2012-05-04

The effect of Chronic Stress on Gill 5HT2A Receptors and Pulsatile Urea Excretion in Opsanus beta

Alexander W. Frere
University of Miami, alexander.frere@gmail.com

UNIVERSITY OF MIAMI

THE EFFECT OF CHRONIC STRESS ON GILL $5HT_{2A}$ RECEPTORS AND PULSATILE UREA EXCRETION IN *OPSANUS BETA*

By
Alexander W. Frere

A THESIS

Submitted to the Faculty

of the University of Miami

in partial fulfillment of the requirements for

the degree of Master of Science

Coral Gables, Florida
May 2012

UNIVERSITY OF MIAMI

A thesis submitted in partial fulfillment of
the requirements for the degree of
Master of Science

THE EFFECT OF CHRONIC STRESS ON GILL $5HT_{2A}$ RECEPTORS AND PULSATILE UREA EXCRETION IN *OPSANUS BETA*

Alexander W. Frere

Approved:

Danielle McDonald, Ph.D. Assistant Professor of Marine Biology & Fisheries	Terri A. Scandura, Ph.D. Dean of the Graduate School
Douglas Crawford, Ph.D. Professor of Marine Biology & Fisheries	Diego Lirman, Ph.D. Associate Professor of Marine Biology & Fisheries
Chris Wood, Ph.D. Research Professor of Marine Biology & Fisheries	Lynne Fieber, Ph.D. Associate Professor of Marine Biology & Fisheries

FRERE, ALEXANDER W. (M.S., Marine Biology & Fisheries)

The Effect of Chronic Stress on Gill $5ht_{2a}$ Receptors and Pulsatile Urea Excretion in *Opsanus beta* (May 2012)

Abstract of a thesis at the University of Miami.

Thesis supervised by Dr. Danielle McDonald.

No. of pages in text. (43)

The Gulf toadfish (*Opsanus beta*) is a facultative ureotele in which urea production via the ornithine-urea cycle (O-UC) and pulsatile urea excretion are initiated by high cortisol levels, but a permissive drop in cortisol is required for the activation of the toadfish urea transporter (tUT) to occur. The goal of this study is to determine the exact relationship between cortisol and regulation of the serotonin 2A ($5\text{-}HT_{2A}$) receptor, which is believed to be responsible for the activation of tUT. A better understanding of the interactions between cortisol and the $5\text{-}HT_2$ receptor family could provide some answers or new treatment methods for humans who suffer from hypercortisol and hypocortisol conditions like Cushing's Syndrome and Addison's Disease, respectively. The hypothesis of the study was that chronically elevated cortisol levels will result in an increase in the gill basolateral membrane population of $5\text{-}HT_{2A}$ receptors through membrane insertion with no significant corresponding change in gill $5\text{-}HT_{2A}$ receptor mRNA expression. Cortisol levels were elevated after one week of crowding compared to uncrowded controls and were reduced by treatment with the cortisol production blocker, metyrapone, compared to saline-treated controls. Crowded fish showed no difference in plasma 5-HT yet had elevated plasma urea concentrations compared to

uncrowded fish; in contrast, plasma urea levels were not different between control and metyrapone -injected fish. Furthermore, no significant difference was measured in gill or brain 5-HT_{2A} mRNA expression levels among uncrowded and crowded, control- or metyrapone-treated fish. A change in [^{3}H] serotonin binding kinetics or in the competition of the 5-HT_2 agonist, α-methyl 5-HT, against [^{3}H] serotonin was also not seen in isolated gill basolateral membranes of uncrowded or crowded toadfish. However, binding kinetics of the 5-HT_{2A} receptor agonist, [^{3}H] ketanserin, was significantly different between the four groups of fish. Specifically, the binding affinity (K_D) values for uncrowded, saline-injected, and metyrapone injected fish were similar to mammalian values for the 5-HT_{2A} receptor, while crowded fish had a K_D that was two-fold higher, suggesting a slight upregulation in a lower affinity receptor in this group of fish. Furthermore, we found the binding maximum (B_{max}) of metyrapone-injected fish to be the greatest followed in descending order by control, crowded, and uncrowded fish. Our results suggest that chronic cortisol elevations may increase 5-HT_{2A} receptor populations on the basolateral membrane. Interestingly, metyrapone-treated fish excreted approximately 2-fold more urea compared to controls when injected with α-methyl 5-HT suggesting that lowering endogenous levels of cortisol on a relatively acute time frame may remove a subsequent inhibition of cortisol on the 5-HT_{2A} receptor or stimulate 5-HT_{2A} recruitment to the basolateral membrane.

Table of Contents

Chapter 1: Stress and 5-HT$_{2A}$

Introduction

Over seagrass beds in the Gulf of Mexico and along the Florida coast, the Gulf toadfish (*Opsanus beta*) undertakes a constant struggle to occupy their ambush predator niche without becoming prey themselves. Predator avoidance is believed to be achieved in part through the cloaking of nitrogenous waste, whereby ammonia is easily detected by chemosensory predators but urea is not (Barimo and Walsh, 2006). Possibly exploiting this, toadfish are facultatively ureotelic, and instead of excreting ammonia alone, toadfish will produce and excrete urea and ammonia, perhaps in an attempt to mask their presence (Barimo and Walsh, 2006).

Another interesting component of toadfish nitrogen waste metabolism and excretion is that when stressed, toadfish do not excrete urea continuously but instead emit pulses of urea (Wood et al. 1997). Their actual production of urea is constant, but the release of urea into the water, usually about once every 12-24 hours, is controlled by a more elusive mechanism (Wood et al. 1997; Wood et al. 1998). Urea does not pass through the gill membrane on its own, rather the toadfish urea transporter (tUT), a facilitated diffusion urea transport mechanism most similar to mammalian UT-A2 urea transporters (Smith et al. 1998; Wood et al. 2001), is responsible for excretion of urea across the gill. Glucocorticoids, more specifically the stress hormone, cortisol, have been shown to have a key role in this pulsatile mechanism (Wood et al. 1997; Wood et al., 2001; McDonald et al. 2004; McDonald et al., 2009; Rodela et al. 2009). While it is the elevation of cortisol from a stressor that initiates the production of urea (Walsh et al. 1994, Hopkins et al. 1995; Laberge et al., 2009) as well as an upregulation in tUT mRNA expression (McDonald et al., 2009), this same elevation of cortisol appears to also inhibit

pulsatile urea excretion (Wood et al. 1997; Wood et al. 2001; McDonald et al. 2004; McDonald et al., 2009; Rodela et al. 2009) as the activation or insertion of tUT into the gill membrane only occurs during reduced levels of cortisol (Wood et al. 1997, Wood et al., 2001; McDonald et al., 2004; Rodela et al. 2009). Since injection with the cortisol production blocker, metyrapone, lowers cortisol levels but leads to pulses of an inadequately small magnitude (Wood et al., 2001) and a natural drop in cortisol can be observed without a corresponding pulse occurring, it is believed that the drop in cortisol alone cannot be the trigger (Wood et al. 1997; Wood et al. 2001). Instead low cortisol levels are hypothesized to allow the activation or the membrane recruitment of tUT to occur.

So, what is responsible for activating or mediating the membrane recruitment of tUT? In mammals, urea transport is regulated by the hormone, arginine vasopressin (AVP or antidiuretic hormone), which increases cAMP and intracellular Ca^{2+} resulting in the relocation of UT-A2 to the plasma membrane and/or its phosphorylation (Potter et al. 2006; Star et al. 1988). However, intravenous injection with the fish analog of AVP, arginine vasotocin (AVT), does not produce a natural-sized urea pulse in toadfish but rather urea pulses that are approximately 10% the size (Wood et al. 2001). Instead, pulsatile urea excretion in toadfish is stimulated by an intravenous injection with the neurotransmitter, serotonin (5-hydroxytryptamine; 5-HT) in approximately 70% of the fish injected (Wood et al. 2003). McDonald and Walsh (2004) went on to investigate which of the seven families of possible 5-HT receptors (Hoyer et al. 2002) could play a role in mediating this response and found that intravenous injection with the 5-HT_2 receptor agonist, α-methyl 5-HT, elicited a pulse in 100% of the fish injected.

Furthermore, intraperitoneal treatment with the 5-HT_{2A} receptor antagonist ketanserin immediately before injection with α-methyl 5-HT resulted in a dose-dependent inhibition of urea excretion and pulse size (McDonald and Walsh, 2004), suggesting that of the three 5-HT_2 receptors (5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C}), 5-HT_{2A} is the most likely in eliciting this response. Interestingly, stimulation of the 5-HT_{2A} receptor leads to the accumulation of IP_3, which stimulates the release of Ca^{2+} stores from the endoplasmic reticulum (Raote et al. 2007), suggesting a potential link between the mode of action of AVP on the mammalian UT-A2 and that of 5-HT mediated by 5-HT_{2A} on the toadfish tUT. Since the early pharmacological work on the toadfish 5-HT_{2A} receptor, the full-length toadfish 5-HT_{2A} receptor has been fully sequenced (Genbank Accession # FJ611960), has been shown to have molecular homology and pharmacological similarity to mammalian 5-HT_{2A} receptors and is expressed in significant quantities in the swim bladder, hindbrain, gill, and other organs (EM Mager, LR Medeiros, A Lange and MD McDonald, unpublished).

Since changes in circulating cortisol are involved in controlling tUT, we hypothesized that the toadfish 5-HT_{2A} receptor, which is believed to mediate the stimulation or membrane recruitment of tUT, might also be the target of cortisol's regulatory action. For example, the chronic administration of corticosterone for one week has been shown to lead to an increase in [^{3}H] ketanserin 5-HT_{2A} binding in the parietal cortex (Kuroda et al. 1992, Fernandes et al. 1997, Hanson et al. 1998). Other studies have shown differential regulation in 5-HT_{2A} mRNA expression depending on whether the stress was acute (e.g., a single shock) or chronic (e.g., repeated shock) (Dwivedi et al. 2005).

The goal of the present study was to investigate the role of cortisol and the toadfish 5-HT_{2A} receptor in the activation of tUT by testing the hypothesis that chronically elevated levels of cortisol, which are associated with the switch to ureotely (Walsh et al. 1994, Hopkins et al. 1995), will result in an increase in the gill basolateral membrane population of 5-HT_{2A} receptors through membrane insertion with no significant corresponding change in gill 5-HT_{2A} receptor mRNA expression. To test this hypothesis, cortisol levels in toadfish were manipulated by exposing fish either to uncrowded (unstressed) or crowded (stressed) conditions for 1 week or exposing fish to 1 week of crowding and a 48 h treatment with the cortisol production blocker, metyrapone. Plasma cortisol, 5-HT, urea and 5-HT_{2A} mRNA expression, binding kinetics and function were measured.

Materials and Methods

Experimental Animals

Commercial shrimpers using roller trawls in Biscayne Bay captured Gulf toadfish (*Opsanus beta*) that were immediately transferred to indoor laboratory holding tanks. Toadfish were treated with malachite green (final concentration 0.05 $mg \cdot l^{-1}$) in formalin (15 $mg \cdot l^{-1}$, Aquavet) on the first day in the lab to prevent contamination with *Cryptocaryon irritans* (Stoskopf, 1993). The fish were kept in 50 l glass aquaria with constant water flow with aerated seawater between 17-24°C. Fish were fed every week with either squid or shrimp.

Experimental Treatments

Experimental Series i investigated differences between toadfish that were randomly placed in either uncrowded (unstressed) or crowded (stressed) conditions. Uncrowded fish were put in a plugged minnow trap (17" by 8" diameter) then placed in an outdoor 6000 l mesocosm for one week; one fish per trap, four traps in each corner of the mesocosm. A total of four mesocosms, with constant aeration and flow-through seawater were used repeatedly throughout the experiment, each containing the seagrass, *Thalassia testudinum*, mimicking the natural environment of toadfish (Serafy et al. 2007). Fish that were stressed by being held in crowded conditions were placed in a 10 l holding tank, 10 toadfish per tank, with three to four, four-inch PVC pipes as shelters, for one week. These smaller tanks were constantly aerated and supplied with either flow-through seawater or experienced a daily 80% water change. After one week, fish were used for quantitative PCR (qPCR) and binding assays as described below.

