The Journal of Experimental Biology 215, 314-323

doi:10.1242/jeb.061895

RESEARCH ARTICLE

Interactions between cortisol and Rhesus glycoprotein expression in ureogenic toadfish, *Opsanus beta*

Tamara M. Rodela[1,*], M. Danielle McDonald[2], Patrick J. Walsh[1] and Kathleen M. Gilmour[1]

[1]Department of Biology, University of Ottawa, Ottawa, Ontario, Canada K1N 6N5 and [2]Rosenstiel School of Marine and Atmospheric Science, University of Miami, Miami, FL 33149-1098, USA

*Author for correspondence (trodela@zoology.ubc.ca)

Accepted 14 October 2011

SUMMARY

In their native environment, gulf toadfish excrete equal quantities of ammonia and urea. However, upon exposure to stressful conditions in the laboratory (i.e. crowding, confinement or air exposure), toadfish decrease branchial ammonia excretion and become ureotelic. The objective of this study was to determine the influences of cortisol and ammonia on ammonia excretion relative to expression of Rhesus (Rh) glycoproteins and the ammonia-fixing enzyme, glutamine synthetase (GS). *In vivo* infusions and/or injections were used to manipulate corticosteroid activity and plasma ammonia concentrations in ureotelic toadfish. Metyrapone treatment to lower circulating cortisol levels resulted in a 3.5-fold elevation of ammonia excretion rates, enhanced mRNA expression of two of the toadfish Rh isoforms (Rhcg1 and Rhcg2), and decreased branchial and hepatic GS activity. Correspondingly, cortisol infusion decreased ammonia excretion 2.5-fold, a change that was accompanied by reduced branchial expression of all toadfish Rh isoforms (Rhag, Rhbg, Rhcg1 and Rhcg2) and a twofold increase in hepatic GS activity. In contrast, maintenance of high circulating ammonia levels by ammonia infusion enhanced ammonia excretion and Rh expression (Rhag, Rhbg and Rhcg2). Toadfish treated with cortisol showed an attenuated response to ammonia infusion with no change in Rh mRNA expression or GS activity. In summary, the evidence suggests that ammonia excretion in toadfish is modulated by cortisol-induced changes in both Rh glycoprotein expression and GS activity.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/215/2/314/DC1

Key words: cortisol, nitrogen excretion, urea, ammonia, plasma, gill, glutamine synthetase, Rhesus proteins, gulf toadfish, *Opsanus beta*.

INTRODUCTION

The gulf toadfish, *Opsanus beta*, is unusual among teleosts in demonstrating the capacity to alter both rates and patterns of nitrogen excretion. Under conditions of minimal stress in the laboratory, toadfish are slightly ammoniotelic (Walsh and Milligan, 1995), resembling the majority of teleost fish that dispose of nitrogenous wastes predominantly as ammonia (the sum of NH_3 and NH_4^+) across the gills (Wood, 1993). However, in response to moderate stress, such as crowding or confinement, toadfish switch to ureotely and excrete the bulk of their waste as urea in a pulsatile manner across the branchial epithelium (Walsh et al., 1990; Walsh et al., 1994; Wood et al., 1995; Wood et al., 1997). The transition to ureotelism is characterized by increases in liver and muscle glutamine synthetase (GS) activity (Walsh et al., 1994; Hopkins et al., 1995; Walsh and Milligan, 1995; Wood et al., 1995; Walsh et al., 2003; Esbaugh and Walsh, 2009). GS converts ammonia into glutamine for use in the piscine ornithine–urea cycle (OUC), and induction of this enzyme results in an increase in urea production and accumulation in the plasma (Walsh et al., 1994; Hopkins et al., 1995; Walsh and Milligan, 1995; McDonald et al., 2009). However, the transition to ureotelism does not involve a significant increase in urea excretion, but rather a substantial reduction in ammonia excretion (Walsh et al., 1994; Walsh and Milligan, 1995). It was postulated that the retention of ammonia is facilitated by the combined activity of hepatic and gill-specific GS isozymes. Although the hepatic GS likely accounts for the bulk of ammonia entry as glutamine into the OUC, the glutamine produced by the gill-specific GS is thought to minimize ammonia leakage across the branchial epithelium of ureotelic toadfish and likely contributes only a small fraction to urea production in the OUC (Wood et al., 1995; Walsh, 1997; Walsh et al., 2003; Esbaugh and Walsh, 2009; McDonald et al., 2009). A recent study by McDonald and colleagues demonstrated that GS expression has a limited distribution in the gills, being localized only in the mitochondrion-rich cells (MRCs) (McDonald et al., 2009), which constitute a very small proportion of the total gill cell population (Perry and Walsh, 1989). Furthermore, branchial GS activity remains unchanged following an acute (24–48 h) crowding and confinement stress (Hopkins et al., 1995; Wood et al., 2003; Esbaugh and Walsh, 2009; Rodela et al., 2012). Increased branchial activity has only been reported following chronic exposure (~1 week) to crowded conditions (McDonald et al., 2009). Collectively, these data suggest that additional mechanisms may play an integral role in modulating ammonia excretion in toadfish.

The recent identification of the ammonia transport (AMT)/methylammonium permease (MEP)/Rhesus (Rh) glycoprotein family of proteins that function as ammonia channels in plasma membranes (Marini et al., 1997; Marini et al., 2006) has led to a re-evaluation of ammonia excretion mechanisms in fish. Vertebrates express three main Rh-associated glycoproteins that facilitate ammonia transport: type A (Rhag), type B (Rhbg) and type C (Rhcg) (Huang and Peng, 2005; Peng and Huang, 2006). With

respect to teleosts, multiple copies of the Rh genes have been isolated in pufferfish (*Takifugu rubripes*) (Nakada et al., 2007), rainbow trout (*Oncorhynchus mykiss*) (Nawata et al., 2007; Nawata and Wood, 2008), mangrove killifish (*Kryptolebias marmoratus*) (Hung et al., 2007) and zebrafish (*Danio rerio*) (Shih et al., 2008). Recently, we cloned four toadfish Rh genes – Rhag, Rhbg, Rhcg1 and Rhcg2 – that show considerable sequence similarity to other piscine Rh proteins (Rodela et al., 2012). These four genes are prominently expressed in the gill and their abundance is correlated with the nitrogen excretion phenotype of the toadfish; unconfined toadfish excreted significant quantities of ammonia and exhibited high levels of Rh mRNA expression (Rodela et al., 2012). By contrast, patterns observed in ureotelic, confined toadfish suggested that Rh abundance in the gill was reduced to minimize the branchial efflux of ammonia. The differential pattern of Rh mRNA expression between the two toadfish phenotypes indicates that confinement stress may provide proximate cues for the downregulation of Rh glycoproteins.

Like most teleosts (Barton and Iwama, 1991), toadfish respond to crowding or confinement with a moderate surge in plasma cortisol levels that peaks at approximately 2 h and returns to normal by 24 h (Hopkins et al., 1995). Studies have consistently found this transient increase in cortisol to be an important regulator of both the ureogenic and the ureotelic capacity of the toadfish. In particular, the rise in cortisol induces transcription of the hepatic GS that results in enhanced enzyme activity (Kong et al., 2000; Esbaugh and Walsh, 2009), an effect eliminated by treatment with the cortisol-synthesis inhibitor metyrapone (Hopkins et al., 1995). Elevated cortisol also causes an upregulation of toadfish urea transporter (tUT) transcript levels (McDonald et al., 2009; Rodela et al., 2011), but inhibits tUT function (McDonald et al., 2004; McDonald et al., 2009), as indicated by a reduction in urea excretion. These observations suggest that cortisol coordinates both transcriptional regulation of tUT and post-translational regulatory events to facilitate the efflux of urea in the pulsatile manner that is unique to the gulf toadfish (Wood et al., 2003; McDonald et al., 2009; Rodela et al., 2011). Based on the strong involvement of cortisol in urea metabolism and excretion, we speculate that glucocorticoids may regulate Rh expression in toadfish to coordinate simultaneous changes in ammonia and urea excretion during the transition to ureotely. The hypothesis of glucocorticoid involvement is consistent with prior observations of differential Rh expression and ammonia excretion between fish that experienced minimal stress and those exposed to an acute stress [i.e. crowding and confinement (Rodela et al., 2012)].

Thus, the main goal of the present study was to address the possibility that Rh mRNA expression in toadfish is regulated by cortisol. The central hypothesis was that cortisol regulates branchial Rh expression and hence ammonia excretion. Assuming Rh protein levels reflect mRNA expression, we predicted that Rh mRNA expression and ammonia excretion would be inhibited by high cortisol but enhanced by low cortisol. To test this hypothesis, nitrogen excretion and Rh mRNA expression were measured in toadfish exposed to treatments that either lowered or increased circulating plasma cortisol levels. Furthermore, data from a companion study indicated that circulating ammonia may also be an important endogenous regulator of Rh mRNA (and hence protein) expression. Following an ammonia load generated *via* feeding, toadfish increased branchial Rh mRNA expression (and presumably Rh protein levels) to enhance post-prandial ammonia efflux rates (Rodela et al., 2012). Therefore, a secondary objective of the study was to examine the influence of circulating ammonia levels on Rh mRNA expression. Plasma ammonia was maintained at levels that were physiologically comparable to those experienced by fed toadfish, and the effect of exogenous ammonia was evaluated in both the absence and presence of cortisol. For all experiments, changes in gill mRNA transcripts were evaluated and correlated with changes in plasma ammonia levels and ammonia excretion. Furthermore, the linkage of ammonia and urea *via* the OUC warranted examination of additional variables including GS mRNA expression and activity.

