SOCIAL SIGNALING AND UREA EXCRETION IN THE GULF TOADFISH, *OPSANUS BETA*

By

Jeremy Fulton

Thesis submitted to the

School of Graduate Studies and Research

University of Ottawa

In partial fulfillment of the requirements for the

M.Sc. Degree in the

Ottawa – Carleton Institute of Biology

ABSTRACT

The gulf toadfish (*Opsanus beta*) is a member of a group of teleosts that have retained their ornithine urea cycle (OUC) allowing them to excrete nitrogenous waste in the form of urea (ureotely). Urea-N for the entire day is excreted in 1-2 quick pulsing events (1-3 h). This study evaluated the hypothesis that urea-N pulsing events in gulf toadfish can be triggered by social signals from conspecifics via a specific waterborne messenger. Using a crowding protocol, we found that pre-conditioned seawater induced a secondary urea pulsing event in naïve conspecifics. Furthermore, it was revealed that other factors such as signal concentration and donor body mass relay information to recipients as well. Fractionation of pre-conditioned seawater was carried out to narrow possible signal candidates and the aqueous portion was found to contain the active molecule. Ammonia was found to be an important factor controlling the response of toadfish to pre-conditioned seawater.

Key words: *Opsanus beta*, Social signaling, Ureotely, Toadfish,

ACKNOWLEDGEMENTS

Throughout my M.Sc. there have been many professors, staff and colleagues who have helped me tremendously through the completion of my degree. First and foremost I like to thank my supervisor Dr. Pat Walsh. Without his patience and continual mentorship it is easy to say my research project would never have gotten off the ground.

I also need to give a massive thank you to all the past and present Walsh lab members. I would especially like to thank Dr. Chris LeMoine, Dr. Carol Bucking and Courtney Deck for all of their endless mentorship in and out of the lab.

I would also like to thank my supervisory committee Drs. Katie Gilmour and Bill Willmore for all their helpful suggestions and constructive criticism. I also need to give a big thank you to Bill Fletcher, without his endless support and trouble-shooting help my experiments would not have been possible.

I completed many of my final experiments at the University of Miami RSMAS campus and as such I have to give a big thank you to Dr. Danielle McDonald for all of her brainstorming help and experimental aid in Miami as well as Dr. Chris Wood for letting me call his house home while I was in Miami.

Lastly, my thesis would not have been possible without the support of my friends and family. I would especially like to acknowledge my parents, Kim and Bill Fulton, for their encouragement and Tanya Nadon for her full support throughout this entire journey.

Thank You!

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

O-UC – ornithine urea cycle

tUT – toadfish urea transporters

5-HT – serotonin; 5-hydroxytryptamine

CPS – carbamoyl phosphate synthetase

OCT – ornithine carbamoyl transferase

ASS – argininosuccinate synthetase

ASL – argininosuccinate lyase

ARG – arginase

GS - glutamine synthetase

GR – Glucocorticoid receptor

MR – Mineralocorticoid receptor

TEM – transmission electron microscope

SEM – scanning electron microscope

AANAT - arylalkylamine-N-acetyltransferase

N – number of fish

S.E.M. – standard error of the mean

ANOVA – analysis of variance

MS–222 – tricaine methanesulfonate

DO – dissolved oxygen

INTRODUCTION

1.1 Nitrogenous Excretion by the Gulf Toadfish (*Opsanus beta*)

The gulf toadfish (*Opsanus beta*) is among the few species of teleost (bony) fish that as mature adults can excrete their nitrogenous waste primarily in the form of urea (ureotely; Walsh *et al.* 1990; Barber and Walsh 1993). Since Mommsen and Walsh (1989) first reported that both *O. beta* and *O. tau* have the ability to synthesize significant amounts of urea via the ornithine-urea cycle (O-UC), there has been much interest in understanding the mechanism behind this phenomenon. Ureotely can be produced in *O. beta* by means of a crowding/confinement protocol (Walsh *et al.* 1994), which has led to many interesting findings. First, in a laboratory setting gulf toadfish will excrete approximately 90% of their nitrogenous waste (combined urea-N and ammonia-N) in the form of urea-N (Wood *et al.* 1995). Second, excretion of urea is in a pulsatile fashion, in that all urea-N excreted takes place one to two times a day, via the gills (Wood *et al.* 1995). Pulse duration ranges from 0.5 to 3 hours and is mediated by specific toadfish urea transporters (tUT's; Wood *et al.* 1997; McDonald *et al.* 2003). Third, cortisol plays a permissive role in the triggering of excretion events by significantly decreasing (from approximately 120 to 40 ng/ml) 2–4 h before a pulse event and rising rapidly thereafter; however the cortisol decrease does not directly induce a urea excretion event (Wood *et al.* 2003; Wood et al., 1997; Wood et al., 2001). Fourth, serotonin (5-HT; 5-hydroxytryptamine) has been shown to directly stimulate urea excretion via specific receptor mediated pathways (5-HT(2)-like receptors;

McDonald and Walsh, 2004). Although there have been large advances in understanding the phenomenon of urea excretion in this teleost, important questions still remain, such as: (1) why did this mechanism evolve at all?; and (2) are there other possible roles these excretions events may play aside from the obvious removal of nitrogen? These questions arise largely due to the considerable expense associated with producing urea (5 mol ATP/ mol urea).

1.2 Ornithine-Urea Cycle

Urea is synthesized in toadfish, similar to most terrestrial vertebrates, via the O-UC. In the mammalian O-UC the entry point is carbamoyl phosphate synthetase (CPS), which introduces a nitrogen donor (ammonia) into the cycle and converts it to carbamoyl phosphate. Carbamoyl phosphate with the addition of ornithine is then transferred into citrulline via ornithine carbamoyl transferase (OCT) in the second step of the cycle. In the third, citrulline and aspartate are catalysed into argininosuccinate by argininosuccinate synthetase (ASS). Argininosuccinate lyase (ASL) then converts argininosuccinate into arginine in the fourth step. Finally, urea and ornithine are synthesized from arginine by arginase (ARG) to complete the cycle (Anderson 1995; Mommsen and Walsh 1991). Notably, in fishes (elasmobranch, lungfish and teleosts) the first enzyme in this cycle is catalyzed by CPS III, which uses glutamine as the nitrogen donor, unlike the mammalian form (CPS I) that utilizes ammonia (Anderson, 1995). This also brings up another important accessory enzyme, glutamine synthetase

(GS), which supplies CPS III with its nitrogen donor by catalyzing the condensation of glutamate and ammonia to form glutamine (Anderson, 2001).

1.3 Feeding Effects on Nitrogenous Excretion

In addition to the crowding/confinement protocol, feeding (nitrogen loading) significantly effects nitrogenous excretion within toadfish. Walsh and Milligan (1995) showed that an unconfined toadfish that was fed prior to water sampling had an approximately 3-fold increase in total nitrogen excreted with little change in the composition of ammonia versus urea (61% ammonia, higher than normal laboratory studies). In confined, unfed, toadfish 90% of nitrogenous waste was excreted as urea. However, when these fish were fed they did not reduce their ammonia output, resulting in an ammonotelic state (55% ammonia of total N-excretion; Rodela *et al.* 2011; Walsh and Milligan 1995).

1.4 Social Signaling

More recently, owing to the fact that cortisol and serotonin are known to be strongly involved with social interaction (Hopkins *et al.* 1995), researchers have been testing the possibility of a social signaling/communication mechanism that may be involved in urea pulsing. A study by Sloman *et al.* (2005) was designed specifically to address this hypothesis. Two toadfish were placed together in an aquarium and one

was injected with radioactive^{14}C-urea to evaluate individual urea excretion during social encounters. A toadfish scoring protocol was used to determine dominance within each pair, and in every pair a hierarchy was demonstrated. After recording interactions and pulsing events for 48 h Sloman and colleagues (2005) concluded that social status did not affect pulsing events; that is, neither dominant nor subordinate fish consistently pulsed urea first. However, Sloman *et al.* (2005) observed another result, which may bring more clarity to the unique ureotely in gulf toadfish, is that in seven out of eight pairs, both fish pulsed within two hours of one another. The timing of the pulses led Sloman *et al.* (2005) to speculate that this was due to a possible social signaling/communication mechanism between conspecifics via specific chemical release events or by behavioural actions during urea pulse emissions.

1.5 Hypotheses

Two hypotheses have formed from the aforementioned knowledge of *O. beta,* as to the reason why ureotely in gulf toadfish is observed. The first describes this action as an anti-predation mechanism in the form of chemical crypsis. A recent study has shown that in a mesocosm setting toadfish pulse urea in a distinctly diurnal pattern (Barimo *et al.* 2010). The majority of nitrogenous excretion was observed at two specific periods of the day (06:00-10:00 and 16:00-18:00), which coincide with the times just before these fish are leaving for, and just after returning from, nightly foraging excursions, during which vocalizations are very pronounced. Furthermore, a strong contrast was shown between laboratory and mesocosm experiments in that the ammonia:urea ratio in

mesocosm analyses was found to be very close to 1:1 (Barimo *et al.* 2010). Barimo and colleague's evidence suggested that in a natural setting toadfish co-excrete urea and ammonia at specific times to conceal their position from predators. This chemical crypsis hypothesis is one way to look at this unusual occurrence but it does not fill in all the gaps surrounding urea pulsing.

The second hypothesis, and the one that I will focus on, describes this ureotely phenomenon as a method of territorial marking between conspecifics. As gulf toadfish are highly territorial animals (McDonald *et al.* 2007), especially when it comes to protecting their shelters (naturally limited resources), it is likely that they are marking their territory prior to leaving to forage for food. Marking their shelters may be accomplished through a pulsing event, where a substance(s) that is released (e.g., ammonia, urea, and/or unknown compounds) could act as a signaling molecule to conspecifics. Signaling molecules could be used for several purposes, for example: (1) as a way for the marking fish to find its way back to a specific shelter at night; (2) as a means to deter any conspecifics from moving into its refuge; or, (3) to display the fitness of the individual by incorporating or removing specific components in its pulses to portray a strong, healthy and fertile individual, or other information such as body size. Lastly, the communication need not be only one way, but could in fact elicit respondent signaling in recipient fish.

While molecules could be released by many potential routes, a particularly intriguing possibility can be found in the work of Laurent *et al.* (2001). Using transmission and scanning electron microscopy (TEM, SEM respectively), Laurent *et al.*

(2001) were able to obtained detailed images of the toadfish gill during pulsing events. Through this technique Laurent and colleagues were able to capture the movement of vesicles carrying a darkly stained (lead-salt staining) substance and releasing it to the external medium upon contact with the apical membrane (Laurent *et al* 2001).

THESIS OUTLINE AND RATIONAL

The purpose of this project was to determine: (1) whether intraspecific-waterborne signals are used during urea pulsing events in gulf toadfish, *Opsanus beta*, to initiate pulsing and marking in *O. beta* individuals; (2) to identify the characteristics of the social signal as well as the response to the signal in naïve conspecifics. Many studies have been conducted to further the understanding of the gulf toadfish's unique ability to pulse urea but very few have specifically addressed the identification and comprehension of social signaling within this process. This study aims to shed light not only on chemical communication between *O. beta* fish but also to understand the aspect of urea pulsing seen in this species.

1.6 Social Signaling Molecule Properties

Many current studies have shown that there is a large number of social signaling molecules being used by aquatic species, many of which have only recently been

identified and understood. These molecules can range from alarm substances in zebrafish (*Danio rerio*; Speedie and Gerlai 2008) to mate choice signals in three-spined sticklebacks (*Gasterosteus aculeatus;* Milinski *et al.* 2005) to migratory pheromones used in lamprey (*Petromyzon marinus;* Stewart *et al.* 2011) to name just a few. In aquatic environments social signaling substances often are derived from bile salts, steroids, nucleotides, amino acids or peptides and in some cases a combination of more than one substance (Wyatt 2003; Serrano *et al.* 2008; Mathuru *et al.* 2012). These chemicals are dispersed, using a wide variety of routes, to individuals or entire populations of conspecifics. The study of social signaling, specifically in fish, is generally an understudied and therefore potentially fruitful area for research.

1.7 Waterborne Chemical Signaling

The chemicals that fish use to communicate with each other are often themselves involved in the specific act that they are communicating. For example, the alarm substance released by many fishes is naturally occurring in the external cells of the animal (Speedie and Gerlai 2008) and is only released when the cell is ruptured usually from a predatory attack (Speedie and Gerlai 2008). Moreover, the chemical released by sticklebacks is produced in the gonads and released by the male prior to spawning events. With these experiments in mind the first place to look for possible signal candidates in gulf toadfish urea pulsing would be molecules involved in urea production or excretion. Studies have confirmed the role of 5-HT as the internal trigger in gulf toadfish pulsing events through the arterial injection of 5-HT and 5-HT2 agonists

(Wood *et al.* 2003). Furthermore, several studies have (McDonald and Walsh 2004; McDonald *et al.* 2010; McDonald *et al.* 2012) shown that these induced pulsing events could also be inhibited by using the 5-HT2 antagonist Ketanserin. Cortisol has also been shown to have an important permissive role in urea pulsing events being involved specifically in urea transport mechanisms (Wood *et al.* 2001). Pulsing toadfish have been shown to up-regulate toadfish urea transporters (tUTs) in response to low cortisol, brought on upon primarily by glucocorticoid receptors (GRs), and to a lesser extent mineralocorticoid receptors (MRs; Rodela *et al.* 2009). However, exogenous waterborne exposure of toadfish to these particularly important substances has not been studied. Furthermore, a 2005 study by Sloman *et al.* exposed toadfish to a waterborne dose of synthetic urea with no response. Additionally the co-excreted component of toadfish pulsing events ammonia, has yet to be tested specifically as an exogenous signaling molecule. Exposure to these substances via a waterborne dose is crucial to the understanding and identification of pulsatile urea behaviour.

1.8 Early fractionation of Signaled Water

Heat-treatment of pre-conditioned water can be used as a tool for early fractionation of signaled water. Although crude, heat-treating water will denature proteins and evaporate aromatic compounds. Many of these heat-labile compounds have been shown to be involved in social communication in other fish (Wyatt 2003), suggesting that heat-treating signaled water could remove the signaling molecule and therefore the response from naïve toadfish. Using this technique is thus an important

preliminary step in the identification process of identifying possible signal molecule candidates.

Behavioural bioassay-guided fractionations have been used in many studies to identify biologically active fractions of conditioned water (Gammon *et al.* 2005; Corkum *et al.* 2006; Katare *et al* 2011). These fractionations can be arduous but are one of the best techniques to use for early identification of aquatic signaling molecules. In the present study, a C18 octadecylsilane solid phase extraction cartridge fractionation technique was used to separate organic vs. aqueous portions as well as fractions of the pre-conditioned water that contain compounds with very small molecular weights. The signaled water fractionation was used in conjunction with a behavioural response protocol to progressively narrow down the bioactive fraction of the pre-conditioned water. Our goal with these techniques was ultimately to identify the specific fraction that was responsible for the urea pulsing response seen in exposed naïve toadfish.

