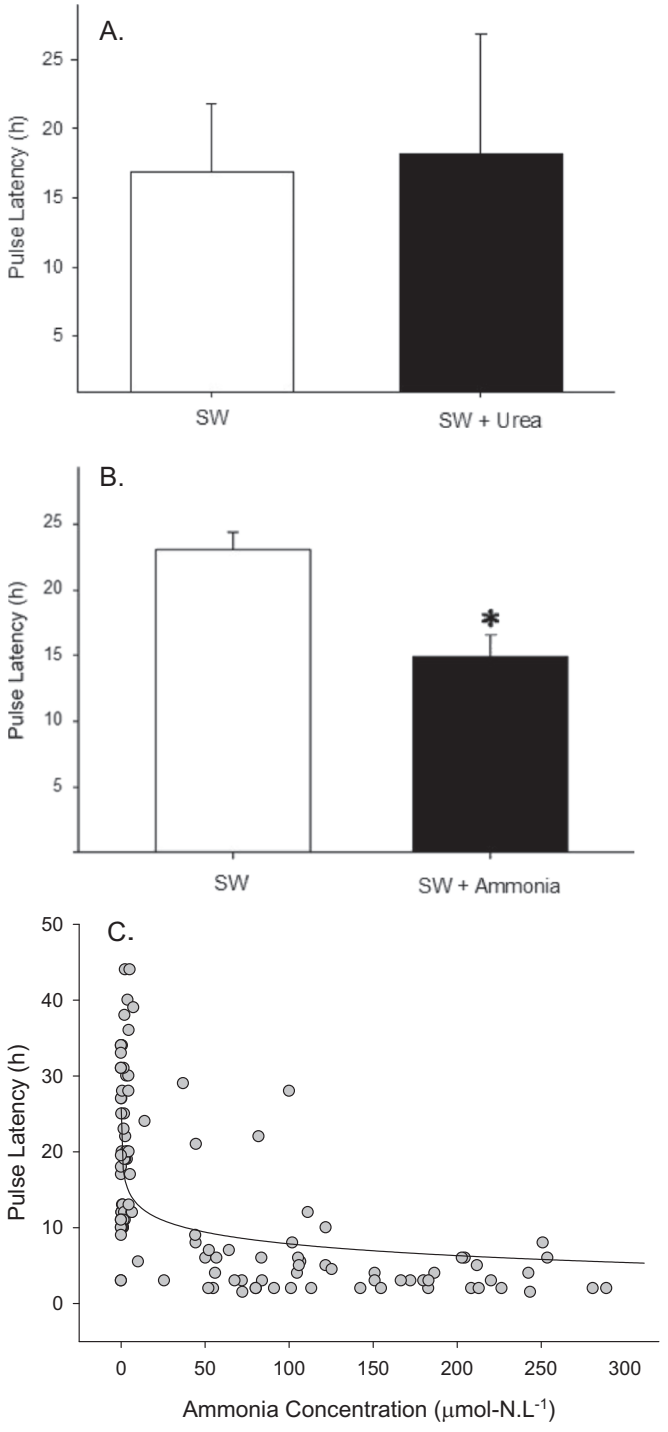

Fig. 5. (A) Pulse latency of toadfish exposed individually to 100 μmol-N·L^{-1} urea or (B) 50 μmol-N·L^{-1} of waterborne ammonia compared to seawater control (SW). Data are shown as means ± 1 SEM; *indicates a statistically significant difference from SW. (C) When taking into account the ammonia concentration of all PC-SW trials, there is a clear relationship between ammonia concentration (μmol-N·L^{-1}) and naïve fish pulse latency (h) ($y = -2.23 \ln(x) + 18.16$, $r^2 = 0.299$; $N = 117$, $P < 0.0001$).

