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Urea transporter and glutamine synthetase regulation and localization in gulf toadfish gill

M. Danielle McDonald[1,*], Branka Vulesevic[2], Steve F. Perry[2] and Patrick J. Walsh[2]

[1]Rosenstiel School of Marine and Atmospheric Science, University of Miami, 4600 Rickenbacker Causeway, Miami, FL 33149 USA and [2]Department of Biology, University of Ottawa, Ottawa, Ontario, Canada K1N 6N5

*Author for correspondence (e-mail: dmcdonald@rsmas.miami.edu)

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SUMMARY

The goal of the present study was to investigate the role of circulating cortisol and urea in the transcriptional regulation of branchial glutamine synthetase (GS), which incorporates NH_3 into glutamate to form glutamine, and the toadfish urea transporter, tUT, which is involved in urea excretion across the gill of the gulf toadfish. GS (of which there are two isoforms, LGS and GGS) and tUT mRNA expression and activity were measured in toadfish exposed to treatments that would induce variable stress responses. In addition, the role of circulating urea in tUT regulation was investigated by infusing toadfish with urea alone or in combination with intraperitoneal injection of RU486, a corticosteroid type II receptor antagonist. There was a 4.8-fold upregulation in the mRNA expression of the gill-specific GS isoform (GGS) in response to cortisol infusion and a similar upregulation in the more ubiquitous isoform (LGS). Furthermore, there was a significant 1.9-fold and 3.3-fold upregulation in the mRNA expression of the toadfish urea transporter, tUT, in response to stress through crowding or exogenous cortisol loading through infusion, respectively. In addition, tUT was found to have a urea-sensitive component to transcriptional regulation that was independent of circulating cortisol concentrations. However, the changes measured in mRNA expression of GGS, LGS and tUT did not correspond with changes in protein activity. To determine the cell type(s) involved in glutamine production and urea excretion, we attempted to localize GGS, LGS and tUT using *in situ* hybridization. This study is the first to show that GGS and tUT expression appear to occur in gill mitochondria-rich cells of toadfish, suggesting that these cells play a combined glutamine production and urea excretion role, which may have implications for predator avoidance.

Key words: facilitated diffusion, urea production, urea excretion, stress, cortisol, glutamine production, *Opsanus beta*.

INTRODUCTION

The gulf toadfish (*Opsanus beta*) has a complete ornithine-urea cycle (O-UC) allowing it to excrete equal quantities of ammonia and urea when in its natural habitat (Hopkins et al., 1997; Hopkins et al., 1999; Barimo and Walsh, 2005). When removed from nature and placed under stressful conditions in the laboratory (crowding, confinement or exposure to air or ammonia), toadfish switch to excreting predominantly urea (Walsh et al., 1990; Walsh et al., 1994; Walsh and Milligan, 1995) in a pulsatile manner (1–2 distinct events of urea excretion per 24 h) (Wood et al., 1995; Wood et al., 1997). The transition to ureotelism is marked by several events, the first being a surge in circulating levels of the stress hormone cortisol (Walsh et al., 1994; Walsh and Milligan, 1995; Hopkins et al., 1995). Second is the induction of glutamine synthetase (GS) activity in the liver, an important enzyme that shuttles ammonia as glutamine into the piscine O-UC, ultimately resulting in an increase in urea production and circulating urea levels (Walsh et al., 1994; Walsh and Milligan, 1995; Hopkins et al., 1995). The final two events marking the transition to ureotelism involve the reduction in ammonia-N excretion (Walsh and Milligan, 1995) and the initiation of pulsatile excretion of urea-N (Wood et al., 1995; Wood et al., 1997).

A potential factor in reducing branchial ammonia excretion is the existence of two GS isoforms in the toadfish gill: a ubiquitous isoform that was initially purified and characterized from the liver (hence called LGS) (Walsh, 1996) and is found in various tissues throughout the body including the liver and the gill, and a second isoform that, to date, has been found only in the gill (GGS) (Walsh et al., 2003). Although the gill is the only organ in toadfish expressing both GS isoforms, total GS activity in the gill contributes only a small percentage (3.3%) to total body GS activity, suggesting a minor role in terms of whole-body glutamine/urea production (Walsh et al., 2003). However, the multiple GS isoforms in the gill could explain the reduction measured in ammonia-N excretion during the switch to ureotely (Walsh and Milligan, 1995), with these isoforms participating in the shuttling of ammonia into glutamine, greatly reducing the amount of ammonia that crosses the gill during stressful situations. Very little is known about the gill GS isoforms in terms of their cortisol sensitivity or, furthermore, the cell type (i.e. pavement cell or mitochondria-rich cell, MRC) (see Evans et al., 2005) in which either GS isoform is expressed. However, with respect to the latter, it has been speculated that GGS may be found in pavement cells, with LGS in MRCs (Walsh et al., 2003).

Extensive research over the past decade has focused on pulsatile urea excretion in toadfish (reviewed by Wood et al., 2003). Excretion is facilitated by a urea transport protein (tUT) that shows greater than 60% identity at the amino acid level to mammalian UT-A2 facilitated diffusion urea transporters (Smith et al., 1998; Walsh et al., 2000) and it is hypothesized that the pulsatile aspect of urea excretion is due to the periodic insertion or activation of tUT. The cellular location of tUT has not been firmly established although changes in pavement cell morphology and increased vesicular trafficking within these cells during urea pulsing led to speculation that tUT and GGS may be localized mainly in pavement cells (Laurent et al., 2001).

Circulating cortisol is an important regulatory component of tUT function that is not completely understood. While it might be beneficial for the surge in cortisol measured in conjunction with the transition to ureotely to prompt an upregulation in tUT transcription as a way to facilitate urea excretion across the gill, high levels of cortisol have consistently been shown to inhibit tUT function on both an acute and a chronic level (Hopkins et al., 1995; Wood et al., 1997; Wood et al., 2001; McDonald et al., 2004). On the acute time course, urea pulses appear only to occur when the normally elevated circulating cortisol concentrations of ureotelic toadfish periodically drop, suggesting inhibition of tUT function at high cortisol concentrations and the reduction in cortisol acting in a permissive manner for tUT activation (Hopkins et al., 1995; Wood et al., 1997; Wood et al., 2001). On a more chronic time course, continuous cortisol infusion (as a way to prevent the pre-pulse drop) results in a significant reduction in the size of the urea pulse (McDonald et al., 2004). Combined, this evidence suggests that elevations in cortisol may affect the number of functional urea transporters, perhaps through transcriptional or post-transcriptional downregulation; however, regulation of tUT could also be an indirect result of cortisol. As mentioned previously, a surge in cortisol results in the switch to ureotelism and a consequent increase in urea production by the liver. Even when already ureotelic, a further increase in circulating cortisol by infusion results in an additional increase in plasma urea concentrations (McDonald et al., 2004). While urea concentration changes have been ruled out as triggering the actual pulse event (Wood et al., 1997), there is a precedent in the mammalian literature for potential urea effects on the transcription of UT message (Klein et al., 1999).