MATERIALS AND METHODS

Experimental Animals

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 5L plastic bags housed within an insulated shipping container to Ottawa, ON. For experiments conducted at the University of Miami Rosenstiel School of Marine and Atmospheric Science, *O. beta* were collected from Biscayne Bay by commercial shrimp trawlers. 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 mgL^{-1}) for 1 h to prevent infection by the ciliate *Cryptocaryon irritans* as described previously (Walsh and Milligan, 1995). In the laboratory, toadfish were transferred to glass aquaria (60 L) with re-circulated, aerated seawater (32ppt) 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 these experiments ranged in mass from 55 g to 345 g (average mass 165.6 g ± 13.5 g; n=117) and gender was recorded once fish were euthanized. 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's Internal Animal Care and Use Committee.

Experimental Protocol:
Social Signaling Experimental Series

Signal Collection

To determine whether toadfish can recognize and respond to pre-conditioned water of a separate ureotelic toadfish, ureotely was induced via a crowding/confinement protocol (6 toadfish in one 8 L aquaria, density = 90gL^{-1}; Walsh *et al.* 1994) following a 48 h fasting period. Crowded toadfish were then transferred into individual aquaria (3 L) for 48 h to collect all excretions (i.e., to pre-condition the water). Fish were monitored during crowding confinement as well as individual confinement for any signs of distress.

Exposing Naïve Fish

Naïve fish (i.e., fish that had previously only been housed in the 60L aquaria at low density) were placed in 3 L of water that contained conditioned water from the initial 48 h period above. 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). Sample collection began one hour prior to fish transfer to record background levels of water chemistry and nitrogenous excretion (salinity, pH, DO, urea, ammonia). 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. Following water

collection, samples were placed in a 4°C cold-room and analyzed for ammonia and urea concentrations no later than one week after collection.

In parallel, control experiments were carried out by transferring naïve fish into 3 L aquaria containing only unaltered fresh seawater and sampling water at identical intervals as treated fish followed by nitrogen excretion assays. Urea and ammonia excretion patterns were analyzed to determine whether a waterborne social signal affecting nitrogen excretion is transferred during a pulsing event.

Signal Dilution Experimental Series

Signal Collection and Exposure

To identify whether there is a specific threshold of signal molecule needed to trigger a pulsing event, a dilution experiment was carried out. To collect signaled water, two fish were placed in a 6 L aquarium supplied with aerated seawater (replenished every 24 h) for 72 h. Collected seawater was then pooled together and analyzed for urea and ammonia content prior to naïve fish exposure. At the end of crowding both fish were euthanized (overdose of MS-222, 5 gL^{-1}, followed by spinal severance) and weight and sex were determined. The signaled water was transferred to individual plastic tubs (3 L) with lids, at different concentrations. There were four dilutions of the signaled water (600x, 60x, 6x, and 3x dilution with seawater) and two controls (negative – only seawater, and positive – only signaled water). Naïve fish were individually placed in aerated exposure tanks (3 L – total aqueous volume), of varying signal concentration, and were housed for a 48 hr exposure period. Water samples (2 mL) were collected continuously over the entire 48 h experiment via fraction collectors

connected to a peristaltic pump (identical to social signaling experimental apparatus). Sampling started one hour prior to fish transfer to collect background levels of water chemistry endpoints and nitrogenous content. Water was placed in a 4°C cold-room and analyzed for urea and ammonia content no later than one week after experiment conclusion. Following the termination of the experimental period all exposed fish were euthanized (overdose of MS-222, 5 gL^{1}, followed by spinal severance). The above experimental design was repeated several times to yield appropriate sample sizes.

Exposure to Heat-Treated Pre-conditioned Water

Collection of pre-conditioned water was carried out similarly to previous experimental series, where two fish (average mass of 117.7 g) were placed in a 6 L aquarium supplied with aerated seawater (replenished every 24 h), for 72 h. Collected seawater, from the pervious 24 h, was then pooled together and analyzed for urea and ammonia content prior to heat-treatment and fish exposure. At the end of crowding both fish were euthanized (overdose of MS-222, 5 gL^{1}, followed by spinal severance) and weight and sex were determined. The pre-conditioned water was then divided into two groups, one group was stored in a 4°C cold-room for approximately two hours and remained unaltered, and the second which was heat-treated by placing it in a 90°C isotemp 220 hot-water bath (Fisher Scientific, Toronto, ON) for 60 min. After heating the water was analyzed for nitrogenous content, pH changes, dissolved oxygen and foam precipitations (denatured protein content).

Exposing Naïve Fish

Naïve fish were separated into three groups; one control group that was only exposed to unaltered fresh 3 ppt seawater, another group that was treated with unaltered pre-conditioned and the last, which was exposed to the pre-conditioned heat-treated seawater. All groups were held at 23°C on a 12/12-hr light/dark photoperiod for the duration of the experiment. Water samples (2 mL) were collected continuously in 1 h intervals over the entire 48 h experiment via fraction collectors connected to a peristaltic pump (identical to social signaling experimental apparatus). Sampling started one hour prior to fish transfer to collect background levels of water chemistry endpoints and nitrogenous content. After sample collection water was placed in a 4°C cold-room and analyzed for urea and ammonia content no later than one week after experiment conclusion. Upon completion all exposed fish were euthanized (overdose of MS-222, 5 gL^{1}, followed by spinal severance) and weight and sex were determined.

Body Mass Experimental Series

To determine the effect of body mass on pulse latency (time-to-first-pulse in hr) pre-conditioned water was obtained from individual fish of known mass prior to exposure to naïve fish. The confined fish in this experimental series were separated by body mass prior to individual confinement in 3 L aquaria. The first “donor” fish was considered a “small donor”, with a body mass of 84.8 g, and was held in a confined 3 L aquaria for 72 h to induce ureotely and pre-condition the seawater. The second “donor” fish was considered large with a body mass of 392.8 g and was also confined to induce ureotely and pre-condition the seawater prior to naïve fish exposure. Three different

concentrations of the "large donor"-conditioned seawater were exposed to naïve fish. The first exposure concentration was a "partial pulse" of urea (collected from the "large donor" 12 h after individual confinement) that contained an equal concentration of urea as the "small donor"-conditioned seawater. The second concentration was a "full urea pulse" pre-conditioned water, collected from the "large donor" 72 h after individual confinement. The third exposure concentration was a dilution of the "full urea pulse" that brought the urea concentration back down to that of the "small donor" urea pulse. This technique was used to collect signaling molecule from both donors at various concentrations.

Naïve Fish Exposure

A large body-mass-range of naïve fish was selected for exposure (body mass ranging from 84.2 g to 360.0 g). With this design it was possible not only to elucidate whether "large donors" elicit different responses from conspecifics as compared to their "small donor" counterparts but also to determine whether large-mass exposed fish reacted differently to the social signal as compared to small-mass exposed fish. Following individual confinement of "donor" fish, water was analyzed for nitrogenous content as well as standard water chemistry end points (pH, DO, salinity) to control for variability. Naïve fish were exposed to pre-conditioned water in 3 L plastic aquaria for 48 h and water samples (2 mL) were taken continuously over the exposure 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 via peristaltic pump (Minipuls 3 peristaltic pump, Gilson Inc. Middleton, WI). Sample

collection began one hour prior to fish transfer to record background levels of water chemistry and nitrogenous excretion (salinity, pH, DO, urea, ammonia). Following water collection, samples were placed in a 4°C cold-room and analyzed for ammonia and urea concentrations no later than one week after collection.

Ammonia Exposure

To determine whether exposure to environmentally relevant concentrations of ammonia evoked a urea pulsing event in naïve gulf toadfish, individual fish were held in 1 L aquaria for 48 h. Water samples were collected in 30 min intervals via fraction collectors attached to a peristaltic pump for 2 h prior to exposure. After background sampling was complete a one-time dose of ammonia was administered to bring the total ammonia concentration in the aquaria to 50 μM. This concentration was chosen from previous studies showing natural shelter ammonia excretion concentrations and rates (e.g. Barimo *et al.* 2011). The exposure ran for 36 h and after initial 6 h exposure period, water collection was reset to intervals of one hour. Samples were then stored at 4°C and analyzed no longer than one week post experimentation for urea and ammonia concentrations. All ammonia exposure experiments were carried out at the University of Miami.

Hormone Exposure

To determine whether cortisol or serotonin (5-HT), which are highly involved in controlling the internal mechanism of urea pulsing events in gulf toadfish, also act on external sites to elicit urea pulsing in conspecifics, fish were exposed to both hormones

separately over 24 h exposure period.

The cortisol concentration used in this experiment was based on average plasma levels in the literature and extrapolated out to be the highest concentration of hormone possible for one toadfish to excrete into the external surroundings. Therefore naïve fish were exposed to a one-time dose of serotonin hydrochloride at 10mg/L two hours after being placed in individual aquaria. Water samples were collected continuously on a 30 min time interval. Samples were then stored at 4°C and analyzed no longer than one week post experimentation for urea and ammonia concentrations. The cortisol experiment was conducted at the University of Ottawa.

Similar to the cortisol experiment, the 5-HT concentration used in this experiment was based on average plasma levels in the literature (Barimo *et al.* 2010; Rodela *et al.* 2009), as well as the concentration needed to elicit a response based on 5-HT caudal artery catheter injection. Thus, naïve toadfish were exposed to one-time dose of 3 μM 5-HT after two hours of background water sampling. Water samples were collected on a 30min 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. The 5-HT experiment was conducted at the University of Ottawa

C18 Column Filtration of Pre-conditioned Water

The following experiments were conducted at the University of Miami. Collection of pre-conditioned water was carried out similar to previous experimental

series, with slight modifications. Two naïve toadfish (average mass of 167.7 g) were placed in a 4 L aquarium supplied with aerated seawater (replenished every 24 h) for 48 h. Pre-conditioned water was accumulated every 48 h until the required volume was collected and then all pre-conditioned water was pooled. At the end of crowding both fish were euthanized (overdose of MS-222, 5 gL^{-1}, followed by spinal severance) and weight and sex were determined. The pre-conditioned water was then divided into two groups, one group was stored for approximately 24 h and remained unaltered, and the second group was passed through an octadecylsilane solid phase extraction cartridge (Seppak C18; Waters, Milford, MA) to remove hydrophobic compounds.

Naïve toadfish were exposed to the aqueous eluate from the column by placing an individual fish in a 1.5 L tank filled with the aqueous phase from C18 filtered pre-conditioned water. Water samples were collected hourly via fraction collectors attached to a peristaltic pump. In parallel individual fish were exposed to the unfiltered pre-conditioned water to compare responses. All water samples were collected for urea and ammonia assays, which were conducted no later than 3 days after collection. All exposed fish were euthanized (overdose of MS-222, 5 gL^{-1}, followed by spinal severance) and weight and sex were determined.

Toadfish were also exposed to organic phase compounds from the C18 column treatment to determine whether a response was mediated through hydrophobic compounds. Organic compounds that had been extracted from the pre-conditioned water by the C18 column were collected by elution with (0.25 L) methanol. The methanol fraction was then dried under vacuum and the residue was reconstituted in

8L of seawater. Naïve toadfish were then exposed to this resulting seawater using the same method as C18 aqueous phase exposure. Water samples were collected hourly and ammonia and urea assays were carried out no later than 3 days after collection.

Nitrogen Quantification

Urea Assay

Water urea concentrations were quantified using a diacetyl monoxime method adapted from Rahmatullah and Boyd (1980). Samples were assayed in duplicate and incubated at 85°C for 30min in a water-bath and were then analyzed on a spectrometer (SpectraMax plus384 absorbance microplate reader, Molecular Devices Corporation, Sunnyvale, CA) at a 540 nm wavelength. All samples were carried out in duplicate and analyzed using SoftMax Pro V5 analysis software (Molecular Devices Corporation). Concentrations were calculated by comparison to a standard curve ranging from 0-120 μM urea.

Ammonia Assay

Water ammonia concentrations were quantified by the indophenol blue method adapted from Ivancic and Degobbis (1984). Samples were assayed in duplicate and incubated for 1 hr at room temperature. SpectraMax Plus recorded absorbance at 635nm and analysis was carried out by SoftMax Pro V5 analysis software and concentrations were calculated by comparison to a standard curve ranging from 0-125 μM ammonium chloride.

Pulse Latency and Ureotelism

The appearance of urea in the sampled water was used to calculate pulse size, and pulse latency, with a threshold of 100 μmol N-$kg^{-1}L^{-1}$ to define a pulsing event as outlined by McDonald *et al.* (2004). Urea and ammonia excretion values (μmol Nkg-L) were calculated as illustrated by McDonald *et al.* (2004) using the equation:

$$\text{Excretion} = (\Delta C \times V_f)/M$$

Where ΔC represents the increase in water urea or ammonia concentration (μmol N), V_f was the volume of the experimental container (in liters) and M was the body mass of the individual fish (in kilograms). To obtain urea-N values urea concentrations were multiplied by two to reflect the fact that urea has two nitrogen atoms. These values were then used to calculate percent ureotelism.

Statistical Analysis

Data are reported as means ± 1 SEM (n= number of fish). Figures are presented as cumulative nitrogen excreted (μmol N-$kg^{-1}L^{-1}$) over a 48 h period as well as means ± 1 SEM of first pulsing event (pulse latency) and percent ureotelism. Unpaired two-tailed Student's t-test as well as analysis of variance (ANOVA) were performed using commercial software (SigmaPlot 10.1, SPSS) to detect significant differences between toadfish exposed to varying experimental conditions vs. pure 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.

RESULTS

Response to pre-conditioned seawater

Gulf toadfish were held separately in aquaria with static 32 ppt seawater (replenished every 48 h if needed) and subjected to a number of specific conditions designed to identify possible signals that can induce a urea pulse. Figure 1.1 shows representative time traces of urea excretion patterns of *O. beta* placed in unaltered seawater vs. seawater containing the excretions of a conspecific (conditioned seawater). From these types of traces, pulse latency was calculated as the time, in hours, it took for an individual toadfish to excrete at least 100 μmol N-$kg^{-1}L^{-1}$ over a period of one hour or less. In Fig 1.1, for example, the signaled toadfish's pulse latency would be reported as 1 h and the individual in unaltered seawater would have a pulse latency of 25 h.