Experimental Series ii investigated differences between toadfish that were initially crowded for one week, then surgically implanted with intraperitoneal (IP) catheters and treated to manipulate circulating cortisol levels as described by McDonald and Walsh (2004). A 15 cm segment of PE50 tubing (Clay-Adams; Sigma), flared at the one end to prevent accidental removal, was fed through a very small incision made into the peritoneal cavity and secured with 3-0 suture silk (Roboz Surgical). A subset of these fish was used in the α-methyl 5-HT challenge as described below. The remaining fish were placed in their own 1 l tanks with water and air supply. After a 24 hour recovery period, toadfish were injected with 1.5 µl saline $\cdot g^{-1}$ fish; or with 20 µg$\cdot$1.5 µl saline$^{-1}\cdot$g fish^{-1} of the cortisol production blocker, metyrapone (2-Methyl-1,2-di-3-pyridinyl-1-propa, Sigma) as described by Rodela et al. (2009). Subsequent injections were administered at t = 48 h and t = 69 h. At 96 h, fish were used for qPCR or binding assays as described below.

Experimental Protocols

i. Quantitative PCR (qPCR)

Total RNA was isolated from gill and brain samples from uncrowded (N = 8), crowded (N = 6), saline-injected controls (0.036 ± 0.003 (7) kg) or metyrapone-injected fish (0.040 ± 0.005 (7) kg) using the protocol provided with Trizol Reagent (Invitrogen). Total RNA was then treated with DNAse I to remove genomic DNA using the Turbo-DNA-free kit (Ambion). cDNA was made using Superscript First Strand Kit (Invitrogen). cDNA from saline and metyrapone-treated fish was then diluted ten-fold (samples from crowded and uncrowded groups were not diluted) with molecular biology grade water (Sigma-Aldrich). Mx3005P Multiple Quantitative PCR system (Stratagene) was used with SYBR Green qPCR Master Mix (Applied Biosystems) as the reporting dye. qPCR was run with the following thermal cycles: 95°C for 10 minutes, 40-50 cycles at 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds. Fold changes between groups were determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Elongation Factor 1 alpha (E1α) was used to normalize expression of each sample using primers outlined in McDonald et al. (2009). Expression of the gene of interest, the $5HT_{2A}$ receptor, was quantified using qPCR primers described by Mager et al. *submitted*. 'No template' and 'no reverse transcriptase' controls were run to check for primer dimers or genomic DNA contamination of samples.

ii. Binding assays using isolated gill basolateral membranes

Gill basolateral membranes (BLMs) were isolated using differential centrifugation as outlined by Rodela et al. (2009), originally sourced from Perry and Flik (1988) and modified by Bury et al. (1999). Uncrowded (105 fish pooled to give N = 6 - 8), crowded (83 fish pooled to give N = 6 - 8), saline-treated (12 fish pooled to give N = 6, of which the 3 with the highest average cortisol levels were used) or metyrapone-treated (12 fish pooled to give N = 6, of which the 3 with the lowest average cortisol levels were used [this was done to assure that toadfish examined were adequately responding to the metyrapone treatment]) toadfish were removed from their tanks, wrapped in a paper towel and a blood sample (0.2 ml) was taken immediately from the caudal vein using a 23 gauge needle on a 1 ml disposable syringe. The blood was spun for 10 minutes at 16,000 rpm, plasma was removed from the top layer with caution so as not to remove any blood cells from the bottom layer, and was promptly frozen in liquid nitrogen and stored at -80°C for later analysis of plasma cortisol, 5-HT and urea. The fish were then overanesthetized in MS222 (3 $g \cdot l^{-1}$; Argent Chemical Laboratories) and subsequently pithed by a scalpel. The dorsal aorta was severed to allow for an outflow of blood and perfusate. The heart was exposed and a small incision was made in the heart ventricle through which a blunted 18g needle attached to a 60 ml syringe filled with modified saline (0.9% NaCl, 0.5$mmol \cdot l^{-1}$ Na_2-EDTA, 20 $IU \cdot l^{-1}$ heparin; adjusted to pH 7.8 with Tris) was inserted. The heart was perfused with approximately 30 ml of modified saline; allowing for the removal of blood from the fish gill. The gill was then excised and placed in ice-cold hypotonic homogenization solution (in mM: 25 NaCl, 1 HEPES, 1

dithiothreitol; adjusted to pH 7.8 with Tris). The gill from 4-8 fish from each respective treatment were pooled together to represent an individual N. Gill tissue was then carefully scraped off the gill arch on ice using a glass microscope slide. Tissue was placed back into the hypotonic homogenization solution and homogenized 30 strokes with a loose-fitting pestle in a Dounce homogenizer. The solution was then topped up to 50 ml and spun at 450 g for 15 minutes at 4°C (Sorvall RC-5B). The supernatant was then spun at 50,000 g for 30 minutes at 4°C which produced a dark bottom layer and a white top layer. The plasma membrane-rich white layer was then carefully isolated from the dark mitochondria-rich bottom layer. The plasma membranes were isolated then suspended in 20 ml of isotonic solution (in mM: 250 sucrose, 5 $MgSO_4$, 5 HEPES; adjusted to pH 8.0 with Tris). After homogenization with a tight-fitting pestle for 100 strokes, plasma membranes were spun at 1,000 g for 10 minutes followed by a 10,000 g spin for 10 minutes both at 4°C. The supernatant was preserved and spun at 38,500 g for 30 minutes at 4°C, isolating the basolateral membranes. This basolateral membrane laden pellet was then resuspended by vortex in 5-8 ml of resuspension buffer (in mM: 250 sucrose, 10 KNO_3, 0.8 $MgSO_4$, 0.5 Na_2-EDTA, 20 HEPES, 0.01 pargyline; adjusted to pH 7.4 with Tris). Protein concentration of the suspension was adjusted to approximately 50 - 100 $\mu g \cdot ml^{-1}$.

Binding assays were run using a modified protocol by Winberg and Nilsson (1996) originally for use on whole brain. Three types of experiments were completed on uncrowded *versus* crowded gill BLM: *(i)* [^{3}H] 5-HT binding saturation kinetics, *(ii)* competition kinetics with α-methyl 5-HT, a 5-HT_2 receptor agonist, and *(iii)* saturation kinetics using [^{3}H] ketanserin, a selective 5-HT_{2A} receptor antagonist. Only experiment

(iii) was completed using saline and metyrapone-treated gill BLM. In *Experiment i*, 0.1 ml of [^{3}H] 5-HT (ranging from 1 x 10^{-11} to 1 x 10^{-3} M) and 0.1 ml of assay medium (in mM: 250 sucrose, 10 KNO_3, 11 $MgSO_4$, 6 Na_2-EDTA, 20 HEPES, 0.01 pargyline; adjusted to pH 7.4 with Tris) were combined with 0.8 ml BLM suspension. In competition experiments *(Experiment ii)*, 0.1 ml of [^{3}H] 5-HT (1.8 x 10^{-8} M) and 0.1 ml of α-methyl 5-HT, a 5-HT_2 receptor family agonist, (ranging from 1 x 10^{-9} M to 1 x 10^{-2} M) were combined with 0.8 ml BLM suspension. In *Experiment iii*, 0.1 ml of [^{3}H] ketanserin (ranging from 5 x 10^{-9} to 5 x 10^{-7} M) and 0.1 ml of assay medium were combined with 0.8 ml BLM suspension. Thus, after being combined in the 1 ml solution, the concentrations of agonist (5-HT, α-methyl 5-HT) or antagonist (ketanserin) was diluted 10-fold. The preparations were incubated for 45 minutes at 25°C. Following incubation, each preparation was filtered, using a Pall Corporation vacuum filtration device, through a Whatman Protran BA 85 nucleic acid and protein transfer media filter that was pre-soaked in ice-cold stop solution (in mM: 250 sucrose, 10 KNO_3, 10 $MgSO_4$, 20 Hepes; adjusted to pH 7.4 with Tris) containing 50 nM cold serotonin for *Experiments i* and *ii* and 1000 nM cold ketanserin for *Experiment* iii; followed by 3 x 5 ml rinses of ice-cold stop solution. Filters were placed in scintillation vials with 10 ml of Ecolume scintillation fluid (MP Biomedicals), left overnight and were then counted in a liquid scintillation counter (Beckman LS3801 Liquid Scintillation Counter). Nonspecific background samples were run using 0.8 ml resuspension buffer in the preparations outlined above instead of BLM suspension. To convert binding on filters from counts per minute (cpm) to fmol·mg protein^{-1}, background radioactivity and non-specific binding

was subtracted from filter cpm values, which were then multiplied by the specific activity ($mol \cdot cpm^{-1}$) of the incubation media and then standardized for protein content (mg).

iii. α-methyl 5-HT challenge experiment

To test whether reduced cortisol concentrations after a chronic elevation by crowding translated into a change in 5-HT_{2A} receptor function, toadfish that were treated with IP-injected saline or metyrapone (see above) were also subjected to an α-methyl 5-HT (alpha-methyl serotonin maleate salt; Sigma) challenge, where α-methyl 5-HT injection has been shown to stimulate urea excretion in toadfish (McDonald and Walsh, 2004). Toadfish were crowded for one week prior to surgery to induce the switch to ureotely (Walsh et al., 1994). After one week, toadfish (0.078 ± 0.005 (31) kg) were anesthetized (1 $g \cdot l^{-1}$ MS222; Argent Chemical Laboratories) and IP catheters were inserted, as described above. In addition, each fish was surgically implanted with a caudal arterial or venous catheter as described by McDonald et al. 2004. This was accomplished by cutting a 3 cm incision along the lateral line, 1 cm posterior to the anus. The site was opened with cotton swabs to prevent cutting the artery and, upon location of the spine and lateral bones, a piece of PE50 tubing (Clay-Adams; Sigma), with a 45 degree angle cut to provide a sharp end, was carefully pushed anteriorly into the caudal vessel for approximately 1-2 cm. The wound was dusted with antibiotic (oxytetracycline; Sigma) and a 2 cm length of PE160 tubing (Clay-Adams; Sigma) flared on both sides was slid onto the catheter so that it sat just inside the wound and glued in place. The wound was then sutured shut using 3-0 suture silk (Roboz Surgical) and three stitches were used to secure the PE160 anchor to the tail. Fish were then placed in their own 1 l

tanks that were constantly aerated and supplied with flow-through seawater, left to recover for 24 h and then injected intraperitoneally at t = -69, -45, -21 and -3 hours with either saline (0.073 ± 0.005 (16) kg) or metyrapone (0.083 ± 0.007 (15) kg). At the time of the last IP injection (t = -3 h), the water supply was stopped to the 1 l tank, and an initial 2 ml water sample was taken. Water samples were then taken every hour for three hours to monitor if the fish experienced a urea pulse prematurely. At t = 0 h, a 0.2 mL blood sample was drawn for analysis of plasma cortisol and urea, and all fish were injected with 0.1 μM α-methyl 5-HT·μl saline^{-1}·g^{-1} fish via the caudal catheter. Water samples were then taken every half hour until t = 4 h. The water samples were assayed for ammonia and urea concentration.

Analytical Techniques

Colorimetric assays were used to measure the appearance of ammonia-N (Ivancic and Degobbis, 1984) and urea-N (Rahmahtullah and Boyde, 1980; Price and Harrison, 1987) in the water and in the blood plasma. Plasma cortisol concentrations were measured using the Cortisol CT Kit (MP Biomedicals). The only change to the standard kit protocol was that cortisol standards were diluted by half to approximate the appropriate plasma protein range for toadfish (Hontela et al. 1992). Protein concentrations were measured using Bradford Reagent (Sigma-Aldrich). Serotonin concentrations were measured by ELISA (ALPCO Diagnostics).

Statistics

Data are presented as mean ± standard error of the mean (N), where N = the number of samples. Multiple comparisons for group means were conducted using a Student's unpaired t-test using the Sigmaplot (SPSS, Inc.) statistical package. Binding curves were determined using GraphPad Prism v5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). Specifically, GraphPad was used to generate hyperbolic or sigmoidal dose-response curves for competition and saturation binding kinetics. Urea excretion over time was analyzed with a one-way ANOVA with time as the main factor and time equals zero as a control using the Dunnett's multiple comparison test. A two-way ANOVA was conducted to test for differences between metyrapone and control groups with time and treatment as the main factors.

Results

Crowded fish had approximately 15-fold higher plasma cortisol (203.4 ± 16.5 (122) $ng \cdot ml^{-1}$) than uncrowded fish (16.3 ± 2.6 (136) $ng \cdot ml^{-1}$); ** $P < 0.001$; Fig. 1A). No significant difference was measured in plasma 5-HT concentrations (uncrowded = 4.9 ± 0.8 (8) $ng \cdot ml^{-1}$; crowded = 5.5 ± 0.7 (7) $ng \cdot ml^{-1}$; $P = 0.596$) in a subset of these fish (Fig. 1B). Crowded fish had significantly higher levels of plasma urea compared to uncrowded fish (uncrowded = 3.0 ± 0.7 (5) $mmol\text{-}N \cdot l^{-1}$; crowded = 8.0 ± 0.6 (9) $mmol\text{-}N \cdot l^{-1}$); ** $P < 0.001$; Fig. 1C). Despite an elevation in plasma cortisol, there was no significant differences in $5\text{-}HT_{2A}$ receptor mRNA expression in the gill or brain of uncrowded compared to crowded fish (Fig. 2), although there was a tendency for higher $5\text{-}HT_{2A}$ receptor expression in crowded fish gill (Fig. 2A).

