THE ROLE OF CORTICOSTEROIDS IN NITROGEN EXCRETION OF THE GULF TOADFISH (*OPSANUS BETA*)

Tamara Rodela

Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
In partial fulfillment of the requirements
For the PhD Degree in the Ottawa – Carleton Institute of Biology

Department of Biology
Faculty of Science
University of Ottawa

ABSTRACT

In contrast to most teleost fish that are ammoniotelic, the gulf toadfish *(Opsanus beta*) is both facultatively ureogenic and ureotelic. *In vivo* pharmacological manipulations were used to show that lowering circulating cortisol levels or blocking glucocorticoid receptors (GR) enhanced both urea excretion and urea pulse size. These findings demonstrated that changes in pulsatile urea excretion in the toadfish are mediated by the permissive action of cortisol through GRs. Measurement of urea transport across isolated basolateral gill membranes revealed a cortisol-sensitive carrier mechanism. Cortisol infusion *in vivo* significantly reduced urea transport capacity, suggesting that cortisol inhibits the recruitment of urea transport proteins (UT) to the basolateral membrane to ultimately decrease the size of the urea pulse in toadfish. A 1.2 kb fragment of the upstream transcription start site for the toadfish urea transporter (tUT) gene was isolated and *in silico* analysis revealed the presence of several putative glucocorticoid response element (GRE) half sites. Toadfish provided with this regulatory sequence in a reporter gene construct showed increased reporter gene transcription driven by cortisol. The data indicated that cortisol-mediated upregulation of tUT mRNA by GREs may be necessary to maintain tUT activity.

Four Rhesus (Rh) glycoproteins (*Rhag*, *Rhbg*, *Rhcg1*, *Rhcg2*) were isolated from toadfish; these sequences grouped with those of other vertebrates coding for membrane channels that transport ammonia. *In vivo* increases in circulating cortisol reduced branchial Rh glycoprotein expression and decreased ammonia excretion. These changes were accompanied by cortisol-induced increases in glutamine synthetase activity, an enzyme that captures ammonia for urea synthesis. Taken

together, the data indicated that cortisol reduces the loss by branchial excretion of ammonia, instead favouring biochemical pathways that convert ammonia to urea.

This thesis confirms that nitrogen excretion in toadfish is controlled and regulated in fashions unlike those in other teleosts. The results demonstrate the importance of the GR signaling pathway in mediating changes in both urea and ammonia transport through molecular mechanisms. As a whole, the data provide a new understanding of branchial nitrogen excretion in the gulf toadfish and enhance our evolutionary perspective of the integrated biological systems involved in nitrogen excretion in fish.

RÉSUMÉ

Contrairement à la plupart des poissons téléostéens qui sont ammoniotélique, le poisson crapaud (*Opsanus beta*) est à la fois facultativement uréogénique et uréotélique. Des manipulations pharmacologique *in vivo* ont permis de démontrer que la réduction des niveaux de circulation de cortisol, ou le bloquage des récepteurs glucocorticoïdes (GR) améliorent à la fois l'excrétion d'urée et la magnitude des pulsations d'urée. Ces résultats ont démontré que les changements dans l'excrétion pulsatile d'urée chez le poisson crapaud sont arbitrés par l'action permissive du cortisol sur les GRs. La mesure du transport d'urée à travers la membrane basolatérale de branchies isolées a révélé un mécanisme de transport sensible au cortisol. Des infusions de cortisol *in vivo* ont fortement réduit la capacité de transport d'urée, suggérant que le cortisol empêche le recrutement des protéines transporteuses d'urée (UT) à la membrane basolatérale, réduisant ainsi la pulsation d'urée chez le poisson crapaud. Un fragment de 1.2 kb en amont du site de départ de transcription du transporteur d'urée chez le poisson crapaud (tUT) a été isolé et les analyses *in silico* ont révélé la présence de multiples moitiés d'éléments de réponse aux glucocorticoïdes (GRE). Les poissons crapauds injectés avec cette séquence régulatrice dans une construction de gène rapporteur ont démontrés une augmentation de la transcription du gène rapporteur promue par le cortisol. Les résultats ont indiqué que l'augmentation de la transcription de l'ARNm du tUT par les GREs est peut être nécessaire pour maintenir l'activité du tUT.

Quatre glycoprotéines rhésus (Rhag, Rhbg, Rhcg1, Rhcg2) ont été isolées du poisson crapaud ; les séquences obtenues se groupent aux autres protéines de

vertébrés encodant pour des protéines-canaux qui transportent l'ammoniac. L'augmentation des niveaux de cortisol *in vivo* ont réduit l'expression branchiale des glycoprotéines rhésus, et réduit l'excrétion d'ammoniac. Ces changements étaient accompagnés d'une augmentation de l'activité de glutamine synthase, un enzyme qui capture l'ammoniac pour la production d'urée. Dans l'ensemble, les données obtenues suggèrent que le cortisol réduit l'excrétion branchiale d'ammoniac, favorisant à la place les réactions biochimiques convertissant l'ammoniac en urée.

La présente thèse confirme que l'excrétion d'azote chez le poisson crapaud est contrôlée et régulée différemment que les autres poissons téléostéens. Les résultats démontrent l'importance de la cascade de signal du GR dans l'orchestration des changements de transport d'urée et d'ammoniac par des mécanismes moléculaires. Dans l'ensemble, ces données offrent une meilleure compréhension de l'excrétion branchiale de l'azote chez le poisson crapaud et améliorent la perspective évolutionnaire des systèmes biologiques intégratifs prenant part à l'excrétion de l'azote chez les poissons.

ACKNOWLEDGEMENTS

I would like to thank my supervisors, Drs. Pat Walsh and Katie Gilmour. Pat and Katie complemented each other well in their co-supervisory roles. Pat's enthusiasm for everything toadfish-related was contagious. Katie's refreshing insight and curiosity motivated me. Their guidance, encouragement, patience, and support have meant a lot to me during the pursuit of my PhD and had a profound effect on my development as a researcher.

The data for this thesis were mostly collected through experiments conducted at the University of Miami. The engaging collaboration and wonderful hospitality of Dr. Danielle McDonald made my research trips to Miami the highlight of my PhD. I would also like to thank my committee members (Dr. Danielle McDonald, Dr. Tom Moon, Dr. Steve Perry, Dr. Bill Willmore) for their feedback and valuable suggestions.

I have been fortunate to come across many funny and good friends in various laboratories both at the University of Ottawa and University of Miami. These companions made the frustrations of labwork endurable and the successes a triumph. Also, I am thankful for a supportive family. My parents, Igor and Helen Rodela, and grandparents, Lusia and Roman Bojmelgrin, provided unwavering support and encouragement during all stages of my education. Although only present for the latter half of my thesis, my daughter Sophie has the remarkable ability to distract me completely and joyously especially after a long arduous day of work. Finally, I thank my husband, Bill Hannah. His intellectual and emotional support kept me grounded and was instrumental in my journey to complete my thesis.

Bill, I dedicate this thesis to you.

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LIST OF ABBREVIATIONS

5-HT, 5-hydroxytryptamine
11-βHSD, 11β-hydroxysteroid dehydrogenase
^{14}C, carbon-14
^{125}I, iodine-125
AMT, ammonium transporter
ANOVA, analysis of variance
AQP, aquaglyceroporin
ASL, argininosuccinate lysase
ASS, argininosuccinate synthetase
ATP, adenosine triphosphate
BLMV, basolateral membrane vesicles
bp, base pairs
CA, carbonic anhydrase
camp, cyclic-3' 5-adenosine monophosphate
cDNA, complementary deoxyribonucleic acid
cpm, counts per minute
CPSase, carbamoyl phosphate synthetase
CR, corticosteroid receptor
CS, corticosteroid
DOC, 11-deoxycorticosterone
DNA, deoxyribonucleic acid
DNase, deoxyribonuclease
dNTP, deoxynucleotide triphosphate
EC_{50}, median effective concentration
ER, endoplasmic reticulum
ERK, extracellular signal-regulated kinase
EtOH, ethanol
g, grams
GLUT, glucose transporter
h, hour
GR, glucocorticoid receptor
GRE, glucocorticoid response element
GRU, glucocorticoid response unit
GSase, glutamine synthetase
HEK, human embryonic kidney
IMCD, inner medullary collecting duct
IP, intraperitoneal
IU, international units
JNK, c-Jun N-terminal kinase
kDa, kilodalton
K_m, Michaelis constant: substrate concentration of half maximal uptake
L, litre
MDCK, Mardin-Darby canine kidney
MEP, methylammonium/ammonium permease

min, minutes
mmol, millimole
MR, mineralocorticoid receptor
MRC, mitochondria rich cell
mRNA, messenger ribonucleic acid
MS-222, Tricane methanesulphonate
n, sample size
NEM, N-ethylamaleimide
nGRE, negative glucocorticoid response element
NHE, Na^+/H^+ exchanger
NKA, Na^+/K^+ ATPase
NKCC, Na^+-K^+-Cl^- transporter
OTCase, ornithine transcarbamylase
O-UC, ornithine-urea cycle
pCMBS, *p*-chloromercuribenezesulfonate
PV, pavement cell
Q_{10}, temperature coefficient
RACE, rapid amplification of cDNA ends
RBC, red blood cell
Rh protein, Rhesus glycoprotein
RM ANOVA, repeated measures analysis of variance
RNA, ribonucleic acid
RNase, ribonuclease
RT-PCR, reverse transcriptase polymerase chain reaction
RU-486, mifepristone
Slc14A, solute carrier 14
s.e.m., standard error of the mean
tUT, toadfish urea transporter
UT, urea transporter
V_{max}, maximal rate of uptake

CHAPTER 1

General Introduction

Nitrogen metabolism and excretion

General overview

Amino acids, unlike lipids and carbohydrates, cannot be stored without detrimental effects on the physiology of the tissues. Amino acids that are not used for growth and maintenance are catabolized into ammonia, a potentially toxic nitrogenous product when concentrated in the tissues of vertebrates. At elevated concentrations, ammonia compromises cell morphology, metabolic function, and transport processes (reviewed by Cooper and Plum, 1987; Wright, 1995). Collectively, these pathological consequences can result in convulsion, coma, and eventually death. Therefore, ammonia must be either eliminated or modified to avoid adverse effects on normal physiological function and homeostasis.

The evolution of nitrogen metabolism and excretion in vertebrates

Aquatic animals, including fish and larval amphibians, typically maintain low internal ammonia levels by excreting ammonia directly into the ambient environment where it can be "infinitely" diluted (Walsh and Mommsen, 2001; Wood, 1993; Wright, 1995). Given the high solubility of ammonia in water, direct excretion is the most efficient way of eliminating ammonia. However, this ammoniotelic strategy is only suitable for aquatic animals; organisms require 400 mL of water to dilute every gram of ammonia and prevent accumulation within the tissues (Wright, 1995). Conversion of ammonia to less toxic compounds like urea and uric acid is a strategy to ameliorate ammonia toxicity when water conservation is essential, e.g. for terrestrial animals (reviewed by Wright, 1995). These alternative waste products require less water for excretion (Wright, 1995); however, the disadvantage of these strategies is that the synthesis of these compounds requires ATP

(Mapes and Krebs, 1978; Wilkie, 2002). Therefore, water availability is clearly an important determinant in the mode of nitrogen excretion in vertebrates.

Although the majority of bony fish are ammoniotelic (Wood, 1993), a few unusual species have adopted unconventional patterns of excretion in response to various environmental constraints (reviewed by Walsh and Mommsen, 2001; Wright, 1995). These species typically encounter conditions that limit ammonia excretion. For example, the Lake Magadi tilapia (*Oreochromis alcalicus grahami*) inhabits an environment characterized by extremely alkaline pH (9.8-10.1) and high buffering capacity (Reite et al., 1974). These conditions disrupt the blood-to-water ammonia diffusion gradient in the fish and make ammonia excretion impossible (Wood et al., 1989). The Lake Magadi tilapia circumvents this problem by synthesizing and excreting urea (Randall et al., 1989). Other exceptions include air-breathing and/or amphibious species such as the Singhi catfish *Heteropneustes fossilis* and the slender lungfish *(Protopterus dolloi*). *H. fossilis* is primarily ammoniotelic but will transition to ureotelism when exposed to elevated ambient ammonia, prolonged emersion, or semiarid conditions in its natural habitat (Saha et al., 1988; Saha and Ratha, 1989; Saha and Ratha, 1998). Similarly, *P. dolloi* synthesizes urea by the ornithine-urea cycle (O-UC) and stores it during aestivation (Chew et al., 2003; Wood et al., 2005). Alternative strategies also are used by teleosts to detoxify ammonia under adverse environmental conditions. The marble goby (*Oxyeleotris marmoratus*), a facultative air-breather, uses glutamine synthetase (GSase) to convert ammonia into glutamine, which then accumulates in the tissues (Jow et al., 1999). The mudskippers, *Periophthalamodon schlosseri* and *Boleophthalmus boddaerti*, adapt to terrestrial conditions by accumulating amino acids in their tissues and greatly reducing protein and amino acid catabolic rates (Ip et al., 2004; Ip et al., 2001b; Lim et al., 2001; Randall et al., 2004). Similar strategies involving

GSase and reduced nitrogen metabolism are used by the cutthroat trout (*Oncorhynchus clarki henshawi*) following a high pH challenge (Wilkie et al., 1994). While each of these strategies raises physiological and phylogenetic questions, for the purpose of this thesis, discussion will be focused on pathways involving ammonia and urea excretion.

Mechanisms of ammonia excretion

In aqueous solution, ammonia exists as either NH_3 gas or ionized NH_4^+. The relative presence of each species is dictated by the pK_a of approximately 9.5 (Wilkie, 2002). At physiological pH (~7.8), more than 95 % of the total ammonia[1] (the sum of NH_3 and NH_4^+) exists as NH_4^+ (Wilkie, 2002). Owing to its charge, NH_4^+ cannot easily penetrate lipid membranes (Knepper et al., 1989) and requires specialized membrane proteins for transport. NH_3 is only moderately lipid soluble with a low lipid partition coefficient (Evans and Cameron, 1986). Until recently, it was generally accepted that the transcellular movement of NH_3 occurred by diffusion through the lipids of the membrane bilayer down a partial pressure gradient (reviewed by Weihrauch et al., 2009; Wright and Wood, 2009). However, specific membrane proteins that facilitate the movement of ammonia across lipid membranes have now been identified.

Less than two decades ago, two studies simultaneously established that the methylammonium/ammonium permeases (MEP) in yeast (*Saccharomyces cerevisiae*; Marini et al., 1994) and the ammonium transporter (AMT) in *Arabidopsis thaliana* (Ninnemann et al., 1994) facilitate the movement of NH_4^+/NH_3 across plasma membranes. A pivotal study by Marini and colleagues revealed that the AMT/MEP family shares significant sequence

[1] Throughout this thesis, the term ammonia will refer to the sum of NH_3 and NH_4^+, whereas the chemical symbols will be used to refer to the individual components.

similarity with the vertebrate Rhesus (Rh) glycoproteins (Marini et al., 1997). Multiple genes belonging to the AMT/MEP/Rh superfamily now have been identified in a wide spectrum of organisms including eubacteria, protists, and animals (Peng and Huang, 2006). Rh genes in vertebrates are separated into four paralogous clusters: Rh-associated glycoprotein (Rhag[2]), Rh glycoprotein type B (Rhbg), Rh glycoprotein type C (Rhcg), and Rh30 (Huang and Peng, 2005). Rhag, Rhbg, and Rhcg are part of the glycosylated group ('G' for glycosylation) of Rh proteins with an approximate molecular mass of 50 kDa (reviewed by Weiner and Verlander, 2010). In mammals, the glycosylated proteins transport ammonia, whereas the non-glycosylated members of Rh30 are associated with immune and other functions (Wright and Wood, 2009). Similar genes are present in teleosts but many piscine species appear to posses multiple copies of the glycosylated Rh genes (Huang and Peng, 2005; Nawata et al., 2007) that also facilitate ammonia transport (Nawata et al., 2010b). In addition, fish and amphibians possess a novel gene, Rhp2, which may represent the ancestral origin of the Rh genes (Huang and Peng, 2005). At present, little is known about the physiological role of Rhp2 in either fish or amphibians.

The mode of transport by these Rh channels remains controversial, studies differ as to the exact molecular species, NH_3 or NH_4^+, transported. Studies of ammonia transport by RhAG have reported facilitated NH_3 transport in human embryonic kidney (HEK) and Mardin-Darby canine kidney (MDCK) cells (Ripoche et al., 2004), electroneutral NH_4^+/H^+ exchange in *Xenopus laevis* oocytes (Westhoff et al., 2002), and enhanced transport of both NH_3 or NH_4^+ in HeLa cells (Benjelloun et al., 2005). Using a *X. laevis* expression system,

[2] By convention, Rh genes and proteins in mammals (e.g. RhAG) are denoted by capital letters, lower case letters are used for all other organisms (e.g. Rhag; Weiner and Hamm, 2007).

two separate studies demonstrated that RhBG mediates electroneutral NH_4^+/H^+ exchange (Ludewig, 2004) and electrogenic NH_4^+ transport (Mak et al., 2006). The data for RhCG is equally unclear. Data collected by various studies that support models of NH_3 gas channels (Zidi-Yahiaoui et al., 2005), dual NH_3 or NH_4^+ transport (Bakouh et al., 2004), and NH_4^+/H^+ exchange resulting in net NH_3 translocation (Mak et al., 2006). Wright and Wood summarized reasons for this disparity among studies and genes and most of the concerns appeared to arise from using mammalian genes in a heterologous expression system (e.g. *X. laevis* oocytes). Some authors question the inherent permeability of the oocyte membrane to ammonia, while other suggest that this system may lack the necessary endogenous proteins and lipids that putatively interact with various Rh proteins to facilitate transport (Wright and Wood, 2009). The hypothetical involvement of additional proteins is a relatively recent idea and is based on structural studies of Rhag in the microbe *Nitrsomonas europaea* and comparisons with related AMT proteins (Li et al., 2007; Lupo et al., 2007). Li and colleagues proposed that the opening of the Rh protein is regulated by the binding of a putative partner protein (Li et al., 2007). However, at present the existence of a putative Rh complex or metabolon has yet to be determined.

Rh glycoproteins are now being incorporated into models of ammonia excretion in fish. In freshwater teleosts, NH_3 is thought to move down a blood-to-water diffusion gradient that is maintained by acidification of the gill boundary layer (Wilkie, 2002). Acidification rapidly protonates excreted NH_3 to NH_4^+; this helps maintain a low NH_3 partial pressure at the gill surface. Recently, Wright and Wood (2009) proposed an updated model where Rh-facilitated NH_3 excretion is linked to sodium uptake and H^+ extrusion (Figure 1.1A). In this model Rhag facilitates NH_3 efflux from the red blood cells to the plasma and across the pillar cells where it diffuses across the basolateral gill membrane via Rhbg. At the

apical surface an "Na^+/NH_4^+ exchange complex" consisting of Rhcg, H^+-ATPase, and Na^+/H^+ exchange proteins (i.e. NHE2/NHE3) facilitate the acid trapping mechanism for ammonia excretion (Figure 1.1; Wright and Wood, 2009). By contrast, there is no evidence to support acidification of the boundary layer in marine teleosts (Weihrauch et al., 2009). This has been attributed to the higher buffering capacity of seawater and also structural and biochemical differences of the gill cells in marine species. While the loss of NH_3 across the gill epithelium is still an important component of ammonia excretion in marine teleost fish, evidence indicates that approximately 40% of ammonia is voided as NH_4^+ (Figure 1.1B; Evans et al., 1989). Significant NH_4^+ diffusion occurs through paracellular routes, facilitated by shallow tight junctions between adjacent gill epithelial cells (Sardet, 1980). Furthermore, NH_4^+ can displace K^+ on basolateral ion exchangers (Figure 1.1B, (Evans et al., 1989). Recently, this model was modified with information on Rh glycoproteins in pufferfish (*Takifugu rubripes*; Nakada et al., 2007b). Based on immunohistochemical evidence, Nakada and colleagues proposed that the excretion of ammonia from the vascular space is facilitated by the expression of Rhag on the plasma membrane of the pillar cells that line the vasculature and lie next to the pavement cells (Figure 1.1B; Nakada et al., 2007). Transport across the pavement cells is accomplished by the basolateral and apical expression of Rhbg and Rhcg2, respectively. They also speculated that the extrusion of ammonia across the mitochondria rich cells (MRCs) is accomplished through the cooperation of basolateral ion pumps or transporters (e.g. Na^+/K^+-ATPase) and an apical Rhcg1. Both freshwater and marine species can modulate Rh gene expression as well as ammonia excretion in response to high ambient ammonia (Braun et al., 2009b; Nawata et al., 2010a; Nawata et al., 2007), however, the precise molecular mechanisms mediating these changes has yet to be determined.

Figure 1.1: An updated model of branchial ammonia excretion in freshwater and marine teleosts. (A) In freshwater fish, the movement of ammonia from the plasma to the ambient water is facilitated by basolateral and apical Rhesus (Rh) glycoproteins that act as conduits for NH_3 transport. This NH_3 is trapped as NH_4^+ at the acidic gill surface, which maintains the transcellular NH_3 gradient. Acidification of the gill surface is accomplished through a complex consisting of H^+-ATPase, Na^+/H^+ exchange (NHE) proteins (modified from Wright and Wood, 2009). The involvement of carbonic anhydrase (i.e. CA-4) in the acidification of the gill boundary layer remains controversial (see Weihrauch et al., 2009). (B) Ammonia excretion in marine fish relies on a combination of paracellular diffusion through shallow tight junctions, NH_4^+ displacing K^+ or H^+ on ion exchangers, and facilitated diffusion through Rh glycoproteins. In pavement (PV) cells, ammonia is transported primarily through Rh glycoproteins. In the mitochondria-rich (MR) cells, basolateral ammonia transport is accomplished by NH_4^+ displacing K^+ on Na^+/K^+-ATPase (NKA) and Na^+-K^+-$2Cl^-$ transporter (NKCC). At the apical surface, NH_4^+ is transported by H^+-ATPase and NH_3 transport is facilitated by an Rh glycoprotein (modified from Weihrauch et al., 2009).

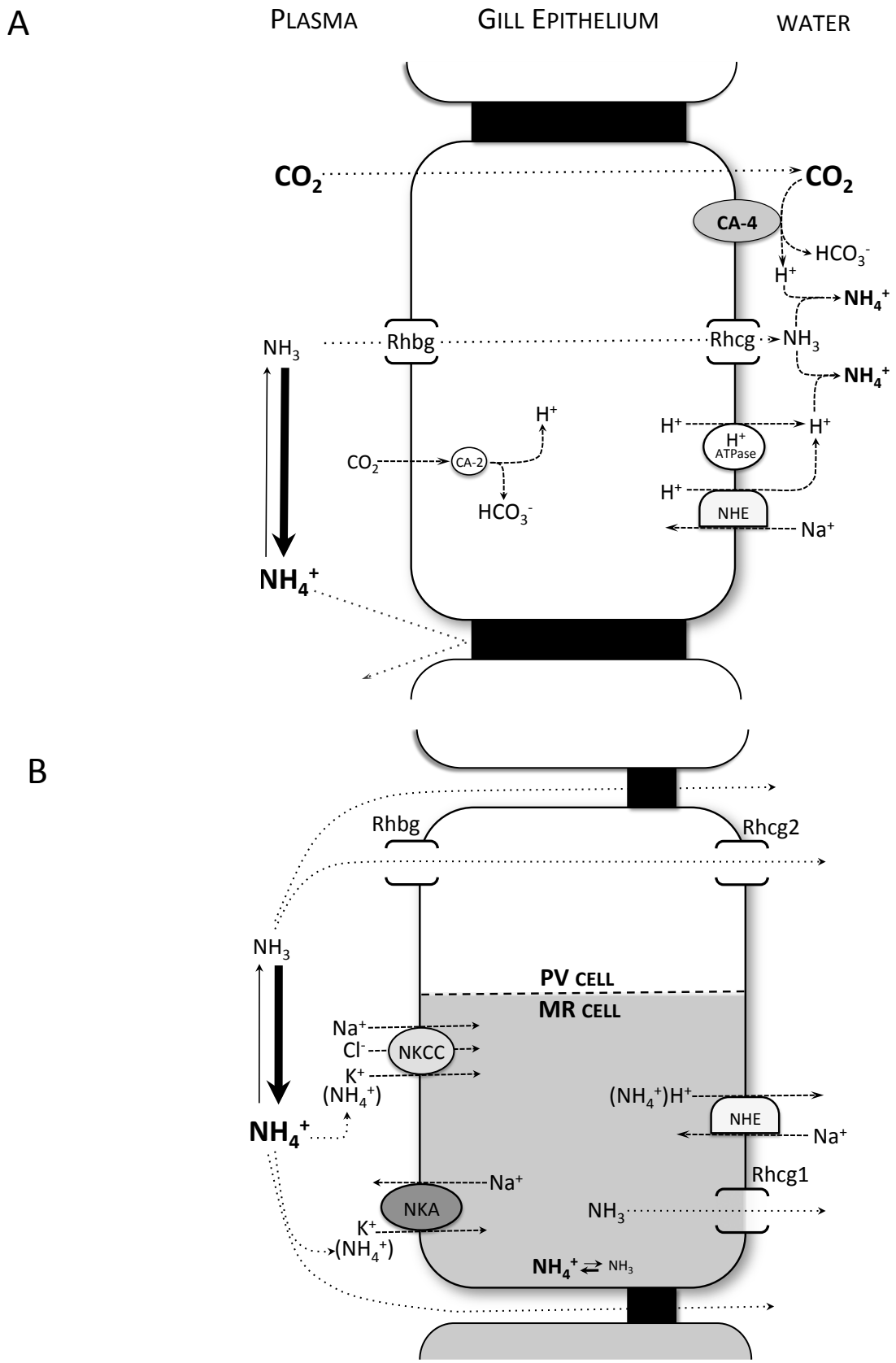

A
PLASMA
GILL EPITHELIUM
WATER
CO2
CO2
CA-4
HCO3-
H+
NH4+
NH3
Rhbg
Rhcg
NH3
NH4+
H+
CO2
CA-2
H+
HCO3-
H+
H+
ATPase
H+
NHE
Na+
NH4+
B
Rhbg
Rhcg2
NH3
PV CELL
MR CELL
Na+
Cl-
NKCC
K+
(NH4+)
NH4+
(NH4+)H+
NHE
Na+
Rhcg1
Na+
NKA
NH3
K+
(NH4+)
NH4+ ⇄ NH3

Figure 1.1

Mechanisms of urea synthesis and excretion

Although they are generally ammoniotelic, most adult teleosts have the capacity to synthesize and excrete small to moderate amounts of urea. Most freshwater species excrete 5-35%, whereas marine species tend to eliminate approximately 40% of the total nitrogenous waste as urea (reviewed by Wood, 2001). In the latter group, urea may be produced and excreted in larger quantities because of its role as a minor organic osmolyte (Wood, 1993; Wright et al., 1995b). Excreted urea in ammoniotelic fish is derived from catabolic routes involving the degradation of dietary arginine by arginase and purines via uricolysis (Anderson, 2001; Korsgaard et al., 1995; Wood, 1993).

Urea production in ureotelic teleosts is attributed to the expression of a fully functional ornithine-urea cycle (O-UC) present in the liver and muscle (Figure 1.2; reviewed by Anderson, 2001). Ureogenesis is a monophyletic trait within teleosts (Mommsen and Walsh, 1989); functional expression of the O-UC is thought to be a trait retained by select adult teleosts from earlier embryonic stages (Wright et al., 1995). Relatively high levels of messenger RNA transcripts and O-UC enzyme activities have been reported in a number of teleost species as early as 3 days post-fertilization (Chadwick and Wright, 1999; Korte et al., 1997; Terjesen et al., 2001; Wright et al., 1995). Urea cycle enzymes are hypothesized to prevent the accumulation of ammonia from protein catabolism during early embryonic stages when ammonia diffusion across the chorion might be problematic (Griffith, 1991; Wright et al., 1995). This capacity appears to be lost shortly after hatch, resulting in minimal or non-detectable levels of O-UC enzymes in most adult teleosts (Wright et al., 1995).

The permeability and transport of urea are tightly controlled processes. Urea permeability across artificial lipid bilayers is relatively low (Galluci et al., 1971), and in many species the movement of urea occurs at much higher rates than can be explained by

simple diffusion alone (Sands, 2002). The data suggest the presence of specialized urea transport proteins within cell membranes. Most information on facilitated urea transporters (UTs) has been generated from mammalian models. Molecular studies have established that there are two similar yet distinct genes, UT-A (Slc14a2) and UT-B (Slc14a1), responsible for the production of mammalian urea transport proteins (reviewed by Sands, 2000; Sands, 2002; Sands, 2003). Originally cloned from rabbit (You et al., 1993) and subsequently from mouse and human (Bagnasco et al., 2001; Fenton et al., 2002a), UT-A is complex (reviewed in Bagnasco, 2005). Two promoters, termed UT-Aα and UT-Aβ, are responsible for the transcription of up to seven UT-A isoforms (Smith and Fenton, 2006). The largest mammalian transcript is UT-A1 that encodes for a protein composed of 927 amino acids (reviewed by Sands, 2002; Walsh and Smith, 2001). In contrast, UT-B is a significantly smaller gene with a single promoter that encodes only one protein with two isoforms (Sands, 2002). Both UT-A and UT-B play crucial roles in renal physiology. UT-A transporters facilitate diffusion of urea across the inner medullary collecting duct and the thin descending limb of the loop of Henle in the mammalian kidney, and UT-B transporters are responsible for the rapid transfer of urea across the vasculature (reviewed by Bagnasco, 2005). These transporters are characterized by their sensitivity to sub-millimolar concentrations of phloretin and a variety of competitive urea analogues such as thiourea and acetamide (Chou and Knepper, 1989). Renal UT abundance is modulated in response to vasopressin (Shayakul et al., 2000; Trinh-Trang-Tan et al., 2002; Wade et al., 2000), glucocorticoids (Peng et al., 2002), mineralocorticoids (Gertner et al., 2004), cAMP (Nakayama et al., 2001), and hypertonicity (Nakayama et al., 2000) through genomic and non-genomic pathways.

In fish, UTs are crucial for the removal of urea in both ureotelic and ammoniotelic species (reviewed by McDonald et al., 2006a). Branchial UTs have been isolated and characterized in the obligate ureotele *A. grahami* (Walsh et al., 2001), the facultative ureotele *P. dolloi* (Wood et al., 2005), and ammoniotelic species such as the rainbow trout (McDonald and Wood, 2003; McDonald and Wood, 2004a), plainfin midshipman (*Porichthys notatus*; (McDonald et al., 2002), zebrafish (Braun et al., 2009a; Braun et al., 2009b), and Japanese eel (*Anguilla japonica*; Mistry et al., 2001). Phylogenetic and structural analyses have revealed that these piscine UTs share a high degree of sequence similarity with the mammalian UT-A2 (Bagnasco, 2003). Recently, a novel urea transporter, UT-C, was identified in *T. rubripes* and *A. japonica*. UT-C is a facilitated urea transporter that is highly expressed in the seawater eel kidney and plays an important role in the transepithelial reabsorption of urea (Mistry et al., 2005). UT-C has minimal identity ($\leq$43%) with other piscine and mammalian UTs. Database searches did not detect UT-C in any mammalian genomes, and at present it is unclear whether UT-C expression is limited to marine teleosts (Mistry et al., 2005). Mistry and colleagues speculate that an ancestral UT was duplicated to produce the UT-C and UT genes, the latter being further duplicated to yield UT-A (Slc14A2) and UT-B (Slc14A1) in the mammalian lineage. Collectively, the identification of these genes and their products raises interesting questions about the evolutionary origins of the UTs.

Figure 1.2: The ornithine-urea (O-UC) cycle consists of carbamoyl phosphate synthetase (CPSase), ornithine transcarbamylase (OTCase), argininosuccinate synthetase (ASS), argininosuccinate lysase (ASL), and arginase (reviewed by (Wright and Fyhn, 2001). Unlike CPSase I in the mammalian O-UC that requires ammonia as a nitrogen-donating substrate, teleost CPSase III uses glutamine and therefore glutamine synthetase (GSase) is a necessary accessory enzyme (Anderson, 2001). Modified from Anderson (2001).

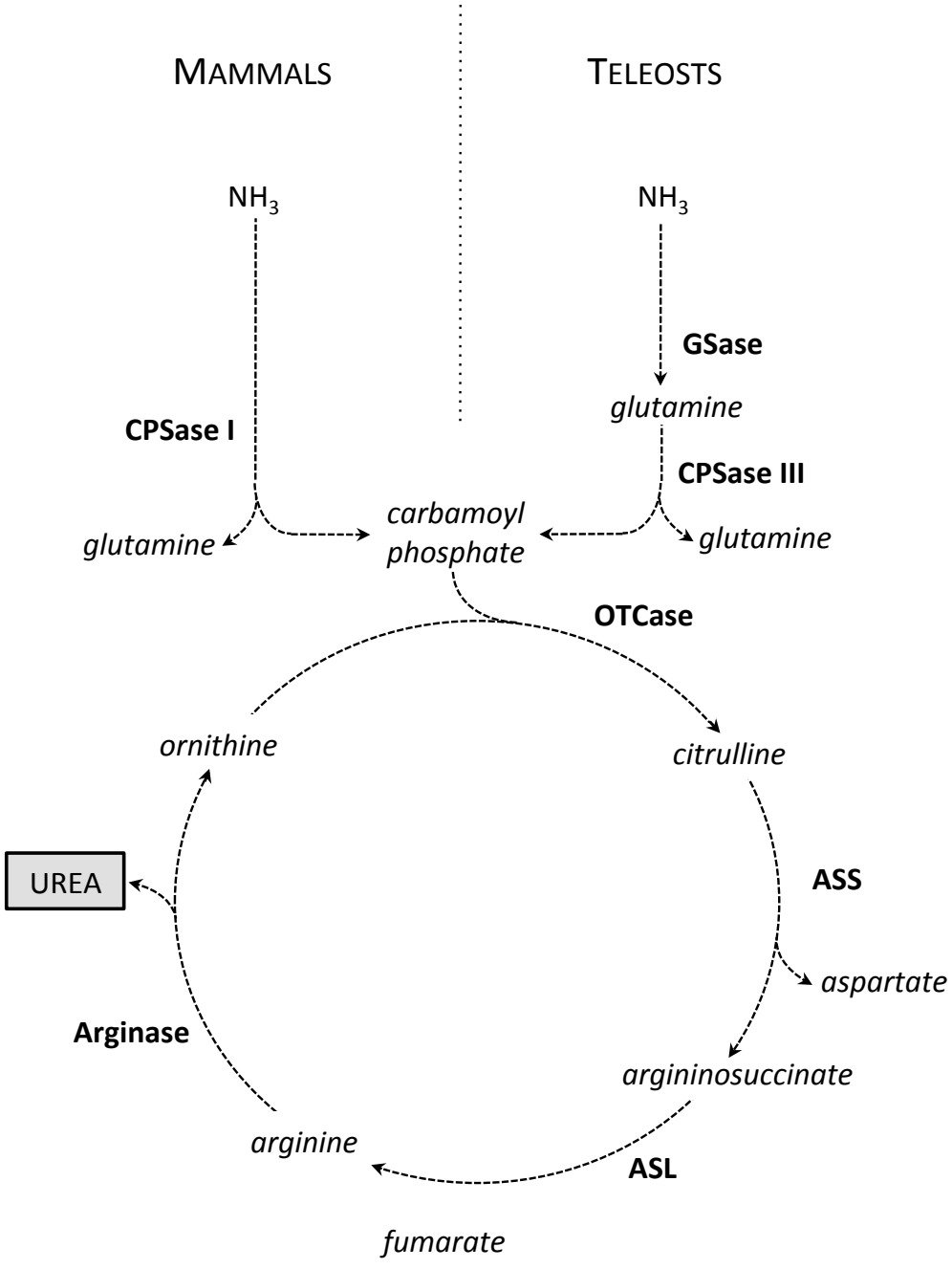

MAMMALS
TELEOSTS
NH3
NH3
GSase
glutamine
CPSase I
CPSase III
glutamine
carbamoyl phosphate
glutamine
OTCase
ornithine
citrulline
UREA
ASS
aspartate
Arginase
argininosuccinate
arginine
ASL
fumarate

Figure 1.2

The corticosteroid-signaling pathway in vertebrates

General overview

In mammals, corticosteroids are essential for the regulation of a variety of physiological functions, including glucose metabolism, lipid and protein homeostasis, and mineral balance (Evans, 1988). These functions are divided between two distinct classes of corticosteroids that are produced and secreted by the adrenal gland. Glucocorticoids, including cortisol and corticosterone, were initially identified for their role in regulating glucose metabolism in mammals, but it is now known that they also mediate the stress response, play important roles in cognition, act as immune suppressants and anti-inflammatories, and regulate salt balance (Burnstein and Cidlowski, 1989; Evans, 1988; Fuller et al., 2000). Mineralocorticoids, primarily aldosterone, were first recognized to regulate sodium and potassium retention in the kidney but also to play a role in regulating blood pressure and perspiration (Johnson, 1992; Sheppard, 2002).

The structure of corticosteroid receptors

The effects of glucocorticoids and mineralocorticoids are mediated by separate, well-characterized receptors that belong to the steroid/thyroid receptor superfamily. The glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) share a standard structure incorporating distinct functional domains, A through E, from the amino terminus to the carboxy terminus, respectively (Evans, 1988). The A/B-domain controls the intracellular localization and nucleocytoplasmic trafficking of the receptor (reviewed by Bury and Sturm, 2007). Within this region, the transactivation site, activation function 1 (AF-1), interacts with basal transcription factors or coactivators to induce ligand-insensitive transactivation (Dahlman-Wright et al., 1995). The A/B-domain is variable in both length and sequence

among different species. By contrast, the C-domain is highly conserved among various receptor subtypes and plays an integral role in DNA binding and receptor dimerization (Bury and Sturm, 2007). In this region there are two clusters of cysteines that facilitate the retention of two zinc atoms, designated as zinc fingers, that are separated by a short sequence (12-21) of amino acids (Baumann et al., 1993; Bury et al., 2003; Ducouret et al., 1995; Freedman et al., 1988; Greenwood et al., 2003). These structures are essential for homodimerization of the GR and the interactions between the receptor protein and the DNA target sequence (a glucocorticoid response element; Baumann et al., 1993; Freedman et al., 1988). The D domain initiates conformation changes that accompany receptor binding. The E-domain is the site for ligand-binding at the carboxyl terminus and within this region there are highly conserved areas that are critical for hormone binding. The similar sequence identity between the GR and MR within this region confers considerable overlap in the pharmacology of these two receptor types (Arriza et al., 1987; Hollenberg et al., 1985). Although aldosterone binds with high specificity only to MRs, glucocorticoids bind to both MRs and GRs (Krozowski and Funder, 1983; Reul et al., 2000). The discrimination of ligands by corticosteroid receptors may relate to the presence of an additional transactivation site (AF-2) within the E-domain and it is believed that interactions between the AF-1 and AF-2 sites modulate gene expression (Glass and Rosenfeld, 2000; Pascual-Le Tallec and Lombes, 2005). However, the precise molecular mechanisms have yet to be determined.

Corticosteroid receptors in teleosts

The first full-length GR, rtGR1$_a$, was isolated in rainbow trout and was highly expressed in the gill, with moderate levels in the liver, kidney, and intestine (Ducouret et al., 1995). A splice variant, rtGR1$_b$, characterized by the absence of nine amino acid residues

within the DNA-binding domain of the receptor, was subsequently identified in the testes of rainbow trout (Takeo et al., 1996). In the presence of cortisol, both splice variants induced transcriptional activity in cells co-transfected with expression vectors containing a GR and a reporter plasmid containing GREs (Ducouret et al., 1995; Takeo et al., 1996). More recently, an additional GR isoform (rtGR2) was identified in rainbow trout (Bury et al., 2003). Functional characterization revealed that rtGR2 was ~60 times more sensitive to cortisol than $rtGR1_a$, with EC_{50} values of 0.72 nM and 46 nM, respectively. Prunet et al. (2006) suggested that each receptor isoform may have a distinct role; rtGR1 may be mobilized at basal circulating cortisol concentrations, whereas rtGR1 and rtGR2 may collectively coordinate cortisol-mediated responses at elevated (stressed) concentrations. A similar suite of glucocorticoid receptors (hbGR1, hbGR2a, hbGR2b) was identified in the cichlid *Haplochromis burtoni* but no differences in sensitivity to cortisol (i.e. EC_{50}) were observed among the various isoforms (Greenwood et al., 2003). In contrast, only one GR with two splice variants, GRα and GRβ, has been discovered in zebrafish (Alsop and Vijayan, 2008a; Schaff et al., 2008). GRα binds cortisol with high affinity, whereas GRβ does not bind cortisol but rather inhibits GRα activity in a system that is reminiscent of the human GR. Phylogenetic analyses of vertebrate GRs suggest that non-functional mutations may have initiated a return to a single gene system within the zebrafish lineage (Alsop and Vijayan, 2009).

Recently, cDNA sequences encoding for an MR homolog have been isolated in several species of teleost (Arterbery et al., 2010; Colombe et al., 2000; Greenwood et al., 2003; Sturm et al., 2005). This receptor is broadly distributed among tissues, with highest expression in the brain (Greenwood et al., 2003; Sturm et al., 2005). Steroidogenesis studies

have consistently reported that fish lack the enzyme aldosterone synthase necessary to produce aldosterone (Balm et al., 1989; Jiang et al., 1998; Sangalang and Uthe, 1994). However, the piscine MR is responsive to a number of corticosteroids. In survey of 8 steroid hormones, Sturm et al. (2005) found that aldosterone and 11-deoxycorticosterone (DOC) were most effective in initiating transactivation of the rainbow trout MR (rtMR) *in vitro*. Because the rainbow trout GR is insensitive to DOC (Bury et al., 2003), Sturm et al. (2005) suggested that DOC may function as an *in vivo* MR agonist; experimental evidence is, however, mixed on this issue (McCormick et al., 2008). Evidence from numerous studies also demonstrates that the piscine MR acts as a high affinity cortisol receptor (Colombe et al., 2000; Greenwood et al., 2003; Sturm et al., 2005), similar to the mammalian MR (Hellal-Levy et al., 2000). However, mammalian MRs are protected from glucocorticoid-induced changes by co-localization of the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2), an enzyme that converts cortisol to cortisone (Edwards et al., 1988; Funder et al., 1988b). A similar enzyme (11βHSD2) has been isolated in fish and the enzyme transcripts exhibit a wide distribution that is in agreement with the localization of MR (Jiang et al., 1998; Kusakabe et al., 2003). Thus, 11βHSD2 in teleosts may prevent cortisol from activating the MR; however, further study is required to confirm this hypothesis.

Mechanisms of corticosteroid action

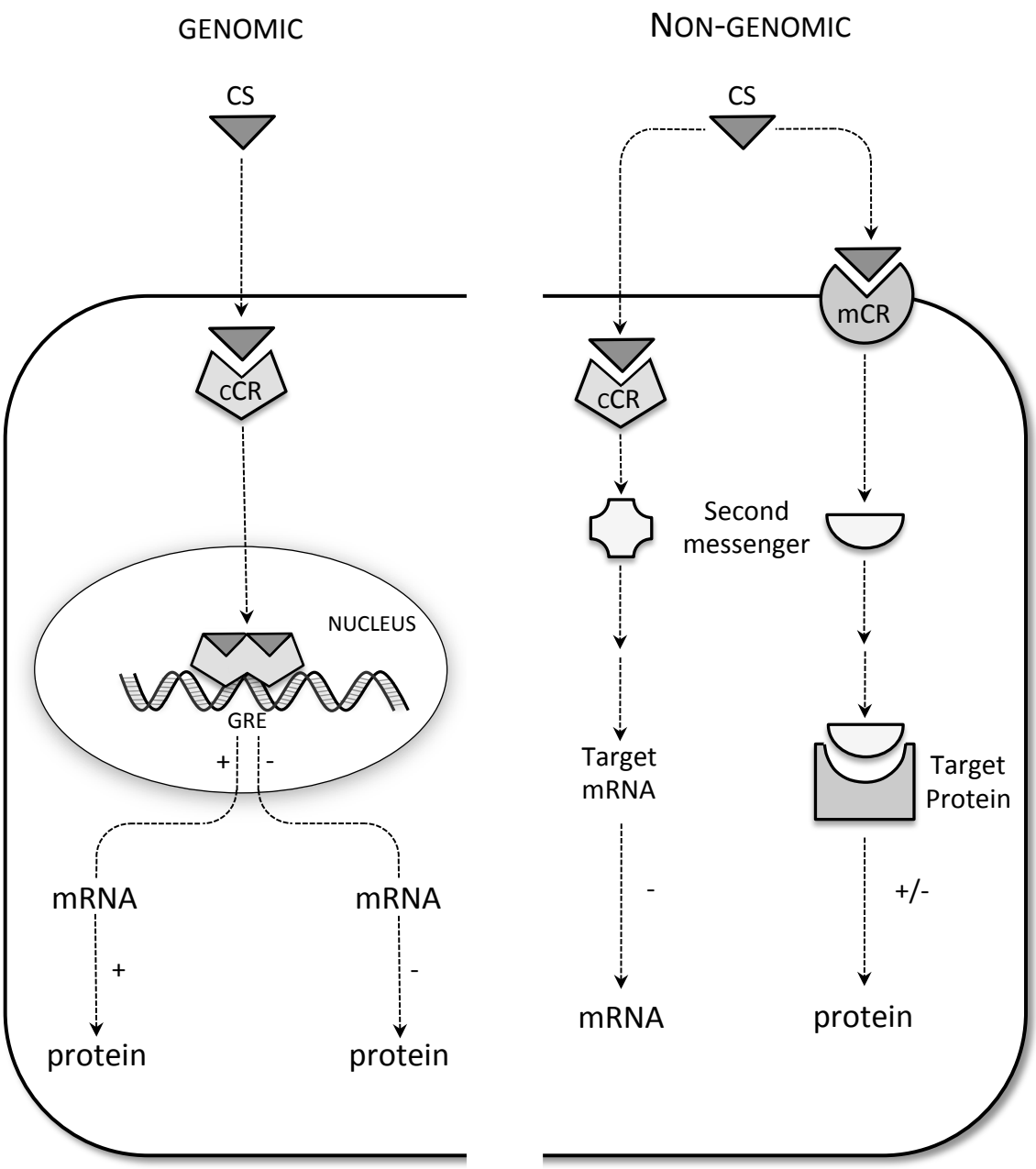
Corticosteroids influence transcription of their target genes through DNA-dependent mechanisms. In the traditional model of corticosteroid action, the binding of a ligand to a cytosolic GR or MR results in receptor activation and translocation of the ligand-receptor complex to the nucleus (Figure 1.3; Fuller et al., 2000). Once in the nucleus, the hormone-ligand receptor complex binds as a dimer to one or more specific DNA motifs, termed

glucocorticoid response elements (GRE; reviewed by Schoneveld et al., 2004). Cooperative binding of homodimers to GREs facilitates the recruitment of additional nuclear transcription factors and initiates transcription of target genes. Four types of DNA motifs have been identified: simple GREs, composite GREs or glucocorticoid response units (GRUs), half site (GRE1/2s), and negative GREs (nGREs). Simple GREs are an imperfect palindrome sequence (e.g. GGTACA nnn TGTTCT) that is recognized by both the GR and the MR to activate transcription (Beato et al., 1995; Bury and Sturm, 2007; Nelson et al., 1999). For some genes, the binding of a GR/MR homodimer to a GRE is not sufficient and additional transcription factors adjacent to the DNA binding sites are required (Schoneveld et al., 2004). These composite GREs, more properly termed glucocorticoid response units (GRUs), exhibit considerable variability in their composition, including 4 to 10 GREs and tissue-specific accessory factors (Schoneveld et al., 2004). Generally, the organization of GRUs allows significantly higher levels of transcription than simple GREs. GRs can also bind as a monomer at GRE half-sites (GRE1/2) to induce transcription, however, they require additional transcriptional elements to mediate a glucocorticoid response (Segard-Maurel et al., 1996). By contrast, nGREs influence gene expression by binding to a consensus sequence that overlaps other regulatory elements (i.e. TATA box) to suppress transcription (Truss and Beato, 1993).The latency time for these molecular mechanisms ranges from 30 min to hours or days, and their effects tend to persist for several hours or days after corticosteroid removal.

The action of corticosteroids is not limited to influences on gene transcription; post-transcriptional modifications increasingly are being recognized as important components of corticosteroid regulation (Figure 1.3). For example, Stellato (2004) summarized the inhibitory role of glucocorticoids using inflammatory pathways as a case study. In particular,

glucocorticoids have been shown to inhibit the P38, extracellular signal-regulated kinase (ERK), and the c-Jun N-terminal kinase (JNK) pathways to inhibit mRNA translation, turnover, and transport, respectively (Stellato, 2004). Under these circumstances, RNA binding factors are considered glucocorticoid targets. In an alternative, non-genomic model, the hormone binds to a membrane-bound corticosteroid receptor (Figure 1.3), initiating a cascade of intracellular events that involves changes in membrane potential, ion fluxes, and/or mobilization of second messengers (reviewed by Borski, 2000). The latency period for non-genomic effects is short, with physiological changes occurring within seconds to minutes (Borski, 2000).

Figure 1.3: The genomic and non-genomic pathways of corticosteroid action. In the genomic model, a corticosteroid steroid (CS) binds to a cytoplasmic corticosteroid receptor (cCR) that acts as a ligand-dependent transcription factor. The ligand-receptor complex is translocated to the nucleus where it interacts with a glucocorticoid response element (GRE) to enhance or inhibit transcription of mRNA, ultimately influencing protein expression. Corticosteroids can also exert non-genomic effects through cytoplasmic CRs or membrane-bound CRs (mCR) that initiate intracellular events involving second messengers (i.e. camp, Ca^{2+}). These non-genomic pathways may decrease mRNA abundance or alter protein expression.


GENOMIC
NON-GENOMIC
CS
CS
cCR
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NUCLEUS
GRE
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Second
messenger
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mRNA
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+
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Figure 1.3

The gulf toadfish (*Opsanus beta*)

General overview

The gulf toadfish *Opsanus beta* (Goode & Bean) is one of the most abundant benthic fish in South Florida estuaries and is commonly found in seagrass beds (Serafy et al., 1997; Sogard et al., 1987). It has been used in studies focusing on aspects of its physiology, toxicology, and ethology (e.g. Bass et al., 2001; Grosell et al., 2004a; Grosell et al., 2004b; Kennedy et al., 1989; Kennedy et al., 1992; Remage-Healey and Bass, 2005; Taylor and Grosell, 2006a; Taylor and Grosell, 2006b; Walsh, 1989). What sets the gulf toadfish apart from other species within the Batrachiodidae family is its unusual nitrogen excretion strategy. Field and outdoor mesocosm studies revealed that toadfish co-excrete equal quantities of ammonia and urea under natural conditions (Barimo et al., 2007; Barimo et al., 2004; Barimo et al., 2010). Exposure to stressful conditions in the laboratory, including crowding, confinement, emersion, or elevated ambient ammonia causes toadfish to excrete predominantly urea in a unique pulsatile fashion (reviewed by Wood et al., 2003a).

Mechanisms of urea excretion

The transition to ureotely in gulf toadfish involves a significant reduction in ammonia excretion coupled with a moderate rise in urea excretion (Walsh and Milligan, 1995; Walsh et al., 1994). The changes are attributed to a stress-induced increase in GSase activity (Hopkins et al., 1995; Wood et al., 1995; Wood et al., 1997) that enables more ammonia to be shuttled into the O-UC to enhance urea production (Hopkins et al., 1995; Walsh and Milligan, 1995; Walsh et al., 1994). Despite the accumulation of urea from continuous synthesis, toadfish excrete urea across the gills in discrete pulses that occur up to

several times a day, with each pulse lasting up to 3 hours (Gilmour et al., 1998; Wood et al., 1995; Wood et al., 1997); between pulses little or no urea is excreted (Wood et al., 2003). The transport of urea is sensitive to classical mammalian UT inhibitors including phloretin (Walsh et al., 2000; Wood et al., 1998) and numerous urea analogues (McDonald et al., 2000). Molecular confirmation of a UT protein was obtained when a cDNA encoding for a 475-amino acid residue was cloned from toadfish gill (Walsh et al., 2000). This protein exhibited over 60% sequence similarity to the mammalian UT-A2 type urea transporter. The periodic increases in branchial urea permeability are thought to be facilitated by the insertion or activation of a urea transport protein (tUT) into the apical membrane of the MRCs in the gill (McDonald et al., 2009; Walsh et al., 2000; Wood et al., 1998; Wood et al., 1995; Wood et al., 1997).

Endocrine control of nitrogen excretion in toadfish

In recent years, significant progress has been made in understanding the regulation of pulsatile urea excretion in the toadfish. The monoamine 5-hydroxytryptamine (5-HT) and the glucocorticoid cortisol are important proximate signals for regulating pulse frequency and size, respectively (McDonald and Walsh, 2004; McDonald et al., 2004; Wood et al., 1997; Wood et al., 2003). Arterial injection of 5-HT and 5-HT_2 receptor agonists elicited substantial urea pulses comparable in size and duration to natural pulse events (McDonald and Walsh, 2004; Wood et al., 2003). and that were inhibited in a dose-dependent fashion by the 5-HT_2 receptor antagonist ketanserin (McDonald and Walsh, 2004). The authors suggested that 5-HT might activate tUT *via* a 5-HT_2 receptor-mediated phosphorylation of two unique sites on the C-terminus of the urea transport

protein (McDonald and Walsh, 2004; Walsh et al., 2000). However, the precise molecular mechanisms involving 5-HT-induced urea excretion have yet to be determined.

Although, cortisol has been implicated in the unique pulsing phenomenon in toadfish for some time (Wood et al., 2001), its regulatory role is not completely understood. In two separate *in vivo* studies, plasma cortisol levels were reported to fall markedly 2-4 h prior to a pulse and rise rapidly thereafter (Wood et al., 1997; Wood et al., 2001). McDonald et al. (2004) demonstrated that this decrease in plasma cortisol was a permissive event, i.e. necessary but not sufficient to trigger a urea pulse (McDonald et al., 2004). Moreover, the amount of urea released during a pulse is dependent on plasma cortisol levels; elevated cortisol inhibits tUT function (McDonald et al., 2009; McDonald et al., 2004). Perfused gill and skin experiments confirmed that the gill is the primary glucocorticoid target; cortisol treatment decreased gill urea permeability by 35-fold (Part et al., 1999; Wood et al., 1998). These findings suggest that high levels of cortisol downregulate the toadfish UT but it is clear that further investigation is necessary to determine the precise mechanisms of corticosteroid action in pulsatile urea excretion in toadfish.

Goals of Thesis

The mechanisms underlying the effects of cortisol on urea excretion in gulf toadfish remain unclear (McDonald et al., 2004). The primary goal of this thesis was to examine the glucocorticoid-sensitive mechanisms involved in regulating nitrogen excretion in gulf toadfish. Chapter 2 examined the roles of the GR and MR in pulsatile urea excretion

in the toadfish. It was hypothesized that cortisol exerts its effects on urea pulse size through the GR. I addressed GR- and MR-mediated *in vivo* changes in urea excretion in toadfish with pharmacologically lowered corticosteroid activity. In chapter 3, I tested the hypothesis that urea movement across the gill basolateral membrane in gulf toadfish occurs through a cortisol-sensitive carrier-mediated mechanism. An *in vitro* approach was used to isolate basolateral membrane vesicles and characterize urea transport in the presence and absence of cortisol through a series of kinetic analyses. Chapter 4 focused on the molecular mechanisms involved in corticosteroid sensitivity in ureotelic toadfish. Two primary objectives were examined using a combination of physiological and molecular techniques. First, changes in tUT transcript levels in response to endogenous and exogenous fluctuations in circulating cortisol were characterized. The second objective tested the hypothesis that cortisol-mediated changes in tUT expression reflected the presence of GRE motif(s) within the promoter region of the tUT gene. The focus of the last two data chapters was ammonia excretion. The literature on nitrogen excretion in toadfish is unbalanced; urea excretion has received considerable attention with little emphasis on ammonia excretion. The recent discovery of Rh glycoproteins is allowing ammonia excretion to be revisited. In chapter 5, a series of *in vivo* experiments was used to test the hypothesis that Rh proteins contribute to ammonia excretion in toadfish. In the final data chapter (6), the hypothesis that cortisol regulates branchial Rh expression, and hence ammonia excretion, was tested.

CHAPTER 2

The regulatory role of glucocorticoid and mineralocorticoid receptors in pulsatile urea excretion of the gulf toadfish, *Opsanus beta*

Notes on Chapter 2

The present chapter has been published in the Journal of Experimental Biology as per the following citation:

Rodela, T.M., McDonald, M.D., Walsh, P.J., and Gilmour, K.M. (2009) The regulatory role of glucocorticoid and mineralocorticoid receptors in pulsatileurea excretion of the gulf toadfish, *Opsanus beta*. *J Exp Biol* 212 (12):1849-1858.

M.D. McDonald was a collaborator at the University of Miami, where *in vivo* experiments were conducted by T.M. Rodela.

Abstract

Gulf toadfish, *Opsanus beta*, are one among a group of unusual teleosts that excrete urea as their predominant nitrogen end product in response to stressful conditions. Under conditions of crowding or confinement, fasted toadfish excrete the majority of their nitrogen waste in large pulses of urea (>90% of total nitrogen) lasting up to 3 hours. An earlier study demonstrated that cortisol has an inhibitory influence on urea pulse size. The present study tested the hypothesis that cortisol mediates changes in urea pulse size in ureotelic toadfish through the glucocorticoid receptor (GR) and not the mineralocorticoid receptor (MR). *In vivo* pharmacological investigations were used to manipulate the corticosteroid system in crowded toadfish, including experimentally lowering plasma cortisol levels by the injection of metyrapone, blocking cortisol receptors through exposure to either RU-486 (GR antagonist) and spironolactone (MR antagonist), or through exogenous infusion of the tetrapod mineralocorticoid aldosterone (tetrapod MR agonist). The data demonstrate that lowering the activity of cortisol, either by inhibiting its synthesis or by blocking its receptor, resulted in a 2- to 3-fold increase in pulse size with no accompanying change in pulse frequency. Treatment with spironolactone elicited a minor (~1.5-fold) reduction in pulse size, as did aldosterone treatment, suggesting that the anti-mineralocorticoid spironolactone exhibits agonistic behaviour in a piscine system. In summary, the evidence suggests that urea transport mechanisms in pulsing toadfish are upregulated in response to low cortisol, mediated primarily by GRs, and to a lesser extent MRs.

Introduction

The gulf toadfish, *Opsanus beta*, is one of several unusual teleosts that have the capacity to synthesize urea de novo *via* the ornithine-urea cycle (O-UC) and excrete substantial quantities of this nitrogen waste. In their natural habitat, toadfish excrete equal amounts of ammonia and urea (Barimo et al., 2004), however, upon transfer to the laboratory there is a transition to predominantly urea excretion, that is triggered by stressful conditions such as crowding, confinement, exposure to air and/or elevated ambient ammonia (Walsh and Milligan, 1995a; Walsh et al., 1994a; Walsh et al., 1990a). This transition to ureotelism is initiated by a moderate and transient (<24 hours) surge in plasma cortisol (Hopkins et al., 1995) that induces hepatic glutamine synthetase (GSase) activity. GSase is a feeder enzyme controlling nitrogen entry as glutamine into the O-UC; induction of GSase causes an increase in production and raises circulating urea levels. A simultaneous decrease in ammonia excretion sets the overall prerequisites for the urea pulsatile excretion mechanism of the gulf toadfish (Walsh and Milligan, 1995). This situation can be contrasted against the majority of teleosts that have a constant branchial clearance of their nitrogenous wastes largely as ammonia. In ureotelic toadfish, urea is voided from the body via the gills a few times daily in distinct pulses that last up to 3 hours. Between pulse events, little to no urea is excreted (Wood et al., 1995; Wood et al., 1997; Wood et al., 1998). Each pulse episode is thought to be the result of the insertion or activation of a facilitated urea transport protein, tUT, that shows high sequence similarity to the mammalian UT-A2 isoform (Part et al., 1999; Walsh et al., 2000; Wood et al., 2003).

The pulse event appears to be under hormonal control *via* the monoamine 5-hydroxytryptamine (5-HT; serotonin) and the corticosteroid cortisol. The initiation of a pulse event ultimately is controlled by 5-HT, as arterial injection of 5-HT or 5-HT_2 agonists elicited urea pulses comparable in size and duration to natural pulse events (McDonald and Walsh, 2004; Wood et al., 2003). These induced urea pulses could be inhibited in a dose-dependent fashion by the 5-HT_2 receptor antagonist ketanserin (McDonald and Walsh, 2004). Experiments employing repetitive blood sampling have demonstrated that plasma cortisol levels fall markedly (from approximately 120 to 40 ng mL^{-1}) 2 to 4 hours before a pulse event and rise rapidly thereafter (Wood et al., 1997; Wood et al., 2001). However, this reduction in cortisol seems to be permissive rather than a causal agent, as plasma cortisol may decline without an associated pulse occurring (Wood et al., 2001). Furthermore, chronically infusing toadfish with cortisol to maintain plasma values at high concentrations (~500 ng mL^{-1}) caused a significant reduction in urea excretion accompanied by a rise in plasma urea-N concentration over the subsequent 36 to 96 hours (McDonald et al., 2004). This decrease was attributed to a reduction in pulse size, but not pulse frequency (McDonald et al., 2004).

In teleosts, the physiological effects of corticosteroids are mediated through specific intracellular receptors that act as ligand-dependent transcription factors. In 1995, the first full-length cDNA of a piscine GR (rtGR1) was isolated from rainbow trout (Ducouret et al., 1995). The discovery of a second trout GR isoform (rtGR2), that showed significant sequence disparity in the A/B domain of the gene, indicated that the two isoforms (GR1 and GR2) are encoded by two distinct genes, both of which are

sensitive to cortisol (Bury et al., 2003). A similar situation has been reported in *Haplochromis burtoni*, but in addition to the two isoforms, a splice variant of the GR2 has also been discovered (Greenwood et al., 2003). The corticosteroid signaling pathway was further complicated by the isolation of a mineralocorticoid receptor (MR) that has higher sensitivity for cortisol than either the rtGR1 or rtGR2 (Alsop and Vijayan, 2008; Colombe et al., 2000; Greenwood et al., 2003; Stolte et al., 2008; Sturm et al., 2005). In tetrapods, the MR is normally activated by the mineralocorticoid aldosterone, and the avoidance of MR activation by cortisol is controlled by co-localization of the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) that catalyzes the conversion of cortisol to receptor-inactive cortisone (Funder et al., 1988). Recent evidence suggests a comparable system may be present in *Salmo salar* where a profound upregulation of 11β-HSD2 may protect branchial MRs from the cortisol surge (Killerich et al., 2007). The same cortisol surge has an essential role in the GR-mediated development of the parr-smolt transformation and saltwater phenotype.

In ureotelic toadfish, the inhibitory influence on urea pulse size of exogenous cortisol loading could be abolished by injection of the potent GR antagonist RU-486 but not the mammalian MR antagonist spironolactone (McDonald et al., 2004). In the present study we sought to extend these findings by investigating in further detail the influence of cortisol on urea excretion in the toadfish. In particular, while McDonald et al. (2004) emphasized hyperactivation of the corticosteroid pathway by *increasing* circulating cortisol levels, we wished to address similar questions of GR- or MR-mediated regulation in fish with physiological and/or experimentally *lowered* corticosteroid action. Based on the evidence to date, the hypothesis we chose to test in

this study is that changes in urea pulse size in ureotelic toadfish are mediated by cortisol through the GR. Although McDonald et al. (2004) found that the effects of cortisol loading in toadfish were unaffected by spironolactone, recent evidence suggests that spironolactone can act as an agonist of the rtMR, suggesting that this compound may exhibit different behaviour in piscine and mammalian systems (Sturm et al., 2005). Therefore, the role of the MR was further investigated using a range of spironolactone concentrations as well as the tetrapod MR ligand aldosterone, a hormone that teleosts cannot synthesize (Prunet et al., 2006).

Materials and Methods

Animals

Sexually mature gulf toadfish (*Opsanus beta* Goode and Bean; 33-179 g) were collected by roller trawl in Biscayne Bay, Florida, USA, by local, commercial shrimpers during the months of January to March 2007. Following capture, toadfish were temporarily housed (<48 hours) in an outdoor tank supplied with running seawater at the shrimpers' holding facility. Prior to transfer to the laboratory, toadfish were given a freshwater dip (2 minutes) followed by a malachite green/formalin treatment (0.05 mg L^{-1}, 15 mg L^{-1}) to prevent infection by the ciliate *Cryptocaryon irritans* (Stoskopf, 1993; Wood et al., 1997a). In the laboratory, toadfish were held in 50 L aquaria, at a density of >10 fish per tank, supplied with aerated flowing seawater (18ºC – 22ºC, pH 8.1) under natural photoperiod. Toadfish were fed with chopped squid once a week.

Experimental protocol

To induce ureotely, groups of 8 to 10 fish were relocated to smaller plastic tubs

(6 L) as outlined in standard crowding protocols (Hopkins et al., 1995; Walsh, 1987; Wood et al., 1997). Fish were maintained under these conditions for 48 hours, until the time of surgery. Caudal artery catheters (McDonald et al., 2000; Wood et al., 1997), and in some cases intraperitoneal (IP) catheters (McDonald and Walsh, 2004; see below), were implanted in fish anaesthetized with MS-222 (1 g L^{-1}, pH 7.8) and covered in moist towels. IP catheters were used to facilitate repeated injection of the different compounds used in this study with minimal handling of the fish. Post-surgery, fish were transferred to individual 2 L flux chambers supplied with flowing, aerated seawater and allowed to recover for 24 hours.

At the initiation of the experiments, 300 μL of blood was withdrawn from each fish *via* the arterial catheter and centrifuged at 10, 000 *g* for 1 minute. An 80 μl aliquot of plasma was removed, flash frozen in liquid nitrogen, and stored at -80°C for later analysis of plasma cortisol and urea. The remaining red blood cells were resuspended in 80 μl of toadfish saline (Walsh, 1987) and re-injected. Water flow to the individual containers was stopped and an initial 2 ml water sample was withdrawn for analysis of urea and ammonia concentrations. A peristaltic pump and fraction collector system was used to continuously collect water samples at a rate of 2 mL per hour for the remainder of the experiment. Every 24 hours boxes were flushed with fresh seawater for 30 min. In between flushes, vigorous aeration was used to ensure oxygen saturation and adequate mixing was maintained in the individual boxes. Four series of experiments were conducted to investigate the involvement of corticosteroids in pulsatile urea excretion.

Series I: Blocking cortisol synthesis with metyrapone

The influence of metyrapone (methyl-1,2-di-3-pyridyl-1-propanone) in diminishing *de novo* cortisol synthesis is well-documented in teleosts (Bennett and Rhodes, 1986; Bernier and Peter, 2001; Milligan, 2003), including the toadfish (Hopkins et al., 1995; Wood et al., 2001). A protocol modified from that of Hopkins et al. (1995) was used. In brief, fish received a dose of either 1.5 μL g^{-1} body mass of saline (150 mmol L^{-1} NaCl) or metyrapone (20 mg mL^{-1} in 150 mmol L^{-1} NaCl) administered *via* IP catheter once every 24 hour period. These treatments were followed by a 1.5 μL g^{-1} body weight saline injection to flush the treatments through the catheter into the peritoneal cavity of the fish. Blood samples were withdrawn at 0 and 24 hours, and immediately following the 24 hour sample fish received their respective treatments; subsequently blood was sampled at 36, 48, 60, 72, 84, and 96 hours.

Series II and III: Blocking GRs with RU-486 *and* MRs with spironolactone

The cortisol receptor antagonist RU-486 (mifepristone) was used to determine the involvement of the GR in regulating pulse size in untreated toadfish in Series II, and in Series III, spironolactone was used to block the MR. Following a 24 hour control period, RU-486 or spironolactone in peanut oil, or peanut oil alone, at a volume of 1.5 μL g^{-1} body weight was administered *via* IP catheter. Each fish in these experiments were exposed to a dose (20, 50, 63, 75, 100, or 125 mg mL^{-1}) of RU-486 (Series II) or spironolactone (Series III). These concentrations were chosen based on similar studies in other fish (McDonald et al., 2004a; Scott et al., 2005; Sloman et al., 2001). The treatment was followed by an additional 1.5 μL g^{-1} body weight peanut oil to flush the

drug or vehicle dose down the length of the IP catheter. Lipid compounds, such as the peanut oil used in the present experiment, are ideal for mediating the slow release of compounds (i.e. RU-486, spironolactone) into the general circulation (Sloman et al., 2001; Vijayan and Leatherland, 1989). Blood samples were withdrawn at 0, 24, 36, 48, 60, and 72 hours.

Series IV: Activating MRs with aldosterone

The aim of the final series of experiments was to investigate the effects on pulsatile urea excretion following activation of the MR *via* infusion of the tetrapod MR agonist aldosterone. Blood samples were first taken from untreated fish at 0 and 24 hours. Following the 24 hour blood sample, the arterial catheter was connected to a Gilson peristaltic pump and fish were infused with either NaCl (150 mmol L^{-1}) or aldosterone (27 μg aldosterone kg^{-1} h^{-1} in 150 mmol L^{-1} NaCl). *In vitro* activation studies on the rtMR have revealed that mineralocorticoids such as aldosterone and 11-deoxycorticosterone (DOC) are 10 times more effective than glucocorticoids in enhancing MR activation in fish (Sturm et al., 2005). Therefore, in the present study the aldosterone concentration (i.e. 9 μg mL^{-1}) was chosen to be 10 times lower than that of the cortisol dose used by McDonald et al. (2004). Furthermore, aldosterone was chosen over DOC as there are diagnostic kits available to measure circulating amounts of aldosterone and thus verify that the fish had received a sufficient dose to elicit a response. Fish in all treatments were infused at a rate of 3 mL kg^{-1} h^{-1}; the rate was verified periodically by measuring the weight of the infusion reservoir. Additional blood samples were taken at 36, 48, 60, 72, 84, 96, 108 and 120 hours.

Analytical techniques

Urea concentrations in seawater and plasma samples were quantified by the diacetyl monoxime method (Rahmatullah and Boyde, 1980) using a ThermoMax microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA). Ammonia content of seawater samples was measured by the indophenol blue method (Ivancic and Degobbis, 1984). The appearance of urea in the water over time was used to calculate both pulse size and pulse frequency, with a threshold value of 40 μmol-N kg^{-1} for a defined urea pulse event as outlined by McDonald et al. (2004). The data are presented as normalized ratios whereby the pulse size or frequency of treatment period was standardized to the respective variables from the control period. Normalized pulse size and frequency are expressed in relative units. In addition, urea or ammonia excretion values (μmol-N kg^{-1}) were calculated as described by (McDonald et al., 2004a) using the following equation:

$$\text{Excretion } (\mu\text{mol-N kg}^{-1}) = [\ \Delta C \times V_f\ ] \div wt\ ,$$

where ΔC represented increases in concentration (μmol/L), V_f was the volume of the experimental flux chambers (L) and the product of both variables was adjusted to the weight (wt) of the individual fish (kg). These values were used to calculate percent ureotelism. Nitrogen excretion (μmol-N kg^{-1}) values during the treatment periods were averaged from multiple 24-hour intervals so they could be compared to the initial 24-hour control period. Excretion values were further divided into total urea (μmol-N kg^{-1}), pulsatile urea (μmol-N kg^{-1}), and non-pulsatile (μmol-N kg^{-1}) components and were reported as normalized ratios whereby the excretion value of the treatment period was standardized to the corresponding component from the control period.

Plasma cortisol and aldosterone (for *Series IV*) concentrations were measured using commercially available ^{125}I radioimmunoassay kits from MP Biomedicals and Diagnostic Systems Laboratories, Inc., respectively.

Statistical analyses

Data are presented as means ± standard error of the mean (s.e.m.). The significance of differences between means for pulse size, frequency, urea excretion components (total and pulsatile), and percent ureotelism were analyzed using a two-way repeated measures (RM) analysis of variance (ANOVA) with time within groups (i.e. before and after application of treatment) as one factor and treatment among or between groups as the other factor. Plasma urea, cortisol, and aldosterone (*Series IV* only) were analyzed using a two-way RM ANOVA with time as one factor and treatment as the second. Where significant differences were detected, a Holm-Sidak post-hoc multiple comparisons test was used to identify differences among groups. All data were checked for normality and equal variances. Where assumptions of normality or equal variances were not satisfied ($p<0.05$), equivalent non-parametric tests were used. Proportional data (i.e. % urea and ammonia) were transformed prior to analysis using $p'=\arcsin\sqrt{p}$ as these values were binomially distributed (Zar, 1999).

Results

Series I

Saline-infused fish were predominantly ureotelic, excreting urea-N at a rate of 87.1± 5.6 μmol-N kg^{-1} hr^{-1} and ammonia-N at 19.3 ± 10.8 μmol-N kg^{-1} hr^{-1} (Table 2.1, RM ANOVA, $p=0.24$). Over the course of the experiment, urea was excreted in a pulsatile

manner with an average pulse size of 866.3 ± 78.6 μmol-N kg^{-1} and mean frequency of 4.5 ± 0.4 per 24 hours; this pulsatile urea excretion accounted for virtually all of the total urea-N excreted. The transition from control period to treatment period (i.e. infusion of saline) was without effect on any of these variables (Figure 2.1A, RM ANOVA, p=0.99; Figure 2.1B, RM ANOVA, p=0.98; Figure 2.1C, RM ANOVA, p=0.99; Table 2.1, RM ANOVA, p=0.97). Plasma urea and cortisol concentrations also remained constant over the duration of the experiment at 5.6 ± 0.2 mmol L^{-1} (Figure 2.1D, RM ANOVA, p=0.96) and 266.4 ± 16.3 ng mL^{-1} (Figure 2.1E, RM ANOVA, p=0.99), respectively.

By contrast, metyrapone treatment caused a significant 0.8-fold reduction and a 4.3-fold increase in, respectively, the percentages of urea and ammonia excreted relative to the pre-treatment period, where urea and ammonia excretion amounted to, respectively, 31.8 ± 3.9 μmol-N kg^{-1} h^{-1} and 2.1 ± 0.2 μmol-N kg^{-1} h^{-1} (Table 2.1, RM ANOVA, p=0.034). Urea excretion occurred in distinct pulses with an average size of 182.9 ± 29.1 μmol kg^{-1} and frequency of 4.9 ± 0.3 per 24 hours. Metyrapone caused a significant 1.7-fold increase in urea pulse size (to 300.8 ± 44.6 μmol kg^{-1}, Figure 2.1A, RM ANOVA, p=0.041) but was without effect on pulse frequency (4.3 ± 0.3 per 24 hours; Figure 2.1B, RM ANOVA, p=0.67). The increase in urea pulse size, in turn, resulted in significant 1.6-fold increase in both total (1314.5 ± 202.1 μmol kg^{-1}, RM ANOVA, p=0.048), pulsatile urea (1304.1 ± 200.4 μmol kg^{-1}, RM ANOVA, p=0.047) excretion relative to control values (Figure 2.1C). The non-pulsatile component (36.3 ± 10.5 μmol kg^{-1}) was unaffected by metyrapone treatment (RM ANOVA, p=0.98). Plasma urea increased significantly by 1.5-fold at 48 hours and by 96 hours was 1.8-fold higher

than the control value (Figure 2.1D, RM ANOVA, p=0.03). Plasma cortisol levels, on the other hand, had decreased significantly after 12 hours of metyrapone treatment and by 96 hours were 6.6-fold lower than the initial control values (Figure 2.1E, RM ANOVA, p<0.001).

Series II

Oil-injected fish excreted urea-N and ammonia-N at a rate of 52.9 ± 5.9 μmol kg^{-1} hr^{-1} and 13.2 ± 3.5 μmol kg^{-1} hr^{-1}, respectively, and the proportion of wastes did not vary over the different phases of the experiment (Table 2.2, RM ANOVA, p=0.64). Pulsatile urea excretion occurred with a frequency of 3.7 ± 0.4 per 24 hours and mean pulse size of 284.2 ± 18.9 μmol kg^{-1} which did change significantly during the trial (Figure 2.2A, t-test, p=0.63 and 2B, t-test, p=0.37, respectively). Total (874.0 ± 107.2 μmol kg^{-1}), pulsatile (832.2 ± 107.1 μmol kg^{-1}), and non-pulsatile (41.8 ± 21.9 μmol kg^{-1}) urea excretion components were unaffected by oil treatment (Figure 2.2C, RM ANOVA, p=0.50). Plasma urea (Figure 2.3A, ANOVA, p=0.17) and cortisol (Figure 2.3B, ANOVA, p=0.85) remained constant over time.

All fish treated with RU-486 remained ureotelic for the duration of the experiment, with urea excretion rates ranging from 39.6 to 112.2 μmol kg^{-1} hr^{-1} (mean = 84.5 ± 5.8 μmol kg^{-1} hr^{-1}) and ammonia excretion ranging from 1.2 to 13.3 μmol kg^{-1} hr^{-1} (mean = 6.7 ± 0.5 μmol kg^{-1} hr^{-1}). Treatment with low concentrations of RU-486 (0 – 63 mg mL^{-1}) did not significantly alter the relative proportions of nitrogenous wastes excreted (Table 2.2), however, RU-486 injections of 75 to 125 mg mL^{-1} significantly increased the percentage of urea excreted and concomitantly reduced the proportion of

ammonia, relative to control values (Table 2.2, RM ANOVA, $p<0.010$). The average urea pulse size of individuals within the RU-486 treatment group ranged from 182.0 to 552.08 μmol kg^{-1} (mean = 347.9 ± 37.6 μmol kg^{-1}) and occurred at frequencies ranging from 3.93 to 6.2 pulses per 24 hours (mean = 4.3 ± 0.2). All RU-486 doses significantly increased urea pulse size by 1.92- to 3.13-fold (Figure 2.2A, RM ANOVA, $p=0.006$), whereas pulse frequency was unaffected (Figure 2.2B, RM ANOVA, $p=0.97$). These increases in pulse size translated into effects on total (mean = 1105.6 ± 400.6 μmol kg^{-1}) and pulsatile (mean = 1085.7 ± 400.8 μmol kg^{-1}) urea excretion only for RU-486 doses of 75 and 100 mg mL^{-1} RU-486, resulting in ~2.4- and ~3.5-fold increases in urea excretion, respectively (Figure 2.2C, RM ANOVA, $p=0.018$). Also in these two treatment groups, plasma urea concentrations fell in a transient fashion relative to the control period and/or the oil-infused treatment group (Figure 2.3A, RM ANOVA, $p=0.031$); plasma urea concentrations were otherwise unaffected in the other treatments (data not shown). Apart from the 125 mg mL^{-1} RU-486 treatment group where initial and final plasma cortisol levels were significantly lower than control values (Figure 2.3B, RM ANOVA, $p=0.025$), cortisol remained constant among treatment groups and did not vary over time (data not shown).

Series III

Fish treated with spironolactone excreted 86.2 ± 1.4 % of their total nitrogenous wastes as urea-N (mean = 46.5 ± 4.2 μmol kg^{-1} hr^{-1}) and 13.8 ± 1.4 % as ammonia –N (mean = 6.5 ± 0.6 μmol kg^{-1} hr^{-1}), with no significant variation in ureotelism between treatment and control periods (data not shown, RM ANOVA, $p=0.51$). Urea pulse size

ranged from 168.2 to 317.3 μmol kg^{-1} (mean = 255.6 ± 18.6) and had a frequency varying from 3.7 to 5.2 (mean = 4.5 ± 0.2) pulses per 24 hours. Treatment had no effect on pulse size for concentrations up to 100 mg mL^{-1} spironolactone (Figure 2.4A, RM ANOVA, p=0.46). Fish treated with 125 mg mL^{-1} of spironolactone had a significant 0.5-fold decrease in pulse size relative to control values (Figure 2.4A, RM ANOVA, p=0.027). Spironolactone treatment had no effect on pulse frequency (Figure 2.4B, RM ANOVA, p=0.729). Total (mean = 1134.4 ± 292.8 μmol kg^{-1}), pulsatile (mean = 997.36 ± 243.8 μmol kg^{-1}), and non-pulsatile (mean = 137.1 ± 16.06 μmol kg^{-1}) urea excretion did not fluctuate significantly between control and treatment periods (Figure 2.4C) for all concentrations except the highest, 125 mg mL^{-1}, which resulted in a significant 0.4-, 0.4-, and 0.5-fold decrease, respectively, relative to control values (Figure 2.4C, RM ANOVA, p=0.018). Plasma urea remained constant over time and treatment (Figure 2.5A, RM ANOVA, p=0.82), excluding the 100 mg mL^{-1} spironolactone treatment group, which had a 1.63-fold higher value at 72 hours than the initial sample at 0 hours (Figure 2.5A, RM ANOVA, p=0.003). Plasma cortisol values remained steady over time and between treatment, excluding the time points at 60 and 72 hours for 20 and 125 mg mL^{-1} that were significantly lower relative to oil control (Figure 2.5B, RM ANOVA, p=0.008).

Series IV

Saline infused fish were ureotelic for the length of the experiment, excreting 85.1 ± 2.5 % as urea-N (mean = 65.3 ± 15.6 μmol kg^{-1} hr^{-1}) and 14.9 ± 2.5 % as ammonia-N (mean = 8.5 ± 1.2 μmol kg^{-1} hr^{-1}). There was no change in the proportion of wastes excreted (data not shown, RM ANOVA, p=0.31). The mean size of the urea pulses

was 376.5 ± 90.7 μmol kg^{-1} and did not significantly change in response to infusion with saline (Figure 2.6A, RM ANOVA, p=0.56). Pulses occurred with a frequency of 4.6 ± 0.4 times per 24 hours, and did not vary with treatment (Figure 2.6B, RM ANOVA, p=0.57). Total (1772.6 ± 478.2 μmol kg^{-1}), pulsatile (1748.6 ± 481.9 μmol kg^{-1}), and non-pulsatile (24.0 ± 12.8 μmol kg^{-1}) urea excretion did not vary in response to saline infusion (Fig 2.6C, RM ANOVA, p=0.50). Plasma urea (Fig 2.7A, RM ANOVA, p=0.96), cortisol (Figure 2.7B, RM ANOVA, p=0.51), and aldosterone (Figure 2.7C, RM ANOVA, p=0.99) were all unaffected by saline treatment.

Aldosterone treatment did not affect the percentage of nitrogenous wastes excreted as urea and ammonia (data not shown, RM ANOVA, p=0.12). Fish excreted 90.4 ± 2.0 % of their wastes as urea-N (mean = 99.0 ± 23.3 μmol kg^{-1} hr^{-1}) and 8.2 ± 2.2 % as ammonia-N (mean = 4.6 ± 1.1 μmol kg^{-1} hr^{-1}). Urea excretion occurred in distinct pulses with a mean size of 648.4 ± 152.4 μmol kg^{-1} hr^{-1} during the control period and was significantly reduced by 0.9-fold following infusion of aldosterone (Figure 2.6A, RM ANOVA, p=0.027). Pulse frequency (mean = 3.67 ± 0.27 per 24 hours) was unaffected by treatment (Figure 2.6B, RM ANOVA, p=0.57). Infusion of aldosterone caused a 0.7-fold decrease in both total (2377.1 ± 259.2 μmol kg^{-1}) and pulsatile (2366.9 ± 258.3 μmol kg^{-1}) urea excretion (Figure 2.6B, RM ANOVA, p<0.001), with no significant change in non-pulsatile components. Plasma urea (Figure 2.7A, RM ANOVA, p=0.96) and cortisol (Figure 2.7B, RM ANOVA, p=0.50) did not vary significantly with time or treatment. Infusion raised and maintained circulating plasma aldosterone levels around 210.91 ± 7.29 ng mL^{-1} (Figure 2.7C, RM ANOVA, p<0.001).

Figure 2.1: *Series I.* (A,B) The effect of a saline vehicle (n=6) or metyrapone (n=7) on urea pulse size (relative units) and frequency (relative units), respectively, in gulf toadfish. (C) Total, pulsatile, and non-pulsatile components of normalized urea excretion (relative units) of gulf toadfish exposed to either saline vehicle or metyrapone. (D, E) Changes in plasma urea concentrations (mmol L^{-1}) and plasma cortisol (ng mL^{-1}), respectively, over time following exposure to a vehicle alone or metyrapone. Metyrapone (20 mg mL^{-1}) was delivered by intraperitoneal injection in a saline vehicle (150 mmol L^{-1} NaCl, 1.5 μL g^{-1} body mass). Data for A,B, and C are presented as the treatment value normalized to the initial 24 hour control period. Values are expressed as means ± s.e.m. Symbols (ϕ,*) represent, respectively, significant differences within the treatment group from control period (>24 hours), and differences between treatment groups at a given time, respectively ($p<0.05$). There were no significant differences in pulse frequency ($p>0.05$).

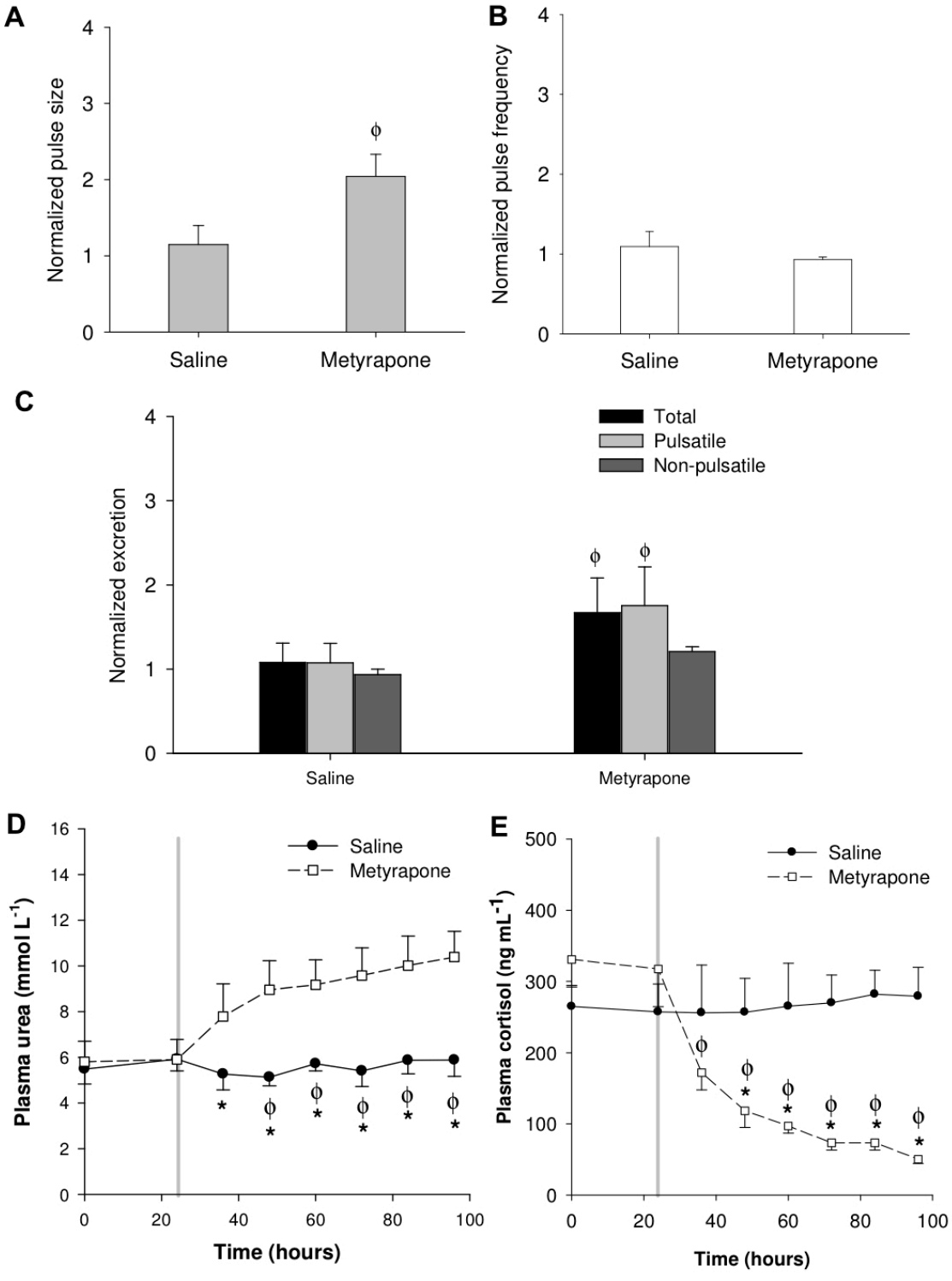
A
Normalized pulse size
Saline
Metyrapone
B
Normalized pulse frequency
Saline
Metyrapone
C
Normalized excretion
Total
Pulsatile
Non-pulsatile
Saline
Metyrapone
D
Plasma urea (mmol L-1)
Saline
Metyrapone
Time (hours)
E
Plasma cortisol (ng mL-1)
Saline
Metyrapone
Time (hours)

Figure 2.1

Figure 2.2: *Series II.* (A,B) The effect of RU-486 (mg mL^{-1}) on urea pulse size (relative units) and frequency (relative units), respectively, in gulf toadfish. (C) Total, pulsatile, and non-pulsatile components of normalized urea excretion (relative units) of gulf toadfish exposed to either oil vehicle (0 mg mL^{-1} RU-486) or RU-486. RU-486 was delivered by intraperitoneal injection in a peanut oil vehicle (1.5 μL g^{-1} body weight). The data are presented as the treatment value normalized to the initial 24 hour control period. Values are expressed as means ± s.e.m. The symbol (ϕ) represents significant differences in pulse size from the initial control period within the treatment group ($p<0.05$). There were no significant differences in pulse frequency ($p>0.05$).

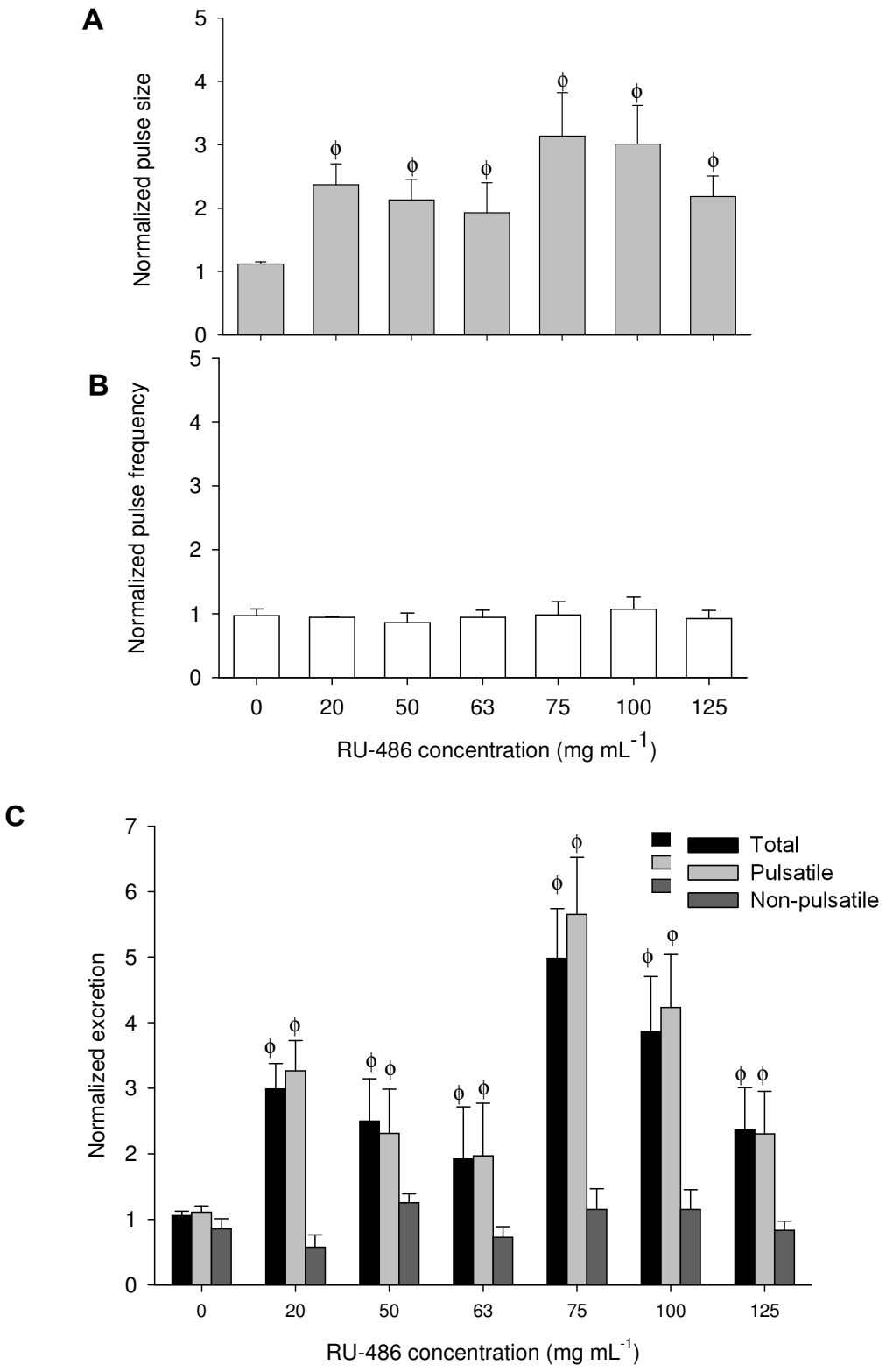
A
Normalized pulse size
B
Normalized pulse frequency
RU-486 concentration (mg mL-1)
C
Normalized excretion
Total
Pulsatile
Non-pulsatile
RU-486 concentration (mg mL-1)

Figure 2.2

Figure 2.3: *Series II.* (A) Changes in plasma urea concentrations (mmol L^{-1}) in gulf toadfish over time (h) following exposure to a vehicle alone or RU-486. (B) Plasma cortisol (ng mL^{-1}) concentrations in gulf toadfish over time (h). RU-486 was delivered by intraperitoneal injection in a peanut oil vehicle (0 mg mL^{-1} RU-486; 1.5 μL g^{-1} body weight). The grey line denotes the start of the treatment period. Values are expressed as means ± s.e.m. The symbol ϕ represents significant differences ($p<0.05$) within the group from the initial sample at 0 hours, and the symbol * represents differences between RU-486 and vehicle control ($p<0.05$).

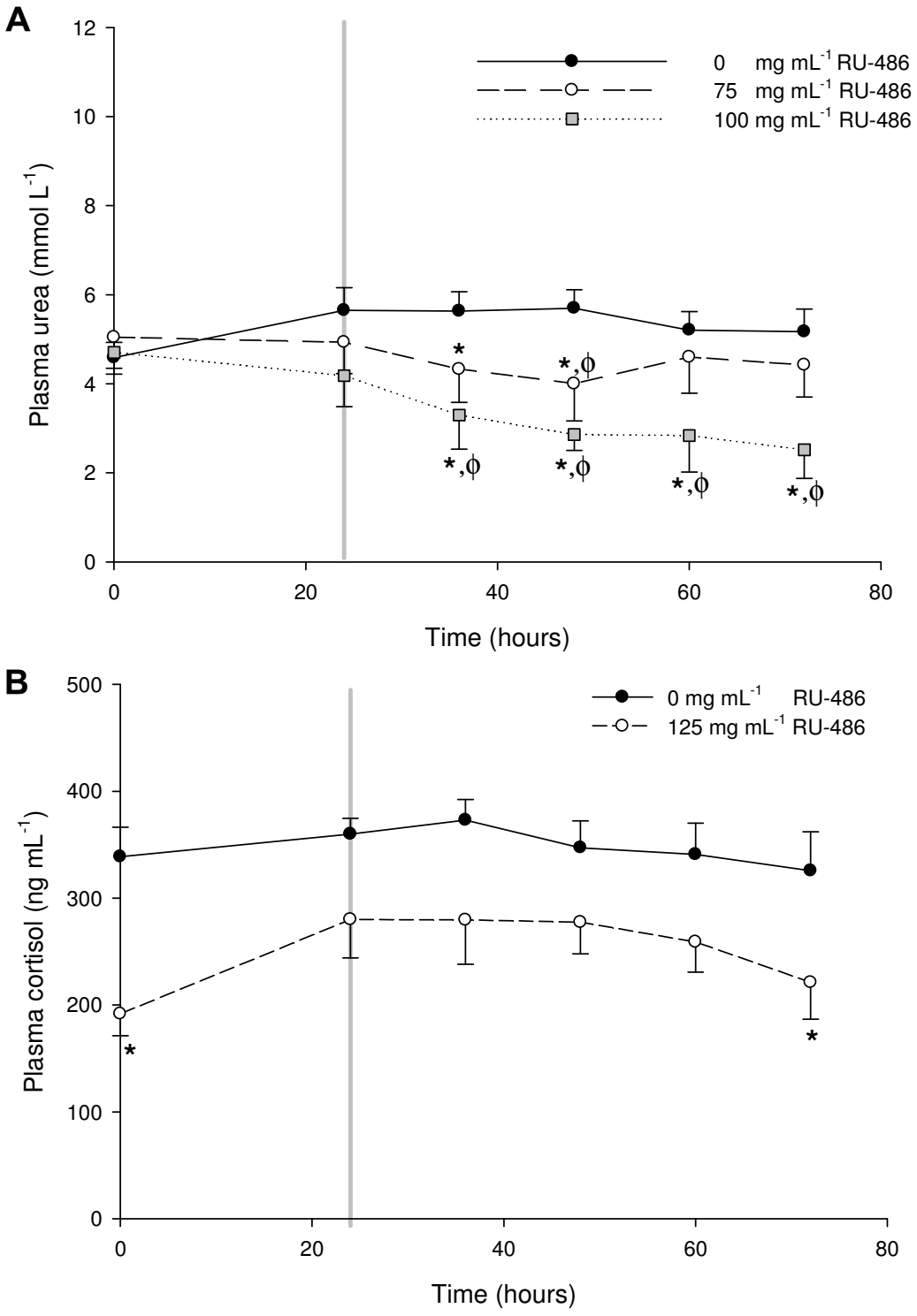
A
Plasma urea (mmol L-1)
Time (hours)
0 mg mL-1 RU-486
75 mg mL-1 RU-486
100 mg mL-1 RU-486
B
Plasma cortisol (ng mL-1)
Time (hours)
0 mg mL-1 RU-486
125 mg mL-1 RU-486

Figure 2.3

Figure 2.4: *Series III.* (A,B) The effect of spironolactone (mg mL^{-1}) on urea pulse size (relative units) and frequency (relative units), respectively, in gulf toadfish. (C) Total, pulsatile, and non-pulsatile components of normalized urea excretion (relative units) of gulf toadfish exposed to either oil vehicle (0 mg mL^{-1} spironolactone) or spironolactone (mg mL^{-1}). The data are presented as the treatment value normalized to the initial 24 hour control period. Values are expressed as means ± s.e.m. The symbol (ϕ) represents significant differences in pulse size from the initial control period within the treatment group ($p<0.05$). There were no significant differences in pulse frequency ($p>0.05$).

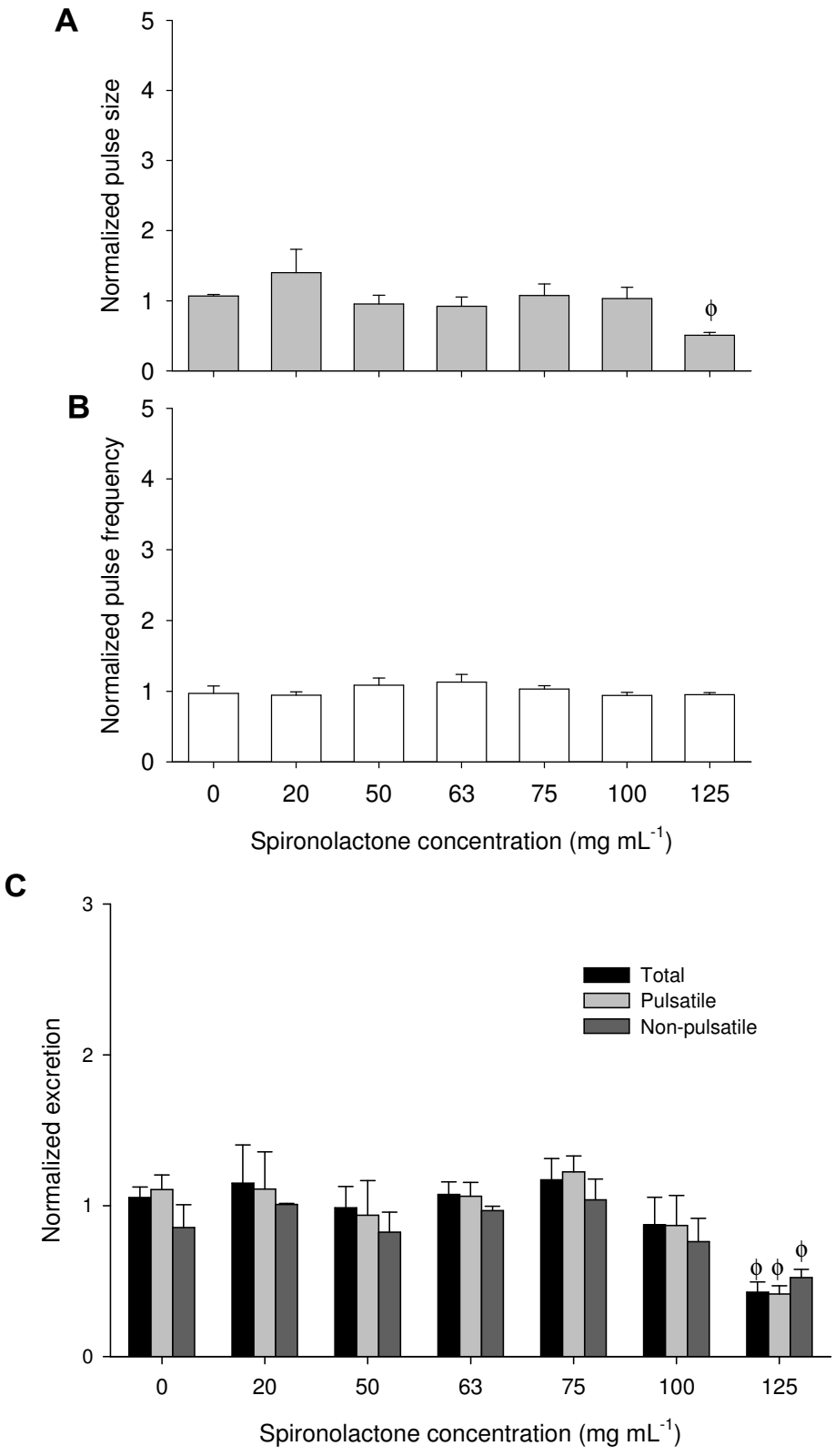
A
Normalized pulse size
0
1
2
3
4
5
ϕ
B
Normalized pulse frequency
0
1
2
3
4
5
0
20
50
63
75
100
125
Spironolactone concentration (mg mL-1)
C
Normalized excretion
0
1
2
3
Total
Pulsatile
Non-pulsatile
ϕ ϕ ϕ
0
20
50
63
75
100
125
Spironolactone concentration (mg mL-1)

Figure 2.4

Figure 2.5: *Series III.* (A) Variation in plasma urea (mmol L^{-1}) from gulf toadfish over time (h) following treatment with spironolactone. (B) Changes in plasma cortisol (mmol L^{-1}) of gulf toadfish over time (h) following spironolactone exposure. The drug was delivered by intraperitoneal injection in a peanut oil vehicle (0 mg mL^{-1} spironolactone; 1.5 μL g^{-1} body weight). The data are presented as the treatment value normalized to the initial 24 hour control period. Values are expressed as means ± s.e.m. The symbol ϕ represents significant differences ($p<0.05$) within the group from the initial sample at 0 hours, and the symbol * represents differences between spironolactone and vehicle control ($p<0.05$).

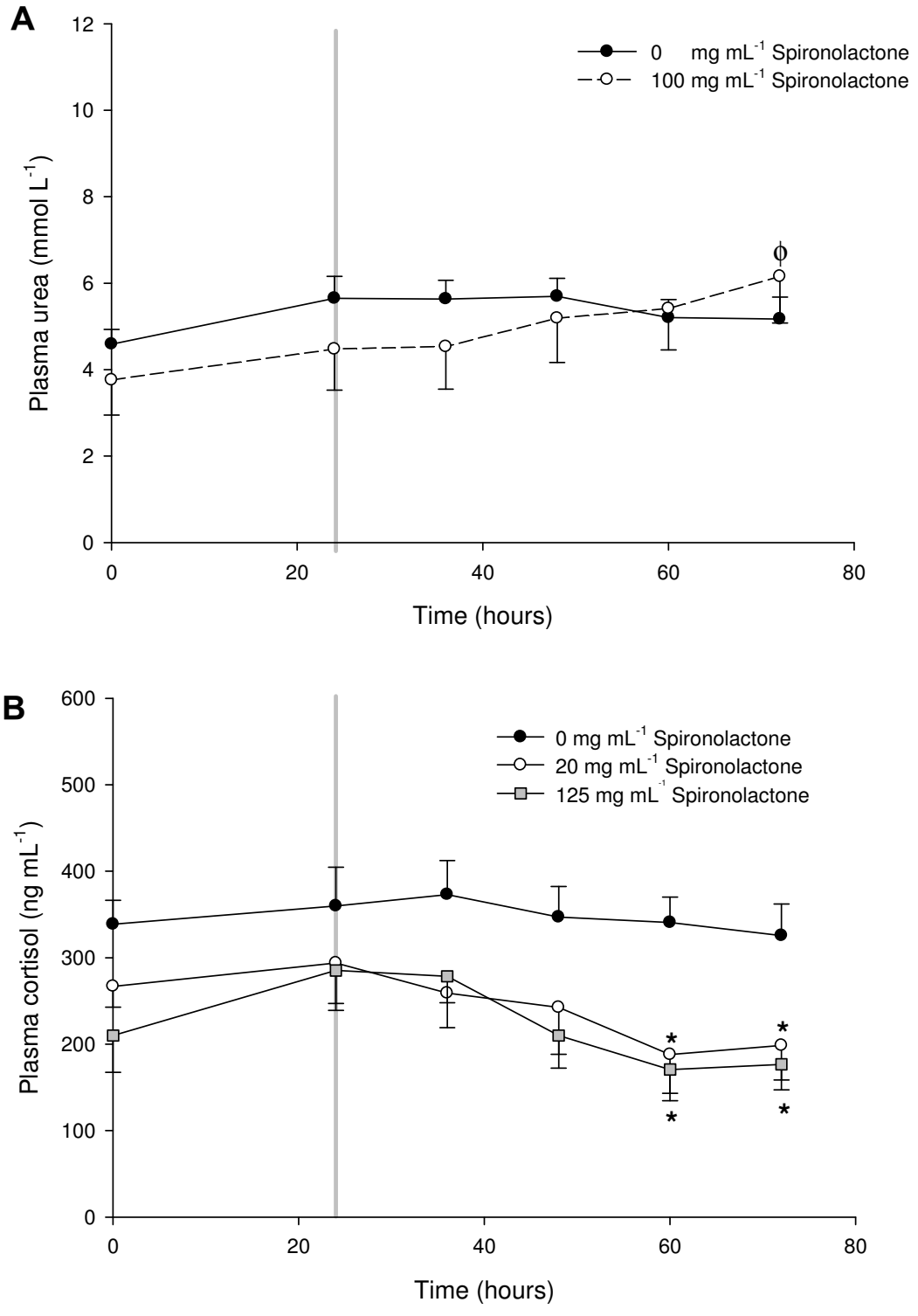
A
Plasma urea (mmol L-1)
0 mg mL-1 Spironolactone
100 mg mL-1 Spironolactone
ϕ
Time (hours)
B
Plasma cortisol (ng mL-1)
0 mg mL-1 Spironolactone
20 mg mL-1 Spironolactone
125 mg mL-1 Spironolactone
*
Time (hours)

Figure 2.5

Figure 2.6: *Series IV.* (A,B) The effect of a saline vehicle (n=8) or aldosterone (n=14) on urea pulse size (relative units) and frequency (relative units), respectively, in gulf toadfish. (C) Total, pulsatile, and non-pulsatile components of normalized urea excretion (relative units) of gulf toadfish exposed to either saline vehicle or aldosterone. Aldosterone (9 μg mL^{-1}) was delivered by caudal artery infusion in a saline vehicle (150 mmol L^{-1} NaCl, 1.5 μL g^{-1} body mass). The data is presented as the treatment value normalized to the initial 24 hour control period. Values are expressed as means ± s.e.m. The symbol (ϕ) represents significant differences in pulse size from the initial control period within the treatment group ($p<0.05$). There were no significant differences in pulse frequency ($p>0.05$).

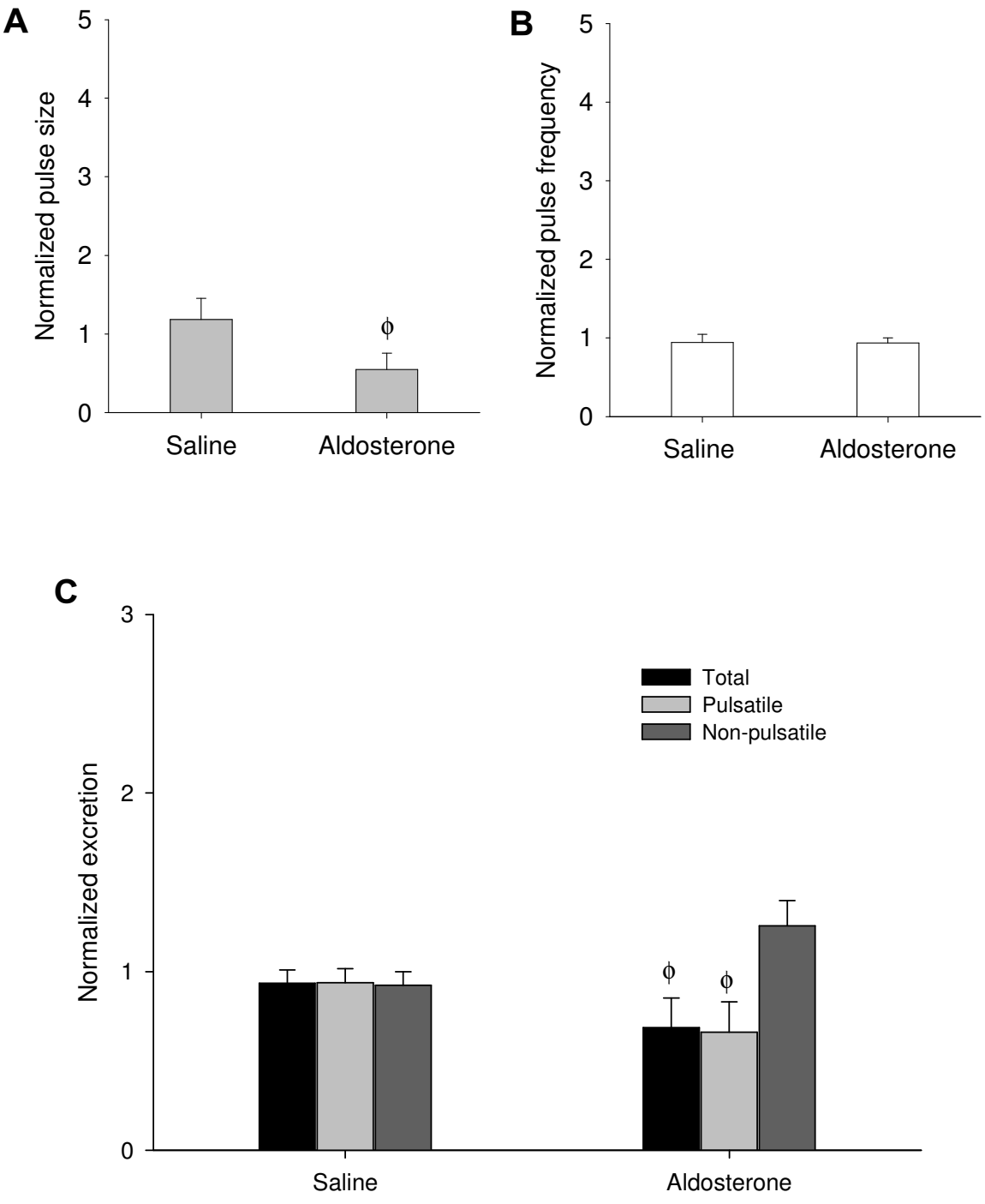
A
Normalized pulse size
5
4
3
2
1
0
ϕ
Saline
Aldosterone
B
Normalized pulse frequency
5
4
3
2
1
0
Saline
Aldosterone
C
Normalized excretion
3
2
1
0
Total
Pulsatile
Non-pulsatile
ϕ
ϕ
Saline
Aldosterone

Figure 2.6

Figure 2.7: *Series IV.* (A) Variation in plasma urea (mmol L^{-1}) from gulf toadfish over time (h) following aldosterone infusion. (B) Changes in plasma cortisol (ng mL^{-1}) of gulf toadfish over time (h) following treatment with aldosterone. (C) Plasma aldosterone levels (ng mL^{-1}) over time (h) in gulf toadfish infused with aldosterone. Aldosterone (9 μg mL^{-1}) was delivered by caudal artery infusion in a saline vehicle (150 mmol L^{-1} NaCl, 1.5 μL g^{-1} body mass). The grey line denotes the start of the treatment period. Values are expressed as means ± s.e.m. The symbol ϕ represents significant differences ($p<0.05$) within the group from the initial sample at 0 hours, and the symbol * represents differences between aldosterone and vehicle control ($p<0.05$). There were no significant differences in plasma urea or cortisol between the vehicle and aldosterone groups ($p>0.05$), nor was there any variation over time ($p>0.05$).

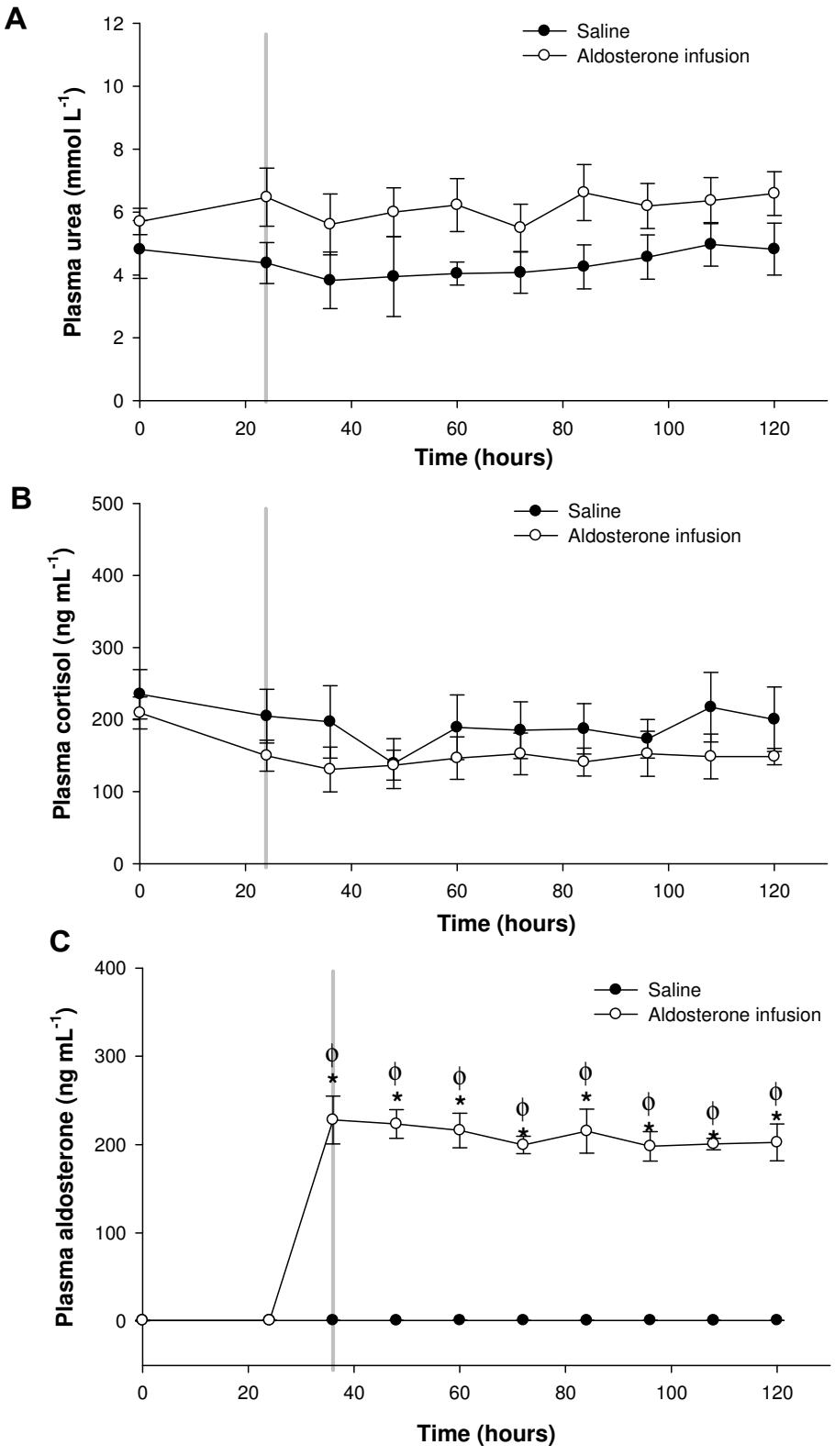
A
Saline
Aldosterone infusion
Plasma urea (mmol L-1)
Time (hours)
B
Saline
Aldosterone infusion
Plasma cortisol (ng mL-1)
Time (hours)
C
Saline
Aldosterone infusion
Plasma aldosterone (ng mL-1)
Time (hours)

Figure 2.7

Table 2.1: *Series I.* The percentage of nitrogenous waste excreted in the form of urea and ammonia for toadfish during an initial 24 hour control period followed by a treatment period with either a saline vehicle (150 mmol L^{-1} NaCl, 1.5 μL g^{-1} body mass; n=6) or 20 mg mL^{-1} metyrapone (in 150 mmol L^{-1} NaCl, 1.5 μL g^{-1} body mass; n=7).

Metyrapone concentration (mg mL^{-1})	Control		Treatment	
	% Urea-N	% Ammonia-N	% Urea-N	% Ammonia-N
0	87.1 ± 5.6	12.8 ± 7.1	88.6 ± 6.2	12.7 ± 6.9
20	93.8 ± 1.1	6.9 ± 1.9	85.2 ± 4.2*	15.3 ± 3.7*

[1] Values are expressed as means ± s.e.m.

* represents a significant difference in percentage excretion from the corresponding control period within the treatment group ($p<0.05$).

Table 2.2: *Series II.* The percentage of nitrogenous wastes excreted in the form of urea and ammonia for toadfish during an initial 24 hour control period followed by a treatment period with either a peanut oil vehicle (1.5 μL g^{-1} body weight) or various concentrations of RU-486 administered in the oil vehicle.

RU-486 concentration (mg mL^{-1})	Control		Treatment	
	% Urea-N	% Ammonia-N	% Urea-N	% Ammonia-N
0	81.3 ± 5.2	18.6 ± 5.2	81.3 ± 6.9	18.7 ± 6.9
20	81.8 ± 5.1	18.6 ± 5.1	94.2 ± 1.1*	5.8 ± 1.1*
50	84.2 ± 2.6	15.8 ± 2.6	94.5 ± 1.0*	5.5 ± 1.0*
63	88.2 ± 1.4	11.8 ± 1.4	96.1 ± 0.7*	3.8 ± 0.7*
75	84.4 ± 6.3	15.6 ± 6.3	93.2 ± 2.1*	6.9 ± 2.1*
100	85.5 ± 3.9	14.5 ± 3.9	92.3 ± 1.8*	7.7 ± 1.7*
125	91.5 ± 4.1	8.5 ± 4.1	97.8 ± 0.8*	2.2 ± 0.8*

[1] Values are expressed as means ± s.e.m.

* represents a significant difference in percentage excretion from the corresponding control period within the treatment group ($p<0.05$).

Discussion

Prior to a natural urea pulse event, plasma cortisol levels decrease markedly and increase sharply thereafter (Wood et al., 1997; Wood et al., 2001). On occasion, cortisol declines without an associated pulse occurring, suggesting that cortisol acts as a permissive rather than causal agent (Wood et al., 2003). Results from the present study support the hypothesis that cortisol regulates urea pulse size is an effect mediated by GRs, as there was a significant increase in pulse size when circulating cortisol levels were reduced by metyrapone treatment or the activity of cortisol was blocked by exposure to the GR antagonist RU-486. These data support earlier studies demonstrating an inverse relationship between cortisol and urea pulse size; the frequency of urea pulsing was not affected by cortisol, rather, the size of the pulses was diminished in ureotelic fish with maintained levels of elevated cortisol (~500-600 ng mL^{-1}; McDonald et al., 2004). Surprisingly, treatment with the MR agonist, aldosterone, caused a decrease in pulse size but these changes were minor compared to those elicited by cortisol (McDonald et al., 2004). Pulse frequency was unchanged by any treatment; implying that cortisol manipulation had no effect on the proximate trigger for pulse events and suggesting that low cortisol may upregulate urea transport activity (i.e. permissive action).

Rates of nitrogen excretion showed considerable variation (urea, 21.2 – 243.2 μmol kg^{-1} hr^{-1}; ammonia, 1.2 – 37.8 μmol kg^{-1} hr^{-1}) among all individuals in *Series I – IV*. Although the animals were fasted for *at least* 72 hours prior to the initiation of experiments, there is evidence that feeding elevates the absolute rates of total nitrogen excretion in both crowded and uncrowded toadfish (Walsh and Milligan, 1995) and

postprandial effects have been shown to persist for up to one week in other species (Fromm, 1963; Kaushik, 1980; Kaushik and Gomes, 1988). Furthermore, previous nutritional history may be exaggerated by the tendency of toadfish to form dominance hierarchies in the laboratory (Sloman et al., 2005). Despite the individual differences, the degree of ureotely (69-99%) in the present study was essentially identical to that measured in previous studies (Gilmour et al., 1998; McDonald et al., 2004; Wood et al., 1995; Wood et al., 1997).

Cannulation chronically raises plasma cortisol levels in fish (Brown et al., 1986; Lo et al., 2003; Sloman et al., 2005). Therefore, metyrapone and RU-486 were used as tools to investigate the effects of lowering cortisol levels by inhibiting endogenous synthesis of cortisol and blocking glucocorticoid receptor activity, respectively. Each of these treatments should have similar effects if indeed cortisol is exerting its effects through GRs. Both compounds have been used successfully in a number of piscine studies (Aluru and Vijayan, 2007; Bennett and Rhodes, 1986; Bernier and Peter, 2001; Killerich et al., 2007; McDonald et al., 2004; Milligan, 2003; Vijayan and Leatherland, 1992). While metyrapone treatment has been shown previously to elicit small urea pulses (Wood et al., 2001), this drug has been used successfully in toadfish to prevent the acute stress-dependent increase in cortisol following crowding (Hopkins et al., 1995). The results from the present study show that chronic administration of metyrapone caused a depression in plasma cortisol accompanied by heightened pulsatile urea excretion. Similarly, inhibiting corticosteroid activity *via* the GR with RU-486 caused a dose-dependent increase in pulse size. Neither treatment caused a change in pulse frequency. These findings are consistent with those of a previous study, in

which a moderate dose of RU-486 (33 mg mL^{-1}) prevented decreases in urea pulse size caused by continuous infusion of cortisol (McDonald et al., 2004). Interestingly, treatment with RU-486 in the 2004 study was so effective it resulted in an up-regulation of urea excretion rates. A recent study documented that toadfish increase tUT mRNA abundance in response to infusion with cortisol with associated decreases in urea excretion (McDonald et al., 2009). However, cortisol-mediated effects on urea excretion rates in toadfish occur fairly rapidly (< 60 min) *in vivo*, and less than 4 hours under more natural conditions (Wood et al., 2001). The timing of this latency period is suggestive of non-genomic modulation based on protein-protein interactions (reviewed by Borski, 2000; Wehling, 1997). Both regulatory mechanisms are reminiscent of systems present in the mammalian kidney, where glucocorticoids regulate the movement of urea in the mammalian kidney by modifying the expression of facilitative UT proteins *and* UT mRNA levels (Klein et al., 1997; Naruse et al., 1997b; Peng et al., 2002). It is possible that both genomic and non-genomic pathways may play a part in regulating pulse size in toadfish. With the former priming the gills for changes in tUT protein expression by increasing the availability of tUT message (mRNA; McDonald et al., 2009), the latter would ultimately modulate pulse size through quicker non-genomic pathways. This uncertainty may become resolved in future experiments looking at changes in tUT protein amount, localization and activation, as molecular analysis of this gene has revealed several phosphorylation and glycosylation sites (Walsh et al., 2000).

Glucocorticoid signaling in fish is more complex than in mammals, owing to the presence of multiple GR isoforms and splice variants (Bury et al., 2003; Greenwood et

al., 2003; Schaff et al., 2008). Functional analysis has demonstrated that GR2 is characterized by a higher sensitivity to cortisol than the GR1 isoform, leading researchers to suggest that these receptors may influence different pathways and coordinate responses during times of stress (Bury et al., 2003; Stolte et al., 2008). It has been suggested that each receptor has a physiologically distinct role, where rtGR2 is responsible for cortisol-mediated gene expression at basal cortisol levels, and during times of stress rtGR1 and rtGR2 may act in tandem to coordinate responses (Bury et al., 2003). It is tempting to speculate that a similar system is present in toadfish, with one isoform mediating acute changes in urea excretion and another taking a more prominent role in the transition to ureotely. The switch to ureotely is characterized by a rise in plasma cortisol and an accompanying reduction in ammonia excretion, mediated by an ammonia-trapping mechanism involving hepatic GSase (Walsh and Milligan, 1995; Walsh et al., 1994). Rates of urea elimination remain unchanged or even decrease moderately at this time. At present, it remains unclear whether maintenance of the ureotelic state relies on a persistent elevation in cortisol levels. Metyrapone caused a significant decrease in endogenous cortisol levels with an associated reduction in the degree of ureotely. Although, a 1.8-fold increase in plasma urea was observed within the first 24 hours following the initial metyrapone injection, it was expected that an increase in the rate of elimination of urea would cause internal levels of urea to fall over time. However, it is possible that potential changes in expression of related urea synthesis enzymes may lag behind removal of the corticosteroid stimulus. One other study has reported a similar trend where toadfish lacked a chronic elevation of cortisol and those individuals reverted to ammoniotelism (Wood et al., 1995). Therefore, it is

possible that cortisol, mediated by the GR, has a role in sustaining ureotelism. However, further experiments are required to resolve this uncertainty and determine whether these influences are exerted through typical corticosteroid pathways. Furthermore, the effects of metyrapone are not fully understood in the piscine system and all the changes associated with this compound, such as those observed in toadfish, may not be solely due to the inhibition of cortisol biosynthesis and may influence other aspects of metabolism (reviewed by Mommsen et al., 1999).

Fish are incapable of synthesizing significant amounts of aldosterone (Jiang et al., 1998; Sangalang and Uthe, 1994), leading to questions about what compound acts as the MR ligand *in vivo*. A number of studies have suggested the possibility that 11-deoxycorticosterone (DOC) may be a physiological ligand of the MR (Prunet et al., 2006; Sturm et al., 2005). Expression studies on the common carp (*Carassius auratus*) and rainbow trout MR indicates this receptor is more sensitive to aldosterone and 11-deoxycorticosterone (DOC), whereas the GR is more sensitive to cortisol (Prunet et al., 2006; Stolte et al., 2008; Sturm et al., 2005). Measurement of aldosterone concentration, prior to infusion, demonstrated that plasma levels of this steroid were undetectable in untreated toadfish. Activating MR pathways in toadfish with a continuous infusion of aldosterone produced decreases in pulsatile urea excretion and pulse size similar to, but less pronounced, than those elicited by cortisol. Furthermore, these effects were not caused by aldosterone-mediated changes in cortisol, as levels of this corticosteroid remained unchanged over time. The aldosterone effect implies that there is an MR sensitive element to urea transport in toadfish that may be analogous to mineralocorticoid-sensitive mechanisms in the mammalian kidney. Gertner et al.

(2004) demonstrated that UT-A1 protein levels were depressed by post-translational modifications following exposure to aldosterone, an effect that was blocked by exposure to spironolactone. It is possible that aldosterone infusion in toadfish may have activated pathways that are normally insensitive to cortisol activation (which is also an agonist of the MR) due to the expression of 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), an enzyme that catalyzes the conversion of cortisol to mineralocorticoid receptor-inactive cortisone (Funder et al., 1988). Increased urea excretion following aldosterone treatment might reflect a secondary rather than direct effect of MR stimulation, i.e. the activation of pathways not normally associated with pulsatile urea excretion.

Due to a high degree of sequence similarity between teleostean GRs and MR, there is a considerable overlap in the pharmacology of these two receptor types (reviewed by Bury and Sturm, 2007). While the piscine MR is activated by aldosterone and 11-deoxycorticosterone, the *in vitro* activation response of the MR to cortisol is equal to or more sensitive than the GRs (Prunet et al., 2006; Stolte et al., 2008; Sturm et al., 2005). With this information in mind, McDonald et al. (2004) examined whether cortisol-induced decreases in urea pulse size could be eliminated by treatment with spironolactone, a classic mammalian MR antagonist (Delyani, 2000). No effect of exposure to a moderate dose (33 mg mL^{-1}) of spironolactone was detected (McDonald et al. 2004). Comparable doses of spironolactone used in the present study yielded a similar lack of effect on pulse size or frequency. However, use of a substantially higher spironolactone dose (125 mg mL^{-1}) caused a significant decrease in pulse size, similar to the response seen in toadfish infused with the MR agonist aldosterone. Interestingly,

agonistic activity of spironolactone (10^{-5} – 10^{-7} M) was reported in an *in vitro* activation study on rainbow trout MRs, with a decreased response at the highest dose (Sturm et al., 2005). The apparent inconsistencies of responses to spironolactone suggest that this compound may exhibit different behaviour in piscine and mammalian systems and the data should be interpreted with caution.

While the precise cellular/sub-cellular location of the toadfish branchial facilitated diffusion mechanism(s) remains uncertain, there is evidence that the apical membrane of pavement cells is active during pulsatile urea excretion. Dense-cored vesicles are translocated to the apical surface of the pavement cells where they come into contact with the branchial membrane (Laurent et al., 2001). It is postulated that this system may aid facilitative urea transport across the membrane following the insertion of tUT (Laurent et al., 2001; McDonald and Walsh, 2004). Less is known about the properties of urea transport across the basolateral membrane of the gill but transport assays have confirmed the presence of a cortisol-sensitive urea transport mechanism (Rodela et al., 2009b). These findings are consistent with the data of the present study, demonstrating that pulsatile urea excretion in toadfish is mediated by the permissive action of cortisol through GRs, and to a lesser extent MRs. This process may involve changes in the abundance of tUT, which may be upregulated in response to low cortisol. The data also suggest that caution should be exercised in using the mammalian anti-MR spironolactone in piscine systems; further study is required to determine if the bi-phasic response detected for toadfish is characteristic of other species of fish. Future investigation should focus on using molecular tools to isolate and assay gene and protein expression patterns (e.g. GRs, MR, tUT) in toadfish gills to

acquire a clearer picture of the physiological and morphological changes associated with the unique pulsing events of this unusual fish.

Acknowledgements

This study was supported by NSERC discovery grants to P.J.W. and K.M.G., and an NSF grant to M.D.M. (IOS-0455904). PJW is supported by the Canada Research Chair program. Special thanks to Jimbo Luznar for collection of toadfish, and Michael B. Morando for care and feeding of the RSMAS toadfish colony.

CHAPTER 3

Cortisol-sensitive urea transport across the gill basolateral membrane of the gulf toadfish, *Opsanus beta*

Notes on Chapter 3

The present chapter has been published in the American Journal of Physiology,

Regulatory, Integrative, and Comparative Physiology as per the following citation:

Rodela, T.M., Gilmour, K.M., Walsh, P.J., and McDonald, M.D. (2009) Cortisol-sensitive urea transport across the gill basolateral membrane of the gulf toadfish, *Opsanus beta*. *Am J Physiol Regul Integr Comp Physiol* 297(2):R313-R22.

M.D. McDonald was a collaborator at the University of Miami, where *in vivo*

experiments were conducted by T.M. Rodela.

Abstract

Gulf toadfish, *Opsanus beta*, employ a unique pulsatile urea excretion mechanism that allows urea to be voided in large pulses via the periodic insertion or activation of a branchial urea transporter. The precise cellular and sub-cellular location of the facilitated diffusion mechanism(s) remains unclear. An *in vitro* basolateral membrane vesicle (BLMV) preparation was used to test the hypothesis that urea movement across the gill basolateral membrane occurs through a cortisol-sensitive carrier-mediated mechanism. Toadfish BLMVs demonstrated two components of urea uptake: a linear element at high external urea concentrations, and a phloretin-sensitive saturable constituent (K_m = 0.24 mmol L^{-1}; V_{max} = 6.95 μmol mg $protein^{-1}$ hr^{-1}) at low urea concentrations (<1 mmol L^{-1}). BLMV urea transport in toadfish was unaffected by *in vitro* treatment with ouabain, N-ethylamaleimide, or the absence of sodium, conditions which are known to inhibit sodium-coupled and proton-coupled urea transport in vertebrates. Transport kinetics were temperature-sensitive with a $Q_{10} > 2$, further suggestive of carrier-mediated processes. Our data provide evidence that a basolateral urea facilitated transporter accelerates the movement of urea between the plasma and gills to enable the pulsatile excretion of urea. Furthermore, *in vivo* infusion of cortisol caused a significant 4.3-fold reduction in BLMV urea transport capacity in lab-crowded fish, suggesting that cortisol inhibits the recruitment of urea transporters to the basolateral membrane which may ultimately affect the size of the urea pulse event in gulf toadfish.

Introduction

With respect to piscine urea transport, the gulf toadfish *Opsanus beta* is among the best-studied teleosts; over two decades of research have chronicled the unusual nature of its nitrogen elimination. In their sub-tidal environment, gulf toadfish excrete equal quantities of ammonia and urea (Barimo et al., 2004). However, during times of stress induced by conditions including crowding, confinement, and exposure to air and/or elevated ambient ammonia, gulf toadfish become ureotelic (Walsh and Milligan, 1995; Walsh et al., 1994; Walsh et al., 1990). This transition to ureotelism is initiated by a moderate and transient (<24 hours) surge in plasma cortisol (Hopkins et al., 1995) that induces hepatic glutamine synthetase (GSase) activity. GSase controls nitrogen entry as glutamine into the ornithine-urea cycle (O-UC); induction of this enzyme causes an increase in urea production and raises circulating plasma urea levels. Accompanied with a simultaneous decrease in ammonia excretion, this sets the conditions for pulsatile urea excretion (Walsh and Milligan, 1995); a feature that is unique to toadfish and sets them apart from other ureotelic teleosts. Gulf toadfish discharge urea in a pulsatile manner; over 90% of the total daily urea is released during short-lasting pulses that may occur once to several times over a 24 hour period (Wood et al., 1995; Wood et al., 1997; Wood et al., 1998). In between these pulse events, little to no urea is excreted. The metabolic production of urea *via* the ornithine-urea cycle (O-UC) is continuous, with plasma and body urea levels steadily rising until a facilitated diffusion mechanism is activated to increase branchial urea permeability 35-fold during a pulse event (Wood et al., 1995; Wood et al., 1997; Wood et al., 1998). This excretion mechanism appears to be very specific for urea as changes in water permeability

during a pulse event are marginal whereas the permeability of structural urea analogues, such as thiourea and acetamide, increases 5- and 18-fold, respectively (Gilmour et al., 1998; McDonald et al., 2000; Part et al., 1999; Wood et al., 1998). A cDNA encoding for a 475 amino acid residue protein was cloned from the branchial tissue of *O. beta* and exhibited over 60 % sequence similarity to mammalian UT-A2 type urea transporters (Walsh et al., 2000). Analysis of gill mRNA of the toadfish urea transporter (tUT) found levels of this transporter to be largely invariant over the pulse cycle (Walsh et al., 2000) suggesting that pulsatile events may be due to the periodic insertion or activation of tUT at the gills.

There is strong evidence that pulsatile urea excretion is under proximate control of the monoamine serotonin (5-hydroxytryptamine, 5-HT) and the corticosteroid cortisol. Arterial injection of 5-HT and a 5-HT_2 receptor agonist elicited substantial urea pulses of comparable size and duration to natural pulse events, with the effect reversed by a 5-HT_2 antagonist (McDonald and Walsh, 2004; Wood et al., 2003). Cortisol appears to play two conflicting roles in terms of pulsatile urea excretion regulation. On the one hand, in addition to mediating an increase in urea production via an increase in GSase activity, the elevated cortisol levels experienced by crowded toadfish also appear to mediate an increase in urea transporter mRNA expression (McDonald et al., 2009). Repetitive blood sampling of pulsatile toadfish has demonstrated that 2 to 4 hours preceding a pulse event, plasma cortisol levels fall markedly (from approximately 120 to 40 ng mL^{-1}) and rise rapidly thereafter (Wood et al., 1997; Wood et al., 2001). This reduction in cortisol seems to be a permissive event rather than a causal agent as often plasma cortisol declines without an associated pulse (Wood et al., 2001). It is

postulated that when cortisol levels are at their minimum, 5-HT initiates a pulse event by activating tUT *via* 5-HT_2 receptor-mediated phosphorylation of two unique sites on the C-terminus of the urea transport protein (McDonald and Walsh, 2004). Following a urea pulse, cortisol returns to pre-pulse concentrations, and the urea pulse is terminated, suggesting that elevated cortisol levels inactivate tUT. Thus, the control of pulse *size* would depend upon the number of urea transport proteins available for phosphorylation. This may be mediated through genomic pathways involving the regulation of the tUT mRNA transcript (McDonald et al., 2009) or another cortisol-sensitive UT isoform, similar to the inner medullary collecting duct (IMCD) of rats (Peng et al., 2002).

The precise cellular and sub-cellular location of the facilitated diffusion mechanism(s) remains uncertain. However, there is evidence that significant ultrastructural rearrangements occur during pulsatile urea excretion. Examination of gill morphology demonstrated an abundance of dense-cored vesicles in the pavement cells of ureotelic toadfish (Laurent et al., 2001), and a similar arrangement is exhibited by the ureotelic Lake Magadi tilapia (*Alcolapia grahami*; Walsh et al., 2001). During urea pulses in the toadfish, these vesicles are translocated to the apical surface where they come into contact with the branchial membrane (Laurent et al., 2001). This scenario is reminiscent of the SNARE (soluble N-ethylmaleimide-sensitive)-mediated vesicular trafficking of UT-A1 in mammalian kidney (Mistry et al., 2007). The vesicular trafficking was inhibited when ureotelic toadfish were treated with colchicine, an inhibitor of microtubule assembly, which ultimately caused a significant reduction in both the rate and frequency of urea pulsing with no discernable changes in ammonia

excretion (Gilmour et al., 1998). Overall, the data suggest that the branchial apical membrane is intimately associated with the release of urea into the environment. However, little attention has been paid to the contribution of urea transport across the basolateral membrane. In recent years, a number of different studies have shown the presence of basolateral UTs, including a salinity-dependent transporter in *Anguilla japonica* (Mistry et al., 2001). A sodium-coupled, secondarily active UT on the branchial basolateral membrane of *Squalus acanthias* plays an integral role in urea retention, an essential osmolyte in their osmoregulatory strategy (Fines et al., 2001). Even the ammoniotelic rainbow trout, *Oncorhynchus mykiss*, has a facilitated diffusion transporter mechanism on the branchial basolateral membrane that saturates at physiologically relevant concentrations (McDonald and Wood, 2004). So it is likely that a similar transport protein is present in the toadfish, and it may facilitate the movement of urea between the plasma and gills, potentially producing an overall increase in basolateral gill urea permeability during pulse events.

In the present study, we tested the hypothesis that urea movement across the gill basolateral membrane in gulf toadfish occurs through a cortisol-sensitive carrier-mediated mechanism. An *in vitro* approach generated basolateral membrane vesicles that were then utilized to characterize urea transport kinetics using a rapid filtration technique (Perry and Flik, 1988). Urea transport was also measured following exposure to various urea transport and energy inhibitors, as well as sodium gradients. Furthermore, urea influx was studied in response to *in vivo* treatments that included manipulation of ambient temperature and chronic infusion of cortisol to artificially raise circulating concentrations.

Materials and Methods

Animals

Gulf toadfish (*Opsanus beta* Goode and Bean; 45-130 g) were collected by commercial shrimpers in Biscayne Bay, Florida, USA during the months of February to April 2007. Toadfish were held for <48 hours at the shrimpers' holding facility in outdoor, flow-through seawater tanks. On the day of transfer to the laboratory, toadfish were given a freshwater dip (2 min) followed by a malachite green/formalin treatment (0.05 mg L^{-1}, 15 mg L^{-1}) as a preventative treatment against ciliate infection of *Cryptocaryon irritans* (Wood et al., 1997). In the laboratory, the fish were kept at a stocking density of 10 fish per 50 L aquaria that were supplied with aerated flowing seawater (18ºC – 22ºC, pH 8.1) under a natural photoperiod. All toadfish, including mesocosm individuals, were fed weekly with chopped squid.

Experimental series

A standardized crowding and confinement procedure (see Hopkins et al., 1995a; Walsh, 1987a) was used to induce and maintain ureotelism for the duration of all experiments, with the exception of mesocosm fish in *Series II*. In brief, 8 to 10 fish were relocated to and held in 6 L tubs supplied with flowing sea water for 48 hours. For terminal sampling of gill tissue, as well as surgical procedures (see *Series III*), all fish were lightly anesthetized with MS-222 (1 g L^{-1}, pH 8.0). For surgical procedures, the animals were returned to anesthetic-free seawater following implantation of a cannula into the caudal artery (McDonald et al., 2004a; Wood et al., 1997a), whereas terminally sampled fish were killed by severance of the spinal cord.

Urea transport across the basolateral gill membrane was measured in three

series of experiments:

Series I: Characterization of urea transport across isolated basolateral membrane vesicles (BLMV) in lab crowded O. beta

Characterization of urea transport across the basolateral membrane of the toadfish gill was measured in lab crowded fish held at 22°C. Initial experiments focused on establishing the concentration dependence relationship of urea transport, for which BLMVs were exposed to physiological urea concentrations ranging from 1 to 20 mmol L^{-1} (0, 0.05, 0.1, 0.175, 0.25, 0.35, 0.5, 0.625, 0.75, 1, 1.25, 1.5, 2, 3.5, 5, 10, 15, 20 mmol L^{-1}) and the rate of urea uptake was assessed (described in *Experimental Protocol*). To further characterize the properties of urea transport, BLMVs were exposed to a number of different non-competitive and competitive inhibitors at a urea concentration of 0.4 mmol L^{-1}, a value below the saturable range of urea uptake (see Results). For all treatments, a 10 μL aliquot of non-competitive or competitive inhibitor or control solution was added to the BLMV suspension and vortexed immediately prior to the initiation of the transport assay. A stock solution of phloretin (9 mmol L^{-1}) was prepared by dissolving phloretin in ethanol, and then it was adjusted to final concentrations ranging from 0 (ethanol alone) to 0.25 mmol L^{-1}. The inhibition of urea uptake in BLMV was further tested following exposure to treatments of phloretin (0.25 mmol L^{-1}), the mercurial compound *p*-chloromercuribenezesulfonate (pCMBS; 0.25 mmol L^{-1}), and a combination of both phloretin and pCMBS (both at 0.25 mmol L^{-1}). Phloretin (Fines et al., 2001; McDonald and Wood, 2004; Morgan et al., 2003a) and pCMBS concentrations (Ogami et al., 2006; Tsukaguchi et al., 1997) were chosen based on previous studies. Final ethanol concentration in the individual solutions did not exceed 0.1% (vol/vol). The effects of urea analogues

(acetamide, thiourea, N-methylurea) were measured at two-fold higher concentrations (0.8 mmol L^{-1}) than urea (0.4 mmol L^{-1}), in accordance with earlier published studies (Pilley and Wright, 2000; Wood et al., 1998; Wright et al., 1995).

The ATP dependence of urea transport was investigated using a variety of compounds that influence ATPase-type proteins. Urea uptake was measured following incubation in solutions containing ATP (10 mmol L^{-1}), ATP (10 mmol L^{-1}) + ouabain (1 mmol L^{-1}; to inhibit Na^+,K^+-ATPase activity), or ATP (10 mmol L^{-1}) + N-ethylamaleimide (NEM; 1 mmol L^{-1}: to inhibit V-type H^+-ATPase activity). Furthermore, sodium specificity of urea transport was also examined in BLMV. For those experiments, BLMV were prepared and isolated in sodium-free solutions.

Series II: The effects of cortisol on urea transport in BLMV in O. beta

This series of experiments manipulated *in vivo* circulating cortisol levels to elicit potential changes in BLMV urea uptake. An *in vivo* approach was used because there is no definitive evidence from the toadfish literature whether cortisol is acting through genomic or non-genomic pathways, thus experiments were designed to ensure that the cellular machinery was present to mediate a response.

Uncrowded toadfish (n=8) were kept, for 1 month prior to sampling, in outdoor mesocosm tanks (~8000 L, 28°C) maintained with flow through seawater (50 L min^{-1}) and aeration. These tanks were enriched with a carbonate sediment and seagrass (*Thalassia testudinum*) to mimic a natural toadfish environment as previously described (Barimo and Walsh, 2006a). In the lab, toadfish (n=8) were kept at a low stocking density in 50 L aquaria (22°C) for 2 weeks prior to undergoing a standardized crowding and confinement procedure. A second group of lab crowded fish (n=7) underwent surgery for implantation of a caudal artery catheter, as outlined by (McDonald et al., 2004). The fish were allowed to recover for 24 hours in 1.5 L containers supplied with flowing seawater. Following the recovery period,

the arterial catheter was connected to one channel of a Gilson 8 channel peristaltic pump and the fish were infused with 0.09 mg cortisol mL^{-1} (hydrocortisone hemisuccinate salt; Sigma Chemicals) in isosmotic NaCl (150 mmol L^{-1}) at a rate of 3 mL kg^{-1} h^{-1} for a 24 hour period. The rate of infusion was verified periodically by measurement of the weight of the infusion reservoir. At the end of the 24 hour exogenous cortisol loading period, fish were terminally sampled as described above.

Mesocosm, lab crowded, and lab infused fish were intended to represent low, intermediate, and high cortisol level groups, respectively. To confirm that these treatments produced the desired effect, at the time of terminal gill extraction, blood (200 μl) was sampled by caudal puncture from lab fish and centrifuged at 10 000 x g for 1 minute. An 80 μL aliquot of plasma was removed, flash frozen in liquid nitrogen and stored at -80°C for later analysis of cortisol content. Reported values for plasma cortisol from mesocosm fish were from a previous study (McDonald et al., 2009). BLMV isolated from these different groups were subjected urea transport assays using the same series of urea concentrations as outlined in *Series I,* ranging from 1 to 20 mmol L^{-1}.

Series III: The effect of temperature on urea transport across BLMV in lab crowded O. beta

Toadfish were housed over the course of this study from February to April and there were significant temperature variations among the different groups. To eliminate any potentially confounding effects of treatment and temperature, as well as to examine the effects of temperature on transport directly, an additional series of experiments was performed to focus on temperature effects on BLMV urea uptake. The first group of fish (n=11), initially used for validation of BLMV preparation, was housed in the lab in 50 L aquaria, supplied with flowing seawater, for a two week period during February, when water temperatures were approximately 18°C. The second group of fish (n=6) was experimentally acclimated to water temperatures of 28°C, which mimicked the acclimation temperature of

the mesocosm fish in *Series II*. These fish were held in 50 L aquaria with a continuous supply of flowing seawater at 22°C. Over the course of 5 days, the temperature was increased at a rate of 1°C day^{-1} to 28°C, at which temperature the animals were maintained for 7 days. All groups were treated with a standard crowding protocol for 48 hours at their respective acclimation temperatures and terminally sampled for gill tissue. Subsequently, kinetic analysis of BLMV urea uptake was carried out over the physiological range of urea concentrations outlined in *Series I*. Furthermore, a cross-comparison of the rates of uptake between different temperatures was used to calculate a Q_{10} for urea transport.

Experimental protocols

Isolation of BLMV

BLMV were isolated following the differential centrifugation protocol of Perry and Flik (1988) as modified by Bury et al. (1999). For each preparation, one toadfish was lightly anesthetized and killed by severance of the spinal cord. The gills were perfused *via* the heart for less than a minute with approximately 60 mL of modified saline (0.9 % NaCl, 0.5 mmol L^{-1} ethylenediaminetetaacetic acid-disodium salt [Na_2-EDTA], 20 IU L^{-1} heparin, 1 mM dithiothreitol, 100 IU ml^{-1} aprotinin; adjusted to pH 7.8 with Tris), and rapidly excised and placed temporarily in ice-cold saline. All subsequent steps were carried out on ice.

The cells of the gill arches were scraped from the cartilaginous filaments with a glass slide and transferred to 20 mL of hypotonic homogenization solution (25 mM NaCl, 1 mM Hepes, 1 mM dithiothreitol, 100 IU mL^{-1} aprotinin; pH adjusted to 7.8 with Tris). Initial homogenization consisted of 30 strokes with a Dounce homogenizer (Kontes) and a loose fitting pestle. A 1 mL aliquot was taken from this homogenate and

frozen at -80°C for later analysis of enzyme activity. Following homogenization, the suspension was transferred to a clean tube, the volume was adjusted to 50 mL, and the suspension was centrifuged at 450 *g* for 15 min at 4°C to separate cellular debris. The supernatant was then centrifuged at 50 000 *g* for 30 min at 4°C. This spin produced a pellet with a light top layer (plasma membranes) and a dark lower layer (mitochondria). The upper layer of the pellet was carefully re-suspended in 2 mL of isotonic solution (250 mM sucrose, 5 mM $MgSO_4$, 5 mM Hepes; adjusted to pH 8.0 with Tris) by gentle agitation. This plasma membrane suspension was then homogenized, in 20 mL of isotonic solution, for 100 strokes using a tight-fitting pestle of a Dounce homogenizer (Kontes). The second homogenate was centrifuged at 1000 *g* for 10 min, followed immediately by a second spin of 10 000 *g* for 10 min at 4°C, which produced a pellet composed of the remaining contaminating membranes. The supernatant was centrifuged at 38 500 *g* for 30 min at 4°C to produce a final pellet enriched in basolateral membranes. The pellet was suspended in 1 - 2 mL of resuspension buffer (250 mM sucrose, 10 mM KNO_3, 0.8 mM $MgSO_4$, 0.5 mM Na_2-EDTA, 20 mM Hepes; adjusted to pH 7.4 with Tris) by passage through a 23-gauge needle (10 times) to aid vesicle formation. Vesicles were allowed to equilibrate for 30 min on ice, then were mixed again by 10 passes through a 23-gauge needle. The suspension was used immediately for vesicle orientation assays, vesicle volume determinations, urea transport assays and protein concentration determinations.

Validation of BLMV preparation

Enzymatic assays performed on the initial and final homogenates were used as an indicator of relative enrichment of the final BLMV preparation. Na^+,K^+-ATPase (McCormick, 1993), cytochrome C oxidase (Blier and Guderley, 1988), glucose-6-phosphate (Stio et al., 1988), and nicotinamide mononucleotide (NMN)-adenylyltransferase (Balducci et al., 1995) were used as marker enzymes for the basolateral membrane, inner mitochondrial membrane, endoplasmic reticulum (ER), and nuclear membrane, respectively. All activities were quantified using published protocols modified for use with a SpectraMax Plus 384 microplate reader (Molecular Devices Corp., Sunnyvale, CA, USA).

The degree of BLMV resealing was assessed by measuring Na^+,K^+-ATPase activity (McCormick, 1993) in the presence or absence of 10 μL of the detergent 0.04% Triton-X (vol/vol), as previously described (Fines et al., 2001). The percentage of resealed vesicles was calculated as the difference between activities of the detergent-treated and intact BLMV. The number of inside-out vesicles was determined by assaying Na^+,K^+-ATPase activity in unmasked and masked BLMV preparations, where masking of inside-out vesicles was accomplished by incubating the vesicles in 10 mM KCl for 10 min on ice, described by Fines et al. (Fines et al., 2001). The percentage of right-side out vesicles was expressed as the difference between resealed vesicles and inside-out vesicles.

Protein-normalized BLMV volumes were measured as an additional indicator of intact and resealed vesicles. In brief, a 40 μL aliquot of BLMV (~0.6 mg protein) was incubated in 1 mL of resuspension buffer containing 10 μL (1 μCi) of ^3H_2O and 10 μL

(0.1 μCi) of ^{14}C-polyethylene glycol-4000 (PEG-4000) for 2 min. Following centrifugation of the BLMV suspension at 12 000 g for 8 min, a 500 μL aliquot of the supernatant was transferred to a 5 mL scintillation vial and 3.5 mL of scintillation fluid (EcoLume, MP Biomedicals) were added. The remaining supernatant was decanted and the tubes were cleaned to ensure the lid and walls were free of any residual supernatant. The pellet was then resuspended in 40 μL of 20% Triton-X (vol/vol) and vortexed. The mixture was then transferred to a 5 mL scintillation vial and 3.5 mL of scintillation fluid were added. The radioactivity (in counts per minute; cpm) of both the supernatant and pellet was measured using a two-channel liquid scintillation counter (Beckman Coulter LS 6500 Multi-Purpose Scinitillation Counter). Protein-normalized vesicle volumes were calculated (Brand, 1995) and expressed as μL mg protein^{-1}.

Urea transport assays

Transport of ^{14}C-urea was measured at 22°C by rapid filtration techniques (McDonald and Wood, 2004a). BLMV urea uptake was initiated by adding 135 μL of assay medium (250 mM sucrose, 10 mM KNO_3, 11 mM $MgSO_4$, 6 mM Na_2-EDTA, 20 mM Hepes; adjusted to pH 7.4 with Tris) to 35 μL of the vesicle suspension. For urea concentration series experiments, sucrose in the assay solution was replaced with urea to keep a constant osmolality across all the different solutions and to reduce the effects of any osmotic differences caused by increasing the urea content. Following immediate mixing (by vortexing), the suspension was incubated for a short timed period. In preliminary experiments, the relationship between incubation time and urea uptake across the vesicular membrane was established. Urea accumulation in the BLMV was

linear until 40 seconds, at which time urea uptake reached a plateau of maximum filling capacity (Figure 3.1). Therefore, a 17 sec incubation period was used for all subsequent experiments. Transport was terminated by quickly transferring the vesicle/assay mixture to nitrocellulose filters (Whatman Protran BA85 filters) pre-incubated in an ice-cold stop solution with a high urea content (250 mM sucrose, 10 mM KNO_3, 10 mM $MgSO_4$, 50 mM urea, 20 mM Hepes; adjusted to pH 7.4 with Tris) so as to reduce non-specific binding of ^{14}C-urea to the filters. Filters were washed twice with 5 mL of ice-cold stop solution to remove any residual free ^{14}C-urea, then transferred to scintillation vials containing 10 mL of scintillation fluid (EcoLume, MP Biomedicals) and counted using a liquid scintillation counter (Beckman LS3801 Liquid Scintillation Counter). The rate of ^{14}C-urea (μmol hr^{-1} mg protein^{-1}) was calculated as:

$$J^{in}_{urea} = cpm_f / (A_{medium} \cdot p \cdot t)$$

where cpm_f represents the counts present on the filters, A_{medium} is the activity of the assay medium in cpm μmol^{-1}, the amount of protein in mg present in the assay suspension is represented by p, and *t* is the incubation time in hours. A_{medium} was calculated for each stock urea concentration as the cpm of the solution divided by total urea (in μmol), i.e. the sum of both non-radiolabelled and radiolabelled (specific activity 54 mCi mmol^{-1}; MP Biomedicals) urea components of the assay medium. Protein concentrations were determined by the Bradford Method (Bradford, 1976) using Bradford reagent (Sigma-Aldrich) with bovine serum albumin standards.

For *Series III*, Q_{10}, defined as the ratio of two rates for a temperature difference of 10°C, was calculated as follows:

$$Q_{10} = [R_{T2} \div R_{T1}] \bullet \exp (10/T2\text{-}T1)$$

where R_{T2} and R_{T1} are the V_{max} values (μmol mg protein^{-1} hr^{-1}) calculated from the Eadie-Hofstee plots for the high (T2) and low (T1) temperature, respectively.

Analytical techniques

Plasma cortisol concentrations (for *Series II*) were measured using a commercially available ^{125}I radioimmunoassay kit from MP Biomedicals.

Statistical analyses

Values are expressed as the mean ± the standard error of the mean (s.e.m). Non-linear or linear regressions, as appropriate, were used to determine the line of best fit for urea concentration dependence experiments. Correlation coefficients (r^2) were generated from regression analysis using SigmaPlot 10.0 software (Systat Software, Inc). Total transport over the entire range of urea concentrations was defined as a sum of two regressions, a linear component ($y = mx+b$) and a saturation component ($y = K_m x / (V_{max} + x)$). An Eadie-Hofstee plot was used to calculate the Michaelis-Menten constant, K_m, and maximum velocity, V_{max}, as the more conventional Lineweaver-Burke method is associated with higher rates of error (Dowd and Riggs, 1965; Raaijmakers, 1987).

For all experiments focusing on the effects of transport and metabolic inhibitors, an estimated diffusion component at the experimental urea concentration was subtracted from the individual values of urea uptake measured at each concentration, thus giving a rate of uptake due to urea transport proteins alone. A one-way analysis of variance (ANOVA) was performed to compare the differences in urea uptake among BLMV exposed to phloretin,

various competitive urea analogues and ATPase inhibtors. In cases where significant differences were detected, a Holm-Sidak test was used for post-hoc multiple comparisons. The dose-dependence curve was plotted as a sigmoidal dose-response regression ($y=a+[(b-a)/(1+(10^{(logIC50-x)*m})]$. Where assumptions of normality or equal variance were not satisfied, equivalent non-parametric tests were used. Results were considered statistically significant when the p-value was less than 0.05. Statistical analyses were performed using SPSS SigmaStat v3.5 (Systat Software Inc.).

Results

Validation of BLMV preparation

Measurement of Na^+,K^+-ATPase activity demonstrated a significant 5.5-fold enrichment of toadfish gill BLMV preparations when compared to the initial crude homogenate (Table 3.1). The same fractions revealed that enzyme activities for cytochrome C oxidase, glucose-6-phosphate, and NMN-adenylyltransferase were reduced to 6.9×10^{-4}, 0.16, and 0.19-fold, respectively, of the crude homogenate (Table 3.1).

Of the total basolateral membrane population, 58.77 ± 8.03% of the membranes formed resealed vesicles, as indicated by Na^+,K^+-ATPase activity. Among resealed BLMV, 11.02 ± 3.86 % were in the inside-out orientation. Furthermore, protein-normalized vesicle volumes averaged 4.21 ± 0.45 μL mg protein^{-1}, providing further evidence for vesicle resealing.

Series I

In vitro BLMV urea uptake measurements over a physiological range of concentrations showed two components of urea influx. At concentrations from 1 to 20 mmol L^{-1}, urea uptake was linearly dependent on the external urea concentration (r^2=0.98, Figure 3.2A). At lower urea concentrations (<1 mmol L^{-1}), urea uptake exhibited saturation kinetics (r^2=0.96, Figure 3.2B). Eadie-Hofstee transformation of these data (Figure 3.2C) revealed a K_m of 0.24 mmol L^{-1} and V_{max} of 6.95 μmol mg protein^{-1} hr^{-1} (Table 3.2).

Saturable urea uptake in toadfish BLMV was sensitive to the transport inhibitor phloretin (Figure 3.3A), with an IC_{50} of less than 0.11 mmol L^{-1}. Urea uptake was significantly reduced by 3.24-fold in the presence of pCMBS ($p<0.05$; Figure 3.3B). Treatment of BLMV with both pCMBS and phloretin resulted in a 4.28-fold reduction ($p<0.05$) in the rate of urea influx relative to the control rate; however, this rate was not significantly different from either phloretin or pCMBS alone ($p>0.05$; Figure 3.3B). Exposure to acetamide or thiourea caused, respectively, 1.5 and 1.74-fold inhibition in urea uptake ($p<0.05$), whereas N-methylurea treatment had no effect on urea movement ($p>0.05$; Figure 3.4). Altering ATP availability in the assay medium by adding ATP alone, ATP + ouabain, or ATP + NEM did not significantly affect urea influx in BLMV compared to control uptake rates (5.0 ± 0.8 μmol mg protein^{-1} hr^{-1}, n=8, $p>0.05$), nor did manipulating the sodium gradient (3.8 ± 0.4 μmol mg protein^{-1} hr^{-1}, n=12, $p>0.05$).

Series II

Fish that underwent a standard crowding protocol in the lab (regardless of whether they were treated with cortisol) showed saturable urea uptake at low urea concentration (<1 mmol L^{-1}), whereas urea uptake in fish held in a mesocosm was linear over all external urea concentrations (Figure 3.5). Cortisol treatment of lab crowded fish caused the K_m value to increase but dramatically lowered V_{max} by 0.23-fold (Table 3.2). Analysis of plasma cortisol at the time of sampling confirmed that infused toadfish exhibited 19-fold higher circulating cortisol concentrations compared to lab crowded fish held at 22°C ($p<0.05$; Figure 3.6).

Series III

Urea uptake in lab crowded fish demonstrated non-linear saturable concentration kinetics, regardless of acclimation temperature (Figure 3.7). However, differences were apparent in the kinetic parameters of the saturation curves (Table 3.2). V_{max} increased as a function of increasing acclimation temperature, yielding a Q_{10} value of 3.33 (Table 3.2). No obvious dependence of K_m on temperature was detected (Table 3.2).

Figure 3.1: *Series I*. The relationship between urea uptake (μmol mg protein^{-1}) and time (seconds) in basolateral membrane vesicles (BLMV) of toadfish gill. Values represent means ± s.e.m (n=11).

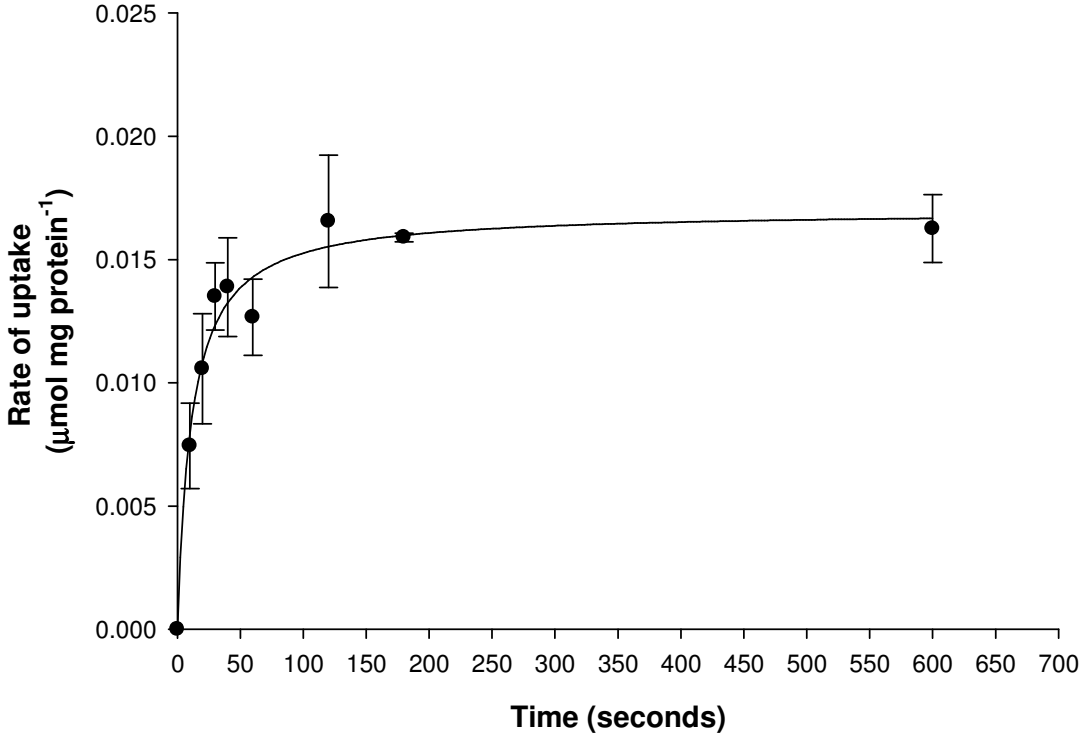
0.025
0.020
0.015
0.010
0.005
0.000
Rate of uptake
(µmol mg protein-1)
0
50
100
150
200
250
300
350
400
450
500
550
600
650
700
Time (seconds)

Figure 3.1

Figure 3.2: *Series I.* (A) The relationship between the rate of urea uptake (μmol mg protein^{-1} hr^{-1}) and external urea concentration (mmol L^{-1}) of basolateral membrane vesicles (BLMV) isolated from the gill of toadfish acclimated to 22°C. The regression is y=9.9032x, r^2=0.98, p<0.05 (n=12). (B) Expansion of the lower range of urea concentrations from A. The regression is y=(6.95x)/(0.24+x), r^2=0.96, p<0.05. (C) Eadie-Hofstee transformation (y=-4.15x+30.69, r^2=0.82, p<0.05) derived from the data in A. All values represent means ± s.e.m (n=12).

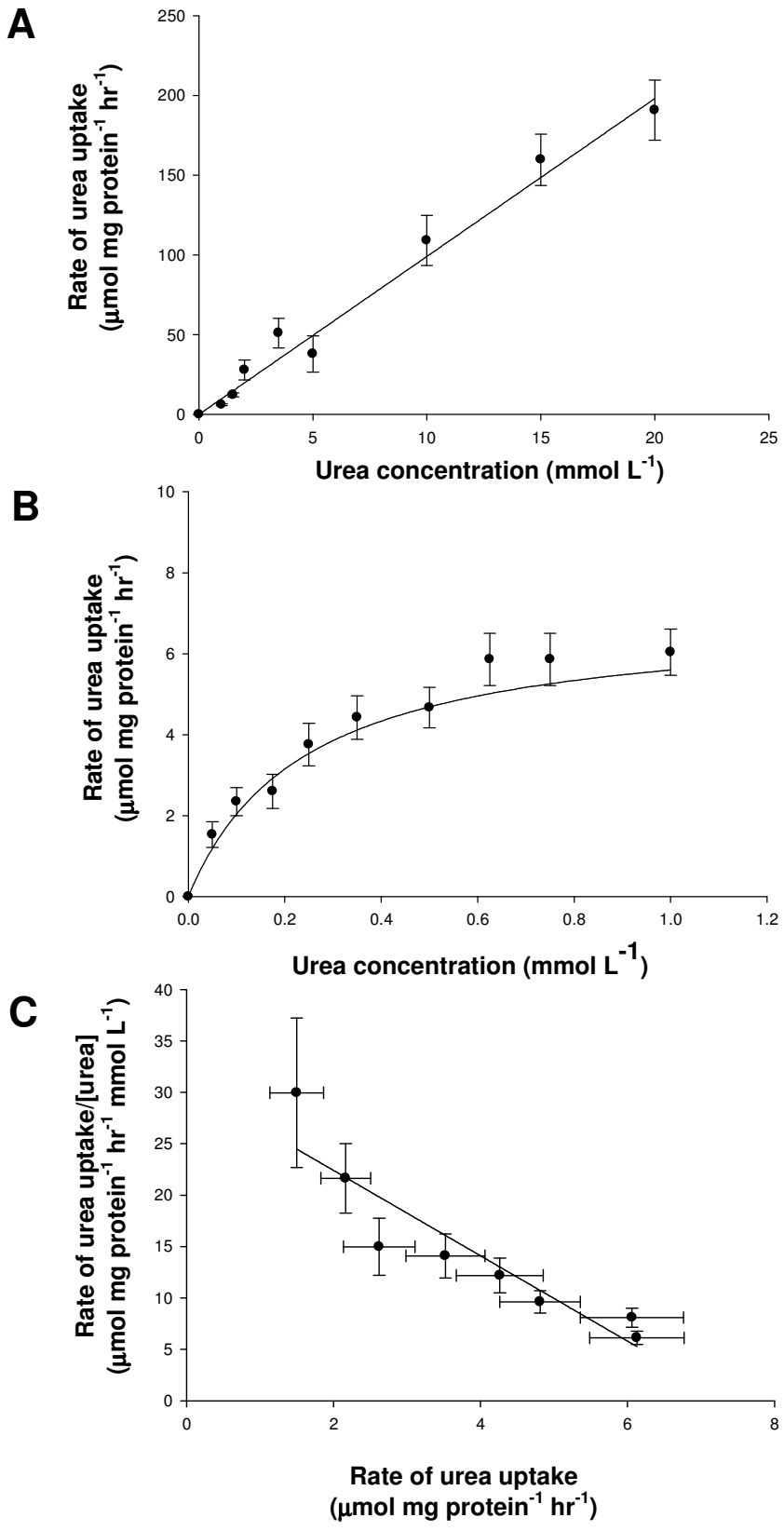
A
Rate of urea uptake
(µmol mg protein-1 hr-1)
Urea concentration (mmol L-1)
B
Rate of urea uptake
(µmol mg protein-1 hr-1)
Urea concentration (mmol L-1)
C
Rate of urea uptake/[urea]
(µmol mg protein-1 hr-1 mmol L-1)
Rate of urea uptake
(µmol mg protein-1 hr-1)

Figure 3.2

Figure 3.3: *Series I.* (A) The effect of phloretin on saturable urea uptake (μmol mg protein^{-1} hr^{-1}) by toadfish gill basolateral membrane vesicles (BLMV) incubated in 0.4 mmol L^{-1} urea (n=10). The regression is $y=0.93+[(1.08)/(1+10^{(\log 0.11-x)*(-27.63)})]$, $r^2=0.90$, $p<0.05$. An asterisk indicates a significant difference from the ethanol control ($p<0.05$). (B) The effects of phloretin (0.25 mmol L^{-1}), pCMBS (0.25 mmol L^{-1}) and a combination of both on urea uptake (μmol mg protein^{-1} hr^{-1}) in BLMV isolated from toadfish gill (n=10). BLMV were incubated in 0.4 mmol L^{-1} urea. Treatments that share a letter are not significantly different from one another ($p>0.05$). Values are expressed as means ± s.e.m.

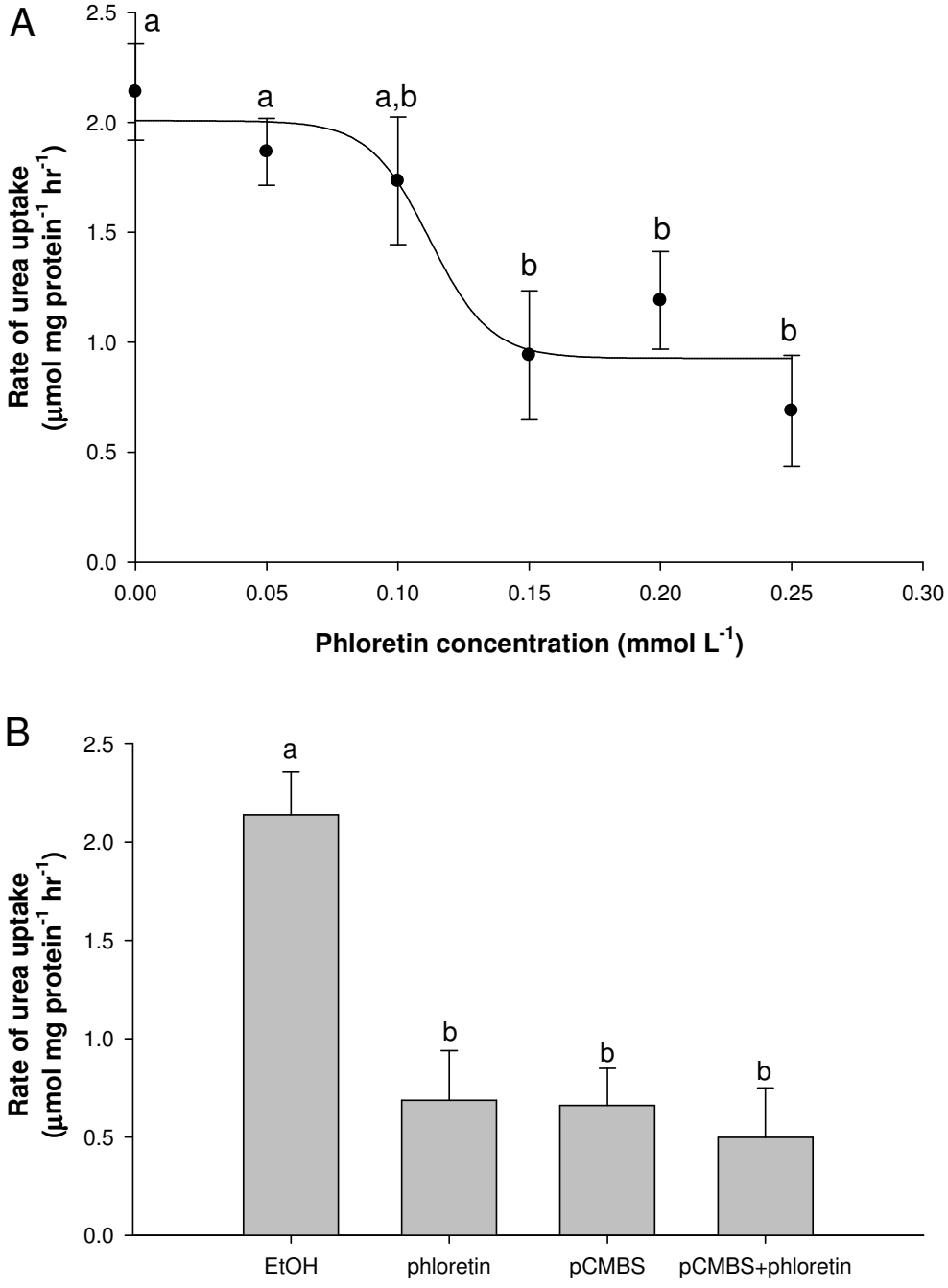
A
a
a
a,b
b
b
b
Rate of urea uptake (µmol mg protein-1 hr-1)
2.5
2.0
1.5
1.0
0.5
0.0
0.00
0.05
0.10
0.15
0.20
0.25
0.30
Phloretin concentration (mmol L-1)
B
a
b
b
b
Rate of urea uptake (µmol mg protein-1 hr-1)
2.5
2.0
1.5
1.0
0.5
0.0
EtOH
phloretin
pCMBS
pCMBS+phloretin

Figure 3.3

Figure 3.4: *Series I.* The effects of analogues, acetamide, thiourea, and N-methylurea, on urea uptake (μmol mg protein^{-1} hr^{-1}) in basolateral membrane vesicles (BLMV) isolated from toadfish gill (n=10). Vesicles were incubated in 0.4 mmol L^{-1} urea and 0.8 mmol L^{-1} analogue compound. Values are expressed as means ± s.e.m. Treatments that share a letter are not significantly different from one another ($p>0.05$).

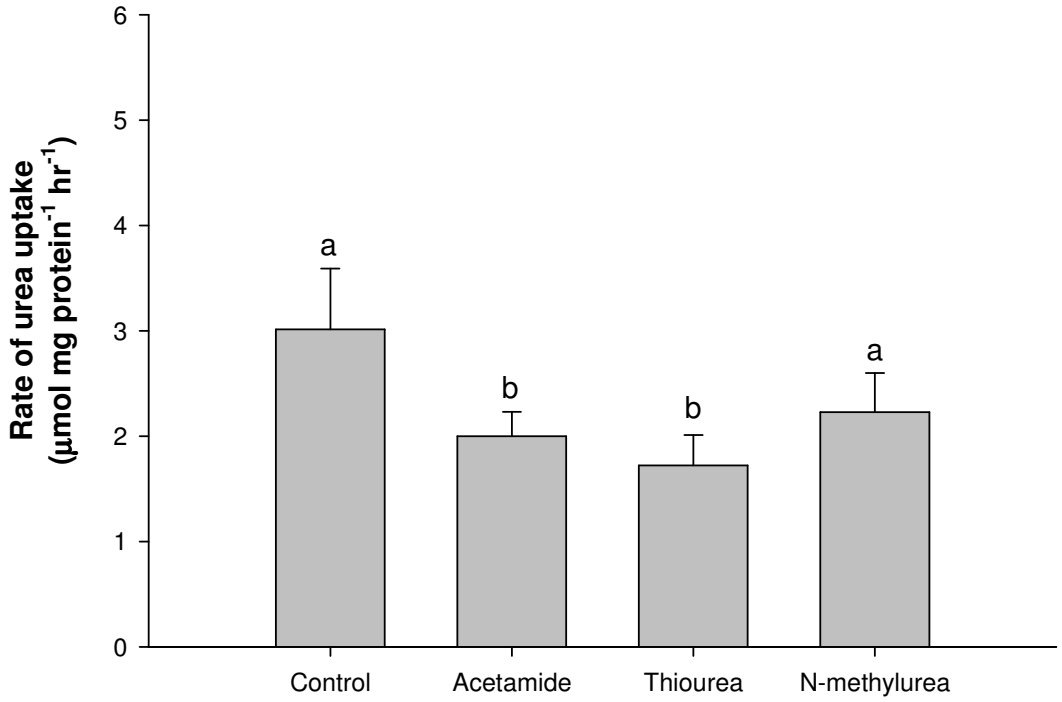
Rate of urea uptake
(µmol mg protein-1 hr-1)
6
5
4
3
2
1
0
a
b
b
a
Control
Acetamide
Thiourea
N-methylurea

Figure 3.4

Figure 3.5: *Series II.* Toadfish gill basolateral membrane vesicle (BLMV) uptake rates at low urea concentrations in response to different treatments including lab crowded fish (n=12), cortisol-infused lab crowded individuals (n=7), and mesocosm fish (n=8). The regression for lab crowded fish is $y=(6.95x)/(0.24+x)$, $r^2=0.96$, $p<0.05$; cortisol fish $y=(1.61)/(0.31+x)$, $r^2=0.96$, $p<0.05$; mesocosm fish $y=3.16x$, $r^2=0.93$, $p<0.05$. Values are expressed as means ± s.e.m.

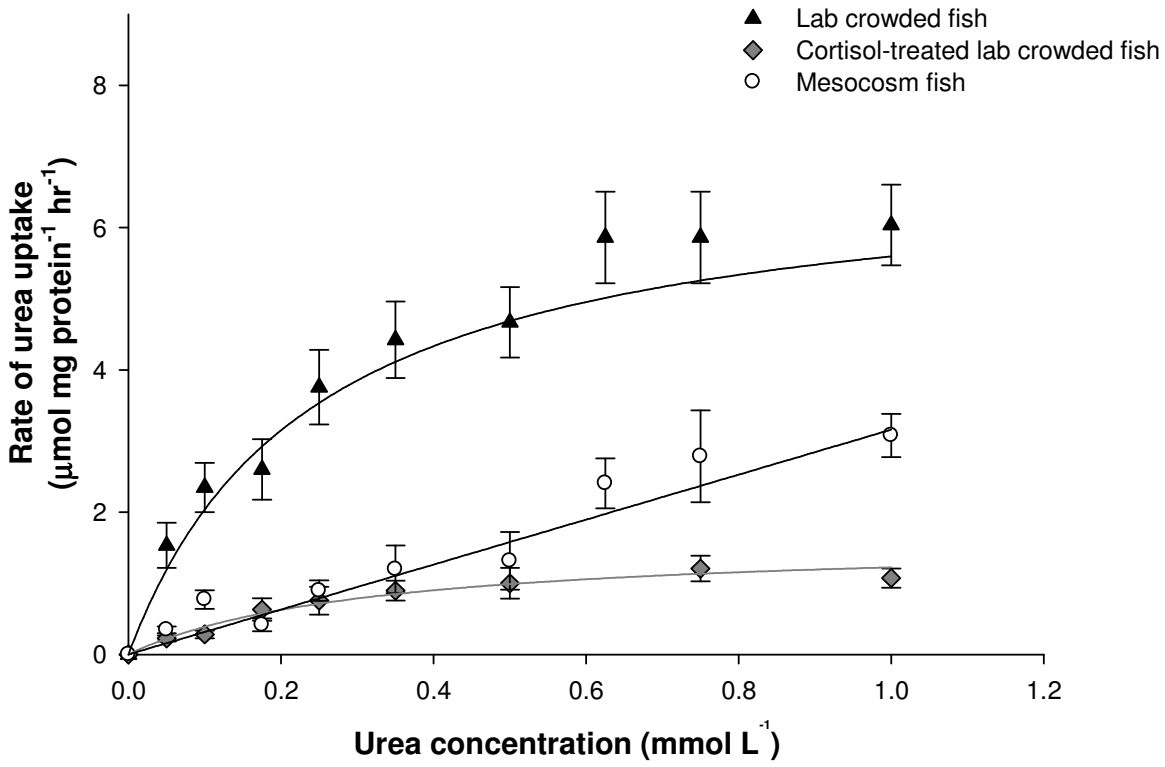
Lab crowded fish
Cortisol-treated lab crowded fish
Mesocosm fish
Rate of urea uptake
(μmol mg protein-1 hr-1)
0
2
4
6
8
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Urea concentration (mmol L-1)

Figure 3.5

Figure 3.6: *Series II.* Plasma cortisol concentrations (ng mL^{-1}) in toadfish in response to different holding, cortisol (n=7) and temperature treatments (n=12, 22°C; n=6, 28°C). Treatments that share a letter are not significantly different from one another ($p>0.05$). Values are expressed as means ± s.e.m.

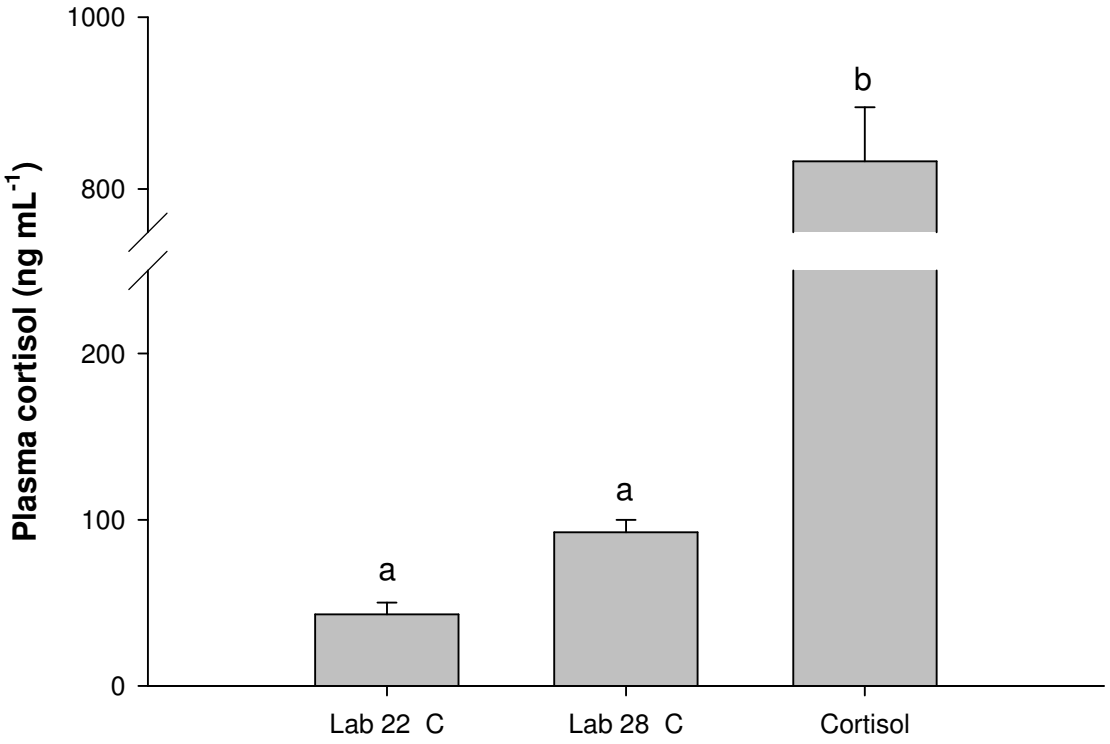
1000
800
200
100
0
Plasma cortisol (ng mL-1)
a
a
b
Lab 22 C
Lab 28 C
Cortisol

Figure 3.6

Figure 3.7: *Series III.* Toadfish gill BLMV uptake at low urea concentrations in response to various acclimation temperatures at 18°C (n=11) and 28°C (n=6). Values are expressed as means ± s.e.m. The regression for 18°C acclimated fish is $y=(1.43x)/(0.34+x)$, $r^2=0.95$, $p<0.05$ and 28°C acclimated fish $y=(10.63x)/(0.37+x)$, $r^2=0.92$, $p<0.05$. Values are expressed as means ± s.e.m.

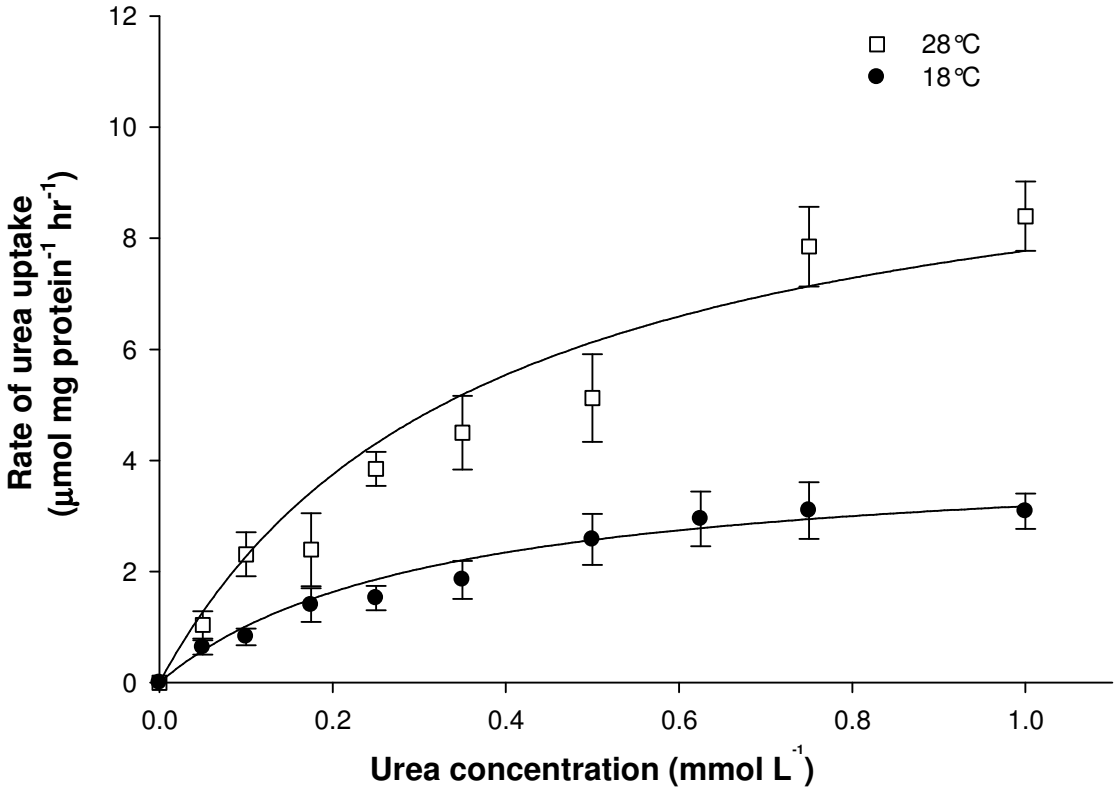
28℃
18℃
Rate of urea uptake
(µmol mg protein-1 hr-1)
Urea concentration (mmol L-1)
0
2
4
6
8
10
12
0.0
0.2
0.4
0.6
0.8
1.0

Figure 3.7

Table 3.1: *Series I.* Specific activities of marker enzymes indicating the degree of purification of basolateral membranes.

	Na^+K^+-ATPase	**Glucose-6-phosphate**	**Cytochrome C oxidase**	**NMN-adenyltransferase**
Crude Homogenate	1.25 ± 0.37	0.085 ± 0.049	1.28 ± 0.54	0.11 ± 0.018
BLMV	6.83 ± 0.87	0.014 ± 0.0017	0.00088 ± 0.00048	0.022 ± 0.0033
Fold Change	5.46	0.16	0.00069	0.19

[1]Values are means ± s.e.m. in μmol substrate mg protein^{-1} hr^{-1} (n=8).

Table 3.2: *Series I-III.* A summary of K_m and V_{max} values for saturable urea uptake by BLMVs calculated from Eadie-Hofstee plots (n=6-12).

Environment	Temperature (°C)	Treatment	K_m (mmol L^{-1})	V_{max} (µmol mg protein^{-1} hr^{-1})	Q_{10}
Laboratory	22	N/A	0.24	6.95	N/A
	22	Cortisol	0.31	1.61	N/A
	18	N/A	0.31	3.19	N/A
	28	N/A	0.37	10.63	3.33[a]
Mesocosm	28	N/A	N/A	N/A	N/A

[a] represents the Q_{10} ratio of the V_{max} values of 18 and 28°C

Discussion

Methodology

In the present study a well-characterized method was employed to prepare plasma membranes from the gill epithelium of toadfish, as previously described in rainbow trout (Bury et al., 1999; McDonald and Wood, 2004a; Perry and Flik, 1988) and little skate (Fines et al., 2001). A 5.5-fold enrichment of Na^+,K^+-ATPase activity indicated that there was a selective isolation of basolateral plasma membranes, similar to other studies (Bury et al., 1999; Fines et al., 2001; McDonald and Wood, 2004a; Perry and Flik, 1988). Only trace amounts of membranes from the ER, nucleus and mitochondria were present in the final homogenate. It is possible that this contamination may have resulted in a slight over-estimation of urea uptake by gill BLMV as some fish mitochondria have the capacity to transport urea (Rodela et al., 2008). The volume measurements confirmed the resealing capacity of the vesicles, which, based on Na^+,K^+-ATPase activity had a resealing efficiency (~59%) comparable to values reported in the literature (Flik et al., 1985).

Urea transport

The data support the hypothesis that urea flux is mediated by a cortisol-sensitive facilitated urea transport mechanism in the basolateral gill membrane of toadfish. Urea uptake exhibited saturation kinetics at low urea concentrations (K_m=0.24 mmol L^{-1}, V_{max}=6.95 μmol mg $protein^{-1}$ hr^{-1}) and a linear component at high concentration that is indicative of simple diffusion or movement through non-specific pathways; both properties are distinctive for piscine urea transport. These high affinity

transporters serve to equilibrate urea concentrations across intracellular compartments in fish (Rodela et al., 2008), play a prominent role in urea retention in elasmobranchs (Fines et al., 2001; Morgan et al., 2003b), and accelerate the excretion of urea in teleosts (McDonald and Wood, 2004a; Pilley and Wright, 2000). Plasma urea levels in crowded toadfish typically fluctuate around 6 mM (McDonald and Wood, 2004b), suggesting that while a basolateral urea transport mechanism in toadfish gill may only represent a fraction of the total urea transport capacity of the gill, in addition to diffusion pathways, such a transporter would greatly facilitate the movement of urea from the plasma into the gills.

Phloretin is commonly used as an analytical tool to characterize both active and facilitated urea transport in vertebrate tissues (Chou and Knepper, 1989; Levine et al., 1973; Smith and Wright, 1999). BLMV urea transport was inhibited by phloretin in a dose dependent fashion; however, this non-competitive inhibitor did not fully abolish urea transport. Phloretin has been shown to increase the fluidity and permeability of cholesterol-containing membranes (Andersen et al., 1976), an effect that may have contributed to increased levels of non-specific transport of urea across the BLMV. Incomplete inhibition of *in vitro* urea transport by similar concentrations of phloretin has been documented in a number of teleost and elasmobranch species (Fines et al., 2001; McDonald and Wood, 2004a; Morgan et al., 2003b; Rodela et al., 2008; Walsh et al., 1994). Interestingly, systemic administration of phloretin to toadfish abolished the pulsatile urea excretion pattern, although urea continued to accumulate in the external water at a steady rate (Wood et al., 1998). This finding should be interpreted with caution, however, as *in vivo* exposure to phloretin tends to yield inconsistent results

owing to its toxicity, which is most likely caused by inhibition of other essential transport systems including those for glucose and chloride (Pilley and Wright, 2000; Wood et al., 1998). *In vitro* experiments focusing on the expression of tUT in *Xenopus laevis* oocytes demonstrated that phloretin caused a significant, nearly complete, reduction in urea uptake (Walsh et al., 2000). However, the clear-cut results of this study may be due to the fact that an isolated part of the urea excretion mechanism was examined, whereas, there may be other branchial influences on urea transport in BLMV. Furthermore, the sub-cellular branchial location of this cloned transporter remains unclear.

In addition to phloretin inhibition, BLMV urea transport was greatly reduced in response to pCMBS. Mercury reagents, such as pCMBS and $HgCl_2$, are inhibitory compounds that target the cysteine residues on channel proteins including urea transporters (Tsukaguchi et al., 1998) and intrinsic water channels called aquaporins (AQP; Kuwahara et al., 1997; Stewart et al., 2006). A subgroup of phloretin-sensitive AQPs (AQP3, AQP9) are permeable to urea and other small neutral solutes, in addition to water (Bagnasco, 2005; Tsukaguchi et al., 1998; Verkman and Mitra, 2000). Although the potential involvement of AQPs in urea pulsing events has not been examined in toadfish, there is evidence of concurrent elevated urea and tritiated water efflux during pulse events in two toadfish studies (Gilmour et al., 1998; Perry et al., 1998). Urea transport in toadfish may be a product of two separate and distinctly located systems involving both UTs and AQPs. Support for this claim comes from immunohistochemical studies on branchial tissue from *Anguilla anguilla* demonstrating that the eel UT (eUT; Mistry et al., 2001) and AQP3 (Lignot et al., 2002) are co-localized in chloride cells, with

basolateral and apical positions, respectively. While the location of the branchial pulsatile urea excretion mechanism in toadfish remains unclear, there is morphological data suggesting the involvement of pavement cells (Laurent et al., 2001) and strong molecular evidence supporting the chloride cells (McDonald et al., 2009). Due to limitations of the techniques used in the present study, it was not possible to differentiate or separate the heterogeneous population of pavement and chloride cells that composed BLMV preparation and therefore it could not be determined which cell types exhibited facilitated basolateral urea transport. Further morphological and immunohistochemical studies following the development of a suitable antibody for both tUT and putative AQPs may resolve these issues.

The differential pattern of BLMV uptake of urea following exposure to urea analogues was consistent with similar *in vivo* studies on branchial excretion (Gilmour et al., 1998; McDonald et al., 2000; Wood et al., 1998). *In vitro* BLMV urea permeability demonstrated similar inhibitory effects with thiourea and acetamide. These compounds act as competitive inhibitors of carrier-mediated urea transport, although the potency of analogues varies among species (Chou and Knepper, 1989; Garcia-Romeu et al., 1981; Shpun and Katz, 1990). For example, *in vivo* and *in vitro* data from rainbow trout demonstrate a pattern of handling wherein urea transport ≥ thiourea uptake > acetamide uptake (McDonald and Wood, 2004a; Pilley and Wright, 2000), whereas *in vitro* preparations from two elasmobranch species, *Squalus acanthias* and *Raja erinacea*, were insensitive to these chemicals (Fines et al., 2001; Morgan et al., 2003b). These quantitative differences in analogue potency may be the product of the regulatory state of the protein (e.g. methylation, phosphorylation) and compositional

differences in lipid content of plasma membranes (Chou and Knepper, 1989; Mayrand and Levitt, 1983). Such factors may be especially relevant when considering the temporal nature of urea excretion in toadfish. Furthermore, unlike urea transporters, AQPs are unable to transport urea analogues such as acetamide or thiourea (Virrki et al., 2002). Although BLMV did demonstrate a sensitivity to these two compounds suggesting that there was a competitive interaction with urea most likely *via* a facilitated urea transporter, the partial inhibition was small (1.5- and 1.7-fold for acetamide and thiourea, respectively) and it may be an indication that two urea transport mechanisms are present: a UT component that is sensitive to urea analogues, and another (possibly AQP) that is insensitive to the same compounds.

Some piscine species such as *Raja erinacea* (Morgan et al., 2003b) and *Squalus acanthias* (Fines et al., 2001) rely on a Na^+-coupled secondarily active transport system to scavenge intracellular urea and actively return it to the blood to maintain low rates of efflux and high rates of retention, the hallmark of the elasmobranch osmoregulatory strategy. The results presented in this study further diminish the possibility of a reabsorptive "back-transport" system in toadfish as was first demonstrated in vivo by Wood et al. (1995). BLMV urea transport in toadfish was unaffected by *in vitro* ouabain, NEM, and sodium treatments; conditions which inhibit sodium-coupled and proton-coupled urea transport in cartilaginous fish (Fines et al., 2001; Morgan et al., 2003b), mammals (Kato and Sands, 1998), and frogs (Rapoport et al., 1989) *via* prevention of endogenous ATPase function. Movement of urea across the basolateral membrane of toadfish appears to be due to a facilitative urea transport mechanism, a finding that

supports the hypothesis first outlined by Wood et al. (1997) whereby, specific urea transport proteins are periodically inserted and/or activated in the gill cells.

In vivo manipulation with cortisol caused a significant 4.3-fold reduction in BLMV urea transport capacity (V_{max}) in lab crowded fish. These findings are in accord with parallel physiological studies demonstrating that infusion of cortisol suppresses urea pulsing in toadfish, specifically by decreasing pulse size, with no discernable influence on pulse frequency (Hopkins et al., 1995; McDonald et al., 2004; Wood et al., 2003; Wood et al., 2001). Glucocorticoids regulate urea movement in the mammalian kidney by reducing permeability in the renal inner medullary collecting duct (IMCD) of rats through down-regulation of the abundance of UT proteins and mRNA (Naruse et al., 1997; Peng et al., 2002). Recent evidence has shown that chronic cortisol infusion causes an increase in tUT mRNA; however, despite this increase in tUT message, these same fish had a significant decrease in rates of urea excretion (McDonald et al., 2009). Examination of the current literature shows that the time course for cortisol-mediated effects on urea excretion rates in toadfish *in vivo* is fairly rapid, with responses elicited in 15 to 60 min following experimentally-induced changes in circulating cortisol levels, and less than 4 hours under more natural circumstances (Wood et al., 2001). This latency is characteristic of non-genomic cascades (reviewed by Borski, 2000; Wehling, 1997) implying that in ureotelic toadfish under resting conditions, the interactions between cortisol and urea excretion may be mediated through non-genomic pathways. Additional supportive evidence comes from molecular analysis, which has identified a number of potential phosphorylation and glycosylation sites on the tUT gene (Walsh et al., 2000). Further experiments focusing on the *in vitro* sensitivity to inhibitors such as

actinomycin D and cyclohexamide may help evaluate the relative contribution of transcriptional and translational pathways, respectively, in the pulsatile discharge of urea in toadfish.

Mesocosm fish were kept at low stocking density in an enclosure that mimicked a natural toadfish environment. Toadfish kept in this uncrowded environment in a previous study had low circulating cortisol levels (23.9 ± 9.5 ng mL^{-1}; McDonald et al., 2009). BLMVs from such fish exhibited a poor kinetic correlation for saturation and Eadie-Hofstee plots but a strong linear relationship between urea uptake and external concentration, characteristics of diffusion or non-specific pathways. Interestingly, field studies have shown that toadfish excrete a mix of urea and ammonia in natural settings (Barimo et al., 2004; Hopkins et al., 1997). The absence of a basolateral UT in mesocosm fish supports the prominent or rate-limiting role of the apical membrane in pulsatile urea excretion as is conserved in uncrowded toadfish (Laurent et al., 2001). A basolateral UT may be advantageous in situations dealing with relatively high physiological concentrations of urea that need to be rapidly equilibrated across the plasma and gill tissue. Lab-crowded fish tend to have a higher degree of ureotely and plasma urea levels than their uncrowded counterparts (Barimo et al., 2004; Hopkins et al., 1997; McDonald et al., 2004).

Urea uptake across the basolateral membrane showed a differential response to varying acclimation temperatures. Most biological reactions are temperature-sensitive and typically have a Q_{10} value greater than 2, whereas purely physical processes, such as diffusion, have values closer 1. Temperature coefficient analysis of V_{max} values demonstrated an increase in BLMV urea influx between temperatures of 18 and 28°C,

with V_{max} undergoing a 3-fold increase. This evidence further supports the hypothesis that a membrane-bound urea transport protein is present on the gill basolateral membrane of toadfish. Similar temperature-sensitive urea transport processes have been reported in toadfish hepatocytes and the gill basolateral membrane of rainbow trout (McDonald and Wood, 2004; Walsh and Wood, 1996). Of further note, in *Series II*, a direct comparison of kinetic urea influx was carried out between mesocosm fish that were acclimated to 28°C and lab-housed individuals acclimated to a temperature of 22°C. While the phase behaviour and physical properties of biological membranes can be modified in response to thermal perturbations, the extent of the compensatory response varies considerably among species, cell types, and particular membranes (reviewed by Hazel, 1995). For the purpose of this study, it appears that temperature had an effect on the absolute rates of urea influx and no discernable effect on the kinetic properties of urea uptake. Differences in the pattern of urea uptake seen between mesocosm fish and lab crowded individuals are due to the cortisol-related effects of crowding, not temperature.

Perspectives and significance

There is considerable diversity of the physiological role of urea transporters characterized from fish, amphibians and mammals, however, structural analysis of UTs demonstrates a high degree of sequence similarity (50 -70%) among all these genes (Bagnasco, 2005; McDonald et al., 2006). Evidence suggests that these UTs all evolved from a common ancestor through several sequence divergence and duplication events, and all current paralogues and orthologues in vertebrates arose from one primordial

system (Minocha, 2003; Sands, 2003). Mammals, reportedly express over 20 isoforms of UT-A and UT-B as products of alternative splicing (Bagnasco, 2003; Minocha, 2003; Sands, 2003). Within the piscine lineage, there is increasing molecular and physiological evidence for the existence of multiple UT isoforms among and within different tissues (McDonald et al., 2000; Mistry et al., 2001; Morgan et al., 2003b). The mechanisms regulating the expression of the non-mammalian UTs are not well characterized, however, evidence from the gulf toadfish demonstrates that it is likely that the system may be similar to the one described in mammals. Glucocorticoids reduce urea permeability in the mammalian kidney by causing a downregulation of the quantity of UT mRNA and proteins (Naruse et al., 1997b; Peng et al., 2002). Evidence in the present study indicates that a similar cortisol-sensitive pathway regulates the insertion or activation of facilitated urea transport protein(s) in the gills of the toadfish; expanding on previous *in vivo* data demonstrating cortisol is involved in regulating urea pulse size in this unusual species (Wood et al., 1998). It is interesting to note that two such distantly related vertebrates (mammals and teleosts) share similar regulating mechanisms for urea excretion, and the similarity raises questions about the evolutionary origins of these systems. Analogous to the UT, the current parsimonious hypothesis for the evolution of the GRs among tetrapods is one of sequence divergence and duplication from a common ancestor with retained functional characteristics (Bury and Sturm, 2007). While the precise mechanisms (genomic vs. non-genomic) of urea transport regulation remain unclear in toadfish, it is clear that future investigations should examine the upstream signal target for cortisol action in the promoter region of

the gene for both putative glucocorticoid response elements and potential phosphorylation/glycosylation sites on the UT protein itself.

Acknowledgements

This study was supported by NSERC discovery grants to P.J.W. and K.M.G., and an NSF grant to M.D. McDonald (IBN-0455904). PJW is supported by the Canada Research Chair program. Special thanks to Jimbo Luznar for collection of toadfish, and Mike Morando for care and feeding of the RSMAS toadfish colony.

CHAPTER 4

Evidence for transcriptional regulation of the urea transporter in the gill of the gulf toadfish, *Opsanus beta*

Notes on Chapter 4

This chapter had multiple authors and will be submitted to a journal as:

Rodela, T.M., Esbaugh, A.J., McDonald, M.D., Gilmour, K.M., and Walsh, P.J. Evidence for transcriptional regulation of the urea transporter in the gill of the gulf toadfish, *Opsanus beta*

The contribution of A.J. Esbaugh was the identification and isolation of the tUT promoter fragment, see first paragraph of *Development of tUT reporter assay* in the Materials and Methods section. M.D. McDonald was a collaborator at the University of Miami, where *in vivo* experiments were conducted by T.M. Rodela.

Abstract

Ureotelic gulf toadfish (*Opsanus beta*) do not excrete urea continuously; instead, urea is accumulated internally until a branchial urea transport mechanism is activated to facilitate the excretion of urea in distinct pulses. This unusual pulsatile urea excretion pattern is regulated, in part, by the permissive action of declines in circulating cortisol concentrations. An earlier study reported that exogenous cortisol loading reduced the rate of urea excretion but also caused an upregulation in toadfish urea transporter (tUT) transcript levels. The current study sought to extend these findings by using a combination of physiological and molecular methods to examine tUT and glucocorticoid receptor (GR) transcript levels in toadfish following chronic and acute changes in corticosteroid action. Experimentally lowering corticosteroid action by inhibiting cortisol synthesis, or by blocking the glucocorticoid or mineralocorticoid receptor did not significantly alter tUT mRNA abundance. *In silico* analysis of an isolated 1.2 kb fragment, upstream promoter region of the tUT gene, revealed 6 putative glucocorticoid response element (GRE) half sites suggesting that cortisol may enhance tUT mRNA expression through genomic pathways. *In vivo* reporter assays of the tUT promoter fragment demonstrated minimal relative luciferase activity in unconfined toadfish with low cortisol. Relative luciferase activity was enhanced 3.4- and 9.8-fold following exposure to moderate (*via* crowding stress) and high (*via* infusion) cortisol. On an acute timescale, real-time PCR analysis of relative mRNA expression revealed a 6.2-fold upregulation of tUT transcript 12 to 18 hours following a urea pulse event. We conclude that an upregulation in tUT mRNA abundance mediated by GREs may be required to maintain tUT activity by offsetting post-transcriptional

and/or post-translational changes that may be associated with chronically elevated plasma cortisol.

Introduction

The excretion of urea, a waste product of nitrogen metabolism, is primarily an adaptation for terrestrial existence that is used by tetrapod vertebrates (Bagnasco, 2005). Yet, there are a few adult teleost fish that have the capacity to generate urea *via* the ornithine-urea cycle (O-UC) and that excrete considerable quantities of this waste product (Anderson, 2001; McDonald et al., 2006; Walsh, 1997). Since the initial studies of Walsh and colleagues (Walsh et al., 1990; Walsh et al., 1994), the gulf toadfish (*Opsanus beta*) has become one of the best characterized piscine models with respect to the excretion of urea, and it is also one of the most unusual. Field and outdoor mesocosm studies have demonstrated that toadfish co-excrete equal quantities of ammonia and urea under natural conditions (Barimo et al., 2007; Barimo et al., 2004; Barimo et al., 2010). Ammoniotelism predominates slightly in the laboratory under conditions of minimal stress (Walsh and Milligan, 1995), but exposure to stressors (e.g. crowding, confinement, air emersion, high ambient ammonia) induces a clear switch to ureotelism in toadfish (Wood et al., 2003). This transition is facilitated by an acute rise in plasma cortisol concentrations accompanied by an increase in liver glutamine synthetase (GSase) mRNA and activity (Esbaugh and Walsh, 2009; Hopkins et al., 1995; Kong et al., 2000; Walsh and Milligan, 1995; Walsh et al., 1994). GSase shuttles ammonia as glutamine into the piscine O-UC, resulting in increases in urea production and plasma urea levels. Despite the accumulation of urea from continuous synthesis, toadfish excrete urea across the gills in discrete pulses that occur up to several times a day, with each pulse lasting up to 3 hours (Gilmour et al., 1998; Wood et al., 1995; Wood et al., 1997). Between pulses, little to no urea is excreted (Wood et al., 2003). The

pulsatile excretion of urea in toadfish is believed to be facilitated by the periodic activation of a urea transport protein (tUT) in mitochondrion-rich cells of the gill (McDonald et al., 2009; Walsh et al., 2000; Wood et al., 1997). In addition to the strong correlation between urea production capacity via GSase activation and elevated plasma cortisol, there is physiological evidence that cortisol is involved in the regulation of tUT/the urea excretion pathway (Hopkins et al., 1995; McDonald et al., 2004; Rodela et al., 2009a; Wood et al., 1997). When circulating cortisol is maintained at a high concentration (> 500 ng mL^{-1}) by chronic infusion, there appears to be an inhibition of tUT function, as indicated by a significant reduction in pulsatile urea excretion and an accompanying rise in plasma urea concentrations (McDonald et al., 2004). However, a recent study documented molecular evidence that appears on the surface to be in opposition to the physiological evidence: toadfish increased tUT mRNA levels in response to elevated cortisol (McDonald et al., 2009). These results suggest a complicated relationship between tUT transcription and tUT protein function, as measured by changes in urea excretion. Beginning several hours prior to a urea pulse, plasma cortisol levels decline significantly (from approximately 120 ng mL^{-1} to 40 ng mL^{-1} in cannulated fish) to be at a minimum coinciding with the peak of the pulse event, and rise rapidly thereafter (Wood et al., 1997; Wood et al., 2001). On occasion, a drop in plasma cortisol occurs without an associated pulse, indicating that cortisol is a permissive rather than causative agent of urea pulses (Wood et al., 2003a; Wood et al., 2001). It is postulated that the acute pre-pulse cortisol decline enables the monoamine serotonin (5-HT; 5-hydroxytryptamine) to initiate a pulse event by activating tUT through 5-HT_2 receptor-mediated phosphorylation (McDonald and Walsh, 2004; Walsh

et al., 2000). The restoration of cortisol to pre-pulse levels inactivates tUT and terminates the pulse event. Taken together, this evidence suggests that cortisol may influence the number of functional transporters by both chronically controlling tUT transcript levels *and* acutely regulate the activity of tUT at the gills through more rapid and separate non-genomic pathways.

In teleost fish, the genomic effects of cortisol are mediated through cytoplasmic glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) that act as ligand-inducible transcription factors (Bury and Sturm, 2007). Upon binding of the hormone to its receptor, two ligand-receptor complexes dimerize and are translocated to the nucleus where they interact with specific DNA motifs termed hormone response elements. The classical glucocorticoid response element (GRE) is an imperfect palindrome GGTACA nnn TGTTCT that is recognized by both the GR and the MR (Beato et al., 1995; Bury and Sturm, 2007; Nelson et al., 1999). Cooperative binding of homodimers to the GRE facilitates the recruitment of other nuclear factors that ultimately induce transcription of target genes. The presence of GREs within the promoter region of a number of piscine genes has been shown to confer cortisol-inducible gene expression (Esbaugh and Walsh, 2009; Schulte et al., 2000). Cortisol-induced changes in pulsatile urea excretion in toadfish are mediated primarily by GRs, and to a much lesser extent, MRs (McDonald et al., 2004; Rodela et al., 2009a). However, the molecular mechanisms of tUT regulation remain unclear.

The main goal of this study was to examine both the chronic and acute changes of tUT in further detail. At present we can only address changes in tUT mRNA expression owing to difficulties in isolating a suitable antibody for quantification of tUT protein to

date. Therefore, as a follow-up to an earlier *in vivo* pharmacological examination (Rodela et al., 2009b), the first objective characterized tUT transcript levels in response to experimentally *lowered* corticosteroid action. The second objective analyzed the 5' flanking region of the tUT gene for promoter activity and identification of factors that may be important in the transcriptional regulation of tUT. Specifically, we hypothesized that cortisol-induced changes in tUT mRNA expression reflect the presence of GRE motif(s) in the promoter region of the tUT gene. This hypothesis was tested using an *in vivo* luciferase reporter assay first described in fish by Schulte et al. (2000). Finally, tUT mRNA transcript levels were monitored in ureotelic toadfish at various times within the pulse cycle as it is uncertain whether acute changes in cortisol influence tUT mRNA expression over a short time course.

Materials and Methods

Animals

For *Series I* and *III*, Gulf toadfish (*Opsanus beta*, Goode and Bean) were acquired from commercial shrimp fishermen in Biscayne Bay, Florida, USA and housed at the Rosenstiel School of Marine and Atmospheric Science (RSMAS), University of Miami, FL, USA during the months of January to March 2007. For *Series II*, toadfish were obtained from Gulf Specimen Marine Lab in Panacea, Florida, USA and housed at the University of Ottawa, Ottawa, ON, Canada. All experiments were conducted under either a protocol approved by the University of Miami IACUC or a protocol (#BL-206) approved by the University of Ottawa's Animal Care Committee. Prior to transfer to holding tanks, toadfish were given a 3 min "freshwater" dip (10 L dechlorinated tapwater plus 500 mL

seawater) followed by either a malachite green-formalin treatment (final concentration 0.05 mg L^{-1}, 15 mg L^{-1}; Aquavet, Hayward, CA, USA; at RSMAS) or a formaldehyde treatment (20 % vol/vol; at the University of Ottawa) to prevent infection by the ciliate *Cryptocaryon irritans* (Wood et al., 1997). At RSMAS, 10 fish were acclimated for at least two weeks to 50 L glass aquaria supplied with flowing, aerated seawater (18-22°C, pH 8.1) and kept under a natural photoperiod. At the University of Ottawa, 2-4 toadfish were held in 20 L glass aquaria supplied with aerated re-circulating artificial seawater (Instant Ocean, Big Al's Aquarium Services, Brampton, ON, Canada) and maintained at a temperature of 24°C; fish were kept on a 12 h:12 h L:D photoperiod. All fish were fed chopped squid once a week.

Experimental protocol

All toadfish were fasted 48 hours prior to experimentation. A standard crowding and confinement protocol was used to induce and maintain ureotelic behaviour (Hopkins et al., 1995b; McDonald et al., 2004; Rodela et al., 2009a; Wood et al., 1997) unless otherwise noted (see below). In brief, toadfish (n=6-8) and their PVC shelters were transferred to plastic tubs (6 L) for 48 hours. Following crowding, fish were relocated to individual 2 L flux chambers. During this time all tanks were supplied with flowing, aerated seawater.

Series I: Chronic changes in tUT and GR expression following in vivo *manipulation*

Rodela et al. (2009a) reported that lowering the action of cortisol, by either inhibiting its synthesis or blocking its receptor, resulted in an increase in pulse size

with no accompanying change in pulse frequency in ureotelic toadfish. The purpose of Series I was to ascertain whether the *in vivo* manipulations published in the 2009 study caused any changes in tUT and GR mRNA expression.

A detailed description of the experimental protocol used in *Series I* is provided by (Rodela et al., 2009a). In brief, caudal artery catheters (McDonald et al., 2004; Wood et al., 1997), and in some cases intraperitoneal (IP) catheters (McDonald et al., 2004), were implanted in toadfish anesthetized with MS-222 (1 g L^{-1}, pH 8.0) prior to transfer to individual flux chambers. After surgery, fish were transferred to their individual flux chambers supplied with flowing, aerated seawater and allowed to recover for 24 hours.

For the first 24 hours of the experiment, cannulated toadfish were left undisturbed in their individual flux chambers. Subsequently, fish received one of three different treatments: metyrapone, RU-486, or spironolactone. Metyrapone (methyl-1,2-di-3-pyridyl-1-propanone; Sigma Chemical Co., St Louis, MO, USA), a potent inhibitor of *de novo* cortisol synthesis (Bennett and Rhodes, 1986; Bernier and Peter, 2001; Milligan, 2003), was delivered as1.5 μL g^{-1} body mass of 20 mg mL^{-1} metyrapone in 150 mmol L^{-1} NaCl *via* IP catheter every 24 hours, up to 96 hours; sham controls received the same volume of 150 mmol L^{-1} NaCl. At the receptor level, the effects of cortisol were blocked by application of RU-486 and spironolactone, GR and MR antagonists, respectively. Although previous studies (McDonald et al., 2004b; Rodela et al., 2009a) demonstrated that cortisol-induced effects on urea excretion are mediated through GRs, we chose to examine the molecular responses to spironolactone in more detail because the highest dose (125 mg mL^{-1}) used previously demonstrated unpredicted, agonistic properties Rodela et al. (2009a). RU-486 or spironolactone in peanut oil, or

peanut oil alone (sham control), was administered by IP catheter at a volume of 1.5 μL g^{-1} body mass. At the end of the experiment (96 hours for metyrapone, and 72 hours for RU-486 and spironolactone) toadfish were euthanized by an overdose of MS-222 (3 g L^{-1}; pH 8.0), and gill tissue was collected and stored at -80°C in RNALater (Sigma, St. Louis, MO, USA) for later analysis of tUT and GR mRNA expression. Results presented in this study will be discussed within the context of the physiological trends reported by Rodela et al. (2009a).

Series II: The regulatory role of the tUT promoter

The aim of this experimental series was to determine whether cortisol targets putative GREs in the promoter region of tUT to modulate its expression. An *in vivo* reporter assay was used as previously described in killifish (Schulte et al., 2000) and toadfish (Esbaugh and Walsh, 2009). In brief, toadfish were assigned to one of three treatment groups: unconfined, crowded, or crowded plus cortisol-infused. Animals in the unconfined group (n=6) were maintained in flowing, aerated seawater for 2 weeks in 20 L aquaria at a stocking density of two individuals per tank to allow recovery from any stress associated with transfer. Following this acclimation period, toadfish were lightly anesthetized with MS-222 (1 g L^{-1}, pH 8.0) and a small incision (< 1 cm) was used to expose the liver. Plasmids were injected directly into the liver with a 50 μL syringe and 28-g needle. The injection cocktail consisted of 40 μg of a tUT promoter construct in a PGL4.10 vector (Promega) and 5 μg control plasmid (PGL4.75, Promega) (see 'Development of tUT reporter assay' below for vector details) re-suspended in 40 μL of 150 mmol L^{-1} NaCl with 2 % vol/vol tempera paint. The inclusion of tempera

paint aided visualization of the injection site for later harvesting. The control PGL4.75 construct contained a cytomegalovirus promoter linked to *Renilla* luciferase, and was co-injected with the PGL4.10 construct to normalize transfection efficiency. The incision site was sutured closed and fish were returned to their tanks for recovery. Crowded and cortisol-infused toadfish were injected with plasmid constructs after undergoing the standard crowding protocol and prior to transfer to 2.5 L confinement aquaria; cortisol-infused fish were fitted with a caudal artery cannula (as described by Rodela et al., 2009a) and infused with 0.09 mg cortisol mL^{-1} in isosmotic NaCl (150 mmol L^{-1}) at a rate of 3 mL kg^{-1} h^{-1} for 48 hours using a Gilson 8-channel peristaltic pump. The infusion rate was periodically verified by weighing the infusion reservoir. After 48 hours, fish in all groups were euthanized, as described above, and blood samples were withdrawn either by caudal puncture or *via* the caudal artery catheter and centrifuged at 10, 000 *g* for 1 min. The plasma was removed, flash frozen in liquid nitrogen, and stored at -80°C for later analysis of plasma cortisol. The injection sites were excised from the liver, including a non-injected portion for measurement of background luminescence. Samples were immediately homogenized in 4 volumes of 1X passive lysis buffer (Promega) using a motor-driven tissue homogenizer (Fisherbrand). The cellular debris was cleared from the homogenate by centrifugation twice at 13,000 *g* for 15 mins. The resulting supernatant was frozen at -20°C for later analysis of luciferase activity. The supernatant was assayed (< 1 month) using published protocols for the Dual Luciferase Assay System (Promega) and an LMax-II luminometer (Molecular Devices). Data are expressed as normalized ratios of firefly luciferase

(PGL4.10 construct) activity to *Renilla* luciferase (PGL4.75) activity and are reported in relative light units.

Series III: Acute temporal changes in tUT and GR expression

This objective was to establish whether GR and tUT mRNA transcript levels change over the pulse cycle. This was achieved using a technique developed by Wood and colleagues (Wood et al., 1998; Wood et al., 1997), wherein toadfish are loaded with ^{14}C-labeled urea or structural analogs (like acetamide and thiourea) and the appearance of ^{14}C in the external water is then indicative of a pulse event.; indeed, radioactive acetamide and thiourea, have been shown to pulse in unison with urea (McDonald et al., 2000; Wood et al., 1998).

Prior to transfer into individual flux chambers, each toadfish received a 200 μL intraperitoneal injection (IP) of 7.6 μCi of ^{14}C-acetamide in 150 mmol L^{-1} isosmotic saline. After a 3-hour post-injection equilibration period, water samples were collected hourly and immediately monitored for ^{14}C radioactivity. Water samples were transferred to scintillation vials containing 10 mL of scintillation fluid (EcoLume, MP Biomedicals) and counted in a liquid scintillation counter (Beckman LS3801 Liquid Scintillation Counter). A regression of counts per min (cpm) against time (hours) allowed the occurrence of pulse events to be pinpointed. Fish were rapidly euthanized by an overdose of MS-222 (3 g L^{-1}; pH 8.0) during a pulse event (n=5), 12 hours post-pulse (n=5), or 18 hours post-pulse (n=5). Gill tissue was collected and stored at -80°C in RNAlater (Sigma, St. Louis, MO, USA) for later analysis of tUT and GR mRNA expression.

Molecular and analytical analyses

Measurement of GR and tUT mRNA expression

Total RNA was extracted from homogenized tissue samples (~100 mg) using TRIzol reagent (Invitrogen) according to the manufacturer's specifications. The quality and concentration of the RNA were assessed at wavelengths of 260 and 280 nm using a NanoDrop 100 spectrophotometer (ND-100; Thermo Scientific). Aliquots containing 2 μg of RNA were treated with amplification grade DNase (Invitrogen) to remove genomic DNA contamination. First strand cDNA was synthesized from the DNase-treated RNA using Revert-Aid H^- M-MuLV Reverse Transcriptase (Fermentas) with oligo-dT primers (500 ng per reaction; cloning) or random hexamers (200 ng per reaction; real-time RT-PCR). The procedure was carried out to the manufacturer's specifications and the final cDNA product was diluted with an equal volume of autoclaved water.

Amplification of a partial fragment of the toadfish GR was accomplished using degenerate primers (Table 4.1) designed from piscine GRs within regions of high sequence similarity and conservation. A 25 μL PCR reaction (2 mmol L^{-1} dNTPs, 0.2 mmol L^{-1} of primer, 0.05 U of *Taq* polymerase and corresponding commercial buffer, Denville Scientific Inc, 0.5 μL of cDNA template) was subjected to initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 sec, primer annealing for 30 sec, 72°C for 60 sec, and completed with a final extension for 15 min at 72°C. Gel-purified PCR products (Minelute gel extraction kit, Qiagen) were ligated into a pDrive vector (Qiagen), and transformed in chemically competent DH5α *Escherichia coli* (Invitrogen); resulting plasmids were then sequenced. A tBlastX search, using the online Pubmed

database, revealed the partial fragments of cDNA shared a high sequence similarity with piscine GRs.

Based on the GR partial fragment , and the published tUT sequence (Genbank Accession # AF165893), gene-specific primers (Table 4.1) for real-time RT-PCR were designed using Genetool software (BioTools Inc.) and selected for optimal amplification at annealing temperatures of 58°C. Primers for 18S (Table 4.1) were designed based on regions of high sequence similarity from vertebrate 18S genes. The specificity of all primer pairs was confirmed by sequencing of amplicons. A 2 µL aliquot of cDNA was used for a 12.5 µL real-time reaction containing forward and reverse primers, and SYBR green master mix kit (Stratagene). Reactions were composed and analyzed according to the manufacturer's specifications using an Mx3000P Real-Time PCR System and associated MxPro 4.1 software (Stratagene). Dissociation curves were used to confirm primer pair specificity and monitor the formation of primer-dimers. To confirm that products did not arise from genomic DNA contamination, a 'no reverse transcriptase' control template, prepared by the exclusion of reverse transcriptase during cDNA synthesis, was included on every plate. Pooled cDNA from toadfish gill was serially diluted (1:5) with RNase/DNase-free molecular grade water (Sigma) to generate standard curves. A linear regression was calculated for the relationship between cycle threshold (Ct) and relative template concentration to assess reaction efficiency. Reaction efficiencies were deemed acceptable if they fell within the range of 85-115% and had an R^2 of at least 0.98. GR and tUT mRNA transcript levels were expressed relative to the control gene 18S (diluted 1,000-fold) using a modification of the delta delta Ct method (Pfaffl, 2001). Prior to real-time analyses of experimental samples, a

set of tissues (brain, gill, heart, stomach, spleen, liver, intestine, muscle, and skin) was collected from laboratory-acclimated (uncrowded) toadfish (n=5) and to assess the mRNA tissue distribution of the GR in toadfish. For the purpose of comparison across tissues, mRNA expression of the GR was calculated relative to expression of GR in the brain. Similarly, expression of tUT and GR mRNA was calculated relative to samples collected from pulsing (*Series III*) or sham control (*Series I*) fish.

Development of tUT reporter assay

Toadfish genomic DNA was extracted from gill using the standard phenol/chloroform protocol. Following the manufacturer's specifications, the upstream promoter region adjacent to the tUT sequence was identified by PCR using the Genome Walker Universal kit (Clontech). The process was repeated until 1.3 kb of sequence upstream of the translation start site was obtained. Using the primers listed in Table 4.1, this promoter fragment was amplified by PCR. Gel-purified PCR products (Minelute gel extraction kit, Qiagen) were ligated into a pDrive vector (Qiagen), and transformed in chemically competent DH5α *E. coli* (Invitrogen); resulting plasmids were then sequenced. The online Transcription Element Search System (http://www.cbil.upenn.edu/cgi-bin/tess/tess) was used to predict transcription factor binding sites in the tUT promoter sequence. Specifically, the program was used to search for putative GRE half sites (i.e. AGAACA).

A reporter plasmid was constructed by sub-cloning the 1.3 kb tUT promoter sequence into the luciferase PGL4.10 vector (Promega). This was accomplished by using PCR to add *BglII* (Fermentas) and *NcoI* (Fermentas) restriction sites to 5' and 3'

ends of the promoter construct, respectively (see Table 4.1 for primers). The construct with the added restriction sites was gel purified, ligated into a pDrive vector (Qiagen), and transformed into DH5α *E. coli* (Invitrogen). The resultant plasmids were verified by sequencing. Approximately 0.4 mg of the tUT reporter PGL4.10 plasmid and a PGL4.75 control construct were isolated, according to the manufacturer's specifications, using a maxi-prep kit (Qiagen).

Sequence analysis

A phylogenetic analysis was carried out on the amino acid sequences of the partial fragment of the toadfish GR and selected piscine GR genes (GenBank accession numbers for each are given in parentheses): *Cyprinus carpio* GR1a (CAI51316.3), *Cyprinus carpio* GR2 (CAJ70650.2), *Danio rerio* GRα (ABR88075.1), *Danio rerio* GRβ (ABR88076.1), *Dicentrarchus labrax* GR (AAS48459.1), *Haplochromic burtoni* GR1 (AF263738.1), *H. burtoni* GR2a (AAM27888.1), *H. burtoni* GR2b (AAM27889.1), *Neolamprologus pulcher* GR1 (ABS11149.1), *Neolamprologus pulcher* GR2 (ABS11150.1), *Oncorhynchus mykiss* GR1 (NP_001118202.1), *O. mykiss* GR2 (NP_001117954.1), *Paralichthys olivaceus* GR (O73673.1), *Porichthys notatus* GR1 (ABK59939.2), *Porichthys notatus* GR2 (ADJ68050.1), *Salmo trutta* GR (AAW56453.1), *Sparus aurata* GR (ABF30967.1), *Tetraodon nigroviridis* GR (CAG11713.1). The toadfish GR sequence was translated using the online Translate tool available from the ExPasy Proteomics Server (http://ca.expasy.org/tools/dna.htm). The full-length piscine GR sequences were truncated appropriately to compare only regions overlapping with the toadfish GR sequence. Comparison sequences for other GRs were obtained by BlastP

search (online Pubmed database) and sequences were aligned using ClustalX (version 2.0.12). The phylogenetic relationship was inferred using the Neighbor-Joining (NJ) method (Saitou and Nei, 1987) on distance matrices (Tamura and Nei, 1993) with Mega4.0 software (Tamura et al., 2007). Gene clusters were analyzed through bootstrap analyses using 1000 replicates (Felsenstein, 1985). All analyses were performed using *H. burtoni* MR (AAM27890.1) as an outgroup.

Statistics

Data are presented as means ± 1 standard error of the mean (s.e.m.). With the exception of the metyrapone treatment, significance differences between means for relative mRNA expression, relative luciferase activity, and plasma cortisol were assessed using one-way analysis of variance (ANOVA). A Student's *t*-test was used to assess significance differences between the metyrapone treated fish and their controls. Where significant differences were detected, a Hold-Sidak *post-hoc* multiple comparisons test was used to identify differences among groups. All data were checked for normality and equal variances. Where assumptions of normality or equal variances were violated ($P<0.05$), ANOVA on ranks followed by a Dunn's multiple comparisons *post-hoc* test was used. Results were considered statistically significant when $P < 0.05$. Statistical analyses were performed using SPSS SigmaStat v3.5 (Systat Software).

Results

Homology cloning of the toadfish GR yielded an 877 bp partial fragment of a gene (GenBank Accession#HQ424878) that codes for 291 amino acids. The predicted

amino acid sequence shares 90% identity to the DNA-binding domain and ligand-binding domain of the cichlid *H. burtoni* GR2a sequence and 88% to the European seabass (*D. labrax*) GR, 87% to the Japanese flounder (*P. olivaceus*). Phylogenetic analyses revealed that the toadfish GR fell within a cluster of genes that included both *P.notatus* GR, *H. burtoni* GR2 isoforms (Figure 4.1). Examination of various toadfish tissues for GR mRNA expression revealed ubiquitous distribution; however, the gene was most highly expressed in gill, liver, and kidney (Figure 4.2). The high levels of GR expression in the gill were in agreement with the presumed role of cortisol in regulating pulsatile urea and tUT, and therefore became a tissue of focus for the present study. Notably, the presence of substantial GR expression in the liver supports its validity as a reporter tissue for tUT.

In *Series I*, tUT relative mRNA expression was found to be unchanged following administration of saline (1.36 $\pm$ 0.52, n=6) or metyrapone (1.30 $\pm$ 0.49; Student's *t*-test, p=0.721, n=7), as well as injection of oil (1.65 $\pm$ 0.62, n=5), RU-486 (63 mg mL^{-1},1.94 $\pm$ 0.86, n=5; 125 mg mL^{-1}, 1.484 $\pm$ 0.81; ANOVA, p=0.689, n=5), or spironolactone (63 mg mL^{-1}, 1.65 $\pm$ 0.62, n=5; 125 mg mL^{-1}, 1.77 ± 0.27; ANOVA, p=0.703, n=5). Inhibiting cortisol synthesis with metyrapone (Rodela et al., 2009a) did cause a significant 3.9-fold upregulation in relative GR mRNA levels (Student's *t*-test, p=0.018, Figure 4.3A). Similarly, RU-486 caused a dose-dependent increase in GR mRNA expression (ANOVA, p= 0.015, Figure 4.3B). Although the lower dose of spironolactone (63 mg mL^{-1}) had no effect on relative expression of GR mRNA, surprisingly, the higher dose (125 mg mL^{-1}) caused a 6.3-fold increase in GR transcript levels (ANOVA, p=0.039, Figure 4.3C).

In *Series II, in silico* sequence analysis revealed a putative TATA-box sequence (consensus sequence TATA(T/A)A(T/A); Juo et al., 1996) and associated transcription factors, TBF (TATA binding factor) and TFIID (transcription factor II D), 523 bp upstream of the tUT transcription start site (+1) (Figure 4.4). One CCAAT box and several associated CCAAT enhancer binding proteins (C/EBP) were identified at -517. Six GRE half sites, each consisting of a hexanucleotide sequence, were identified within the 5' flanking region of the tUT sequence (Figure 4.4). Also present were sequences for well-characterized transcription factors stimulating protein-1 (SP1), and nuclear factor-1 (NF-1), as identified by the TESS program (Figure 4.4).

Functional analysis of the putative GRE sequences was carried out using the *in vivo* reporter assay. Minimal relative luciferase activity was detected in unconfined fish injected with the tUT reporter construct (n=6, Figure 4.5A). Crowding and cortisol infusion caused significant 3.4- and 9.8-fold increases, respectively, in luciferase activity relative to the unconfined group (ANOVA, $p=0.002$, n=6, Figure 4.5A). A similar trend was observed for plasma cortisol concentrations (ANOVA, $p<0.001$, n=6, Figure 4.5B).

Analysis of relative mRNA expression over the pulse cycle, in *Series III*, revealed significant 6.2-fold increases in tUT transcript levels at both 12 and 18 hours following a pulse (ANOVA, $p=0.032$, Figure 4.6A), whereas GR mRNA levels were largely invariant over the pulse cycle (ANOVA, $p=0.905$, Figure 4.6B).

Figure 4.1: A phylogenetic tree illustrating the relationship between toadfish glucocorticoid receptor (GR; highlighted in black) and other piscine GR isoforms. The phylogenetic tree was constructed using neighbour-joining analysis with *Haplochromi burtoni* MR 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 for the teleost GRs and *H. burtoni* MR were obtained from GenBank (see Materials and Methods for accession numbers).

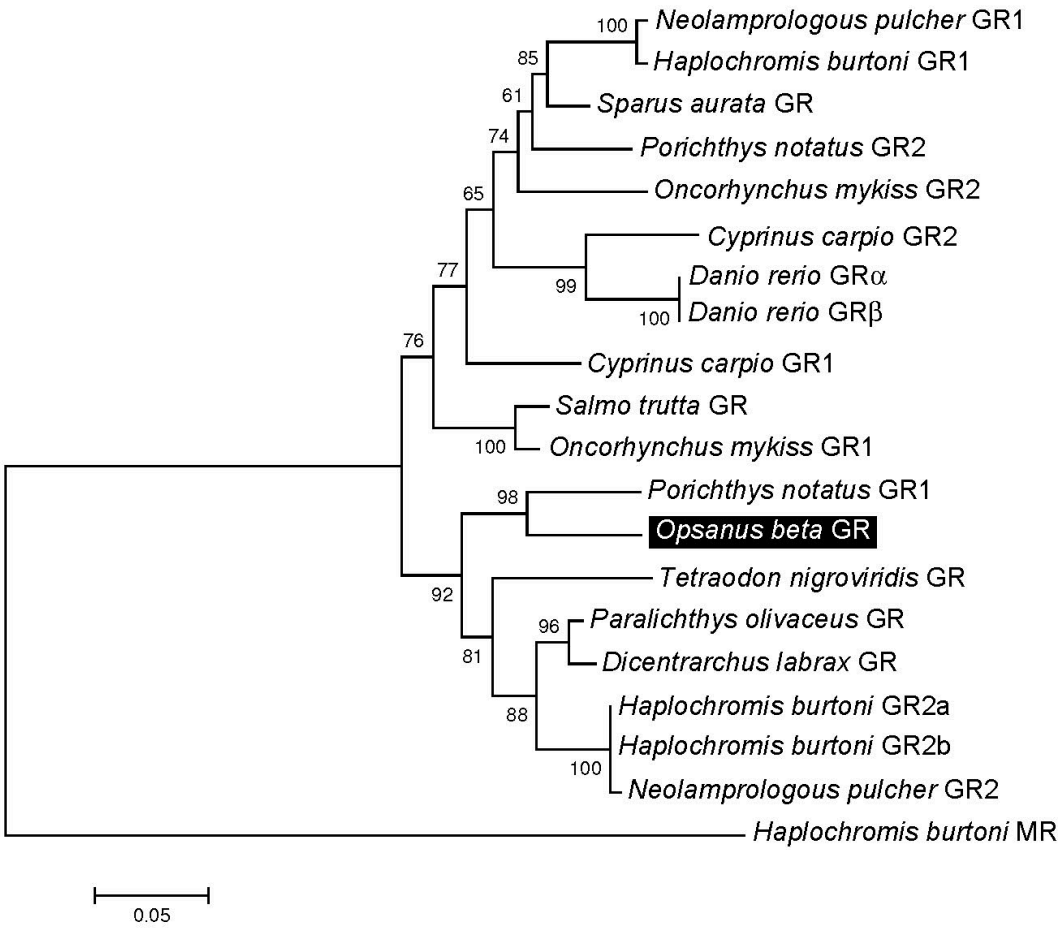
100
Neolamprologous pulcher GR1
Haplochromis burtoni GR1
85
61
Sparus aurata GR
74
Porichthys notatus GR2
Oncorhynchus mykiss GR2
65
Cyprinus carpio GR2
77
99
Danio rerio GRα
100
Danio rerio GRβ
76
Cyprinus carpio GR1
Salmo trutta GR
100
Oncorhynchus mykiss GR1
98
Porichthys notatus GR1
Opsanus beta GR
Tetraodon nigroviridis GR
92
96
Paralichthys olivaceus GR
81
Dicentrarchus labrax GR
88
Haplochromis burtoni GR2a
Haplochromis burtoni GR2b
100
Neolamprologous pulcher GR2
Haplochromis burtoni MR
0.05

Figure 4.1

Figure 4.2: Tissue distribution of the gulf toadfish (*Opsanus beta*) glucocorticoid receptor (GR) mRNA expression, as determined using real-time RT-PCR (n=5). All values are relative to mRNA expression of the control gene 18S, and were expressed relative to the value for GR in brain, which was assigned a value of 1. Values are expressed as means $\pm$ s.e.m. RBCs, red blood cells.

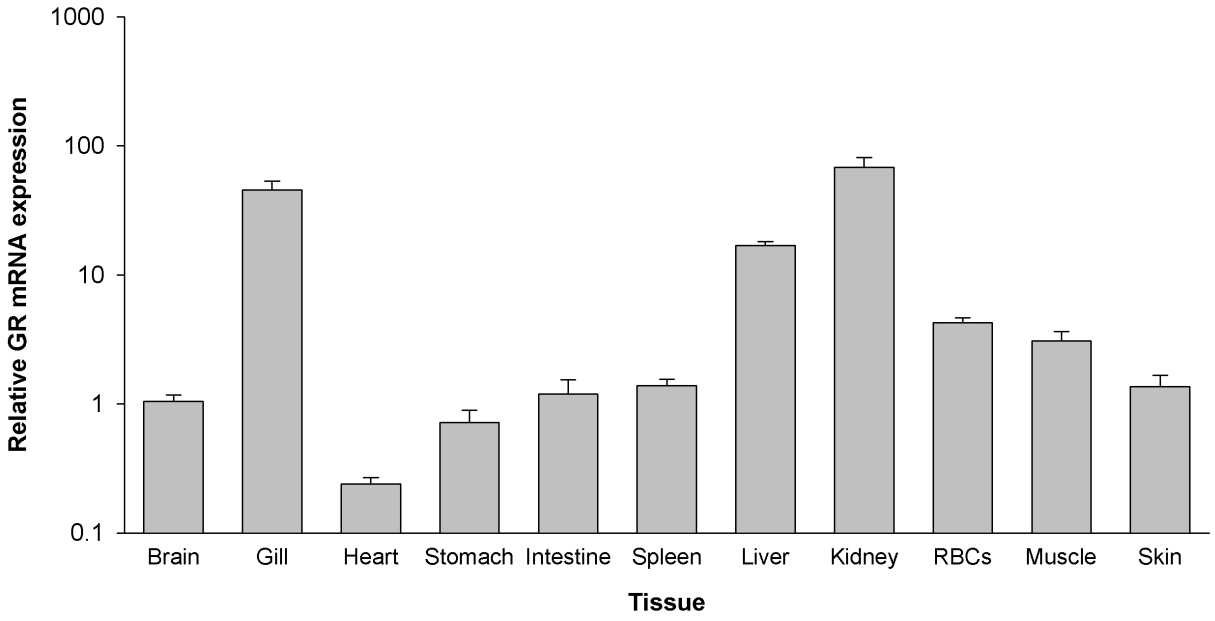
1000
100
10
1
0.1
Relative GR mRNA expression
Brain
Gill
Heart
Stomach
Intestine
Spleen
Liver
Kidney
RBCs
Muscle
Skin
Tissue

Figure 4.2

Figure 4.3: The effects of (A) metyrapone (20 mg mL^{-1}, n=7), (B) RU-486 (n=6), and (C) spironolactone (n=6) on relative mRNA expression of the toadfish (*Opsanus beta*) glucocorticoid receptor (GR), as determined by real-time RT-PCR. All values are relative to mRNA expression of the control gene 18S, and were expressed relative to the injection vehicle for the respective treatments, which was assigned a value of 1. Values are expressed as means $\pm$ s.e.m. Columns that do not share a letter are significantly different from one another (Student's *t*-test for panel A, ANOVA for panels B and C; $p<0.05$).

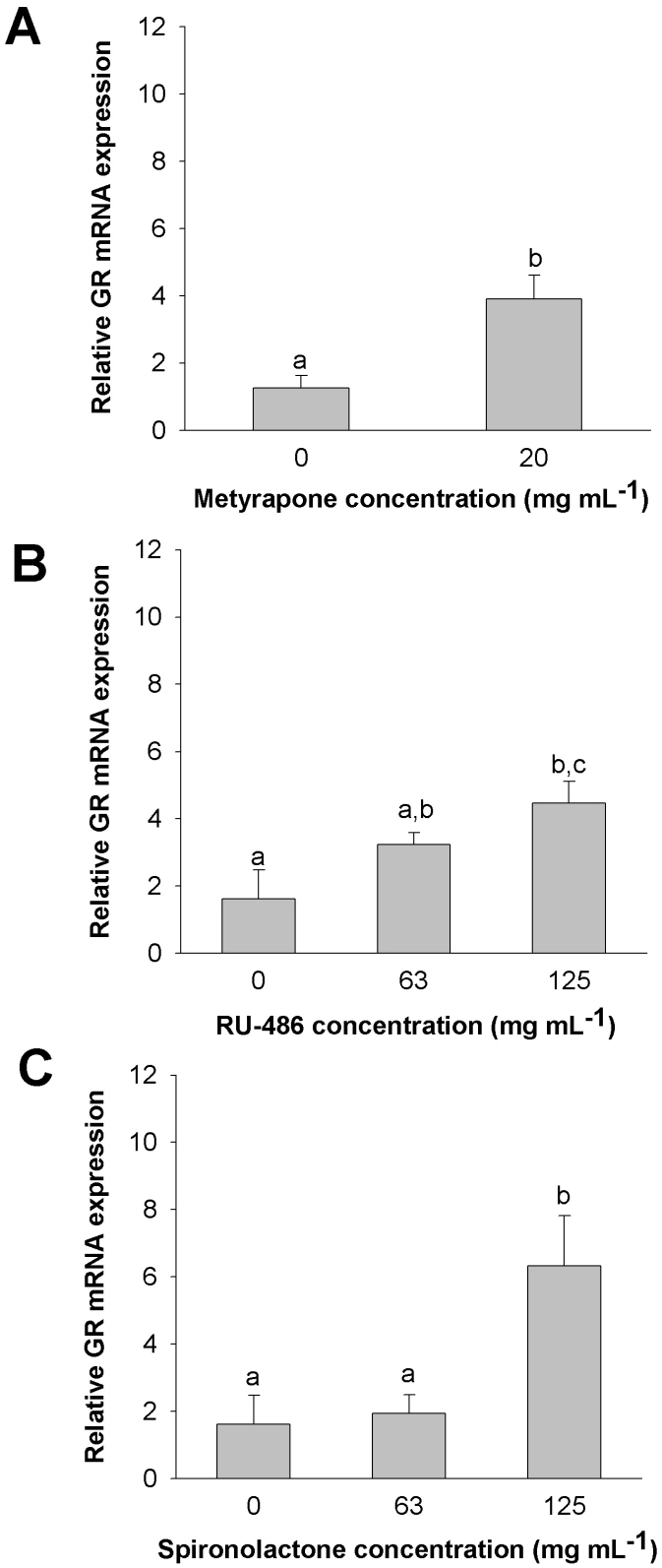
A
Relative GR mRNA expression
12
10
8
6
4
2
0
a
b
0
20
Metyrapone concentration (mg mL-1)
B
Relative GR mRNA expression
a
a,b
b,c
0
63
125
RU-486 concentration (mg mL-1)
C
Relative GR mRNA expression
a
a
b
0
63
125
Spironolactone concentration (mg mL-1)

Figure 4.3

Figure 4.4: The DNA sequence for the promoter region of gill urea transporter (UT) from toadfish *(Opsanus beta*). Predicted GRE half sites are highlighted in black with white text, basal promoter sites (TATA-box) and CCAAT box are shown in bold, and additional transcription factor targets are also indicated; SP-1. highlighted in grey boxes; NF-1. a solid outline; and C/EBP, a dotted outline.


TCTTCAGGGACATATGGGTAGATCACACGGCACAAGGTAGGAAAGCTTTCGCTATATTCCACAAAAACACTTA
CACCATCACTGCACACAATCATGCTCTTTTTAATTGTTTACAGAAAGTCACTGGTGTAATGTTGGAACAAGTGT
ATAATTTTTTTCCCTCAAAAATCATAAAATCACTGAATGGGGACGATTGGTTTTGAAAAAAAAAATAACTCAAA
ACATAGAAAAGGTGAATCAAAAATTTAGATTTATATGAAATTATTAGCACCAAATATGTCTTCCTCAGCAAAGT
TTATAATTTTCATTTAAAAAAATTTATTTTTGGTAAATTTAATGTCATGAACAGTGGCCACACCATCGTTAATA
TACAAAAGCTACATAATGAATAATGGGAGTGAATGACGTTCGATGGATAAGCAAGATACAAAGCTGCGTGTTAG
GAGGGTTTTTTGTTTTTGCTGTTTTTTTGAAGATCCACTGGCTTGAAAAATGTAAGAGACCCATTGTTAGACAT
ATAGATGGGTCAAAGAGAGAAGACAGGGCACGAAAAATGTGAATGACTTTGCAGCACAGGTAGATGATTAAATG
ATGAGTGGATGAGGTGTAGTCAGCTAACTTTATTAAAAACCAATAGTATTGTGCAAAATATTGCACCATAATAT
TTATTAATAAATATATATATAATTAACATATTTTCACCATAAAATAAAAAGTGTATATAGTGTTTTTAAAAGTATT
AAAAACATTTCTTTTTGGTGATAAAACTACATAATTCTTTTTAGGTGGTCTGCAATTTATTTGAAATTCAAAGA
AAATGGAATTGTATTCAATCAGATTTTTACAGTCAATGTTATTATTGCATGATCATCAGCATTTGTCTTTAAAT
GGGCAGGCACCAACAAAGAATTCAAGATGTTTTTCTTTTTCAAATTAAGATTATAACCTTGTAACTGGTTACTT
TCCTTCATGATTTTCATTTGATTTTTGTTGAAATAATCATGTTTAGACATTTAAAAATCCATTTGCCCCATTGA
TGAAGATTTAAAAAATAAATAAAAAATCATCCCCTAAAGTCATGAAAATTCCAGCAGGCCTAAAGCTCACTTGT
CTTCCACCAGGATATTCAGCTGAATATCACCTCATCTGCTGTTGTGCAAACACACGGGCTTCCTGTCACAACCT
TTACAGAACTCCAGCCA

Figure 4.4

Figure 4.5: (A) Relative luciferase activity (relative light units) of liver tissue injected with the gill urea transporter (tUT) promoter construct, and (B) plasma cortisol concentrations for unconfined (n=6), crowded (n=6), and cortisol-infused crowded (n=6) gulf toadfish (*Opsanus beta*). Values are expressed as means $\pm$ s.e.m. Columns that do not share a letter are significantly different from one another (ANOVA, $p<0.05$).

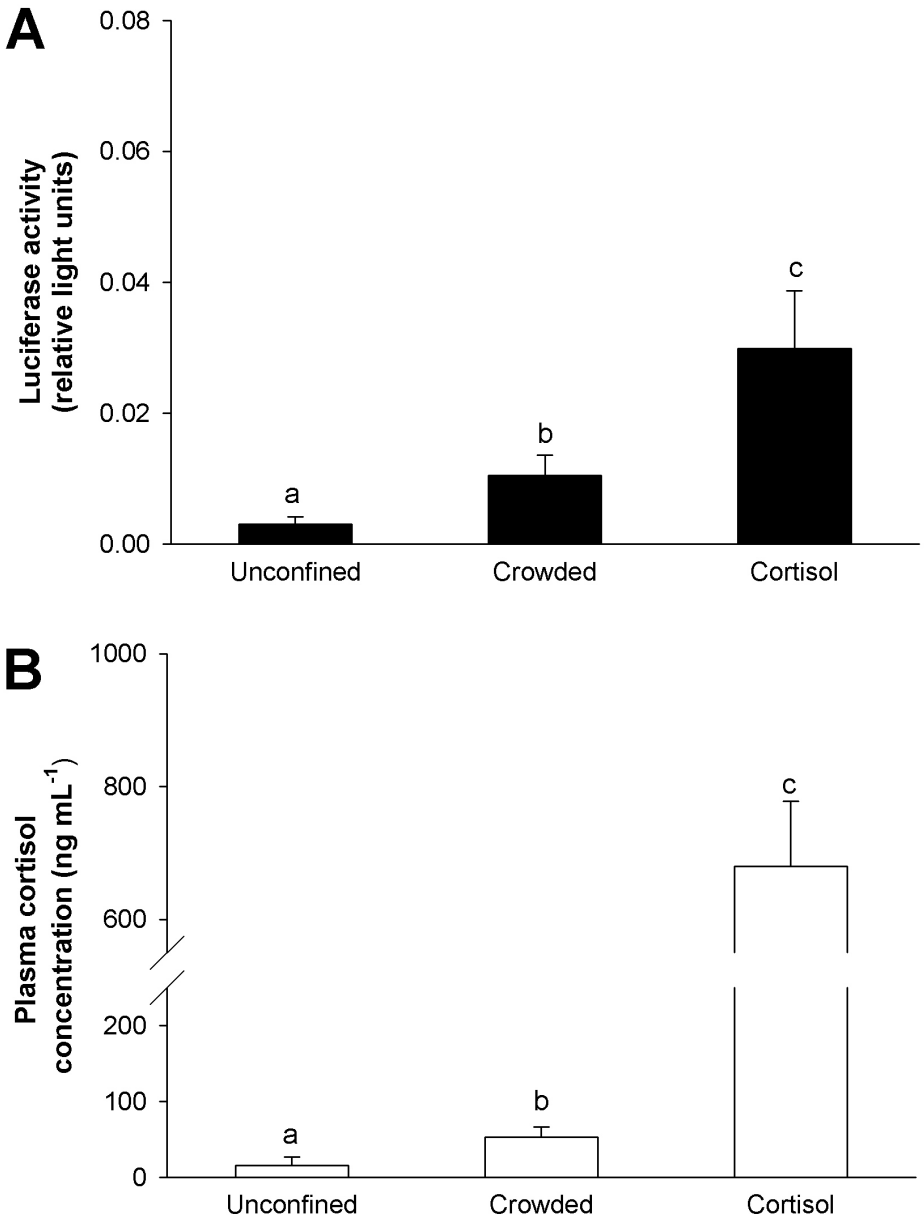
A
Luciferase activity (relative light units)
0.08
0.06
0.04
0.02
0.00
a
b
c
Unconfined
Crowded
Cortisol
B
Plasma cortisol concentration (ng mL-1)
1000
800
600
200
100
0
a
b
c
Unconfined
Crowded
Cortisol

Figure 4.5

Figure 4.6: Temporal changes in the relative expression of urea transporter (tUT;A) and glucocorticoid receptor (GR; B) mRNA in the gill of the gulf toadfish (*Opsanus beta*), as determined by real-time RT-PCR (n=5). All values are relative to mRNA expression of the control gene 18S, and were expressed relative to the "pulse" time point, which was assigned a value of 1. Values are expressed as means $\pm$ s.e.m. Columns that do not share a letter are significantly different from one another (ANOVA, $p<0.05$).

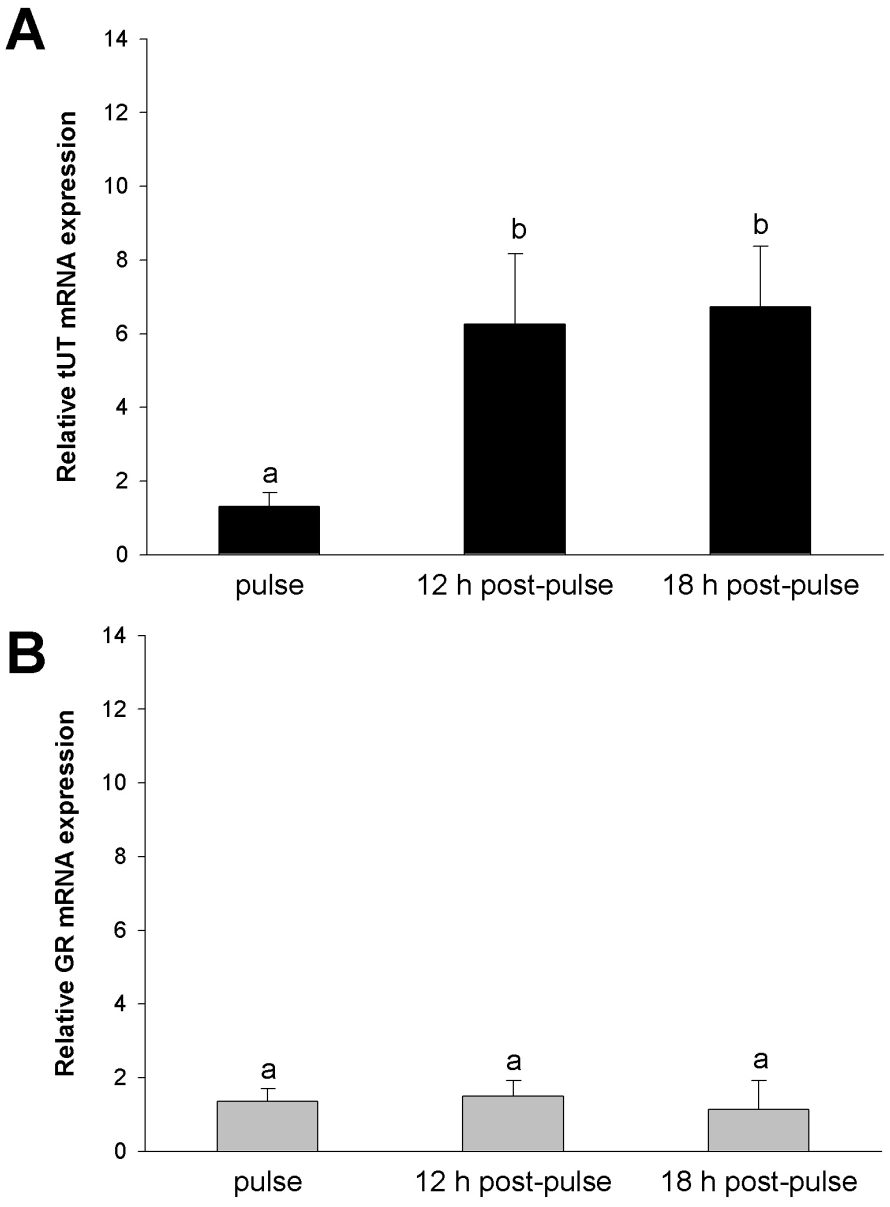
A
Relative tUT mRNA expression
14
12
10
8
6
4
2
0
a
b
b
pulse
12 h post-pulse
18 h post-pulse
B
Relative GR mRNA expression
14
12
10
8
6
4
2
0
a
a
a
pulse
12 h post-pulse
18 h post-pulse

Figure 4.6

Table 4.1: Primers used to amplify products for the cloning of the glucocorticoid receptor (GR); real-time PCR of the urea transporter (tUT), GR, and 18S; and amplification of the promoter region of tUT from the gulf toadfish (*Opsanus beta*).

Gene	Application	Sequence (5' – 3')
GR	*cloning - forward	TGG TGT GYT CNG AYG ARG C
GR	*cloning - reverse	TAG AAG CGY TGC CAG TTC T
tUT	real time PCR -forward	TTT CAA CAT CCT GGT GTG TCT C
tUT	real time PCR - reverse	GGA GAT GAA GAG GGA GAT GAT G
GR	real time PCR -forward	TGC CCA CCA TGC TGT CCT TGC TGA A
GR	real time PCR - reverse	AAG ACA TGA GAA AAA GCC ACG ACG AC
18S	real time PCR -forward	GCT CGT AGT TGG ATC TCG G
18S	real time PCR - reverse	GGC CTG CTT TGA ACA CTC
tUT promoter	cloning - forward	TCT TCA GGG ACA TAT GGG TAG A
tUT promoter	cloning - reverse	TGG CTG GAG TTC TGT AAA GGT T

* designed from piscine GRs within regions of high sequence similarity and conservation

Discussion

Cortisol and tUT regulation

The role of cortisol in regulating toadfish urea excretion is complex. Previous studies have consistently demonstrated the inhibitory effects of cortisol on urea transport, as interpreted by decreases in the appearance of urea in the ambient water (Hopkins et al., 1995b; McDonald et al., 2009; McDonald et al., 2004; Rodela et al., 2009a; Wood et al., 2001). At the same time, however, tUT mRNA abundance was increased following exogenous elevation of plasma cortisol (McDonald et al., 2009). The results indicate that tUT protein activity does not correspond to tUT mRNA expression in a direct manner in pulsing toadfish. The present study demonstrated that lowering circulating cortisol did not change tUT mRNA transcripts even though these treatments enhanced urea excretion in the same fish (Rodela et al., 2009a). Taken together, the evidence suggests that high cortisol is necessary for inhibition of tUT function *and* accumulation of tUT transcript, thus signifying that the influence of cortisol on urea excretion needs to be examined in both chronic and acute contexts.

In the mammalian kidney, glucocorticoid treatment reduces urea permeability through a decrease in UT-A protein expression (Klein et al., 1997; Naruse et al., 1997a) and repression of transcription (Peng et al., 2002). However, in contrast to the response in mammals (Peng et al., 2002), McDonald and colleagues noted an increase in tUT mRNA following exposure to moderate stress (*via* crowding), an effect that was enhanced by cortisol infusion (McDonald et al., 2009). These effects were accompanied by significant decreases in pulsatile urea excretion. Taken together, the low tUT function despite high tUT mRNA levels suggest that cortisol may regulate tUT

expression through two distinct pathways; protein regulation and/or transcription. It is clear from the literature that cortisol can elicit a broad range of responses *via* GRs to regulate gene expression (Stellato, 2004). At the post-transcriptional level, glucocorticoids have been shown to inhibit translational initiation factors and ribosomal genes resulting in a downregulation of protein synthesis (Huang and Hershey, 1989). Alternatively, cortisol may cause accelerated protein degradation, which has been observed with inducible nitric oxide synthase (iNOS; (Kunz et al., 1996), acetylcholinesterase (Brank et al., 1998), and the glucose transporter 2 (GLUT2; (Gremlich et al., 1997) in various murine tissues. Elevated cortisol in toadfish may repress tUT protein expression through one of these mechanisms. Therefore, we speculated that lowered cortisol would restore normal tUT protein expression and result in higher urea excretion rates, independent of changes in tUT mRNA. Although we are currently unable to directly measure changes in tUT protein expression, the physiological and molecular data collected from the toadfish in the present study support this interpretation. In particular, chronic administration of metyrapone caused a 6.6-fold depression in plasma cortisol accompanied by a 1.6-fold increase in both urea excretion rate and urea pulse size (Rodela et al., 2009b). A comparison of tUT mRNA revealed similar transcript levels in metyrapone- and saline-injected fish, despite different patterns of urea excretion. Likewise, both RU-486 doses (63 and 125 mg mL^{-1}) increased urea excretion approximately 2-fold (Rodela et al., 2009a), but mRNA transcript levels of tUT were not significantly different between RU-486- and vehicle-treated fish. While it may be surprising that both metyrapone- and RU-486-treated fish had levels of tUT mRNA transcripts comparable to control fish, it should be noted that

all fish in this experimental series were chronically cannulated, a treatment that consistently raises plasma cortisol in fish (Brown et al., 1986; Lo et al., 2003; Sloman et al., 2005). These initial cortisol concentrations (>200 ng mL^{-1}; (Rodela et al., 2009a) may have raised tUT transcript levels (McDonald et al., 2009). Such genomic effects of cortisol may have persisted over the experimental period due to the lengthy process of altering cortisol synthesis (*via* metyrapone; Mommsen et al., 1999) and the slow release of the receptor antagonists *via* lipid vehicle (Sloman et al., 2001; Vijayan and Leatherland, 1989). Collectively, the data suggest that the chronic regulation of tUT expression may involve post-transcriptional and/or post-translational events integrated with transcriptional regulation.

The regulation of gene transcription relies on the recognition of specific DNA motifs by regulatory proteins. *In silico* analysis of a 1.2 kb fragment of the 5' flanking region of the tUT gene revealed the presence of a basal promoter site approximately 500 bp upstream of the transcriptional start site. Structural studies indicate that the TATA-box position can vary 28-34 bp from the transcriptional start site; however, if the TATA site is outside these boundaries the gene may be controlled by other transcription factors (Sandelin et al., 2007). Therefore, the tUT promoter resembles a TATA-less gene, similar to the promoter controlling expression of the mammalian, cortisol-sensitive UT-A1 and UT-A3 isoforms (Fenton et al., 2002b; Smith and Fenton, 2006). Within the analyzed fragment of the tUT promoter, six putative GRE half sites were identified by the transcription element search system. However, none of these hexanucleotide sequences were part of a consensus 15 bp GRE palindrome sequence, imperfect or otherwise. While many vertebrate GRE motifs exhibit the canonical

sequence (GGTACA nnn TGTTCT), there is increasing evidence that many GR DNA binding sites lack one half of the palindrome (Truss and Beato, 1993). In the simple GRE model (Schoneveld et al., 2004), the 3' half of the hexanucleotide motif is the more conserved and acts as a binding site for a monomeric form of the GR (Eriksson and Wrange, 1990; Scheidereit and Beato, 1984). Formation of the homodimer is completed with the binding of an additional GR monomer that accommodates greater deviations within the less conserved, 5' half of the GRE palindrome (Truss and Beato, 1993). In the complex model (Schoneveld et al., 2004), glucocorticoid-mediated activation requires GR binding to GRE half site(s) as well as binding of additional regulatory elements to adjacent sites within the promoter region, or glucocorticoid response units (GRUs; Bristeau et al., 2001; Danesch et al., 1987; Faust et al., 1996; Massaad et al., 2000; Miksicek et al., 1987). Within GRUs, there is considerable diversity among genes and tissues in the composition and spatial positioning of response elements and transcription binding sites (Schoneveld et al., 2004). Examination of the ~1.2 kb tUT promoter fragment revealed binding sites for SP-1, NF-1, and C/EBP transcription factors (Figure 4.4), similar to sites described in the mammalian phenylalanine hydroxylase (Bristeau et al., 2001; Faust et al., 1996) and Type II phopholipase A2 (Massaad et al., 2000) promoters. Such models may explain putative glucocorticoid signaling within the 1.2 kb promoter region of the toadfish UT regardless of the absence of consensus palindrome GRE motifs. Functional analysis of this region using a luciferase reporter construct and reporter gene assays confirmed that this promoter fragment initiates gene transcription in a cortisol-dependent fashion. The levels of luciferase activity measured were similar to those reported in previous studies using

the same techniques with different genes (Esbaugh and Walsh, 2009; Schulte et al., 2000). In general, these results provide strong evidence that one or more of the predicted GRE motifs is involved in regulating cortisol-sensitive tUT transcription. However, additional work is required to identify the specific response element(s) involved.

All urea pulses in toadfish are preceded by a decline in plasma cortisol (Wood et al., 1997; Wood et al., 2001) that may be permissive for post-transcriptional modification of tUT mediated by 5-HT_2 receptors (Hopkins et al., 1995; McDonald et al., 2004; Wood et al., 1997; Wood et al., 2001). This explanation is supported by molecular analysis revealing several phosphorylation and glycosylation sites within the tUT amino acid sequence (Walsh et al., 2000). However, our findings suggest that the cortisol drop is also associated with genomic changes in tUT expression, and can be correlated to the lowest levels of tUT mRNA expression. The return of cortisol to pre-pulse concentrations was associated with a delayed upregulation of tUT transcript that was observed 12 to 18 hours following a urea pulse. A similar (non-significant) trend was seen in an earlier study using Northern blot analysis (Walsh et al., 2000). This upregulation of tUT transcript may be to prepare for the next urea pulse following increased rates of tUT protein degradation during termination of the prior pulse event. The protein degradation pathway warrants further examination as there may be similarities between pathways controlling mammalian UT localization and tUT. The cell surface regulation of UT-A1, UT-A2, and UT-A3 is under control of the ubiquitin-proteasome pathway, a mechanism that removes proteins from the cell membrane and tags them for degradation (Chen et al., 2008; Stewart et al., 2008). Covalent conjugation

with ubiquitin and subsequent proteasomal degradation results in decreased urea transport activity. Furthermore, glucocorticoids cause an upregulation of the ubiquitin-proteasomal pathway (Auclair et al., 1997; Price et al., 1994; Sathiyaa and Vijayan, 2003; Tiao et al., 1996). Potentially, the return of cortisol to pre-pulse levels may enhance the removal of tUT protein from the branchial membrane of the toadfish, necessitating the post-pulse accumulation of tUT transcripts in preparation for the next urea pulse. Further experiments examining the sensitivity of pulsatile urea excretion to transcriptional and proteasomal inhibitors may help elucidate the relative importance of the post-pulse increase in tUT mRNA.

Glucocorticoid receptors and urea excretion

The DNA-binding domain and ligand-binding domain of the toadfish GR exhibited high identity with other piscine GRs. Glucocorticoid signaling in teleosts is complicated by the identification of multiple glucocorticoid receptors in many species that differ in agonist specificity (reviewed by Bury and Sturm, 2007). For example, *O. mykiss* rtGR2 is 60-fold more sensitive to cortisol than rtGR1 (Bury et al., 2003). It is postulated that each receptor has a distinct role, where cortisol preferentially binds to the more sensitive isoforms (i.e. rtGR2) during low or mild stress conditions, and both receptors (i.e. rtGR1 and rtGR2) may act in tandem to coordinate responses during severe stress (Bury et al., 2003; Prunet et al., 2006). This interpretation may be especially relevant to the toadfish model where modulation of urea transport is intimately linked to chronic stress and may involve a glucocorticoid receptor with a low affinity for cortisol. The toadfish GR fragment cloned in the present study clustered

phylogenetically with the less sensitive GR isoforms, specifically HbGR2a. However, the sensitivity of the toadfish GR to cortisol remains to be determined. Furthermore, we were unable to isolate other GR isoform(s) with conventional homology cloning. Although zebrafish have only a single GR (Alsop and Vijayan 2008), we cannot at present rule out the possibility of additional toadfish GR genes as seen in most other teleosts (Bury et al., 2003; Greenwood et al., 2003; Stolte et al., 2008).

Examination of mRNA expression revealed a lack of variation in GR mRNA transcripts present in the gills over a pulsecycle. Although the level of steroid receptor protein expression in target cells can modulate corticosteroid sensitivity, it does not appear that this process is involved in regulating the acute response to cortisol during a pulse event. Increases in GR mRNA abundance in response to elevation of plasma cortisol have been reported in a number of teleost studies (Arterbery et al., 2010; Sathiyaa and Vijayan, 2003; Takahashi et al., 2006; Vazzana et al., 2010). The increase in GR mRNA counterbalances higher rates of GR protein degradation caused by elevated cortisol (Sathiyaa and Vijayan, 2003). By contrast, ureotelic toadfish experience a transient *decline* in plasma cortisol. Although there is no evidence of GR mRNA regulation following acute changes in cortisol during a pulse cycle, chronic inhibition of cortisol synthesis or blocking its interaction with the receptor caused increases in GR mRNA abundance. Although changes in GR protein content were not evaluated, an inverse relationship between circulating cortisol and GR protein content has been reported in several teleost species (Maule and Schreck, 1991; Pottinger, 1990; Sathiyaa and Vijayan, 2003; Vazzana et al., 2010). Therefore, the initial cannulation stress may have lowered GR protein, but subsequent lowering of corticosteroid action

with metyrapone or RU-486 may require an upregulation of GR protein, necessitating the increase in GR mRNA. Surprisingly, the highest dose of spironolactone, a mammalian MR antagonist, also increased GR mRNA transcripts while simultaneously decreasing circulating cortisol, resulting in a decrease in pulse size and rates of urea elimination (Rodela et al., 2009a). This result suggests that the influence on urea excretion may have been due to spironolactone-mediated effects on GR activity, a finding that lends further evidence for caution when using this antagonist in a piscine system.

Conclusions

In conclusion, cortisol appears to be necessary for the modulation of tUT transcript levels in both acute and chronic contexts. We have identified several GRE motifs within the upstream promoter region of tUT that may be responsible for glucocorticoid-induced changes in gene expression. These genomic trends differ from physiological data that show chronic elevation of cortisol inhibits tUT function, an effect that can be blocked by reducing corticosteroid activity. Taken together, we speculate that the upregulation in tUT mRNA abundance with cortisol may be necessary to offset the physiological consequences of high cortisol, and therefore may be crucial for maintaining tUT activity.

Acknowledgements

This study was supported by NSERC Discovery grants awarded to P.J.W. and K.M.G., and an NSF grant to M.D.M. (IOS-0455904). P.J.W. is supported by the Canada Research

Chair program. Special thanks to Jimbo Luznar for collection of toadfish, and Michael B. Morando for care and feeding of the RSMAS toadfish colony.

CHAPTER 5

The effects of feeding and crowding in the gulf toadfish, *Opsanus beta* re-visited: the role of Rhesus glycoproteins in nitrogen metabolism and excretion.

Notes on Chapter 5

This chapter had multiple authors and will be submitted to a journal as:

Rodela, T.M., Esbaugh, A.J., Weihrauch, D., Veauvy, C.M., McDonald, M.D., Gilmour, K.M., and Walsh, P.J. The effects of feeding and crowding in the gulf toadfish, *Opsanus beta* re-visited: the role of Rhesus glycoproteins in nitrogen metabolism and excretion.

The contribution of A.J. Esbaugh, D. Weihrauch, and C.M. Veauvy was the isolation and full length sequencing of all the Rh glycoprotein isoforms, see first paragraph of *Molecular analysis of gene expression* in the Materials and Methods section. M.D. McDonald was a collaborator at the University of Miami, where *in vivo* experiments were conducted by T.M. Rodela.

Abstract

Proposed 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/NH_4^+ across cell membranes. This study examines 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 (GSase). Four Rh isoforms (*Rhag*, *Rhbg*, *Rhcg1*, *Rhcg2*) were identified from toadfish. Tissue distributions showed higher levels of mRNA expression of all Rh isoforms in the gills and liver, 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 two-fold, paralleled by similar increases in branchial Rhcg1 mRNA, gastric Rhcg1 mRNA, and mRNA of all intestinal Rh isoform. 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.

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*) 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, 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 in 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., 1993). This is remarkable considering that continued ventilation and perfusion of the gills with blood ($[ammonia]_{plasma} \sim 200$ μmol L^{-1}) maintains a substantial, outwardly directed P_{NH3} 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 (GSase), an enzyme responsible for the conversion of ammonia to glutamine (Walsh, 1996; Walsh et al., 1999; Walsh et al., 2003). GSase activities increase 5- and 2-fold in the liver and muscle,

respectively, accompanied by comparable increases in protein and mRNA levels (Esbaugh and Walsh, 2009; Hopkins et al., 1995; Kong et al., 2000; McDonald et al., 2009; Walsh et al., 1999; Walsh and Milligan, 1995; Walsh et al., 1994; Wood et al., 1995). 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 GSase isoform is exclusively expressed at the gill and is thought to play a role in trapping ammonia to minimize its leakage across the gill (Walsh et al., 2003; Wood et al., 1995). Calculations by Walsh (1997) suggested that there is sufficient gill GSase activity to support this mechanism. However, a recent study by McDonald and colleagues (McDonald et al 2009) demonstrated that gill GSase expression is limited to the mitochondrion-rich cells (MRC), 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 evolved rapidly since the discovery of Rhesus (Rh) glycoproteins. Members of the ammonia transport (AMT)/methylammonium permease (MEP)/Rh glycoprotein superfamily facilitate the movement of NH_4^+/NH_3 across the plasma membrane (Marini et al., 1997; Peng and Huang, 2006). 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; Nakhoul et al., 2005; Westhoff et al., 2002). Similar Rh-like sequences were found in both the Japanese pufferfish (*Takifugu*

rubripes) and the green spotted pufferfish (*Tetraodon nigroviridis*; (Huang and Peng, 2005), 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 hydration of excreted CO_2 as well as the active extrusion of protons (Lin et al., 1994; Lin and Randall, 1990; Playle and Wood, 1989; Wright et al., 1986; Wright et al., 1989). 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 identified a role for Rh glycoproteins as NH_3 conduits in this freshwater model (Nawata et al., 2007; Nawata and Wood, 2009; Nawata et al., 2010b) that has been incorporated into a new freshwater model of branchial ammonia excretion (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 accounts for approximately 79% of the total ammonia that is excreted (Evans et al., 1989). Nakada and colleagues established the differential distribution and cellular polarization of several Rh proteins in the branchial epithelium of pufferfish (*T.rubripes*) and confirmed that these proteins can transport an ammonia analog (Nakada et al., 2007). Therefore, one can envision a role for Rh

glycoproteins in mediating NH_3/ NH_4^+ excretion via transcellular pathways in the marine fish gill.

The goal of this study was to re-examine the effects of feeding and crowding/confinement in toadfish (studied earlier by 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 (Alsop and Wood, 1997; Brett and Zala, 1975; Bucking and Wood, 2008; Kaushik and Teles, 1985; Wicks and Randall, 2002), 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 the months of January and February 2009. Toadfish were immediately transferred to the laboratory and given a freshwater dip (2 min) following by a malachite green-formalin treatment (final concentrations 0.05 mg L^{-1}, 15 mg L^{-1}; Aquavet, Hayward, CA, USA) to prevent infection by the ciliate *Cryptocaryon irritans* (Stoskopf, 1993; Wood et al., 1997a). Fish were acclimated to the laboratory for at least a 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 one week prior to experimental manipulation. A second group of fish ("crowded"; n=15) was subjected to a standard crowding protocol (Hopkins et al., 1995a; Walsh, 1987a; Wood et al., 1997a) wherein 7-8 fish were held in 6 L plastic tubs for 48 h to induce ureotely. All fish were deprived of food during this time to eliminate prior feeding effects on nitrogen metabolism and excretion. 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 (McDonald et al., 2000; Wood et al., 1997). Post-surgery, unconfined and crowded toadfish were transferred to individual 13 L 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 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. 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 RNA*Later* (Sigma); 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, RBCs, muscle, and skin) were dissected, placed in RNA*Later* and 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, a focus on transcript levels was emphasized. Total RNA was extracted from homogenized tissues

(~100 mg) using Trizol reagent (Invitrogen) 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). 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, Rhcg1; Fermentas) and oligo dT primers (500 ng per reaction; cloning and 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 that was cloned from RBC cDNA, all Rhs were cloned from gill tissue using degenerate primers (Table 5.1) 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) 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 sec, primer annealing for 30 sec, 72°C for 60 sec, and were completed with a final extension for 15 min at 72°C. Gel-purified PCR products (*via* QIAex II, Minelute gel extraction kit, both from Qiagen) 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 *Eschericia 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 5.1) using Oligo 6.87 (Molecular Biology Insights, Inc) and Gene Tool software (BioTools Inc) for use in 3' and 5' RACE (rapid amplification of cDNA ends) and nested RACE PCR reactions. Poly A^+ RNA was generated from gill total RNA using the PolyATract mRNA isolation kit (Promega). 5' and 3' sequences for Rhbg and Rhcg2 were amplified from adaptor-ligated gill cDNA using the Marathon cDNA Amplification Kit (Clonetech). Cycle parameters for the initial RACE PCR reactions were: 94°C (30 sec), followed by 5 cycles of 94°C (5 sec), 70°C (4 min), and 30 cycles of 94°C (20 sec), 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 rounds (Rhag) of nested PCR. The first round of PCR used the primary gene-specific primer (Table 5.1) and oligo dT or oligo dT anchor primer (Table 5.1) 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 GSase mRNA transcript levels, primers (Table 5.1) were designed using Gene Tool software and selected for optimal amplification at an annealing temperature of 58°C. The specificity of individual primer pairs for Rh and GSase genes was confirmed by sequencing of amplicons. Primer sequences for tUT and 18S were designed and validated in a previous study and yielded products of 219 and 166 bp, respectively (Rodela et al., in preparation-a). All real-time RT-PCR reactions were performed on samples of cDNA (2 μL) using a SYBR green master mix kit (Stratagene) and analyzed using an Mx3000P Real-Time PCR System and 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 (Ct) 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 genes 18S (template diluted 1000-fold) and calculated according to the ΔΔCt method of Pfaffl (Pfaffl, 2001). For purposes of comparison, mRNA expression was calculated relative to the respective gene from unconfined fasted individuals. In addition, five fish from general holding tanks were euthanized and tissues (brain, gill, heart, stomach, intestine, spleen, liver, kidney, RBCs, muscle, and skin) were sampled for preliminary experiments examining the mRNA distribution of toadfish Rhag, Rhbg, Rhcg1, and Rhcg2. Of further note, RhAG expression in mammals is strongly associated with red blood cells and erythropoietic tissues (Huang and Liu, 2001; Huang and Peng, 2005), as well as in fish studied to date. Therefore, the co-amplification of Rhag and hemoglobin (Hb) mRNA (see Table 5.1 for real time PCR primers) was measured to establish the degree of Rhag mRNA expression due to red blood cell 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.htm). Sequence alignments for the Rh genes were obtained using ClustalX (version 2.0.12). 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 (μmol-N kg^{-1}) as previously described by McDonald et al. (2004) using the following equation:

$$\text{Excretion} = (\Delta C \times V_f) \div M_f$$

where ΔC represents the increase in concentration (μmol L^{-1}) over time, V_f corresponds to the volume of the individual flux chambers (in litres), and M_f is the mass of the individual fish (in kg). Nitrogen excretion values (μmol-N kg^{-1}) were averaged over the entire treatment interval to facilitate a direct comparison with the initial 24-hour control period. These values were used to analyze the percent ureotelism for each treatment. The changes in water urea concentration over time were used to calculate both the frequency and size of urea pulses, with a threshold value for a pulse of 40 μmol-N kg^{-1} as defined by (McDonald et al., 2004b). Values were then further analyzed as total (μmol-N kg^{-1}), pulsatile (μmol-N kg^{-1}), and non-pulsatile (μmol-N kg^{-1}) components of urea excretion. 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.

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. A separate aliquot of plasma was used to quantify plasma urea concentration (μmol L^{-1}) according to the diacetyl monoxime method (Rahmatullah and Boyde, 1980). Plasma cortisol concentration was measured

using a commercially available ^{125}I radioimmunoassay kit (MP Biomedicals; Santa Anna, CA, USA).

GSase 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 5 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 GSase activity at 540 nm using a Spectramax Plus 384 plate spectrophotometer (Molecular Devices). GSase activity is reported in units of μmol min^{-1} g^{-1}.

Statistical analyses

All data are presented as means $\pm$ 1 standard error of the mean (s.e.m.). The effects of treatments on normalized nitrogen excretion were statistically analyzed using a two-way repeated measures (RM) analysis of variance (ANOVA) on normalized data with crowding status and feeding regimen as the two factors. Differences within each group were tested using paired t-test 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 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. Prior to analysis, proportional data (% urea and ammonia) were transformed using $p'=arcsin\sqrt{p}$ as these values were binomially distributed (Zar, 1999).

Results

Molecular analysis of toadfish genes

The physiological effects of crowding

For the first 24 h of the experiment, unconfined fasted toadfish excreted almost equal quantities of ammonia and urea (Table 5.2), with ammonia-N eliminated at a rate of 89.7 ± 5.4 μmol-N kg^{-1} h^{-1}. The majority of urea (≥ 93 %; 1948.5 ± 324.9 μmol-N kg^{-1}, n=7) was excreted in a pulsatile manner, with a mean pulse size of 830.6 ± 134.4 μmol-N kg^{-1} and average frequency of 2.9 ± 0.41 pulses per 24 h. The non-pulsatile contribution to urea excretion was relatively small with a mean value of 4.1 ± 1.1 μmol-N kg^{-1}. The values of these parameters did not change significantly between the initial 24 h (control period) of the experiment and the subsequent 72 h of the experiment during which the toadfish continued to be fasted (Table 5.2, p=0.58; Figure 5.1A, p=0.43; pulse frequency, p=0.45; Figure 5.1C total, p=0.42; pulsatile, p=0.43, non-pulsatile, p=0.62; Figure 5.1E, p=0.75, n=7). Both plasma ammonia (Figure 5.1B, p=0.99) and urea (Figure 5.1D, p=0.98) remained constant over the entire 96 h of the experiment. Plasma cortisol remained constant over time (p=0.82), averaging 211.3 ± 17.2 ng mL (n=7).

On average, crowded fasted fish excreted 2107.9 ± 436.1 μmol-N kg^{-1} of urea, and >96% of this urea was discharged in distinct pulses with a mean size of 613.0 ±

85.3 μmol-N kg^{-1} and an average frequency of 3.4 ± 0.3 pulses per 24 h. The non-pulsatile component of urea excretion was 6.9 ± 1.8 μmol-N kg^{-1}. Urea accounted for most of the total nitrogenous wastes excreted (Table 5.2); ammonia was excreted at a relatively low rate of 15.5 ± 2.5 μmol-N kg^{-1} h^{-1}. Measurements from the initial 24 h control period were not significantly different from the following 72 h of the experiment (Table 5.2, p=0.96; Figure 5.1A, p=0.80; pulse frequency, p=0.65; Figure 5.1C total, p=0.96; pulsatile, p=0.97, non-pulsatile, p=0.42; Figure 5.1E, p=0.62, n=8). Both plasma ammonia (Figure 5.1B, p=0.97) and plasma urea (Figure 5.1D, p=0.80) remained steady for the entire duration of the experiment. Plasma cortisol did not change over the entire 96 h period of the experiment (p=0.71), averaging 261.6 ± 19.2 ng mL (n=8).

A comparison between unconfined and crowded fish revealed that crowding significantly lowered the proportion of ammonia that is excreted by crowded fish in both the initial control ($p<0.001$) and subsequent 72 h period ($p<0.001$; Table 5.2). Crowding did not have a significant effect on cortisol concentrations at any of the measured time points (0 h, p=0.48; 36 h, p=0.61; 48 h p=0.25; 96 h, p=0.48). Crowding also significantly elevated GSase activity in the liver (p=0.038) and stomach ($p<0.001$) but had no significant impact on branchial (p=0.071) or intestinal (p=0.13) GSase activity (Figure 5.2A).

The physiological effects of feeding

Initial (control) fasted rates of ammonia excretion from individuals in the unconfined fed group were approximately 108.8 ± 16.2 μmol-N kg^{-1} h^{-1} and feeding

resulted in 2.6-fold increase, raising the rates to 249.4 ± 35.4 μmol-N kg^{-1} h^{-1} (Figure 5.1A, $p<0.024$, n=6). Although feeding did not significantly alter the proportion of total nitrogenous waste excreted as urea in unconfined fish (Table 5.2, p=0.40, n=6), it significantly elevated all components of urea excretion within the unconfined group (Figure 1C; total, p=0.005; pulsatile, p=0.006; non-pulsatile, p=0.031). The total and pulsatile components of urea excretion were raised from initial fasted values of 1960.6 ± 542.4 and 1956.4 ± 543.1 μmol-N kg^{-1} to 5053.7 ± 378.8 and 4991.8 ± 383.8 μmol-N kg^{-1}, respectively. Feeding to satiation caused a significant increase in urea pulse size within the unconfined group, elevating values from 580.6 ± 83.5 to 1502.2 ± 74.7 μmol-N kg^{-1} (Figure 5.1E, $p<0.001$) without any significant change in mean pulse frequency (3.3 ± 0.3 pulses per 24 h, p=0.64). The non-pulsatile component of urea excretion was also elevated 11.9-fold in response to feeding (Figure 5.1C, p=0.004), with post-prandial values of 61.9± 14.4 μmol-N kg^{-1}. Plasma ammonia (Figure 5.1B) and urea (Figure 5.1C) were significantly elevated at 36 h (ammonia, p=0.002; urea, $p<0.001$) and 40 h (ammonia, p=0.03; urea , $p<0.01$) compared to the initial plasma values at 0 h. Plasma cortisol did not vary significantly over the 96 hour experiment (p=0.84), averaging 175.9 ± 15.3 ng mL (n=6).

Parallel trends were observed with crowded toadfish following feeding. Ammonia excretion rates were significantly augmented approximately 3-fold between the initial fasted and subsequent fed periods (Figure 5.1A, p=0.16, n=7), increasing from 21.9 ± 3.7 to 46.8 ± 7.5 μmol-N kg^{-1} h^{-1}. The proportion of waste excreted in the forms of ammonia and urea was maintained through the experiment (Table 5.2, p=0.31). The total (p=0.030) and pulsatile (p=0.031) components of urea excretion

were significantly elevated following feeding within the crowded group (Figure 5.1C). The total (2069.5 ± 437.6 μmol-N kg^{-1}) and pulsatile (2037.8 ± 436.7 μmol-N kg^{-1}) components of urea excretion increased to 6682.4 ± 1389.9 and 6654.9 ± 1387.7 μmol-N kg^{-1}, respectively, after toadfish were fed to satiation. These changes translate into a significant increase in urea pulse size (Figure 5.1E, p=0.02), from 647.3 ± 119.1 to 2234.7 ± 426.3 μmol-N kg^{-1} with no associated change in pulse frequency (mean=3.4 ± 0.3, p=0.68). The non-pulsatile component of urea excretion was unaffected by feeding (38.4 ± 7.6 μmol-N kg^{-1}, Figure 5.1C, p=0.94). Plasma ammonia (Figure 5.1B) and urea (Figure 5.1C) in crowded toadfish were significantly elevated at 36 h (ammonia, p<0.001; urea, p<0.002) and 40 h (ammonia, p=0.02; urea , p=0.04) following feeding compared to the initial plasma values at 0 h. Plasma cortisol in crowded fed toadfish remained constant during the experiment (p=0.84), averaging 173.4 ± 15.6 ng mL (n=6).

A comparison between fasted and fed toadfish demonstrated that feeding did not significantly alter the proportion of nitrogenous wastes excreted as ammonia and urea during the control (p=0.48) and fed (p=0.47) periods (Table 5.2). Additional contrasts between fasted and fed groups of toadfish revealed feeding significantly raised ammonia excretion (Figure 5.1A, p=0.26), all components of urea excretion (Figure 5.1C; total, p=<0.001; pulsatile, p<0.001; non-pulsatile, p=0.001), and urea pulse size (Figure 5.1E, p<0.001). Feed fish also had higher plasma ammonia concentrations at 36 (p<0.001) and 40 h (p<0.001) than their fasted counterparts (Figure 5.1B). Plasma urea concentrations were higher at 36 (p<0.001), 40 (p<0.001), 48 (p<0.001), and 72 h (p=0.028) in the fed groups of toadfish *vs* the fasted groups

(Figure 5.1D). Feeding did not alter plasma cortisol concentrations at any of the measured time points (0 h, $p=0.062$; 36 h, $p=0.094$; 48 h $p=0.10$; 96 h, $p=0.33$). Stomach GSase activity was significantly elevated by feeding (Figure 5.2A, $p<0.001$). There were no significant interactions between confinement and crowding in all the physiological parameters measured ($p>0.05$).

Molecular analysis of toadfish Rh genes

A combination of homology cloning and RACE techniques was used to 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 (Figure 5.3). A gene isolated from toadfish RBCs was found to group strongly with piscine Rhag genes (Figure 5.3). This toadfish Rhag sequence (GenBank accession # HQ424874), which was 1515 nucleotides long and was predicted to code for 434 amino acids, shared 88% sequence similarity with *G. aculeatus* Rhag, 83% with Rhag from *T. rubripes* and *O. mykiss*, and 78% with the *D. rerio* Rhag. The largest transcript (1673 nucleotides) isolated from toadfish gill grouped phylogenetically with the Rhbg subgroup (Figure 5.3). The predicted 458 amino acid product of the toadfish Rhbg gene (GenBank accession # HQ424875) showed 84% sequence identity to Rhbg from *K. marmoratus*, 83% to both Rhbg-a and –b orthologs from *O. mykiss*, and 82% to Rhbg from *T. nigroviridis*. A second gene isolated from toadfish gill was 1519 nucleotides long and phylogenetic analysis showed that it fell within the clade of Rhcg1 genes (Figure 5.3). Toadfish Rhcg1 (GenBank accession # HQ424876) coded for a predicted sequence that was 482 amino acids long and was 93% similar to *T. rubripes* Rhcg1, 91% to *T.*

nigroviridis Rhcg1, and 87% to *K. marmoratus* Rhcg1. Examination of the third sequence isolated from gill tissue showed that this gene fell within a cluster of piscine Rhcg2 homologs (Figure 5.3). This gene (GenBank accession # HQ424877), 1647 nucleotides in length with a predicted sequence of 482 amino acids, was 88% similar to *T. rubripes* Rhcg2, 87% to *G. aculeatus* Rhcg2, and 79% to *K. marmoratus*.

Rh transcripts were expressed in all tissues examined (Figure 5.4). 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 red blood cell contamination (Figure 5.4A). However, higher relative expression of Rhag mRNA over Hb mRNA was observed in gill and intestine, suggesting tissue-specific expression of Rhag in these organs. Rhbg mRNA transcript levels were similar among tissues, with the exception of stomach, which demonstrated low levels of expression, and liver, in which expression was somewhat higher than in other tissues (Figure 5.4B). Both Rhcg1 (Figure 5.4C) and Rhcg2 (Figure 5.4D) exhibited relatively high expression in gill, and low levels in stomach. Intestine and liver exhibited moderate abundance of Rhcg1 mRNA expression (Figure 5.4B). Based on these tissue expression profiles, gill, stomach, intestine, and liver became the focus of further analysis.

The effects of crowding on gene expression

In general, a comparison of all unconfined (fasted and fed) and crowded (fasted and fed) toadfish revealed that branchial expression of all Rh isoforms are all significantly influenced by crowding (Rhag, $p=0.001$; Rhbg, $p<0.001$; Rhcg1, $p<0.001$; Rhcg2, $p=0.026$). A post-hoc analysis within the fasted group of fish, crowded toadfish

had significantly lower expression of Rhag (p=0.002), Rhbg (p=0.002), Rhcg1 (p=0.015), and Rhcg2 (p=0.025) than unconfined toadfish (Figure 5.5A). A similar general comparison of all unconfined and crowded groups showed the only Rh isoform in the stomach affected by crowding was Rhcg2 (p=0.036; Figure 5.5B). Expression of Rhag (p=0.075), Rhbg (p=0.15), and Rhcg1 (p=0.13) in the stomach was unaffected by crowding (Figure 5.5B). A detailed analysis of mRNA abundance in the stomach of fasted toadfish showed that crowding lowered Rhcg1 (p=0.012) and Rhcg2 (p<0.001) abundance. In the intestine, Rhag (p=0.72), Rhbg (p=0.35), Rhcg1 (p=0.63), Rhcg2 (p=0.56) mRNA expression generally did not increase in crowded individuals. Post-hoc statistical tests revealed significantly higher levels of Rhbg (p=0.045), Rhcg1 (p=0.035), and Rhcg2 (p=0.021) were present in the intestine of crowded fasted fish relative to unconfined fasted toadfish (Figure 5.5C). Post-hoc statistical analysis in fasted fish showed Rhbg (p=0.012), Rhcg1 (p=0.020), and Rhcg2 (p=0.042) were higher in crowded individuals (Figure 5.5D).

Glutamine synthetase mRNA abundance in the liver was elevated in response to crowding (p<0.001; Figure 5.2B) but gill (p=0.80), stomach (p=0.28), and intestine (p=0.83) mRNA expression was unaffected. Similarly, crowding did not have any significant effects on relative tUT mRNA expression in the gill (data not shown).

The effects of feeding on gene expression

A comparison between all fasted and fed toadfish revealed that feeding significantly increases branchial Rhag (p=0.033), Rhbg (p=0.023), Rhcg1 (p=0.019). Rhcg2 expression was unaffected by feeding (p=0.074). Post-hoc statistical tests

revealed that branchial Rhag mRNA increased in response to feeding in crowded fish ($p=0.028$, Figure 5.5A), whereas it was unaffected in unconfined toadfish ($p=0.40$, Figure 5.5A). Branchial Rhbg and Rhcg1 mRNA abundance was elevated following feeding in unconfined fish (Rhbg, $p=0.030$; Rhcg1, $p=0.030$; Figure 5.5A) but unaltered in crowded fish (Rhbg, $p=0.26$; Rhcg1, $p=0.009$). Levels of Rhcg2 mRNA were higher in unconfined toadfish following feeding ($p=0.046$; Figure 5.5A) but similar in crowded toadfish ($p=0.68$).

In the stomach Rhcg1 mRNA abundance was altered in response to feeding ($p<0.001$). Statistical analyses showed that there was a significant interaction between crowding and feeding for most Rh isoforms in the stomach (Rhbg, $p<0.001$, Rhcg1, $p=0.029$; Rhcg2, $p<0.001$). A post-hoc analysis showed that feeding elevated Rhcg1 mRNA expression in both unconfined ($p<0.001$) and crowded ($p=0.025$) toadfish (Figure 5.5B). Rhag ($p=0.22$), Rhbg ($p=0.23$), and Rhcg2 ($p=0.36$) mRNA levels in the stomach were not altered in response to feeding. Measurements of mRNA expression in the intestine showed that only Rhcg1 and Rhcg2 are increased following feeding (Rhcg1, $p=0.035$; Rhcg2, $p=0.021$; Figure 5.5C). Rhag was unaffected by feeding ($p=0.16$). Feeding and crowding had a significant interactive effect ($p=0.032$) on intestinal Rhbg expression, resulting in a significant increase in Rhbg mRNA expression in crowded fish ($p=0.034$; Figure 5.5C).

A general comparison of hepatic expression of Rhbg and Rhcg2 in all unconfined and crowded toadfish showed that the expression of these Rh isoforms is altered in response to feeding (Rhbg, $p=0.007$; Rhcg2, $p=0.032$; Figure 5.5D). Mostly Rhag ($p=0.84$) and Rhcg2 ($p=0.69$) mRNA abundance in the liver was not altered by feeding.

Statistical analyses revealed a significant interaction between crowding and feeding for all Rh isoforms in the liver (Rhag, $p=0.046$; Rhbg, $p<0.001$, Rhcg1, $p=0.003$; Rhcg2, $p=0.042$). Post-hoc analyses revealed that feeding elevated Rhag ($p=0.007$), Rhbg ($p=0.014$), Rhcg1 ($p=0.043$), and Rhcg2 ($p=0.006$) mRNA abundance in unconfined fish (Figure 5.5D). Feeding tended to lower Rhbg ($p<0.001$) and Rhcg1 ($p=0.02$) expression in crowded toadfish (Figure 5.5D).

Glutamine synthetase mRNA abundance (Figure 5.2B) was unaffected by feeding in the gill ($p=0.72$), stomach ($p=0.43$), stomach ($p=0.11$), and intestine ($p=0.78$). Analysis of tUT mRNA abundance revealed that feeding did not influence expression in the gill ($p=0.69$).

Figure 5.1: The effects of crowding and/or feeding on ammonia excretion (relative units; A) and plasma ammonia (μmol L^{-1}; B) in gulf toadfish (*Opsanus beta*), as well as total, pulsatile, and non-pulsatile components of normalized urea excretion (relative units; C) and plasma urea concentrations (μmol L^{-1}; D). (E) The effect of crowding and/or feeding on urea pulse size (relative units) in toadfish. Data in A, C, amd E are presented as the treatment value normalized to the initial 24 h control period. Values are expressed as means $\pm$ s.e.m. (n=6-8). Significant differences are labeled by different letters between groups in panels A and E ($p<0.05$). In panel C, significant differences are denoted by different letters within each variable (i.e. total, pulsatile, non-pulsatile; $p<0.05$). Symbols (ϕ,*), in panels B and D, indicate, respectively, significant differences within the treatment group from the control period (>24 h), and differences between treatment groups at a given time ($p<0.05$). The dotted lines represent the times at which fed groups received a meal.

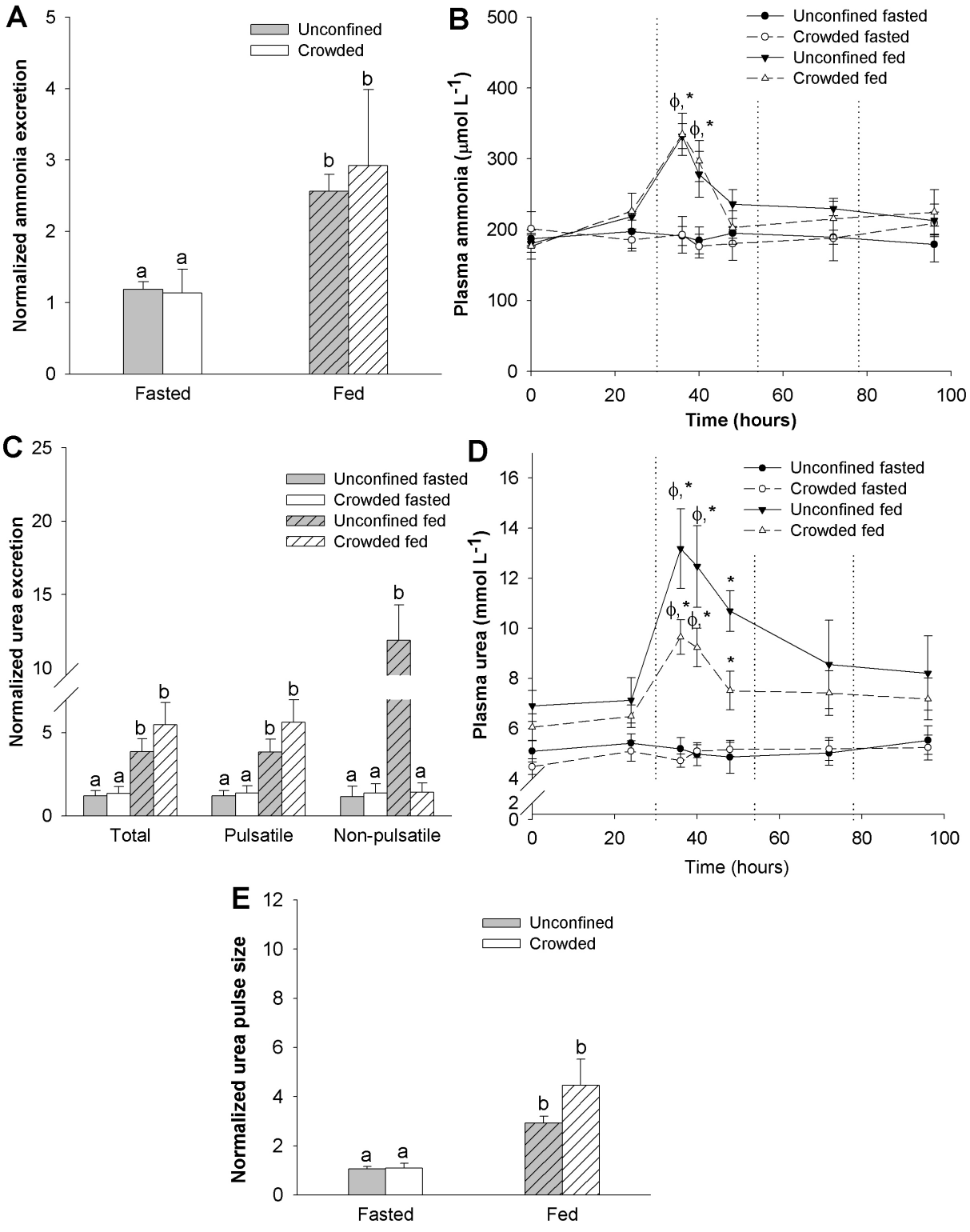
A
Normalized ammonia excretion
Unconfined
Crowded
Fasted
Fed
B
Plasma ammonia (μmol L-1)
Unconfined fasted
Crowded fasted
Unconfined fed
Crowded fed
Time (hours)
C
Normalized urea excretion
Total
Pulsatile
Non-pulsatile
D
Plasma urea (mmol L-1)
E
Normalized urea pulse size

Figure 5.1

Figure 5.2: The influence of crowding and/or feeding on (A) glutamine synthetase (GSase) activity and (B) relative GSase mRNA expression in gulf toadfish (*Opsanus beta*) as measured by real-time RT-PCR. 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 $\pm$ s.e.m. (n=6-8). Significant differences for each tissue are denoted by different letters ($p<0.05$).

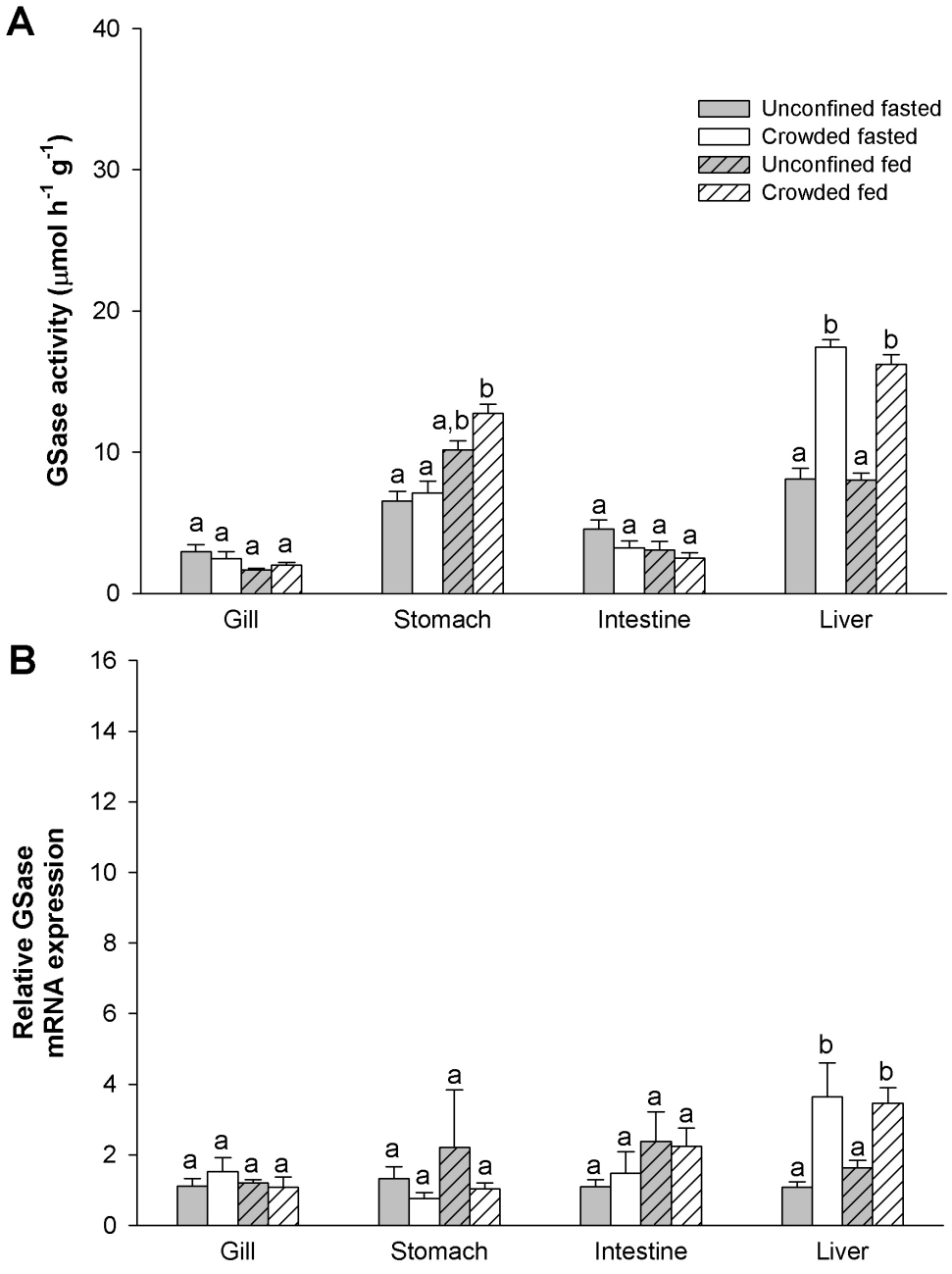
A
GSase activity (μmol h-1 g-1)
40
30
20
10
0
Unconfined fasted
Crowded fasted
Unconfined fed
Crowded fed
a
a
a
a
a
a
a
b
a
a
a,b
b
a
a
a
a
a
b
a
b
Gill
Stomach
Intestine
Liver
B
Relative GSase mRNA expression
16
14
12
10
8
6
4
2
0
a
a
a
a
a
a
a
a
a
a
a
a
a
b
a
b
Gill
Stomach
Intestine
Liver

Figure 5.2

Figure 5.3: A phylogenetic tree illustrating the relationship between toadfish 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).

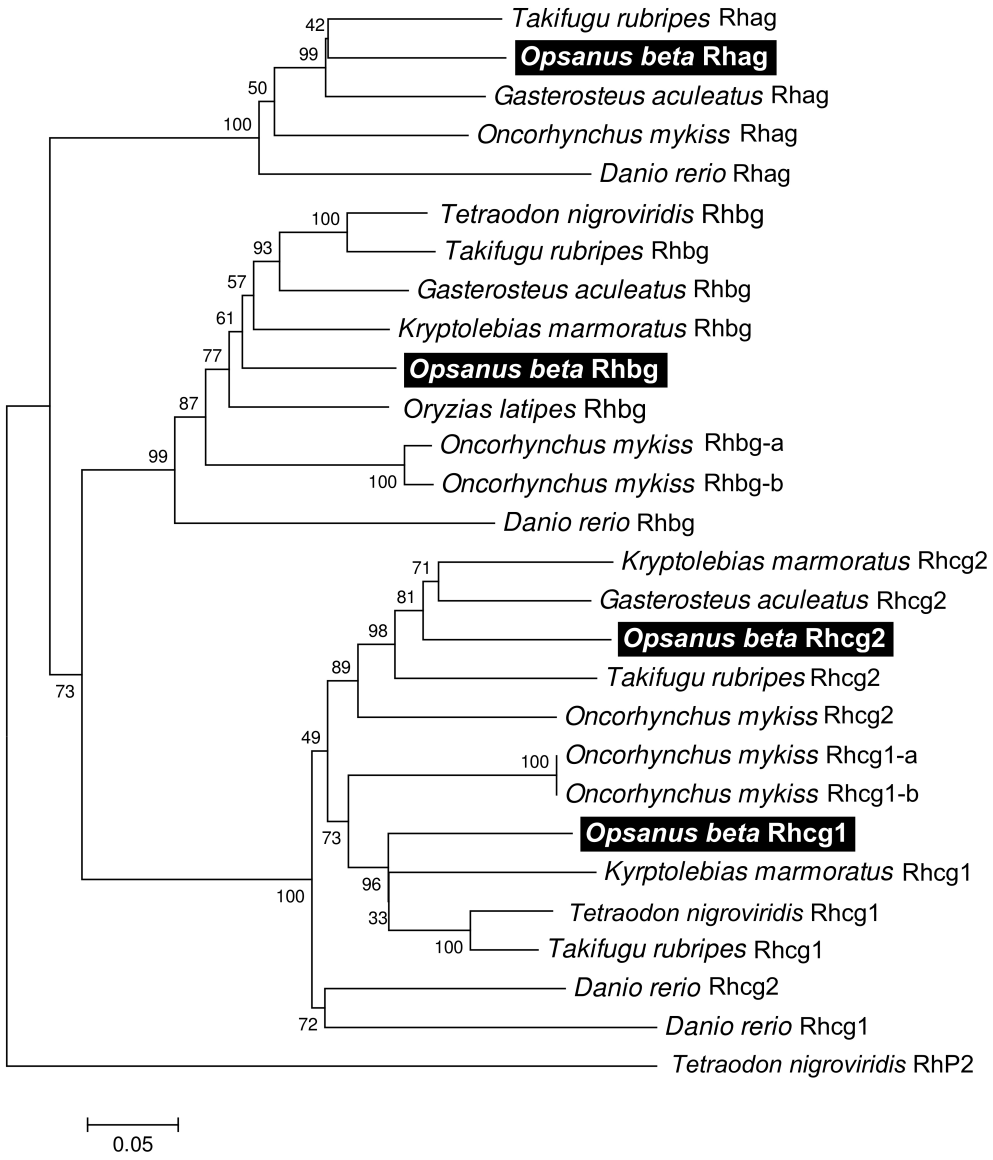
Takifugu rubripes Rhag
Opsanus beta Rhag
Gasterosteus aculeatus Rhag
Oncorhynchus mykiss Rhag
Danio rerio Rhag
Tetraodon nigroviridis Rhbg
Takifugu rubripes Rhbg
Gasterosteus aculeatus Rhbg
Kryptolebias marmoratus Rhbg
Opsanus beta Rhbg
Oryzias latipes Rhbg
Oncorhynchus mykiss Rhbg-a
Oncorhynchus mykiss Rhbg-b
Danio rerio Rhbg
Kryptolebias marmoratus Rhcg2
Gasterosteus aculeatus Rhcg2
Opsanus beta Rhcg2
Takifugu rubripes Rhcg2
Oncorhynchus mykiss Rhcg2
Oncorhynchus mykiss Rhcg1-a
Oncorhynchus mykiss Rhcg1-b
Opsanus beta Rhcg1
Kyrptolebias marmoratus Rhcg1
Tetraodon nigroviridis Rhcg1
Takifugu rubripes Rhcg1
Danio rerio Rhcg2
Danio rerio Rhcg1
Tetraodon nigroviridis RhP2
42
99
50
100
100
93
57
61
77
87
99
100
71
81
98
89
73
49
100
73
96
33
100
72
0.05

Figure 5.3

Figure 5.4: Tissue distribution of relative mRNA expression of (A) Rhag and hemoglobin (Hb), (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 which was set to a value of 1. Values represent means $\pm$ s.e.m (n=5).

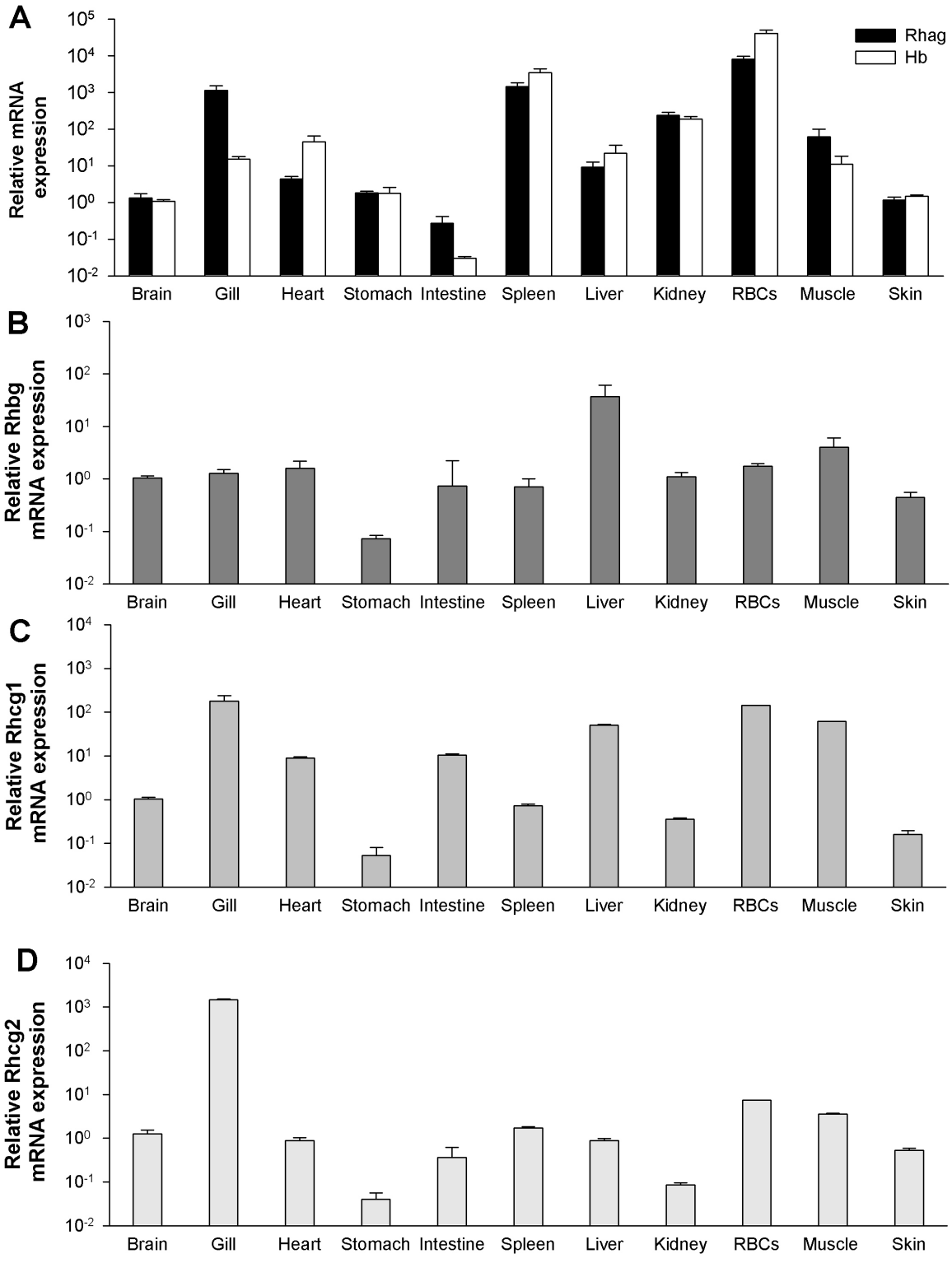
A
Relative mRNA expression
Rhag
Hb
Brain
Gill
Heart
Stomach
Intestine
Spleen
Liver
Kidney
RBCs
Muscle
Skin
B
Relative Rhbg mRNA expression
C
Relative Rhcg1 mRNA expression
D
Relative Rhcg2 mRNA expression

Figure 5.4

Figure 5.5: The effects of crowding and/or feeding on relative mRNA expression of Rhag, Rhbg, Rhcg1, and Rhcg2 in the gill (A), stomach (B), intestine (C), and liver (D) of gulf toadfish (*Opsanus beta*) as measured by real-time RT-PCR. 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 $\pm$ s.e.m. (n=6-8). Significant differences between treatments for each gene are denoted by different letters ($p<0.05$).

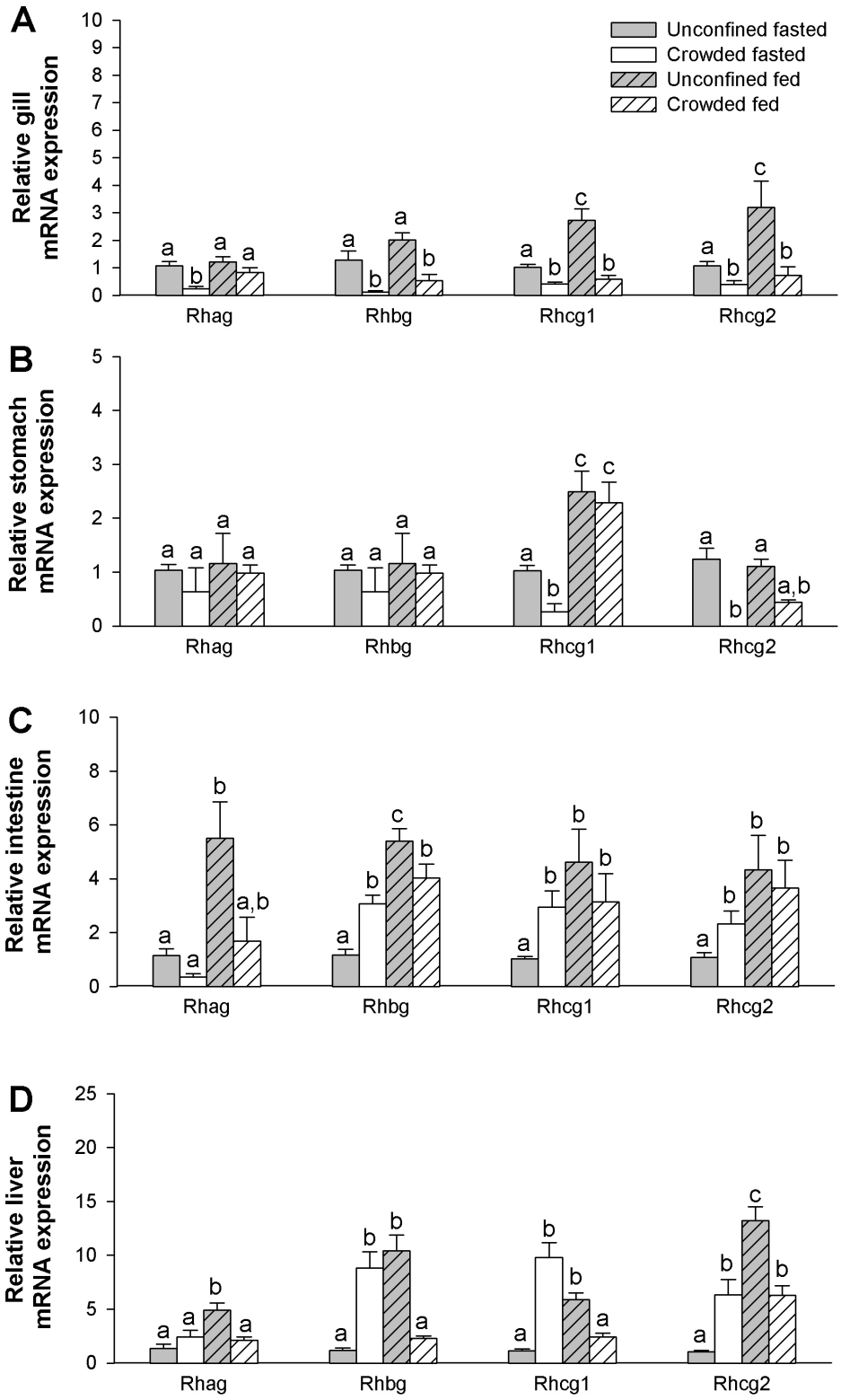
A
Relative gill mRNA expression
Unconfined fasted
Crowded fasted
Unconfined fed
Crowded fed
Rhag
Rhbg
Rhcg1
Rhcg2
B
Relative stomach mRNA expression
C
Relative intestine mRNA expression
D
Relative liver mRNA expression

Figure 5.5

Table 5.1: Primers used to amplify products for cloning, RACE PCR, and real-time PCR of the Rhesus (Rh) glycoprotein isoforms in toadfish.

Gene	Application	Sequence (5` - 3`)
Rhag	cloning - forward	GCC TTC GSY YTG CAG TGG
Rhag	cloning - reverse	TTR AAR CTR GGC CAG AAC ATC C
Rhbg	cloning - forward	CGC TGT TTG CTG TGA ACG AA
Rhbg	cloning - reverse	ATC CAG GTT GAG CCG ATA GA
Rhcg1	cloning - forward	CGA RGA GKC DGA YAC DCA CTG G
Rhcg1	cloning - reverse	CWG GCA TGG CRT GCA GGT TRT G
Rhcg2	cloning - forward	TGG TGG TAT TGG CTG TAA CTT
Rhcg2	cloning - reverse	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
tUT[1]	real time PCR - forward	TTT CAA CAT CCT GGT GTG TCT C
tUT[1]	real time PCR - reverse	GGA GAT GAA GAG GGA GAT GAT G
18S[1]	real time PCR - forward	GCT CGT AGT TGG ATC TCG G
18S[1]	real time PCR - reverse	GGC CTG CTT TGA ACA CTC

[1]Primers were designed and validated in a previous study (see Rodela et al., in preparation).

Table 5.2: 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.

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

Values are means ± s.e.m.
Significant differences between groups are labeled with different letters (2-way ANOVA, $p<0.05$).
No significant differences were detected within groups between the control and treatment period within each
group (paired t-test, $p>0.05$).

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 (Braun et al., 2009b; Hung et al., 2007; Nakada et al., 2007; Nawata et al., 2007). However, we also demonstrated 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 (Handlogten et al., 2005; Liu et al., 2000; Liu et al., 2001; Nakhoul and Hamm, 2004; Weiner et al., 2003; Weiner and Verlander, 2003). Mammalian Rh proteins enhance the movement of NH_3/NH_4^+ 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

Walsh and Milligan (1995) reported that the transition to ureotely represented a rapid shutting down of ammoniotely and only a moderate increase in ureotely. Earlier reports have shown that plasma ammonia levels do not change following crowding/confinement in toadfish (Wood et al., 1995). It is well established that the rise in plasma ammonia is prevented by increases in hepatic GSase activity and

accompanied by comparable increases in transcript levels (Hopkins et al., 1995; Kong et al., 2000; Walsh et al., 2003; Walsh and Milligan, 1995; Wood et al., 1995). Identical trends for both enzyme activity and mRNA expression were observed in the current study. The transcriptional upregulation of GSase is attributed to the activity of glucocorticoid response elements within the promoter region of the GSase gene that respond to the transient increase in plasma cortisol associated with crowding stress (Esbaugh and Walsh, 2009; Hopkins et al., 1995b; Kong et al., 2000; McDonald et al., 2009). The enhanced enzyme activity increases the conversion rate of ammonia into glutamine, the nitrogen-donating substrate for piscine CPSase III in the O-UC, and accounts for increased rates of urea synthesis in the liver (Wood et al., 2003). In addition to these well-documented changes, 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 it also applies at the protein level) may facilitate the equilibration of ammonia between the plasma and the cytosolic compartment of the hepatocytes. Although the localization of hepatic Rh proteins in teleosts is currently unknown, there is evidence from the mammalian literature documenting 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. In comparison, the roles for Rhcg1 and Rhcg2 are less clear, as evidence from murine studies shows that mammalian RhCG is expressed in the bile duct epithelium where it facilitates ammonia secretion into the biliary fluid (Weiner et al., 2003). One further interesting aspect of

GSase in toadfish is that the ubiquitous (or hepatic) GSase 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/confinement (Walsh and Milligan, 1995b). While 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 GSase 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 it has yet to be characterized at the molecular level, evidence confirms that sub-cellular sites involved in nitrogen metabolism warrant further attention.

In the present study, we report that crowded fish excreted 7-fold lower quantities of ammonia than unconfined fish. We had hypothesized that the expression of Rh glyoproteins in the gill of crowded (ureotelic) toadfish would be significantly lower than unconfined (ammoniotelic) individuals. Our results support this hypothesis and provide correlative evidence that toadfish use Rh glycoproteins to excrete ammonia. Crowding/confinement stress significantly lowered the abundance of all Rh transcripts in the gill of unfed toadfish. Although we lack information about the precise 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). 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. The diffusion of ammonia across the pavement cell is dependent on basolateral Rhbg and apical Rhcg2. It is speculated that the pavement cells are the dominant transcellular pathway for net ammonia excretion due to the relatively high surface area of the gill that is covered by this cell type (Weihrauch et al., 2009). By contrast, no Rh proteins were detected on the basolateral membrane of the MRCs and the basolateral transport of ammonia into the MRCs is accomplished by NH_4^+ displacement for K^+ on a basolateral Na^+/K^+-ATPase and from the cytosol (Nakada et al., 2007; Weihrauch et al., 2009; Wilkie, 2002). Transport of ammonia from the cytosol of the MRCs occurs *via* an apical Rhcg2 (Nakada et al., 2007). If the Fugu Rh protein model applies to the gill of toadfish, a putative reduction in Rhbg and Rhcg2 would greatly reduce the quantity of ammonia excreted across the abundant pavement cell population. This effect would be further enhanced by a decline in Rhag abundance in the pillar cells, thereby decreasing the amount of ammonia that is transported from the vasculature. The elimination of ammonia across the MRCs is achieved by a reduction in Rhcg1 expression. Enhanced ammonia trapping by gill GSase may also contribute to reduced ammonia efflux across the gill, but likely over a longer time course (e.g. a week, McDonald et al., 2009b) than that used in the present study (Figure 5.5A, see also Esbaugh and Walsh, 2009; Walsh et al., 2003). Taken together, the evidence suggests that the ammonia-sequestering mechanism in the gill appears to be constitutively present in laboratory-acclimated toadfish but may demonstrate a latent upregulation in response to prolonged stress (e.g. crowding/confinement).

Therefore, we speculate that ammonia excretion in crowded toadfish is initially lowered by a rapid downregulation of Rh expression in both pavement cells and MRCs and 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 liver Rh mRNA expression in unconfined and crowded toadfish. Unconfined individuals displayed a 3.7- to 12.5-fold upregulation in liver mRNA expression for all Rh isoforms, perhaps to enhance ammonia efflux from the liver for subsequent elimination from the body. This scenario is similar to that of most teleosts that simply excrete the majority of excess ammonia across the gills (Bucking et al., 2009; Bucking and Wood, 2008; Kaushik and Teles, 1985; Wicks and Randall, 2002). Indeed, physiological data from the present study confirm that unconfined toadfish excrete the majority of their wastes as ammonia (Table 5.2), and also have higher rates of ammonia excretion than their crowded counterparts. By contrast, crowded fed fish showed a four-fold downregulation in Rhbg and Rhcg1 transcript abundance relative to fasted individuals, while Rhag and Rhcg2 abundance was unchanged. Increased ammonia production coupled with reduced efflux from the hepatocytes would presumably shuttle ammonia towards metabolic pathways involving GSase (i.e. urea production). This interpretation is supported by data showing that fed, crowded toadfish accumulate more glutamine in their liver than unfed, crowded toadfish, despite similar levels of GSase 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).

Information on post-prandial plasma urea concentration in teleosts is limited; most of the focus has been placed on ammonia because of its dominant role in nitrogen excretion. However, the few studies that have monitored plasma urea following rationed feeding revealed no significant variation over time in this variable or urea excretion (Bucking et al., 2009; Bucking and Wood, 2008). Other experiments, however, have reported elevated urea excretion rates in response to alterations in ration and dietary composition (Alsop and Wood, 1997; Kaushik, 1980; Wright, 1993); plasma urea was not monitored in these experiments. In the present study we observed an ~1.7-fold elevation in plasma urea in both unconfined and crowded toadfish fed to satiation (Figure 5.1D). The time course and magnitude of the response was similar between the two groups, and it was accompanied by an ~5-fold increase in urea excretion rates, with the majority of urea voided *via* pulsatile routes following feeding in both groups of fish. While parallel patterns of urea handling between crowded and unconfined fish may be surprising, it does suggest that toadfish use the urea synthesis pathway in a post-prandial state to ameliorate feeding-induced ammonia loads. An examination of additional teleost species is required to determine if this is a strategy used by other teleosts or whether it is limited to the facultative ureotelic toadfish.

Although toadfish have the capacity for de novo urea synthesis, a previous study reported that a nitrogen load induced by feeding may exceed the capacity of the ammonia trapping mechanism in the liver of the ureotelic toadfish (Walsh and Milligan, 1995). Under these circumstances, most of the 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 current study. This scenario is reminiscent of most teleosts who simply excrete excess ammonia across the gills (Alsop and Wood, 1997; Bucking et al., 2009; Bucking and Wood, 2008; Kaushik and Gomes, 1988; Kaushik and Teles, 1985; Wicks and Randall, 2002; Zimmer et al., 2010). In addition, this explanation also clarifies why similar magnitude increases in both plasma ammonia (Figure 5.1B) and excretion rates (Figure 5.1A) were observed in crowded and unconfined fish during the post-prandial period. However, a direct comparison revealed the absolute rates of ammonia efflux from crowded fish were ~6-fold lower than unconfined fed individuals (see text in *Results*). These decreased rates were correlated with lower branchial mRNA expression of Rhbg, Rhcg1, and Rhcg2 in crowded fed fish compared to unconfined fed fish (Figure 5.5A), a pattern that matched 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 select Rh isoforms (Rhbg, Rhcg1, and Rhcg2) in unconfined toadfish. A trend that was similar to those described in the ammoniotelic rainbow trout, in which elevated ammonia excretion and branchial Rhcg2 mRNA are observed approximately 4-6 hours following feeding (Zimmer et al., 2010). Yet, we also showed that Rh mRNA expression in gill of crowded fish was unaltered in response to feeding (Figure 5.5A), suggesting that there may be endogenous factors that prevent increases in Rh expression in crowded fish following feeding. Likely candidates include cortisol, however, further study is required to resolve this uncertainty.

In addition to a prominent role in digestion and nutrient absorption, the fish gastrointestinal tract is an important site of ammonia transport and a major contributor to post-prandial ammonia release (Brown and Cameron, 1991; Kajimura et al., 2004). At present, information about gastrointestinal Rh proteins comes from a single mammalian study by Hanglogten and colleagues, 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). As in other tissues, RhBG has a basolateral localization, whereas RhCG is limited to the apical membrane. 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 (Figure 5.3B) versus moderate levels in the intestine (Figure 5.3C). This expression profile may reflect the relative secretory and absorptive roles of the stomach versus intestine. In the present study, plasma ammonia concentrations were approximately 200 μmol L^{-1} (Figure 5.1B), and 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. These low levels of Rh expression may reduce ammonia secretion into the lumen of the stomach, an effect that may be enhanced by the generally high levels of GSase present in the stomach (Figure 5.5A; Walsh et al., 2003). In contrast, the alkaline intestinal lumen (pH ~8.5, Taylor and Grosell, 2006) and high intestinal ammonia concentrations (207 $\pm$ 101 μmol L^{-1}; Walsh et al.,

1990)indicate that there would be an NH_3 gradient that facilitates ammonia absorption from the intestine fluid into the plasma. Interestingly, Rhbg, Rhcg1, and Rhcg2 mRNA expression in fasted fish were higher in crowded fish than in unconfined fish (Figure 5.4C), and coupled with generally low levels of intestinal GSase activity (Figure 5.5A), suggests that intestinal ammonia absorption rates are enhanced. These patterns are consistent with the trend for crowded fish to "trap" ammonia for urea synthesis. However, minimizing fecal ammonia loss becomes more difficult during the post-prandial period because changes in pH influence the species (ionic *vs* non-ionic) of ammonia that is present (Wilkie, 2002) and, in addition, piscine Rh proteins function as bi-directional transporters (Nawata et al., 2010). Acidification of the gastric lumen to a pH of 3 (Taylor and Grosell, 2006b) and elevation of plasma ammonia to ~350 μmol L^{-1} create an even stronger gradient for ammonia secretion into the stomach. Rhcg1 mRNA expression was significantly higher in fed fish regardless of crowding status (Figure 5.4B). This suggests that 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 secretion of ammonia into the intestine. Kajimura and co-workers demonstrated that significant quantities of ammonia are lost through gastrointestinal routes in fed rainbow trout (Kajimura et al., 2004). In keeping with this hypothesis, the relative mRNA expression of all Rh isoforms in the intestine was significantly higher in fed than fasted unconfined fish. Within crowded fish, intestinal Rh mRNA expression was not affected by feeding, perhaps reflecting a compromise

between ammonia trapping for urea synthesis and the need to eliminate the post-prandial nitrogen load.

Conclusion

In the present study, we identified four Rh sequences that were differentially expressed in tissues that are involved in nitrogen excretion and metabolism. In unconfined individuals, branchial Rh expression was high to facilitate the excretion of ammonia from the tissues into the ambient environment. Patterns observed in ureotelic crowded toadfish suggest that Rh abundance in the gill is reduced to minimize the efflux of ammonia, which supports our hypothesis that Rh glycoproteins contribute to ammonia excretion. These data add to the current literature implicating Rh glycoproteins as important mediators of branchial ammonia transport in many aquatic species (reviewed by Weihrauch et al., 2009; Wright and Wood, 2009). However, it is clear that the literature on piscine Rh glycoproteins is unbalanced, with limited information on Rh expression in non-branchial tissues. In our examination of feeding we provide evidence for the involvement and regulation of Rh glycoproteins in the gastrointestinal tract that, to our knowledge, has not been previously studied in teleosts. Our observations revealed differential expression of Rh proteins along the stomach and gut, a pattern reminiscent of reminiscent of the functional zonation previously described in the teleostean digestive tract (Bakke-McKellep et al., 2000; Harpaz and Uni, 1999; Mommsen et al., 2003). These data provide a good foundation for future studies that should focus on examining physiological and molecular changes on a finer spatial and temporal scale during the post-prandial period. Finally, while our

study provided a broad picture of molecular changes associated with crowding and feeding, the results also indicate that there may be several endogenous factors (e.g. cortisol) that regulate Rh expression. Future investigations should focus on using pharmacological and molecular tools to isolate the regulatory mechanisms that enable toadfish to tightly control their patterns of nitrogen excretion.

Acknowledgements

This study was funded by NSERC Discovery Grants awarded to P.J.W. and K.M.G., an NSF grant to M.D.M. (IOS-0455904), and a Student Travel Research grant to T.M.R. from the Canadian Society of Zoologists. P.J.W. is supported by the Canada Research Chair program. Special thanks to Bill Hannah for his help with sampling, and care and feeding of the RSMAS toadfish colony.

CHAPTER 6

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

Notes on Chapter 6

This chapter had multiple authors and will be submitted to a journal as:

Rodela, T.M., McDonald, Walsh, P.J. M.D., and Gilmour, K.M. Interactions between cortisol and Rh glycoprotein expression in ureogenic toadfish, Opsanus beta

M.D. McDonald was a collaborator at the University of Miami, where *in vivo*

experiments were conducted by T.M. Rodela.

Abstract

In their native environment, gulf toadfish excrete equal quantities of ammonia and urea. However, upon exposure to stressful conditions in the laboratory (i.e. crowding, confinement, air exposure), toadfish decrease branchial ammonia excretion and become ureotelic. The objective of this study was to determine the influence of cortisol and ammonia on ammonia permeability relative to expression of Rhesus (Rh) glycoproteins and the ammonia-fixing enzyme, glutamine synthetase (GSase). *In vivo* infusions/injections were used to manipulate corticosteroid action and plasma ammonia concentrations in ureotelic toadfish. Metyrapone treatment to lower circulating cortisol levels resulted in a 3.5-fold elevation of ammonia excretion rates, enhanced mRNA expression of two of the toadfish Rh isoform (Rhcg1 and Rhcg2), and decreased branchial and hepatic GSase activity. Alternatively, cortisol infusion decreased ammonia excretion by 2.5-fold, a change that was accompanied by reduced branchial expression of all toadfish Rh isoforms (Rhag, Rhbg, Rhcg1 and Rhcg2) and a 2-fold increase in hepatic GSase activity. In contrast, maintenance of high circulating ammonia by ammonia infusion enhanced ammonia excretion and Rh expression (Rhag, Rhbg, and Rhcg2). Toadfish treated with cortisol showed an ameliorated response to ammonia infusion with no change in Rh mRNA expression or GSase activity. In summary, the evidence suggests that ammonia excretion in toadfish is decreased by cortisol-induced changes in both Rh glycoprotein mRNA abundance and GSase activity.

Introduction

The gulf toadfish (*Opsanus beta*) is unusual among teleosts in demonstrating the capacity to alter both rates and patterns of nitrogen excretion. Under conditions of minimal stress in the laboratory, toadfish are slightly ammoniotelic (Walsh and Milligan, 1995), resembling the majority of teleost fish that dispose of nitrogenous wastes predominantly as ammonia (the sum of NH_3 and NH_4^+) across the gill (Wood, 1993). However, in response to moderate stress, such as crowding or confinement, toadfish switch to ureotely and excrete the bulk of their waste as urea in a pulsatile manner across the branchial epithelium (Walsh et al., 1990; Walsh et al., 1994; Wood et al., 1995; Wood et al., 1997). The transition to ureotelism is characterized by increases in liver and muscle glutamine synthetase (GSase) activity (Esbaugh and Walsh, 2009; Hopkins et al., 1995; Walsh et al., 2003; Walsh and Milligan, 1995; Walsh et al., 1994; Wood et al., 1995). GSase converts ammonia into glutamine for use in the piscine ornithine-urea cycle (O-UC), and induction of this enzyme results in an increase in urea production and accumulation in the plasma (Hopkins et al., 1995; McDonald et al., 2009; Walsh and Milligan, 1995; Walsh et al., 1994). However, the transition to ureotelism does not involve a significant increase in urea excretion, but rather a substantial reduction in ammonia excretion (Walsh and Milligan, 1995; Walsh et al., 1994). It was postulated that the retention of ammonia is facilitated by the combined activity of hepatic and gill-specific GSase isozymes. While the hepatic GSase likely accounts for the bulk of ammonia entry as glutamine into the O-UC, the glutamine produced by the gill-specific GSase is thought to greatly minimize ammonia leakage across the branchial epithelium of ureotelic toadfish and likely contributes only a small

fraction to urea production in the O-UC (Esbaugh and Walsh, 2009; McDonald et al., 2009; Walsh, 1997; Walsh et al., 2003; Wood et al., 1995). A recent study by McDonald and colleagues demonstrated that GSase expression has a limited distribution in the gills, being localized only in the mitochondrion rich cells (MRCs; McDonald et al., 2009), which constitute a very small proportion of the total gill cell population (Perry and Walsh, 1989). Furthermore, branchial GSase activity remains unchanged following an acute (24 - 48 h) crowding/confinement stress (Esbaugh and Walsh, 2009; Hopkins et al., 1995; Rodela et al., in preparation-b; Walsh et al., 2003). Increased activity has only been reported following chronic exposure (~ 1 week) to crowded conditions (McDonald et al., 2009). Collectively, these data suggest that additional mechanisms may play an integral role in modulating ammonia excretion in toadfish.

The recent identification of the Amt/MEP/Rhesus (Rh) glycoprotein family of proteins that function as ammonia channels in plasma membranes (Marini et al., 2006; Marini et al., 1997) has led to a re-evaluation of ammonia excretion mechanisms in toadfish. Vertebrates express three main Rh-associated glycoproteins that facilitate ammonia transport; type A (Rhag), type B (Rhbg), and type C (Rhcg; Huang and Peng, 2005; Peng and Huang, 2006). With respect to teleosts, multiple copies of the Rh genes have been isolated in pufferfish (*Takifugu rubripes*) (Nakada et al., 2007), rainbow trout (*Oncorhynchus mykiss*) (Nawata et al., 2007; Nawata and Wood, 2008), mangrove killifish (*Kryptolebias marmoratus*) (Hung et al., 2007), and zebrafish (*Danio rerio*) (Shih et al., 2008). Recently, we cloned four toadfish Rh genes - Rhag, Rhbg, Rhcg1, and Rhcg2 - that show considerable sequence similarity to other piscine Rh proteins (Rodela et al., in preparation-b). These four genes are prominently expressed in the gill

and their abundance is correlated with the nitrogen excretion phenotype of the toadfish; unconfined toadfish excreted significant quantities of ammonia which were correlated with high levels of Rh mRNA expression (Rodela et al., in preparation-b). By contrast, patterns observed in ureotelic confined toadfish suggested that Rh abundance in the gill was reduced to minimize the branchial efflux of ammonia. The differential pattern of Rh mRNA expression between the two toadfish phenotypes indicates that confinement stress may provide proximate cues for the downregulation of Rh glycoproteins.

Like most teleosts (Barton and Iwama, 1991), toadfish respond to crowding or confinement with a moderate surge in plasma cortisol levels that peaks at approximately 2 h and returns to normal by 24 h (Hopkins et al., 1995). Studies have consistently found this transient increase in cortisol to be an important regulator of both the ureogenic and ureotelic capacity of the toadfish. In particular, the rise in cortisol induces transcription of the hepatic GSase that results in enhanced enzyme activity (Esbaugh and Walsh, 2009; Kong et al., 2000), an effect eliminated by treatment with the cortisol-synthesis inhibitor metyrapone (Hopkins et al., 1995). Elevated cortisol also causes an upregulation of toadfish urea transporter (tUT) messenger RNA transcript levels (McDonald et al., 2009; Rodela et al., in preparation-a), but inhibits tUT function (McDonald et al., 2009; McDonald et al., 2004), as indicated by a reduction in urea excretion. These observations suggest that cortisol coordinates both transcriptional regulation of tUT and post-translational regulatory events to facilitate the efflux of urea in a pulsatile manner that is unique to the gulf toadfish (McDonald et al., 2009; Rodela et al., in preparation-a; Wood et al., 2003). Based on the strong

involvement of cortisol in urea metabolism and excretion, we speculate that glucocorticoids may regulate Rh expression in toadfish to coordinate simultaneous changes in ammonia and urea excretion during the transition to ureotely. The hypothesis of glucocorticoid involvement is consistent with prior observations of differential Rh expression and ammonia excretion between fish that experienced minimal stress and those exposed to an acute stress (i.e. crowding and confinement; (Rodela et al., in preparation-b).

Thus, the main goal of the present study was to address the possibility that Rh expression in toadfish is regulated by cortisol. The central hypothesis was that cortisol regulates branchial Rh expression and hence ammonia excretion. We predicted that Rh expression and ammonia excretion would be inhibited by high cortisol but enhanced by low cortisol. To test this hypothesis, nitrogen excretion and Rh mRNA expression were measured in toadfish exposed to treatments that either lowered or increased circulating plasma cortisol. A secondary objective of the study was to examine the influence of circulating ammonia levels on Rh mRNA expression. Plasma ammonia was maintained at levels that were physiologically comparable to those experienced by fed toadfish, and the effect of exogenous ammonia was evaluated in both the absence and presence of cortisol. For all experiments, changes in gill mRNA transcripts were evaluated and correlated with changes in plasma ammonia levels and ammonia excretion. Furthermore, the linkage of ammonia and urea *via* the O-UC warranted examination of additional variables including plasma urea levels, urea excretion, and GSase mRNA expression and activity.

Material and Methods

Animals

Gulf toadfish (*Opsanus beta* Goode and Bean) were collected by roller trawl in Biscayne Bay, Florida by local commercial shrimpers during January-February, 2009. Immediately following capture, toadfish were transferred to the laboratory and treated with a freshwater dip for 2 minutes, followed by a malachite green-formalin treatment (final concentration 0.05 mg L^{-1}, 15 mg mL^{-1}, Aquavet, Hayward, CA, USA) to prevent infection by the ciliate *Cryptocaryon irritans* (Stoskopf, 1993; Wood et al., 1997). Toadfish were acclimated to laboratory conditions for a minimum of one week prior to experimental manipulation. During this period, fish were kept at low stocking density (> 10 fish per tank) in 50 L glass aquaria supplied with flowing, aerated seawater (18-22°C, pH 8.1) and kept under a natural photoperiod. Toadfish were fed chopped squid once a week.

Experimental protocol

All fish were exposed to a standard crowding and confinement procedure protocol to induce and maintain ureotely for the duration of the experiments (Hopkins et al., 1995; Walsh, 1987; Wood et al., 1997). In brief, 7-8 fish were transferred to a 6 L plastic tub supplied with flowing seawater for 48 h; fish were deprived of food. Following this period, fish were lightly anesthetized with MS-222 (1 g L^{-1} buffered with 2 g L^{-1} sodium bicarbonate, pH 7.8), covered with moist towels, and surgically implanted with caudal artery catheters (McDonald et al., 2000; Wood et al., 1997) and in select cases, intraperitoneal (IP) catheters (see below) (McDonald and Walsh, 2004).

Catheters were used to facilitate delivery of pharmacological agents and/or for blood sampling with minimal handling stress. Following surgery, toadfish were allowed to recover for 24 h in individual 2 L flux chambers supplied with flowing, aerated seawater.

For all experiments, an initial 200 μL blood sample was withdrawn from each fish *via* the caudal artery catheter at time 0 h. For *Series I* the blood sample was centrifuged at 10,000 *g* for one minute and an 80 μL aliquot of plasma was removed, flash frozen in liquid nitrogen, and stored at -80°C for later analysis of ammonia, urea, and cortisol levels. The aliquot was replaced with toadfish saline (Walsh, 1987) and the re-suspended red cells were re-injected into the fish. For *Series II* and *III*, blood pH was measured over the experimental period to monitor changes in acid-base status that may result following infusion of ammonia. In brief, an initial 300μL blood sample was withdrawn from each fish *via* the caudal catheter at time 0 h. Approximately 80 μL of the whole blood was immediately used to measure blood pH using an Accumet pH electrode inserted into a tightly sealed glass chamber that was maintained at the temperature of ambient seawater (18-22°C). The pH electrode was connected to a Radiometer PHM220 pH meter (Radiometer, Copenhagen, Denmark). The remaining blood was centrifuged as described above to obtain a plasma sample. Following each of these procedures, the whole blood used for pH measurements was recovered and combined with the re-suspended red cells remaining from the plasma samples and re-injected into the fish.

Concurrently, water flow to the individual flux chambers was stopped and an initial 2 mL water sample was removed by pipette. Additional water samples were

collected hourly using a peristaltic pump (2 mL h^{-1}) and fraction collector for the remainder of the experiment. Every 24 h, boxes were flushed with fresh seawater for 30 min. In between flushes, oxygen saturation and adequate mixing in the flux chambers were maintained by vigorous aeration. For the first 24 h of all experimental treatments, toadfish were left undisturbed in their individual flux chambers to collect baseline measurements of nitrogen excretion and plasma variables. Three series of experiments were conducted.

Series I: Metyrapone treatment

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

later analysis of mRNA expression of all toadfish Rh isoforms, GR, and gill and hepatic GSase isoforms.

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

Toadfish were separated into 4 treatment groups. Using a Gilson 8 channel peristaltic pump connected to the arterial catheter, after the 24 h control period one group of toadfish (mean mass ± SEM; 0.070 $\pm$ 0.001 kg; n=8) was infused with iso-osmotic saline (150 mmol L^{-1} NaCl) at a rate of 3 mL kg^{-1} h^{-1} for the duration of the experiment. A second group (mean masss 0.062 $\pm$ 0.003 kg; n=8) was infused with cortisol (0.09 mg mL^{-1}; hydrocortisone hemisuccinate salt, Sigma Chemicals, St. Louis, MO) in iso-osmotic saline at a rate of 3 mL kg^{-1} h^{-1}. Cortisol concentration was chosen based on previous studies (McDonald et al., 2004; Rodela et al., 2009a). A third group (mean mass 0.065 $\pm$ 0.003 kg; n=8) was infused with ammonia [$(NH_4)_2HPO_4$); Sigma-Aldrich] in isosmotic saline. The concentration of infused ammonia was chosen based on a preliminary experiment demonstrating that circulating ammonia in laboratory-acclimated toadfish increased from 191.3 $\pm$ 26.6 μmol L^{-1} to 503.4 $\pm$ 58.2 μmol L^{-1} within 6 h following feeding. An intermediate value of 300 μmol L^{-1} was chosen to avoid potential long-term toxicity effects associated with elevated ammonia. Maintenance of plasma levels was achieved by adjusting the infusion rates following periodic evaluation of plasma ammonia concentrations. Furthermore, ammonium phosphate was chosen for infusion to avoid the alteration of acid-base status that is associated with the infusion of similar compounds such NH_4HCO_3 or $(NH_4)_2SO_4$ (see Salama et al., 1999). Accumulation of phosphate in the blood was not a concern because renal

excretion is capable of handling the phosphate load introduced by infusion (Kaune and Hentschel, 1987; Maren et al., 1992). The final group of fish (mean mass 0.078 $\pm$ 0.008 kg; n=8) was infused with a combination of cortisol and ammonia. Cortisol (0.09 mg mL^{-1}) and ammonia infusions were achieved as described above, with both compounds dissolved in iso-osmotic saline. Blood samples were withdrawn at 24, 30, 36, 48, 72, and 96 h during the experiment and an aliquot was immediately used to measure blood pH and another aliquot was removed for later physiological analyses of plasma variables. Following termination of the experiment, toadfish were anesthetized and sampled as described in *Series I*.

Series III: Inhibition of GSase activity with methionine sulfoximine

The branchial permeability to ammonia in crowded toadfish is lower than in most other teleosts (Part et al., 1999; Wood, 1993) and is attributed to a reduction in Rh expression (Rodela et al., in preparation-b) and enhanced ammonia-trapping *via* GSase (Walsh, 1997; Wood et al., 1995). The relative contribution of ammonia-trapping by GSase is currently unknown. Therefore, the aim of the final series of experiments was to investigate the effects of GSase inhibition on nitrogen excretion and Rh expression. Following the 24 h control period, toadfish received one of three treatments. The sham control fish for this series was the group used in *Series I*; i.e. fish received 1.5 μL g^{-1} body mass of 150 mmol L^{-1} NaCl injected by IP catheter every 24 h. The second group was dosed with L-methionine sulfoximine (MSO; 50 mg mL^{-1} in isosmotic saline) delivered as 1.5 μL g^{-1} body mass every 24 h, up to 96 h. The concentration and frequency of administration were determined from a previous study

(Veauvy et al., 2005). Veauvy and colleagues demonstrated that a single dose (150 mg kg^{-1}) of MSO diminished GSase activity by ~88 % after 40 h, and based on these data we selected a concentration of 75 mg kg^{-1} to be administered daily for 72 h. Individuals in the final experimental group were injected with MSO (50 mg mL^{-1} in isosmotic saline) and infused with cortisol as described in *Series II*. All treatments received an additional injection of saline (1.5 μL g^{-1} body mass) to advance the compounds through the IP catheter into the peritoneal cavity of the fish. Blood samples were collected at 24, 30, 36, 48, 72, and 96 h and one was aliquot was used to measure whole blood pH and the other aliquot was used to acquire a plasma sample for later analyses. At the end of the experiment, toadfish were anesthetized and sampled as described in *Series I*.

Physiological assays

Ammonia content of water samples was measured according to the indophenol blue method (Ivancic and Degobbis, 1984) using a ThermoMax microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA). The concentration of urea in water samples was determined using the diacetyl monoxime method described by Rahmatullah and Boyde (Rahmatullah and Boyde, 1980) adapted for use with a microplate reader. The excretion (μmol-N kg^{-1}) of ammonia or urea was calculated according to the equation:

$$\text{Excretion} = (\Delta C \times V_f) \div M_f$$

where ΔC was the difference in concentration (μmol L^{-1}) over a sampling interval, V_f represented the volume of the individual flux chamber in litres, and M_f was the mass (kg) of the toadfish (McDonald et al., 2004). These values were used to calculate the

percent ureotelism. Nitrogen excretion (μmol-N kg^{-1}) values from the treatment period were averaged from multiple 24-h intervals to facilitate a comparison with the initial 24 h control period. In addition, water urea content was used to analyze the size and frequency of pulsatile urea excretion events. A threshold pulse size of 40 mmol-N kg^{-1} was used, as defined by (McDonald et al., 2004). Urea efflux was then examined in more detail to identify total (mmol-N kg^{-1}), pulsatile (mmol-N kg^{-1}), and non-pulsatile (mmol-N kg^{-1}) components. All excretion measurements are presented graphically as ratios that were calculated by normalizing the value from the treatment period to the value from the control period for that same treatment.

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

GSase activity was measured according to the transferase protocol (Walsh, 1996). In brief, individual gill and liver samples were homogenized separately using a motor-driven tissue homogenizer (Fisherbrand) in 5 volumes of 50 mmol L^{-1} HEPES buffer (pH 7.4) and then centrifuged at 13,000 g for 8 min. The resulting supernatant

was decanted and assayed for GSase activity at 540 nm using a Spectramax Plus 384 plate spectrophotometer (Molecular Devices). GSase activity is reported in units of μmol min^{-1} g^{-1}.

Molecular analysis of gene expression

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

For real time PCR analysis of gene transcript levels, primers designed and validated in previous studies were used. In particular, the primers for the toadfish GR (GenBank accession #HQ424878) and 18S were obtained from (Rodela et al., in preparation-a). Similarly, primers for Rhag (GenBank accession #HQ424874), Rhbg (GenBank accession #HQ424875), Rhcg1 (GenBank accession #HQ424876), Rhcg2 (GenBank accession #HQ424877), gill GSase (GenBank accession # AF532312), and hepatic GSase (GenBank accession #AF118103) were obtained from Rodela et al. (in

preparation-b). All primer pairs were chosen for optimal amplification at an annealing temperature of 58°C.

All real time PCR reactions were performed using a SYBR green master mix (Stratagene) with a Mx3000P Real-Time PCR System and associated MxPro 4.1 software (Stratagene). A 2 µL aliquot of template was used for a 12.5 µL reaction that contained both forward and reverse primers, and SYBR master mix. The composition of the reactions and the thermocycler settings were based on those suggested by the manufacturer. Dissociation curves were generated and evaluated for each reaction to verify the specificity of the primers pairs and monitor the formation of primer dimers. 'No reverse transcriptase' and no template control templates were used to ensure that amplicons did not arise from genomic DNA or reagent contamination, respectively. These control samples were generated by omission of reverse transcriptase during cDNA synthesis. Standard curves were used to assess the reaction efficiency of all primer pairs. Templates for the standard curves were derived from pooled gill cDNA samples and serially diluted (1:5) with RNase/DNase-free molecular grade water (Sigma). Efficiencies were assessed by linear regression of cycle threshold (Ct) values on relative template concentration. Primer pair efficiencies were considered satisfactory when they fell within the range of 85-115% with $R^2 \geq 0.99$. All transcript levels were expressed relative to the control gene 18S (diluted 1,000-fold) and calculated according to the delta delta Ct method outlined by Pfaffl (Pfaffl, 2001). For purposes of comparison, the mRNA expression of each gene was calculated relative to the corresponding control samples.

Statistical analyses

All data are presented as means ± 1 standard error (s.e.m.). Differences among treatments were statistically analyzed using a two-way repeated measures (RM) analysis of variance (ANOVA) with time within groups (i.e. before and after application of treatment) as one factor and treatment among or between groups as the second factor. Gene expression was analyzed by Student's *t*-test or one-way ANOVA, as appropriate. A Holm-Sidak test was used for *post-hoc* multiple comparisons. Equivalent non-parametric tests were used when assumptions of normality and equal variance were not satisfied ($p<0.05$). Prior to analysis, proportional data (% urea and ammonia) were transformed using $p' = arcsin\sqrt{p}$ as these values were binomially distributed (Zar, 1999). All statistical analyses were carried out using SPSS SigmaStat software (version 3.5).

Results

Series I

Prior to saline injection, toadfish excreted approximately 32% of their waste as ammonia (Table 6.1) at a rate of 37.3 ± 3.2 μmol kg^{-1} h^{-1} (n=8). The majority of urea was excreted in a pulsatile manner (≥ 96 %; 2172.7 ± 299.6 μmol-N kg^{-1}), with a mean pulse size of 540.3 ± 214.9 μmol kg^{-1} and average frequency of 3.6 ± 0.4 pulses per 24 h. The non-pulsatile component of urea excretion was relatively small (157.8 ± 75.4 μmol-N kg^{-1}). Daily injections of isosmotic saline did not alter the proportion of urea and ammonia excreted over time (Table 6.1, p=0.70). Ammonia (Figure 6.1A, p=0.24) and urea excretion (Figure 6.1C, $p \geq 0.89$; Figure 6.1E, p=0.46) were also unaffected by

saline treatment. Similarly, plasma ammonia (Figure 6.1B, $p \geq 0.062$) and urea (Figure 6.1D, $p=0.40$) were unchanged in response to IP saline treatment.

As expected metyrapone treatment was effective at reducing circulating cortisol concentrations. Measurable differences in cortisol levels between saline- and metyrapone-treated individuals were observed as early 36 hours and persisted until 96 hours (Figure 6.2B, $p \leq 0.002$). Metyrapone treatment elevated ammonia excretion by 3.5-fold (Figure 6.1A, $p=0.016$), raising rates from 4.7 $\pm$ 0.6 to 15.8 $\pm$ 1.9 $\mu mol\ kg^{-1}\ h^{-1}$. This change in ammonia excretion translated into an increase in the proportion of nitrogenous waste excreted as ammonia (Table 6.1, $p=0.013$). Metyrapone injection also caused an approximately 2-fold increase in total (1618.0 $\pm$ 150.5 $\mu mol\ kg^{-1}$) and pulsatile components of urea excretion (1483.0 $\pm$ 51.5 $\mu mol\ kg^{-1}$; Figure 6.1C, $p \leq 0.014$), whereas the non-pulsatile component was unaffected (55.9 $\pm$ 23.8 $\mu mol\ kg^{-1}$; Figure 6.1C, $p=0.75$). Pulse size increased by approximately 1.8-fold, with the average pulse size being 393.6 $\pm$ 48.3 $\mu mol\ kg^{-1}$ following treatment. Pulse frequency was unchanged and remained at 3.4 $\pm$ 0.3 pulses per 24 h. Metyrapone treatment had no effect on plasma ammonia (Figure 6.1B, $p=0.40$) and minimal effect on plasma urea, with only the 72 h value being different from the initial treatment value (Figure 6.1D, $p<0.001$).

The mRNA expression of select Rh glycoprotein isoforms in the gill was affected by metyrapone treatment. Rhag mRNA abundance was lowered by 4.8-fold (Figure 6.3A, $p=0.007$) and Rhcg1 and Rhcg2 mRNA expression was elevated 2.2- and 4-fold, respectively, in metyrapone treated toadfish relative to saline injected individuals (Figure 6.3A, $p \leq 0.038$ in each case). Branchial Rhbg was unaffected (Figure 6.3A, $p=0.073$). Glutamine synthetase activities in both the gill and liver were 1.6- and 1.8-

fold lower in metyrapone treated fish (Figure 6.4A, $p \leq 0.023$ in each case). These differences were not reflected in mRNA levels, as mRNA abundance of neither the gill-specific nor the hepatic isoform of GSase was affected by metyrapone treatment (Figure 6.4B, $p \geq 0.86$). GR mRNA transcript levels in the gill were raised in response to metyrapone treatment (Figure 6.2A, $p=0.005$), however, GR mRNA expression in the liver remained unchanged (Figure 6.2A, $p=0.91$).

Series II

Saline infused fish excreted ammonia at a rate of 19.7 $\pm$ 2.6 μmol kg^{-1} h^{-1}, which accounted for approximately 25% of the total nitrogenous wastes excreted during both the control and treatment periods (Table 6.1, $p=0.83$). The rate of ammonia excretion was not affected by infusion of isosmotic saline (Figure 6.5A, $p=0.85$). Similarly, urea excretion was unaltered by saline treatment (Figure 6.5C, $p \geq 0.16$), with rates remaining around 60.6 $\pm$ 4.7 μmol kg^{-1} h^{-1} over the duration of the experiment. Approximately 99% of that urea (1454.5 $\pm$ 113.8 μmol kg^{-1}) was excreted in discrete pulses ranging in size from 116.7 to 569.7 μmol kg^{-1}, with a frequency of 2.9 $\pm$ 0.3 events per 24 h. Furthermore, plasma variables including ammonia (Figure 6.5B, $p \geq 0.50$) and urea (Figure 6.5D; $p \geq 0.52$) concentrations, and plasma pH (7.87 $\pm$ 0.013; $p=0.50$) also were unaffected by saline treatment.

Toadfish infused with cortisol experienced a reduction in ammonia excretion over the experimental period (Figure 6.5A, $p=0.034$), with rates decreasing from 18.9 $\pm$ 5.3 to 7.3 $\pm$ 1.3 μmol kg^{-1} h^{-1}. Ammonia excretion accounted for 31.4 $\pm$ 6.3 % of total nitrogen excreted over the entire experimental period in cortisol-infused fish (Table

6.1; p=0.83). Urea was excreted in a pulsatile fashion; however, cortisol infusion significantly decreased the initial total (679.2 ± 74.3 μmol kg^{-1}), pulsatile (628.7 ± 47.2 μmol kg^{-1}), and non-pulsatile components (50.5 ± 2.6 μmol kg^{-1}) by 34 (p=0.046), 33 (p=0.043), and 44% (p=0.040), respectively (Figure 6.5C). This change also resulted in a ~50% reduction in urea pulse size (Figure 6.5E, p=0.036). Urea pulse frequency, however, increased significantly from 2.25 ± 0.16 to 2.79 ± 0.14 pulses per 24 h (p=0.030). Plasma ammonia concentration did not vary significantly from initial values; however, levels were significantly lower than saline-treated fish at 96 h (Figure 6.2C, p=0.006). Plasma urea level rose over time (Figure 6.2D, $p \leq 0.002$), and was significantly higher than values for saline-treated fish starting at 36 h ($p \leq 0.002$). Cortisol infusion had no effect on plasma pH (p=0.75), which remained at 7.86 ± 0.013 for the duration of the experiment.

Prior to ammonia infusion, toadfish were excreting approximately 19% of their total nitrogenous waste as ammonia (Table 6.1); ammonia was excreted at a rate of 16.0 ± 5.2 μmol kg^{-1} h^{-1} and urea at a rate of 75.9 ± 11.7 μmol kg^{-1} h^{-1}. Urea was eliminated in a pulsatile manner with 3.1 ± 0.2 pulses occurring every 24 h and an average pulse size of 603.4 ± 223.2 μmol kg^{-1}. As a result of infusion, plasma ammonia was significantly elevated ($p<0.001$) and maintained around a concentration of 300 μmol L^{-1} (Figure 6.5B). Maintenance of plasma ammonia at these concentrations elevated ammonia efflux by 1.7-fold (Figure 6.5A, p=0.012), raising excretion rates to 23.3 ± 6.5 μmol kg^{-1} h^{-1}. All other variables, including the components of urea excretion (Figure 6.5C, $p \geq 0.90$), urea pulse size (Figure 6.5E, p=0.99) and frequency (p= 0.17),

and plasma urea concentration (figure 6.2D, $p>0.36$), and plasma pH (7.87 $\pm$ 0.015; $p=0.50$) were unaffected by the infusion of ammonia.

Infusing toadfish with cortisol and ammonia together reduced ammonia excretion by 50% (Figure 6.5A, $p=0.53$), lowering rates from 67.1 $\pm$ 16.0 to 27.8 $\pm$ 4.3 $\mu mol\ kg^{-1}\ h^{-1}$. This change was accompanied by a ~50% decrease from the initial levels of total (5349.1 $\pm$ 1251.2 $\mu mol\ kg^{-1}$), pulsatile (5246.2 $\pm$ 1259.9 $\mu mol\ kg^{-1}$), and non-pulsatile (103.3 $\pm$ 23.7$\mu mol\ kg^{-1}$) urea excretion (Figure 6.5C, $p<0.001$). These changes translated into a 50% reduction in pulse size (Figure 6.5E, $p=0.009$), decreasing values from 1819.4 $\pm$ 251.2 to 788.9 $\pm$ 199.1 $\mu mol\ kg^{-1}$. Pulse frequency was unaffected by the infusion and remained at 2.8 $\pm$ 0.3 pulses per 24 h. The infusion successfully elevated plasma ammonia and maintained concentrations at ~300 $\mu mol\ L^{-1}$ (Figure 6.5B, $p\leq0.002$). Plasma urea concentrations were slightly elevated during the infusion period (Figure 6.5D, $p\leq0.020$). Plasma pH was unaffected by the combined cortisol and ammonia infusion (7.85 $\pm$ 0.015, $p=0.50$).

Exogenous elevation of plasma cortisol through infusion caused a decrease in mRNA expression for all Rh isoforms relative to expression in saline infused toadfish (Figure 6.3B, $p\leq0.033$). Ammonia infusion caused significant 5.5-, 5.5-, and 11.7-fold increases in Rhag, Rhbg, and Rhcg2 expression relative to saline controls (Figure 6.3B); Rhcg1 mRNA expression was unaffected by ammonia infusion ($p=0.17$). In the final group in this series, no significant difference in mRNA abundance of any Rh isoform was detected between cortisol + ammonia-infused individuals and saline-infused toadfish (Figure 6.3B).

With respect to urea synthesis capacity, ammonia-infused fish exhibited lower levels of branchial GSase activity than cortisol-, saline- or cortisol + ammonia-infused fish (Figure 6.3C). Toadfish infused with cortisol or a combination of cortisol and ammonia exhibited approximately 2-fold higher levels of branchial GSase mRNA expression than saline- or ammonia-infused fish (Figure 6.3D, $p=0.041$). A similar trend was observed in the liver (Figure 6.3D), however, mRNA abundance of hepatic GSase in cortisol and ammonia infused fish was significantly different to that in saline- ($p=0.12$) and ammonia-infused fish ($p=0.15$). In the liver, comparable levels of GSase activity were measured in saline-, ammonia-, and cortisol+ammonia-treated fish, but approximately 2-fold higher activity was observed in cortisol-infused toadfish (Figure 6.3C, $p<0.001$).

Both cortisol infusion alone and the combination of cortisol and ammonia were successful in raising and maintaining plasma cortisol concentrations at high levels for the duration of the treatment period (Figure 6.2D, $p<0.001$). Cortisol concentrations in saline- ($p>0.45$) and ammonia-infused fish ($p>0.67$) did not deviate significantly from their initial values. Furthermore, neither GR mRNA expression in the gill ($p=0.10$) nor that in the liver ($p=0.11$) was altered in response to any of the treatments used in this experimental series (Figure 6.2C).

Series III

The group of saline-injected fish was the same as that used in *Series I* (see *Series I* for values). Prior to injection with the GS inhibitor MSO, toadfish excreted approximately 22% of their waste as ammonia (Table 6.1), at a rate of 25.5 ± 7.4 μmol

kg^{-1} h^{-1}. IP injections of MSO resulted in significant increases in the percentage of waste as ammonia and in the rate of ammonia excretion (Figure 6.6A), with post-injections values of 57% (Table 6.1, $p<0.001$) and 88.2 $\pm$ 16.1 μmol kg^{-1} h^{-1} ($p=0.002$). Treatment with MSO elevated plasma ammonia concentrations (Figure 6.6B, $p<0.001$). Urea (78.6 $\pm$ 10.0 μmol kg^{-1} h^{-1}) accounted for 78% of total nitrogenous waste (Table 6.1) and was excreted in a pulsatile fashion with an average pulse size of 549.6 $\pm$ 180.3 μmol kg^{-1} and mean frequency of 3.8 $\pm$ 0.3 pulses per 24 h. The pulsatile component accounted for 99% of total urea excretion (1886.2 $\pm$ 147.06 μmol kg^{-1}); non-pulsatile urea excretion was relatively small with a mean value of 17.3 $\pm$ 3.4 μmol kg^{-1}. No urea excretion variable changed in response to treatment with MSO (Figure 6.6C, $p>0.36$; Figure 6.6E, $p=0.21$). Plasma urea was significantly lower than the initial plasma urea sample by the end of the treatment period (Figure 6.6D, $p<0.001$). Plasma pH did not vary significantly following treatment with MSO (7.85 $\pm$ 0.0088; $p=0.16$).

Toadfish in the final experimental group excreted ammonia at an initial rate of 47.9 $\pm$ 6.3 μmol kg^{-1} h^{-1} that increased significantly to a mean rate of 93.1 $\pm$ 9.6 μmol kg^{-1} h^{-1} following treatment with MSO and cortisol (Figure 6.6A, $p<0.001$). The percentage of nitrogenous waste that was excreted as ammonia increased from 23% to 47% following treatment (Table 6.1, $p=0.044$). During the treatment period, plasma ammonia concentration in the MSO + cortisol-treated group was significantly higher than that of saline-injected individuals (Figure 6.6B, $p<0.001$). The total and pulsatile components of urea excretion were affected following treatment, decreasing from 7953.2 $\pm$ 1378.1 and 7811.2 $\pm$ 1378.2 to 4127.8 $\pm$ 429.3 and 3778.2 $\pm$ 396.8 μmol kg^{-1}, respectively (Figure 6.6C, $p<0.001$). The non-pulsatile component of urea excretion was

unaffected and remained at 142.1 ± 16.6 μmol kg^{-1} for the duration of the experiment (Figure 6.6C, p=0.65). Urea pulse size decreased from an initial value of 1548.3 ± 788.3 to 669.6 ± 250.9 μmol kg^{-1} (Figure 6.3E, p=0.006) with an average frequency of 2.7 ± 0.3 pulses per 24 h that remained unchanged during the experimental period (p=0.21). Plasma urea concentration was significantly elevated by MSO + cortisol treatment (Figure 6.6D, p<0.001). Plasma pH (7.83 ± 0.0038) was unaffected by the combination of MSO injection coupled with cortisol infusions (p=0.16).

Treatment with MSO resulted in significantly lower branchial mRNA expression of all Rh isoforms, relative to saline-injected fish (Figure 6.4C, p≤0.025). Whereas significantly lower Rhcg1 mRNA expression was detected in MSO + cortisol-treated individuals (Figure 6.4D, p<0.001), the other Rh isoforms were unaffected. Furthermore, assays demonstrated that MSO treatment knocked down GSase activity to levels below detection (data not shown). GSase mRNA expression was not measured for *Series III* as we were not interested in the effects of MSO on GSase expression.

Branchial GR mRNA transcript levels were lower in toadfish treated with MSO (Figure 6.6E, p=0.007) than in fish treated with MSO + cortisol or saline. Analysis of hepatic GR mRNA expression revealed similar levels in saline- and MSO-treated fish, but fish infused with cortisol and injected with MSO exhibited approximately 3-fold higher levels of expression (Figure 6.6E, p=0.001). The MSO+cortisol-treated fish exhibited elevated levels of cortisol that were maintained around 800 ng mL^{-1} for the duration of the infusion period (Figure 6.6F, p<0.001). Saline- and MSO-treated fish had comparable concentrations of plasma cortisol.

Figure 6.1: Series I. The effects of intraperitoneal injections of saline (150 mmol L^{-1} NaCl, 1.5 µl g^{-1} body mass, n=8) or metyrapone (20 mg mL^{-1} in 150 mmol L^{-1} NaCl, 1.5 µl g^{-1} body mass, n=8) on ammonia excretion (relative units; A) and plasma ammonia levels (mmol L^{-1}; B) in gulf toadfish (Opsanus beta). Total, as well as pulsatile and non-pulsatile components of normalized urea excretion (relative units; C) and plasma urea concentrations (mmol L^{-1}; D) are also depicted. (E) The effect of saline or metyrapone injections on urea pulse size (relative units) in toadfish. Data in A, C, and E are presented as the treatment value normalized to the initial 24 h control period. Values are expressed as means $\pm$ s.e.m. In panels A, C, and E, different letters denote significant differences between treatment groups, whereas an asterisk (*) indicates a significant difference within a group between the control and treatment periods ($p<0.05$). There were no significant differences in panel B for any of the variables measured. Symbols (*,ϕ) in panel D indicate significant differences within the treatment group from the control period (< 24 h), and differences between treatment groups at a given time, respectively ($p<0.05$). The dotted lines indicate the start of the treatment period.

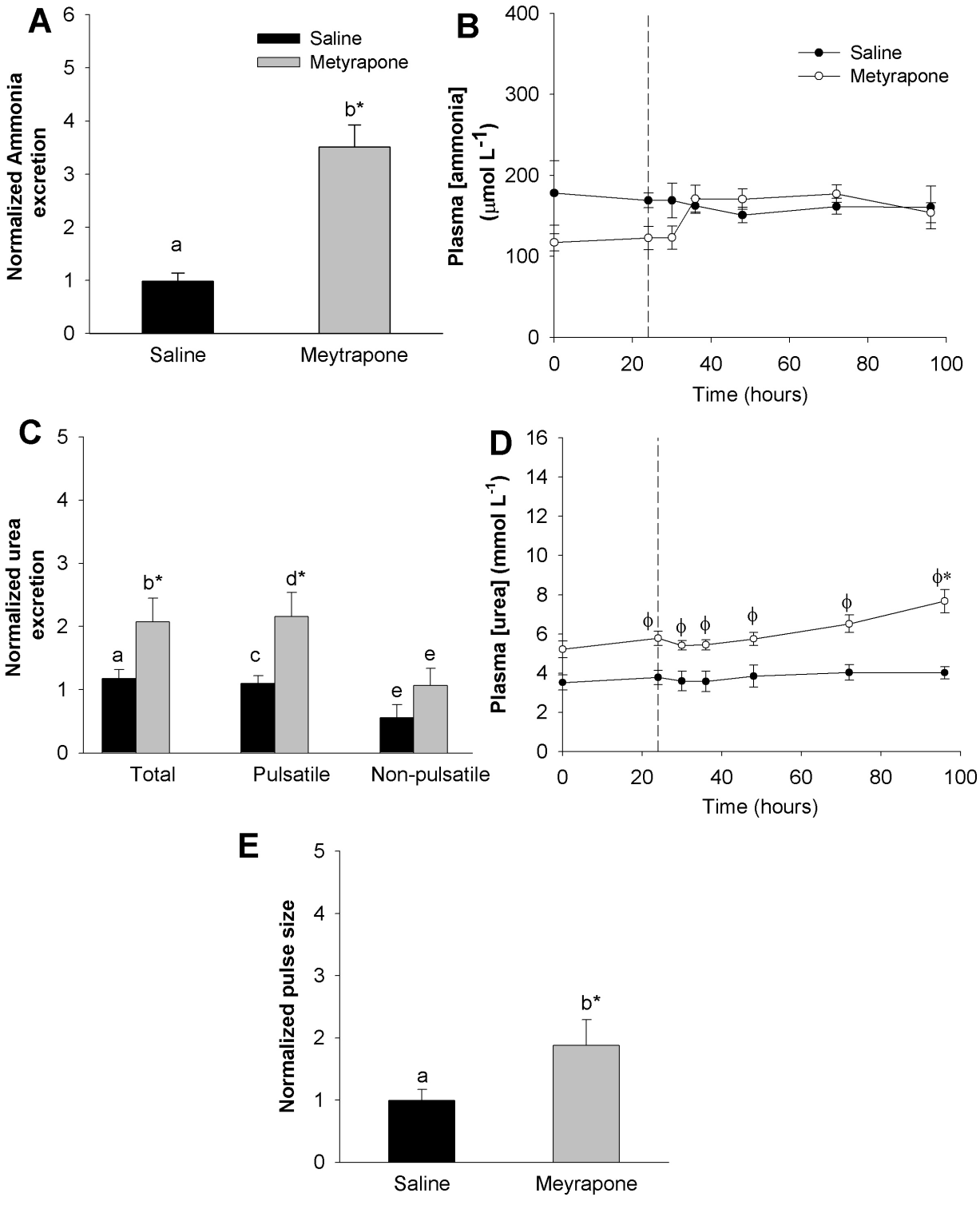
A
Normalized Ammonia excretion
Saline
Metyrapone
b*
a
Saline
Meytrapone
B
Plasma [ammonia] (μmol L-1)
Saline
Metyrapone
Time (hours)
C
Normalized urea excretion
b*
d*
a
c
e
e
Total
Pulsatile
Non-pulsatile
D
Plasma [urea] (mmol L-1)
ϕ*
ϕ
Time (hours)
E
Normalized pulse size
b*
a
Saline
Meyrapone

Figure 6.1

Figure 6.2: Series I-III: The effects of experimental treatments on the relative mRNA expression of the toadfish (Opsanus beta) glucocorticoid receptor (GR) as measured by real-time RT-PCR (A,C,E), and on circulating cortisol concentrations (ng mL^{-1}) (B,D,F). (A,B) In Series I, toadfish were injected with saline (150 mmol L^{-1} NaCl, 1.5 µl g^{-1} body mass, n=8) or metyrapone (20 mg mL^{-1} in 150 mmol L^{-1} NaCl, 1.5 µl g^{-1} body mass, n=8). (C,D) In Series II, one group of toadfish was infused with isosmotic saline (150 mmol L^{-1} NaCl, n=8), a second with hydrocortisone hemisuccinate salt (cortisol; 0.09 mg mL^{-1}), the third with ammonia alone ($(NH_4)_2HPO_4$, n=8), and the fourth with ammonia and cortisol ($(NH_4)_2HPO_4$ + 0.09 mg mL^{-1} hydrocortisone hemisuccinate salt in 150 mmol L^{-1} NaCl, n=8). All fish were infused at a rate of 3 mL kg^{-1} h^{-1}. (E,F) In Series III, toadfish were injected with saline (150 mmol L^{-1} NaCl, 1.5 µl g^{-1} body mass, n=8), methionine sulfoximine (MSO; 50 mg mL^{-1} in 150 mmol L^{-1} NaCl, 1.5 µl g^{-1} body mass, n=8), or MSO and infusion with cortisol (0.09 mg mL^{-1} in 150 mmol L^{-1} NaCl infused at a rate of 3 mL kg^{-1} h^{-1}, n=7). The mRNA levels for each gene were normalized against the control gene 18S and expressed relative to the control group for each series, which was set to a value of 1. Values are means $\pm$ s.e.m. Different letters, in panels A, C, and E, denote significant differences among or between treatments ($p<0.05$) within a tissue. Symbols (*,ϕ) in panels B, D, and F indicate significant differences within the treatment group from the control period (< 24 h), and differences between treatment groups at a given time, respectively ($p<0.05$). The dotted lines indicate the start of the treatment period.

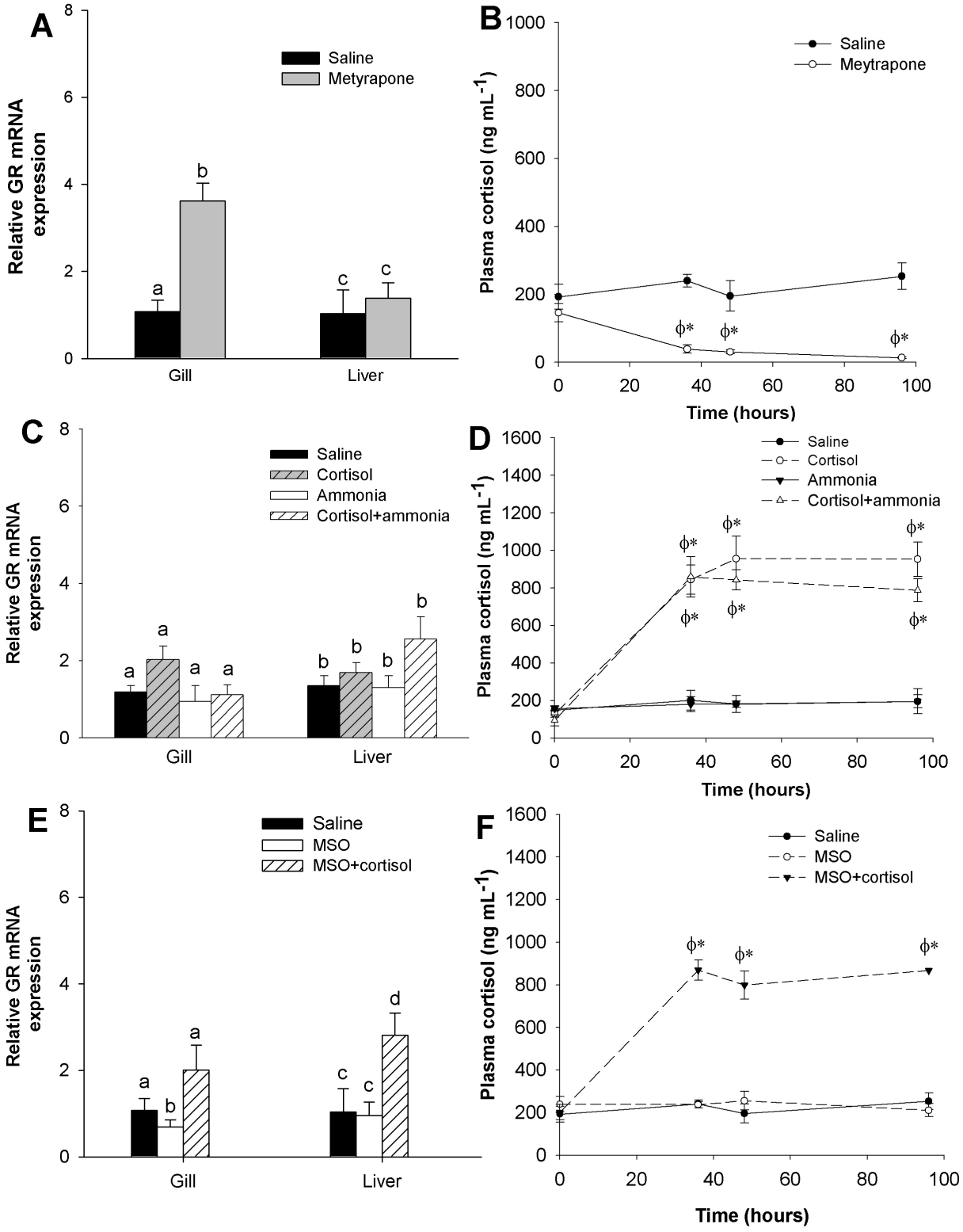
A
Relative GR mRNA expression
Saline
Metyrapone
Gill
Liver
B
Plasma cortisol (ng mL-1)
Saline
Meytrapone
Time (hours)
C
Relative GR mRNA expression
Saline
Cortisol
Ammonia
Cortisol+ammonia
Gill
Liver
D
Plasma cortisol (ng mL-1)
Saline
Cortisol
Ammonia
Cortisol+ammonia
Time (hours)
E
Relative GR mRNA expression
Saline
MSO
MSO+cortisol
Gill
Liver
F
Plasma cortisol (ng mL-1)
Saline
MSO
MSO+cortisol
Time (hours)

Figure 6.2

Figure 6.3: Series I-III: The effects of cortisol and/or ammonia manipulation on branchial and hepatic glutamine synthetase (GSase) activities (A,C) and relative GSase mRNA expression as measured by real-time RT-PCR (B,D) in gulf toadfish (Opsanus beta). (A,B) In Series I, toadfish were injected with saline (150 mmol L^{-1} NaCl, 1.5 µl g^{-1} body mass, n=8) or metyrapone (20 mg mL^{-1} in 150 mmol L^{-1} NaCl, 1.5 µl g^{-1} body mass, n=8). (C,D) In Series II, one group of toadfish was infused with isosmotic saline (150 mmol L^{-1} NaCl, n=8), a second with hydrocortisone hemisuccinate salt (cortisol; 0.09 mg mL^{-1}), the third with ammonia alone ($(NH_4)_2HPO_4$, n=8), and the fourth with ammonia and cortisol ($(NH_4)_2HPO_4$ + 0.09 mg mL^{-1} hydrocortisone hemisuccinate salt in 150 mmol L^{-1} NaCl, n=8). All fish were infused at a rate of 3 mL kg^{-1} h^{-1}. The mRNA transcript levels for each tissue were normalized against the control gene 18S and expressed relative to the control group for each series, which was set to a value of 1. Values are means $\pm$ s.e.m. Different letters denote significant differences between or among treatments ($p<0.05$) within a tissue.

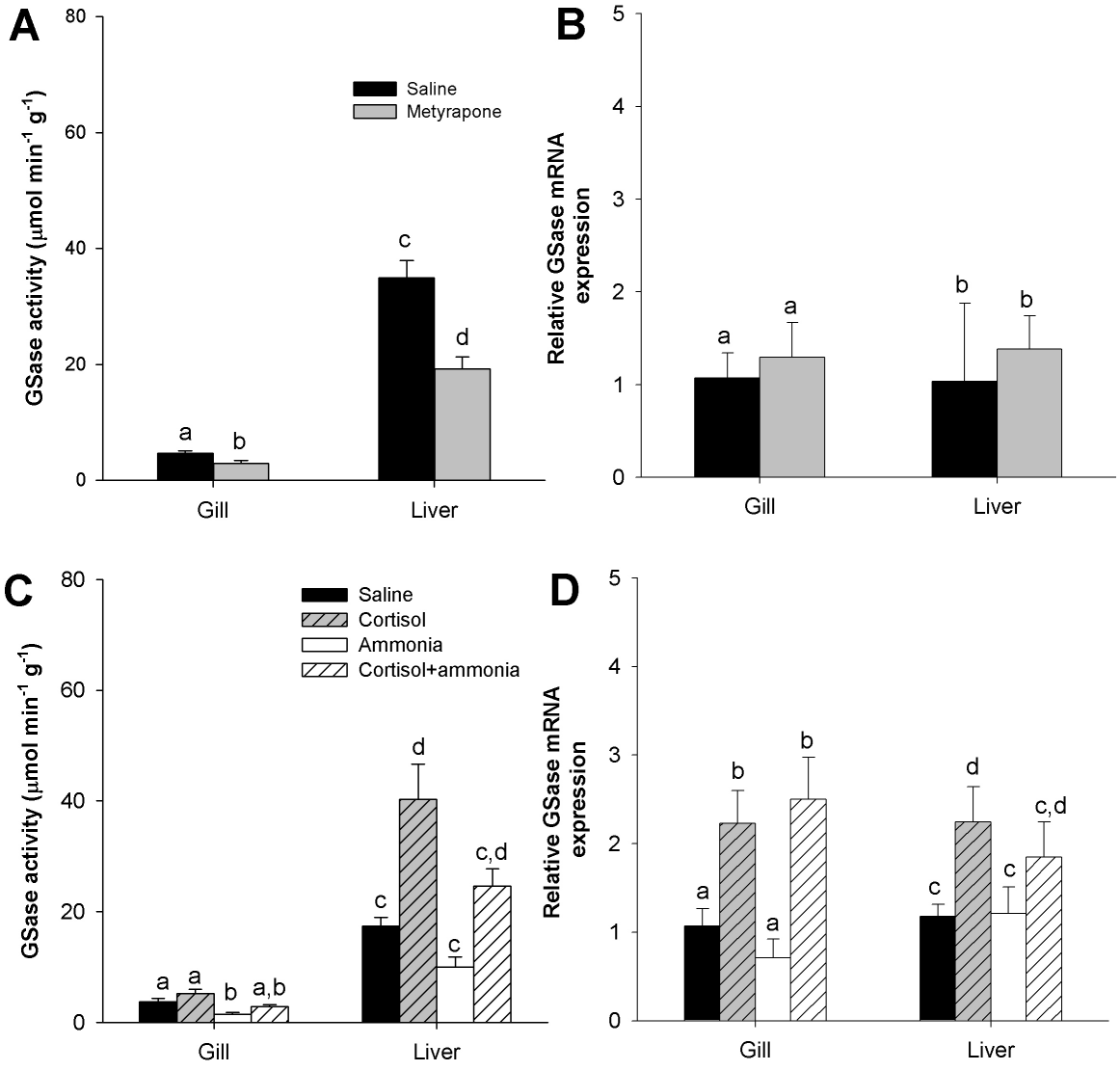
A
GSase activity (μmol min-1 g-1)
Saline
Metyrapone
Gill
Liver
B
Relative GSase mRNA expression
C
Saline
Cortisol
Ammonia
Cortisol+ammonia
D

Figure 6.3

Figure 6.4: Series I-III: Relative branchial mRNA expression of Rhag, Rhbg, Rhcg1, and Rhcg2 from the gill of ureotelic toadfish (Opsanus beta) as measured by real-time RT-PCR. (A) In Series I, toadfish were injected with saline (150 mmol L^{-1} NaCl, 1.5 µl g^{-1} body mass, n=8) or metyrapone (20 mg mL^{-1} in 150 mmol L^{-1} NaCl, 1.5 µl g^{-1} body mass, n=8). (B) In Series II, one group of toadfish was infused with isosmotic saline (150 mmol L^{-1} NaCl, n=8), a second with hydrocortisone hemisuccinate salt (cortisol; 0.09 mg mL^{-1}), the third with ammonia alone ($(NH_4)_2HPO_4$, n=8), and the fourth with ammonia and cortisol ($(NH_4)_2HPO_4$ + 0.09 mg mL^{-1} hydrocortisone hemisuccinate salt in 150 mmol L^{-1} NaCl, n=8). All fish were infused at a rate of 3 mL kg^{-1} h^{-1}. (C) In Series III, toadfish were injected with saline (150 mmol L^{-1} NaCl, 1.5 µl g^{-1} body mass, n=8), methionine sulfoximine (MSO; 50 mg mL^{-1} in 150 mmol L^{-1} NaCl, 1.5 µl g^{-1} body mass, n=8), or MSO and infusion with cortisol (0.09 mg mL^{-1} in 150 mmol L^{-1} NaCl, n=7). The mRNA levels for each gene were normalized against the control gene 18S and expressed relative to the control group for each series, which was set to a value of 1. Values are means $\pm$ s.e.m. Different letters denote significant differences between or among treatments ($p<0.05$) within a gene.

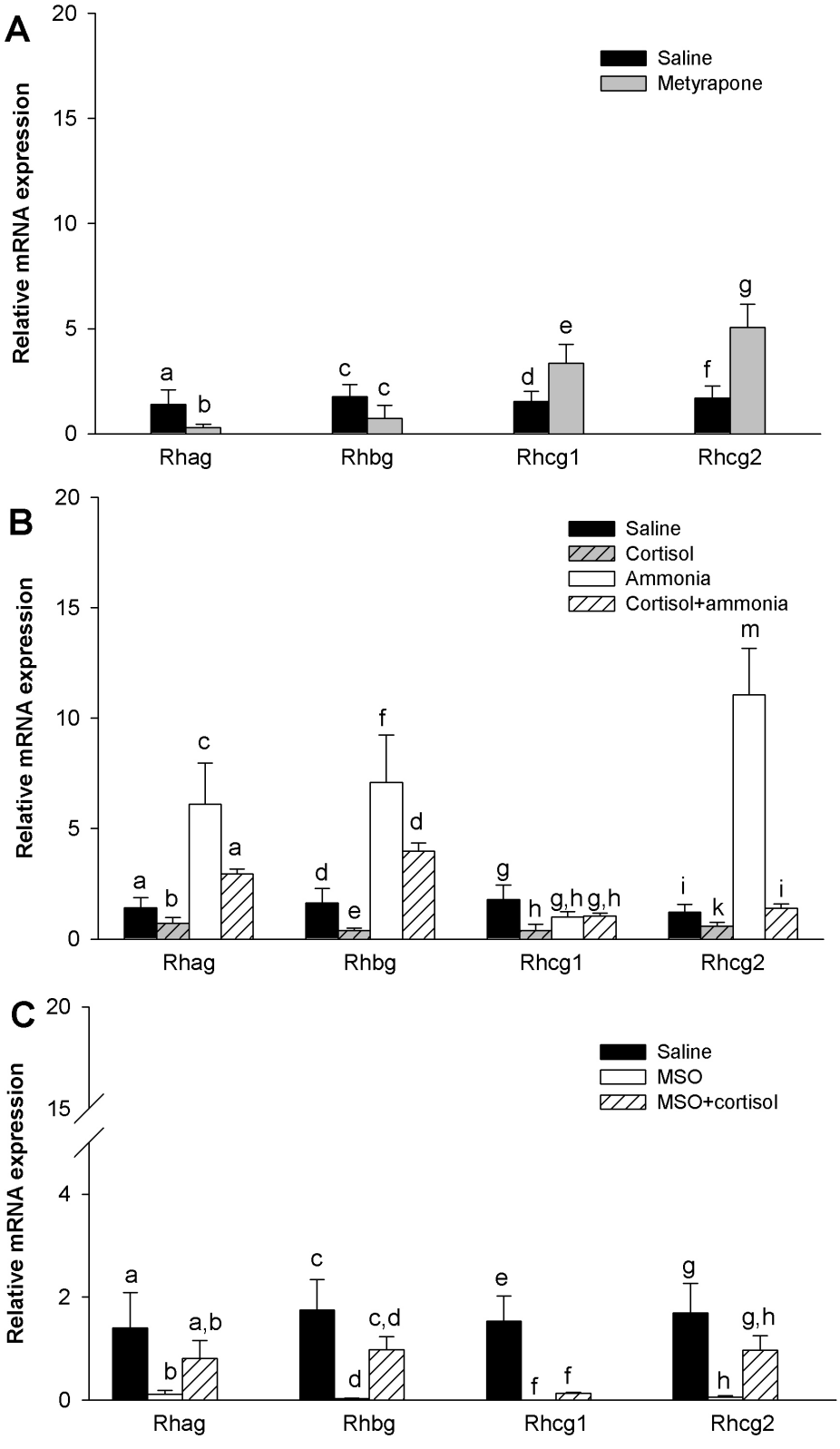
A
Relative mRNA expression
20
15
10
5
0
Saline
Metyrapone
a
b
c
c
d
e
f
g
Rhag
Rhbg
Rhcg1
Rhcg2
B
Relative mRNA expression
Saline
Cortisol
Ammonia
Cortisol+ammonia
a
b
c
a
d
e
f
d
g
h
g,h
g,h
i
k
m
i
C
Relative mRNA expression
Saline
MSO
MSO+cortisol
a
b
a,b
c
d
c,d
e
f
f
g
h
g,h

Figure 6.4

Figure 6.5: Series II. The effects of infusion with isosmotic saline (150 mmol L^{-1} NaCl, n=8), hydrocortisone hemisuccinate salt (cortisol; 0.09 mg mL^{-1} in 150 mmol L^{-1} NaCl, n=8), ammonia alone ($(NH_4)_2HPO_4$, n=8), or ammonia and cortisol ($(NH_4)_2HPO_4$ + 0.09 mg mL^{-1} hydrocortisone hemisuccinate salt in 150 mmol L^{-1} NaCl, n=8) on nitrogen excretion and metabolism in toadfish (Opsanus beta). Fish were infused at a rate of 3 mL kg^{-1} h^{-1}. Measurements include ammonia excretion (relative units; A) and plasma ammonia concentration (mmol L^{-1}; B), pulsatile and non-pulsatile components of normalized urea excretion (relative units; C), plasma urea concentrations (mmol L^{-1}; D), and urea pulse size (relative units, E). Data in A, C, and E are presented as the treatment value normalized to the value for the initial 24 h control period. Values are expressed as means $\pm$ s.e.m. In panels A, C, and E, different letters denotes significant differences among treatment groups, whereas the asterisk (*) indicates differences with a group between the control and treatment periods ($p<0.05$). Symbols (*,ϕ) in panels B and D indicate significant differences within the treatment group from the control period (< 24 h), and differences between treatment groups at a given time, respectively ($p<0.05$). The dotted lines indicate the start of the treatment period.

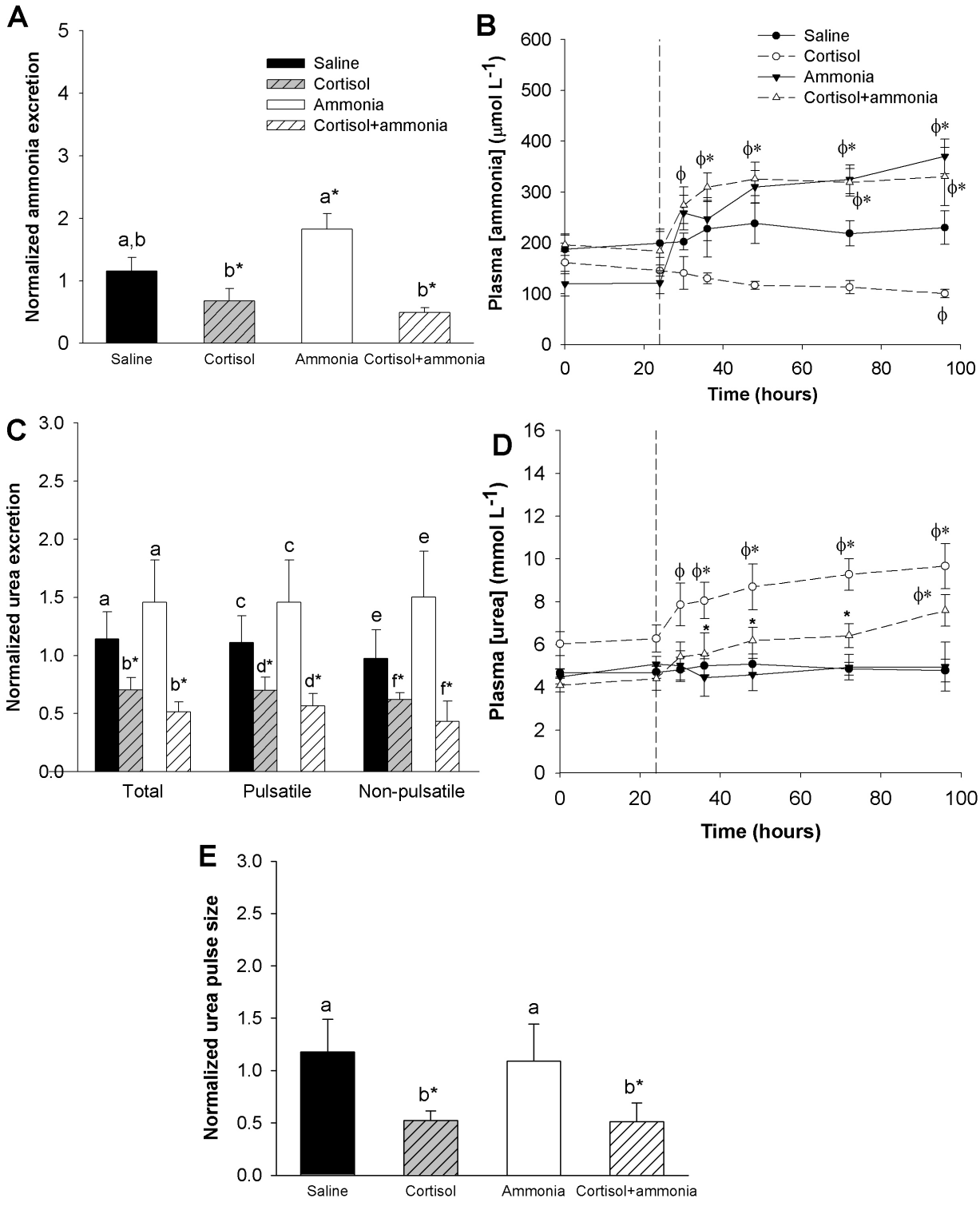
A
Normalized ammonia excretion
Saline
Cortisol
Ammonia
Cortisol+ammonia
a,b
b*
a*
b*
B
Plasma [ammonia] (μmol L-1)
Time (hours)
C
Normalized urea excretion
Total
Pulsatile
Non-pulsatile
D
Plasma [urea] (mmol L-1)
Time (hours)
E
Normalized urea pulse size

Figure 6.5

Figure 6.6: Series III. The effects of intraperitoneal (IP) injections of saline (150 mmol L^{-1} NaCl, 1.5 µl g^{-1} body mass, n=8), methionine sulfoximine (MSO; 50 mg mL^{-1}in 150 mmol L^{-1} NaCl, 1.5 µl g^{-1} body mass, n=8), or MSO and together with cortisol infusion (0.09 mg mL^{-1} hydrocortisone hemisuccinate salt in 150 mmol L^{-1} NaCl, n=7) on nitrogen excretion and metabolism in toadfish (Opsanus beta). Measurements include ammonia excretion (relative units; A), plasma ammonia concentration (mmol L^{-1}; B), pulsatile and non-pulsatile components of normalized urea excretion (relative units; C), plasma urea concentration (mmol L^{-1}; D), and urea pulse size (relative units, E). Data in A, C, and E are presented as the treatment value normalized to the value for the initial 24 h control period. Values are expressed as means $\pm$ s.e.m. In panels A, C, and E, different letters denote significant differences among treatment groups, and the asterisk (*) indicates differences with a group between the control and treatment periods ($p<0.05$). Symbols (*,ϕ) in panels B and D indicate significant differences within the treatment group from the control period (<24 h), and differences among treatment groups at a given time, respectively ($p<0.05$). The dotted lines indicate the start of the treatment period.

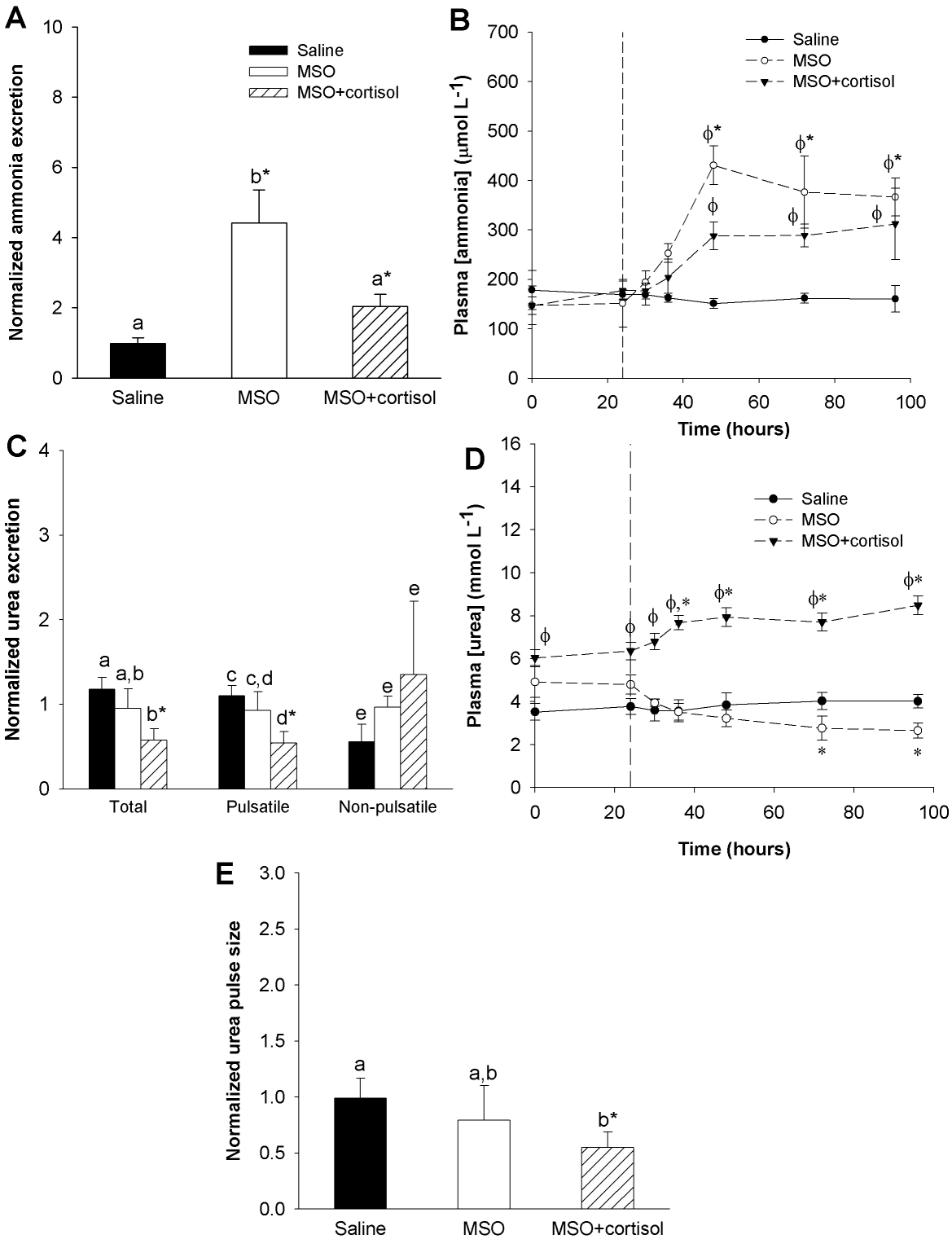
A
Normalized ammonia excretion
Saline
MSO
MSO+cortisol
a
b*
a*
Saline
MSO
MSO+cortisol
B
Plasma [ammonia] (μmol L-1)
Saline
MSO
MSO+cortisol
Time (hours)
C
Normalized urea excretion
a
a,b
b*
c
c,d
d*
e
e
e
Total
Pulsatile
Non-pulsatile
D
Plasma [urea] (mmol L-1)
Saline
MSO
MSO+cortisol
Time (hours)
E
Normalized urea pulse size
a
a,b
b*
Saline
MSO
MSO+cortisol

Figure 6.6

Table 6.1: The percentage of nitrogenous waste excreted in the form of urea and ammonia by toadfish during an initial 24 hour control period following by a treatment period where fish were either infused and/or injected with various compounds. See text in *Materials and Methods* for details about infusion/injection conditions.

Series	Group	Control period		Treatment period	
		% Urea-N	% Ammonia-N	% Urea-N	% Ammonia-N
I	Saline[1] (n=8)	68.3 ± 4.2[a]	31.7 ± 3.2[d]	67.3 ± 4.7[a]	32.7 ± 3.6[d]
	Metyrapone (n=8)	89.5 ± 2.7[b]	10.7 ± 2.1[e]	79.7 ± 2.3[c]	20.3 ± 1.8[f]
II	Saline (n=8)	74.7 ± 4.6[g]	25.2 ± 3.5[i]	72.3 ± 7.8[g]	28.3 ± 4.2[i]
	Cortisol (n=8)	68.6 ± 8.2[g]	31.4 ± 6.3[i]	64.2 ± 13.2[g]	35.7 ± 8.5[i]
	Ammonia (n=8)	81.4 ± 6.6[h]	18.6 ± 5.0[k]	60.9 ± 14.2[g]	39.1 ± 9.2[i]
	Cortisol + ammonia (n=8)	67.9 ± 9.4[g]	28.1 ± 7.4[i]	77.5 ± 5.2[g]	19.7 ± 3.8[i]
III	MSO	77.8 ± 5.1[m]	22.2 ± 3.8[o]	42.8 ± 10.7[n]	57.2 ± 9.9[p]
	MSO+cortisol	76.4 ± 6.8[m]	23.6 ± 5.2[o]	52.8 ± 10.8[n]	47.2 ± 7.0[p]

Values are means ± s.e.m.
[1]Toadfish injected with saline served as a control group for both *Series I* and *Series III*
[a]Significant differences within each series are labeled by different letters (2-way ANOVA, $p<0.05$).

Discussion

Toadfish respond to crowding or confinement with an acute rise in plasma cortisol that is accompanied by a decrease in ammonia excretion (Hopkins et al., 1995; Walsh and Milligan, 1995). Previously, it was postulated that the combined activity of branchial and hepatic GSase is sufficient to trap all the ammonia as glutamine, thereby reducing ammonia excretion by metabolic means (Hopkins et al., 1995; Walsh, 1997; Walsh et al., 2003; Walsh and Milligan, 1995; Wood et al., 1995). However, the recent discovery of Rh glycoprotein ammonia transporters has led to a re-evaluation of ammonia transport in fish (reviewed by Weihrauch et al., 2009; Wright and Wood, 2009). Our recent identification of four Rh glycoproteins in toadfish revealed that these isoforms were differentially regulated in response to crowding and feeding, and indicated that there may be additional mechanisms controlling ammonia excretion in toadfish (Rodela et al., in preparation-b). In the present study, we provide evidence to support the hypothesis that cortisol may play a role in decreasing the rate of ammonia excretion by mediating the downregulation of Rh protein expression. Cortisol-induced effects on ammonia efflux were coordinated with well-documented cortisol-mediated changes in both urea metabolism and excretion (Hopkins et al., 1995; McDonald et al., 2009; McDonald et al., 2004; Rodela et al., 2009; Rodela et al., 2009; Wood et al., 2001). The synthesis of these results reveals that there are numerous factors that contribute to the capacity of the toadfish to tightly control and regulate nitrogen excretion.

Cannulation, for repetitive non-invasive blood sampling, results in a chronic elevation of plasma cortisol in teleost fish (Brown et al., 1986; Lo et al., 2003; Sloman et al., 2005). In the present study, initial plasma cortisol concentrations ranged from 86 –

376 ng mL^{-1} approximately 24 h following surgery. These values are comparable to previous reports where endogenous concentrations in cannulated toadfish have been shown to exceed 400 ng mL^{-1} (McDonald et al., 2004; Sloman et al., 2005). These elevated concentrations necessitated the use of a pharmacological rather than physiological dose of exogenous cortisol to measure the effects of enhanced corticosteroid activity on nitrogen excretion in toadfish (*Series II* and *Series III*). While elevated cortisol has been linked to increased proteolytic activity and ammonia excretion, values for both plasma ammonia concentration (103.2 – 211.2 μmol L^{-1}) and ammonia excretion (5.7 – 48.7 μmol kg^{-1} h^{-1}) were within the range reported for non-cannulated ureotelic toadfish (plasma ammonia, 100-200 μmol L^{-1}; ammonia excretion 5 – 50 μmol kg^{-1} h^{-1}; Walsh and Milligan, 1995; Wood et al., 1995). These results suggest that the observed changes in ammonia excretion can be attributed to changes in excretion capacities and not metabolic rearrangements due to chronically elevated cortisol.

Cortisol infusion decreased Rh mRNA expression in ureotelic toadfish (Figure 6.4B). In the model of ammonia movement across the branchial pavement cells of the marine teleost *Takifugu rubripes* proposed by Nakada and colleagues (Nakada et al., 2007), the expression of Rhag in the pillar cells enables the movement of ammonia from the vasculature to the pavement cells, and transcellular transport across the pavement epithelium is accomplished through Rhbg and Rhcg2 expressed on the basolateral and apical membranes, respectively (Nakada et al., 2007). If a similar arrangement is present in toadfish, then a cortisol-induced reduction in Rh transport pathways across the pavement cell would greatly reduce the rate of ammonia excretion.

This reduction likely would be further enhanced by a decrease in Rhcg1, the isoform putatively located in the mitochondrion-rich cells (MRCs) based on the model described by Nakada and colleagues (Nakada et al., 2007). In the present study, cortisol-induced decreases in Rh mRNA expression were correlated with lower ammonia excretion rates (Figure 6.5A), providing support for this model. These findings are consistent with our earlier study that demonstrated that confinement stress (elevated plasma cortisol) resulted in lower levels of all Rh mRNA transcripts as well as ammonia excretion compared to unconfined fish (Rodela et al., in preparation-b). By contrast, lowering circulating cortisol with metyrapone had the opposite effect, resulting in higher mRNA transcript levels of Rhcg1 and Rhcg2 and corresponding increases in ammonia efflux (*Series I*). The inverse relationship between circulating cortisol concentration and Rh mRNA expression/ammonia excretion indicates that cortisol may be an important proximate cue for mediating changes in branchial ammonia permeability in toadfish.

Aside from the present investigation, only three other studies have addressed the relationship between cortisol and Rh expression in fish. These studies, carried out on rainbow trout, reported rather different patterns (Nawata and Wood, 2008; Nawata and Wood, 2009; Tsui et al., 2009), differences that likely reflect the nitrogen handling capacities of each species. Rainbow trout, like most teleosts, must quickly excrete ammonia to avoid accumulation and maintain normal physiological function (Ip et al., 2001; Wood, 1993). In comparison, toadfish can tolerate relatively high internal concentrations of ammonia and also have the ability to detoxify this nitrogenous waste through multiple mechanisms (Walsh et al., 2003; Wang and Walsh, 2000). In addition

to direct excretion across branchial surfaces, toadfish can sequester ammonia as glutamine and/or use it for the synthesis of urea v*ia* a fully functional O-UC (reviewed by Wood et al., 2003).

GSase, the enzyme that feeds ammonia into the piscine O-UC, is considered to be a rate-limiting enzyme for ureagenesis in the toadfish (Mommsen and Walsh, 1991). Earlier studies have consistently demonstrated that acute elevation of endogenous plasma cortisol increases both mRNA expression and enzyme activity of GSase in the liver and muscle (Esbaugh and Walsh, 2009; Hopkins et al., 1995; Kong et al., 2000; McDonald et al., 2009; Walsh et al., 2003; Walsh and Milligan, 1995; Walsh et al., 1994; Wood et al., 1995). The enhanced activity of hepatic GSase increases substrate mobilization for urea synthesis and leads to a slow accumulation of urea in the plasma (Hopkins et al., 1995; Walsh and Milligan, 1995; Walsh et al., 1994; Wood et al., 2003). It is likely that the activity of hepatic GSase contributed (in part) to the reduction in branchial ammonia efflux observed in our study (Figure 6.5A). Administration of cortisol resulted in a 2-fold increase in both GSase mRNA abundance and enzyme activity in the liver (Figure 6.3D and 6.3C, respectively) together with a slow decline in circulating ammonia concentration (Figure 6.5B) and a rise in plasma urea content (Figure 6.5D). The observed accumulation of plasma urea likely represented the net influence of enhanced urea production and decreased rates of urea excretion, both pulsatile and non-pulsatile (Figure 6.5C and 6.5E). Previous studies have revealed that the amount of urea released during the discrete urea pulse events is dependent on the concentration of plasma glucocorticoids; in particular, elevated cortisol inhibits tUT function (McDonald et al., 2009; McDonald et al., 2004; Rodela et al., 2009).

Branchial tissue expresses a gill-specific GSase that is the product of a separate gene and lacks a close biochemical association with ureagenic enzymes (McDonald et al., 2009; Walsh et al., 2003; Wood et al., 1995). GSase within the gill is positioned in the MRCs to act as a high-affinity trap for ammonia and minimize the amount of ammonia that is lost across the branchial epithelium (Walsh, 1997; Walsh et al., 2003). Examination of gill tissue revealed a significant increase in GSase mRNA abundance (Figure 6.3D) with no detectable difference in enzyme activity following cortisol administration (Figure 6.3C). A similar trend in mRNA expression was observed following a shorter period (~ 48 h) of cortisol infusion, whereas enhanced enzyme activity was associated with prolonged periods of stress (e.g. a week; McDonald et al., 2009). Taken together, the cortisol-induced upregulation of GSase in the liver and constitutive activity in the gill likely contributed to patterns of ammonia excretion in cortisol-treated toadfish in the present study.

It is clear that GSase plays a prominent role in the detoxification of endogenous ammonia during periods of fasting and quiescence (Hopkins et al., 1995; Walsh, 1997; Walsh et al., 2003; Walsh and Milligan, 1995; Walsh et al., 1994). However, toadfish experience elevated plasma ammonia shortly following feeding (~ 300 – 500 μmol L^{-1}; Rodela et al., in preparation-b) or during exposure to high ambient ammonia (~ 1000 μmol L^{-1}; Veauvy et al., 2002; Wang and Walsh, 2000). In these circumstances, internal ammonia concentrations likely exceed the ammonia trapping capacity of GSase and as a result toadfish may rely more heavily on transport pathways to eliminate ammonia. Our results demonstrate that maintaining plasma ammonia at physiologically relevant concentrations (~300 μmol L^{-1}) caused an approximately two-fold increase in ammonia

excretion (Table 6.1, Figure 6.5A) and occurred with significant increases in the mRNA expression of Rhag, Rhbg, and Rhcg2 (Figure 6.4B), which may facilitate the transcellular transport of excess ammonia across the pavement cells. The mRNA expression of Rhcg1, the isoform thought to be associated with MRCs, was not affected by ammonia infusion (Figure 6.4B), but the reduction in branchial GSase activity (Figure 6.3) might serve to promote ammonia efflux through MRCs. Collectively, the data from ammonia-infused toadfish indicate that they upregulate and maintain high branchial Rh expression, and ammonia itself may be a potent regulator of Rh transcription. These molecular changes were accompanied by increased rates of ammonia excretion. Similar trends have been reported for rainbow trout, which increase both Rhbg and Rhcg2 mRNA to augment excretion following exogenous loading of ammonia *via* NH_4HCO_3 infusion (Nawata and Wood, 2009). Therefore, the data indicate that both facultatively ureotelic and ammoniotelic species mount similar responses to high internal concentrations of ammonia.

Administration of both ammonia and cortisol decreased ammonia excretion (Figure 6.5A). Analysis of gene expression for this group revealed lower levels of mRNA expression for Rhag, Rhbg, and Rhcg2 compared to ammonia infused fish (Figure 6.4B), a trend similar to the effect of cortisol infusion alone in lowering Rh expression relative to saline-infused fish. We speculate that increases in Rh mRNA expression associated with ammonia infusion were suppressed by the elevated concentrations of circulating cortisol. Furthermore, it is unlikely that changes in the hepatic conversion of ammonia to urea contributed to the reduction in ammonia excretion because no significant changes in GSase activity or mRNA were observed following infusion of ammonia alone

(Figure 6.3C and 6.3D, respectively). Therefore, it appears that the changes in ammonia efflux are the product of strong yet opposing influences of cortisol and ammonia on Rh glycoprotein expression in toadfish.

In teleosts, the physiological effects of cortisol are mediated through corticosteroid receptors that include the mineralocorticoid (MR) and glucocorticoid receptor (GR; reviewed by Bury and Sturm, 2007). In an earlier study, we isolated a GR from toadfish that shared significant sequence similarity with piscine GRs that are thought to play a prominent role during stress (Rodela et al., in preparation-a). We measured changes in branchial GR expression to determine whether cortisol-induced changes in our present experiments were the result of altered steroid receptor expression and/or circulating cortisol concentrations. Most treatments did not significantly alter GR mRNA expression in either the gill or liver (Figure 5.2A, C, and E), but higher GR mRNA content was measured in the gill of metyrapone-treated toadfish, lower branchial GR mRNA content occurred in MSO-treated individuals, and elevated hepatic GR mRNA was detected in fish administered both MSO and cortisol (Figure 5.2A and 5.2E). Although we are currently unable to measure GR protein expression in toadfish, the observed trends in GR mRNA expression do not correspond to observations from another study. Sathiyaa and Vijayan (2003) reported that increased circulating cortisol in rainbow trout leads to decreased GR protein but increased GR mRNA expression, which they attributed to autoregulation of mRNA abundance by protein content through circulating cortisol. Given the transient nature of circulating cortisol, it is possible that changes in GR mRNA following the various treatments were not accurately captured by single measurement at the end of the experiment.

Therefore, the data collected on GR mRNA expression in the present study should be interpreted with caution.

In *Series III* we manipulated GSase activity in an attempt to determine the relative contribution of ammonia trapping to ammonia excretion. L-Methionine sulfoximine is a toxic and potent irreversible inhibitor of GSase in mammalian systems (Liaw and Eisenberg, 1994; Pace and McDermott, 1952; Ronzio and Meister, 1968; Ronzio et al., 1969; Rowe and Meister, 1970) and also has been used effectively in several fish species (Ip et al., 2005; Sanderson et al., 2010; Veauvy et al., 2005; Wee et al., 2007). Our results confirmed that MSO effectively inhibited both branchial and hepatic GSase, similar to earlier *in vivo* and *in vitro* studies on toadfish (Veauvy et al., 2005; Walsh, 1996). The inhibition of GSase activity raised plasma ammonia concentrations to ~400 μmol L^{-1} (Figure 6.3B) and enhanced ammonia excretion (Figure 6.3A). Surprisingly, measurement of mRNA expression revealed low levels of Rh glycoprotein transcripts in the gill (Figure 6.4C). This finding contrasted with the significant upregulation of Rh mRNA transcript levels correlated with augmented ammonia excretion following infusion of ammonia (Figure 6.4B). Although the MSO concentration (75 mg kg^{-1}) was not as high as those used in other teleost studies (100 mg kg^{-1}, Ip et al., 2005; 150 mg kg^{-1}, Veauvy et al., 2005; Wee et al., 2007), chronic administration of MSO clearly adversely affected pathways responsible for the transcription of Rh glycoproteins and may or may not also have affected Rh protein expression. Sellinger and colleagues reported that MSO increased the phosphatidylethanolamine content in the neural membranes of mice, resulting in increased membrane fluidity (Sellinger et al., 1984). If MSO similarly influenced the

fluidity of the toadfish gill, greater NH_3 leakage across the branchial epithelium could explain the augmentation of ammonia excretion. Interestingly, the effects of MSO on Rh expression were ameliorated when toadfish were treated with a combination of MSO and cortisol (Figure 6.4C). Collectively, the data indicate that caution needs to be exercised when using MSO for longer-term experiments.

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

provide valuable insight and enhance our understanding of the ability of the toadfish to tightly control and regulate nitrogen excretion.

Acknowledgements

NSERC Discovery Grants awarded to P.J.W. and K.M.G., an NSF grant to M.D.M. (IOS-0455904), and a Canadian Society of Zoologists Student Travel Research grant to T.M.R, funded this study. P.J.W. is supported by the Canada Research Chair program. Special thanks to Bill Hannah for his help with sampling, and care and feeding of the RSMAS toadfish colony.

CHAPTER 7

General Conclusion

General Discussion

A review of the fish literature indicates that the pattern of urea elimination in the gulf toadfish (*Opsanus beta*) is unprecedented. The facultative capacity of the toadfish to alter its nitrogen excretion involves the adjustment of numerous metabolic and excretion pathways (reviewed by Wood et al., 2003). The role of corticosteroids in regulating these pathways was explored extensively in this thesis, and the data reveal that cortisol is an important proximate cue to coordinate numerous changes in nitrogen excretion in toadfish. This thesis expands on previous work and provides new insight into the underlying molecular mechanisms that control the transport of nitrogenous wastes in teleosts. In this chapter, the experimental results are synthesized into an updated model of branchial nitrogen excretion mechanisms and their regulation in the gulf toadfish.

A new model for branchial nitrogen excretion in gulf toadfish

Although high activities of ornithine-urea cycle (O-UC) enzymes and substantial rates of urea synthesis occur in toadfish hepatocytes (Mommsen and Walsh, 1989), initial studies did not observe significant rates of urea efflux. It was believed that toadfish, like most teleosts, were ammoniotelic. In 1989, Evans et al. (1989) outlined a simple model of ammonia excretion across the gill epithelium in gulf toadfish. Physiological measurements indicated that approximately 21% of total ammonia efflux occurred *via* paracellular diffusion of NH_4^+, 22% as NH_4^+ through K^+ displacement on a basolateral Na^+/K^+-ATPase, and ~57% through paracalleular and passive transcellular NH_3 diffusion (Evans et al., 1989). With the recent identification of Rh glycoproteins,

this model of ammonia excretion in toadfish needs to be revised. Based on the observations of Nakada et al. (2007), who reported a detailed description of the cellular locations of Rh glycoproteins in *Takifugu rubripes* (a marine teleost), a picture of ammonia excretion in toadfish can be postulated. Figure 7.1 outlines the various pathways available for ammonia excretion across the toadfish gill. NH_3 is depicted as the product channeled through all Rh proteins, which is based on recent results favouring NH_3 rather than NH_4^+ transport . An *in vitro* study on rainbow trout reported that Rh glycoproteins have a higher affinity for NH_4^+ than NH_3 and are sensitive to pH gradients (Nawata et al., 2010). This finding led Nawata et al. (2010) to propose that NH_4^+ may initially bind to the Rh protein but that it is subsequently deprotonated and transported as NH_3. This hypothesis may explain how NH_4^+ can leave the mitochondrion rich cells (MRCs) despite the lack of evidence for an apical outlet for NH_4^+ (see Evans et al., 1989).

Initially reported as anecdotal observations (Barber and Walsh, 1993; Mommsen and Walsh, 1989; Walsh et al., 1990; Walsh et al., 1994), the unique pulsatile efflux of urea in gulf toadfish is becoming one of the best-characterized piscine models of urea excretion and transport. Until recently, a great deal of uncertainty surrounded the cellular location of tUT. Using *in situ* hybridization, McDonald et al. (2009) demonstrated that tUT mRNA transcripts were exclusively localized to the MRCs of the toadfish gill. Similar distributions were reported for zebrafish (*Danio rerio;* Braun et al., 2009b) and Japanese eel (*Anguilla japonica*; Mistry et al., 2001). The immunohistochemical staining pattern for the Japanese eel was interpreted as basolateral expression of eel UT (eUT) in MRCs (Mistry et al., 2001). Collectively, these

results provide further evidence in support of a urea facilitated transporter in toadfish (see Chapter 3). Presently, it is unclear whether the basolateral UT-like protein is the product of tUT. Regardless, it is likely that this putative UT protein, when present in the basolateral membrane, accelerates the movement of urea between the plasma and gill epithelial cells (Figure 7.1). This mechanism likely creates a substantial influx of urea into the MRCs. How urea exits the apical side of gill epithelial cells remains an open question. Notably, there is no physiological evidence for an apical UT in the MRCs of any fish species, but there are a number of reports of apically located aquaglyceroporins (AQP). AQPs form a subgroup of intrinsic membrane channels that are permeable to urea and other small neutral solutes, including water (Bagnasco, 2005; Cutler et al., 2007; Tsukaguchi et al., 1998; Verkman and Mitra, 2000). In several teleost species, immunohistochemistry revealed intense AQP3 staining within the apical regions of MRCs (Hirata et al., 2003; Lignot et al., 2002; Tse et al., 2006). A similar urea transport mechanism could conceivably be present in toadfish, but this model needs to be validated with physiological and molecular experiments.

An abundance of dense-cored vesicles in the branchial pavement cells (PVC) were reported in early studies on ureotelic toadfish (Laurent et al., 2001). During urea pulse events, these vesicles translocated to the apical region of the cell where they came into contact with the plasma membrane (Laurent et al., 2001). These ultrastructural rearrangements are reminiscent of the vesicular trafficking of UT-A1 in the mammalian kidney (Mistry et al., 2007). When ureotelic toadfish were treated with colchicine, an inhibitor of microtubule assembly and hence of vesicular trafficking, pulsing was abolished and urea accumulated in the plasma (Gilmour et al., 1998).

These data suggest that ultrastructural rearrangements are a significant component of pulsatile urea excretion; such ultrastructural rearrangements may involve sequestering cytosolic urea that is eventually excreted into the ambient water, or may involve recruiting urea transporter proteins into the apical membrane. Although molecular evidence indicates that it is unlikely that tUT is present in pavement cells (McDonald et al., 2009), the recent identification of a novel urea transporter, UT-C (Mistry et al., 2005), suggests that additional transporter isoforms may be involved. Originally cloned from pufferfish (*Takifugu rubripes*), UT-C has been isolated and characterized in the Japanese eel and found to have ~40% sequence similarity with other piscine UTs (Mistry et al., 2005). A similar UT-C recently was cloned from toadfish (Marcus and Walsh, unpublished data), and preliminary experiments revealed a detectable mRNA signal from the gill. Therefore, it is possible that UT-C may be associated with the cytosolic vesicles in the PVCs of ureotelic toadfish.

Glucocorticoids and branchial ammonia excretion

Earlier studies on toadfish consistently demonstrated that the transition to ureotely involved a significant reduction in ammonia excretion and only a moderate rise in urea efflux (Walsh and Milligan, 1995; Walsh et al., 1994). Walsh calculated that the glutamine synthetase (GSase) activity present among different tissues is sufficient to account for the extremely low rates of ammonia excretion in ureotelic toadfish (Walsh, 1997). However, reductions in ammonia efflux occurred 4 h following exposure to acute stress (Walsh et al., 1994), much earlier than enzymatic changes in hepatic GSase, which were observed to occur at 24 h (Hopkins et al., 1995; Walsh et al., 1994).

Recently, Esbaugh and Walsh (2009) demonstrated that changes in hepatic GSase activity are mediated by glucocorticoid receptors (GR) that bind to specific DNA motifs within the GSase promoter region to enhance gene transcription. This cortisol-induced upregulation in mRNA expression takes at least 24 h to manifest significant increases in enzyme activity (Hopkins et al., 1995; Walsh et al., 1994). The mechanisms of cortisol sensitivity for gill GSase are less clear. An *in silico* analysis of the promoter region of gill GSase did not reveal response elements that could confer cortisol-induced changes in gene expression (Esbaugh and Walsh, 2009). Yet, cortisol-induced increases in branchial GSase activity were observed in an *in vivo* study, although the changes were not apparent until 96 h after a chronic confinement stress (McDonald et al., 2009). Taken as a whole, these results suggest that cortisol-mediated changes in GSase activity are too slow to account for the rapid reduction in ammonia excretion that occurs in the transition to ureotely. Therefore, it is likely that the rapid reduction in ammonia excretion can be attributed to another pathway. The prominent expression of Rh glycoproteins in the toadfish gill (Figure 5.3) presented the possibility of a regulated ammonia facilitated transport mechanism within the gill of the toadfish. Rh abundance in the gill is reduced in ureotelic toadfish (Figure 5.4), presumably to minimize ammonia efflux (see text in Chapter 5 Results). These changes may be mediated, in part, by cortisol-induced decreases in Rh glycoprotein expression (Figure 6.4). Based on the current evidence, I speculate that acute modifications in ammonia efflux during the transition to ureotely might be the result of rapid (< 4 h) decreases in Rh protein expression; these changes could be regulated by cortisol but through non-genomic pathways, the least understood route of cortisol action that is thought to be rapidly

mediated by membrane-bound GRs. A long-term reduction of ammonia excretion rates may be achieved through cortisol acting *via* slower, genomic pathways to elevate GSase activity and lower Rh mRNA expression. Although the data collected in chapters 5 and 6 support this hypothesis for the long-term maintenance of low ammonia excretion during ureotely, the acute regulation of ammonia excretion needs to be investigated with studies focusing on the physiological and molecular changes that occur within the short period (a few hours) following an acute stress.

Glucocorticoids and branchial urea excretion

Through a series of carefully conducted experiments, Wood et al. (1997; 1998) demonstrated that the pulsatile clearance of urea in toadfish may be attributed to the periodic activation or insertion into the gill membrane of a urea transporter (tUT) . The recruitment or activation of these transporters for a pulse event is triggered by the local release of 5-HT within the gills, targeting 5-HT_2 receptors to mediate post-translational modifications (McDonald and Walsh, 2004; Wood et al., 2003). However, the number of transporters available for recruitment/activation is controlled by cortisol. McDonald et al. (2009) demonstrated that chronic elevation of circulating cortisol causes an increase in tUT mRNA. An *in silico* analysis of an upstream promoter region for tUT revealed numerous glucocorticoid response elements (GREs) that could putatively interact with cortisol via a glucocorticoid receptor (GR) to enhance transcription (Figure 4.4). Injection of a tUT promoter construct into the liver of toadfish conferred inducible gene expression in response to elevated cortisol *via* infusion in an *in vivo* reporter assay (Figure 4.5). These data provide evidence for a

direct interaction between cortisol and tUT mediated through cytosolic GRs that act as ligand-dependent transcription factors. While the upregulation of tUT mRNA may allow more urea transporters to be translated, elevated cortisol levels also appear to inhibit tUT function. Exogenous administration of cortisol suppressed tUT function, as indicated by a reduction in urea excretion (McDonald et al., 2004). This effect was attributed to a decrease in pulse size rather than a change in pulse frequency. Conversely, when circulating cortisol was lowered, either by inhibiting cortisol synthesis or by blocking the glucocorticoid receptor (GR), a dramatic elevation in urea pulse size was observed (Figure 2.1A and Figure 2.2A, respectively). Similarly, urea transport capacity across the basolateral membrane (assessed *in vitro* using basolateral membrane vesicles) was reduced following *in vivo* infusion of cortisol (Figure 3.5), which also represents a decrease in UT protein in the toadfish gill. These trends emphasize the permissive role of cortisol in pulsatile urea excretion. At low circulating concentrations, the inhibitory effects of cortisol on tUT protein are removed, thus enabling 5-HT to recruit/activate the transporter to/in the gill membrane. These effects are likely mediated through post-transcriptional modifications of tUT (see Discussion sections of Chapters 2- 4), although the precise mechanisms have yet to be determined. Future study on branchial urea transport in toadfish would benefit from the development of tUT-specific antibodies to examine changes in protein expression. Altogether, the molecular and physiological evidence indicates that the regulation of tUT by cortisol is complex, involving regulation of both tUT messenger RNA transcript and protein, the latter through non-genomic mechanisms that have yet to be determined.

Evolutionary perspectives

The first UT to be identified and characterized came from the inner renal medulla of rabbit (You et al., 1993). The protein encoded by the 3 kb cDNA came to be known as UT-A2, and is derived from the *Slc14a2* gene. The molecular organization and regulation of the UT-A gene is complex (see Chapter 1), but the mammalian kidney has become one of the best-characterized models of urea transport (see Bagnasco, 2005; Sands, 2007; Smith and Fenton, 2006). Glucocorticoids inhibit urea transport in the mammalian kidney by reducing the abundance of UT-A1 type protein (Klein et al., 1997; Naruse et al., 1997; Sands, 1999); glucocorticoids decrease UT-A1 and UT-A3 transcription through inhibition of the UT-Aα promoter. Although the precise mechanisms have yet to be determined, a recent study demonstrated that this repression is not mediated by the glucocorticoid response elements (GRE) within the UT-Aα promoter (Peng et al., 2002). A single GRE-half site is also present within the UT-Aβ promoter that controls the transcription of UT-A2 (Nakayama et al., 2001); however, the expression of mammalian UT-A2 is insensitive to cortisol (Peng et al., 2002). Similar DNA motifs were identified in toadfish (Chapter 3). However, by contrast with the apparently non-functional glucocorticoid response elements in mammals, cortisol exposure enhanced tUT promoter activity (Figure 3.5) and increased mRNA abundance (McDonald et al., 2009), strongly suggesting that the GRE half-sites within the tUT promoter confer glucocorticoid-inducible UT transcription in toadfish. