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RESEARCH ARTICLE

Revisiting the effects of crowding and feeding in the gulf toadfish, *Opsanus beta*: the role of Rhesus glycoproteins in nitrogen metabolism and excretion

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SUMMARY

Models of branchial transport in teleosts have been reshaped by the recent discovery of Rhesus (Rh) glycoproteins, a family of proteins that facilitate the movement of NH_3 across cell membranes. This study examines the effects of crowding and feeding on ammonia excretion in gulf toadfish (*Opsanus beta*) within the context of Rh glycoproteins and the ammonia-fixing enzyme, glutamine synthetase (GS). Four Rh isoforms (Rhag, Rhbg, Rhcg1 and Rhcg2) were isolated from toadfish. Tissue distributions showed higher levels of mRNA expression in the gills and liver, moderate levels in the intestine and lower levels in the stomach. Crowding significantly lowered branchial Rh expression and ammonia excretion rates in fasted toadfish. A comparison of Rh expression in the digestive tract revealed relatively low levels of Rhcg1 and Rhcg2 in the stomach and high mRNA abundance of Rhbg, Rhcg1 and Rhcg2 in the intestine of fasted, crowded toadfish. We speculate that these trends may reduce secretion and enhance absorption, respectively, to minimize the amount of ammonia that is lost through gastrointestinal routes. By contrast, these patterns of expression were modified in response to an exogenous ammonia load *via* feeding. Post-prandial ammonia excretion rates were elevated twofold, paralleled by similar increases in branchial Rhcg1 mRNA, gastric Rhcg1 mRNA and mRNA of all intestinal Rh isoforms. These changes were interpreted as an attempt to increase post-prandial ammonia excretion rates into the environment owing to a gradient created by elevated circulating ammonia concentrations and acidification of the digestive tract. Overall, we provide evidence that toadfish modulate both the expression of Rh isoforms and urea synthesis pathways to tightly control and regulate nitrogen excretion.

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

INTRODUCTION

The deamination of excess amino acids leads to the production of ammonia, a highly toxic compound that must either be excreted or converted to less toxic products for the maintenance of normal physiological function. In contrast to the majority of adult teleost fish that dispose of their nitrogenous waste predominantly as ammonia [the sum of NH_3 and NH_4^+ (Wood, 1993)], the gulf toadfish, *Opsanus beta* (Goode and Bean), can facultatively change its nitrogen excretion pattern (the partitioning of ammonia and urea) across the gills in response to environmental conditions. Toadfish are ammoniotelic under conditions of minimal disturbance in the laboratory (Walsh and Milligan, 1995). However, when subjected to stressors (e.g. crowding, confinement, emersion and high ambient ammonia), gulf toadfish predominantly excrete urea (Walsh et al., 1990; Walsh et al., 1994; Wood et al., 1997). Crucial to understanding this facultative ureotelism is that the transition involves a significant reduction in ammonia excretion coupled with an elevation of urea excretion (Walsh et al., 1994; Walsh and Milligan, 1995). Ammonia excretion is nearly eliminated in ureotelic toadfish; the absolute rates are relatively low even for fasted teleosts (Wood et al., 2003). This is remarkable considering that continued ventilation and perfusion of the gills with blood ($[ammonia]_{plasma} \approx 200\,\mu mol\,l^{-1}$) maintains a substantial, outwardly directed NH_3 partial pressure gradient (Wang and Walsh, 2000).

The reduction in branchial ammonia excretion in ureotelic toadfish may be explained (in part) by the upregulation of glutamine synthetase (GS), an enzyme responsible for the conversion of ammonia to glutamine (Walsh, 1996; Walsh et al., 1999; Walsh et al., 2003). GS activities increase twofold and fivefold in muscle and liver, respectively, accompanied by comparable increases in protein and mRNA levels (Walsh et al., 1994; Walsh and Milligan, 1995; Hopkins et al., 1995; Wood et al., 1995; Walsh et al., 1999; Kong et al., 2000; Esbaugh and Walsh, 2009; McDonald et al., 2009). Induction of this ubiquitous enzyme increases the production of urea, and also helps maintain plasma ammonia levels at pre-ureotelic values (Wood et al., 2003). A second GS isoform is exclusively expressed at the gill and is thought to play a role in trapping ammonia to minimize its leakage across the gill (Wood et al., 1995; Walsh et al., 2003). Calculations by Walsh (Walsh, 1997) suggested that there is sufficient gill GS activity to support this mechanism. However, a recent study by McDonald and colleagues (McDonald et al., 2009) demonstrated that gill GS expression is

limited to the mitochondrion-rich cells (MRCs), a cell type that represents a relatively small proportion (~10%) of the total gill cell population (Perry and Walsh, 1989). These results suggest a need for re-evaluation of gill ammonia transport mechanisms, with particular emphasis on comparisons between ammoniotelic and ureotelic toadfish.

The prevailing view that the ammonia permeability of a membrane was due to the passive diffusion of NH_3 and carrier-mediated exchange of NH_4^+ has changed since the discovery of Rhesus (Rh) glycoproteins as gas channels. Members of the ammonia transport (AMT)/methylammonium permease (MEP)/Rh glycoprotein superfamily facilitate the movement of NH_3 across the plasma membrane (Marini et al., 1997; Peng and Huang, 2006; Gruswitz et al., 2010). In mammals, three isoforms have been shown to transport ammonia: Rh-associated glycoprotein (RhAG), Rh type B glycoprotein (RhBG) and Rh type C glycoprotein (RhCG) (Marini et al., 2000; Westhoff et al., 2002; Nakhoul et al., 2005). Similar Rh-like sequences were found in Japanese pufferfish (*Takifugu rubripes*) and green spotted pufferfish (*Tetraodon nigroviridis*) (Huang and Peng, 2005), rainbow trout (*Oncorhynchus mykiss*) (Nawata et al., 2007), zebrafish (*Danio rerio*) (Nakada et al., 2007; Braun et al., 2009) and mangrove killifish (*Kryptolebias marmoratus*) (Hung et al., 2007), suggesting that these piscine homologs may play a fundamental role in the movement of ammonia in fish. This new paradigm is being incorporated into the teleost model of ammonia excretion. In freshwater fish, the trans-branchial NH_3 gradient is maintained by acidification of the gill boundary layer, a microenvironment created by the accumulation of H^+ from the combined activity of H^+-ATPases and Na^+/H^+ exchange protein(s) and the hydration of excreted CO_2 (Wright et al., 1986; Wright et al., 1989; Playle and Wood, 1989; Lin and Randall, 1990; Lin et al., 1994; Wright and Wood, 2009). As NH_3 diffuses across the gill and boundary layer, it is trapped as NH_4^+ (Wright et al., 1986; Wright et al., 1989). The identification and characterization of Rhag, Rhbg, and splice variants Rhcg1 and Rhcg2 in the gill of rainbow trout has established a role for Rh glycoproteins as NH_3 conduits in this freshwater model (Nawata et al., 2007; Nawata and Wood, 2009; Nawata et al., 2010a) (reviewed by Wright and Wood, 2009). Less is known about mechanisms of branchial ammonia transport in marine teleosts. However, evidence indicates that the movement of ammonia occurs down favourable blood-to-water diffusion gradients, and relies on a combination of paracellular NH_4^+ diffusion, basolateral Na^+/NH_4^+ exchange and transcellular NH_3 diffusion (reviewed by Wilkie, 2002). In gulf toadfish, the transcellular movement of NH_4^+ and NH_3 was reported to account for approximately 79% of the total ammonia that is excreted (Evans et al., 1989). Recently, the differential distribution and cellular polarization of several Rh proteins in the branchial epithelium of pufferfish (*T. rubripes*) was established and it was confirmed that these proteins can transport an ammonia analog, methylammonium (Nakada et al., 2007; Nawata et al., 2010b).

The goal of this study was to re-examine the effects of feeding and crowding and confinement in toadfish [studied previously by Walsh and Milligan (Walsh and Milligan, 1995)] within the new paradigm of Rh glycoprotein-mediated ammonia transport. We hypothesized that Rh glyoproteins contribute to toadfish ammonia excretion and, in particular, that unconfined (ammoniotelic) fish would exhibit higher gill Rh expression than crowded (ureotelic) individuals. Furthermore, as feeding is associated with increases in both plasma ammonia and ammonia excretion in ammoniotelic teleosts (Brett and Zala, 1975; Kaushik and Teles, 1985; Alsop and Wood, 1997; Wicks and Randall, 2002; Bucking and Wood, 2008) and has, more recently, been shown to induce Rhcg2 mRNA expression (Zimmer et al., 2010), we anticipated that feeding would differentially modulate Rh expression in the gill and along the gastrointestinal tract in unconfined and crowded toadfish.

MATERIALS AND METHODS

Animals

Gulf toadfish were caught by roller trawl in Biscayne Bay, Florida, by commercial shrimpers during January and February 2009. Toadfish were immediately transferred to the laboratory and given a freshwater dip (2 min) followed by a Malachite-Green–formalin treatment (final concentrations 0.05 mg l^{-1} Malachite Green, 15 mg l^{-1} formalin; Aquavet, Hayward, CA, USA) to prevent infection by the ciliate *Cryptocaryon irritans* (Stoskopf, 1993; Wood et al., 1997). Fish were acclimated to the laboratory for at least 1 week in 50 l glass aquaria supplied with flowing, aerated seawater (18–22°C, pH 8.1) and kept under a natural photoperiod. Initially, toadfish were kept at a stocking density of less than 10 fish per tank and fed chopped squid once a week.

Experimental protocol

'Unconfined' toadfish (N=14) were kept individually in 13 l plastic containers for 1 week prior to experimental manipulation. A second group of fish ('crowded'; N=15) was subjected to a standard crowding protocol (Walsh, 1987; Hopkins et al., 1995a; Wood et al., 1997a) wherein seven to eight fish were held in 6 l plastic tubs for 48 h to induce ureotely. All fish were deprived of food to eliminate feeding effects on nitrogen metabolism and excretion for at least 48 h prior to surgery and experimentation. Following the acclimation period, fish were anesthetized with MS-222 (1 g l^{-1} buffered with 2 g l^{-1} sodium bicarbonate, pH 7.8) and fitted with caudal artery catheters (Wood et al., 1997a; McDonald et al., 2000). Post-surgery, unconfined and crowded toadfish were transferred to individual 13 and 1.5 l flux chambers, respectively. Chambers were supplied with flowing, aerated seawater and the fish were allowed to recover for 24 h.

An initial 200 µl blood sample was taken from each fish *via* the caudal artery catheter. The blood was centrifuged at 10,000 ***g*** for 1 min, and an 80 µl aliquot of plasma was removed and flash frozen in liquid nitrogen for later analysis of plasma ammonia, urea and cortisol concentrations. The red blood cells (RBCs) were re-suspended in toadfish saline (Walsh, 1987) and the blood/saline mix was re-injected into the fish. At the same time, seawater flow to the individual chambers was stopped; mixing was achieved by aeration. An initial 2 ml water sample was removed by pipette. A peristaltic pump and fraction collector system was then used to continuously collect water samples at a rate of 2 ml h^{-1} for the duration of the experiment. Following the initial 30 h food-deprivation control period, fish from each group were divided between food-deprived (fasted) and fed treatments. Fasted individuals received no food for the duration of the experiment. Fed individuals were provided daily with live pink shrimp, *Panaeus duorarum*, also from Biscayne Bay (Crook and Crook Marina), until satiated. All uneaten food was removed after 12 h. The flux chambers of all groups were flushed with fresh seawater for 30 min every 24 h. In addition to the initial aliquot, blood was sampled at 24, 36, 40, 48, 72 and 96 h. At 96 h, fish were euthanized (MS-222; 3 g l^{-1}) and gill, stomach, intestine and liver tissue were dissected. Gut contents were scrutinized to verify that toadfish in the fed groups had digested or were in the process of digesting their meal. Prior to freezing, the dissected fragments of the gastrointestinal tract were cleaned of any remaining shrimp with a combination of a saline wash (150 mmol l^{-1} NaCl) and gentle compression. Half of the tissue was frozen immediately

in liquid nitrogen, while the remaining half was transferred to a manufacturer-specified volume of RNA*later*® (Sigma-Aldrich, St Louis, MO, USA); both were stored at –80°C for later analysis of enzyme activity and mRNA expression, respectively. In addition, five fish from general holding tanks were euthanized and tissues (brain, gill, heart, stomach, intestine, spleen, liver, kidney, muscle and skin) were dissected and placed in RNA*later*®, and an aliquot of RBCs was transferred to a clean tube. All tissues were stored at –80°C for preliminary experiments examining the mRNA distribution of toadfish Rhag, Rhbg, Rhcg1 and Rhcg2.

Molecular analysis of gene expression

As suitable antibodies to toadfish Rh proteins are not yet available, transcript levels were emphasized. Total RNA was extracted from homogenized tissues (~100 mg) using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's specifications. The RNA was quantified at a wavelength of 260 nm using a NanoDrop 100 spectrophotometer (ND-100; Thermo Scientific, Waltham, MA, USA). Prior to cDNA synthesis, samples were treated with amplification grade DNase (Invitrogen) as described by the manufacturer to eliminate genomic DNA contamination. First-strand cDNA was synthesized from 2 µg of DNase-treated total RNA using either Superscript II (for Rhbg and Rhcg2; Invitrogen) or RevertAid H^- M-MuLV Reverse Transcriptase (for Rhag and Rhcg1; Fermentas, Glen Burnie, MD, USA) and oligo dT primers [500 ng per reaction; cloning and rapid amplification of cDNA ends (RACE) techniques] or random hexamers (200 ng per reaction; real-time RT-PCR). The protocol was carried out as specified by the manufacturer, and the final cDNA samples were diluted with an equal volume of autoclaved water.

With the exception of Rhag, which was cloned from RBC cDNA, all Rh isoforms were cloned from gill tissue using degenerate primers (Table 1) designed from vertebrate Rh sequences in regions with a high degree of amino acid and nucleotide conservation. PCR was performed using 0.5 µl of cDNA template in a 25 µl reaction composed of 2 mmol l^{-1} dNTPs, 0.2 mmol l^{-1} of primer, and 0.05 U of *Taq* polymerase (Denville Scientific Inc., Metuchen, NJ, USA) in buffer supplied with the enzyme. All PCR reactions were subjected to an initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, primer annealing for 30 s and 72°C for 60 s, and were completed with a final extension at 72°C for 15 min. Gel-purified PCR products (*via* QIAex II and Minelute gel extraction kit, Qiagen, Germantown, MD, USA) for Rhbg and Rhcg2 were ligated into PCR 2.1 vectors (TOPO TA cloning kit, Invitrogen), transformed into TOP10 chemically competent cells and sequenced. Rhag and Rhcg1 were ligated into the pDrive vector (Qiagen), transformed in DH5a chemically competent *Escherichia coli* (Invitrogen) and sequenced. A BlastX search revealed that the cloned fragments of cDNA shared highest amino acid identity with known teleost Rh isoform sequences.

Based on the partial sequences, gene-specific primers for the Rh genes were designed (Table 1) using Oligo 6.87 (Molecular Biology Insights, Inc., Cascade, CO, USA) and GeneTool software (BioTools, Inc., Jupiter, FL, USA) for use in 3′ and 5′ RACE and nested RACE PCR reactions. Poly A^+ RNA was generated from gill total RNA using the PolyATract mRNA isolation kit (Promega, Madison, WI, USA). 5′ and 3′ sequences for Rhbg and Rhcg2 were amplified from adaptor-ligated gill cDNA using the Marathon cDNA Amplification Kit (Clonetech, Mountain View, CA, USA). Cycle parameters for the initial

Table 1. Primers used to amplify products for cloning, RACE PCR, and real-time PCR of the Rhesus (Rh) glycoprotein isoforms in gulf toadfish, *Opsanus beta*

Gene	Application	Sequence (5′–3′)
Rhag	Cloning – forward[b]	GCC TTC GSY YTG CAG TGG
Rhag	Cloning – reverse[b]	TTR AAR CTR GGC CAG AAC ATC C
Rhbg	Cloning – forward[b]	CGC TGT TTG CTG TGA ACG AA
Rhbg	Cloning – reverse[b]	ATC CAG GTT GAG CCG ATA GA
Rhcg1	Cloning – forward[b]	CGA RGA GKC DGA YAC DCA CTG G
Rhcg1	Cloning – reverse[b]	CWG GCA TGG CRT GCA GGT TRT G
Rhcg2	Cloning – forward[b]	TGG TGG TAT TGG CTG TAA CTT
Rhcg2	Cloning – reverse[b]	AAA CTA TGT GAG TCC GAA GAG
Rhag	5′ RACE PCR	GGG CCA GAA CAT CCA CAG G
Rhag	3′ RACE PCR	GCC CTG TGC AGC TCC TCA TCA T
Rhbg	5′ RACE PCR	TGT TCA TTG CTG TTC GGT GCT G
Rhbg	3′ RACE PCR	ATG TTA GAG GTC ACG CTG TTT GCT G
Rhcg1	5′ RACE PCR	CCC GCC ACA CAG AAA TCA G
Rhcg1	3′ RACE PCR	CGG ACA CGC ACT GGA TAG AAT TTA G
Rhcg2	5′ RACE PCR	CTT CTG TCT TCA AAG CGT CCT TCA A
Rhcg2	3′ RACE PCR	GCG GCA AAG CAA TCG ACT CAA T
Rhag	Real-time PCR – forward	TGG GAG GAC TGG CAG GCA TTG TAG
Rhag	Real-time PCR – reverse	TCC AAC CAA AGC GAA CCC AAG TGA
Rhbg	Real-time PCR – forward	TGG CCT GAC GGT TAC ACG GAT CCT
Rhbg	Real-time PCR – reverse	TTG CTG TGC GGT GCT GGT CGT C
Rhcg1	Real-time PCR – forward	CAT CGG TGG AAT TGT GGG AGC C
Rhcg1	Real-time PCR – reverse	GGC AAC ATG TTT TGA AAG CAC CCT C
Rhcg2	Real-time PCR – forward	GGG TCC ACA GGC GCC ATC GTT G
Rhcg2	Real-time PCR – reverse	GCC CAA ATG CAA TCG CCA CAC
Hb	Real-time PCR – forward	CCG CCA AAT CTG AGT CCG TGA AAA A
Hb	Real-time PCR – reverse	CCG GCC CAG GTG AGA GTC GCA TTT
18S[a]	Real-time PCR – forward	GCT CGT AGT TGG ATC TCG G
18S[a]	Real-time PCR – reverse	GGC CTG CTT TGA ACA CTC

[a]Primers were designed and validated in a previous study (see Rodela et al., 2011).
[b]Degenerate nucleotides (S=C+G, Y=C+T, R=A+G, K=G+T, D=A+G+T).