MATERIALS AND METHODS

Animals

Gulf toadfish, *Opsanus beta* (Goode and Bean), were collected by roller trawl in Biscayne Bay, Florida, by local commercial shrimpers during January–February 2009. Immediately following capture, toadfish were transferred to the laboratory and treated with a freshwater dip for 2 min, followed by a Malachite-Green–formalin treatment (final concentration 0.05 mg l^{-1} Malachite Green, 15 mg ml^{-1} formalin; Aquavet, Hayward, CA, USA) to prevent infection by the ciliate *Cryptocaryon irritans* (Stoskopf, 1993; Wood et al., 1997). Toadfish were acclimated to laboratory conditions for a minimum of 1 week prior to experimental manipulation. During this period, fish were kept at low stocking density (<10 fish per tank) in 501 glass aquaria supplied with flowing, aerated seawater (18–22°C, pH 8.1) under a natural photoperiod. Toadfish were fed chopped squid once a week.

Experimental protocol

All fish were exposed to a standard crowding and confinement procedure to induce and maintain ureotely for the duration of the experiments (Walsh, 1987; Hopkins et al., 1995; Wood et al., 1997). In brief, seven to eight fish were transferred to a 61 plastic tub supplied with flowing seawater for 48 h; fish were deprived of food during this time and for the remainder of the experiment. Following this period, fish were lightly anesthetized with MS-222 (1 g l^{-1} buffered with 2 g l^{-1} sodium bicarbonate, pH 7.8), covered with moist towels and surgically implanted with caudal artery catheters (Wood et al., 1997; McDonald et al., 2000) and, in some cases, intraperitoneal catheters (see below) (McDonald and Walsh, 2004). Catheters were used to facilitate delivery of pharmacological agents and/or for blood sampling with minimal handling stress. Following surgery, toadfish were allowed to recover for 24 h in individual 21 flux chambers supplied with flowing, aerated seawater.

Two series of experiments were conducted. For all experiments, an initial 200 µl blood sample was withdrawn from each fish *via* the caudal artery catheter at time 0 h. For Series I, the blood sample was centrifuged at 10,000 ***g*** for 1 min and an 80 µl aliquot of plasma was removed, flash frozen in liquid nitrogen and stored at –80°C for later analysis of ammonia, urea and cortisol levels. The aliquot was replaced with toadfish saline (Walsh, 1987) and the re-suspended red blood cells were re-injected into the fish. For Series II, blood pH was measured over the experimental period to monitor changes in acid–base status resulting from infusion of ammonia. In brief, an initial 300 µl blood sample was withdrawn from each fish *via* the caudal catheter at time 0 h. Approximately 80 µl of the whole blood was immediately used to measure blood pH with an Accumet microprobe combination pH electrode (Cole-Parmer, Model EW-55500-40, Vernon Hills, IL, USA) inserted into a tightly sealed plexiglass chamber that was maintained at the temperature of ambient seawater (18–22°C). The pH electrode was connected to a Radiometer PHM220 pH meter (Radiometer, Copenhagen, Denmark). The remaining blood was centrifuged as described above to obtain a plasma sample. Following each of these procedures, the whole blood used for pH measurements was recovered and

combined with the re-suspended red blood cells remaining from the plasma samples and re-injected into the fish.

Concurrently, water flow to the individual flux chambers was stopped and an initial 2 ml water sample was removed by pipette. Additional water samples were collected hourly using a peristaltic pump (2 ml h^{-1}) and fraction collector for the remainder of the experiment. Every 24 h, boxes were flushed with fresh seawater for 30 min. Between flushes, air saturation and adequate mixing in the flux chambers were maintained by vigorous aeration. For the first 24 h of all experimental treatments, toadfish were left undisturbed in their individual flux chambers to collect baseline measurements of nitrogen excretion and plasma variables.

Series I: metyrapone treatment

Metyrapone (methyl-1,2-di-3-pyridyl-1-propanone) is a useful pharmacological tool for lowering circulating cortisol levels through inhibition of 11β-hydroxysteroid dehydrogenase in the cortisol synthesis pathway (Bennett and Rhodes, 1986; Mommsen et al., 1999; Bernier and Peter, 2001; Milligan, 2003). Toadfish were given a dose of either 1.5 µl g^{-1} body mass of saline (150 mmol l^{-1} NaCl) or metyrapone (20 mg ml^{-1} in 150 mmol l^{-1} NaCl) delivered by intraperitoneal catheter every 24 h [as per Hopkins et al. (Hopkins et al., 1995)], for up to 96 h. The treatments were followed by injection of an additional 1.5 µl g^{-1} body mass of saline to advance the compounds through the catheter into the peritoneal cavity of the fish. Blood samples were withdrawn at 24, 30, 36, 48, 72 and 96 h. The experiment was terminated at 96 h, at which time toadfish were terminally anesthetized with an overdose of MS-222 (3 g l^{-1}) and gill and liver tissue were collected. Half of the tissue was frozen immediately in liquid nitrogen and stored at –80°C for later analysis of branchial and hepatic GS enzyme activity. The remaining tissue was frozen in RNA*later*® (Sigma-Aldrich, St Louis, MO, USA) for later analysis of mRNA expression of toadfish Rh isoforms, and gill and hepatic GS isoforms.

Series II: exogenous cortisol and/or ammonia loading through arterial infusion

Toadfish were separated into four treatment groups. Using a Gilson eight-channel peristaltic pump connected to the arterial catheter, after the 24 h control period, one group of toadfish (mean ± s.e.m. mass=0.070±0.001 kg, *N*=8) was infused with iso-osmotic saline (150 mmol l^{-1} NaCl) at a rate of 3 ml kg^{-1} h^{-1} for the duration of the experiment. A second group (mean mass=0.062±0.003 kg, *N*=8) was infused with cortisol (0.09 mg ml^{-1}, hydrocortisone hemisuccinate salt; Sigma-Aldrich) in isosmotic saline at a rate of 3 ml kg^{-1} h^{-1}. The cortisol concentration was chosen based on previous studies (McDonald et al., 2004; Rodela et al., 2009a). A third group (mean mass=0.065±0.003 kg, *N*=8) was infused with ammonia [$(NH_4)_2HPO_4$; Sigma-Aldrich] in isosmotic saline. The concentration of infused ammonia was chosen based on a preliminary experiment demonstrating that circulating ammonia in laboratory-acclimated toadfish increased from 191.3±26.6 to 503.4±58.2 µmol l^{-1} within 6 h following feeding. An intermediate value of approximately 300 µmol l^{-1} was chosen to avoid potential long-term toxicity effects associated with elevated ammonia. Maintenance of plasma levels was achieved by adjusting the infusion rates following periodic evaluation of plasma ammonia concentrations. Furthermore, ammonium phosphate was chosen for infusion to avoid the alteration of acid–base status that is associated with the infusion of similar compounds such NH_4HCO_3 or $(NH_4)_2SO_4$ (see Salama et al., 1999). Accumulation of phosphate in the blood was not a concern because renal excretion is capable of handling the phosphate load introduced by infusion (Kaune and Hentschel, 1987; Maren et al., 1992). The final group of fish (mean mass=0.078±0.008 kg, *N*=8) was infused with a combination of cortisol and ammonia. Cortisol (0.09 mg ml^{-1}) and ammonia infusions were as described above, with both compounds dissolved in iso-osmotic saline. Blood samples were withdrawn at 24, 30, 36, 48, 72 and 96 h and an aliquot was immediately used to measure blood pH; another aliquot was removed for later analyses of plasma variables. Following termination of the experiment, toadfish were terminally anesthetized and sampled as described in Series I.

Physiological assays

Ammonia content of water samples was measured according to the Indophenol Blue method (Ivancic and Degobbis, 1984) using a ThermoMax microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA). The concentration of urea in water samples was determined using the diacetyl monoxime method described by Rahmatullah and Boyde (Rahmatullah and Boyde, 1980), adapted for use with a microplate reader. The excretion (µmol N kg^{-1}) of ammonia or urea was calculated as reported in previous studies (Wright and Wood, 1985; McDonald et al., 2004; Rodela et al., 2009a). These values were used to calculate the percent ureotelism. Nitrogen excretion (µmol N kg^{-1}) values from the treatment period were averaged from multiple 24 h intervals (24–48, 48–72 and 72–96 h) to facilitate comparison with the initial 24 h control period. In addition, water urea content was used to analyze the size and frequency of pulsatile urea excretion events. A threshold pulse size of 40 µmol N kg^{-1} was used, as defined by McDonald et al. (McDonald et al., 2004). Urea efflux (µmol N kg^{-1}) was then examined in more detail to identify total, pulsatile and non-pulsatile components. Urea-related data were measured to verify that the toadfish were responding in the same manner as reported in previous studies. However, as ammonia was the primary focus of this study, the data pertaining to urea excretion are reported in supplementary material Figs S1 and S2. All excretion measurements are presented graphically as ratios that were calculated by normalizing the value from the treatment period to the value from the control period for that same treatment. Data were normalized to reduce the natural variability in nitrogen excretion rates that can occur among different batches of wild-caught toadfish.

Prior to measurement of plasma ammonia concentrations (µmol l^{-1}), an aliquot of plasma was deproteinized in two volumes of 8% perchloric acid, vortexed, and centrifuged at 16,000 ***g*** for 10 min (4°C). The resulting supernatant was neutralized with saturated $KHCO_3$ and centrifuged at 16,000 ***g*** for 10 min (4°C) to remove the precipitate. The ammonia content of the final supernatant was measured using a Sigma Diagnostics ammonia (L-glutamate dehydrogenase) kit modified for use with a microplate reader. A second plasma aliquot was used to quantify plasma urea (mmol l^{-1}) according to the diacetyl monoxime method (Rahmatullah and Boyde, 1980); plasma urea values are reported in supplementary material Figs S1 and S2. Finally, plasma cortisol concentration was measured using a commercial ^{125}I radioimmunoassay kit from MP Biomedicals (Santa Ana, CA, USA).