Therefore, the goal of the present study was to investigate in more detail the potential for transcriptional regulation of GS, involved in the incorporation of ammonia into glutamine, and tUT, involved in urea excretion in the gill of the gulf toadfish. The central hypothesis is that, in response to stress, the toadfish gill exhibits increased expression of GS and tUT so as to conserve ammonia yet excrete urea. An alternative hypothesis is that tUT mRNA expression is actually downregulated by elevated plasma cortisol and/or plasma urea levels. To test these hypotheses, GGS, LGS and tUT mRNA expression and activity were measured in toadfish exposed to treatments to induce variable stress responses. To determine the cell type(s) involved in glutamine production and urea excretion, we attempted to localize GGS, LGS and tUT mRNA expression using *in situ* hybridization. The role of circulating urea in tUT regulation was also investigated by infusing toadfish with urea alone or in combination with intraperitoneal injection of RU486, a corticosteroid type II receptor antagonist (Bertagna et al., 1984; Gaillard et al., 1985) that has been shown to prevent cortisol-induced urea excretion effects in toadfish (McDonald et al., 2004).

MATERIALS AND METHODS

Experimental animals

Gulf toadfish (*Opsanus beta*, Goode and Bean; 0.067±0.004 kg ranging from 0.044 to 0.191 kg, *N*=55) were caught by commercial shrimp fishermen in Biscayne Bay, FL, USA, in the spring and summer of 2004. The toadfish were held in an outdoor tank at the shrimpers' holding facility with running sea water (ambient seasonal conditions) for no longer than 24 h following capture, then transferred to the laboratory. Fish were treated with a dose of malachite green (final concentration 0.05 mg l^{-1}) in formalin (15 mg l^{-1}) (AquaVet, Hayward, CA, USA) on the day of transfer to the laboratory in order to prevent infection by the cilate *Cryptocaryon irritans* (Stoskopf, 1993). Initially the fish were kept in 501 glass aquaria with flowing, aerated seawater (24–26°C) and fed once weekly with previously frozen squid.

Experimental protocol

Series i: endogenous cortisol elevation by crowding

Uncrowded toadfish (*N*=6) were kept individually in large, mesocosm tanks (1 m^3) that simulated the natural toadfish environment, for 1 week prior to sampling. Crowded toadfish (*N*=8) were maintained together in 101 plastic containers for 1 week prior to sampling. After the acclimation period, toadfish were removed from either the mesocosm or the crowding tanks, wrapped in wet paper towels and the blood quickly sampled by caudal puncture. Fish were then anesthetized with a lethal dose of MS-222 (3 g l^{-1}) and gill tissue dissected. Blood samples were centrifuged at 10,000 ***g*** for 1 min and the plasma decanted. Plasma and gill samples were frozen immediately in liquid nitrogen and stored at –80°C for no longer than 1 month before analysis of plasma urea and cortisol, gill tUT and GS mRNA expression, and GS activity.

Series ii: exogenous cortisol loading through arterial infusion

Caudal arterial catheterizations and recovery were performed as described previously (McDonald et al., 2000). In a protocol similar to that described before (McDonald et al., 2004), the arterial catheter was connected to one channel of a Gilson 8-channel peristaltic pump and fish were infused for 48 h with isosmotic NaCl (150 mmol l^{-1}) at an infusion rate of 3 ml kg^{-1} h^{-1}; the rate was checked by periodic measurement of the mass of each individual infusion reservoir. After the 48 h infusion with NaCl, a blood sample was taken. Toadfish were then separated into two treatment groups. Saline-infused fish (mean mass ± s.e.m. 0.065±0.006 kg, *N*=10) continued to be infused with isosmotic NaCl at an infusion rate of 3 ml kg^{-1} h^{-1} for a second 48 h. Fish treated with cortisol (mean mass 0.060±0.003 kg, *N*=10) were infused with cortisol (0.19 mmol l^{-1}; hydrocortisone hemisuccinate salt; Sigma-Aldrich Chemicals, St Louis, MO, USA) in isosmotic NaCl at a rate of 0.56 μmol cortisol kg^{-1} h^{-1} for 48 h. In both groups, after the second 48 h infusion, a blood sample was taken through the arterial catheter; fish were then anesthetized with a lethal dose of MS-222 (3 g l^{-1}) and gill tissue was dissected. Blood samples were centrifuged at 10 000 ***g*** for 1 min and the plasma decanted. Plasma and gill samples were frozen immediately in liquid nitrogen and stored as described above. The concentration of infused cortisol was chosen to raise circulating cortisol levels to approximately 5-fold higher than in a typical ureotelic toadfish; levels which have been shown in a previous study to result in an inhibition of pulsatile urea excretion in toadfish (McDonald et al., 2004).

Series iii: exogenous urea loading through arterial infusion

Arterial catheter implantation was as described above. At the same time, intraperitoneal (IP) catheters (Clay-Adams PE160, Franklin Lakes, NJ, USA) filled with peanut oil were inserted through a small ventral incision and threaded approximately 4 cm inside the body cavity as described by McDonald and Walsh (McDonald and Walsh, 2004). The fish were left to recover undisturbed for 24 h, water flow to the fish box was stopped, set to a known volume and an initial water sample was taken for the measurement of urea concentration. Water samples were continued as described above.

The arterial catheter was connected to one channel of a Gilson 8-channel peristaltic pump and fish were infused for 48 h with an isosmotic load of NaCl (150 mmol l^{-1}) at an infusion rate of 3 ml kg^{-1} h^{-1}. After the 48 h infusion with NaCl, a blood sample was taken and toadfish were then separated into two treatment groups.