However, there was a significant correlation between pulse latency and naïve fish body mass whether naïve fish were exposed to PC-SW that contained 10 μmol-N·L^{-1} urea from a small donor fish ($r^2 = 0.604$, $P = 0.007$; Fig. 6B) or exposed to PC-SW that contained 200 μmol-N·L^{-1} urea from a large donor fish ($r^2 = 0.612$, $P = 0.0001$; Fig. 6C). Overall, small naïve fish had a low pulse latency when exposed to PC-SW, a response that was independent of the size of the donor fish or the intensity, measured as urea concentration, of the PC-SW (Fig. 6B–D). Specifically, for small naïve fish, pulse latency was 5.4 ± 1.5 h ($N = 5$) in response to PC-SW that contained 10 μmol-N·L^{-1} urea from a small donor fish, and 5.7 ± 1.5 h ($N = 6$) and 8.5 ± 2.5 h ($N = 2$) when exposed to PC-SW that contained 200 or 3000 μmol-N·L^{-1} urea from a large donor fish, respectively. Furthermore, the pulse latency of small naïve fish exposed to 10 μmol-N·L^{-1} urea from a small donor fish or 200 μmol-N·L^{-1} urea from a large donor fish was significantly lower than the response of large naïve fish (Fig. 6B and C). In contrast, pulse latency in response to PC-SW appeared to be dependent on both the size of the donor fish as well as intensity of the PC-SW amongst larger naïve fish (Fig. 6B–D). Larger naïve fish responded with a high pulse latency to PC-SW containing 10 μmol-N·L^{-1} urea from a small donor fish (38.8 ± 1.6 h, $N = 10$; Fig. 6B) but responded with a significantly lower pulse latency to PC-SW with 200 μmol-N·L^{-1} urea from a large donor fish (13.2 ± 2.0, $N = 12$; Fig. 6C) that was further reduced (5.75 ± 2.3, $N = 4$; Fig. 6D) when the chemical cue intensity increased to 3000 μmol-N·L^{-1} urea.

4. Discussion

In the present study, the consistent response of a lower urea pulse latency in Gulf toadfish exposed to seawater that previously held a conspecific (*i.e.*, PC-SW) compared to control fish held in clean seawater supports the hypothesis of a waterborne chemical cue for this species. As toadfish were held individually in shielded chambers, the waterborne chemical cue alone was able to prompt a physiological response (the induction of ureotelism and a large urea pulse within approximately 7 h of PC-SW exposure) without additional visual, auditory or tactile cues. This pulse latency is higher than that measured when toadfish are allowed to fully interact in the same aquarium; Sloman et al. [60] demonstrated that one toadfish will respond to the urea pulse of the other fish within 2 h when allowed to fully interact. Combined, these data suggest that while toadfish are sensitive to the chemical cue alone, other sensory stimuli (visual, auditory and tactile) may be important contextual cues that allow the fish to assign relevance and interpret whether the cue is reproductive (inter-sexual) or social/territorial (intra-sexual) [24,36].

Chemical cues can be released through the urine, intestinal fluid, gill and/or glandular secretions (reviewed by [68]). Freshwater fish have been well-documented to use the urine to transmit chemical cues for the purpose of signaling social/territorial or reproductive status, and oftentimes the frequency of urine pulses increases when freshwater fish are presented with the appropriate context [2,4–6,35,36,55]. In a marine environment dehydration is a constant challenge; consequently, urine flow rates in marine fish are approximately 10% those of freshwater fish (reviewed by [37]). These low urine flow rates in marine fish could possibly limit the transmission of chemical cues *via* the urine and chemical communication *via* a mechanism other than the urine has been documented in several cases (reviewed by [27]). Specifically, male sea lamprey (*Petromyzon marinus*), which are predominantly marine but spawn in freshwater, secrete a bile acid that acts as a sex pheromone *via* a specific transport mechanism across the gill [33,59]. Secretions from the anal gland of male peacock blennies (*Salaria pavo*), which have similar male parental behavior as toadfish, have been shown to attract females ([57]; reviewed by [27]). Eel skin mucus or bile may act as sexual attractants ([28]; reviewed by [27]) and Pacific herring (*Clupea harengus pallasi*) milt allows for spawning synchronization ([18,67]; reviewed by [27]).