GS activity was measured according to the transferase protocol (Walsh, 1996). In brief, individual gill and liver samples were homogenized separately using a motor-driven tissue homogenizer (Fisherbrand, Waltham, MA, USA) in five volumes of 50 mmol l^{-1} HEPES buffer (pH 7.4) and then centrifuged at 13,000 ***g*** for 8 min. The resulting supernatant was decanted and assayed for GS activity at 540 nm using a Spectramax Plus 384 plate spectrophotometer (Molecular Devices). GS activity is reported in units of µmol min^{-1} g^{-1}.

Molecular analysis of gene expression

At present, a suitable antibody for Rh glycoprotein quantification in toadfish does not exist and we were therefore only able to measure changes in Rh mRNA expression. Total RNA was extracted from gill using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The quantity and quality of the resulting RNA were assessed using a Nanodrop spectrophotometer (ND-100; Thermo Scientific, Waltham, MA, USA). Prior to cDNA synthesis, 2 µg of total RNA were treated with amplification grade DNase (Invitrogen) to remove any remaining genomic DNA. First-strand cDNA synthesis was performed on Dnase-treated total RNA using RevertAid H$^-$ MULV reverse transcriptase (Fermentas, Glen Burnie, MD, USA) with random hexamers (200 ng per reaction; Integrated DNA Technologies, Coralville, IA, USA), following the manufacturer's protocol. The final cDNA product was diluted in an equal volume of autoclaved water.

For real-time PCR analysis of gene transcript levels, primers designed and validated in previous studies were used; in particular, primers for Rhag (GenBank accession no. HQ424874), Rhbg (GenBank accession no. HQ424875), Rhcg1 (GenBank accession no. HQ424876), Rhcg2 (GenBank accession no. HQ424877), gill GS (GenBank accession no. AF532312) and hepatic GS (GenBank accession no. AF118103) were obtained from Rodela et al. (Rodela et al., 2012). Similarly, primers for 18S were designed and validated from an earlier study (Rodela et al., 2011). All primer pairs were chosen for optimal amplification at an annealing temperature of 58°C. The mRNA of the liver-specific isoform of GS was not measured in the gill as previous studies have shown levels to be negligible (Walsh et al., 2003; McDonald et al., 2009). Similarly, the mRNA expression of the gill-specific GS isoform is relatively small in non-branchial tissues (Walsh et al., 2003; McDonald et al., 2009) and therefore was not measured in liver in the present study.

All real-time PCR reactions were performed using a SYBR Green master mix (Stratagene, Santa Clara, CA, USA) with an Mx3000P Real-Time PCR System and associated MxPro 4.1 software (Stratagene). A 2 µl aliquot of template was used for a 12.5 µl reaction that contained both forward and reverse primers and SYBR master mix. The composition of the reactions and the thermocycler settings were based on those suggested by the manufacturer. Dissociation curves were generated and evaluated for each reaction to verify the specificity of the primer pairs and monitor the formation of primer-dimers. 'No reverse transcriptase' (generated by the omission of reverse transcriptase during cDNA synthesis) and no template control templates were used to ensure that amplicons did not arise from genomic DNA or reagent contamination, respectively. Standard curves were used to assess the reaction efficiency of all primer pairs. Templates for the standard curves were derived from pooled gill cDNA samples and serially diluted (1:5) with RNase/DNase-free molecular grade water (Sigma-Aldrich). Efficiencies were assessed by linear regression of cycle threshold (C_t) values on relative template concentration. Primer pair efficiencies were considered satisfactory when they fell within the range of 85–115% with R^2>0.99. All transcript levels were expressed relative to the control gene 18S (diluted 1000-fold) and calculated according to the $\Delta\Delta C_t$ method outlined by Pfaffl (Pfaffl, 2001). For purposes of comparison, the mRNA expression of each gene was calculated relative to the corresponding control samples.

Statistical analyses

All data are presented as means ± s.e.m. Differences among treatments for nitrogen excretion data were statistically analyzed using a two-way repeated-measures (RM-) ANOVA with time within groups (i.e. before and after application of treatment) as the first factor and treatment among or between groups as the second factor. Gene expression was analyzed by Student's *t*-test or one-way ANOVA, as appropriate. A Holm–Sidak test was used for *post hoc* multiple comparisons. Equivalent non-parametric tests were used when assumptions of normality and equal variance were not satisfied (P<0.05). Prior to analysis, proportional data (% urea and ammonia) were transformed using $p'=\arcsin\sqrt{p}$, as these values were binomially distributed (Zar, 1999). All statistical analyses were carried out using SPSS SigmaStat software (version 3.5, San Jose, CA, USA).

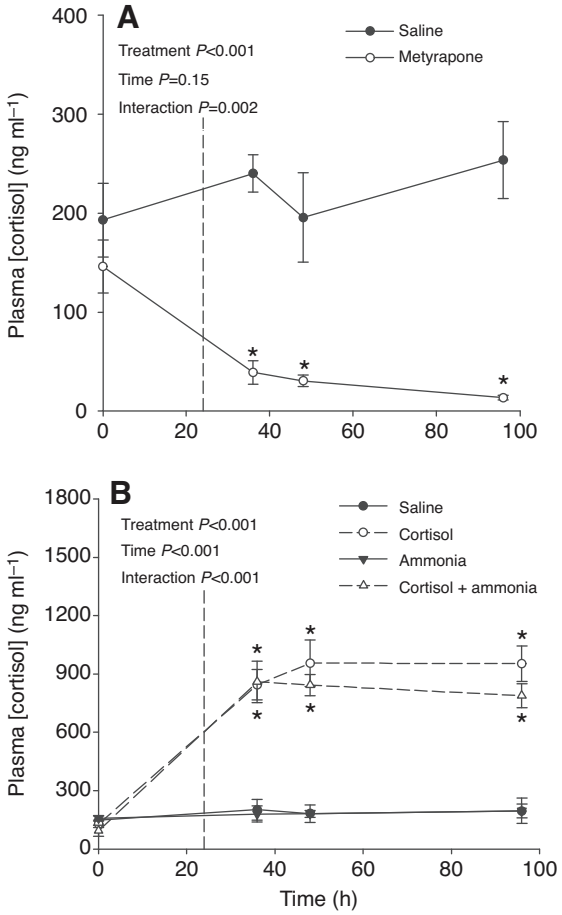


Fig. 1. The effects of experimental treatments on circulating cortisol concentrations (ng ml^{-1}) in gulf toadfish, *Opsanus beta*. In Series I (A), toadfish were injected with saline (N=8) or metyrapone (N=8). In Series II (B), toadfish were infused with saline (N=8), hydrocortisone hemisuccinate salt (cortisol; N=8), ammonia (N=8) or ammonia and cortisol combined (N=7). See Materials and methods for further details of treatment procedures. All values are expressed as means ± s.e.m. Data were analyzed by two-way RM-ANOVA with treatment group and sampling time as factors; P-values are indicated on the figure. Asterisks indicate a significant difference from the initial (0 h) sample within a given treatment group (Holm–Sidak *post hoc* test, P<0.05). Dashed vertical lines indicate the start of the treatment period.

RESULTS

Series I

As expected, metyrapone treatment was effective at reducing circulating cortisol concentrations. Measurable differences in cortisol levels were detected at 36, 48 and 96 h relative to the initial 0 h cortisol concentration (Fig. 1A). Within the metyrapone group,

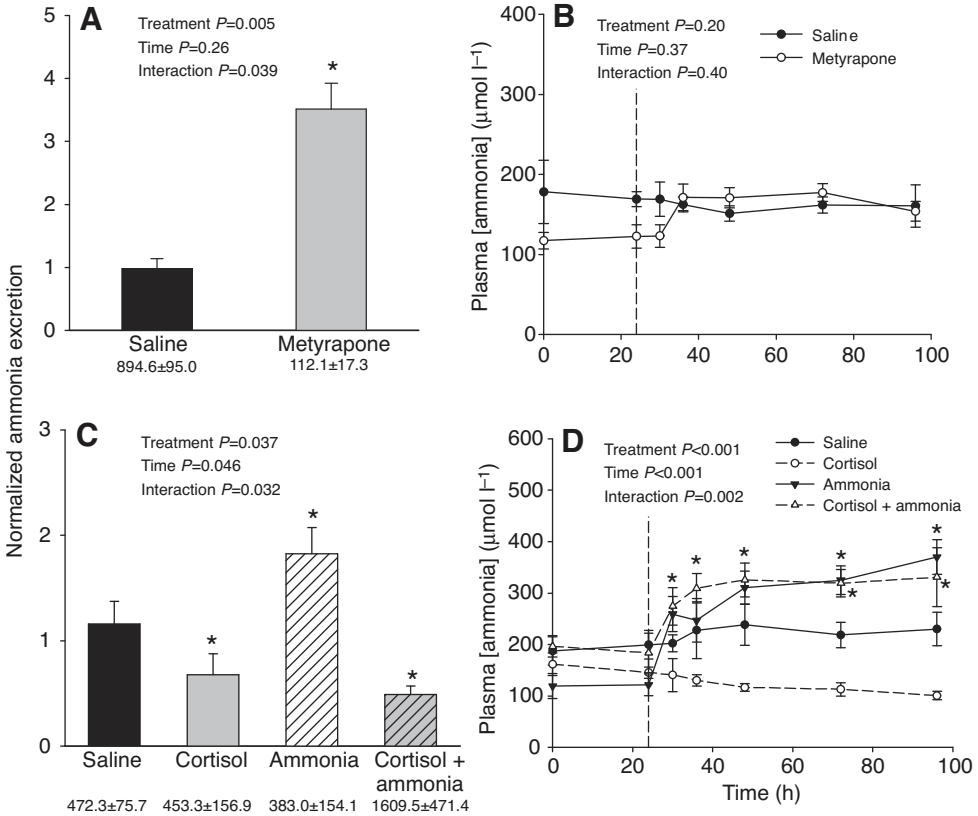


Fig. 2. Measurements of (A,C) ammonia excretion (relative units) and (B,D) plasma ammonia levels (mmol l^{-1}) in gulf toadfish, *Opsanus beta*, following various corticosteroid and ammonia treatments. In Series I (A,B), toadfish were treated with saline (N=8) or metyrapone (N=8). In Series II (C,D), toadfish were infused with saline (N=8), hydrocortisone hemisuccinate salt (cortisol; N=8), ammonia (N=8) or ammonia and cortisol combined (N=8). See Materials and methods for further details of treatment procedures. All ammonia excretion data are presented as the treatment value normalized to the initial 24 h control period. In A and C, numerical values presented under the data bars represent absolute values (μmol N kg^{-1}) of ammonia excretion during the initial 24 h control period. All values are expressed as means ± s.e.m. Data were analyzed by two-way RM-ANOVA with treatment group and sampling time as factors; P-values are indicated on the figure. Asterisks indicate a significant difference between initial control and treatment values within a given treatment group (Holm–Sidak *post hoc* test, P<0.05). In B and D, the dashed vertical lines indicate the start of the treatment period.

treatment elevated ammonia excretion 3.5-fold (Fig. 2A), raising rates from 4.7±0.6 to 15.8±1.9 μmol N kg^{-1} h^{-1} (N=8). Metyrapone injection also increased total urea excretion, which was attributed to an increase in the pulsatile component of excretion as the non-pulsatile component was unaffected by treatment (supplementary material Fig. S1A,B). Metyrapone treatment was without effect on plasma ammonia concentrations (Fig. 2B) and had a minimal effect on plasma urea; only the 72 and 96 h values were different from the initial treatment value (supplementary material Fig. S2A). Saline injection had no significant impact on any of these variables (Table 1, Fig. 1A, Fig. 2A,B, supplementary material Fig. S1A,B and Fig. S2A). Fish in the saline treatment group exhibited a lower degree of ureotelism than fish in the metyrapone treatment group, a difference that was not affected by experimental period (Table 1).

In vivo treatment with metyrapone did not significantly influence mRNA abundance of the gill-specific or hepatic isoforms (*t*-tests, P=0.97, 0.86, respectively; Fig. 3A) of GS compared with saline-injected toadfish. However, GS activities in the gill and liver were 1.6- and 1.8-fold lower in metyrapone-treated fish, respectively (*t*-tests, P=0.023, P<0.001, respectively; Fig. 3B). The mRNA expression of Rh glycoprotein isoforms in the gill was affected by metyrapone treatment. Rhag mRNA abundance was lowered by 4.8-fold whereas Rhcg1 and Rhcg2 mRNA expression was elevated 2.2- and 4-fold, respectively, in metyrapone-treated toadfish relative to saline-injected individuals (*t*-tests, $P \leq 0.038$ in each case; Fig. 4A). Branchial Rhbg mRNA expression tended to be lower in metyrapone-treated fish, although the effect did not reach statistical significance (*t*-test, P=0.073; Fig. 4A).

Series II

Saline-infused fish excreted ammonia at a rate of 19.7±2.6 μmol kg^{-1} h^{-1} (N=8). Ammonia excretion, plasma ammonia concentrations, urea excretion, plasma urea concentrations and plasma cortisol concentrations were not significantly affected by saline infusion (Fig. 1B, Fig. 2C,D, supplementary material Fig. S1B, Fig. S2B).

Infusion with cortisol raised circulating concentrations significantly (Fig. 1B). Toadfish infused with cortisol experienced a reduction in ammonia excretion over the experimental period (Fig. 2C), with rates decreasing from 18.9±5.3 to 7.3±1.3 μmol kg^{-1} h^{-1} (N=8). Plasma ammonia concentrations did not vary significantly from initial values (Fig. 2D). Urea was excreted in a pulsatile fashion; however, cortisol infusion significantly decreased the total and pulsatile components of urea excretion (supplementary material Fig. S1C), as well as urea pulse size (supplementary material Fig. S1D). Plasma urea levels rose over time (supplementary material Fig. S2B).

Infusion of toadfish with $(NH_4)_2HPO_4$ raised circulating ammonia to a concentration of 300 μmol l^{-1}, where it was maintained (Fig. 2D). Maintenance of plasma ammonia at elevated concentrations increased ammonia excretion 1.7-fold (Fig. 2C), raising excretion rates from 16.0±5.2 to 23.3±6.5 μmol kg^{-1} h^{-1} (N=8). None of the urea-related variables (supplementary material Fig. S1C,D, Fig. S2B) or plasma cortisol concentrations (Fig. 1B) were affected by ammonia infusion.

Infusion of toadfish with cortisol and ammonia together raised circulating concentrations of both compounds (cortisol, Fig. 1B; ammonia, Fig. 2D). The combined infusion reduced ammonia excretion by 50% (Fig. 2C), lowering rates from 67.1±16.0 to 27.8±4.3 μmol kg^{-1} h^{-1} (N=8). This effect was accompanied by a ~50% decrease in total and pulsatile urea excretion (supplementary material Fig. S1C) that translated into a 50% reduction in pulse size (supplementary material Fig. S1D). Plasma urea concentrations were slightly elevated during the infusion period (supplementary material Fig. S2B).

Table 1. Percentage of nitrogenous waste excreted in the form of urea and ammonia by gulf toadfish during an initial 24 h control period followed by a treatment period where fish were either infused and/or injected with various compounds

		Control period		Treatment period	
Series	Group	% Urea-N	% Ammonia-N	% Urea-N	% Ammonia-N
I	Saline[a]	68.3±4.2	31.7±3.2	67.3±4.7	32.7±3.6
	Metyrapone	89.5±2.7	10.7±2.1	79.7±2.3	20.3±1.8
II	Saline	74.7±4.6	25.2±3.5	72.3±7.8	28.3±4.2
	Cortisol	68.6±8.2	31.4±6.3	64.2±13.2	35.7±8.5
	Ammonia	81.4±6.6	18.6±5.0	60.9±14.2	39.1±9.2
	Cortisol + ammonia	67.9±9.4	28.1±7.4	77.5±5.2	19.7±3.8

See Materials and methods for details about infusion/injection conditions.
Values are means ± s.e.m. (N=8).
Data were analyzed by two-way RM-ANOVA with treatment group and time (control, treatment) as factors (Series I: time P=0.061, treatment P=0.014, time–treatment interaction P=0.12; Series II: time P=0.83, treatment P=0.34, time–treatment interaction P=0.18).
[a]Toadfish injected with saline served as a control group for both Series I and Series II.

Neither the proportion of nitrogenous waste excreted as urea (Table 1) nor plasma pH (mean=7.86±0.002) was significantly affected by treatment or time (two-way RM-ANOVA, treatment P=0.76, time P=0.47, treatment–time interaction P=0.50).

Significant effects on both gill (one-way ANOVA, P=0.041) and liver (one-way ANOVA, P=0.011) GS mRNA expression were detected (Fig. 3C). Toadfish infused with cortisol (Holm–Sidak *post hoc* test, P=0.025) or a combination of cortisol and ammonia (Holm–Sidak *post hoc* test, P=0.014) exhibited approximately twofold higher levels of branchial GS mRNA expression than saline-infused fish (Fig. 3C). A similar trend was observed in the liver (Fig. 3C); mRNA abundance of hepatic GS in fish infused with cortisol and cortisol + ammonia was elevated relative to saline controls. One-way ANOVA analysis revealed significant differences among GS enzyme activities in the gill (P<0.001) and liver (P<0.001; Fig. 3D). Ammonia infusion significantly reduced enzyme activity in the gill relative to saline controls (Fig. 3D). In the liver, cortisol and cortisol + ammonia significantly increased GS activity relative to saline-infused individuals (Fig. 3D).

Exogenous manipulation of plasma cortisol and/or ammonia through infusion significantly altered mRNA expression of Rhag (one-way ANOVA, P=0.007), Rhbg (one-way ANOVA, P=0.043), Rhcg1 (one-way ANOVA, P=0.038) and Rhcg2 (one-way ANOVA, P=0.004) in the gill (Fig. 4B). In particular, cortisol infusion caused a decrease in mRNA expression for all Rh isoforms relative to expression in saline-infused toadfish. By contrast, ammonia infusion