Fish treated with urea+peanut oil (mean mass 0.066±0.005 kg, N=10) were infused with urea (100 mmol l^{-1}) in isosmotic NaCl at a rate of 300 μmol urea kg^{-1} h^{-1} for 48 h during which the toadfish were injected through the IP catheter with 0.4 ml of peanut oil starting immediately before the urea infusion. Injections through the IP catheter were repeated every 12 h for the remainder of the experiment while fish were continuously infused with urea. This treatment group served as a vehicle and IP injection control for fish infused with urea+RU486 (mean mass 0.067±0.008 kg, N=11). Fish in this group were infused with urea during which they were injected intraperitoneally with 1.5 mg RU486, a glucocorticoid receptor antagonist (mifepristone, 11β–[4-dimethylamino]phenyl-17β-hydroxy-17[1-propynyl]estra-4,9-dien-3-one; Sigma-Aldrich Chemicals) in 0.1 ml peanut oil followed by 0.3 ml of peanut oil, starting immediately before the urea infusion. In both groups, after the second 48 h infusion, a blood sample was taken through the arterial catheter; fish were then anesthetized with a lethal dose of MS-222 (3 g l^{-1}) and gill tissue was dissected. Blood samples were centrifuged at 10 000 ***g*** for 1 min and the plasma decanted. Plasma and gill samples were frozen immediately in liquid nitrogen and stored as described above.

The concentration of infused urea was chosen to raise circulating urea levels to approximately 5-fold higher than in a typical ureotelic toadfish as described previously (McDonald et al., 2003). The amount of RU486 injected was chosen such that circulating levels of antagonist were 10-fold greater than circulating cortisol concentrations. This concentration had also been shown previously to inhibit a cortisol-induced reduction in urea excretion in toadfish (McDonald et al., 2004). The IP injection of lipophilic compounds (such as RU486) in oil vehicles (i.e. peanut oil, coconut oil, corn oil) mediates the slow release of these substances into the circulation (Vijayan and Leatherland, 1989; Christensen et al., 1999; McDonald et al., 2004).

Quantitative PCR

Total RNA was isolated from whole gill within 1 month of obtaining the sample following the protocol provided with Trizol reagent (Invitrogen, Carlsbad, CA, USA) and treated with DNAse to remove potential residual genomic DNA (TurboDNA-free kit; Ambion, Austin, TX, USA). cDNA synthesis using random hexamers was performed according to the protocol provided with the SuperScript first-strand synthesis system for RT-PCR (Invitrogen). Quantitative PCR (qPCR) was performed for *tUT*, *GGS* and *LGS* (genes of interest; GOI) using a Mx4000 multiple quantitative PCR system (Stratagene, La Jolla, CA, USA) with SYBR Green. For elongation factor 1α (EF1α; forward primer 5′-GTT GGT GTC ATC AAG GCT GTT A-3′, reverse primer 5′-TGA ACT CTG CCT TGA AGA TGA A-3′), tUT (forward primer 5′-CAT CAT CTC CCT CTT CAT CTC C-3′, reverse primer 5′-GTA TCC CCA CAA GCC AAA ATA A-3′), GGS (forward primer 5′-AAA CCC AGG TCA CCT ACA TCT G-3′, reverse primer 5′-GCA CAC TGG GAT GAG GTA CAT A-3′) and LGS (forward primer 5′-TTG AGT AAA GCT GTC AAG AAG CA-3′, reverse primer 5′-AAC CAA GTA CAT GTC GCT GTT G-3′), primers were designed based on published toadfish sequences (GenBank accession nos AF165893, AF532312 and AF118103 for tUT, GGS and LGS, respectively). The amount of cDNA for the GOI was expressed relative to the amount of cDNA from a normalizer gene (*18S* for *tUT* and *GGS*, and *EF1α* for *LGS*). Two different normalizers were used because the abundance of the normalizer gene should be similar to that of the GOI as evidenced by similar *Ct* values. The stability of normalizer gene expression with treatment was tested by comparing normalizer gene expression in fish from different treatment groups, using cDNA obtained from normalized quantities of total RNA as described in Schmittgen and Zakrajsek (Schmittgen and Zakrajsek, 2000). To determine whether the amplification/detection efficiencies of the GOI and the normalizer gene were similar, a standard curve was generated with known quantities of cDNA for the GOIs and the normalizer gene plotted *versus* their *Ct* values. The standard curves of the GOIs and normalizer genes in the present studies gave PCR efficiencies of 100% (tUT), 100% (GGS), 99.8% (LGS), 80.6% (18S) and 74.2% (EF1α). To ensure that the amplification of only one PCR product was contributing to the measured *Ct* value, a dissociation curve was established for each product, which revealed only a single peak signifying only one amplified product. No-template controls were also run to ensure that primer concentrations were optimized and primer-dimers were not contributing to the fluorescence. To verify that the correct product for each primer pair was being amplified, the size of the PCR product for a subset of samples was determined using gel electrophoresis. Gel-extracted PCR products (with Qiaex II from Qiagen, Valencia, CA, USA) were then cloned and amplified in *Escherichia coli* according to the protocol provided with the TOPO TA cloning kit for sequencing using TOP10 chemically competent one shot cells (Invitrogen). The plasmid cDNA was isolated (Qiagen miniprep) and the clones sequenced and identified (Geneway LLC, CA, USA).

Tissue preservation

Gill filaments were removed from freshly dissected gill arches collected from uncrowded and crowded fish as described in series i. The filaments were placed in ice-cold 4% paraformaldehyde (pH 7.4) and kept at 4°C overnight. They were then transferred to phosphate-buffered saline (PBS) containing 15% sucrose for 2 h at 4°C and, finally, transferred to PBS containing 30% sucrose. Tissue samples were embedded in Shandon Cryomatrix embedding medium (Fisher Scientific, Pittsburgh, PA, USA), and sections (10 μm) were prepared using a Leica CM 1850 cryostat at –22°C. Sections were placed on SuperFrost^{++} (Fisher Scientific) microscope slides, air dried for 30 min, and stored at –20°C until use.

Immunocytochemistry

Sections were washed *in situ* (3×5 min) with a washing buffer containing 0.1 mol l^{-1} PBS, and 0.9% Triton X-100. They were then incubated for 2 h at 37°C, in a humidified chamber, with primary antibodies diluted in the buffer: α5, a mouse monoclonal antibody against the α_1-subunit of chicken Na^+/K^+-ATPase (1:100; University of Iowa Hybridoma Bank). For negative controls, sections were incubated with washing buffer lacking primary antibodies. The α5 antibody has been used in numerous previous studies to localize Na^+/K^+-ATPase in fish tissues (e.g. Wilson et al., 2000). The slides were then washed (3×5 min) in 0.1 mol l^{-1} PBS. The α5 antibody was detected with a 1:400 dilution of Alexa 546-coupled goat anti-mouse IgG (Fisher Scientific). Slides were incubated in a humid chamber for 1 h at room temperature. They were then washed (3×5 min) in 0.1 mol l^{-1} PBS and mounted with a mounting medium (Vector Laboratories, Burlingame, MA, USA) with or without 4′,6′-diamidino-2-phenylindole (DAPI) to stain nuclei.