The initial experimental series was carried out to determine if there was a response by an individual toadfish exposed to conditioned water from a conspecific. Naïve toadfish did in fact respond excretions by conspecifics. Fish exposed to conditioned seawater had significantly lower pulse latency (Fig 1.2, student's t-test, p=0.003),on average pulsing 4.5 h after being placed in pre-conditioned water when compared to individuals placed in seawater alone. Fig. 1.3 shows that toadfish exposed to pre-pre-conditioned water became ureotelic. When compared to fish placed in seawater alone there may be a slight increase in their percent ureotelism, but this difference is not significant (student's t-test, p=0.07).

Dilution of pre-conditioned seawater

Reproducibly significant results were produced from placing fish in diluted pre-conditioned water as well. Figure 1.4 demonstrates that unaltered pre-conditioned water can be diluted as much as 6-fold and still allow toadfish to respond in the same manner (mean pulse latency for undiluted, 3-fold and 6-fold dilutions are significant compared to unaltered seawater, ANOVA followed by Holm-Sidak post-hoc, $p=0.001$). However when individuals are placed into water that has been diluted by 12-fold or greater this response is lost (ANOVA followed by Holm-Sidak post-hoc, $p>0.05$ for all other groups compared to control). Furthermore, when measuring percent ureotelism in fish that were exposed to diluted pre-conditioned seawater a significant increase was seen in the 3-fold group as well as the undiluted group compared to control fish (ANOVA followed by Holm-Sidak post-hoc, $p=0.017$ and $p=0.035$ respectively; Fig 1.5).

Response to heat-treated pre-conditioned seawater

Significant results were also observed when individual fish were exposed to pre-conditioned seawater that had been heat-treated for 60min at 90°C. The pulse latency of fish exposed to heat-treated pre-conditioned seawater the average initial urea pulse was excreted significantly faster when compared to fish place in seawater alone (30.5hr vs. 11hr for seawater and heat-treated pre-conditioned seawater respectively; ANOVA followed by Holm-Sidak post-hoc, $p=0.006$, Fig 1.6). Furthermore when comparing fish placed in heat-treated pre-conditioned seawater to conspecifics placed in untreated pre-conditioned seawater both groups began to pulse on average within 2 h of each

other, therefore no significant change in pulse latency was detected (Fig 1.6). There was no significant effect of heat-treatment on percent ureotelism (ANOVA followed by Holm-Sidak post-hoc, p=0.359; Fig 1.7).

Effect of body mass on response times

Body was shown to play a key role in the response times of toadfish exposed to pre-conditioned seawater. Body mass (kg) formed a clear relationship with the concentration of the pre-conditioned seawater and the pulse latency of the exposed individuals. Figure 1.8 shows a series of linear regressions depicting the relationship of toadfish body mass to their pulse latency dependent on the urea concentration in the pre-conditioned water to which they were exposed. Urea concentration in the pre-conditioned water was used as a proxy measurement to quantify the 'intensity' of the pulse because the concentration(s) of any potential signaling molecule(s) is (are) unknown. Figure 1.8.A-C describes the pulse latency (hr), with respect to body mass (kg), of individual toadfish exposed to pre-conditioned water with a urea concentration of 10 μM, 200 μM and 3000 μM, respectively. Figure 1.8.D describes pulse latency with respect to body mass of toadfish exposed to unaltered seawater. Through panels A-C it is clear that smaller naïve fish decrease pulse latency whether they are exposed to excretions from small or large donor fish, as well as low versus high concentrations from large donor fish. However, larger naïve fish respond only to excretions from larger donor fish and higher concentrations of these excretions.

Response to waterborne cortisol

No significant differences in pulse latency or percent ureotelism were detected between fish exposed to waterborne cortisol (10 mg L^{-1}) and control individuals exposed to unaltered seawater (Student's t-test, p=0.833 and p=0.201; Fig 1.9 and Fig 1.10, respectively).

Response to waterborne serotonin

We examined the role that 5-HT may play on the pulse response times of toadfish by exposing individual fish to a one-time dose of 3 μM serotonin. There was a decrease in pulse latency in 5-HT exposed fish, however when compared to control fish pulse times are not significantly different from each other (student's t-test, p=0.181, Fig 1.11). There was no significant difference observed in fish exposed to 5-HT compared to unaltered seawater (student's t-test, p=0.086, Fig 1.12).

Response to waterborne ammonia

Ammonia is another substance that is known to be involved in the nitrogenous excretions of toadfish, and as such we examined its possible effects on individual toadfish. When exposed to 50 μM ammonia fish significantly decreased their pulse latency by 8 h compared to control fish latency (Student's t-test, p=0.001, Fig 1.13). When looking at the percent ureotelism of exposed individuals no significant results were observed (Student's t-test, p=0.804, Fig 1.14).

Ammonia concentration was also calculated prior to each urea pulsing event observed in experimental toadfish, both pre-conditioned water groups and control animals. Figure 1.15 describes the relationship between ammonia concentration within the experimental tank and the pulse latency found in naïve toadfish during experimentation.

Response to fractionated pre-conditioned seawater

Exposure of the aqueous fraction of the C18 reverse phase filtration produced a significant decrease in pulse latency of exposed individuals when compared to unaltered seawater exposure (ANOVA followed by Holm-Sidak post-hoc, $p=0.001$, Fig 1.16). Furthermore there was a significant increase in pulse latency observed in fish exposed to the organic phase of the C18 fractionation compared to fish that were exposed to unaltered pre-conditioned seawater (ANOVA followed by Holm-Sidak post-hoc, $p=0.001$, Fig 1.16). When fish were exposed to either the aqueous or the organic phase of the fractionation percent ureotelism was not significantly altered when compared to both signaled and control fish (ANOVA followed by Holm-Sidak post-hoc, $p=0.421$, Fig 1.17).

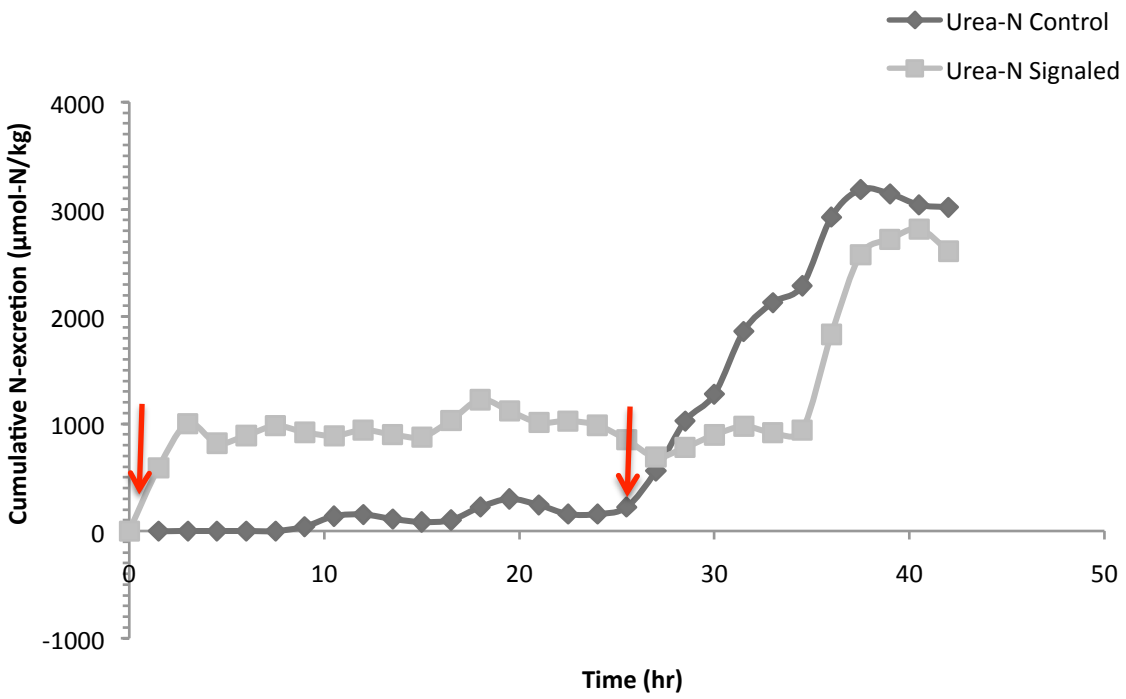


Figure 1.1. Representative traces of the pulsatile urea-N excretion of *Opsanus beta* exposed to pre-conditioned water (Signaled) from a conspecific and exposed to 32 ppt seawater (control). Red arrows depict the start of pulsing events. Data are presented as cumulative urea-N excretion adjusted for the body mass of exposed fish.

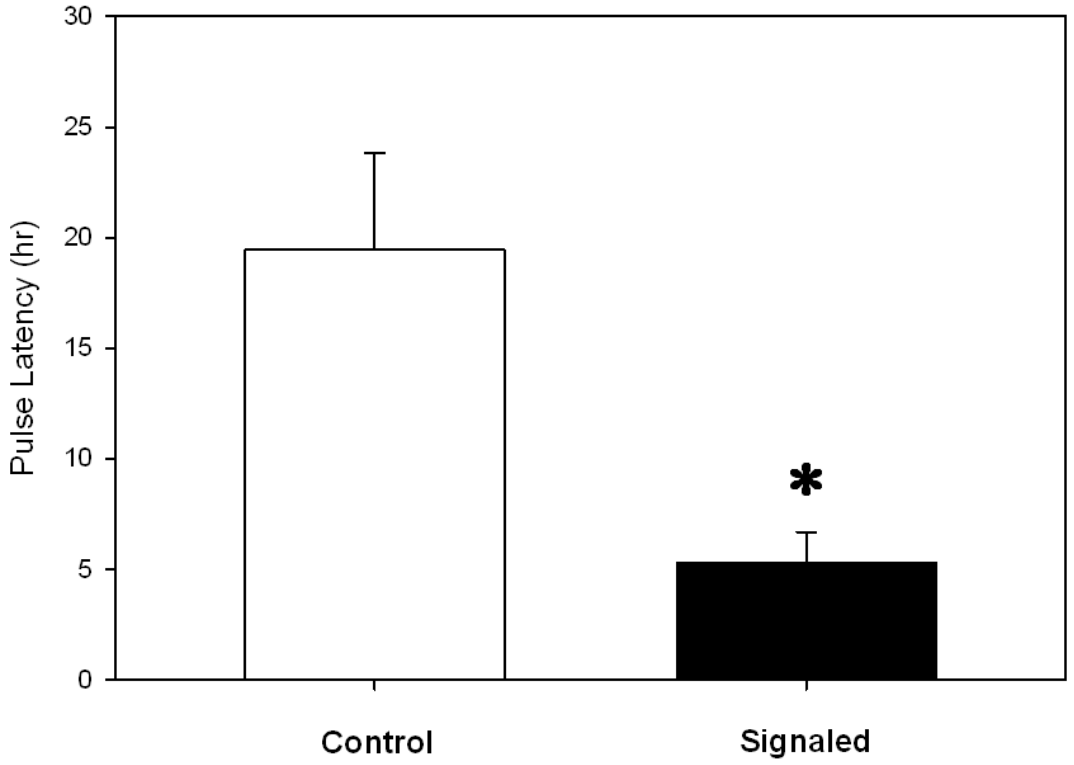


Figure 1.2. Urea pulse latency (time-to-first-pulse; hr) of gulf toadfish (*Opsanus beta*) exposed individually to conditioned water from a conspecific (black bar; N=14) or to control seawater (white bar; N=14). Data are shown as means ±1 SEM; significant differences from control values are denoted by an asterisk (P = 0.003; unpaired students t-test Mann-Whitney method).

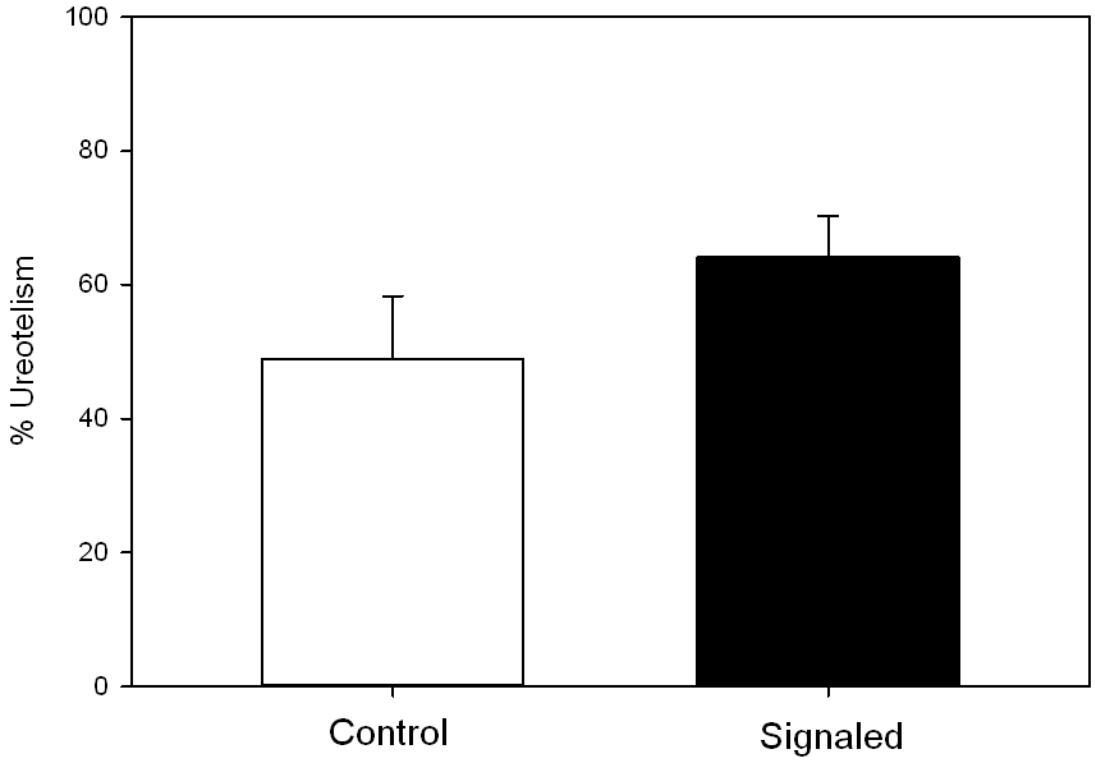


Figure 1.3. The percent ureotelism of gulf toadfish (*Opsanus beta*) exposed individually to conditioned water from a conspecific (black bar; N=14) or to control seawater (white bar; N=14). Data are shown as means ±1 SEM; no significant differences were found (P=0.07; unpaired students t-test).