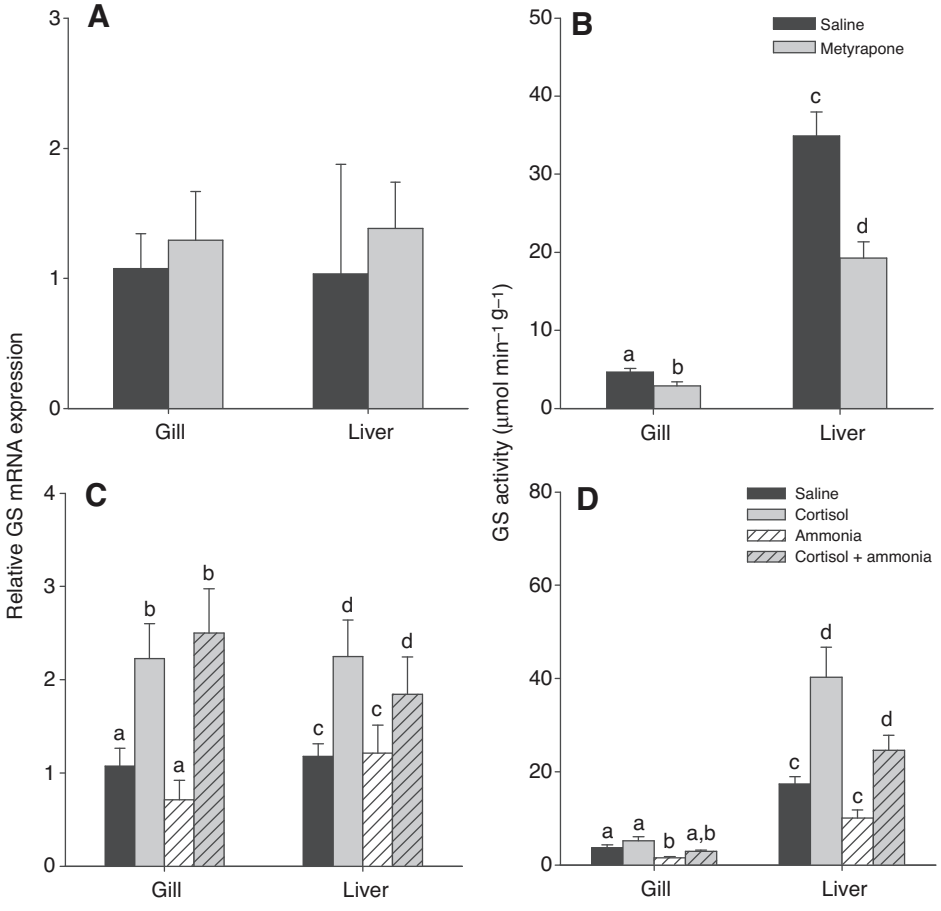


Fig. 3. The effects of cortisol and/or ammonia manipulation on branchial and hepatic glutamine synthetase (GS) relative mRNA expression as measured by (A,C) real-time RT-PCR and (B,D) GS activity (mmol min^{-1} g^{-1}) in gulf toadfish, *Opsanus beta*. In Series I (A,B), toadfish were injected with saline (N=8) or metyrapone (N=8). In Series II (C,D), toadfish were infused with saline (N=8), hydrocortisone hemisuccinate salt (cortisol; N=8), ammonia (N=8) or ammonia and cortisol combined (N=7). See Materials and methods for further details of treatment procedures. The mRNA transcript levels for each tissue were normalized against the control gene 18S and expressed relative to the saline-treated group for each series, which was set to a value of 1. Values are means ± s.e.m. For Series I, data were analyzed by Student's *t*-tests; see Results for *P*-values. No significant differences were detected for GS mRNA expression. For Series II, data were analyzed by one-way ANOVA; see Results for *P*-values. Statistical differences within a given tissue are indicated by different letters; different sets of letters were used between tissues (Holm–Sidak *post hoc* tests, P<0.05).

caused significant 5.5-, 5.5- and 11.7-fold increases in Rhag, Rhbg and Rhcg2 mRNA expression, respectively, relative to saline controls (Fig. 4B). No significant difference in mRNA abundance of any Rh isoform was detected between cortisol + ammonia-infused individuals and saline-infused toadfish (Holm–Sidak *post hoc* test; Rhag, P=0.11; Rhbg, P=0.12; Rhcg1, P=0.32; Rhcg2, P=0.61; Fig. 4B).

DISCUSSION

Toadfish respond to crowding or confinement with an acute rise in plasma cortisol levels that is accompanied by a decrease in ammonia excretion (Hopkins et al., 1995; Walsh and Milligan, 1995). Previously, it was postulated that the combined activity of branchial and hepatic GS is sufficient to trap all ammonia as glutamine, thereby reducing ammonia excretion by metabolic means (Hopkins et al., 1995; Walsh and Milligan, 1995; Wood et al., 1995; Walsh, 1997; Walsh et al., 2003). However, the recent discovery of Rh glycoprotein ammonia transporters has led to a re-evaluation of ammonia transport in fish (reviewed by Weihrauch et al., 2009; Wright and Wood, 2009). Our recent identification of four Rh glycoproteins in toadfish revealed that these isoforms were differentially regulated in response to crowding and feeding, and indicated that there may be additional mechanisms controlling ammonia excretion in toadfish (Rodela et al., 2012). In the present study, we provide indirect evidence to support the hypothesis that cortisol may play a role in decreasing the rate of ammonia excretion by mediating the downregulation of Rh protein expression. Cortisol-induced effects on ammonia efflux were coordinated with well-documented cortisol-mediated changes in both urea metabolism and excretion. The synthesis of these results reveals that there are numerous factors that contribute to the capacity of the toadfish to tightly control and regulate nitrogen excretion *via* different nitrogen end products.

Cannulation, for repetitive non-invasive blood sampling, results in a chronic elevation of plasma cortisol in teleost fish (Brown et al., 1986; Lo et al., 2003; Sloman et al., 2005). In the present study, initial plasma cortisol concentrations ranged from 86 to 376 ng ml^{-1} approximately 24 h following surgery. In the laboratory, resting cortisol levels in uncannulated fish are typically around 40 ng ml^{-1} as measured by caudal artery puncture (Hopkins et al., 1995; Wood et al., 1995). However, endogenous concentrations can exceed 500 ng ml^{-1} following extended periods of confinement [>1 week (McDonald et al., 2009)]. Thus, the values reported in the present study are comparable to those of previous reports, where endogenous cortisol concentrations in cannulated toadfish have exceeded 400 ng ml^{-1} (McDonald et al., 2004; Sloman et al., 2005). These elevated concentrations necessitated the use of a pharmacological rather than physiological dose of exogenous cortisol to measure the effects of enhanced corticosteroid activity on nitrogen excretion (Series II). Although elevated cortisol levels have been linked to increased proteolytic activity and ammonia excretion in many teleosts (see Mommsen et al., 1999), cortisol does not seem to elicit the same extent of proteolysis and endogenous nitrogen production in toadfish. Toadfish reduce nitrogen excretion when exposed to elevated cortisol (Hopkins et al., 1995; Wood et al., 2003; McDonald et al., 2004; Rodela et al., 2009a). At extremely high concentrations of circulating cortisol (~1500 ng ml^{-1}), the proteolytic effect swamps the ammonia-trapping mechanisms in the toadfish and high rates of ammonia excretion resume (see McDonald et al., 2004). In the present study, circulating cortisol levels were maintained below 1000 ng ml^{-1} and values for both plasma ammonia concentration (103.2–211.2 µmol l^{-1}) and rates of ammonia excretion (5.7–48.7 µmol kg^{-1} h^{-1}) were within the range reported for non-cannulated ureotelic toadfish [plasma ammonia, 100–200 µmol l^{-1}; ammonia excretion, 5–50 µmol kg^{-1} h^{-1} (Walsh and Milligan, 1995; Wood et al., 1995)]. Therefore, the observed changes in ammonia excretion were attributed primarily to changes in excretion capacity and not to increased rates of proteolysis elicited by chronically elevated cortisol.

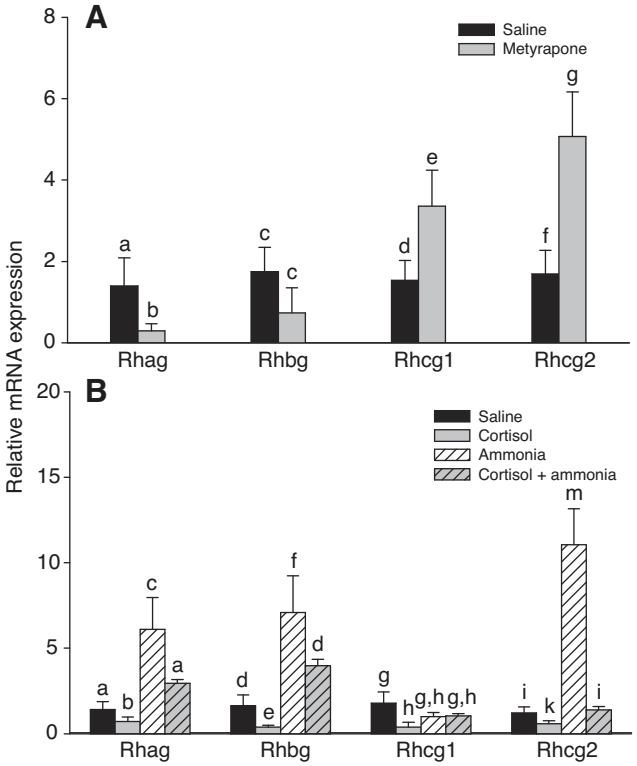


Fig. 4. Relative mRNA expression of Rhag, Rhbg, Rhcg1 and Rhcg2 in the gill of ureotelic gulf toadfish, *Opsanus beta*, as measured by real-time RT-PCR. In Series I (A), toadfish were injected with saline (N=8) or metyrapone (N=8). In Series II (B), toadfish were infused with saline (N=8), hydrocortisone hemisuccinate salt (cortisol; N=8), ammonia (N=8) or ammonia and cortisol combined (N=7). See Materials and methods for further details of treatment procedures. The mRNA levels for each gene were normalized against the control gene 18S and expressed relative to the saline-treated group for each series, which was set to a value of 1. Values are means ± s.e.m. For Series I, data were analyzed by Student's t-tests, and for Series II, data were analyzed by one-way ANOVA; see Results for P-values. Statistical differences for a given Rh isoform are indicated by different letters; different sets of letters were used between Rh isoforms (Series I, P<0.05; Series II, Holm–Sidak *post hoc* tests, P<0.05).