In situ hybridization

For *in situ* studies, digoxigenin-labeled RNA probes were prepared by *in vitro* transcription using linearized plasmid cDNA and SP6 RNA polymerase (for antisense) or T7 RNA polymerase (for sense). To generate a homologous probe for Na^+/K^+-ATPase, primers were

designed against conserved regions of the α_1 subunit. These primers (Na^+/K^+-ATPase forward 5′-TAC TAC CAA GAR GCC AAG AGC T-3′; Na^+/K^+-ATPase reverse 5′-GTT CTG GGT CAG GGT GC-3′) corresponded to nucleotides 487–508 and 1181–1197 of the α_1a isoform of rainbow trout (*Oncorhynchus mykiss*) Na^+/K^+-ATPase (GenBank accession no. AY319391.1). The resultant 587 bp PCR product was ligated into PCR II vector (Invitrogen) and transformed into competent DH5α *E. coli* cells. Purified plasmids were sequenced to confirm that the cloned PCR product was homologous to Na^+/K^+-ATPase. For tUT (forward primer 5′-ATC ACA CGG CAC AAA GG AT-3′, reverse primer 5′-ATG AAC AGC TTG GGC AAA T-3′), GGS (forward primer 5′-CGC TGT TTG GTA CAG ATG GA-3′, reverse primer 5′-GTA CGG GTC ACA GTT TGC AG-3′) and LGS (forward primer 5′-TCT TCC GGA ATG GAA CTT TG-3′, reverse primer 5′-CTT CTC CTG GCC GAC ACT AC-3′), primers were designed based on published toadfish sequences.

These primers were used to amplify selected regions of full-length cDNAs from previously prepared plasmids. The PCR products (tUT 783 bp, GGS 635 bp and LGS 832 bp) were cloned and sequenced as described above. Sections on slides were hydrated (2×15 min) in 1× PBST (PBS with 0.1% Tween 20). Proteinase K (20 μg ml^{-1} in 1× PBST; Gibco-BRL, Orand Island, NY, USA) was used to deproteinate samples for 20 min at room temperature. Following deproteination, samples were fixed in 4% formaldehyde (in PBS) for 5 min. Fixed tissues were subsequently rinsed twice (10 min per wash) with 1× PBST and air dried at 60°C for 15 min.

Probes (approximately 200 ng per reaction) were denatured for 3 min at 94°C in a solution containing 250 μg ml^{-1} salmon sperm DNA, 250 μg poly(A)$^+$, topped up to 12.5 μl with DEPC (diethyl pyrocarbonate) H_2O. Probes were then quickly chilled on ice and centrifuged (7500 ***g***) for 1 min. Hybridization buffer (100 μl of 4× SSC, 20% dextran sulfate, 50% formamide, 250 μg ml^{-1} poly(A)$^+$, 250 μg ml^{-1} ssDNA, 0.1 mol l^{-1} DTT, 250 μg ml^{-1} tRNA, 0.5× Denhardt's solution) was added to each probe. Each probe was then mixed well by vortexing and placed onto sections. Hybridization was performed for 48 h at 57°C in a humid chamber. Following overnight hybridization, sections were washed twice (15 min per wash, 58°C) with 2× SSC and twice (15 min per wash, 58°C) with 0.2× SSC, followed by one wash in 0.1× SSC for 10 min at room temperature and two washes in 0.1× PBS (10 min per wash, room temperature). To detect hybridization, sections were incubated for 1 h at room temperature with 1% goat serum, 2 mg ml^{-1} BSA in 0.1 mol l^{-1} PBS with 0.3% Triton X-100, followed by overnight incubation at 4°C in anti-digoxigenin antibody conjugated to alkaline phosphatase (1:1000 dilution; Roche Molecular Biochemicals, Temecula, CA, USA). Slides were washed at room temperature in 0.1 mol l^{-1} PB for 15 min and then briefly rinsed in water. The slides were next washed twice (5 min per wash) in coloration buffer (100 mmol l^{-1} Tris pH 9.5, 50 mmol l^{-1} $MgCl_2$, 100 mmol l^{-1} NaCl, 0.1% Tween 20). Nitroblue tetrazolium (NBT) and a single 5-bromocresyl-3-indolyl phosphate (BCIP) tablet (Sigma-Aldrich Chemicals) were dissolved in 10 ml of H_2O and layered over the sections. Color was allowed to develop in a humid chamber at room temperature for at least 4 h or until satisfactory coloration was observed. The slides were then washed twice with 0.1 mol l^{-1} PBS (15 min per wash). Coverslips were placed on the slides using 60% glycerol as mounting medium.

Once prepared, all specimens were observed and photographed using a Zeiss Axiophot microscope (Zeiss, Jena, Germany) equipped with a Hamamatsu C5985 chilled CCD camera, using Metamorph imaging software 4.01 (Molecular Devices, Dowingtown, PA, USA).

Assays

Urea concentrations in plasma and water were measured using the diacetyl monoxime method of Rahmatullah and Boyde (Rahmatullah and Boyde, 1980) with appropriate adjustments of reagent strength for the different urea concentration ranges in water and blood plasma. Total urea excretion was measured and calculated as described previously (McDonald et al., 2004). GS activity in gill tissue was measured using the transferase assay as described by Walsh (Walsh, 1996). Plasma cortisol concentrations were measured using a commercial ^{125}I radioimmunoassay kit (MP Biomedical, Solon, OH, USA) with standards diluted to the same protein range as toadfish plasma.

Statistics

Data are reported as means ± 1 s.e.m. (*N*=number of fish). The significance of differences between means was evaluated using Student's unpaired two-tailed *t*-test ($P<0.05$). When data were still not normally distributed upon log transformation, a Mann–Whitney rank sum test was used.