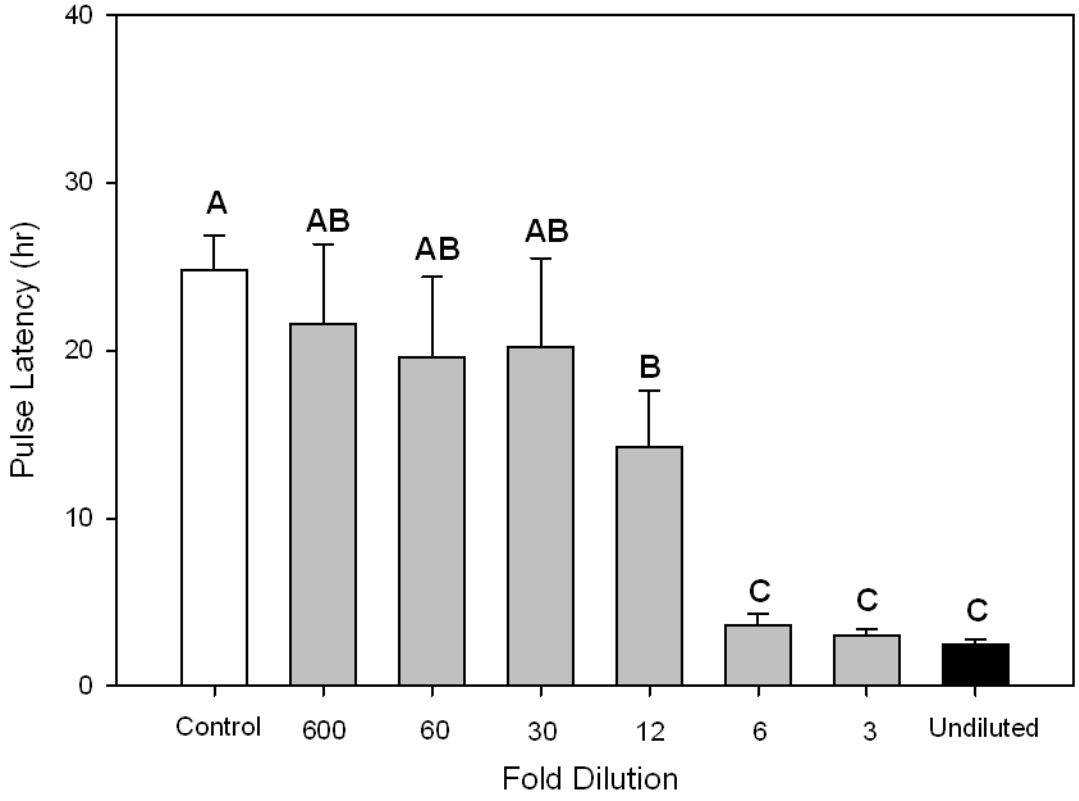


Figure 1.4. Urea pulse latency (hr) of gulf toadfish (*Opsanus beta*) exposed individually to varying dilutions of pre-conditioned water (grey bars, N=6 for each group; Black bar, N=9) from a conspecific and compared to seawater control (white bar; N=9). Data are shown as means ±1 SEM; groups that share a letter are not significantly different from one another (p=0.001, ANOVA followed by Holm-Sidak post-hoc).

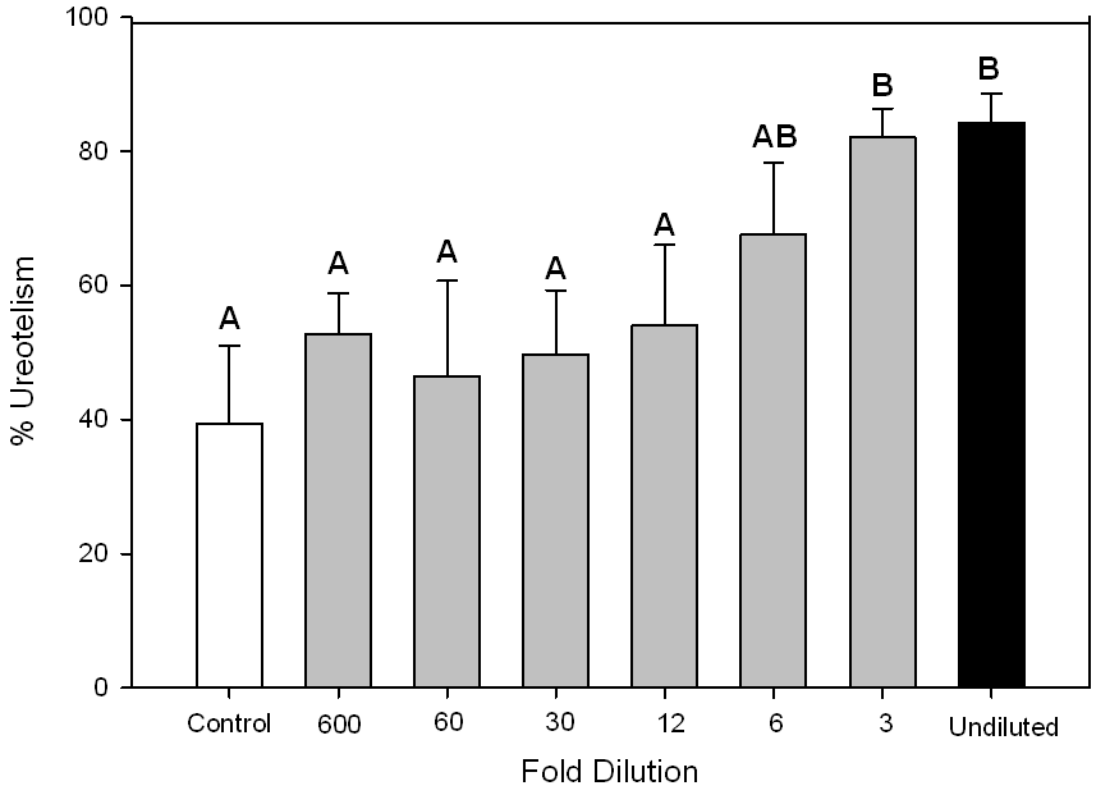


Figure 1.5. Percent ureotely of gulf toadfish (*Opsanus beta*) exposed individually to varying dilutions of pre-conditioned water (grey bars, N=6;Black bar, N=9) from a conspecific and compared to seawater control (white bar; N=9). Data are shown as means ±1 SEM; lettering indicates significant differences as compared to control values ($p=0.017$ and $p=0.035$ for undiluted and 3-fold dilution, respectively, ANOVA followed by Holm-Sidak post-hoc).

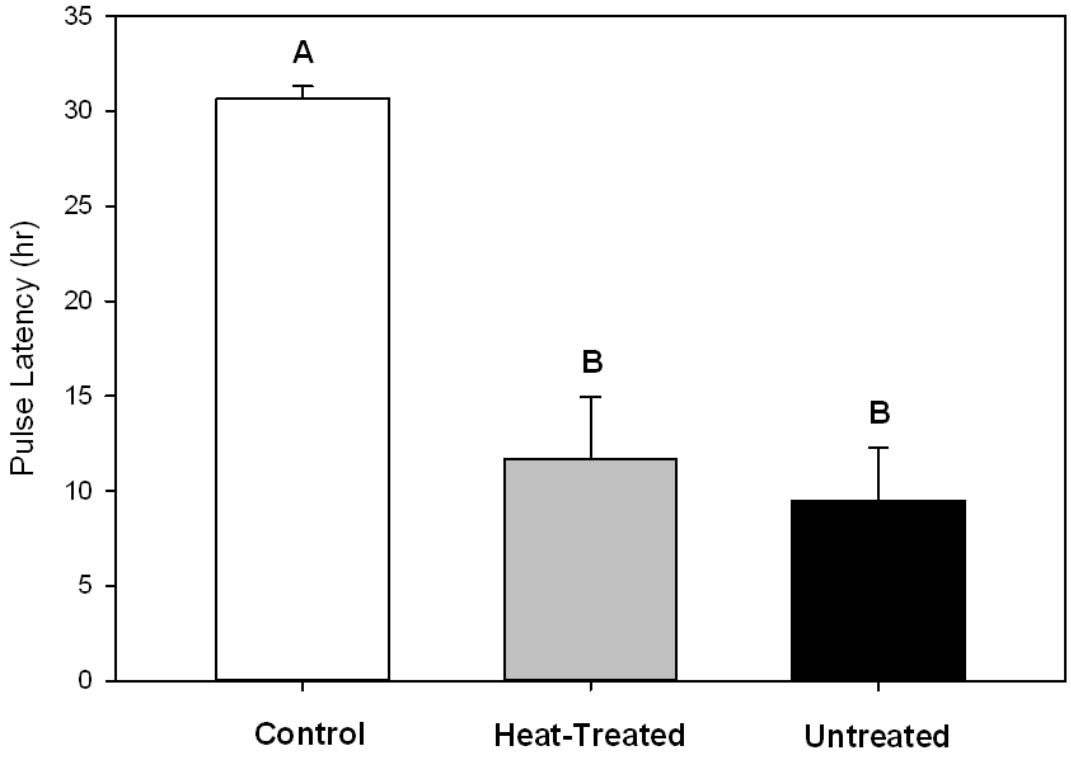


Figure 1.6. Pulse latency (hr) of gulf toadfish (*Opsanus beta*) exposed individually to heat-treated pre-conditioned water (grey bar; N=6) or untreated pre-conditioned water (black bar; N=6) and compared to seawater control (white bar; N=6). Data are shown as means ±1 SEM; lettering indicates significant differences as compared to control values (ANOVA followed by Holm-Sidak post-hoc, P =0.006).