There was no significant difference in [^{3}H] serotonin binding saturation kinetics, which potentially takes into account the gill's entire population of basolateral membrane-bound 5-HT receptors, in uncrowded (B_{max} = 32322 ± 8264 fmol·mg protein^{-1}, K_D = 8.2 ± 4.4 x 10^{-8} M, h coefficient = 1.3 ± 0.7; Fig 3A) compared to crowded (B_{max} = 36295 ± 7120 fmol·mg protein^{-1}, K_D = 6.0 ± 3.6 x 10^{-8} M; Fig. 3B) toadfish. There was also no significant difference in the competitive binding kinetics of α-methyl 5-HT, which targets the 5-HT_2 family of receptors, between uncrowded (IC_{50} = 6.4 ± 1.6 x 10^{-7}; Min. = 31.8 ± 4.7 %, Max. = 87.2 ± 3.7 %; Fig. 4A) and crowded (IC_{50} = 1.1 ± 0.3 x 10^{-6}; Min. = 42.6 ± 7.5 %, Max. = 95.6 ± 4.6 %; Fig. 4B) isolated gill BLM. However, a significant difference in both B_{max} (P = 0.019) and K_D (P = 0.046) was measured in [^{3}H] ketanserin binding saturation kinetics, which has the highest specificity for the 5-HT_{2A} receptor (Fig. 5). Specifically, gill BLM from uncrowded fish had an almost 50% lower B_{max} (39395 ± 1577 fmol·ml protein^{-1}) and a 4-fold lower K_D (5.5 ± 0.3 x 10^{-9} M; Fig. 5A), signifying a higher binding affinity, than crowded toadfish (B_{max} = 79922 ± 7070 fmol·ml protein^{-1}, K_D = 1.3 ± 0.2 x 10^{-8} M; Fig. 5B). The Hill coefficients were not significantly different between the two groups of fish.

With respect to the pharmacologically-manipulated fish, control fish had approximately three times higher cortisol levels (146.2 ± 16.6 (16) ng·ml^{-1}) than those of fish injected with the cortisol-production blocker, metyrapone (50.4 ± 9.3 (12) ng·ml^{-1}) (Fig. 6A). Furthermore, there was no significant difference in plasma urea concentrations (control = 6.3 ± 0.6 (7) mmol-N·l^{-1}; metyrapone = 6.9 ± 0.8 (6) mmol-N·l^{-1}) (Fig. 6B). Similar to findings in uncrowded and crowded fish, these differences in plasma cortisol did not translate to differences in 5-HT_{2A} receptor transcript levels as there was no

significant difference in $5HT_{2A}$ mRNA expression in the gill or brain of the two groups of fish (Fig. 7). When [^{3}H] ketanserin binding saturation kinetics were investigated, two significantly different curves were produced ($P = 0.0095$), with the metyrapone-treated fish having a tendency for a higher B_{max} (362 008 ± 84 030 fmol·mg protein^{-1}) than the controls (139 331 ± 25 704 fmol·mg protein^{-1}) (Fig. 8). The binding affinities and hill coefficients were consistent between the two groups (control $K_D = 4.1 \pm 1.8 \times 10^{-9}$ M, h coefficient = 1.5 ± 0.7; metyrapone-treated $K_D = 3.7 \pm 1.6 \times 10^{-9}$ M, h coefficient = 2.1 ± 1.4). Compared to both uncrowded and crowded fish, the B_{max} of both control- and metyrapone-treated fish were substantially higher, with the K_D values of these two groups falling in the range similar to that of the uncrowded fish (Fig. 5 *cf* Fig. 8; Fig. 10).

When injected with α-methyl-5-HT, a 5-HT_2 receptor agonist that stimulates urea excretion in toadfish, there was an approximate doubling of urea excretion in response to the injection in metyrapone-treated fish compared to controls (Fig. 9A *cf.* 9B, Table 1). This was reflected in a significant elevation in the size of a urea pulse (666.9 ± 154.9 (12) μmol-N·kg^{-1}) in metyrapone-treated fish compared to control individuals (253.8 ± 60.4 (16) μmol-N·kg^{-1}) (Fig. 9C) with no significant difference in the rate of ammonia excretion or in the % ureotely between the two groups (Table 1). Furthermore, slightly more metyrapone-treated fish responded to α-methyl 5-HT compared to controls (100% or 12/12 fish *vs*. 81.25% or 13/16 fish, respectively) (Fig. 9D).

Discussion

This investigation presented data suggesting a relationship between the toadfish 5-HT_{2A} receptor in the gill, the stress hormone, cortisol, and pulsatile urea excretion. In the present study, crowding and saline-treatment raised cortisol levels in toadfish while toadfish kept in uncrowded conditions and those injected with metyrapone demonstrated significantly lower cortisol levels. These data concur with previous studies (Wood et al. 1997, Wood et al. 1998; Rodela et al. 2009). The elevation in plasma cortisol demonstrated that the crowded and saline-injected control groups were likely under a larger amount of stress and, based on previous studies, we would predict that these fish would demonstrate an upregulation in all processes related to pulsatile urea excretion (Walsh et al. 1994; Hopkins et al. 1995; Laberge et al. 2009; McDonald et al., 2009), including the 5-HT_{2A} receptor. No change in circulating 5-HT levels was measured in crowded versus uncrowded fish, providing evidence that fluctuating levels of circulating neurotransmitter did not account for the changes measured, but rather fluctuations in circulating cortisol are at the heart of this study.

Laberge et al. (2009) found that after one week of crowding, four key enzymes in the ornithine-urea cycle (O-UC), liver glutamine synthetase, carbamoyl phosphate synthetase III, ornithine carbamoyl transferase and arginase, showed increased enzyme activity despite highly variable mRNA expression levels. Similarly, the present study also showed highly variable 5-HT_{2A} mRNA expression data resulting in no measurable difference in 5-HT_{2A} transcript levels in either the brain or the gill with any treatment. This finding is in contrast to a study in rats that showed differential regulation in 5-HT_{2A} mRNA expression depending on whether the stress was acute (e.g., a single shock) or

chronic (e.g., repeated shock) (Dwivedi et al. 2005). The large variation in 5-HT_{2A} receptor mRNA expression measured in toadfish could be explained by the fact that these fish are not genetically identical. Another theory for the large variation in 5-HT_{2A} receptor mRNA expression could be attributed to variations in the digestion and replacement of the receptor *i.e.*, regulation could be due to receptor relocation from the plasma membrane by clathrin-mediated endocytosis, after which time, the 5-HT_{2A} receptor-containing endosome can either be relocated back to the membrane if needed or digested by lysozymes (Gray and Roth 2001). It is currently unknown how many times each receptor is excised from the membrane before it is digested and recycled (Gray and Roth 2001).

Chronic elevations in glucocorticoids are known to increase binding maximum (B_{max}) of [^{3}H] ketanserin to the 5-HT_{2A} receptor (Kuroda et al. 1992, Fernandes et al. 1997, Hanson et al. 1998). In all three studies, rats were injected with pellets of corticosterone, their main glucocorticoid. After one week of treatment, there were increases in [^{3}H]ketanserin binding and function, as measured by the "wet dog shakes" behavior, known to be controlled by the 5-HT_{2A} receptor. In the present study, isolating the basolateral membrane of the gill through differential centrifugation allowed for the removal of any non-membrane bound 5-HT receptors that may have been internalized either prior to membrane insertion or post-activation (Perry and Flik 1988; Bury et al. 1999; Gray and Roth 2001; Rodela et al. 2009). Using this technique, it was established that there was no difference measured in 5-HT binding to gill basolateral membranes of uncrowded and crowded toadfish, representing no net change in the gill's entire 5-HT receptor population. Perhaps whatever upregulation in 5-HT_{2A} receptor levels that may

be occurring in response to elevations in cortisol is counteracted by a reciprocal change in at least one other 5-HT receptor expressed in the gill, if not more. For example, the toadfish 5-HT_{1A} receptor is expressed in the gill (Medeiros et al., 2010) and has been shown to have protein binding that is negatively correlated to plasma cortisol levels (Medeiros and McDonald, 2012). It is also possible that any change in the 5-HT_{2A} receptor was undetectable due to changes in other gill 5-HT receptors having a higher affinity for 5-HT, *e.g.* the affinity of the toadfish 5-HT_{1A} receptor ($K_D = 5.0 \times 10^{-10}$ M; Medeiros et al. 2010) is approximately 10-fold higher to that of the mammalian 5-HT_{2A} receptor (K_D = 4.5 x 10^{-9} M (canine); 1.8 x 10^{-9} M (human); Bonaventure et al., 2005). Interestingly, the K_D of the mammalian 5-HT_{2A} receptor for 5-HT is an order of magnitude higher than the K_D (7 x 10^{-8} M) measured in the present experiment, suggesting that the gill population of 5-HT receptors is not dominated by the 5-HT_{2A} receptor.

The 5-HT binding data are supported by the α-methyl 5-HT competition data. Emphasizing that there are more than just 5-HT_{2A} receptors in the gill, in both uncrowded and crowded fish gill about 30-40% of receptors were not bound by α-methyl 5-HT reflecting the percentage of the total population of 5-HT receptors in the gill that are not in the 5-HT_2 family. The IC_{50} values for α-methyl 5-HT determined from uncrowded and crowded fish gill were consistent with mammalian 5-HT_{2A} receptor values (http://pubchem.ncbi.nlm.nih.gov). Furthermore, no significant difference was measured in the binding kinetics of the gill from the two treatments, suggesting that if a change occurred in the 5-HT_{2A} receptor, an opposite and equal reaction was also occurring in the 5-HT_{2B} and/or 5-HT_{2C} receptors as α-methyl 5-HT binds to all three types. To our

knowledge, interactions between cortisol and 5-$HT_{2B/C}$ have not been investigated. The binding affinity for α-methyl 5-HT is highest for the 5-HT_{2B} receptor ($K_I = 4 \times 10^{-9}$ M) which is 10-fold higher than the affinity for 5-HT_{2C} ($K_I = 5 \times 10^{-8}$ M) and 100-fold higher than the affinity for 5-HT_{2A} ($K_I = 7.9 \times 10^{-7}$ M; Baxter et al., 1995; reviewed by Barnes and Sharp 1999). While not directly comparable, the IC_{50} value found in the present study ($6 - 11 \times 10^{-7}$ M) is within the same order of magnitude as the the K_I for the 5-HT_{2A} receptor in the literature, implying a gill 5-HT_2 receptor family population dominated by the 5-HT_{2A} receptor.

The ketanserin binding data helped to tease out the effects of cortisol on the 5-HT_{2A} receptor in particular. While ketanserin, like α-methyl 5-HT, does bind to all three 5-HT_2 receptors, it does so with different affinities, where the binding affinity of ketanserin for the mammalian 5-HT_{2A} ($K_I = 1.26 \times 10^{-9}$ M) is 100-fold greater than its affinity for 5-HT_{2C} ($K_I = 1 \times 10^{-7}$ M) and 1000-greater than its affinity for 5-HT_{2B} ($K_I = 3.98 \times 10^{-6}$ M, Baxter et al. 1995). The mammalian K_D value for the 5-HT_{2A} receptor ($2.9 \pm 0.9 \times 10^{-9}$ M; Knight et al. 2004) is very close to those determined for gill from uncrowded, saline- and metyrapone-injected fish. Interestingly, gills from crowded fish demonstrate a K_D that is almost double the other treatment groups, suggesting that in crowded gill tissue, there was a possible upregulation in another receptor from the 5-HT family, either 5-HT_{2C} or 5-HT_{2B}. Both of these receptors have been implicated in vasoconstriction associated with the hypoxia response, which crowded toadfish could have been experiencing (McDonald et al. 2010). Alternatively, these data may support the ideas proposed by Gray and Roth (2001). Specifically, that activation of the 5-HT_{2A} receptor leads to a downregulation via endocytosis from the cell membrane. If all of the

5-HT_{2A} receptors have been activated recently, as could be the case in crowded, ureotelic fish since they are highly pulsatile (Wood et al. 1997), then the 5-HT_{2A} receptors could be removed from the basolateral membrane, leaving 5-HT_{2B} and/or 5-HT_{2C} behind and resulting in the K_D shift.