RESULTS

Plasma cortisol concentrations were lowest in fish that were uncrowded in the mesocosm tanks (Fig. 1A). In comparison, fish that were kept at a high density for 1 week had cortisol concentrations that were more than 10-fold higher (Fig. 1A). Saline infusion resulted in cortisol concentrations that were significantly higher than those in uncrowded toadfish but significantly lower than those in fish that were infused with cortisol, which had concentrations that exceeded those of uncrowded fish by approximately 17-fold (Fig. 1A). Fish infused with urea or urea+RU486 had plasma cortisol concentrations that were significantly higher than those of uncrowded fish but were similar to those of crowded and saline-infused fish (Fig. 1A). However, urea-infused fish with or without RU486 treatment had significantly lower cortisol concentrations than fish that were cortisol infused.

Like circulating cortisol concentrations, plasma urea concentrations were also lowest in uncrowded toadfish (Fig. 1B). Urea concentrations were significantly increased in fish that were saline infused, but fish that were just crowded were not different from uncrowded or saline-infused fish. Fish that were infused with cortisol had even higher circulating urea concentrations than those infused with saline and a further increase was measured in fish that were urea infused either with or without RU486 treatment (Fig. 1B).

Total GS activity was significantly higher in all treatment groups compared with that in the uncrowded, control fish, with the greatest elevation being measured in the crowded toadfish (Fig. 2A). The relative expression of GGS mRNA did not show the same level of sensitivity to treatment as GS activity (Fig. 2B). Specifically, GGS mRNA expression in crowded, saline-infused or urea-infused ±RU486 fish was not significantly different from that measured in uncrowded fish (Fig. 2B). However, a 4.8-fold elevation in GGS expression was measured in fish that were infused with cortisol (Fig. 2B). The mRNA expression of the LGS isoform showed slightly more sensitivity to treatment than that of the GGS isoform. Similar to GGS, crowding had no effect on LGS mRNA expression; however, saline infusion resulted in a significant 4.7-fold increase in LGS mRNA expression, which remained elevated in the other treatments (Fig. 2C). Nonetheless, a rough comparison of normalized GGS and LGS transcript abundance indicates that the GGS isoform was approximately 20-fold more abundant in the gill under all conditions tested than the LGS isoform (data not shown).

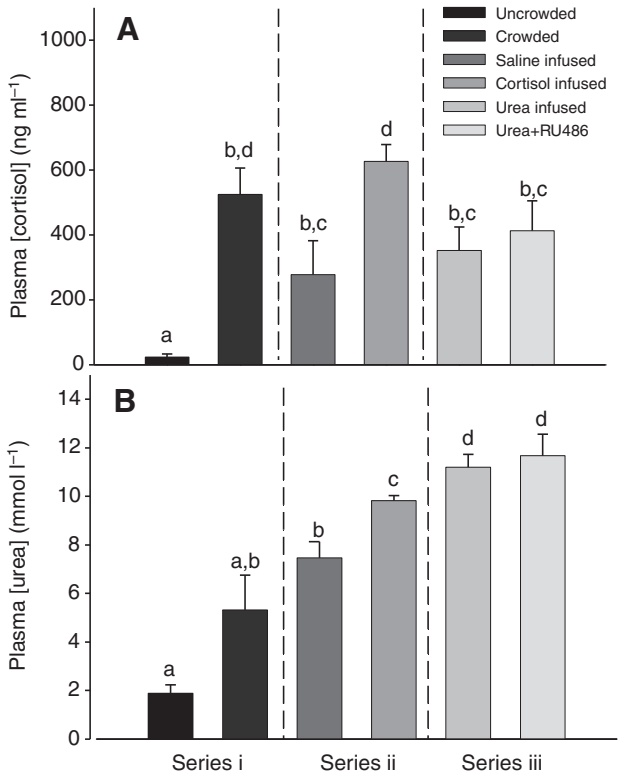

Fig. 1. (A) Plasma cortisol concentrations and (B) plasma urea concentrations in toadfish that were relatively unstressed (uncrowded) compared with those under different experimental treatments. Values are means + 1 s.e.m. Different letters depicted significant difference, $P<0.05$.

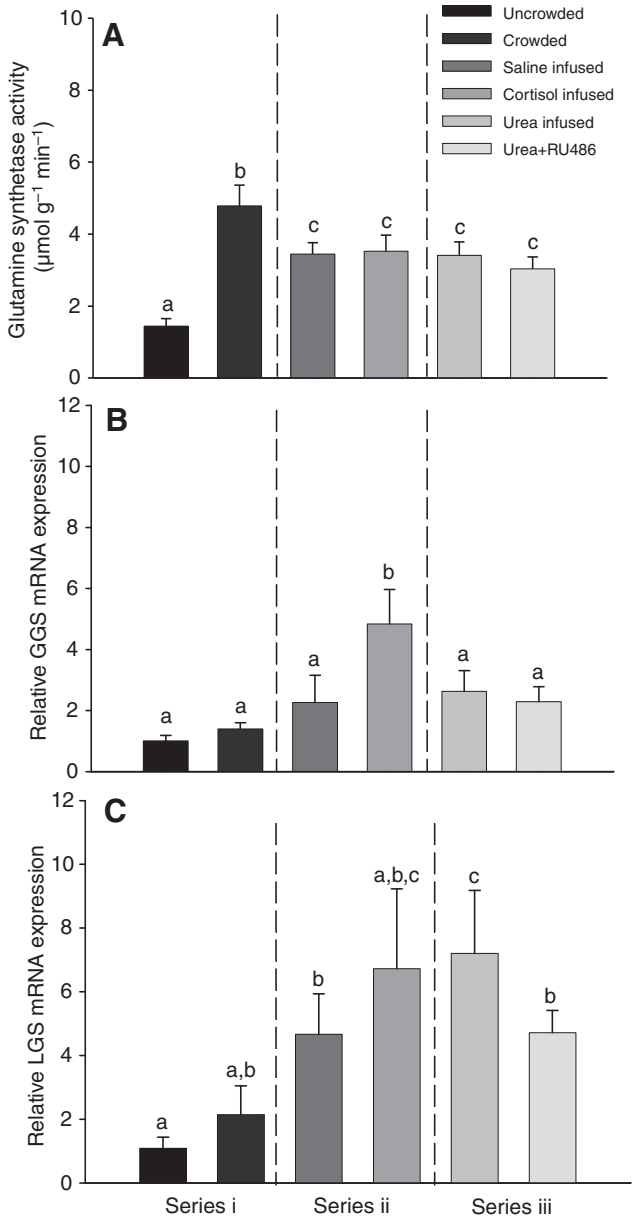

Fig. 2. (A) Total glutamine synthetase (GS) activity, (B) relative mRNA expression of the gill-specific GS isoform (GGS) and (C) relative mRNA expression of the ubiquitous isoform (LGS). Notably, treatments that resulted in changes in enzyme activity do not correspond to mRNA expression changes in either GS isoform. Values are means + 1 s.e.m. Different letters depict significant difference, $P<0.05$.