RACE PCR reactions were: 94°C (30 s), followed by five cycles of 94°C (5 s), 70°C (4 min) and 30 cycles of 94°C (20 s), and 65°C (4 min). Subsequent nested RACE PCR was performed using primary PCR product diluted (1:50) in tricine-EDTA buffer. The procedures used in 5′ and 3′ RACE for Rhag and Rhcg1 differed from the previous Rh RACE reactions. cDNA for 5′ and 3′ RACE was synthesized from total blood (for Rhag) and gill (Rhcg1) RNA using an oligo dT primer (0.5 µg per reaction) and RevertAid H$^-$ M-MuLV Reverse Transcriptase (Fermentas), purified using a QIAquick PCR purification kit (Qiagen) and then tailed with dATP using a terminal transferase (TdT; Invitrogen) with final reaction conditions of 17 µl cDNA, 0.25 mmol l^{-1} dATP, 0.5 µl TdT and 5 µl of buffer supplied with the enzyme; the reaction was incubated according to the manufacturer's specifications. The tailed cDNA was used for one (Rhcg1) or two (Rhag) rounds of nested PCR. The first round of PCR used the primary gene-specific primer (Table 1) and oligo dT or oligo dT anchor primer (Table 1) for 3′ and 5′ RACE, respectively, followed by another PCR using the secondary gene-specific primer with the oligo dT primer. All RACE PCR products were purified, cloned, and sequenced as described above. Sequenced products were aligned with the appropriate initial cDNA fragments for the construction of consensus sequences for all Rh glycoproteins.

For real-time RT-PCR analysis of Rh and GS mRNA transcript levels, primers (Table 1) were designed using GeneTool software and selected for optimal amplification at an annealing temperature of 58°C. The specificity of individual primer pairs for Rh and GS genes was confirmed by sequencing of amplicons. Primer sequences for 18S were designed and validated in a previous study and yielded a product size of 166 bp (Rodela et al., 2011). All real-time RT-PCR reactions were performed on samples of cDNA (2 µl) using a SYBR Green master mix kit (Stratagene, Santa Clara, CA, USA) and analyzed using an Mx3000P Real-Time PCR System with associated MxPro 4.1 software (Stratagene). The thermocycler settings and composition of the reaction were those suggested by the manufacturer, with the exception that the reaction volume was scaled to 12.5 µl from 25 µl. Dissociation curves were created and evaluated for each reaction to assess the specificity of primers pairs and monitor the formation of primer-dimers. 'No reverse transcriptase' and no template control samples were included in every plate to confirm that the product was not amplified from genomic DNA or contaminated reagents, respectively. The 'no reverse transcriptase' control templates were created by omission of reverse transcriptase during cDNA synthesis. Reaction efficiencies were assessed by analysis of standard curves generated from a pool of cDNA from toadfish gill/RBCs that was serially diluted (1:5) with molecular grade RNase/DNase-free water (Sigma). A linear regression of the resulting cycle thresholds (C_t) against relative template concentration was plotted and primer pair efficiencies were considered acceptable if they fell within the range of 85–115% and had an R^2 of at least 0.98. Transcript levels of the genes of interest were expressed relative to the reference gene 18S (template diluted 1000-fold) and calculated according to the $\Delta\Delta C_t$ method of Pfaffl (Pfaffl, 2001). For purposes of comparison, mRNA expression was calculated relative to the respective gene from unconfined fasted individuals. Of further note, RhAG expression in mammals is strongly associated with RBCs and erythropoietic tissues (Huang and Liu, 2001; Huang and Peng, 2005); this has also been the case in fish studied to date. Therefore, the co-amplification of Rhag and hemoglobin (Hb) mRNA (see Table 1 for real-time PCR primers) was measured to establish the degree of Rhag mRNA expression due to RBC contamination in the various toadfish tissues.

Sequence analysis

Phylogenetic analyses were conducted on the amino acid sequences of the toadfish and selected piscine Rh genes including (GenBank accession numbers for each are given in parentheses): Rhag *Danio rerio* (AF531094), *Gasterosteus aculeatus* (DQ523516), *Oncorhynchus mykiss* (NM_001124674), *Takifugu rubripes* (AY618933); Rhbg *D. rerio* (NM_200071), *G. aculeatus* (DQ523517), *Kryptolebias marmoratus* (DQ995211), *O. mykiss* Rhbg (NM_001124662) and Rhbg2a (NM_001136097), *Oryzias latipes* (AY353247), *Tetradon nigroviridis* (AY865614), *T. rubripes* (NM_001032646); Rhcg1 *D. rerio* (NM_001109843), *K. marmoratus* (DQ995210), *O. mykiss* Rhcg1-a (NM_001124577) and Rhcg1-b (EF051115), *T. nigroviridis* (AY865615), *T. rubripes* (NM_001032647); and Rhcg2 *D. rerio* (NM_207082), *G. aculeatus* (DQ523518), *K. marmoratus* (DQ423779), *O. mykiss* (NM_001124523) and *T. rubripes* (AB218982). The toadfish Rh genes were translated using the online Translate tool available from the ExPasy Proteomics Server (http://ca.expasy.org/tools/dna.html). Sequence alignments for the Rh genes were obtained using ClustalW2 (version 2.0.12, www.ebi.ac.uk/Tools/msa/clustalw2). The phylogenetic relationship was inferred using the neighbor-joining (NJ) method (Saitou and Nei, 1987) and conducted using Mega4.0 software (Tamura et al., 2007). NJ was performed using distance matrices derived from the Tamura–Nei model (Tamura and Nei, 1993). Support for gene clusters was obtained through bootstrap analyses using 1000 replicates (Felsenstein, 1985). All analyses were performed using *T. nigroviridis* RhP2 (DQ013062) as an outgroup, as this gene is thought to be ancestral to the Rhag, Rhbg and Rhcg clades (Huang and Peng, 2005).

Assay techniques

Ammonia concentration in seawater was measured by the Indophenol Blue method (Ivancic and Degobbis, 1984) with a ThermoMax microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA). Urea concentration in seawater was quantified according to the diacetyl monoxime method (Rahmatullah and Boyde, 1980) with a microplate reader. Water ammonia and urea concentrations were also used to calculate nitrogen excretion values (E; µmol N kg^{-1}), as previously described by McDonald et al. (McDonald et al., 2004) using the following equation:

$$E = (\Delta C V_f) / M_f, \quad (1)$$

where ΔC is the increase in concentration (µmol N l^{-1}) over time, V_f is the volume (l) of the individual flux chambers, and M_f is the mass (kg) of the individual fish. Nitrogen excretion values were averaged over the entire treatment interval to facilitate a direct comparison with the initial 24 h control period. These values were used to analyze the percent ureotelism for each treatment. All excretion measurements are presented as normalized ratios whereby the value for the treatment period was expressed relative to the corresponding control period for that treatment. Data were normalized to reduce the natural variability in nitrogen excretion rates that is present among different batches of wild-caught toadfish.

Plasma ammonia content (µmol l^{-1}) was measured using an aliquot of plasma that was deproteinized in two volumes of 8% perchloric acid, vortexed and centrifuged at 16,000 ***g*** for 10 min (4°C). The supernatant was neutralized with saturated $KHCO_3$ and centrifuged at 16,000 ***g*** for 10 min (4°C). The final supernatant was analyzed for ammonia content using a Sigma Diagnostics ammonia (L-glutamate dehydrogenase) kit, modified for use with a microplate reader. Plasma cortisol concentration was measured using a

Table 2. The percentage of nitrogenous waste excreted in the form of urea and ammonia by toadfish during an initial 24 h control period following by a treatment period where fish were either fasted or fed every 24 h

		Control period		Treatment period	
	Group	% Urea	% Ammonia	% Urea	% Ammonia
Fasted	Unconfined	45.3±4.9	54.8±4.9	46.6±3.2	53.5±3.2
	Crowded	82.5±3.4	17.5±3.4	82.7±2.8	17.3±4.3
Fed	Unconfined	39.5±5.8	60.5±5.8	48.0±4.3	52.0±4.3
	Crowded	79.9±4.9	20.1±3.7	87.3±2.5	12.7±1.6

Values are means ± s.e.m.