Similar to these other marine fish, we hypothesize that the cue present in PC-SW from ureotelic toadfish is transmitted across the gill and not in the urine, as a consequence of limited urine production [39,44]. Indeed, pulsatile excretion of urea occurs in a hormonally-controlled manner across the gill and not *via* the urine or another pathway [82,83]. Furthermore, pulsatile urea excretion appears to be coordinated when two toadfish are allowed to fully interact [60]. Therefore, the chemical cue may be released *via* the same mechanism or at the same

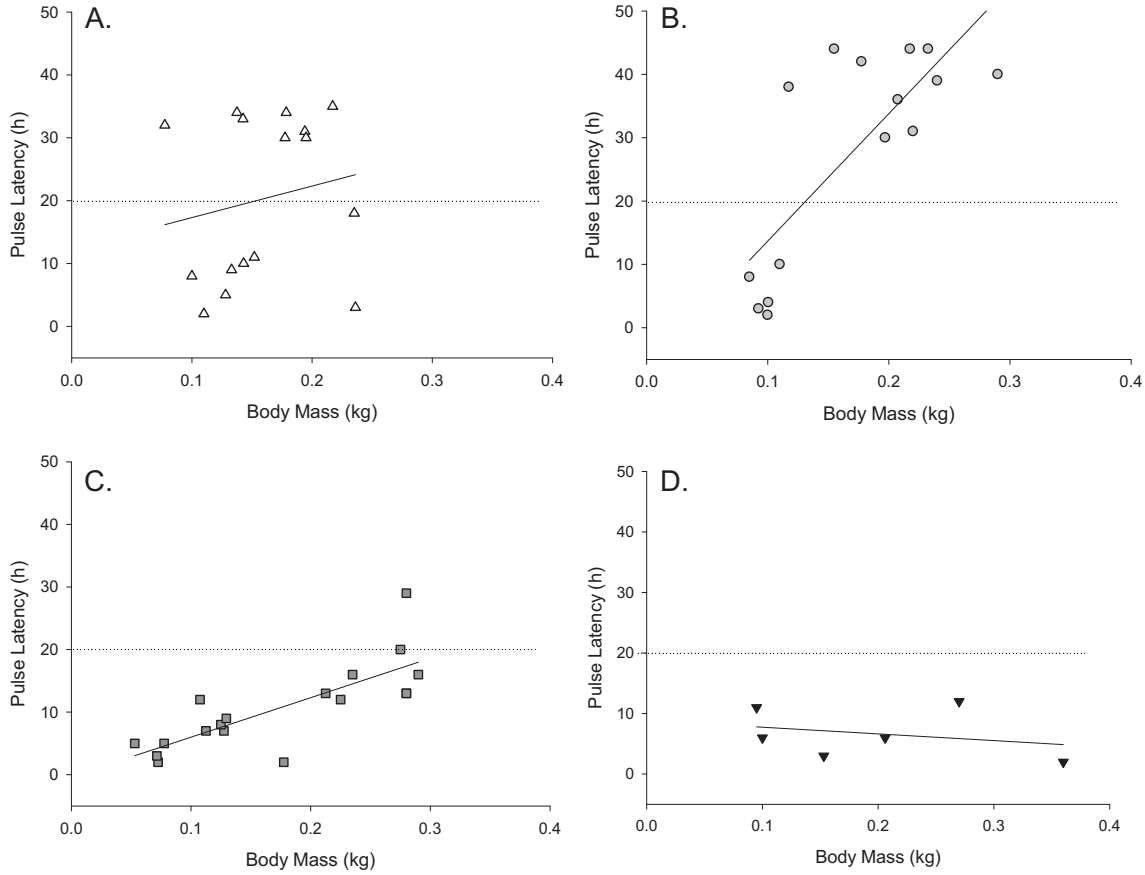

Fig. 6. Relationship of pulse latency to naïve fish body mass grouped by concentration of urea in PC-SW. (A) Naïve fish held in SW control with 0 μmol-N·L^{-1} urea ($y = 50.12x + 12.30$, $r^2 = 0.033$, $P = 0.5$; $N = 16$); (B) naïve fish exposed to PC-SW from a small donor fish with 10 μmol-N·L^{-1} urea ($y = 201.31x - 6.46$, $r^2 = 0.604$, $P = 0.007$; $N = 16$); (C) naïve fish exposed to PC-SW from the large donor fish with 200 μmol-N·L^{-1} urea ($y = 63.05x - 0.30$, $r^2 = 0.612$, $P = 0.0001$; $N = 16$); (D) naïve fish exposed PC-SW from the large donor fish with 3000 μmol-N·L^{-1} urea ($y = -11.04x + 8.85$, $r^2 = 0.079$, $P = 0.590$; $N = 6$). Dotted line signifies average response to SW control measured in panel A.

time as the urea pulse. Before being exposed to PC-SW, naïve fish in the present study were housed in uncrowded conditions and, under those conditions, are not found to be ureotelic [25,74]. The delayed pulse latency response of naïve fish exposed to PC-SW could be partially due to the time required to upregulate the pulsatile urea excretion mechanisms necessary to respond [25,31,40,52,73,74]. However, the possibility that the cue is not part of the pulsatile urea excretion process but transmitted *via* some other pathway such as urine, intestinal fluid or glandular secretions was not directly tested and should be ruled out in future studies.

The detection and recognition of a chemical cue in an aqueous environment is complicated by the dilution and disturbance of the cue as it is transmitted through water ([77]; reviewed by [68]). In the present study, ≥8.3% of PC-SW was able to elicit a significant decrease in urea pulse latency in naïve fish, suggesting that cue detection would occur in a natural setting within a limited transmission range. Prior