Cortisol infusion decreased Rh mRNA expression in ureotelic toadfish. In the model of ammonia movement across the branchial pavement cells of the marine teleost *T. rubripes* proposed by Nakada and colleagues (Nakada et al., 2007), the expression of Rhag in the pillar cells enables the movement of ammonia from the vasculature to the pavement cells, and transcellular transport across the pavement epithelium is then accomplished through Rhbg and Rhcg2 expressed on the basolateral and apical membranes, respectively (Nakada et al., 2007). If a similar arrangement were present in toadfish, then a cortisol-induced reduction in Rh transport capacity across the pavement cell would be expected to reduce the rate of ammonia excretion. This reduction likely would be further enhanced by a decrease in Rhcg1, the isoform putatively located in the MRCs in

the model of Nakada and colleagues (Nakada et al., 2007). In the present study, cortisol-induced decreases in Rh mRNA expression were correlated with lower ammonia excretion rates, providing support for this model. These findings are consistent with our earlier study that demonstrated that confinement stress (elevated plasma cortisol) resulted in lower levels of all Rh mRNA transcripts as well as lower ammonia excretion compared with unconfined fish (Rodela et al., 2012). By contrast, lowering circulating cortisol with metyrapone had the opposite effect, resulting in higher mRNA transcript levels of Rhcg1 and Rhcg2 and a corresponding increase in ammonia efflux (Series I). It was surprising that metyrapone treatment did not also increase Rhag mRNA expression as expected and, at present, we require more information about the role of Rhag in the toadfish gill to fully understand this response. However, the inverse relationship between circulating cortisol concentration and most of the Rh mRNA transcripts as well as ammonia excretion indicates that cortisol may be an important proximate cue for mediating changes in branchial ammonia transport in toadfish.

Aside from the present investigation, three other studies have addressed the relationship between cortisol and Rh expression in fish. These studies, carried out on rainbow trout, reported rather different patterns (Nawata and Wood, 2008; Nawata and Wood, 2009; Tsui et al., 2009). In particular, Nawata and Wood (Nawata and Wood, 2009) reported that cannulation stress resulted in an elevated ammonia excretion rate accompanied by upregulation of Rhbg, Rhcg1 and Rhcg2 in the gill, a trend opposite to that observed in toadfish in the present study. These differences likely reflect the nitrogen-handling capacities of each species. Rainbow trout, like most teleosts, excrete ammonia to avoid its accumulation and maintain normal physiological function (Wood, 1993; Ip et al., 2001). In comparison, toadfish can tolerate relatively high internal concentrations of ammonia and also have the ability to detoxify this nitrogenous waste through multiple mechanisms (Wang and Walsh, 2000; Walsh et al., 2003). In addition to direct excretion across branchial surfaces, toadfish can sequester ammonia as glutamine and/or use it for the synthesis of urea *via* a fully functional OUC (reviewed by Wood et al., 2003).

GS, the enzyme that feeds ammonia into the piscine OUC, is considered to be a rate-limiting enzyme for ureagenesis in the toadfish (Mommsen and Walsh, 1991). Earlier studies have consistently demonstrated that acute elevation of endogenous plasma cortisol increases both mRNA expression and enzyme activity of GS in the liver and muscle (Walsh et al., 1994; Hopkins et al., 1995; Walsh and Milligan, 1995; Wood et al., 1995; Kong et al., 2000; Walsh et al., 2003; Esbaugh and Walsh, 2009; McDonald et al., 2009). The enhanced activity of hepatic GS increases substrate mobilization for urea synthesis and leads to a slow accumulation of urea in the plasma (Walsh et al., 1994; Hopkins et al., 1995; Walsh and Milligan, 1995; Wood et al., 2003). It is likely that the modulation of hepatic GS activity contributed to the changes in branchial ammonia efflux observed in our study. This statement is supported by data from metyrapone-treated toadfish that demonstrated lower hepatic GS activity that was accompanied by higher rates of ammonia excretion. In contrast, administration of cortisol resulted in a twofold increase in both GS mRNA abundance and enzyme activity in the liver together with decreased rates of ammonia efflux and a slow decline in circulating ammonia concentration. These changes were also accompanied with a rise in plasma urea content. The observed accumulation of plasma urea likely represented the net influence of enhanced urea production and decreased rates of urea excretion. Previous studies have reported that the amount of urea released during discrete urea pulse events is dependent on the concentration of plasma glucocorticoids; in particular, elevated cortisol inhibits tUT function (McDonald et al., 2004; McDonald et al., 2009; Rodela et al., 2009b).

Branchial tissue expresses a gill-specific GS that is the product of a separate gene and lacks a close biochemical association with OUC enzymes (Wood et al., 1995; Walsh et al., 2003; McDonald et al., 2009). GS within the gill is positioned in the MRCs and is believed to act as a high-affinity trap for ammonia, thereby minimizing the amount of ammonia that is lost across the branchial epithelium (Walsh, 1997; Walsh et al., 2003). Examination of gill tissue revealed a significant increase in GS mRNA abundance, with no detectable difference in enzyme activity following cortisol administration. A similar trend in mRNA expression was previously observed following a shorter period (~48 h) of cortisol infusion, whereas enhanced enzyme activity was associated with prolonged periods of stress (e.g. 1 week) (McDonald et al., 2009). Taken together, the cortisol-induced upregulation of GS in the liver and constitutive GS activity in the gill likely contributed to the patterns of ammonia excretion in cortisol-treated toadfish observed in the present study.

It is clear that GS plays a prominent role in the detoxification of endogenous ammonia during periods of fasting and quiescence (Walsh et al., 1994; Hopkins et al., 1995; Walsh and Milligan, 1995; Walsh, 1997; Walsh et al., 2003). However, toadfish experience elevated plasma ammonia shortly following feeding [~300–500 μmol l^{-1} (Rodela et al., 2012)] or during exposure to high ambient ammonia [~1000 μmol l^{-1} (Wang and Walsh, 2000; Veauvy et al., 2002)]. In these circumstances, internal ammonia concentrations likely exceed the ammonia-trapping capacity of GS and, as a result, toadfish may rely more heavily on transport pathways to eliminate ammonia. Our results demonstrate that maintaining plasma ammonia at physiologically relevant concentrations (~300 μmol l^{-1}) caused an approximately twofold increase in ammonia excretion and occurred with significant increases in the mRNA expression of Rhag, Rhbg and Rhcg2, which may facilitate the transcellular transport of excess ammonia across the pavement cells. The mRNA expression of Rhcg1, the isoform thought to be associated with MRCs, was not affected by ammonia infusion, but the reduction in branchial GS activity might serve to promote ammonia efflux through MRCs. Collectively, the data from ammonia-infused fish indicate that toadfish upregulate and maintain high branchial Rh expression in response to elevated plasma ammonia concentrations, and that ammonia itself may be a potent regulator of Rh transcription. These molecular changes were accompanied by increased rates of ammonia excretion. Similar trends have been reported for rainbow trout, which increase both Rhbg and Rhcg2 mRNA to augment excretion following exogenous loading of ammonia *via* NH_4HCO_3 infusion (Nawata and Wood, 2009). Exposure to elevated environmental ammonia has been reported to cause corresponding increases in both tissue ammonia and branchial Rhcg2 mRNA expression in mangrove killifish [*K. marmoratus* (Hung et al., 2007)], and Rhag, Rhbg and Rhcg1 mRNA abundance in zebrafish larvae [*D. rerio* (Braun et al., 2009)]. Therefore, the data indicate that both facultatively ureotelic and ammoniotelic species mount similar responses to high internal and/or external concentrations of ammonia.

Administration of ammonia and cortisol together decreased ammonia excretion. Analysis of gene expression for this group revealed lower levels of mRNA expression for Rhag, Rhbg and Rhcg2 compared with ammonia-infused fish, a trend similar to the effect of cortisol infusion alone in lowering Rh expression relative to saline-infused fish. We speculate that increases in Rh mRNA

expression associated with ammonia infusion were suppressed by the elevated concentrations of circulating cortisol. Furthermore, it is unlikely that changes in the hepatic conversion of ammonia to urea contributed to the reduction in ammonia excretion because no significant changes in GS activity or mRNA were observed following infusion of ammonia alone. Therefore, it appears that the changes in ammonia efflux were the product of strong yet opposing influences of cortisol and ammonia on Rh glycoprotein expression in toadfish.

Our study provided strong evidence that changes in ammonia excretion in toadfish are mediated by cortisol-induced modifications in both Rh glycoprotein expression and GS activity. Elevated plasma cortisol caused a significant reduction in Rh mRNA expression that was accompanied by an increase in the ammonia-trapping activity of hepatic GS, to ultimately reduce the amount of ammonia excreted into the environment. This regulatory pathway may be of ecological relevance in predator avoidance by the gulf toadfish. A recent study revealed that ammonia is an important chemical odorant that is used by the grey snapper, *Latjanus griseus*, to detect its prey, gulf toadfish (Barimo and Walsh, 2006). Reduced ammonia excretion and a subsequent increase in urea efflux were postulated to minimize the detection of toadfish by predators, as urea masked the ammonia odour detected by *L. griseus*. The ability of the toadfish to regulate both ammonia and urea excretion in response to stress may enable a quicker, coordinated response to facilitate this 'chemosensory-cloaking' tactic. Further studies should examine the rapidity with which these cortisol-induced changes in ammonia excretion occur as well as the relative importance of Rh glycoproteins *versus* GS in the initiation of these physiological changes. Clarification of the precise mechanisms (rapid non-genomic *versus* slower genomic) would provide valuable insight and enhance our understanding of the unique ability of the toadfish to tightly control and regulate nitrogen excretion *via* different nitrogen end products.

ACKNOWLEDGEMENTS

Special thanks to Bill Hannah for his help with sampling, and for care and feeding of the Rosenstiel School of Marine and Atmospheric Science toadfish colony.

FUNDING

This study was funded by Natural Sciences and Engineering Research Council of Canada Discovery Grants awarded to P.J.W. and K.M.G., a National Science Foundation grant to M.D.M. (IOB-0920547), and a Student Travel Research grant to T.M.R. from the Canadian Society of Zoologists. P.J.W. is supported by the Canada Research Chair program.

REFERENCES

Barimo, J. F. and Walsh, P. J. (2006). Use of urea as a chemosensory cloaking molecule by a bony fish. *J. Exp. Biol.* **209**, 4254-4261.

Barton, B. A. and Iwama, G. K. (1991). Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Annu. Rev. Physiol.* **1**, 3-26.