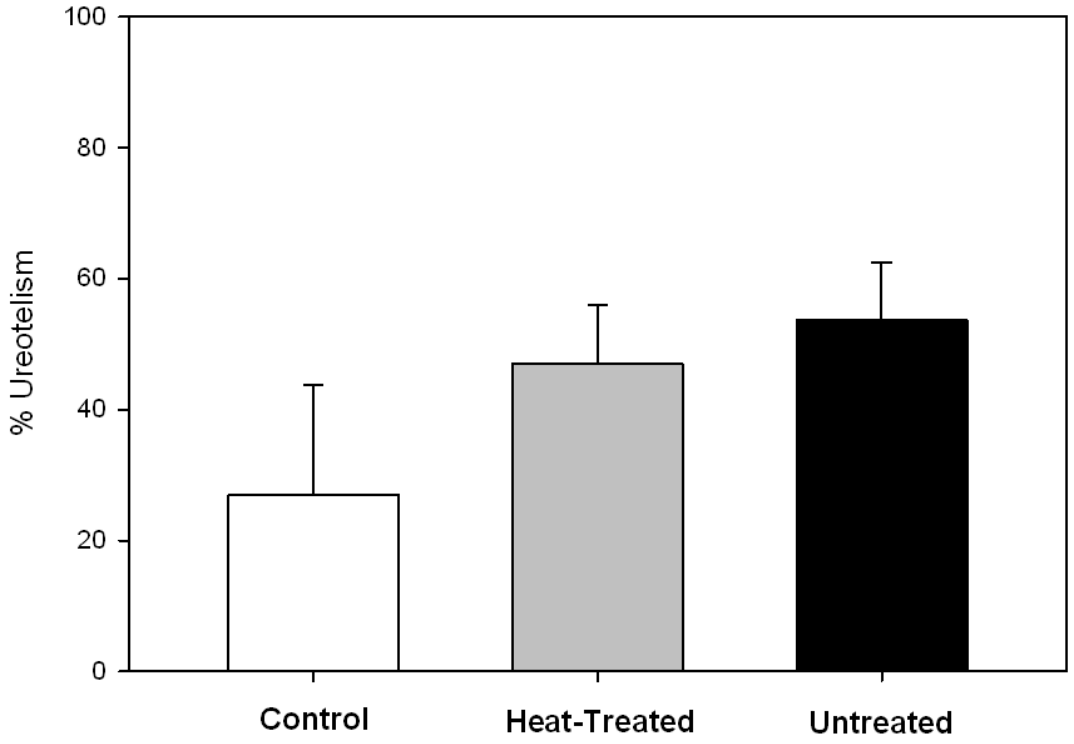
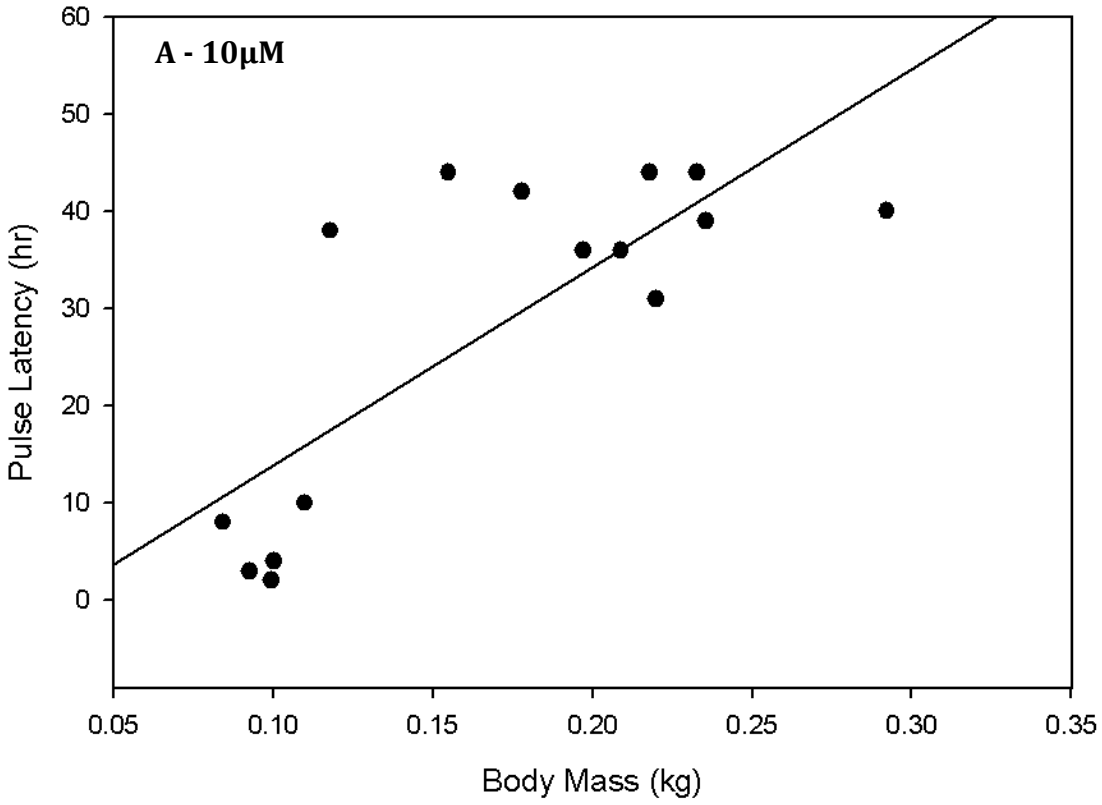
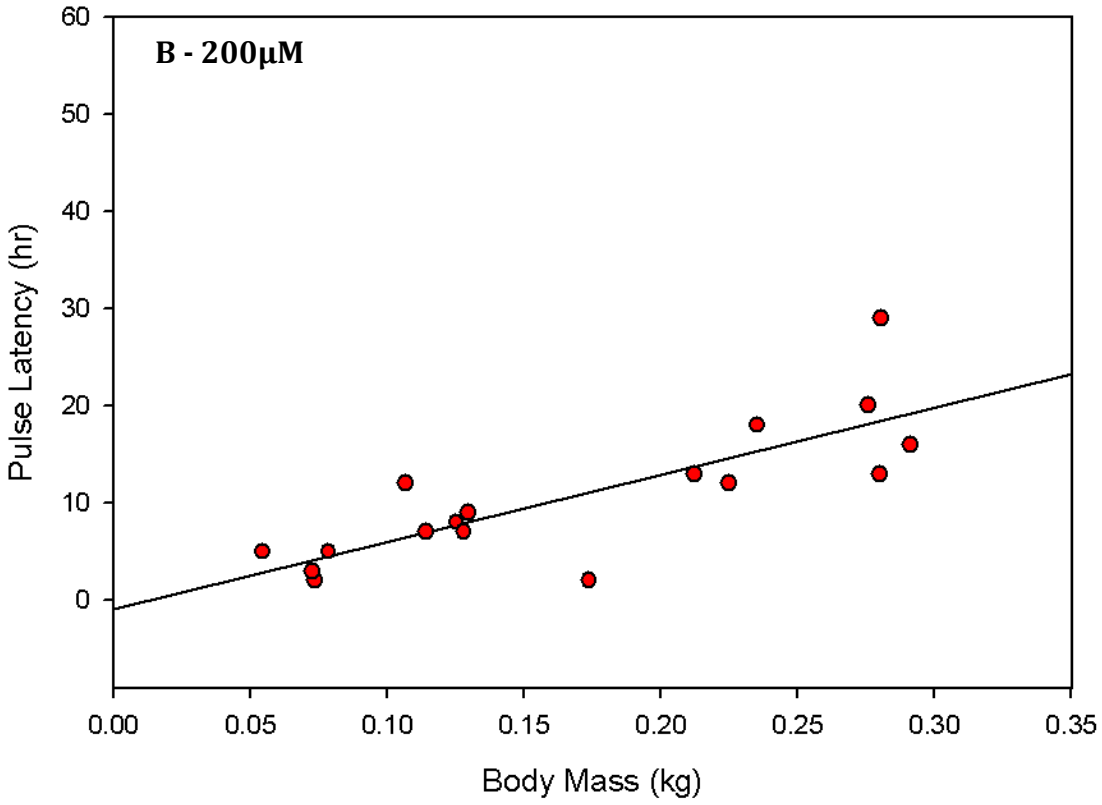
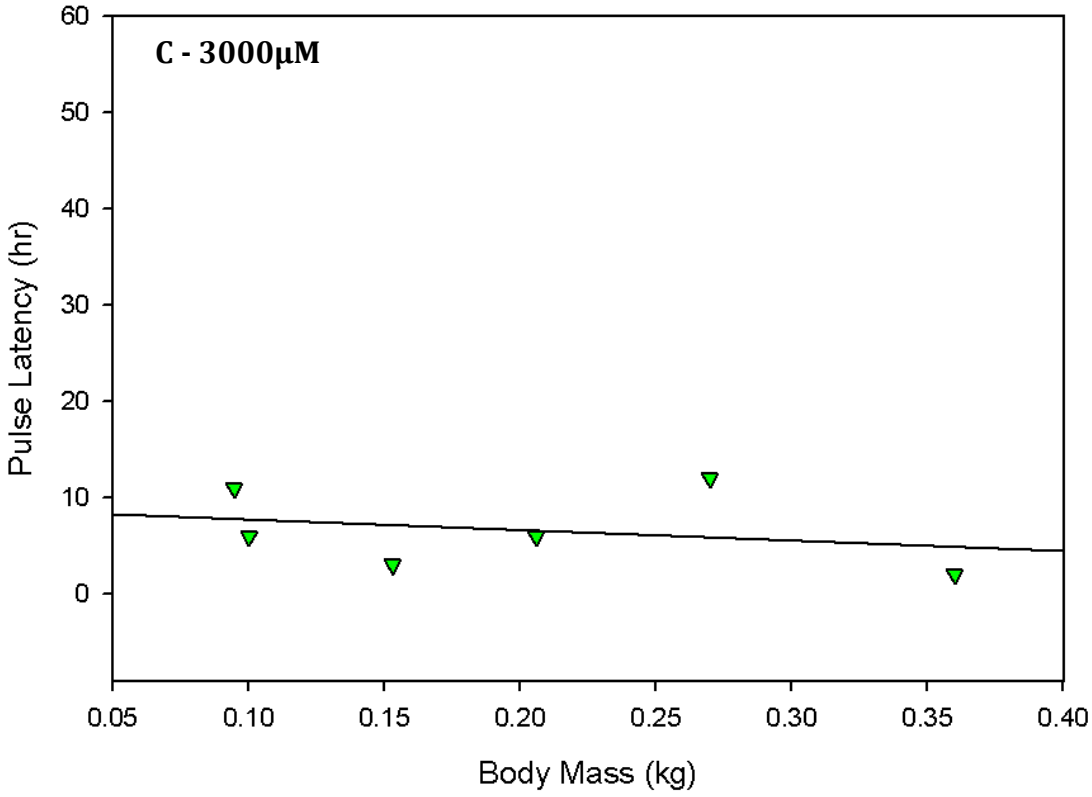


Figure 1.7. Percent ureotelism of gulf toadfish (*Opsanus beta*) exposed individually to heat-treated pre-conditioned water (grey bar; N=6) or untreated pre-conditioned water (black bar; N=6) and compared to seawater control (white bar; N=6). Data are shown as means ±1 SEM, no significant differences were found (P =0.359; ANOVA).


A - 10µM
Pulse Latency (hr)
Body Mass (kg)
60
50
40
30
20
10
0
0.05
0.10
0.15
0.20
0.25
0.30
0.35


B - 200µM
Pulse Latency (hr)
Body Mass (kg)
60
50
40
30
20
10
0
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35


C - 3000µM
Pulse Latency (hr)
60
50
40
30
20
10
0
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
Body Mass (kg)

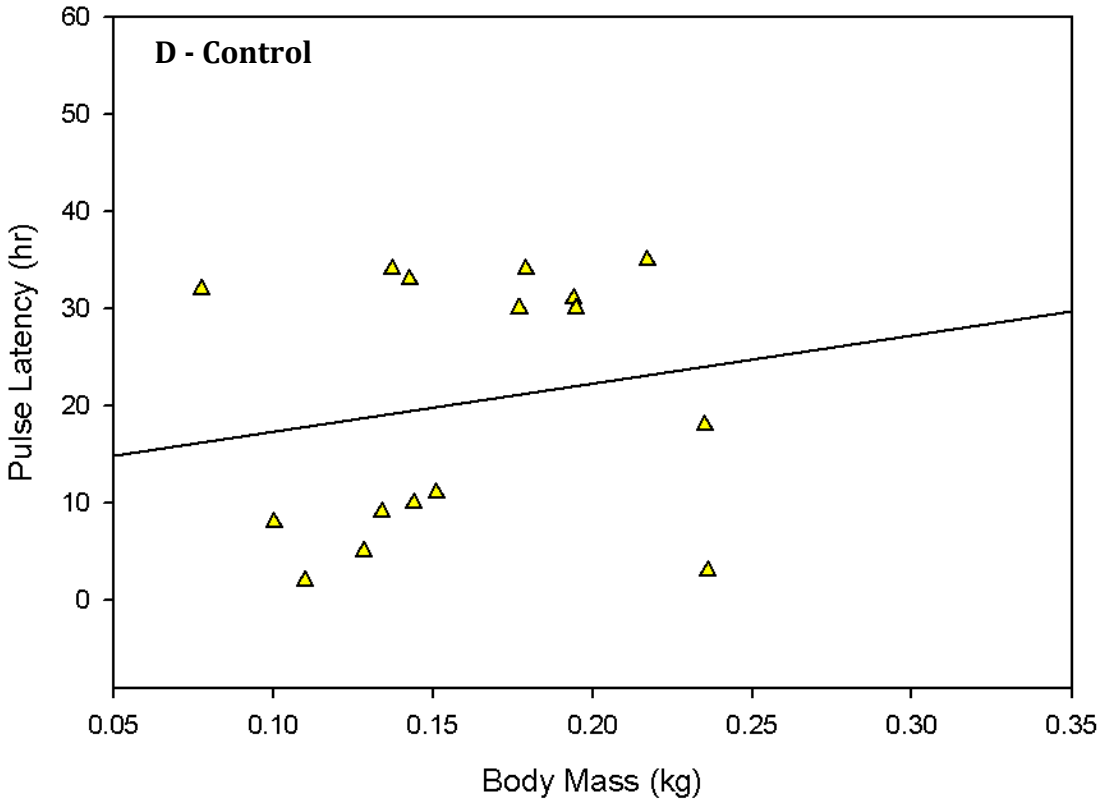


Fig 1.8. A-D: Relationship of pulse latency (hr) to naïve (recipient) fish body mass (kg) grouped by concentration of urea in pre-conditioned water. (A) Black dots (N=16) show individuals exposed to pre-conditioned water from a small donor fish (84.8 g) yielding 10µM urea (y = 203.93x – 6.493); (B) red dots (N=16) describe individuals exposed to pre-conditioned water from a large donor fish (392.8 g) yielding 200µM urea (y=69.123x - 0.968), (C) green triangles (N=6) show individuals exposed pre-conditioned water from the same large fish as in panel B, but yielding 3000 µM (y=11.092x + 8.856). (D) Shows control values for all individuals held in unaltered seawater (y=49.671x + 12.37).

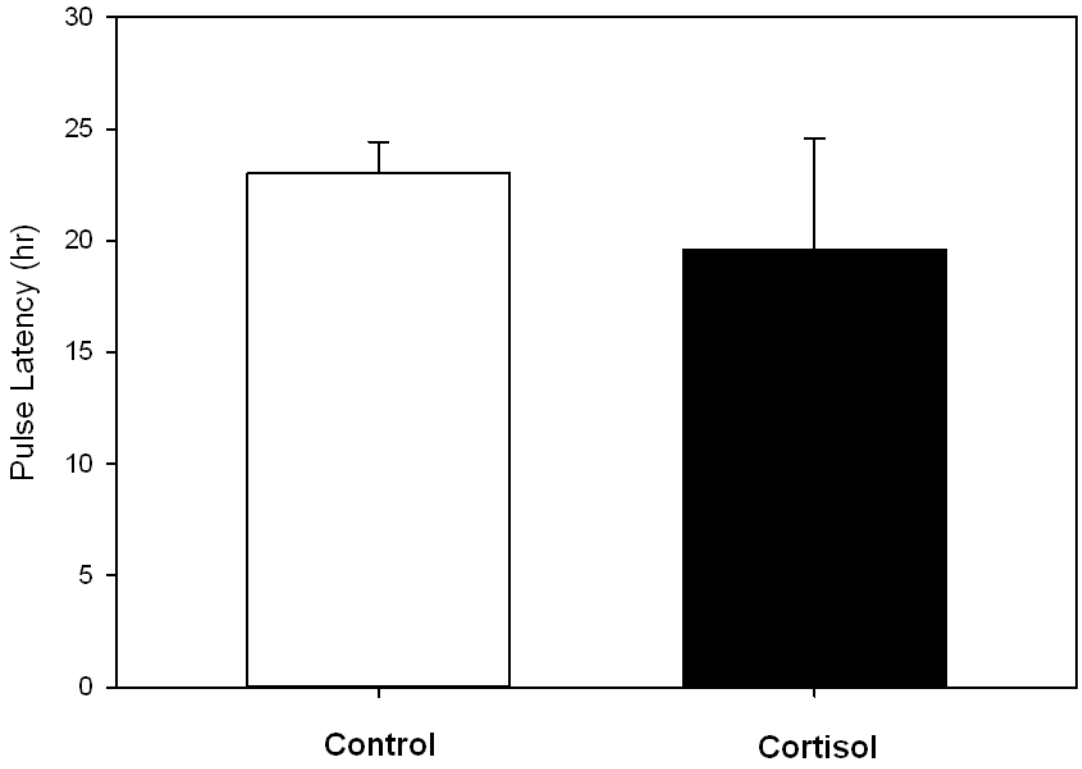


Figure 1.9. Pulse latency (hr) of gulf toadfish exposed individually to 10 mg L^{-1} of waterborne cortisol (black bar; N=6) and compared to seawater control (white bar; N=9). Data are shown as means ±1 SEM; no significant differences were found compared to control values (P =0.833; unpaired students t-test).

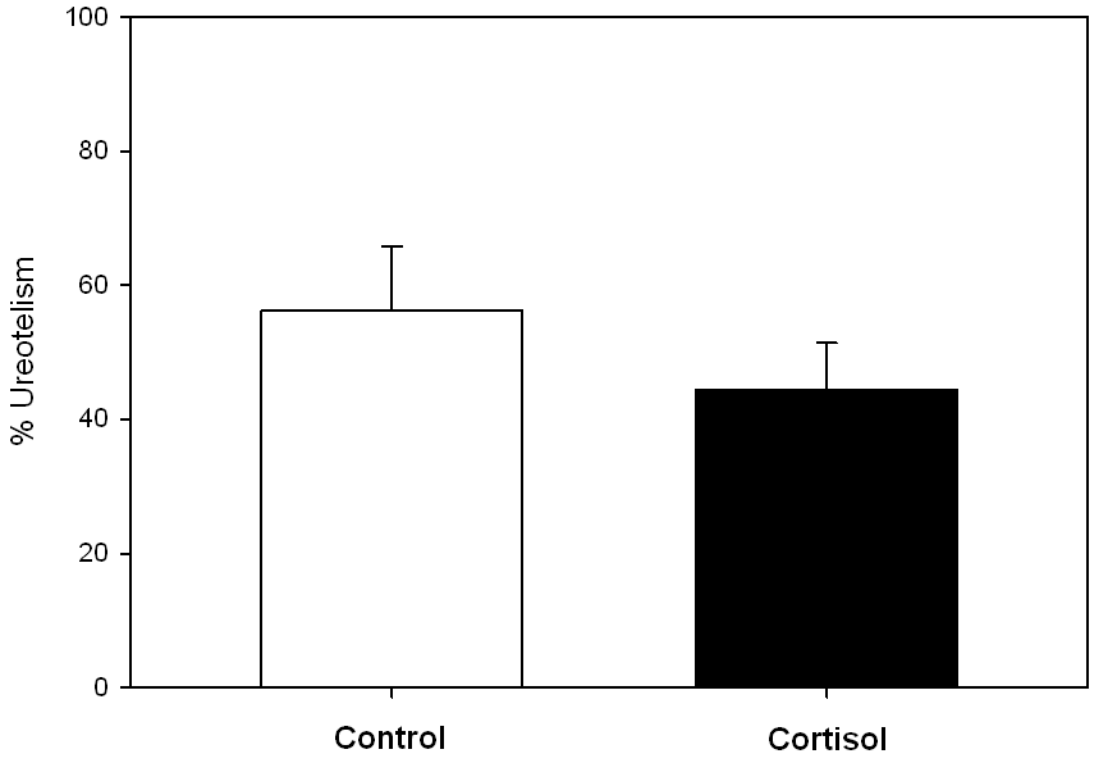


Figure 1.10. Percent ureotelism of gulf toadfish exposed individually to 10 mg $^{-}$1L of waterborne cortisol (black bar; N=6) and compared to seawater control (white bar; N=9). Data are shown as means ±1 SEM; no significant differences were found between groups (P =0.201; unpaired students t-test).