Uncrowded fish are not ureotelic and have not activated the components required for pulsatile urea excretion (Wood et al. 1997, Wood et al. 1998, Wood et al., 2001; McDonald et al. 2004). Consistent with this, the lowest B_{max}, which reflects the amount of receptor protein on the basolateral membrane potentially available for activation, is measured in these fish. One week of crowding produces more receptors in the 5-HT_2 family, but, based on the higher K_D value, it is difficult to conclude what proportion of these receptors are 5-HT_{2A}, as it likely includes at least a slight upregulation in another 5-HT_2 receptor subtype (in addition to a downregulation within the 5-HT_2 family, as there was no net difference in α-methyl 5-HT competition kinetics). While the data from crowded fish are complex, we believe that the complicated relationship between the 5-HT_{2A} receptor and cortisol becomes clearer when comparing uncrowded fish to fish treated with either saline or metyrapone. Fish in the saline control- and metyrapone-treated groups experienced the same week of crowding as the crowded treatment group, but following the surgical implantation of a venous and an intraperitoneal catheter, these fish were isolated, and in this way, removed from social stress for three days while being treated with either saline or metyrapone. Saline-treated controls had B_{max} values which were higher than the uncrowded (and crowded) fish while having K_D values that, similar to uncrowded fish, were more characteristic of the 5-HT_{2A} receptor, suggesting an upregulation in the 5-HT_{2A} receptor in association with the increase in plasma cortisol

associated with crowding, saline-treatment and, ultimately, a switch to ureotely (McDonald et al. 2004).

After one week of crowding and the subsequent reduction in cortisol levels for 48 h with metyrapone treatment, the gill basolateral membrane ended up with almost twice as many 5-HT_{2A} receptors compared to saline-treated controls in which cortisol levels remained elevated for the entire period. This suggests that perhaps the removal of cortisol in metyrapone-treated fish over this shorter time-frame has resulted in a further upregulation in the 5-HT_{2A} receptor, suggesting a dual role for cortisol in the control of the 5-HT_{2A} receptor. There is evidence in the literature of acute changes in stress mediating changes in the 5-HT_{2A} receptor that are opposite to the chronic stress effects, specifically, Dwivedi et al. (2005) found that in opposition to chronic cortisol that resulted in increased 5-HT_{2A} receptor protein levels, acute treatment initiated by a single shock paradigm resulted in a decrease in 5-HT_{2A} receptor protein. That a relatively acute reduction in cortisol after a chronic elevation in toadfish may instigate a further increase in the number of 5-HT_{2A} receptors is supported by the finding that α-methyl 5HT injection in these fish results in a urea pulse that is double the size of that elicited in saline-injected control fish. This matched increase in 5-HT_{2A} receptor B_{max} and function, where function here is being measured as activation or insertion of tUT, suggests that the number of 5-HT_{2A} receptors activated on the basolateral membrane is in direct correlation to the size of the urea pulse and the number of tUTs activated. Upon activation, we hypothesize that the 5-HT_{2A} receptor is either phosphorylating the already membrane-bound tUT or it is mediating the relocation of the transporter to the membrane in a similar

fashion as the possible mechanism of upregulation of the 5-HT_{2A} receptor itself (Gray and Roth 2001).

Our data provide evidence suggesting that the 5-HT_{2A} receptor may be sensitive to cortisol in a similar way as tUT, where cortisol elevation results in both an upregulation and a downregulation in tUT (McDonald et al., 2004; McDonald et al., 2009; Rodela et al., 2009). In the case of the 5-HT_{2A} receptor, chronic cortisol associated with the switch to ureotely may be required for recruiting the 5-HT_{2A} receptor to the basolateral membrane initially as is evident when comparing the B_{max} of uncrowded fish to that of saline-injected fish; however, sustained elevations in cortisol may then inhibit the continued installment of the 5-HT_{2A} receptor to the basolateral membrane. One possibility is that the continued elevation in cortisol is upregulating 5-HT_{2A} receptor degradation, perhaps through the ubiquitin-proteasome pathway or an analogous mechanism (Tiao et al. 1997, Gray and Roth 2001). During the relatively acute absence of cortisol elicited by metyrapone treatment, the degradation of the 5-HT_{2A} receptor may have slowed down resulting in a higher B_{max}.

The enhanced membrane recruitment or reduced turnover of the 5-HT_{2A} receptor in metyrapone treated fish may be initiated by the lowering of cortisol past an endogenous threshold. Under resting conditions, an endogenous drop in cortisol, from approximately 100 $ng \cdot ml^{-1}$ to 40 $ng \cdot ml^{-1}$, occurs naturally in ureotelic toadfish and appears to control the size of the urea pulse (Wood et al. 1997; McDonald et al., 2004). In the present study, the cortisol values for metyrapone are approximately 50 $ng \cdot ml^{-1}$, which could indicate that these fish are now under this permissive threshold, leading to increased recruitment/reduced degradation of the 5-HT_{2A} receptor, the increased

activation of tUT and larger urea pulses. This hypothesis is supported by data from the present study, which show that the size of 5-HT_{2A} receptor-mediated urea pulses through α-methyl 5-HT injection increases in metyrapone-treated fish and also by Rodela et al. (2009) who showed an increase in the size of natural urea pulses during a metyrapone treatment. When toadfish are infused with cortisol, which reduces pulse size despite increases in tUT mRNA expression (McDonald et al. 2004; McDonald et al., 2009), cortisol levels are likely pushed above the cortisol threshold necessary to recruit the 5-HT_{2A} receptors to the membrane, resulting in a reduction in the activation or relocation of tUT and smaller urea pulses. The location of cytoplasmic *versus* membrane-bound tUT could be tested with an effective tUT antibody, but the production of a specific antibody against tUT has not been successful thus far.

A greater understanding of cortisol will do more than just benefit the scientific community. Cushing's syndrome affects the pituitary's ability to function normally. Most cases involving people affected by Cushing's syndrome are caused by a pituitary adenoma that produces too much adrenocorticotropic hormone (ACTH) (Nieman and Ilias, 2005). This in turn results in excessively high levels of cortisol that lead to the symptoms of excessive weight gain, excessive sweating, dilation of capillaries and polyuria. This hypercortisolism stands in contrast to the hypocortisolism of Addison's disease. This disease results in weight loss, fatigue, fever, and muscle weakness and can lead to an Addisonian crisis where extremely low blood pressure can lead to a coma (Stewart and Krone, 2011). This is caused by a lack of ACTH, which would stimulate the release of cortisol. To compensate, the body upregulates ACTH's precursor, proopiomelanocortin (POMC), which will inadvertently result in excess melanocyte-

stimulating hormone (MSH), another hormone derived from POMC. Excess MSH results in a darkening of the skin. Interestingly, we observed that upon injection with α-methyl 5-HT, toadfish went from their natural dark brown state to a pale white within one minute of the injection. While this change could not be adequately quantified, it could be hypothesized that the activation of the 5-HT_2 family of receptors in toadfish is possibly inhibiting the production of either POMC or MSH altogether, perhaps reducing cortisol which might be why we observe a urea pulse following injection. A better understanding of this interaction between 5-HT_2 receptors and POMC and its derivatives could greatly benefit those individuals stricken with either Cushing's syndrome or Addison's disease, and could greatly improve their quality of life.

Consistent with our hypothesis, this investigation shows that chronic increases in cortisol may lead to an upregulation of the 5-HT_{2A} receptor through basolateral membrane recruitment and not through changes in mRNA expression. Interestingly, sustained elevations in cortisol may inhibit the continued installment of the 5-HT_{2A} receptor to the basolateral membrane, perhaps due to an increase in 5-HT_{2A} receptor degradation, and further membrane recruitment of the 5-HT_{2A} receptor may require a permissive drop in cortisol. Future studies should investigate the mechanism responsible for the chronic *versus* acute cortisol-mediated regulation of the 5-HT_{2A} receptor. Also, an exact mechanism for 5-HT_{2A} receptor-mediated activation or membrane recruitment of tUT should be established. Furthermore, it is not yet clear if the 5-HT_{2A} receptor acts alone, or in conjunction with other 5-HT receptors and signaling pathways in the toadfish gill.

Acknowledgements

Thank you to my advisor, my professor and my mentor Dr. M. Danielle McDonald for her guidance, knowledge and assistance over the last two years. And sincere thanks to Mr. Ray Hurley and Ms. Debbie Fretz for providing toadfish. This work was funded by a National Science Foundation grant (# IOS-0920547) to MDM.

Bibliography

1 Barimo JF and Walsh PJ. Use of urea as a chemosensory cloaking molecule by a bony fish. J of Exp Biol 75, 225-237. 2006.

2 Barnes N and Sharp T. A review of central 5-HT receptors and their function. Neuropharmacol 38:8, 1083-1152. 1999.

3 Baxter G, Kennett G, and Blackburn T. 5-HT2 receptor subtypes: A family re-united? Trend Pharm Sci 16:3, 105-110. 1995.

4 Bury NR, Sturm A, Le Rouzic P, Lethimonier C, Ducouret B, Guiguen Y, Robinson-Rechavi M, Laudet V, Rafestin-Oblin ME, and Prunet P. Evidence for two distinct functional glucocorticoid receptors in teleost fish. J.Mol.Endocrinol. 31, 141-156. 2003.

5 Dwivedi Y, Mondal AC, Payappagoudar, and Rivazi. Differential regulation of serotonin (5HT)2A receptor mRNA and protein levels after single and repeated 2 stress in rat brain: role in learned helplessness behavior. Neuropharmacol 48, 204-214. 2005.

6 Fernandes C, McKittrick C, File S, and McEwen B. Decreased 5-HT1A and increased 5-HT2A receptor binding after chronic corticosterone associated with a behavioural indication of depression but not anxiety. Psychoneuroendocrinology 22:7, 477-491. 1997.

7 Flik G and Perry SF. Cortisol stimulates whole body calcium uptake and the branchial calcium pump in freshwater rainbow trout. J Endocrinol 120, 75-82. 1989.

8 Gray J and Roth B. Paradoxical trafficking and regulation of 5-HT2A receptors by agonists and antagonists. Brain Res 56:5, 441-451. 2001.

9 Hanson L, Gorzalka B, and Brotto L. The antidepressant, nefazodone, attenuates corticosterone-induced increases in 5-HT2A receptor-mediated behaviors in the female rat. Eur J of Pharmacol 342:2-3, 163-165. 1998.

10 Hontela A, Rasmussen J, Audet C, and Chevalier G. Impaired cortisol stress response in fish from environments polluted by PAHs, PCBs, and mercury. Arch Environ Contam Toxic 22, 278-283. 1992.

11 Hopkins TE, Wood CM, and Walsh PJ. Interactions of cortisol and nitrogen metabolism in the ureogenic gulf toadfish *Opsanus beta*. J of Exp Biol 198, 2229-2235. 1995.

12 Hoyer D, Hannon J, and Martin G. Molecular, pharmacological and functional diversity of 5-HT receptors. Pharm Biochem Behavior 71-4, 533-554. 2002.

13 Julius D, Huang KN, Livellie TJ, Axel R, and Jessell TM. The 5HT2 receptor defines a family of structurally distinct but functionally conserved serotonin receptors. EMBO 7:13, 4135-4140. 1988.

14 Kitano T, Liu YH, Ueda S, and Saitou N. Human-specific amino acid changes found in 103 protein-coding genes. Mol Biol Evol 21:5, 936-944. 2004.

15 Knight A, Misra A, Quirk K, Benwell K, Revell D, Kennet G, and Bickerdike M. Pharmacological characterisation of the agonist radioligand binding site of 5-HT 2A, 5-HT 2B and 5-HT 2C receptors. 2004.

16 Kuroda Y, Mikuni M, Ogawa T, and Takahashi K. Effect of ACTH, adrenalectomy and the combination treatment on the density of 5-HT2 receptor binding sites in neocortex of rat forebrain and 5-HT2 receptor-mediated wet-dog shake behaviour. Psychopharmacol 108, 27-32. 1992.

17 Laberge T, Walsh PJ, and McDonald MD. Effects of crowding on ornithine–urea cycle enzyme mRNA expression and activity in gulf toadfish (Opsanus beta). J of Exp Biol 212, 2394-2402. 2009.

18 Livak K and Scmittgen T. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Applied Biosystems . 2001.

19 McDonald MD and Walsh PJ. 5-HT2A-like receptors are involved in triggering pulsatile urea excretion in the gulf toadfish, *Opsanus beta*. J of Exp Biol 207, 2003-2020. 2004.

20 McDonald MD, Wood CM, Grosell M, and Walsh PJ. Glucocorticoid receptors are involved in the regulation of pulsatile urea excretion in toadfish. J Comp Physiol 174B, 649-658. 2004.