Bennett, R. O. and Rhodes, R. C., III (1986). Evaluation of oral administration of cortisol and metyrapone: the effects on serum cortisol in rainbow trout (*Salmo gairdneri*). *Comp. Biochem. Physiol.* **83A**, 727-730.

Bernier, N. J. and Peter, R. E. (2001). Appetite-suppressing effects of urotensin I and corticotropin-releasing hormone in goldfish (*Carassius auratus*). *Neuroendocrinology* **73**, 248-260.

Braun, M. H., Steele, S. L. and Perry, S. F. (2009). The responses of zebrafish (*Danio rerio*) to high external ammonia and urea transporter inhibition: nitrogen excretion and expression of rhesus glycoproteins and urea transporter proteins. *J. Exp. Biol.* **212**, 3846-3856.

Brown, S. B., Eales, J. G. and Hara, T. J. (1986). A protocol for estimation of cortisol plasma clearance in acid-exposed rainbow trout (*Salmo gairdneri*). *Gen. Comp. Endocrinol.* **62**, 493-502.

Esbaugh, A. J. and Walsh, P. J. (2009). Identification of two glucocorticoid response elements in the promoter region of the ubiquitous isoform of glutamine synthetase in gulf toadfish, *Opsanus beta*. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **297**, R1075-R1081.

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.

Huang, C. H. and Peng, J. (2005). Evolutionary conservation and diversification of Rh family genes and proteins. *Proc. Natl. Acad. Sci. USA* **102**, 15512-15517.

Hung, C. Y., Tsui, K. N., Wilson, J. M., Nawata, C. M., Wood, C. M. and Wright, P. A. (2007). Rhesus glycoprotein gene expression in the mangrove killifish *Kryptolebias marmoratus* exposed to elevated environmental ammonia levels and air. *J. Exp. Biol.* **210**, 2419-2429.

Ip, Y. K., Chew, S. F. and Randall, D. J. (2001). Ammonia toxcitiy, tolerance, and excretion. In *Nitrogen Excretion* (ed. P. A. Wright and P. M. Anderson), pp. 109-148. New York: Academic Press.

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

Kaune, R. and Hentschel, H. (1987). Stimulation of renal phosphate secretion in the stenohaline freshwater teleost: Carassius auratus gibelio Bloch. *Comp. Biochem. Physiol.* **87A**, 359-362.

Kong, H. Y., Kahatapitiya, N., Kingsley, K., Salo, W. L., Anderson, P. M., Wang, Y. X. S. and Walsh, P. J. (2000). Induction of carbamoyl phosphate synthetase III and glutamine synthetase mRNA during confinement stress in gulf toadfish (*Opsanus beta*). *J. Exp. Biol.* **203**, 311-320.

Lo, W. Y., Chang, C. F. and Song, Y. L. (2003). Evaluation of dorsal aorta cannulation for immunological studies of grouper (*Epinephelus malabaricus*). *Fish Shellfish Immunol.* **14**, 289-303.

Maren, T. H., Fine, A., Swenson, E. R. and Rothman, D. (1992). Renal acid-base physiology in marine teleost, the long-horned sculpin (*Myoxocephalus octodecimspinosus*). *Am. J. Physiol. Renal Physiol.* **263**, F49-F55.

Marini, A. M., Urrestarazu, A., Beauwens, R. and Andre, B. (1997). The Rh (rhesus) blood group polypeptides are related to NH_4^+ transporters. *Trends Biochem. Sci.* **22**, 460-461.

Marini, A. M., Boeckstaens, M. and Andre, B. (2006). From yeast ammonium transporters to Rhesus proteins, isolation and functional characterization. *Transfus. Clin. Biol.* **13**, 95-96.

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.

McDonald, M. D., Wood, C. M., Wang, Y. X. and Walsh, P. J. (2000). Differential branchial and renal handling of urea, acetamide and thiourea in the gulf toadfish *Opsanus beta*: evidence for two transporters. *J. Exp. Biol.* **203**, 1027-1037.

McDonald, M. D., Wood, C. M., Grosell, M. and Walsh, P. J. (2004). Glucocorticoid receptors are involved in the regulation of pulsatile urea excretion in toadfish. *J. Comp. Physiol. B* **174**, 649-658.

McDonald, M. D., Vulesevic, B., Perry, S. F. and Walsh, P. J. (2009). Urea transporter and glutamine synthetase regulation and localization in gulf toadfish gill. *J. Exp. Biol.* **212**, 704-712.

Milligan, C. L. (2003). A regulatory role for cortisol in muscle glycogen metabolism in rainbow trout *Oncorhynchus mykiss* Walbaum. *J. Exp. Biol.* **206**, 3167-3173.

Mommsen, T. P. and Walsh, P. J. (1991). Metabolic and enzymatic heterogeneity in the liver of the ureogenic teleost *Opsanus beta*. *J. Exp. Biol.* **156**, 407-418.

Mommsen, T. P., Vijayan, M. M. and Moon, T. W. (1999). Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Rev. Fish Biol. Fish.* **9**, 211-268.

Nakada, T., Westhoff, C. M., Kato, A. and Hirose, S. (2007). Ammonia secretion from fish gill depends on a set of Rh glycoproteins. *FASEB J.* **21**, 1067-1074.

Nawata, C. M. and Wood, C. M. (2008). The effects of CO_2 and external buffering on ammonia excretion and Rhesus glycoprotein mRNA expression in rainbow trout. *J. Exp. Biol.* **211**, 3226-3236.

Nawata, C. M. and Wood, C. M. (2009). mRNA expression analysis of the physiological responses to ammonia infusion in rainbow trout. *J. Comp. Physiol. B* **179**, 799-810.

Nawata, C. M., Hung, C. C., Tsui, T. K., Wilson, J. M., Wright, P. A. and Wood, C. M. (2007). Ammonia excretion in rainbow trout (*Oncorhynchus mykiss*): evidence for Rh glycoprotein and H^+-ATPase involvement. *Physiol. Genomics* **31**, 463-474.

Peng, J. and Huang, C. H. (2006). Rh proteins vs Amt proteins: an organismal and phylogenetic perspective on CO_2 and NH_3 gas channels. *Transfus. Clin. Biol.* **13**, 85-94.

Perry, S. F. and Walsh, P. J. (1989). Metabolism of isolated fish gill cells: contribution of epithelial chloride cells. *J. Exp. Biol.* **144**, 507-520.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, e45.

Rahmatullah, M. and Boyde, T. R. (1980). Improvements in the determination of urea using diacetyl monoxime; methods with and without deproteinisation. *Clin. Chim. Acta* **107**, 3-9.

Rodela, T. M., Gilmour, K. M., Walsh, P. J. and McDonald, M. D. (2009a). Cortisol-sensitive urea transport across the gill basolateral membrane of the gulf toadfish (*Opsanus beta*). *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **297**, R313-R322.

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

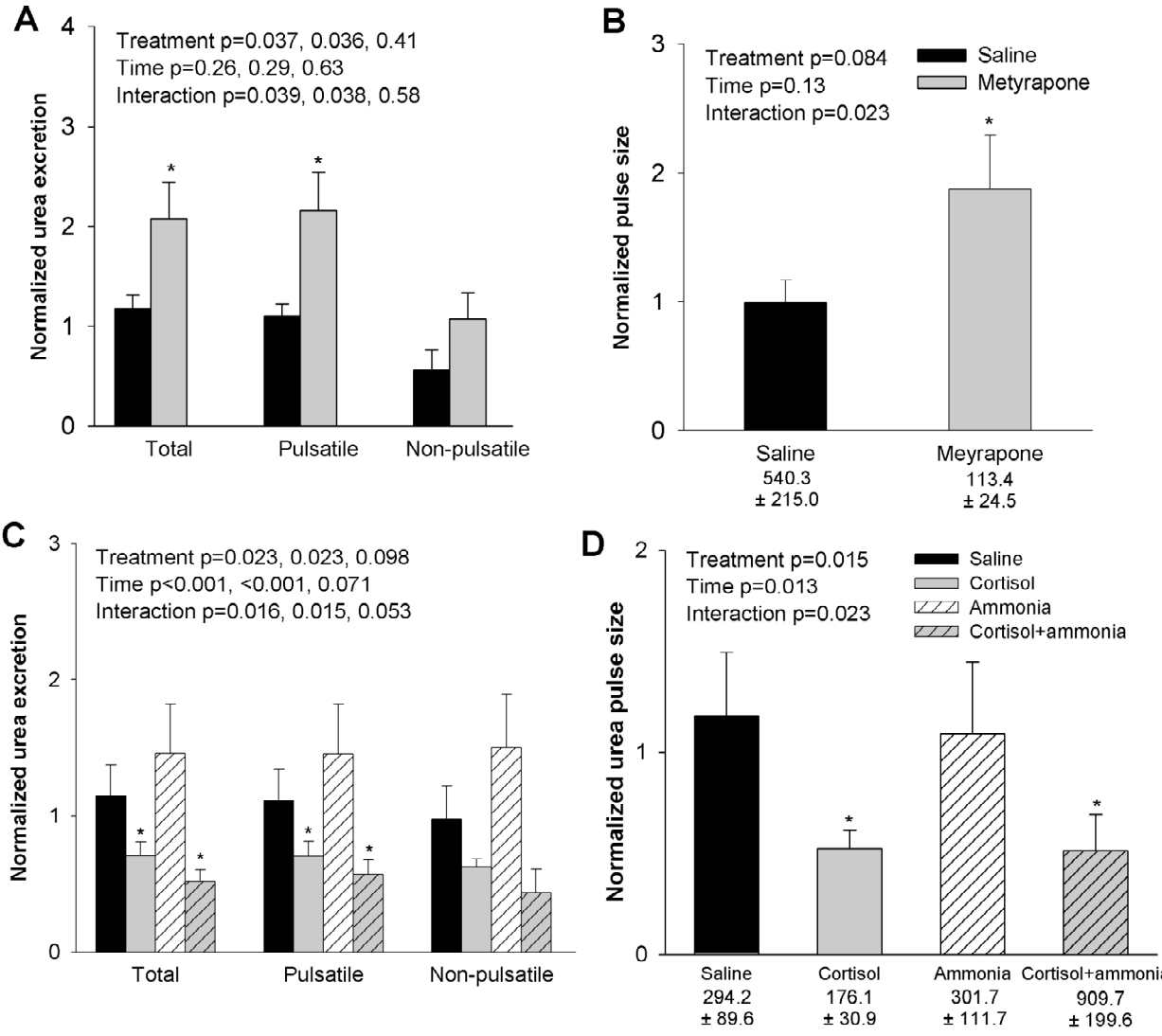
Rodela, T. M., Esbaugh, A. J., McDonald, M. D., Gilmour, K. M. and Walsh, P. J. (2011). Evidence for transcriptional regulation of the urea transporter in the gill of the gulf toadfish, *Opsanus beta*. *Comp. Biochem. Physiol.* **160B**, 72-80.