Figure 1.11. Pulse latency (hr) of gulf toadfish exposed individually to 3 µM serotonin (waterborne delivery, black bar; N=6) and compared to seawater control (white bar; N=9). Data are shown as means ±1 SEM; no significant differences were found (p=0.181; unpaired students t-test).

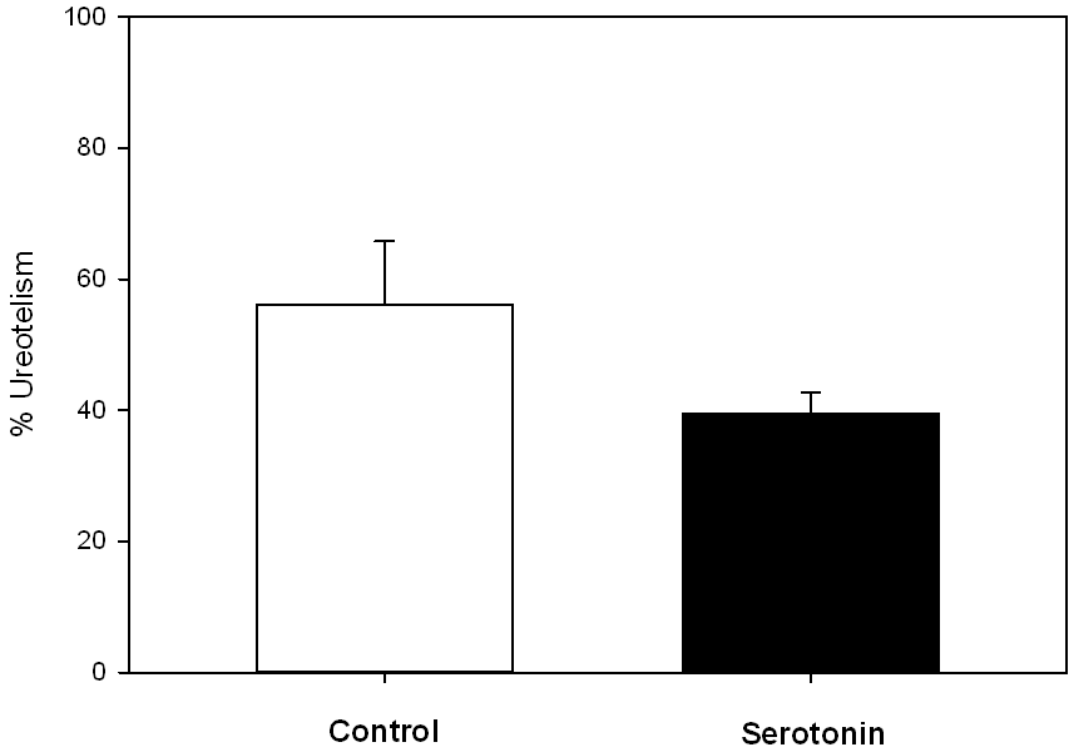


Figure 1.12. Percent ureotelism of gulf toadfish exposed individually to 3 μM serotonin (waterborne delivery, black bar; N=6) compared to seawater control (white bar; N=9). Data are shown as means ±1 SEM; no significant differences were found (p=0.086; unpaired students t-test).

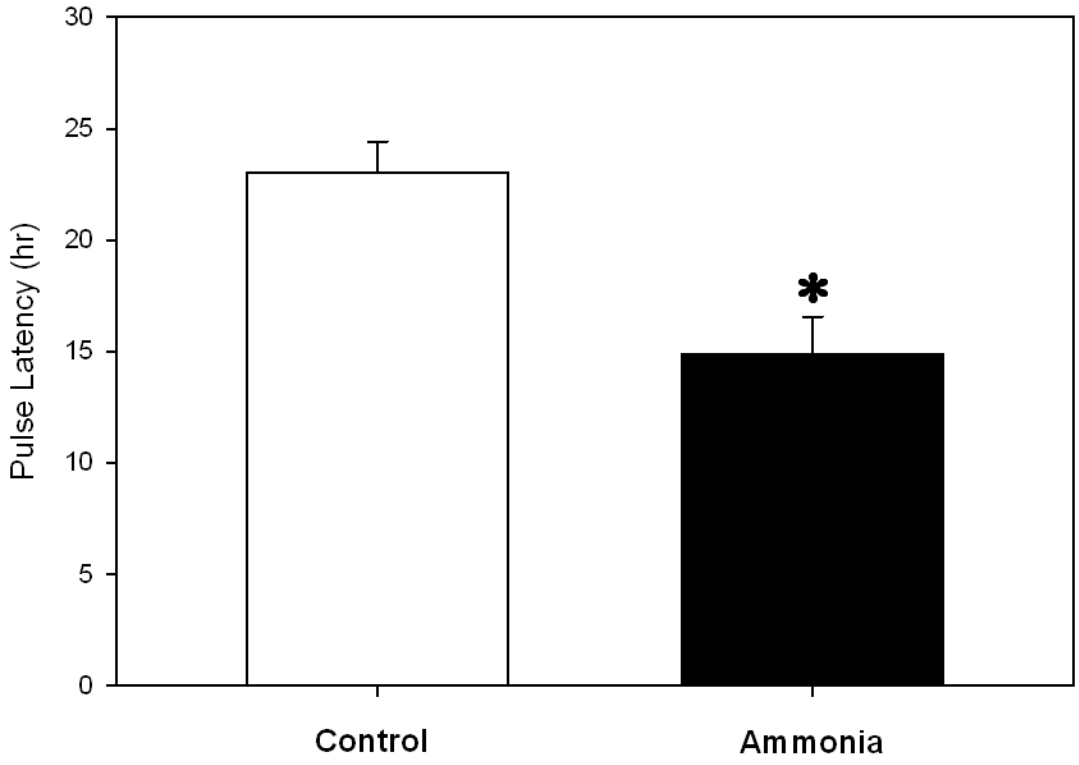


Figure 1.13. Pulse latency (hr) of gulf toadfish exposed individually to 50 μM of waterborne ammonia (black bar; N=7) and compared to seawater control (white bar; N=8) Data are shown as means ±1 SEM; asterisks denote significant differences between groups (P = 0.001; students t-test, Mann-Whitney method).

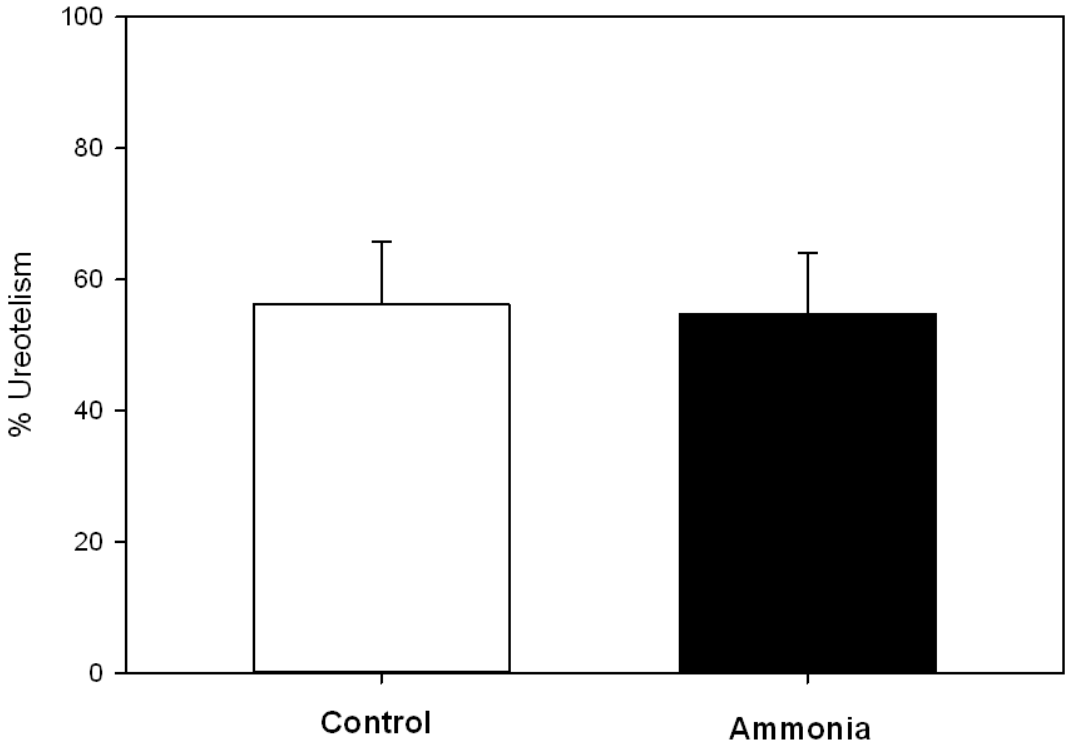


Figure 1.14. Percent ureotelism of gulf toadfish exposed individually to 50 μM of waterborne ammonia (black bar; N=7) and compared to seawater control (white bar; N=8). Data are shown as means ±1 SEM; no significant differences were found between the groups (Student's t-test, P=0.804).

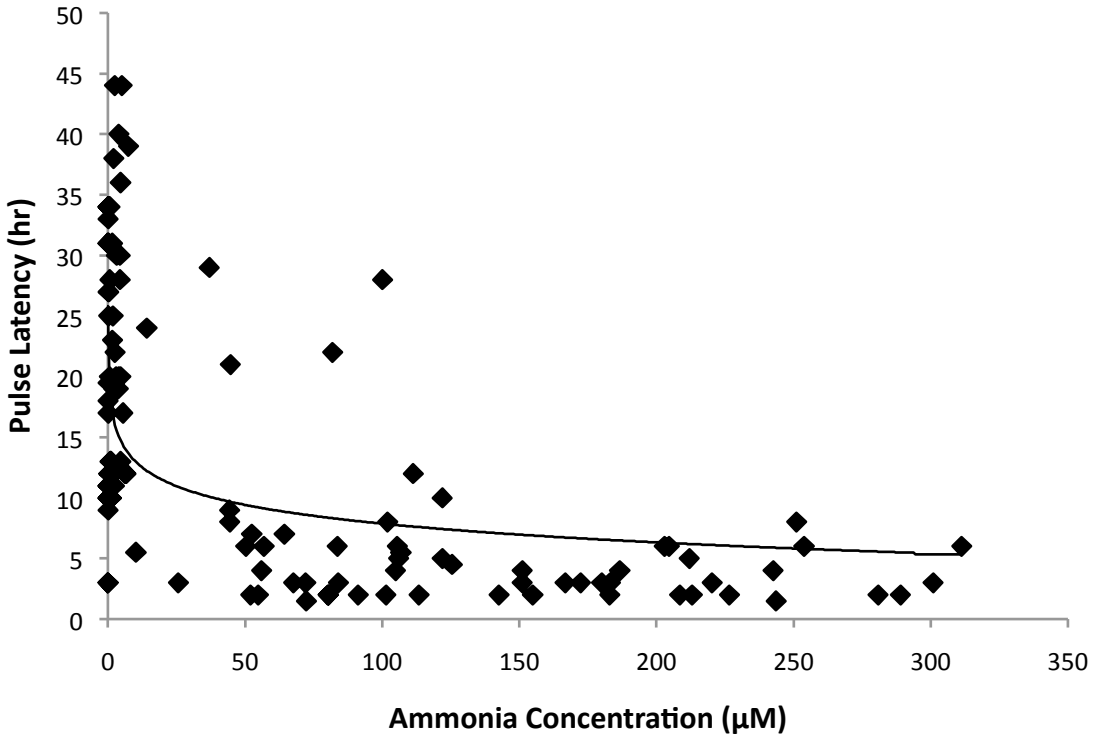


Figure 1.15. Relationship of ammonia concentration (µM) to naïve (recipient) fish pulse latency (hr). Black dots (N=117) show both male and female individuals exposed to pre-conditioned water ($y = -2.23\ln(x) + 18.15$; $R^2 = 0.299$).

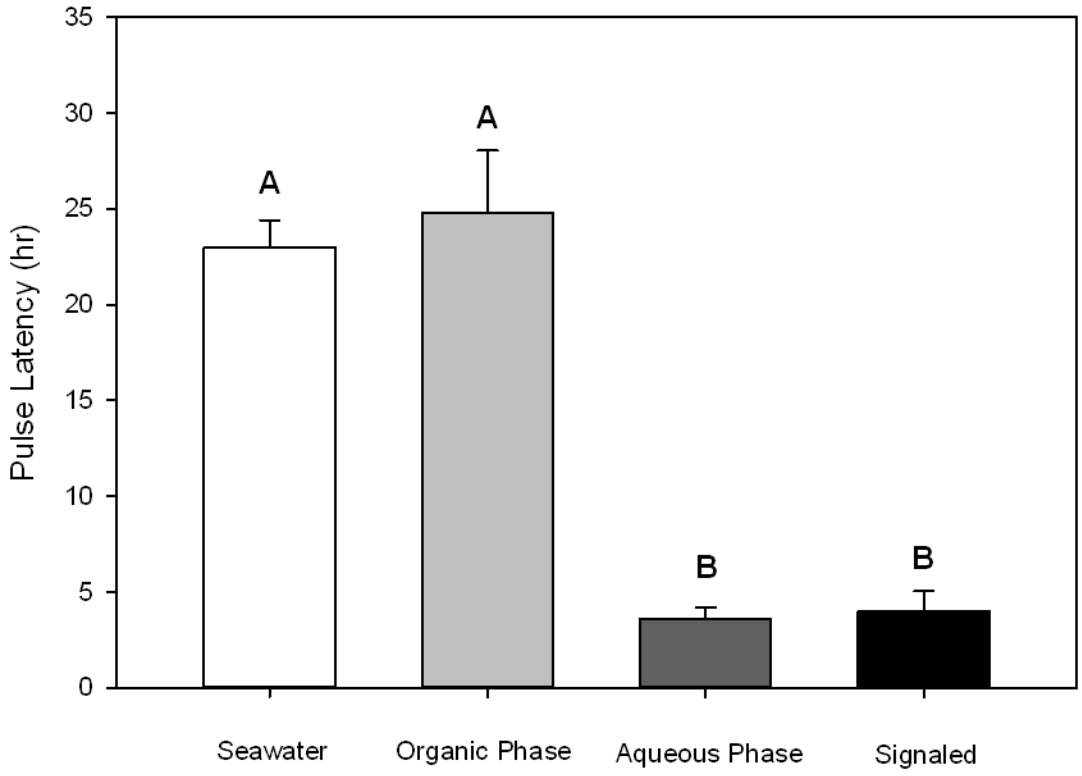


Figure 1.16. Pulse latency of gulf toadfish exposed individually to the aqueous and organic phases of C18 column filtration (N=9, N=5 respectively) compared to unaltered seawater (N=6). Data are shown as means ±1 SEM; lettering indicates significant differences as compared to control values (P=0.001; one way ANOVA, Holm-Sidak method;).