21 McDonald MD, Vulsevic B, Perry SF, and Walsh PJ. Urea transporter and the glutamine synthetase regulation and localization in gulf toadfish gill. J of Exp Biol 212:5, 704-712. 2009.

22 McDonald MD, Gilmour KM, Walsh PJ, and Perry SF. Cardiovascular and respiratory reflexes of the gulf toadfish (Opsanus beta)
during acute hypoxia. Resp Phys Neurobiol 170, 59-66. 2010.

23 Medeiros L and McDonald MD. Cortisol-mediated downregulation of the serotonin receptor subtype 1A in the Gulf toadfish, *Opsanus beta*. Gen Comp Endocrinol , Submitted. 2010.

24 Medeiros L, Mager EM, Grossell M, and McDonald MD. The serotonin subtype 1A receptor regulates cortisol secretion in the Gulf toadfish, Opsanus beta. Gen Comp Endocrinol 168(3), 377-387. 2010.

25 Morando MB, Medeiros L, and McDonald MD. Fluoxetine treatment affects nitrogen waste excretion and osmoregulation in a
marine teleost. Aquatic Toxicology 95, 164-171. 2009.

26 Nieman LK and Ilias I. Evaluation and treatment of Cushing's syndrome. J of Amer Med 118(12), 1340-1346. 2005.

27 Perry SF and Flik G. Characterization of branchial transepithelial calcium fluxes in freshwater trout, Salmo gairdneri. Am J Physiol Regul Integr Comp Physiol 254, R491-R498. 1988.

28 Potter EA, Stewart G, and Smith CP. Urea flux across MDCK-mUT-A2 monolayers is acutely sensitive to AVP, cAMP and $[Ca^{2+}]i$. Am J Physiol 291, F122-F128. 2006.

29 Raote I, Bhattacharya A, and Panicker M. Chapter 6 Serotonin 2A (5-HT2A) Receptor Function: Ligand-Dependent Mechanisms and Pathways. http://www.ncbi.nlm.nih.gov/books/NBK1853/ . 2007.

30 Rodela TM, Gilmour KM, Walsh PJ, and McDonald MD. Cortisol-sensitive urea transport across the gill basolateral membrane of the gulf toadfish, *Opsanus beta*. Am J Physiol 297, R313-R322. 2009.

31 Rodela TM, McDonald MD, Walsh PJ, and Gilmour KM. The regulatory role of glucocorticoid and mineralocorticoid receptors in pulsatile urea excretion of the gulf toadfish, *Opsanus beta*. Journal of Experimental Biology 212, 1849-1858. 2009.

32 Serafy JE, Hopkins TE, and Walsh PJ. Field studies on the ureogenic gulf toadfish in a subtropical bay. I. Patterns of abundance, size composition and growth. J Fish Biol 50, 1258-1270. 1997.

33 Sloman K, McDonald MD, Barimo JF, Lepage O, Winberg G.E., Wood CM, and Walsh PJ. Does pulsatile urea excretion serve as a social signal in the gulf toadfish Opsanus beta? 78:5, 724-735. 2012.

34 Smith CP, Heitz MJ, Wood CM, and Walsh PJ. Molecular identification of a gulf toadfish (*Opsanus beta*) urea transporter. J Physiol Lond 511:33p. 1998.

35 Stewart PM, Krone NP. The adrenal cortex. Kronenberg H, Melmed S Polonsky K Larsen PR. Williams Textbook of Endocrinology. (12th ed.). 2011. Philadelphia, Pa, Saunders Elsevier.

36 Tiao G, Hobler S, Wang JJ, Meyer TA, Luchette FA, Fischer JE, and Hasselgren PO. Sepsis is associated with increased mRNAs of the ubiquitin-proteasome proteolytic pathway in human skeletal muscle. J Clin Invest 99(2), 163-168. 1997.

37 Walsh PJ, Tucker B, and Hopkins TE. Effects of confinement/crowding on ureogenesis in the gulf toadfish, *Opsanus beta*. J of Exp Biol , 195-206. 1994.

38 Watanabe Y, Sakai RR, McEwen BS, and Mendelson S. Stress and antidepressant effects on hippocampal and cortical 5-HT1a and 5-HT2 receptors and transport sites for serotonin. Brain Res 615, 87-94. 1993.

39 Winberg S and Nilsson G.E. Multiple high affinity binding sites for [3H]serotonin in the brain of a teleost fish, the Arctic charr (Salvelinus alpinus). J of Exp Biol 199, 2429-2435.

40 Wood CM, Hopkins TE, and Walsh PJ. Pulsatile urea excretion in gulf toadfish (*Opsanus beta*) is due to a pulsatile excretion mechanism, not a pulsatile production mechanism. J of Exp Biol 200, 1039-1046. 1997.

41 Wood CM, Gilmour KM, Perry SF, Part P, and Walsh PJ. Pulsatile urea excretion in gulf toadfish (*Opsanus beta*): evidence for activation of a specific facilitated diffusion transport system. J of Exp Biol 201, 805-817. 1998.

42 Wood CM, Warne JM, Wang Y, McDonald MD, Balment RJ, Laurent P, and Walsh PJ. Do circulating plasma AVT and/or cortisol levels control pulsatile urea excretion in the gulf toadfish (*Opsanus beta*)? Comp Biochem Physiol 129A, 859-872. 2001.

43 Wood CM, McDonald MD, Sundin L, Laurent P, and Walsh PJ. Pulsatile urea excretion in the gulf toadfish: mechanisms and controls. Comp Biochem Physiol 136B, 667-684. 2003.

Figure Legends

Figure 1: (A) An elevation in plasma cortisol ($ng \cdot ml^{-1}$) is measured in crowded fish compared to uncrowded fish. (B) No significant difference was measured in plasma serotonin concentrations ($ng \cdot ml^{-1}$). (C) Plasma urea concentrations were approximately 2.5-fold higher in the crowded fish over the uncrowded. Values are means ± S.E.M.; ** $P < 0.001$ compared to uncrowded toadfish.

Figure 2: There is no significant difference in 5-HT_{2A} receptor mRNA expression between uncrowded and crowded fish (A) in the gill or (B) in the brain. Values are means ± S.E.M.

Figure 3: There is no significant difference in [^{3}H] serotonin binding saturation kinetics in (A) uncrowded compared to (B) crowded toadfish isolated gill basolateral membrane. The equation of the uncrowded line was $y = 32322x/(8.2 \times 10^{-8} + x)$, ($B_{max}$ = 32322 ± 8264 fmol·ml protein^{-1}, $K_D = 8.2 \pm 4.4 \times 10^{-8}$ M, h coefficient = 1.3 ± 0.7) and the equation of the crowded line was $y=36295x/(6.0 \times 10^{-8} + x)$ (B_{max} = 36295 ± 7120 fmol·ml protein^{-1}, $K_D = 6.0 \pm 3.4 \times 10^{-8}$ M).

Figure 4: There is no significant difference in the competitive binding kinetics of α-methyl 5-HT against [^{3}H] serotonin between (A) uncrowded and (B) crowded fish isolated gill basolateral membrane. The equation of the uncrowded line was $y = 31.8 + (87.2\text{-}31.8)/(1+10^{(x+6.2)})$ ($IC_{50} = 6.5 \pm 0.2 \times 10^{-7}$ M) and the equation of the crowded line was $y = 42.6 + (95.6\text{-}42.6)/(1+10^{(x+6.0)})$ ($IC_{50} = 1.1 \pm 0.2 \times 10^{-6}$ M).

Figure 5: A significant difference in both B_{max} and K_D was measured in [^{3}H] ketanserin binding saturation kinetics between (A) uncrowded fish and (B) crowded fish isolated

gill basolateral membrane suggesting a shift from a low amount of high affinity receptors to a high amount of lower affinity receptors. The equation of the uncrowded line (shown in inset) was $y = 39395x^{2.3}/((5.53 \times 10^{-9})^{2.3} + x^{2.3})$ (B_{max} = 39395 ± 1577 fmol·ml protein^{-1}, $K_D = 5.5 \pm 0.4 \times 10^{-9}$ M, h coefficient = 2.3 ± 0.3) and the equation of the crowded line was $y = 79922*x^{1.5}/((1.3 \times 10^{-8})^{1.5} + x^{1.5})$ (B_{max} = 79922 ± 7070 fmol·ml protein^{-1}, $K_D = 1.3 \pm 0.2 \times 10^{-8}$ M, h coefficient = 1.5 ± 0.2); P = 0.046.

Figure 6: (A) An elevation in plasma cortisol (ng·ml^{-1}) is measured in crowded fish compared to metyrapone-injected fish. (B) Plasma urea concentrations are not significantly different between metyrapone and saline-injected treatments. Values are means ± S.E.M.; **P < 0.001 compared to control.

Figure 7: There is no significant difference in 5-HT_{2A} receptor mRNA expression between control- and metyrapone-treated fish (A) in the gill or (B) in the brain. Values are means ± S.E.M.

Figure 8: A significant difference was measured in [^{3}H] ketanserin binding saturation kinetics between (A) control and (B) metyrapone-treated fish isolated gill basolateral membrane. The equation of the control line was $y = 139\ 331*x^{1.5}/((4.12 \times 10^{-9})^{1.5} + x^{1.5})$ (B_{max} = 139 331 ± 25704 fmol·ml protein^{-1}, $K_D = 4.1 \pm 1.8 \times 10^{-9}$ M, h coefficient = 1.5 ± 0.7) and the equation of the metyrapone line was $y = 362\ 008x^{2.1}/(3.7 \times 10^{-9})^{2.1} + x^{2.1})$ (B_{max} = 362 008 ± 84030 fmol·ml protein^{-1}, $K_D = 3.7 \pm 1.6 \times 10^{-9}$ M, h coefficient = 2.1 ± 1.4); P = 0.0095.

Figure 9: The appearance of urea (μmol-N·kg^{-1}) in the water in (A) control- and (B) metyrapone-treated fish during the α-methyl 5-HT challenge. At t = 0 h fish of both

groups were injected with α-methyl-5-HT, resulting in a significant increase in the excretion of urea. Metyrapone-treated fish show a significant elevation in (C) pulse size and a (D) slightly higher frequency in their response to α-methyl 5-HT compared to controls. Values are means ± S.E.M.; * $P < 0.05$ compared to control, ** $P < 0.001$, *** $P < .0001$ compared to respective t = 0 h.

Figure 10: (A) A higher B_{max} for [^{3}H] ketanserin was measured in gill basolateral membranes in metyrapone-treated fish compared to all other treatments. (B) K_D values indicate that differences in the type of stressor result in a membrane made up of different types of 5-HT_2 receptors. (C) Corresponding cortisol values are relisted to demonstrate that cortisol levels alone do not predict K_D or B_{max}. Values are means ± S.E.M.; a different letter denotes a significant difference, $P < 0.05$.