ecological surveys have shown that toadfish are highly abundant within *Thalassia* seagrass beds of Florida Bay, with densities of on average one fish per square meter [61,56]. During the spawning season, toadfish males are known to attract females by singing at nesting sites, typically large gastropod shells or other sheltered areas with only one opening [12,13], increasing the potential for the chemical cue to be concentrated. Whether toadfish optimize the efficiency of chemical transmission, for example, taking advantage of the prevailing current, directing the chemical towards a conspecific, or orienting towards plumes (reviewed by [68]) is yet to be determined.

Sloman et al. [60] demonstrated that toadfish presented with urea alone tended to show avoidance behavior; however, the ability for urea alone to prompt a urea pulse in a conspecific had, up until now, not been directly tested. While urea alone does not appear to be the cue, the cue used in toadfish chemical communication could be any number of unidentified compounds that are co-excreted with urea to elicit a response in a conspecific. Teleost fish have been shown to use a wide variety of chemical cues including bile salts such as taurochloric acid (reviewed by [16]), steroids and prostaglandins or their metabolites [32,63] as well as peptides and amino acids such as L-kynurenine (reviewed by [20]). Heat treatment of PC-SW in the present study allowed for various compounds to be removed by volatilization or denaturation such as large proteins and steroids, aromatics, as well as low molecular weight organics such as ketones. The data of the present study clearly show that the signaling mechanism used by toadfish is not heat-labile as naïve toadfish still responded to heat-treated, PC-SW with a low pulse latency. In addition to exhibiting heat stability, the active cue compounds(s) were found to be located within the polar, aqueous fraction and not the non-polar fraction, further eliminating steroids, hydrophobic amino acids and most organic compounds as possible candidates. The elimination of steroid compounds by heat and solid-phase extraction is consistent with the additional finding that waterborne exposure to the steroid hormone, cortisol, does not result in a decrease in pulse latency, despite its involvement in the control of toadfish pulsatile urea excretion and social behavior (reviewed by [42,62,84]).