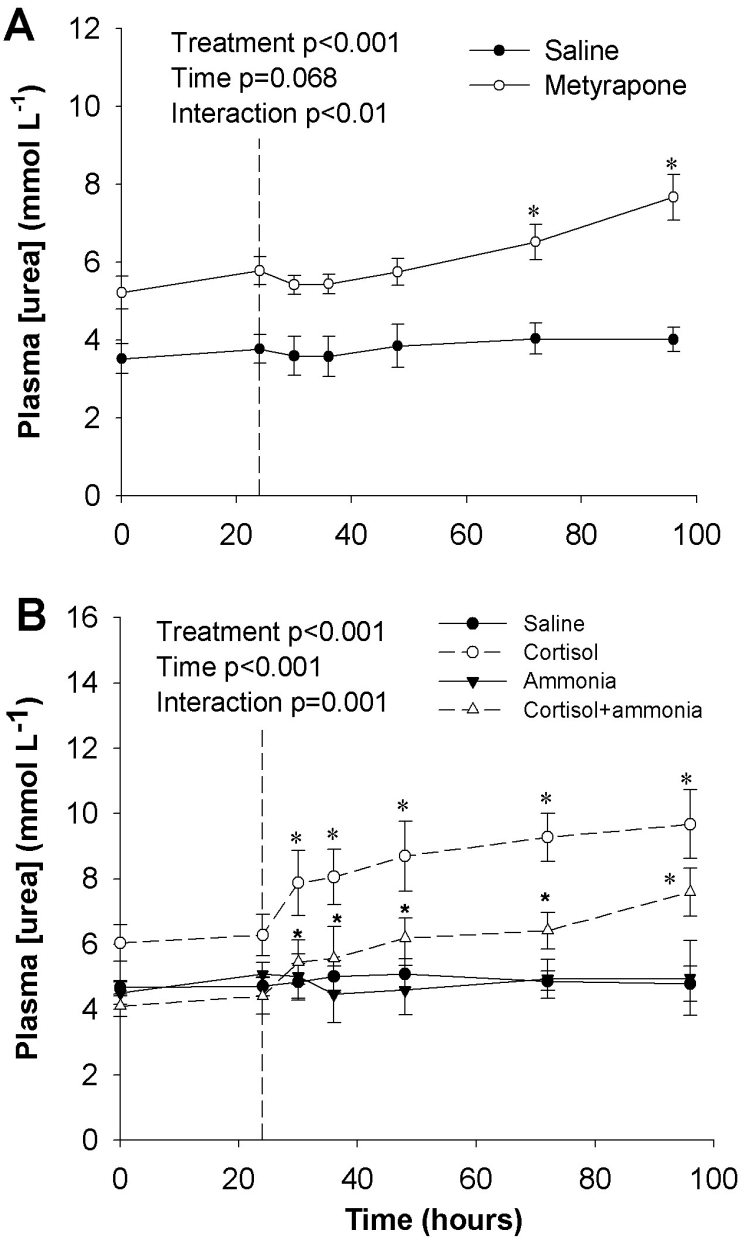
Rodela, T. M., Esbaugh, A. J., Weihrauch, D., Veauvy, C. M., McDonald, M. D., Gilmour, K. M. and Walsh, P. J. (2012). Revisiting the effects of feeding and crowding in the gulf toadfish, *Opsanus beta*: the role of Rhesus glycoproteins in nitrogen metabolism and excretion. *J. Exp. Biol.* **215**, 301-313.

Salama, A., Morgan, I. J. and Wood, C. M. (1999). The linkage between Na^+ uptake and ammonia excretion in rainbow trout: kinetic analysis, the effects of $(NH_4)_2SO_4$ and NH_4HCO_3 infusion and the influence of gill boundary layer pH. *J. Exp. Biol.* **202**, 697-709.

Shih, T. H., Horng, J. L., Hwang, P. P. and Lin, L. Y. (2008). Ammonia excretion by the skin of zebrafish (*Danio rerio*) larvae. *Am. J. Physiol. Cell Physiol.* **295**, C1625-C1632.

Sloman, K. A., McDonald, M. D., Barimo, J. F., Lepage, O., Winberg, S., Wood, C. M. and Walsh, P. J. (2005). Does pulsatile urea excretion serve as a social signal in the gulf toadfish opsanus beta? *Physiol. Biochem. Zool.* **78**, 724-735.
Stoskopf, M. K. (1993). *Fish Medicine.* Philadelphia, PA: Saunders.
Tsui, T. K., Hung, C. Y., Nawata, C. M., Wilson, J. M., Wright, P. A. and Wood, C. M. (2009). Ammonia transport in cultured gill epithelium of freshwater rainbow trout: the importance of Rhesus glycoproteins and the presence of an apical Na^+/NH_4^+ exchange complex. *J. Exp. Biol.* **212**, 878-892.
Veauvy, C. M., Wang, Y. X., Walsh, P. J. and Perez-Pinzon, M. A. (2002). Comparison of the effects of ammonia on brain mitochondrial function in rats and gulf toadfish. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **283**, R598-R603.
Walsh, P. J. (1987). Lactate uptake by toadfish hepatocytes – passive diffusion is sufficient. *J. Exp. Biol.* **130**, 295-304.
Walsh, P. J. (1996). Purification and properties of hepatic glutamine synthetases from the ureotelic gulf toadfish, *Opsanus beta*. *Comp. Biochem. Physiol.* **115B**, 523-532.
Walsh, P. J. (1997). Evolution and regulation of urea synthesis and ureotely in (batrachoidid) fishes. *Annu. Rev. Physiol.* **59**, 299-323.
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.
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., 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., Mayer, G. D., Medina, M., Bernstein, M. L., Barimo, J. F. and Mommsen, T. P. (2003). A second glutamine synthetase gene with expression in the gills of the gulf toadfish (*Opsanus beta*). *J. Exp. Biol.* **206**, 1523-1533.
Wang, Y. X. and Walsh, P. J. (2000). High ammonia tolerance in fishes of the family Batrachoididae (toadfish and midshipmen). *Aquat. Toxicol.* **50**, 205-219.
Weihrauch, D., Wilkie, M. P. and Walsh, P. J. (2009). Ammonia and urea transporters in gills of fish and aquatic crustaceans. *J. Exp. Biol.* **212**, 1716-1730.
Wood, C. M. (1993). *Ammonia and Urea Metabolism and Excretion.* Boca Raton, FL: CRC Press.
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.
Wood, C. M., Warne, J. M., Wang, Y. X., McDonald, M. D., Balment, R. J., Laurent, P. and Walsh, P. J. (2001). Do circulating plasma AVT and/or cortisol levels control pulsatile urea excretion in the gulf toadfish (*Opsanus beta*)? *Comp. Biochem. Physiol.* **129A**, 859-872.
Wood, C. M., McDonald, M. D., Sundin, L., Laurent, P. and Walsh, P. J. (2003). Pulsatile urea excretion in the gulf toadfish: mechanisms and controls. *Comp. Biochem. Physiol.* **136B**, 667-684.
Wright, P. A. and Wood, C. M. (2009). A new paradigm for ammonia excretion in aquatic animals: role of Rhesus (Rh) glycoproteins. *J. Exp. Biol.* **212**, 2303-2312.
Zar, J. H. (1999). *Biostatistical Analysis.* Upper Saddle River, NJ: Prentice Hall.


A
Treatment p=0.037, 0.036, 0.41
Time p=0.26, 0.29, 0.63
Interaction p=0.039, 0.038, 0.58
Normalized urea excretion
Total
Pulsatile
Non-pulsatile
B
Treatment p=0.084
Time p=0.13
Interaction p=0.023
Saline
Metyrapone
Normalized pulse size
Saline
540.3
± 215.0
Meyrapone
113.4
± 24.5
C
Treatment p=0.023, 0.023, 0.098
Time p<0.001, <0.001, 0.071
Interaction p=0.016, 0.015, 0.053
Normalized urea excretion
Total
Pulsatile
Non-pulsatile
D
Treatment p=0.015
Time p=0.013
Interaction p=0.023
Saline
Cortisol
Ammonia
Cortisol+ammonia
Normalized urea pulse size
Saline
294.2
± 89.6
Cortisol
176.1
± 30.9
Ammonia
301.7
± 111.7
Cortisol+ammonia
909.7
± 199.6


A
Treatment p<0.001
Time p=0.068
Interaction p<0.01
Saline
Metyrapone
Plasma [urea] (mmol L-1)
0
2
4
6
8
10
12
0
20
40
60
80
100
B
Treatment p<0.001
Time p<0.001
Interaction p=0.001
Saline
Cortisol
Ammonia
Cortisol+ammonia
Plasma [urea] (mmol L-1)
0
2
4
6
8
10
12
14
16
0
20
40
60
80
100
Time (hours)