Figure 1

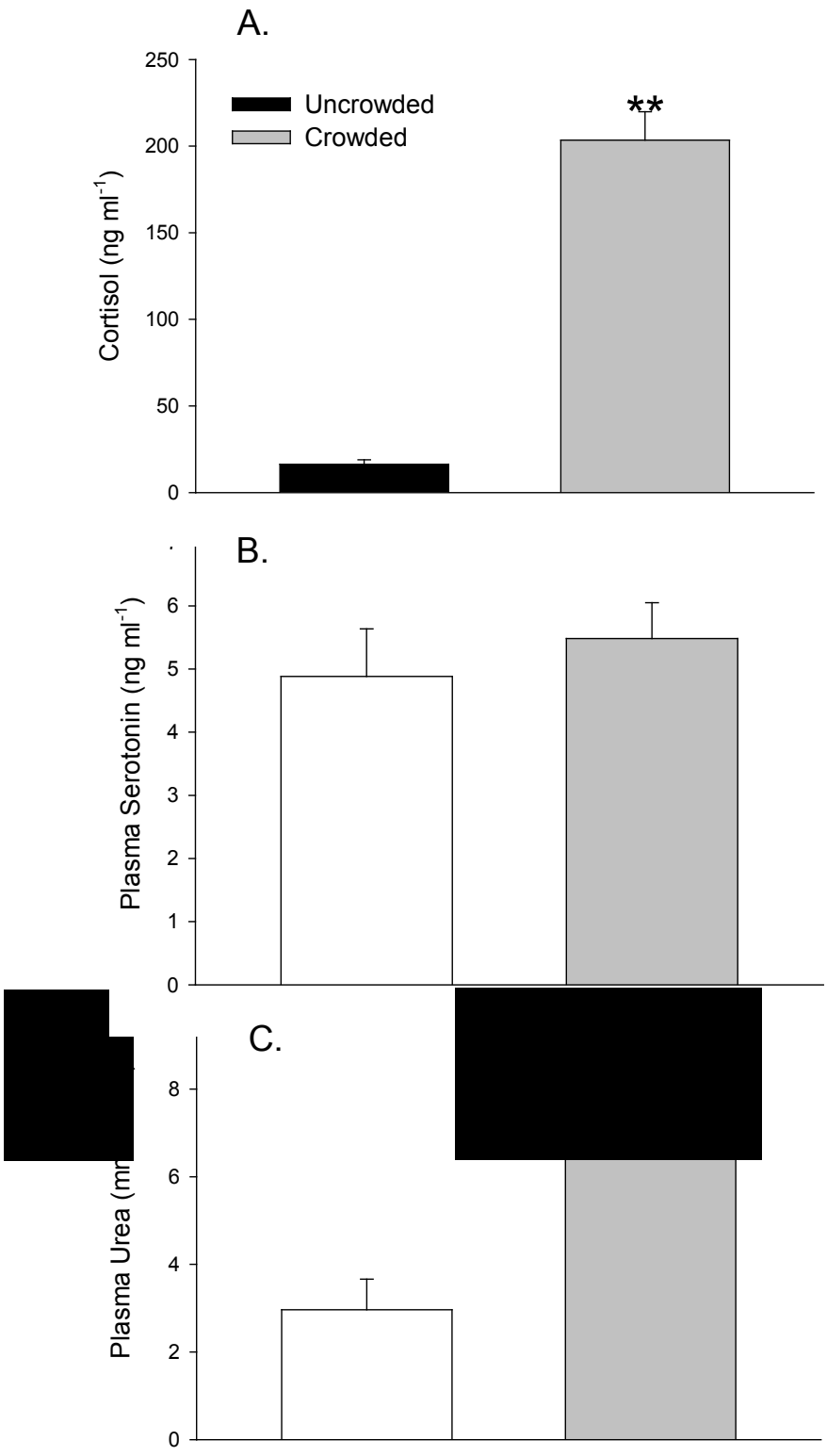
A.
Uncrowded
Crowded
**
Cortisol (ng ml-1)
250
200
150
100
50
0
B.
Plasma Serotonin (ng ml-1)
6
5
4
3
2
1
0
C.
Plasma Urea (m
8
6
4
2
0

Figure 2

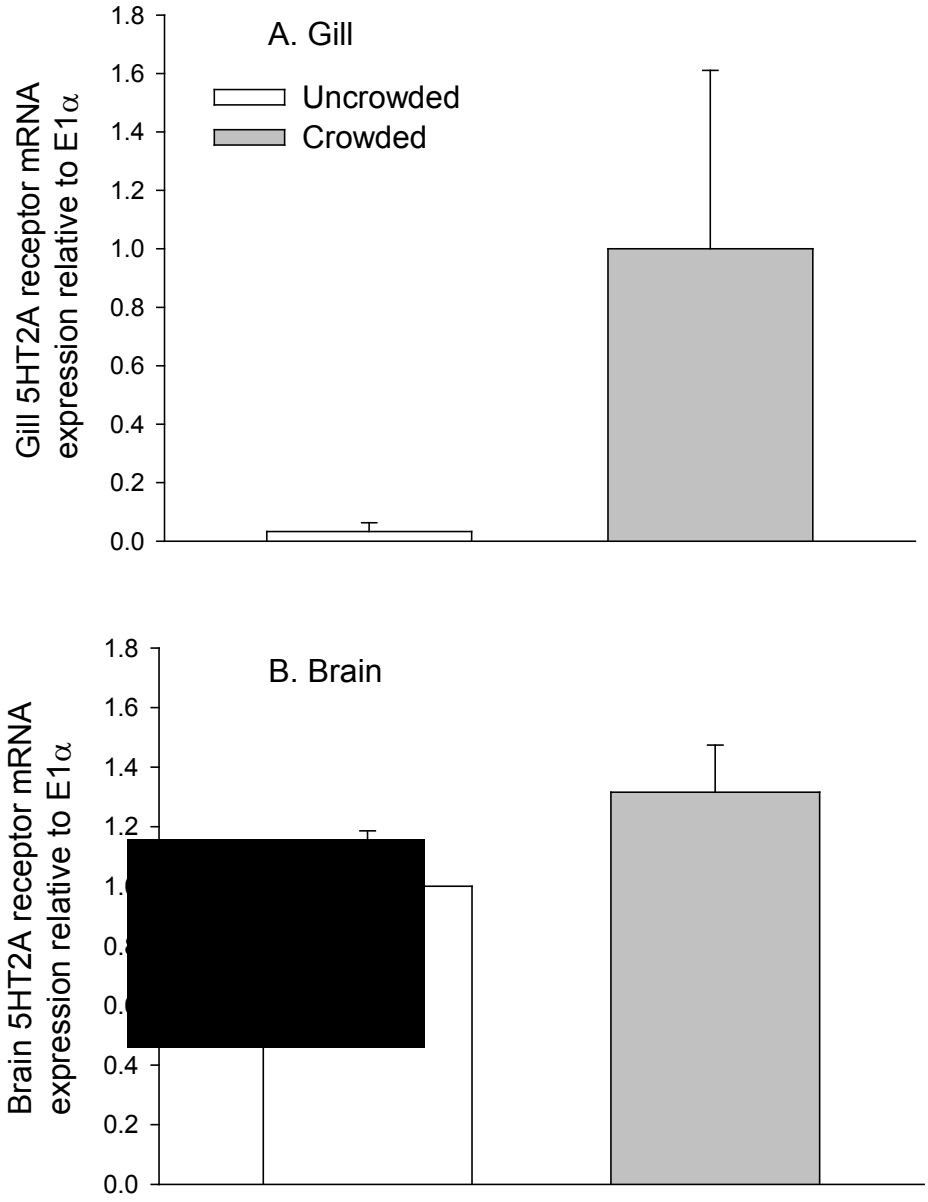
A. Gill
Uncrowded
Crowded
Gill 5HT2A receptor mRNA expression relative to E1α
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
B. Brain
Brain 5HT2A receptor mRNA expression relative to E1α
1.8
1.6
1.4
1.2
0.4
0.2
0.0

Figure 3

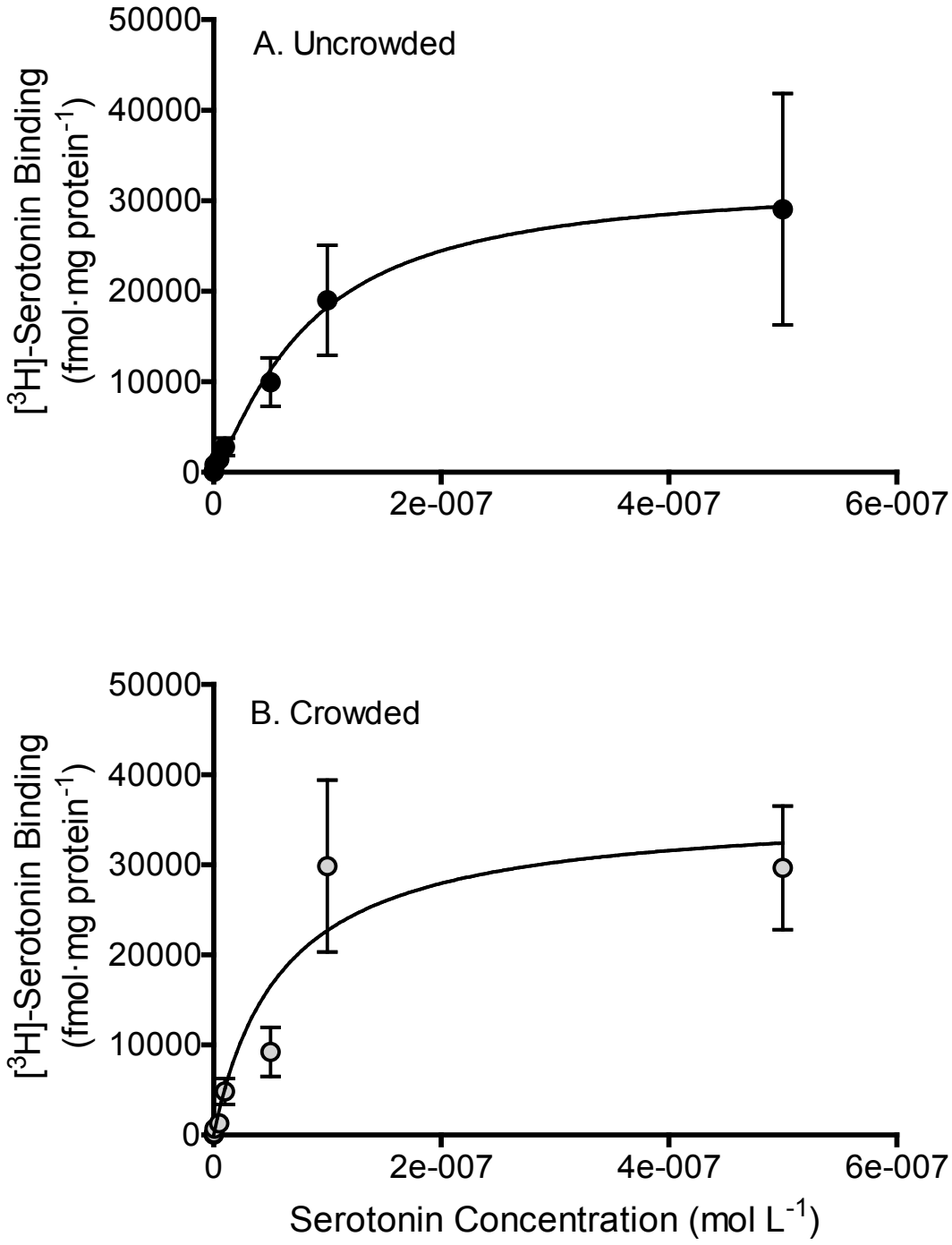
A. Uncrowded
B. Crowded
[3H]-Serotonin Binding (fmol·mg protein-1)
50000
40000
30000
20000
10000
0
0
2e-007
4e-007
6e-007
Serotonin Concentration (mol L-1)

Figure 4

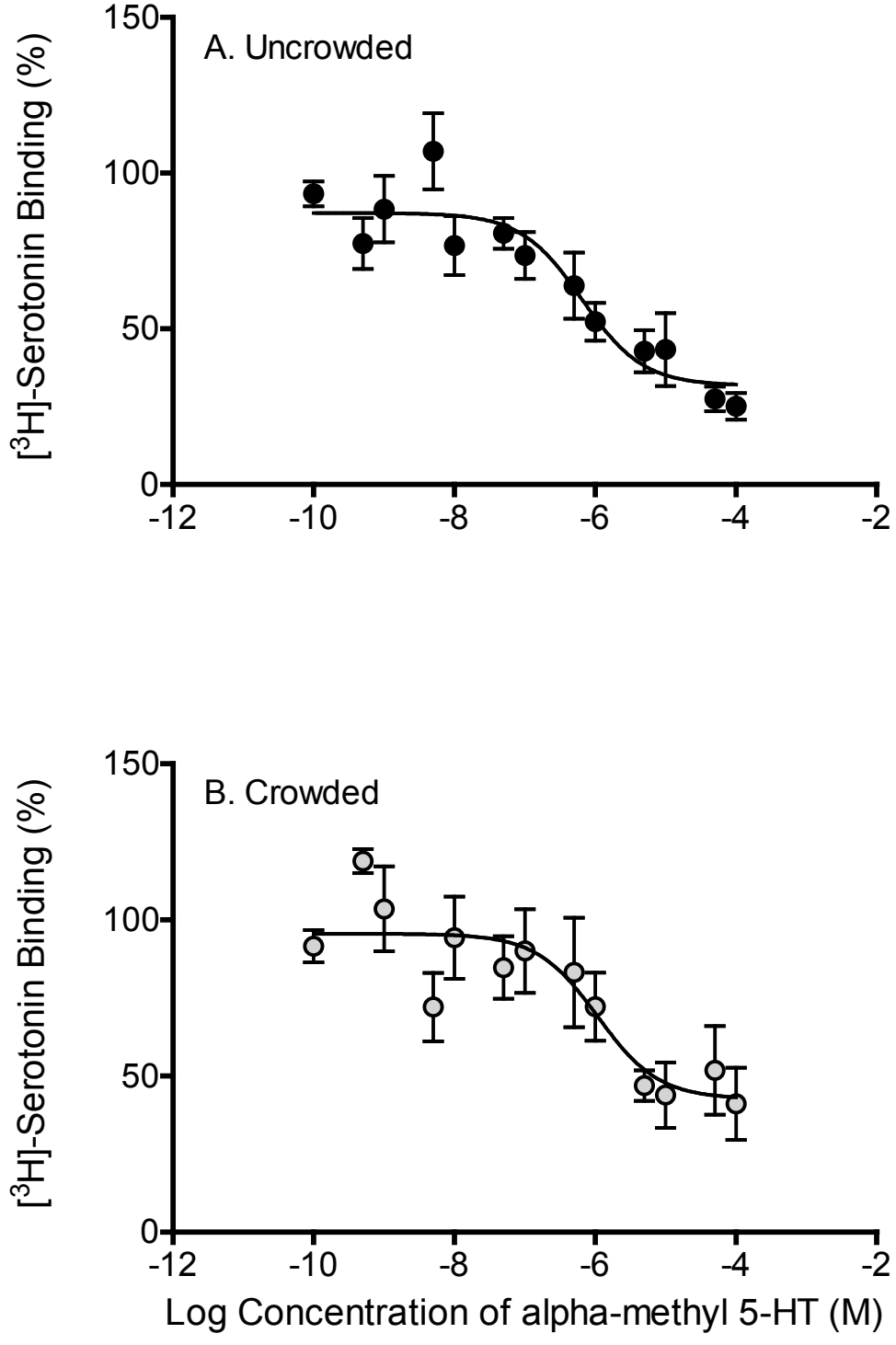
A. Uncrowded
B. Crowded
[3H]-Serotonin Binding (%)
150
100
50
0
-12
-10
-8
-6
-4
-2
Log Concentration of alpha-methyl 5-HT (M)