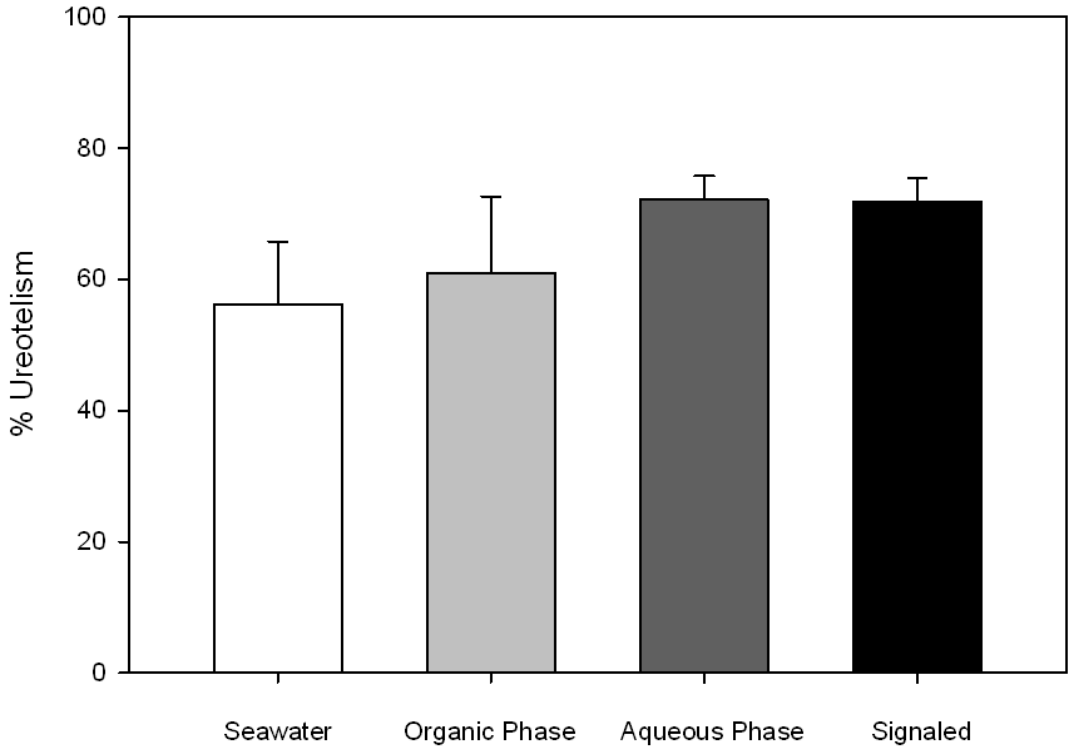


Figure 1.17. Percent ureotelism of gulf toadfish exposed individually to the aqueous and organic phases of C18 column filtration (N=9, N=5 respectively) compared to unaltered seawater (N=6). Data are shown as means ±1 SEM; no significant differences were found among groups (P=0.420; ANOVA).

DISCUSSION

Toadfish exposed to excretions of a conspecific showed a marked response in the form of large pulse of urea released shortly (on average 5 h) after exposure (fig 1.2). This study confirmed the presence of a waterborne chemical messenger that is transmitted and detected by the gulf toadfish. Due to visual barricades as well as individually confined exposures periods, *O. beta* could only have received social cues if they were released within the excretions of a donor fish. Figures 1.1-1.3 show the response of naïve toadfish to pre-conditioned water from a conspecific donor. The consistent response of lowered urea pulse latency compared to control fish supports the hypothesis for waterborne social-signal within this species.

The secretions produced during pulsing events are known to contain both forms of nitrogenous waste excreted by this fish. Previous studies have tested the hypothesis that the urea released in these events signal other toadfish to begin pulsing, however no response to this molecule was observed (Sloman *et al.* 2005). In addition to ammonia (and urea in the case of *O. beta*) teleost excretions have been shown to contain a wide variety of molecules having social effects including steroids, amino acids, nucleotides and bile salts (Wyatt 2003). This is the first study to specifically investigate the social nature and communication capability of molecules other than urea in the pulsatile urea behaviour seen in *O. beta.* The social signal used in toadfish communication could be any number of unidentified compounds that are co-excreted within pulsing events or possibly a combination of factors released together that elicits this response. This study aimed to shed light on the factor(s) responsible for signal transmission.

The detection and recognition of a social signaling molecule in an aqueous environment is complicated by dilution of the signal as it is transmitted to other individuals. To test the chemical communication hypotheses in the gulf toadfish it was first necessary to confirm whether signal detection could potentially occur in a natural environment where dilution would take place. In this study seawater containing the signaling molecule(s) was diluted by as much as 6-fold and still consistently elicited a dramatic decrease in urea pulse latency in naïve fish. Furthermore, many fish exposed to pre-conditioned seawater diluted by a factor of 12 still showed a decrease in pulse latency compared to control fish. However, this value was also significantly higher than that seen in fish exposed to un-diluted pre-conditioned water (Fig 1.4). The fact that this social compound can be diluted suggests that signal detection could potentially occur in a natural setting accounting for diminishing signal strength as it is propagated to conspecifics. Many studies have found teleost social chemicals to be diluted to very low amounts and still be detected by others (Sorensen and Caprio 1997; Wyatt 2003). Relevant to the current study, Barimo et al., (2011) were able to show that toadfish predators in mesocosms were responding to ammonia concentrations calculated to be as low as 50-250 nM. Although this is not quite what we have seen in our results for a dilution profile for *O. beta*, it is possible that toadfish in mesocosms might have higher sensitivity when stress is at a much lower level in this near-natural environment. It would be of great interest to test a putative signaling molecule in a mesocosm setting.

To identify signaling molecule(s) there are several procedures one can make use of. The process of molecule identification, specifically the classification of an aqueous signaling molecule, can be an arduous task due mainly due to the fact that there are so

many potential signaling molecules, many of which have been only recently identified. The method of heat-treatment was utilized in this study to begin the identification process by evaluating the signaling molecule's potential to be disrupted at high temperatures. Depending on the amount of heat introduced into an aqueous sample various compounds will be removed by volatilization or through heat denaturation. In the case of the protocol used in the present study, large proteins and volatile compounds, as well as low molecular weight organics such as keytones would be removed from the water sample effectively eliminating the communication mechanism if it is being propagated through one or more of the removed compounds. Figure 1.6 clearly shows that the signaling mechanism used by gulf toadfish is not heat liable. After exposure to heat-treated pre-conditioned water naïve toadfish significantly decrease pulse latency, as seen in fish exposed to untreated pre-conditioned water. This suggests that the molecule(s) in question is likely not a large steroid or aromatic compound. Furthermore, although insignificant, there was a slight increase in percent ureotelism in both heat-treated pre-conditioned water and untreated pre-conditioned water exposures further suggesting that the signaling compound(s) are not removed or altered enough to remove their bioactivity (fig 1.7).

It has been shown previously that social status did not play a role in the initiation and response of urea pulsing events (Sloman *et al.* 2005), although the pairs used in their (Sloman *et al.* 2005) study were very closely size-matched. However we do know that both serotonin and cortisol are highly involved in internal mechanisms triggering toadfish pulsing events and that these molecules themselves are known to be involved in social communication and hierarchy formation (Winberg et al. 1997;

Sloman and Armstrong 2002). In this project a specific waterborne dose of cortisol was administered based on previous findings of confined toadfish plasma concentrations (McDonald *et al.* 2003; Laberge *et al.* 2009). This value was then used to calculate the concentration that would be present if all plasma cortisol could be excreted during one pulsing event. However, waterborne cortisol exposure did not produce a decrease in urea pulse latency or an increase in percent ureotelism (Fig 1.9 and 1.10). The decrease in percent ureotelism, seen in cortisol exposed fish, as compared to control fish can be attributed to the decrease in pulse size (on average 892 μmol –N Kg^{-1}) as seen in previous studies (McDonald *et al.* 2004).

Similar results were obtained when naïve toadfish were exposed to an exogenous dose of 5-HT. Dosing concentration of 5-HT was also calculated from whole body 5-HT plasma concentrations found from previous research and estimated conservatively by summating all plasma 5-HT that could be excreted during one pulsing event (Wood *et al.* 2003). Pulse latency was not significantly decreased by the 5-HT waterborne dose. Furthermore, although statistically insignificant, there was a trend of decreasing percent ureotelism in fish exposed to 5-HT compared to control fish ($p=0.086$; Figure 1.12). These observations suggest that exogenous 5-HT does not play a major role in social communication for urea excretion in gulf toadfish.

One must also consider the fact that the percent ureotelism found in many of these experiments vary quite markedly. The reason we have documented this unpredictable observation is still not completely known. However, we do know that in mesocosm studies toadfish ammonia-N and urea-N are excreted in a ratio close to 1:1

however, in most laboratory studies this value is often reported closer to a minimum of 70% urea-N 30% ammonia-N (Barimo *et al.* 2011; Wood *et al.* 2003; Walsh *et al.* 1997). Nevertheless, in this project few experimental conditions resulted in a significant difference in percent ureotelism between control and exposure groups. This result may be attributed naïve (recipient) fish becoming ureotelic because of confinement in a small chamber even without exposure to conditioned water, and it is for this reason that the percent ureotelism data did not differ substantially between fish exposed to control seawater and fish exposed to conditioned seawater. If these periods were decreased to 24 h (as was conducted in many previous studies) fish exposed to the signaling compound would almost certainly have a significantly increased ureotely. Furthermore, in the hormonal exposure periods, where we see the largest discrepancy in ureotely, results can easily be skewed as even small amounts of absorption of these molecules can alter stress levels which are know to affect toadfish pulse behaviour (Walsh *et al.* 1997).

Octadecylsilane solid phase extraction cartridges are another method to refine candidate signaling-molecules and in fact have been used previously to fractionate bioactive teleost excretions (Kartae *et al.* 2011; Tierney *et al.* submitted). In their (Kartae *et al.* 2011; Tierney *et al.* submitted) studies the separation and re-exposure of organic and aqueous fractions of toadfish urea pulse components clearly showed that naïve fish do not respond to the organic phase, instead only reacting to the aqueous portion of the pulse (Figure 1.15). First, these findings suggest that the active signal component(s) are located within the aqueous phase. This understanding allows us to eliminate most steroids, bile salts and hydrophobic amino acids as possible candidates

for the signaling molecule. Secondly, this result further supports our observation that cortisol and 5-HT do not act as the signal messenger as both would be contained in the organic phase after filtration.

As described by figure 1.13 ammonia when administered exogenously to a concentration of 50 uM does significantly decrease pulse latency in exposed toadfish from 23.5 h, in control individuals, to 15 h. This is an interesting result clearly suggesting that ammonia may play some role in the communication of pulsatile urea action in gulf toadfish. Furthermore, due to the fact that ammonia resides in the aqueous portion this result is consistent with the result of the C18 column experiments. However, the decrease in latency attributed to ammonia is not as large as what would be expected from a pure pulse exposure including all secretions from the donor toadfish, i.e., down to 5 h in typical experiments (Fig 1.2). Therefore further data analysis was warranted on ammonia exposure to better understand the role it plays in this phenomenon. Plotting the pulse latencies of all experimental fish vs. the ammonia concentration within their water immediately prior to their pulse event was a way to directly examine whether ammonia was the single factor causing the decrease in pulse latency in previous experiments as well. Figure 1.15 shows that there is some merit to ammonia being the social signal, as in all experiments conducted there is a tendency for fish to pulse when ambient ammonia concentrations are high. However these data again make it clear that the concentration of ammonia in the exposure water does not account for the complete decrease in pulse latency (4.5 h pulse latency) as seen in pure pulse exposure. Using the equation that describes the line fitted to figure 1.15 one can calculate the predicted effect of ammonia on latency. At an ammonia concentration of 0

μM pulse latency is predicted to be 27.52 h. Contrasting this to the predicted latency time of fish exposed to 50μM ammonia concentrations, one would expect to see a decrease in latency to an average of 11.8 h, again a lower value, but not as low as the 5h typical of exposure to pulse water in its entirety. This analysis suggests that ammonia may play a role in signal detection and operates as either: (1) a single component of the multi-component excretion used for social communication in *O. beta* or (2) that ammonia is responsible for reducing the sensitivity to the signaling molecule(s). One could envision a role for exogenous ammonia in potentially changing the resting membrane potential and/or intracellular pH of gill cells via entry through apical Rh proteins (responsible for transport of ammonia). Interestingly, in the mitochondrial rich cells (MRC) of toadfish gills, the cells responsible for urea excretion, while no Rh proteins exist on the basolateral membrane, Rhcg1 is expressed on the apical membrane (Bucking et al., submitted), presenting a potential path for ammonia entry in to MR cells.

In many fish hierarchies the largest individual holds the dominant position and therefore it was natural to ask if body mass of the donor or recipient fish alters the response of urea pulses. Not surprisingly we did see a strong correlation between body mass and pulse latency in fish that were exposed to pre-conditioned water. The smaller the body mass of the recipient fish the more sensitive it was to detection of the social signal. That is to say that with a high concentration (3000 μM) of urea in the pre-conditioned water or a low concentration (10 μM) of urea in pre-conditioned water, fish with a body mass of approximately less than ~150.0 g reacted the same (pulse latency of 4.5 h on average). However, the individuals with a larger body mass did not

react at all to pre-conditioned water with a low concentration of urea but, responded quickly (same as a low body mass fish) to an exposure of pre-conditioned water that contained a high concentration of urea. This observation suggests that there are other mechanisms in place within this species that allow them to respond differently to this social stimulus under different situations. Furthermore, this also suggests that fish may gather other information from the detection of this social signaling compound, such as the size/social status of the donor (as larger fish excrete larger amounts of urea in their pulses, on average). Due to the observation that larger fish do not respond in the same manner to the signal of small donors it may also imply a possible dominance relationship if this is in fact a territorial marking adaptation.