Percent ureotelism was affected by crowding but not feeding in both the control (two-way ANOVA, crowding $P<0.001$, feeding $P=0.74$, crowding–feeding interaction $P=0.77$) and treatment period (two-way ANOVA, crowding $P<0.001$, feeding $P=0.47$, crowding–feeding interaction $P=0.72$).

No significant differences were detected within a group between the control and treatment periods (paired *t*-test, $P>0.05$).

commercially available ^{125}I radioimmunoassay kit (MP Biomedicals, Santa Ana, CA, USA).

GS activity was measured using a transferase protocol (Walsh, 1996). In brief, individual gill and liver samples were homogenized separately using a motor-driven tissue homogenizer (Fisherbrand) in five volumes of 50 mmol l^{-1} HEPES buffer (pH 7.4). Following centrifugation for 8 min at 13,000 ***g***, the resulting supernatant was removed 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}.

Statistical analyses

All data are presented as means ± s.e.m. The effects of treatments on normalized nitrogen excretion were statistically analyzed using a two-way ANOVA on normalized data with crowding status and feeding regimen as the two factors. Differences within each group were tested using paired *t*-tests on values in the control and treatment periods. For plasma variables, time points among the groups were analyzed by two-way ANOVA with defined factors of crowding and feeding. Differences within each group over time were analyzed with a one-way repeated-measures (RM-) ANOVA. The statistical significance of treatment effects on gene expression was assessed using a two-way ANOVA, with crowding status and feeding regimen as the two factors. Where significant differences were detected ($P<0.05$), 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 ($P<0.05$). All statistical analyses were carried out using SPSS SigmaStat v3.5 (San Jose, CA, USA). Prior to analysis, proportional data (% urea and ammonia) were transformed using $p'=\arcsin\sqrt{p}$, as these values were binomially distributed (Zar, 1999).

RESULTS

The physiological effects of crowding and feeding

Analysis of the physiological parameters revealed that although both crowding and feeding had significant effects, there were no significant interactions between these factors (data analyzed by two-way RM-ANOVA with crowding and feeding as factors, $P>0.05$ for the interaction term in all cases); consequently, the physiological effects of crowding and feeding will be considered separately.

The effects of crowding were most easily observed by comparing the initial 24 h (control period) of the experimental period between unconfined and crowded toadfish. This analysis revealed that crowding significantly lowered ammonia excretion; unconfined toadfish excreted approximately equal quantities of ammonia and urea (Table 2), with ammonia-N being eliminated at a rate of 98.5±10.0 μmol N kg^{-1} h^{-1} over a 24 h period ($N=13$). The majority of urea (≥93%) was excreted in a pulsatile manner. In crowded fish, however, urea accounted for most of the nitrogenous waste excreted (Table 2); ammonia was excreted at a relatively low rate (21.3±3.1 μmol N kg^{-1} h^{-1}) over a 24 h period ($N=14$). On average, >96% of the urea was excreted in distinct pulses. In fish that were fasted throughout the experimental period, the values of these parameters did not change over the course of the experiment (paired Student's *t*-tests, $P>0.05$ in all cases; Table 2, Fig. 1A, supplementary material Fig. S1A,B). Plasma ammonia (Fig. 1B) and urea (supplementary material Fig. S1C) concentrations did not differ between unconfined and crowded toadfish (two-way ANOVA at each time point, $P>0.05$ for the main effect of crowding in all cases). Plasma cortisol concentrations did not differ between unconfined (192.7±12.1 ng ml^{-1}, $N=13$) and crowded (219.4±13.3 ng ml^{-1}, $N=14$) toadfish (two-way ANOVA at each time point, $P>0.05$ for the main effect of crowding in all cases). Hepatic GS mRNA abundance was elevated in response to crowding but GS mRNA expression in gill, stomach and intestine was unaffected (Fig. 2). Glutamine synthetase enzyme activity was elevated in response to crowding in stomach and liver, but the activity in gill and intestine did not change in response to crowding (Fig. 2B).

Feeding did not significantly alter the proportion of nitrogenous wastes excreted as ammonia and urea (Table 2), but raised ammonia excretion rates (two-way ANOVA, $P=0.026$; Fig. 1A) from 62.1±13.8 mol N kg^{-1} h^{-1} ($N=12$) to 139.3±31.8 mol N kg^{-1} h^{-1} over a 24 h period ($N=12$). Urea excretion was also elevated [from 84.1±16.8 mol N kg^{-1} h^{-1} ($N=12$) to 247.1±37.6 mol N kg^{-1} h^{-1} ($N=12$)]. All components (total, pulsatile and non-pulsatile) of urea excretion were significantly elevated in fed individuals (supplementary material Fig. S1A), including urea pulse size (two-way ANOVA, $P<0.028$; supplementary material Fig. S1B). Fed fish had higher plasma ammonia concentrations at 36 and 40 h relative to their fasted counterparts (Fig. 1B). Similarly, plasma urea concentrations at 36, 40 and 48 h were higher in fed fish *vs* fasted individuals (supplementary material Fig. S1C). Plasma cortisol concentrations did not differ between fasted (241.9±30.5 ng ml^{-1}, $N=14$) and fed (199.9±29.5 ng ml^{-1}, $N=12$) toadfish (two-way ANOVA at each time point, $P>0.05$ for the main effect of crowding in all cases). Glutamine synthetase mRNA abundance (Fig. 2A) was unaffected by feeding and, with the exception of elevated activity in the liver, GS enzyme activity was also unaffected (Fig. 2B).

Molecular analysis of toadfish Rh genes

A combination of homology cloning and RACE techniques was used to isolate and identify four distinct toadfish Rh glycoproteins. The full-length coding sequences of these genes were included in a phylogenetic analysis of piscine Rh glycoprotein genes (Fig. 3). A

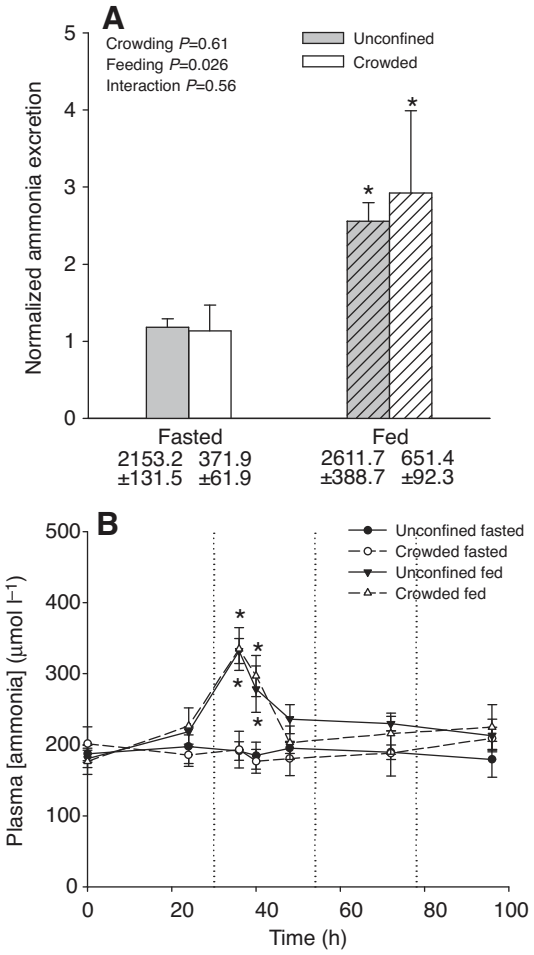

Fig. 1. Effects of crowding and/or feeding on (A) ammonia excretion (relative units) and (B) plasma ammonia (mmol l^{-1}) in gulf toadfish, *Opsanus beta*. In B, the dotted lines represent the times at which fed groups received a meal. The data in A are presented as the treatment value normalized to the initial 24 h control period. In A, numerical values reported below the bars represent absolute values (μmol N kg^{-1}) of ammonia excretion during the initial 24 h control period. Values are expressed as means ± s.e.m. (N=6–8). Data were analyzed by two-way ANOVA with defined factors of crowding and feeding. Statistics for panel A are indicated on the figure. Asterisks in A denote a significant difference within a group between the initial control period (<24 h) and the treatment period (>24 h) as determined by paired t-test (P<0.05). Two-way ANOVAs with crowding and feeding as factors were carried out at each sample time. With two exceptions, P>0.05 for the effects of crowding, feeding and the interaction of these two factors at every sample time. The two exceptions were at 36 and 40 h, where significant effects of feeding were detected (P<0.001 and P=0.004, respectively); these points are indicated with asterisks in B.