Figure 5

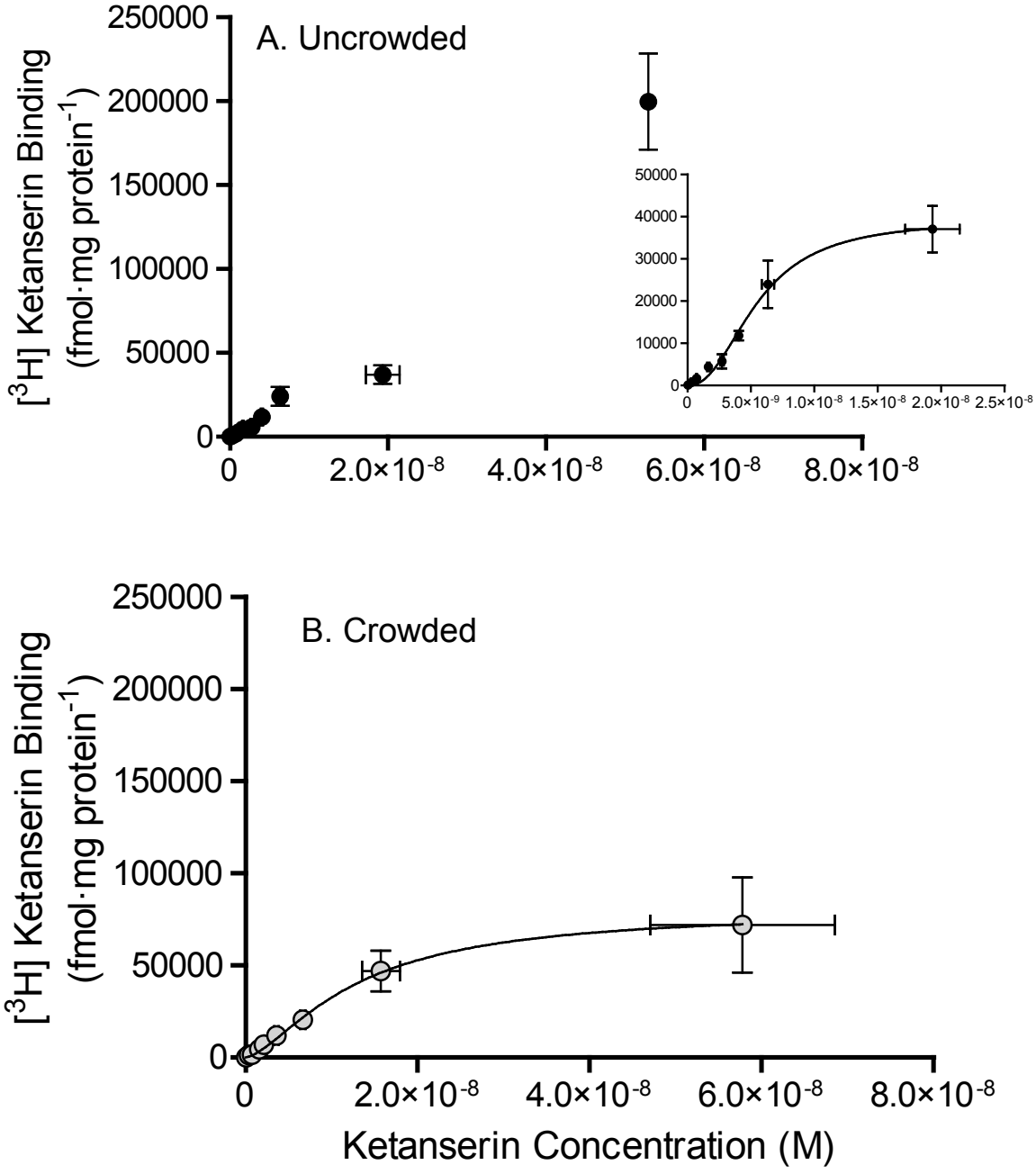
A. Uncrowded
B. Crowded
[3H] Ketanserin Binding (fmol·mg protein-1)
Ketanserin Concentration (M)
250000
200000
150000
100000
50000
0
2.0×10-8
4.0×10-8
6.0×10-8
8.0×10-8
50000
40000
30000
20000
10000
5.0×10-9
1.0×10-8
1.5×10-8
2.0×10-8
2.5×10-8

Figure 6

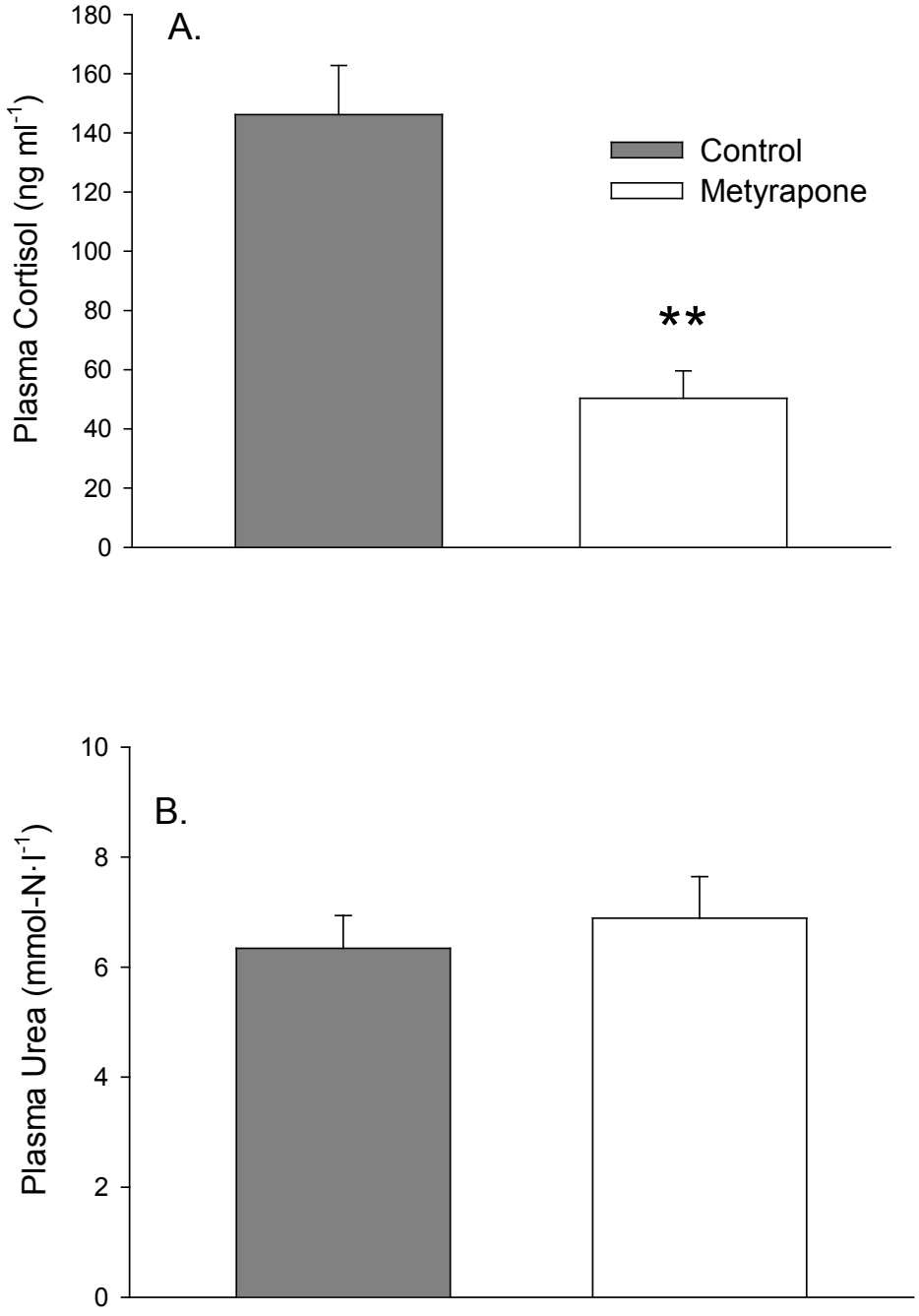
A.
Plasma Cortisol (ng ml-1)
180
160
140
120
100
80
60
40
20
0
Control
Metyrapone
**
B.
Plasma Urea (mmol-N·l-1)
10
8
6
4
2
0

Figure 7

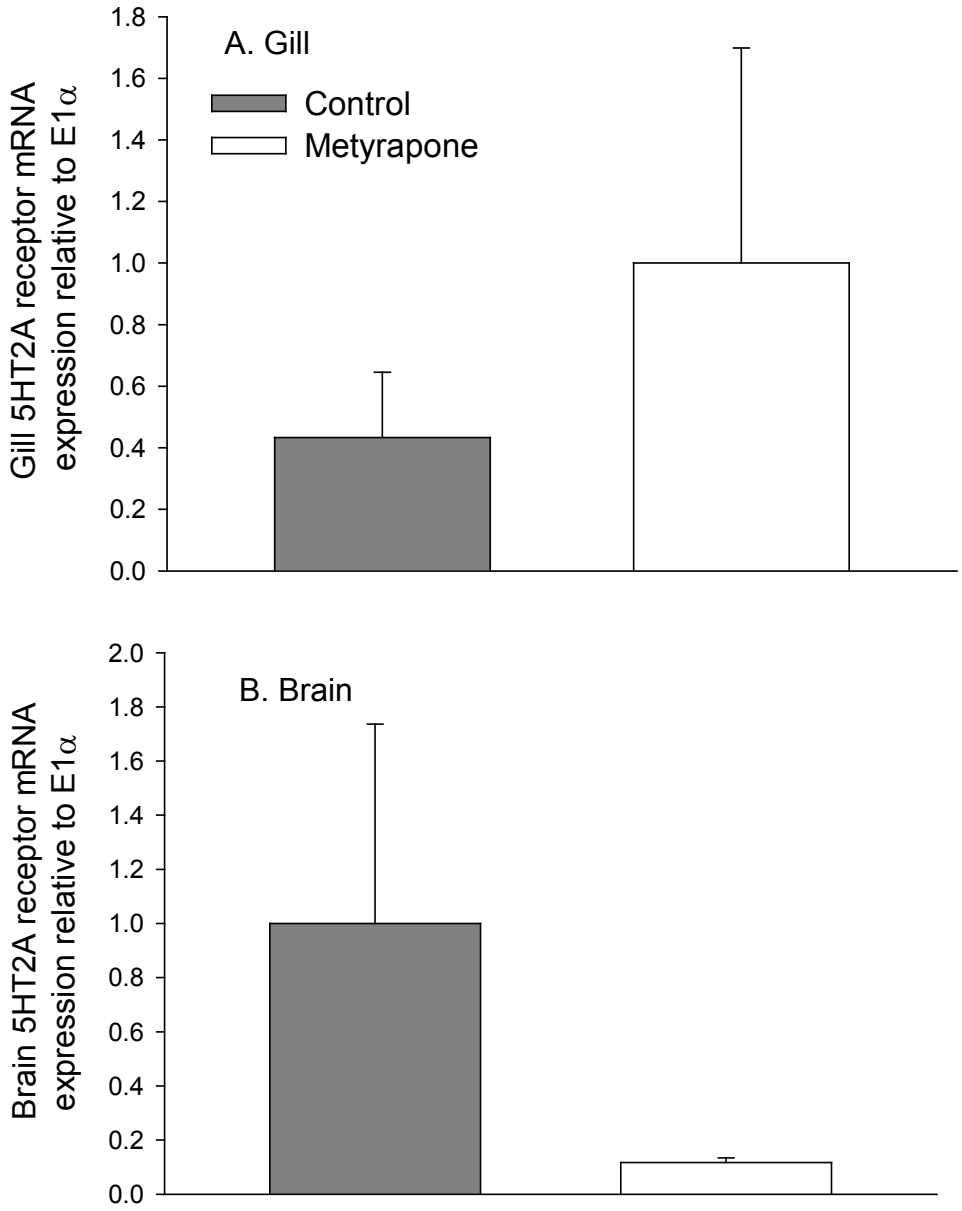
A. Gill
Control
Metyrapone
Gill 5HT2A receptor mRNA expression relative to E1α
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
B. Brain
Brain 5HT2A receptor mRNA expression relative to E1α
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0