OVERALL SUMMARY AND SIGNIFICANCE OF THESIS

In summary we have shown that a waterborne social signal does exist in the gulf toadfish. This signal is delivered to conspecifics via a mechanism that could potentially be passed through closely spaced toadfish, which is what has been observed in their natural setting. In this regard, prior ecological surveys have shown typical toadfish densities in Florida Bay to be approximately 2 m^{-2} (Sogard et al., 1987), This spatial packing suggests that the signaling compound(s) could be diluted, and still potentially elicit a response, especially if sensitivities in wild fish are higher compared to laboratory fish. Notably, signaling could be 'daisy-chained' in that toadfish B could react to toadfish A within a certain radius, and then B could transmit information to C also within its radius even if C were spatially further away from A, and so forth. The molecule(s) that make up this signal are heat-stable up to at least 90°C. Chemicals

highly involved in internal urea pulsing events (cortisol and 5-HT) are not responsible for propagating this message through the water to the rest of the population. Using C18 column filtration and behavioural bioassays, we have shown that the signaling compound does not reside in the organic fraction of the donor's excretion. Ammonia has been shown to play a partial role in signal detection in one or two possible ways. Firstly, ammonia may reduce the recipient fish's sensitivity to the toadfish-signaling compound or secondly, the social signaling mechanism may have at least two components. In the latter hypothesis ammonia is expected to be one component of the multi-component signal and the remaining molecules are likely to reside within the aqueous portion of a toadfish pulse.

UNANSWERED QUESTIONS AND FUTURE DIRECTIONS

Although the present thesis advances our understanding of the urea pulse phenomenon in toadfish, there still exists a considerable amount of uncertainty with respect to the adaptive significance of pulsatile urea events in the gulf toadfish. We have now confirmed the presence of a social messenger co-excreted during the urea pulse event but many questions still remain. Earlier I presented two working hypotheses for the role of pulsatile urea excretions. With this research we suggest that the latter, a territorial marking mechanism, seems a more likely explanation to describe the natural synthesis and pulsatile emission of urea as well as the social communication that takes place via urea excretion. With the additional involvement of a body mass factor controlling some aspects of this mechanism, it is possible that toadfish are both

marking their territory, especially in socially crowded environments, and also communicating their social status to others within the population. Further studies are needed to completely unravel the adaptive significance of this behaviour as well as the full compliment used for signal propagation in *O. beta*. Two key sets of experiments should be conducted next. Namely, the aqueous portion of signal water should be concentrated and subjected to chemical analyses (by for example HPLC-Mass Spectrometry) to determine whether there are molecules in this fraction not present in control seawater. If molecules other than ammonia can be identified, they should be purified/synthesized (or potentially purchased commercially) and used in mesocosm based experiments to see whether, and at what concentrations, they can elicit urea pulses in recipient fish. These experiments could be conducted in combination with various concentrations of ammonia. Further, mesocosm experiments could also focus on varying the numbers, sizes and densities of toadfish in the enclosure with and without signal molecules.

References

Anderson, P. M. (1995). Urea cycle in fish: molecular and mitochondrial studies. In ionoregulation: *Cellular and Molecular Approaches*, vol. 14 (ed. C. M. Wood and T.J. Shuttleworth), pp. 57-83. New York: Academic Press.

Anderson, P. M. (2001). Urea and glutamine synthesis: environmental influences of nitrogen excretion. In Nitrogen Excretion (ed. P. A. Wright and P. M. Anderson), pp. 239-277. San Diego, CA: Academic Press.

Barber, M. L. and Walsh, P. J. (1993). Interactions of acid–base status and nitrogen excretion and metabolism in the ureogenic teleost *Opsanus beta.* J. Exp. Biol. **185**, 87–105.

Barimo, J.F. Walsh, P.J. and McDonald,M.D. (2011). Diel patterns of nitrogen excretion, plasma constituents and behavior in the gulf toadfish (*Opasnua beta*) in laboratory versus outdoor mesocosm settings. Physiol. Biochem. Zool. **83,** 958-972.

Bucking, C., S.L. Edwards, P. Tickle, C.P. Smith, M.D. McDonald, S.F. Perry and P.J. Walsh. Immunohistochemical localization of urea and ammonia transporters in two confamilial fish species, the ureotelic gulf toadfish (*Opsanus beta*) and the ammonotelic plainfin midshipman (*Porichthys notatus*), *Cell Tiss. Res.*, in review.

Corkum, L. D., Arbuckle, W. J., Belanger, A. J., Gammon, D. B., Li, W., Scott, A. P. and Zeilinski, B. (2006). Evidence of a male sex pheromone in the round goby (Neogobius *melanostomus).* Biological Invasions, **8,** 105–112.

Falcón, J. (1999). Cellular circadian clocks in the pineal. Prog. Neurobiol. **8,** 121–162.

Vancic, I. and Degobbis, D. (1984). An optimal manual procedure for ammonia analysis in natural waters by the indophenol blue method. Water Res. **1B,** 1143-1147.

Gammon, D.B., Li, W., Scott, A.P., Zielinski, B.S. and Corkum, L.D. (2005). Behavioural responses of female *Neogobius melanostomus* to odours of conspecifics. J. Fish Bio*.,* **67,** 615–626.

Gilmour, K.M., Perry, S.F., Wood, C.M., Henry, R.P., Laurent, P., Pärt P. and Walsh, P.J. (1998). Nitrogen excretion and the cardiorespiratory physiology of the gulf toadfish, *Opsanus beta*. Physiol. Zool. **71,** 492-505.

Hopkins, T.E., Wood C.M. and Walsh P.J . (1995). Interactions of cortisol and nitrogen metabolism in the ureogenic gulf toadfish, Opsanus beta. J. Exp. Biol. **198**, 2229-2235.

Katare, Y.K., Scott, A.P., Laframboise, A.J., Li, W., Alyasha'e, Z., Caputo, C.B., Loeb, S.J., Zielinski, B. (2011). Release of free and oncjugated froms of the putative pheromonal steroid 11-oxo-etiocholanolone by reproductively mature male round goby (*Neogobius melanostomus*). Biol Reprod. **84,** 288-98.

Laberge, T., Walsh, P.J. and McDonald, M.D. (2009). Effects of crowding on ornithine-urea cycle enzyme gene expression and activity of the gulf toadfish (Opsanus *beta).* J Exp Biol **212,** 2394–2402.

Laurent, P., Wood, C.M., Wang, Y., Perry, S.P., Gilmour, K.M., Part, P., Chevalier, C., West, M. and Walsh, P.J. (2001). Intracellular vesicular trafficking in the gill epithelium of urea-excreting fish. *Cell Tiss. Res.* **303,** 197–210.

Mathuru, A.S., Kibat, C., Cheong, W., Shui, G., Wenk, M.R., Friedrich, R.W. and Jesuthasan, S. (2012) Chondroitin fragments are odorants that trigger fear behavior in fish. *Curr Biol*, **22,** 538-544.

McDonald, M.D., Gilmour, K.M., Barimo, J.F., Frezza, P.E., Walsh, P.J. and Perry, S.F. (2007). Is urea pulsing in toadfish related to environmental O2 or CO2 levels? Comp. Biochem. Physiol. **146A**, 366-374.

McDonald, M.D., Gilmour, K.M., Walsh, P.J. and Perry, S.F. (2010). Cardiovascular and respiratory reflexes of the gulf toadfish, Opsanus beta, during acute hypoxia: the role of 5-HT2 receptors, Respir. Physiol. Neurobiol. **170**, 59-66.

McDonald, M.D., Walsh, P.J. and Wood, C.M. (2012). 5-Hydroxytryptamine initiates pulsatile urea excretion from perfused gills of the Gulf toadfish (Opsanus beta), Comp. Biochem. Physiol. Part A: Molecular and Integrative Physiology, **163**, 7-30.

McDonald, M.D., Wood, C.M., Grosell, M., Walsh, P.J. (2003). Glucocorticoid receptors are involved in the regulation of pulsatile urea excretion in toadfish. J. Comp. Physiol. **174B,** 649–658.

McDonald M.D. and Walsh, P.J. (2004). Dogmas and controversies in the handling of nitrogenous wastes: 5-HT2-like receptors are involved in triggering pulsatile urea excretion in the gulf toadfish, *Opsanus beta*. J. Exp. Biol. **207,** 2003–2010.

Milinski, M., Griffiths, S., Wegner, K.M., Reusch, T.B.H., Haas-Assenbaum, A., Boehm, *T*. (2005). Mate choice decisions of stickleback females predictably modified by MHC peptide ligands. *Proc. Natl Acad. Sci.* USA **102** 4414–4418.

Mommsen, T.P. and Walsh, P.J. (1989). Evolution of urea synthesis in vertebrates: the piscine connection. Science **243,** 72-75.

Mommsen, T. P. and Walsh,P. J. (1991). Urea synthesis in fishes: evolutionary and biochemical perspectives. *In Phylogenetic and Biochemical Perspectives*, vol. 1 (ed. P. W. Hochachka and T. P. Mommsen), pp. 138-162. New York: Elsevier.

Mutoh, T., Shibata, S., Korf, H-W. and Okamura, H. (2003). Melatonin modulates the light-induced sympathoexcitation and vagal suppression with participation of the suprachiasmatic nucleus in mice. J. Physiol. **547,** 317

Rahmatullah, M. and T. Boyd. (1980). Improvements in the determina- tion of urea using diacetyl monoxime methods without deproteini- sation. Clin. Chem. **107,** 3–9.

Rodela, T.M., McDonald, M.D., Walsh, P.J. and Gilmour, K.M. (2009). The regulatory role of glucocorticoid and mineralocorticoid receptors in pulsatile urea excretion of the gulf toadfish, *Opsanus beta.* J. Exp. Biol. **212,** 1849-1858.

Rodela, T.M., Esbaugh, A.J. Weihrauch, D. Veauvy, C.M. McDonald, M.D. Gilmour, K.M. and Walsh P.J. (2011). Revisiting the effects of feeding and crowding in the gulf toadfish, Opsanus beta: the role of Rhesus glycoproteins in nitrogen metabolism and excretion. J. Exp. Biol. **205**, 301-313.

Serrano, R., Blanes, M.A. and Lopez, F.J. (2008). Maternal transfer of organochlorine compounds to oocytes in wild and farmed gilthead sea bream (Sparus aurata). Chemosphere **70,** 561–566.

Sloman, K.A. and Armstrong, J.D. (2002). Physiological effects of dominance hierarchies: laboratory artefacts or natural phenomena? J Fish Biol **61,** 1–23.

Sloman, K.A., McDonald, M.D., Barimo, J., FLepage, O., Winberg, S., Wood, C.M. and Walsh, P.J. (2005). The role of pulsatile urea excretion in the gulf toadfish, *Opsanus beta*, as a social signal. Physiol. Biochem. Zool. **78,** 724-735.

Sogard, S.M., Powell, G.V.N. and Holmquist, J.G. (1987). Epibenthic fish communities on Florida Bay banks: relations with physical parameters and seagrass cover. Mar. Ecol. Prog. Ser. **40,** 25-39.

Sorensen, P.W. and Caprio, J. (1997). Chemoreception. *In* The phys- iology of fishes. 2nd ed. *Edited by* D.H. Evans. CRC Press, Boca Raton. pp. 375–405.

Speedie, N. *and* Gerlai, R. *(*2008*).* Alarm substance induced behavioral responses in zebrafish (*Danio rerio*). Behav. Brain. Res. ***188,*** *168–177.*

Stewart, M., Baker, C. and Cooney, T. (2011). A Rapid, Sensitive, and Selective Method for Quantitation of Lamprey Migratory Pheromones in River Water. J. Chem. Ecol. **37,** 1203–1207.

Tierney, K.B., Kereliuk, M., Katare, Y.K., Scott, A.P., Loeb, S.J. and Zielinski, B. Extractable components in reproductive male urine attract females in the invasive round goby (*Neogobius melanostomus*). J. Chem. Ecol. In review.

Walsh, P.J., Tucker, B.C. and Hopkins, T.E. (1994). Effects of confinement/crowding on ureogenesis in the gulf toadfish Opsanus beta. J. Exp. Biol. **191,** 195-206.

Walsh, P. J., Danulat, E. and Mommsen, T. P. (1990). Variation in urea excretion in the gulf toadfish, *Opsanus beta*. Mar. Biol. **106,** 323–328.

Walsh, P.J. and Milligan, C.L. (1995). Effects of feeding and confinement on nitrogen metabolism and excretion in the gulf toadfish *Opsanus beta*. J. Exp. Biol. **198,** 1559-1566.

Winberg, S., Nilsson, A., Hylland, P., Soderstom, V. and Nilsson, G.E. (1997). Serotonin as a regulator of hypothalamic–pituitary–interrenal activity in teleost fish. Neurosci. Lett. **230,** 113–116.

Wood, C. M. (1993). Ammonia and urea metabolism and excretion. In *The Physiology of Fishes*, chapter 13 (ed. D. H. Evans), pp. 379–425. Boca Raton: CRC Press.

Wood, C.M., McDonald, M.D., Sundin, L., Laurent, P. and Walsh, P.J. (2003). Pulsatile urea excretion in the gulf toadfish: mechanisms and control. Comp. Biochem. Physiol. **136B,** 667-684.

Wood, C.M., Warne, J.M., Wang, Y., McDonald, M.D., Balment, R.J., Laurent, P., Walsh, P.J. (2001). Do circulating plasma AVT andyor cortisol levels control pulsatile urea excretion in the gulf toadfish (Opsanus *beta)?* Comp. Biochem. Physiol. **129,** 859–872.

Wood, C.M., Hopkins, T.E., Hogstrand, C. and Walsh, P.J. (1995). Pulsatile urea excretion in the ureagenic toadfish *Opsanus beta*: an analysis of rates and routes. J. Exp. Biol. **198,** 1729–1741.

Wood, C.M., Hopkins, T.E. and Walsh, P.J. (1997). Pulsatile urea excretion in the toadfish (Opsanus beta) is due to a pulsatile excretion mechanism, not a pulsatile production mechanism. J. Exp. Biol. **200,** 1039-1046.

Wyatt T.D. (2003). Pheromones and animal behaviour: communication by smell and taste. Cambridge University Press, Cambridge