Following this analysis, possible chemical cue candidates within PC-SW can be narrowed down to heat-stable, water-soluble compounds. While the neurotransmitter, 5-HT, is relatively water-soluble and, like cortisol, strongly linked to both the control of toadfish pulsatile urea excretion [23,34,41,84] and behavior (reviewed by [62]), waterborne 5-HT did not elicit a decrease in pulse latency. Interestingly, exposure to water-soluble ammonia did result in a significant decrease in pulse

latency, and a positive correlation between ammonia exposure and urea excretion has been noted previously, with ammonia concentrations as low as 10 μmol-N·L^{-1} resulting in a significant increase in urea excretion [8,72]. While ammonia gas (NH_3) is heat labile, inorganic ammonium salts, such as the NH_4Cl present in seawater and added in the present study (as well as [72]) have melting/boiling points that exceed 300 °C and thus would withstand heat exposure. That being said, the pulse latency in response to ammonia exposure alone is not as low as would be expected from a 100% PC-SW exposure (15 h compared to 7 h in typical experiments). Interestingly, the effect of ammonia alone is emulated when analyzing the pulse latencies of all PC-SW-exposed fish *vs*. ammonia concentration of the PC-SW immediately prior to their pulse event. These data show a tendency for fish to have a lower pulse latency when ambient ammonia concentrations are higher than typically measured in the water column (0–10 μmol-N·L^{-1}; [8]) but not high enough to interfere with ammonia excretion [75]. Using the equation of the fitted line, the effect of 0 μmol-N·L^{-1} ammonia on pulse latency is calculated to be 27.5 h, similar to the response to seawater alone, whereas the pulse latency at 50 μmol-N·L^{-1} ammonia is 11.8 h, similar to the 15 h achieved by exposure to 50 μmol-N·L^{-1} ammonia alone. Together, these findings suggest that ammonia may be one component of a multi-component cue used for chemical communication in toadfish. It is possible that the ammonia component of the cue could play a role in changing the resting membrane potential and/or intracellular pH of gill cells *via* entry through apical Rh proteins that are responsible for ammonia transport [17,51,54,87], triggering a response in a naïve fish. Indeed, toadfish gill mitochondrial rich cells (MRCs) that are responsible for urea excretion also express Rhcg1 on the apical membrane [17].

Finally, there were clear differences in the responsiveness of small compared to large toadfish in that regardless of the concentration of urea within PC-SW or the size of the donor fish, small naïve toadfish reacted to the PC-SW with a pulse latency of ~6 h. In contrast, larger toadfish only responded to PC-SW that was made by a large donor fish and pulse latency fell as the "intensity" of the chemical cue, measured by the concentration of urea within the PC-SW, increased. Since smaller fish reacted most consistently, perhaps the urea pulse in response to the PC-SW is a means to communicate subordinance and circumvent an aggressive interaction similar to crustaceans, such as crayfish and lobster, that release chemicals in the urine to reduce physical fight duration and intensity [3,11,14,48,58]. Supporting this hypothesis is a study by McDonald et al. [38] that suggested that the increase in aggression experienced by dominant toadfish treated with fluoxetine (the active ingredient in the selective serotonin reuptake inhibitor antidepressant, Prozac™) may be because fluoxetine-treated subordinates were unable to adequately pulse urea [43,49].

In summary, one or more heat-stable, water-soluble compounds are released by Gulf toadfish that elicit pulsatile urea excretion in a conspecific. Small toadfish show a greater tendency to respond to this chemical cue suggesting that toadfish may use pulsatile urea excretion as a cue to communicate social status to conspecifics. At this point, urea, cortisol and 5-HT have been ruled out as possible cues, but ammonia, possibly in the form of inorganic ammonium salts (*e.g.*, NH_4Cl), has been shown to play at least a partial role. However, the longer pulse latency in response to NH_4Cl alone compared to PC-SW as well as preliminary evidence indicating the cue maybe >700 Da (C.M.R. Lemoine, C. Bucking, P.J. Walsh, personnel communication) suggests that ammonia may be one component of a multi-component cue. However, further studies are needed to fully identify the chemical cue and determine whether it has any adaptive significance for this marine teleost fish.

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