Figure 8

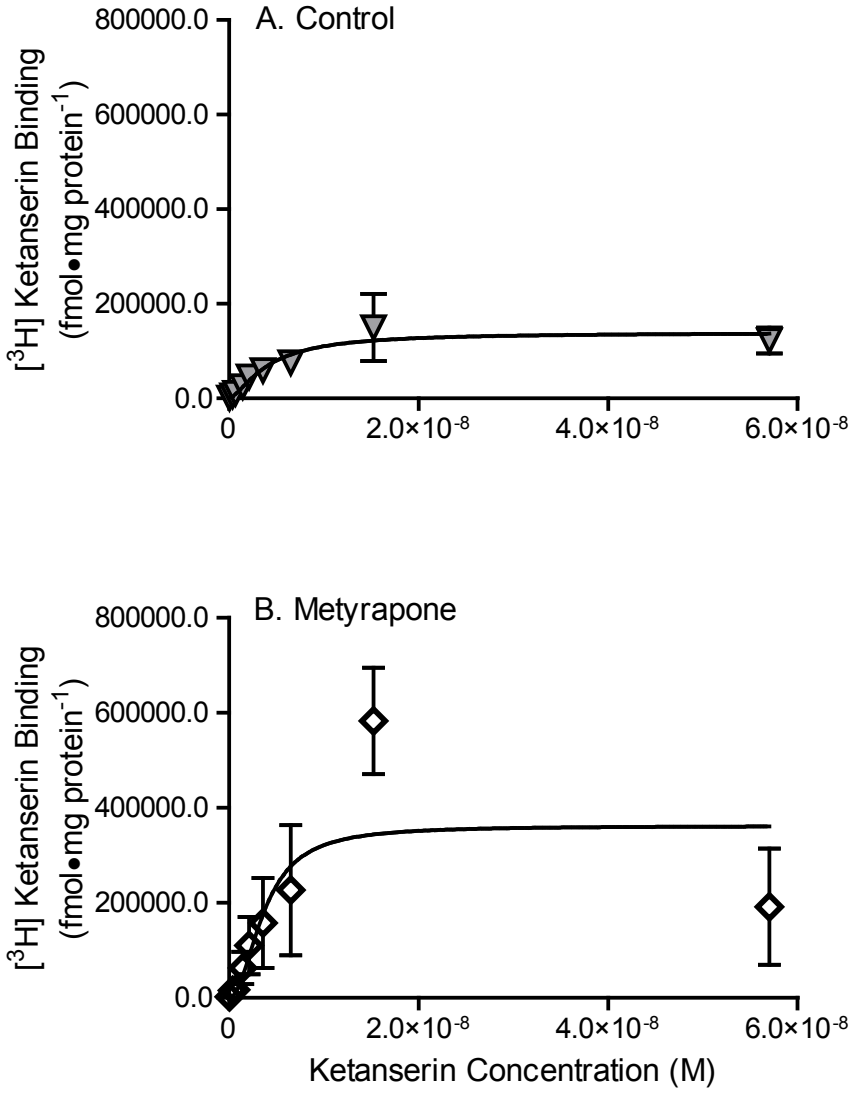
A. Control
B. Metyrapone
[3H] Ketanserin Binding (fmol•mg protein-1)
800000.0
600000.0
400000.0
200000.0
0.0
0
2.0×10-8
4.0×10-8
6.0×10-8
Ketanserin Concentration (M)

Figure 9

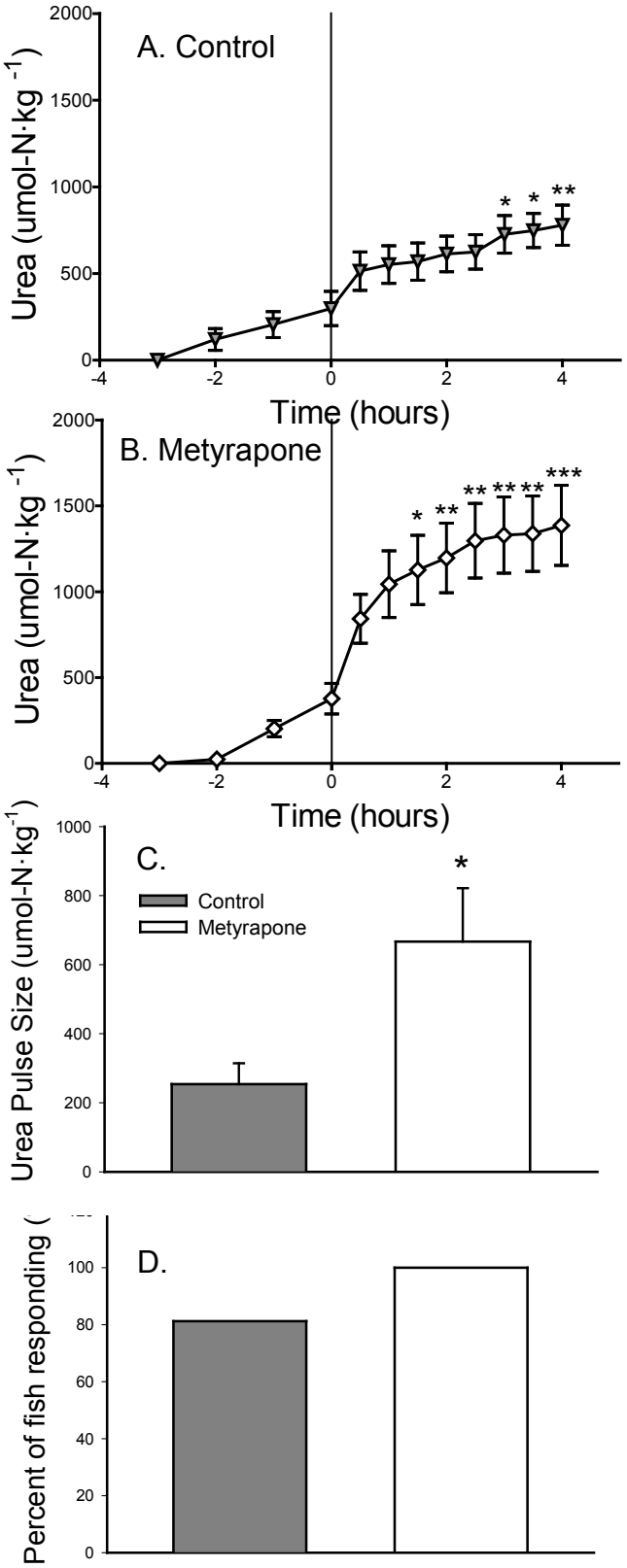
A. Control
Urea (umol-N·kg -1)
Time (hours)
B. Metyrapone
Urea (umol-N·kg -1)
Time (hours)
C.
Control
Metyrapone
Urea Pulse Size (umol-N·kg-1)
D.
Percent of fish responding

Figure 10

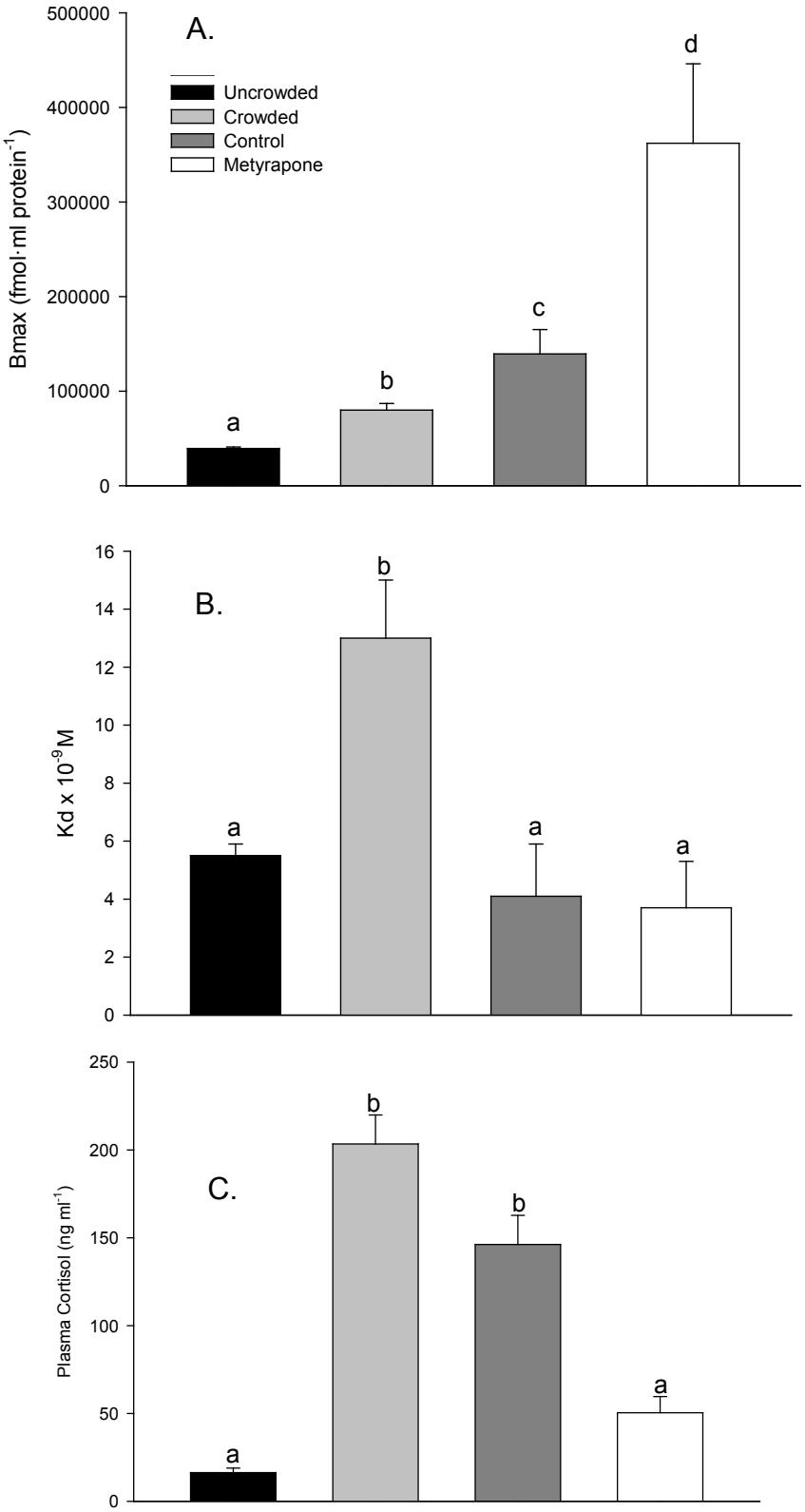
A.
Uncrowded
Crowded
Control
Metyrapone
Bmax (fmol·ml protein-1)
500000
400000
300000
200000
100000
0
a
b
c
d
B.
Kd x 10-9 M
16
14
12
10
8
6
4
2
0
a
b
a
a
C.
Plasma Cortisol (ng ml-1)
250
200
150
100
50
0
a
b
b
a

Table 1: Nitrogen excretion rates and overall % ureotely for both control and metyrapone-treated toadfish.

	Control	Metyrapone
Urea excretion rate ($\mu mol\text{-}N \cdot kg^{-1} \cdot h^{-1}$)	97.4 ± 14.5 (16)	173.3 ± 29.1 (14)*
Ammonia excretion rate ($\mu mol\text{-}N \cdot kg^{-1} \cdot h^{-1}$)	2.46 ± 0.63 (16)	3.31 ± 0.82 (14)
% Ureotely	95.8 ± 1.4 (16) %	97.0 ± .9 (14) %

Values are means ± S.E.M. (N); * $P < 0.05$ compared to corresponding control value.