The relative expression of tUT mRNA showed a 1.9-fold and 1.7-fold elevation in crowded and saline-infused fish, respectively, compared with fish that were held in uncrowded conditions (Fig. 3). Fish that were cortisol infused had tUT mRNA levels that were 3.3-fold higher than those of uncrowded fish and significantly greater than those of both crowded and saline-infused fish. Interestingly, a 6.0-fold increase was seen in urea-infused fish compared with uncrowded fish, which was reduced to a 3.2-fold increase in fish that were treated with urea+RU486 (Fig. 3).

Despite having higher levels of tUT mRNA expression, fish that were infused with cortisol had significantly lower rates of urea excretion than fish infused with saline alone (Fig. 4A cf. Fig. 3). Along the same lines, fish infused with urea+RU486 treatment showed an elevation in urea excretion compared with the 48 h control NaCl infusion that was similar to that in fish infused with urea alone, despite RU486-treated fish having lower tUT mRNA expression levels than fish infused with urea alone (Fig. 4B cf. Fig. 3).

Based on the results of immunocytochemistry and *in situ* hybridization (Fig. 5), it was apparent that the MRCs (identified on the basis of Na^+/K^+-ATPase enrichment) of the gill epithelium typically were scattered along the lamellae (Fig. 5A,B). Notably, in a single fish, it was observed that the MRCs were specifically localized to the interlamellar regions within the filament epithelium. There was no obvious effect of holding conditions (crowded *versus* non-crowded) on the pattern of MRC distribution. The distribution pattern of GGS and tUT mRNA was similar to that of Na^+/K^+-ATPase, suggesting their exclusive localization to the MRCs (Fig. 5C,D). Positive staining for LGS was undetectable.

DISCUSSION

The present study is the first to demonstrate that toadfish experience an upregulation in the mRNA expression of branchial GS (both GGS and LGS isoforms) and the toadfish urea transporter, tUT, in response to crowding or exogenous cortisol loading through infusion. Furthermore, tUT appears to have a 'urea-sensitive' component to transcriptional regulation as well. GGS and tUT expression appear to occur in gill MRCs, suggesting that these cells play a combined glutamine production/ammonia sequestration and urea excretion role, while LGS was found in low transcript levels in the gill by qPCR and was undetectable with *in situ* hybridization.

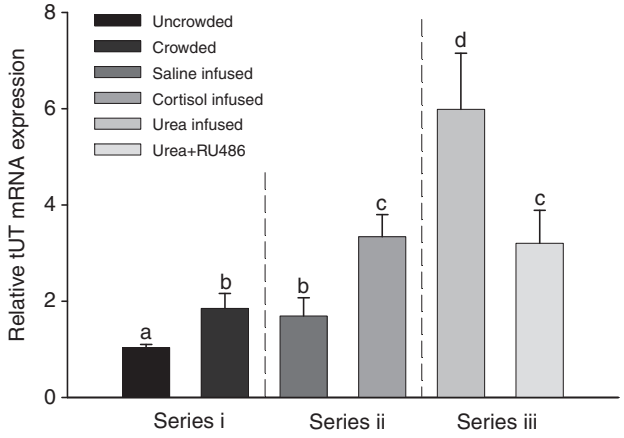

Fig. 3. Relative tUT mRNA expression in gill of toadfish that are unstressed (uncrowded) compared to those under different experimental treatments. In addition to elevations in tUT mRNA expression observed in cortisol-infused fish, the greatest elevation in expression was measured in fish that were infused with urea. Values are means + 1 s.e.m. Different letters depicted significant difference, $P<0.05$.

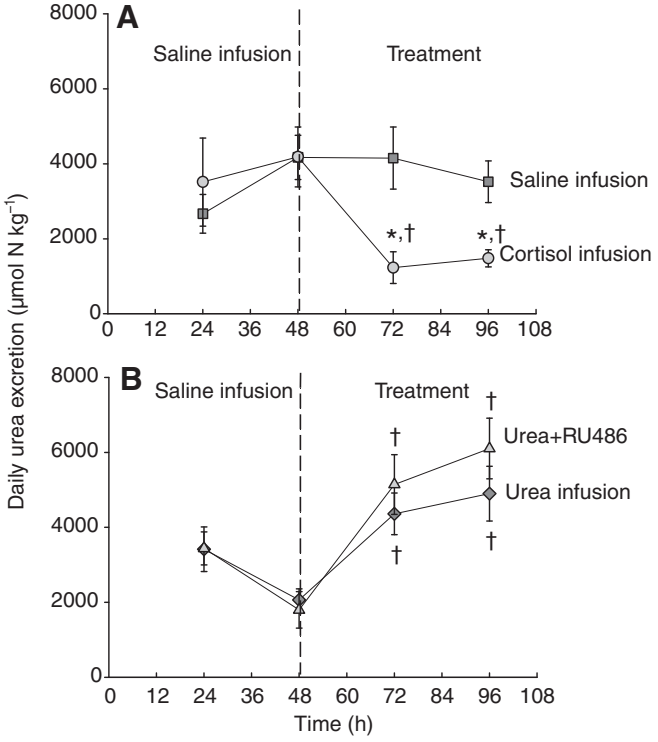

Fig. 4. (A) Daily excretion of urea in toadfish that were saline infused for an initial 48 h control period and were then switched to being infused either with saline or with cortisol for a subsequent 48 h. A significant decrease in excretion was measured in cortisol-treated fish compared with their urea excretion values measured during the control period and compared with saline-infused fish at the same time point. (B) Daily excretion of urea in toadfish that were saline infused for an initial 48 h control period and were then switched to being infused with either urea alone or urea+RU486. Both groups showed a significant elevation in urea excretion compared with the initial saline control period; however, the treatments were not significantly different from one another. Values are means ± 1 s.e.m.; *$P<0.05$, significantly different from corresponding treatment group; †$P<0.05$, significantly different from control infusion period.