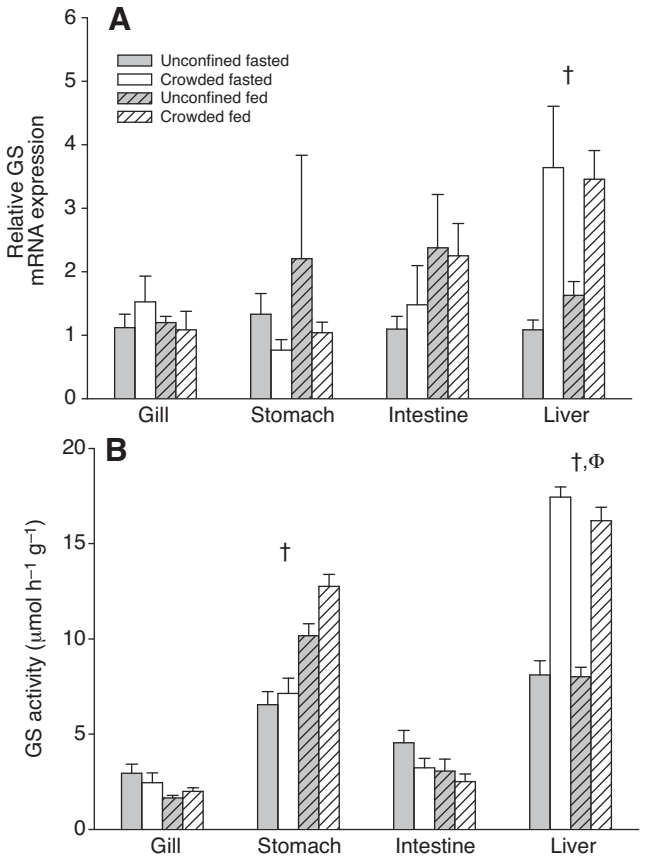

Fig. 2. Influence of crowding and/or feeding on (A) relative glutamine synthetase (GS) mRNA expression as measured by real-time RT-PCR and (B) GS activity (μmol h^{-1} g^{-1}) in gulf toadfish, *Opsanus beta*. The mRNA transcript levels for each tissue were normalized against the control gene 18S and expressed relative to the unconfined unfed group, which was set to a value of 1. Values are means ± s.e.m. (N=6–8). Data were analyzed by two-way ANOVA with defined factors of crowding and feeding. Symbols indicate significant effects of crowding (†) or feeding (Φ) within a tissue. For GS mRNA expression, the only significant effect detected was an effect of crowding in the liver (P<0.001). For GS enzyme activity, a significant effect of crowding was detected in stomach (P<0.001), and significant effects of both crowding (P=0.038) and feeding (P<0.001) were detected in liver; in all other cases, P>0.05.

gene isolated from toadfish RBCs was found to group strongly with piscine Rhag genes (Fig. 3). This toadfish Rhag sequence (GenBank accession no. HQ424874) was 1515 nucleotides long and was predicted to code for 434 amino acids. The largest transcript (1673 nucleotides) isolated from toadfish gill grouped phylogenetically with the Rhbg subgroup (Fig. 3) and yielded a predicted product of 458 amino acids (GenBank accession no. HQ424875). A second gene isolated from toadfish gill was 1519 nucleotides long and phylogenetic analysis showed that it fell within the clade of Rhcg1 genes (Fig. 3). The toadfish Rhcg1 gene (GenBank accession no. HQ424876) coded for a predicted sequence that was 482 amino acids long. Examination of the third sequence isolated from gill tissue revealed that this gene fell within a cluster of piscine Rhcg2 homologues (Fig. 3). This gene (GenBank accession no. HQ424877) was 1647 nucleotides in length with a predicted sequence of 482 amino acids. All toadfish Rh sequences displayed 75 to 88% sequence similarity to all available fish homologues.

Rh transcripts were expressed in all tissues examined (Fig. 4). Equivalent relative expression of Rhag and Hb mRNA in the majority of tissues examined suggested that expression of Rhag in these tissues was a result of RBC contamination (Fig. 4A). However, higher relative expression of Rhag mRNA over Hb mRNA was observed in gill and intestine tissues, suggesting tissue-specific expression of Rhag in these organs. Although the detection of Rhcg1 and Rhcg2 mRNA in the blood was somewhat surprising, it should be noted that the RBC preparation is crude and may include other cell types that may be responsible for the expression of these Rh isoforms. Rhbg mRNA transcript levels were similar among tissues, with the exception of stomach, which demonstrated qualitatively

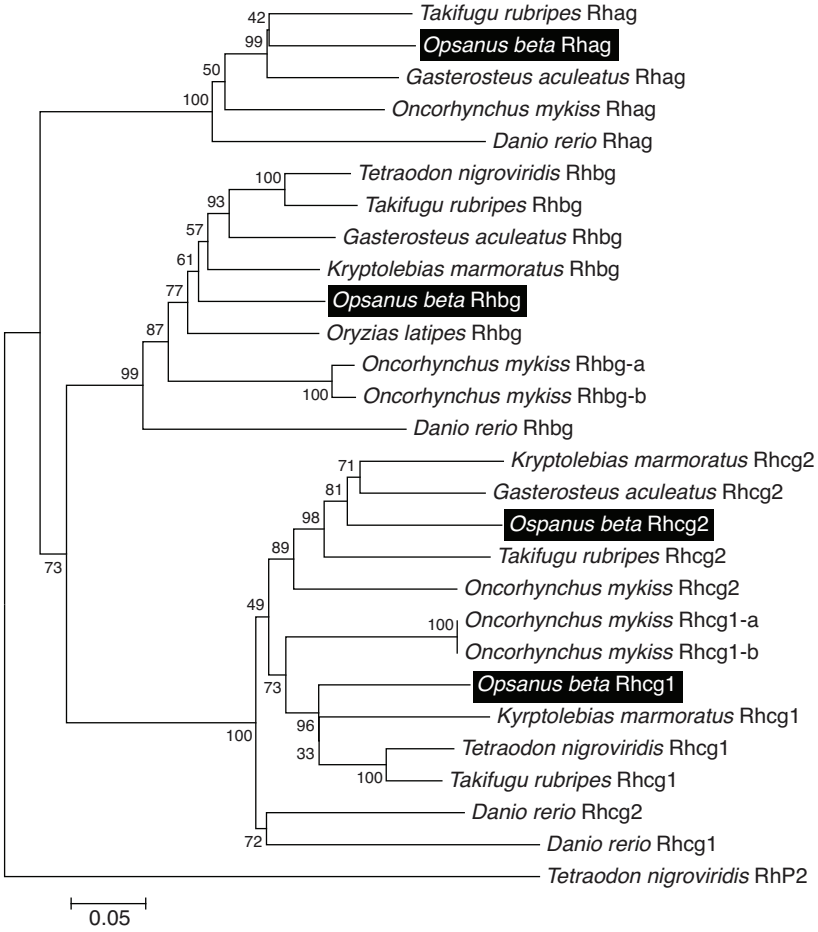

Fig. 3. Phylogenetic tree illustrating the relationship between gulf toadfish, *Opsanus beta*, Rhesus (Rh) glycoproteins (highlighted in black) and other piscine Rh isoforms. The phylogenetic tree was constructed using neighbour-joining analysis with *Tetraodon nigroviridis* RhP2 as an outgroup. The branch lengths are scaled to represent the relative number of substitutions occurring along each branch. The statistical support for the nodes is indicated as percentage obtained from bootstrap analysis using 1000 replicates. Sequences were obtained from GenBank (see Materials and methods for accession numbers).

low levels of expression, and liver, in which expression was somewhat higher than in other tissues (Fig. 4B). Both Rhcg1 (Fig. 4C) and Rhcg2 (Fig. 4D) exhibited relatively high expression in gill tissues and low levels in stomach tissues; this pattern was especially evident for Rhcg1. Intestine and liver tissues exhibited moderate Rhcg1 mRNA expression (Fig. 4C). Based on these tissue expression profiles and their prominent role in handling nitrogenous wastes following feeding, gill, stomach, intestine and liver tissues became the focus of further analysis.

In gill tissues, confinement and fasting had significant effects on the relative mRNA expression of all four Rh isoforms, and there was no interaction between these factors (Fig. 5A; see Table 3 for *P*-values). Whereas confinement significantly decreased the branchial mRNA expression of Rh isoforms, their mRNA expression was increased by feeding (Fig. 5A). Significant interactions between confinement and fasting were detected with respect to the relative mRNA expression of Rh isoforms in stomach (Fig. 5B, Table 3; note that Rhag was not examined in stomach tissue as expression was likely the result of blood contamination as noted above). For Rhbg, *post hoc* comparisons could not reveal the origin of the significant differences. For Rhcg1 and Rhcg2, gastric mRNA expression was significantly lowered by crowding within fasted fish; this was true within fed fish for Rhcg2 alone. Feeding resulted in significantly higher mRNA expression of Rhcg1 for both unconfined and crowded fish, but only within crowded fish for Rhcg2 (Fig. 5B). Within intestine tissue, relative mRNA expression of Rhag was unaffected by crowding but significantly altered by feeding (Fig. 5C, Table 3). Fed fish exhibited significantly higher relative mRNA expression of Rhcg1 and Rhcg2 in the absence of any effect of crowding or interaction between crowding and feeding (Fig. 5C, Table 3). A significant interaction between crowding and feeding was detected for the relative mRNA expression of Rhbg in intestine tissue (Fig. 5C, Table 3); feeding had a significant effect within unconfined but not crowded fish whereas confinement exhibited the opposite effects depending on whether the fish were fasted (increased expression) or fed (decreased expression). The relative mRNA expression of Rhag in liver tissue was not examined owing to the likelihood that it reflected blood contamination (see above). Significant interactions between feeding and confinement were detected for the other three Rh isoforms in liver (Table 3). Feeding resulted in significantly higher mRNA expression of Rhbg, Rhcg1 and Rhcg2 in unconfined fish, but significantly lower mRNA expression of Rhbg and Rhcg1 in crowded fish (Fig. 5D, Table 3). Similarly, confinement exhibited opposite effects depending on whether the fish were fasted (higher expression in confined fish for all three isoforms) or fed (lower expression in crowded fish for all three isoforms) (Fig. 5D, Table 3).

DISCUSSION

In the present study we identified four Rh sequences that shared a strong identity with teleost genes encoding Rhag, Rhbg, Rhcg1 and Rhcg2, and were differentially expressed in tissues that are involved in nitrogen excretion and metabolism. We observed high mRNA signals in the gill for Rhag, Rhcg1 and Rhcg2, which is consistent with previous teleost studies (Hung et al., 2007; Nakada et al., 2007; Nawata et al., 2007; Braun et al., 2009). Also as in previous studies (Hung et al., 2007; Nakada et al., 2007; Nawata et al., 2007), Rh gene expression was detected across a wide range of tissues. The

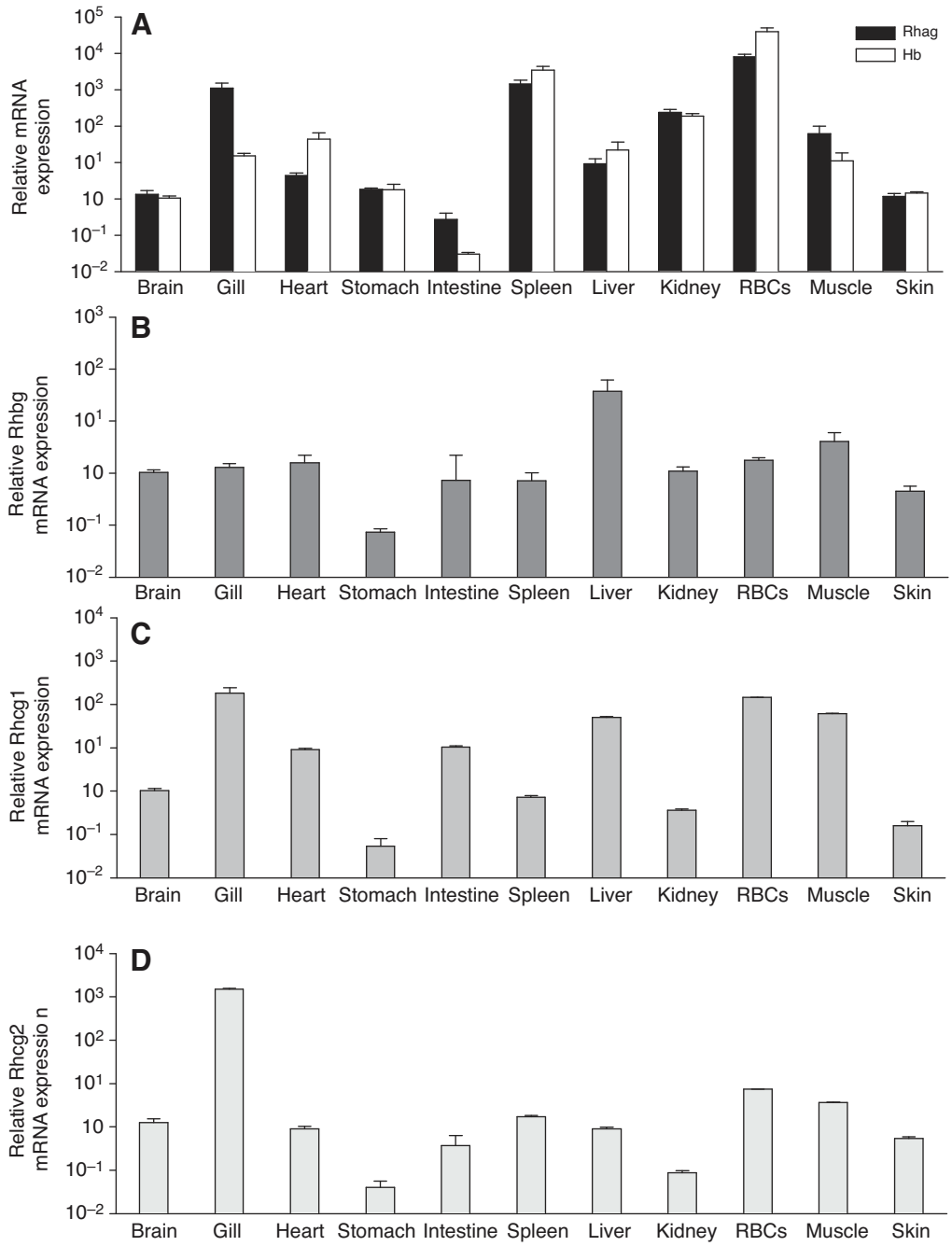

Fig. 4. Tissue distribution of relative mRNA expression (relative units) of (A) Rhag, (B) Rhbg, (C) Rhcg1 and (D) Rhcg2 from laboratory crowded gulf toadfish, *Opsanus beta*, as determined by real-time RT-PCR. All tissue mRNA levels were normalized against the control gene 18S and expressed relative to the level in the brain that was set to a value of 1. In A, relative hemoglobin (Hb) mRNA was co-amplified with Rhag to establish the level of red blood cell contamination within the tissues. Values are means ± s.e.m. (*N*=5).

present study provides in particular a detailed examination of Rh gene expression in tissues of the digestive system. The results revealed that moderate levels of Rhbg and Rhcg1 were present in the liver and intestine, and consistently low levels of Rhbg, Rhcg1 and Rhcg2 were expressed in the stomach. This Rh expression profile in toadfish is reminiscent of human and murine models that maintain significant quantities of RhBG and RhCG mRNA and protein in the liver, gastrointestinal tract, kidney and brain (Liu et al., 2000; Liu et al., 2001; Weiner et al., 2003; Weiner and Verlander, 2003; Nakhoul and Hamm, 2004; Handlogten et al., 2005). Mammalian Rh proteins enhance the movement of NH_3 in tissues that play a prominent role in ammonia secretion and uptake. We provide evidence that a similar arrangement may contribute to the ability of the toadfish to tightly control and regulate ammonia excretion, and results are discussed below in this context.

The effects of crowding on nitrogen excretion

Our results show that there is a significant elevation in hepatic Rhbg and Rhcg1 mRNA expression following crowding in unfed toadfish, a response, that assuming mRNA abundance reflects a corresponding changes a the protein level (which may not always be the case), may facilitate the equilibration of ammonia between the plasma and the cytosolic compartment of the hepatocytes. Although the subcellular localization of hepatic Rh proteins in teleosts is currently unknown, there is evidence from the mammalian literature that RhBG is present on the basolateral membrane of perivenous hepatocytes (Liu et al., 2001; Weiner et al., 2003). If a similar arrangement is present in toadfish, then an upregulation of Rhbg mRNA and, putatively, protein following crowding may help channel NH_3 into the hepatocytes for conversion to urea. Such a response is consistent with earlier work demonstrating that the

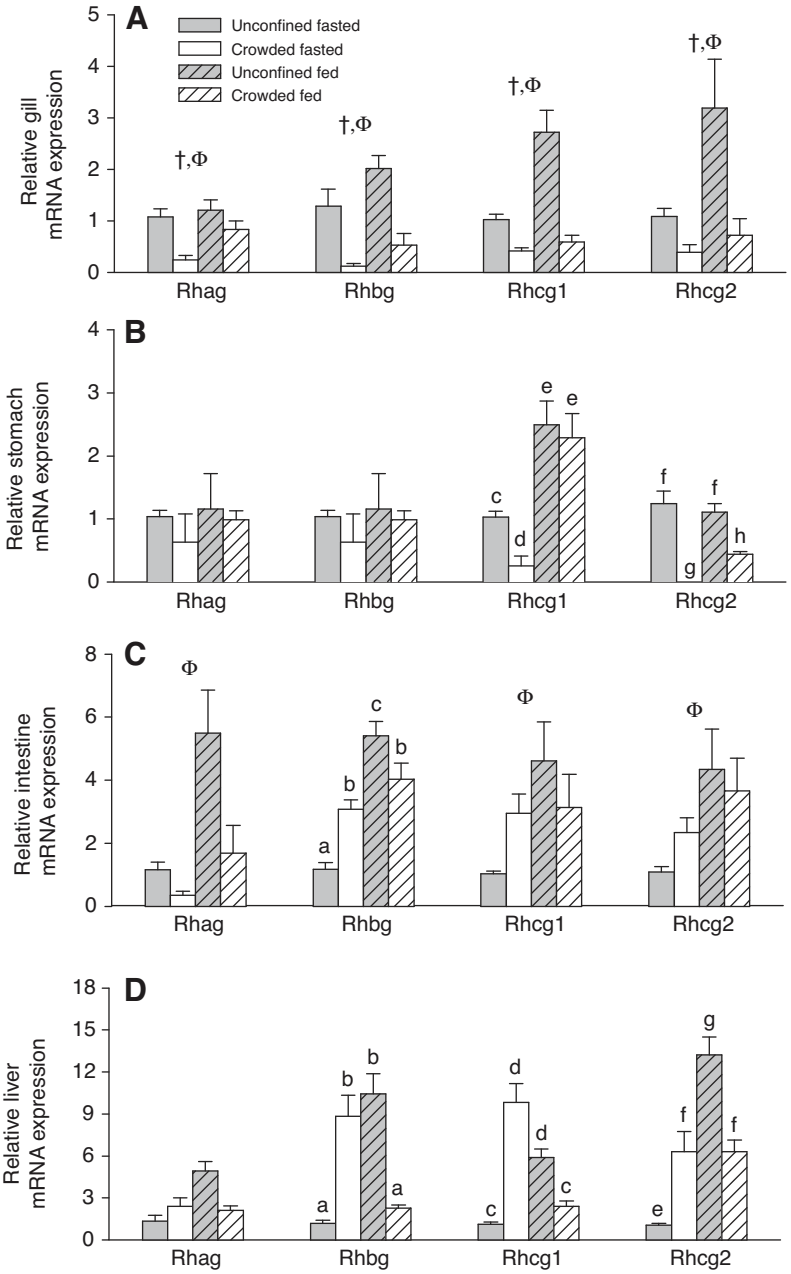

Fig. 5. The effects of crowding and/or feeding on relative mRNA expression of Rhag, Rhbg, Rhcg1 and Rhcg2 in the (A) gill, (B) stomach, (C) intestine and (D) liver of gulf toadfish, *Opsanus beta*, as measured by real-time RT-PCR. (Note that Rhag expression in the stomach and liver was assumed to be the result of blood contamination and these data are therefore not presented.) The mRNA levels for each gene were normalized against the control gene 18S and expressed relative to the unconfined unfed group, which was set to a value of 1. Values are means ± s.e.m. (N=6–8). Data were analyzed by two-way ANOVA with defined factors of crowding and feeding. Where the interaction of these two factors was not significant and the main effects can therefore be interpreted, symbols indicate significant effects of crowding (†) or feeding (Φ) for each gene within a tissue. See Table 3 for P-values for Rh protein isoforms. For B and C, select genes displayed a significant interaction between crowding and feeding, and for those isoforms in each tissue, values that do not share a letter are significantly different from one another (Holm–Sidak *post hoc* test, see Results for reported P-values).

transition to ureotely represents a rapid shutting down of ammoniotely and only a moderate increase in ureotely (Walsh and Milligan, 1995). Plasma ammonia levels do not change following crowding and confinement in toadfish (Wood et al., 1995) owing to increases in hepatic GS activity that are accompanied by comparable increases in transcript levels (Hopkins et al., 1995b; Walsh and Milligan, 1995; Wood et al., 1995; Kong et al., 2000; Walsh et al., 2003). Identical trends for both enzyme activity and mRNA expression were observed in the present study. The transcriptional upregulation of GS is attributed to the activity of glucocorticoid response elements within the promoter region of the GS gene that respond to the transient increase in plasma cortisol associated with crowding stress (Hopkins et al., 1995b; Kong et al., 2000; Esbaugh and Walsh, 2009; McDonald et al., 2009). The enhanced enzyme activity increases the rate of conversion of ammonia into glutamine, the nitrogen-donating substrate for piscine CPSase III in the ornithine–urea cycle, and accounts for increased rates of urea synthesis in the liver (Wood et al., 2003). One further interesting aspect of GS in toadfish is that the ubiquitous (or hepatic) GS gene generates two products, cytoplasmic and mitochondrial isoforms (Walsh, 1996; Walsh et al., 1999; Walsh et al., 2003). The former is the enzyme that is significantly upregulated in response to crowding and confinement (Walsh and Milligan, 1995). Although the cytoplasmic isozyme would clearly benefit from an Rh glycoprotein present on the plasma membrane, the limited permeability of the inner mitochondrial membrane suggests that a similar facilitative ammonia transporter may be required to supply the mitochondrial form of GS with ammonia. However, further experiments are necessary to determine the relative roles of Rhbg, Rhcg1 and Rhcg2 and their cellular and sub-cellular locations in relation to hepatic ammonia metabolism. A carrier-mediated urea transport mechanism has been characterized in the hepatic mitochondria of rainbow trout (Rodela et al., 2008). Although this mechanism has yet to be characterized at the molecular level,

Table 3. Results from a two-way ANOVA performed on Rh protein mRNA expression (relative units) collected from four groups of toadfish: unconfined fasted (*N*=7), crowded fasted (*N*=8), unconfined fed (*N*=6) and crowded fed (*N*=6)

		Gene			
Tissue	Factor	Rhag	Rhbg	Rhcg1	Rhcg2
Gill	Crowding	0.001	<0.001	<0.001	0.026
	Feeding	0.033	0.023	0.019	0.044
	Interaction	0.28	0.43	0.13	0.27
Stomach	Crowding	–	0.15	0.13	0.036
	Feeding	–	0.023	<0.001	0.062
	Interaction	–	<0.001	0.029	<0.001
Intestine	Crowding	0.079	0.98	0.72	0.66
	Feeding	0.014	<0.001	0.043	0.0030
	Interaction	0.10	<0.001	0.054	0.29
Liver	Crowding	–	<0.001	0.15	0.45
	Feeding	–	0.007	0.69	0.037
	Interaction	–	<0.001	0.003	0.042

Note that Rhag expression in the stomach and liver was assumed to be the result of blood contamination and the *P*-values pertaining to that data are therefore not presented. *P*-values are reported for the main effects (confinement and feeding) and the interaction between the two factors. See Fig. 5 for data.

evidence confirms that sub-cellular sites involved in nitrogen metabolism warrant further attention.

In the present study, we report that crowded fish excreted fivefold less ammonia than unconfined fish. We had hypothesized that the mRNA expression of Rh glyoproteins in the gill of crowded (ureotelic) toadfish would be significantly lower than that in unconfined (ammoniotelic) individuals. Our results support this hypothesis and provide correlative evidence that toadfish use Rh glycoproteins to excrete ammonia. Crowding and confinement stress significantly lowered the abundance of all Rh transcripts that may have resulted in lower protein levels in the gill of toadfish. Although we lack information about the cellular and sub-cellular locations of Rh glycoproteins in the gill of the toadfish, they are likely similar to the distribution described in *T. rubripes* (Nakada et al., 2007; Nawata et al., 2010b). In brief, Nakada and co-workers demonstrated that Rhag proteins present on the pillar cells that line the vasculature are ideally positioned to facilitate the movement of ammonia from the plasma to the adjacent pavement cells (Nakada et al., 2007). The diffusion of ammonia across the pavement cell is dependent on basolateral Rhbg and apical Rhcg2. By contrast, MRCs express only apical Rhcg1 (Nakada et al., 2007; Nawata et al., 2010b). If the fugu Rh protein model applies to the gill of toadfish, a reduction in the various Rh isoforms could greatly reduce the quantity of ammonia excreted across the gills. Enhanced ammonia trapping by gill GS may also contribute to reduced ammonia efflux across the toadfish gill, but likely over a longer time course [e.g. 1 week (McDonald et al., 2009)] than that used in the present study (Fig. 5A) (see also Walsh et al., 2003; Esbaugh and Walsh, 2009). Taken together, the evidence suggests that the ammonia-sequestering mechanism in the gill appears to be constitutively present in laboratory-acclimated toadfish but may be upregulated in response to prolonged stress (e.g. crowding and confinement). Therefore, we speculate that ammonia excretion in crowded toadfish is initially lowered by a rapid downregulation of Rh expression in pavement cells, pillar cells and MRCs, which is then maintained by a combination of low Rh expression and enhanced ammonia conversion to urea in the liver.

The effects of feeding on nitrogen excretion

Ingested amino acids in excess of requirements for protein synthesis are converted to ammonia in the liver (Ballantyne, 2001; Wood, 2001). Our data demonstrate two distinct patterns of feeding effects on liver Rh mRNA expression in unconfined and crowded toadfish. Unconfined individuals displayed a 3.7- to 12.5-fold upregulation (Fig. 5D) in liver mRNA expression for all Rh isoforms, except Rhag, in response to feeding, perhaps to enhance ammonia efflux from the liver for subsequent elimination from the body. Higher hepatic Rh mRNA expression was accompanied by an upregulation of both Rhcg1 and Rhcg2 at the gill (Fig. 5A). This scenario is similar to that of most teleosts that simply excrete the majority of excess ammonia across the gills (Kaushik and Teles, 1985; Wicks and Randall, 2002; Bucking and Wood, 2008; Bucking et al., 2009). Indeed, physiological data from the present study confirmed that unconfined toadfish excreted the majority of their wastes as ammonia (Table 2), and also had higher rates of ammonia excretion than their crowded counterparts. By contrast, crowded fed fish showed a fourfold downregulation in hepatic Rhbg and Rhcg1 transcript abundance relative to fasted individuals, whereas Rhcg2 abundance was unchanged (Fig. 5D). Increased ammonia production coupled with reduced efflux from hepatocytes would presumably shuttle ammonia towards metabolic pathways involving GS (i.e. urea production). This interpretation is supported by data showing that crowded fed toadfish accumulate more glutamine in their liver than crowded unfed toadfish, despite similar levels of GS activity and mRNA in both groups (Walsh and Milligan, 1995). Enhanced glutamine availability and utilization by the liver, in turn, result in increased urea production (Wood et al., 2003).

Although toadfish synthesize urea *de novo*, a previous study reported that a nitrogen load induced by feeding may exceed the capacity of the ammonia-trapping mechanism in the liver of ureotelic (crowded) toadfish (Walsh and Milligan, 1995). Under these circumstances, ammonia liberated from post-prandial catabolic processes would begin to accumulate in the plasma and eventually translate into elevated rates of ammonia excretion, as was the case in the present study. This pattern is also observed in other ammoniotelic teleosts following feeding (Kaushik and Teles, 1985; Kaushik and Gomes, 1988; Alsop and Wood, 1997; Wicks and Randall, 2002; Bucking and Wood, 2008; Bucking et al., 2009; Zimmer et al., 2010). In addition, this explanation also clarifies why the increases in plasma ammonia levels (Fig. 1B) and ammonia excretion rates (Fig. 1A) observed in crowded and unconfined fish during the post-prandial period were of similar magnitude. However, a direct comparison revealed that the absolute rates of ammonia efflux from crowded fish were approximately fourfold lower than

those of unconfined fed individuals. These lower rates were correlated with lower branchial mRNA expression of Rhbg, Rhcg1 and Rhcg2 in crowded fed fish compared with unconfined fed fish (Fig. 5A), a pattern that matched that of fasted fish. Collectively, the data indicate that the differences in excretion rates likely reflect the effects of crowding on Rh expression and the associated activation of ureotely. Of further note, feeding appeared to upregulate branchial mRNA expression of all Rh isoforms. This trend was similar to those described in the ammoniotelic rainbow trout, in which elevated ammonia excretion and branchial Rhcg2 mRNA were observed approximately 4–6 h following feeding (Zimmer et al., 2010). Yet, we also showed that Rh mRNA expression in gills of crowded fish was unaltered in response to feeding (Fig. 5A), suggesting that there may be endogenous factors that prevent increases in Rh expression and ammonia excretion in crowded fish following feeding. A likely candidate would be cortisol. Although no measurable differences in plasma cortisol were detected among the various treatments in the present study, it should be noted that, prior to experimentation, unconfined and crowded fish experienced different endogenous cortisol levels, which may have resulted in different patterns of Rh mRNA expression. However, further study is required to resolve this uncertainty.

In addition to playing a prominent role in digestion and nutrient absorption, the gastrointestinal tract is an important site of ammonia transport and a major contributor to post-prandial ammonia release in fish (Brown and Cameron, 1991; Kajimura et al., 2004). At present, information about gastrointestinal Rh proteins comes from a single mammalian study by Handlogten and colleagues (Handlogten et al., 2005), who demonstrated that both RhBG and RhCG mRNA and protein were prominently expressed in the proximal regions of the stomach and intestinal tract, with a trend for decreasing expression along the duodenum, jejenum, ileum and colon (Handlogten et al., 2005). By contrast, Rh mRNA expression in the toadfish digestive tract exhibited the opposite pattern: low levels of Rhbg, Rhcg1 and Rhcg2 mRNA expression were detected in the stomach (Fig. 4B) compared with moderate levels in the intestine (Fig. 4C). This expression profile may reflect the relative roles of the stomach *versus* the intestine. In the present study, plasma ammonia concentrations were approximately 200 μmol l^{-1} (Fig. 1B). Taking into consideration blood plasma pH (~7.9) and stomach pH [~5.75 (Taylor and Grosell, 2006)], a substantial NH_3 partial pressure gradient exists, favouring NH_3 entry into the lumen of the stomach. In fasted fish, Rhcg1 and Rhcg2 mRNA expression were significantly lower in the stomach of crowded relative to unconfined fish. The low levels of Rh mRNA expression may result in reduced protein expression and, coupled with high levels of GS activity, may prevent the movement of NH_3 into the stomach. Similarly, low intestinal Rh mRNA abundance and putatively protein suggests that intestinal ammonia efflux rates are reduced in fasted fish despite low levels of GS activity. These patterns are consistent with the trend for fasted fish to minimize the amount of NH_3 lost via gastrointestinal routes and 'trap' ammonia for urea synthesis.

However, minimizing fecal ammonia loss becomes more difficult during the post-prandial period as the amount of ammonia liberated from catabolic processes may overwhelm the ammonia 'trapping' capacity. Acidification of the gastric lumen to a pH of 3 (Taylor and Grosell, 2006) and elevation of plasma ammonia to ~350 μmol l^{-1} create an even stronger gradient for ammonia secretion into the stomach. Given that piscine Rh proteins function as bi-directional transporters (Nawata et al., 2010a) and that Rhcg1 mRNA expression in the stomach was significantly higher in fed fish, toadfish may manage the post-prandial nitrogen load by increasing ammonia secretion into the stomach. This trend carries over into the intestine, where mild post-prandial acidification [pH ~7.5; (Taylor and Grosell, 2006)] coupled with the elevated circulating ammonia concentration creates an NH_3 partial pressure gradient for the movement of ammonia into the intestine. In keeping with this hypothesis, the relative mRNA expression and presumably protein of all Rh isoforms in the intestine was significantly higher in fed than in fasted fish. These findings are consistent with a previous study by Kajimura and co-workers, who demonstrated that significant quantities of ammonia are lost through gastrointestinal routes in fed rainbow trout (Kajimura et al., 2004).

Conclusions

The results of the present study implicate Rh proteins in the unusual nitrogen excretion patterns of the gulf toadfish. Branchial Rh expression was low in crowded (ureotelic) animals relative to unconfined (ammonotelic) fish. Assuming Rh protein levels reflect mRNA expression, low Rh abundance in the gill of crowded animals likely minimizes ammonia efflux so as to favour urea production, whereas high Rh abundance in the gill of unconfined toadfish will facilitate nitrogen excretion as ammonia. These data add the unique toadfish perspective to the current literature on Rh glycoproteins as important mediators of branchial ammonia transport in many aquatic species (reviewed by Weihrauch et al., 2009; Wright and Wood, 2009). To date, however, this literature has been dominated by consideration of the gill. Our study broke new ground by providing evidence for the involvement and regulation of Rh glycoproteins in the gastrointestinal tract. Toadfish exhibited differential expression of Rh proteins along the stomach and gut, a pattern reminiscent of the functional zonation previously described in the teleostean digestive tract (Harpaz and Uni, 1999; Bakke-McKellep et al., 2000; Mommsen et al., 2003). Regional specificity of Rh proteins along the gastrointestinal tract may help toadfish deal with post-prandial nitrogen loads in a controlled manner. Finally, in addition to providing a broad picture of molecular changes associated with crowding and feeding, the present study also indicated that endogenous factors (e.g. cortisol) regulate Rh expression. Future investigations should focus on characterizing the regulatory mechanisms that enable tight control of nitrogen excretion patterns in toadfish.

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