Gill GS activity and mRNA expression

An endogenous elevation in circulating cortisol levels in response to crowding under laboratory conditions has been shown to result in a significant increase in the activity and mRNA expression of GS in the liver of toadfish (Hopkins et al., 1995; Walsh et al., 2003). In correspondence with these documented changes in urea production by the liver, toadfish in the present study showed a significant elevation in the total GS activity within the gill (a measure that includes the activity of both LGS and GGS isoforms). Sensitivity of non-hepatic GS to cortisol has been demonstrated in tilapia gastrointestinal tract, stomach and muscle (Mommsen et al., 2003) as well as in mammalian astrocytes (O'Banion et al., 1994) and intestine (Sarantos et al., 1994). In toadfish, the increase in gill total GS activity in response to crowding alone is in contrast to previous findings, which showed no change (Walsh et al., 2003); however, the inconsistency between the two studies could be explained by protocol differences; toadfish in the previous study were only crowded for 48 h (and levels of stress were not estimated by measurement of cortisol levels) compared with the present 1 week crowding protocol. While crowding resulted in an increase in the total GS activity in the gill, there was no corresponding increase in the mRNA expression of either LGS or GGS with crowding. Induction of the GS enzyme without changes in transcription has been measured in the mammalian jejunum; in this case it was postulated that glucocorticoids increased GS levels by accelerating protein translation (Sarantos et al., 1994).

In contrast to fish that were simply crowded, an increase in the transcription of both LGS and GGS isoforms was measured in fish that were infused with saline or cortisol, respectively. This result is unlikely to be explained by measured differences in plasma cortisol concentrations amongst the three groups (the crowded and cortisol-treated fish had similar levels of circulating cortisol). The upregulation of LGS mRNA expression could be in response to the volume loading experienced by infused fish; however, this would not explain the upregulation measured in GGS, which appears only to be sensitive to cortisol infusion. Another difference between crowded, saline-infused and cortisol-infused fish is the stressor involved; the social stress experienced by crowded fish is very intense and, in addition to cortisol elevation, could result in changes in many other physiological parameters that may interfere with the changes in GGS gene expression measured in fish infused with cortisol alone (Sloman et al., 2005). It also cannot be ruled out that the varied exposure to different acute stressors experienced by saline- and cortisol-infused fish (i.e. surgery) may have resulted in periods of much greater cortisol levels that were not captured by the single post-infusion blood sample. Nevertheless, the pattern of transcriptional upregulation measured in GGS and LGS suggests that a more chronic stressor may be required to increase GGS transcription than is necessary for the upregulation of LGS. Interestingly, total GS enzyme activity was not different between saline- and cortisol-infused fish, which is reflected in similar branchial ammonia excretion rates between the two groups (data not shown); however, this may be a consequence of relatively small transcriptional changes not translating to detectable changes in protein activity.

The significantly higher GGS compared with LGS transcript levels suggests that GGS probably makes up a greater proportion of the total GS activity of the gill, providing functional significance to this gill-specific isoform and putting into context the differences measured in the apparent cortisol sensitivity of the two isoforms. Walsh and colleagues (Walsh et al., 2003) determined that the

cellular compartmentation of GGS differs from that of LGS, because GGS is missing the mitochondrial leader sequence that would target it to the mitrochondrial compartment, and it had an exclusively soluble/cytosolic distribution, potentially increasing its direct contact with ammonia. The demonstrated cytosolic location of GGS combined with the higher transcript levels of GGS compared with those of LGS in the gill supports an ammonia-trapping function of the gill itself that differs from the function of GS in other organs. That higher cortisol concentrations are required to increase GGS transcription than are necessary for the upregulation of LGS suggests that glutamine production may occur secondary to the increase in urea production in response to stress by liver-bound LGS, as the pattern of mRNA expression measured in LGS probably reflects what is going on in the liver. Gill-bound LGS probably does not play a major role in gill glutamine production as indicated by the low transcript levels and lack of LGS signal using *in situ* hybridization.

tUT activity and mRNA expression

The findings of the present study suggest that both cortisol and urea have potential regulatory effects on toadfish gill tUT activity and mRNA expression. Previous studies investigating the role of cortisol in the regulation of toadfish pulsatile urea excretion have consistently demonstrated an inhibitory effect of both acute and chronic elevations of cortisol on tUT function (Hopkins et al., 1995; Wood et al., 1997; Wood et al., 2001; McDonald et al., 2004). These past results were further supported by the present study, in which exogenous cortisol loading through infusion resulted in a significant decrease in urea excretion, suggesting a potential downregulation of tUT mRNA expression or function. A decrease in mRNA abundance in response to glucocorticoid treatment was measured in several mammalian facilitated diffusion urea transporters, namely UT-A1, UT-A3 and UT-A3b found in the inner medullary collecting duct (IMCD); glucocorticoids suppressing the activity *via* the promoter region responsible for transcription (Knepper et al., 1975; Naruse et al., 1997; Peng et al., 2002). However, Peng and colleagues (Peng et al., 2002) did not find any change in the transcription of UT-A2 in response to glucocorticoids, which is the mammalian isoform that most closely resembles toadfish tUT (Walsh et al., 2000). In contrast to the hypothesized downregulation or potential insensitivity measured by Peng and colleagues (Peng et al., 2002), the mRNA abundance of tUT in fish with either endogenous (through crowding) or exogenous (through infusion) elevations in cortisol was significantly increased compared with that of uncrowded fish, revealing a clear distinction between the transcription of tUT and the capacity of the fish to excrete urea, a measure of tUT protein function.

An increase in tUT mRNA abundance in association with the surge in cortisol during the transition to ureotely would be adaptive for timing the increase in urea production with an increased ability to excrete urea across the gill. What has become apparent, however, is that cortisol may have two roles in tUT regulation (Hopkins et al., 1995; Wood et al., 1997; Wood et al., 2001; McDonald et al., 2004). It appears that a chronic elevation in circulating cortisol serves to increase tUT mRNA expression, which may in fact allow more urea transporter to be translated. However, elevated cortisol levels measured in ureotelic toadfish also appear to prevent urea from being excreted. When circulating cortisol concentrations periodically drop, a pulse of urea occurs, suggesting that the cortisol drop is permissive to the post-transcriptional modification of tUT (Hopkins et al., 1995; Wood et al., 1997; Wood et al., 2001). Without the periodic, natural drop in cortisol, as observed in toadfish infused with cortisol, the newly transcribed tUTs might never be utilized. Alternatively, maybe the increase in tUT mRNA abundance is simply a (failed) attempt to offset an increased degradation of tUT protein that may be occurring in response to elevated cortisol concentrations, as suggested by Kong and colleagues (Kong et al., 2000) in the case of toadfish carbamoyl phosphate synthetase III. Then again, there could be a second urea transporter in the gill, as evidenced by Walsh and colleagues (Walsh et al., 2000) using northern analysis, that responds to cortisol with a downregulation in transcription which overwhelms the upregulation measured in tUT, resulting in the measured overall decrease in urea excretion.

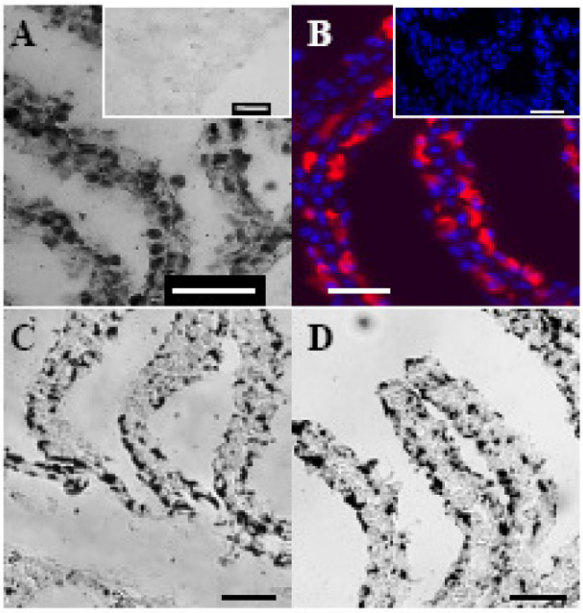

Fig. 5. The pattern of Na^+/K^+-ATPase (A,B), GGS (C) and tUT (D) localization in the toadfish gill whether by *in situ* hybridization (A,C,D) or immunocytochemistry (B), demonstrating Na^+/K^+-ATPase-positive mitochondria-rich cells (MRCs) scattered along the lamellae (A,B). The insets in A and B represent a no-probe negative control and a negative control in which primary antibody was omitted, respectively. GGS (C) and tUT mRNA (D) showed a similar pattern of staining to Na^+/K^+-ATPase, scattered along the lamellae and within the interlamellar regions. The scale bars indicate 50 μm.

The differing transcriptional responses of tUT and the closely related mammalian UT-A2 isoform to cortisol begged the question of whether cortisol was acting directly on tUT or whether its apparent sensitivity to cortisol was instead due to the elevation in circulating urea concentrations that is often measured in stressed toadfish and other teleosts (Vijayan et al., 1996; McDonald and Wood, 2004). An upregulation of mammalian UT-A protein in response to uremia, a pathological condition resulting in higher circulating urea concentrations, was originally documented in rats subjected to nephrectomy (Klein et al., 1999). However, a later study by Klein and colleagues (Klein et al., 2002) determined that it was the acidosis that occurred in response to nephrectomy that directly resulted in the increase in UT-A2 abundance and not the elevation in urea concentrations that was a byproduct of the acidosis (Klein et al., 2002). In the present study, there did appear to be a urea-sensitive component to tUT mRNA expression; however, blood pH in toadfish infused with urea was not measured and thus it cannot be conclusively ruled out that acidosis was a contributing factor.

An increased expression of UT-A2 transporters has also been shown to occur in response to the hydration state of the animal (Smith et al., 1995), with the increase in transcription resulting from the activation of the UT-A promoter by cAMP-dependent pathways (Nakayama et al., 2001). Changes in hydration status based on the volume loading experienced by both cortisol-infused and urea-infused fish would not explain the significant difference in tUT transcription measured between these two groups. Furthermore, a significant difference in tUT mRNA expression is not observed between crowded toadfish and those that are saline infused, two groups that have similar plasma cortisol and urea levels but differ in that the latter is volume loaded. Thus, it appears that the hydration status of toadfish is probably not a regulatory component of tUT transcription.

In situ hybridization and colocalization of GGS and tUT

Because of the lack of a mitochondrial leader sequence in GGS, Walsh and colleagues (Walsh et al., 2003) speculated that the gill and ubiquitous forms of GS might be expressed in different cell types; GGS in pavement cells and LGS in MRCs. In marked contrast to their speculation, GGS showed a similar pattern of staining to Na^+/K^+-ATPase, which is expressed in MRCs, while we were unable to determine the location of LGS, probably due to its markedly lower transcript abundance as demonstrated by qPCR measurements. Similar to GGS, and in contrast to speculation by Laurent and colleagues (Laurent et al., 2001), tUT also showed a similar pattern of staining to Na^+/K^+-ATPase, suggesting MRC localization. Changes in pavement cell morphology and increased vesicular trafficking during urea pulsing suggested that tUT may be found in gill pavement cells (Laurent et al., 2001). However, the present findings are in agreement with immunohistochemistry on a largely ammoniotelic species, the Japanese eel (*Anguilla japonica*) urea transporter (eUT), which was localized to the basolateral membrane of MRCs (Mistry et al., 2001). Thus, the toadfish MRCs express both GGS and tUT and these cells probably have a combined function to reduce ammonia excretion by producing glutamine while at the same time excreting urea in times of stress. Interestingly, the elaboration of the pavement cells suggests excretion of an electron-dense material to the apical surface *via* vesicles (Laurent et al., 2001) and, in the light of recent behavioral studies in toadfish (Sloman et al., 2005; Barimo and Walsh, 2006), it would be of interest to examine these materials for molecules involved in communication that might accompany urea and ammonia excretion as regulated by the chloride cells.

Recent evidence has outlined an important role for the combined reduction of ammonia excretion and elevation in urea excretion in toadfish survival. Toadfish have been shown to excrete a combination of urea and ammonia in the wild (Hopkins et al., 1997; Hopkins et al., 1999). A recent study has revealed ammonia waste to be an important chemical attractant in the aquatic environment which becomes undetectable if excreted in combination with urea (Barimo and Walsh, 2006). Having the combined control to upregulate urea-N production in the liver, and upregulate urea-N excretion across the gill while decreasing ammonia-N excretion across the gill when under stressful conditions ensures that the fish will be excreting the appropriate mix of ammonia:urea as a predator-avoidance tactic. When under conditions of very high stress, such as in a laboratory environment, evidence shows that fish almost shut down ammonia excretion entirely, becoming predominantly ureotelic (Walsh, 1997; Wood et al., 1995; Wood et al., 1997, Wood et al., 1998; Wood et al., 2001). In terms of predator avoidance where a natural analog to the high stress laboratory model might be a fish under repeated attack by predators, this 'full ureotely' response is not disadvantageous; Barimo and Walsh (Barimo and Walsh, 2006) did not find a significant difference in the ability of ammonia+urea or urea alone to attract/avoid predators.

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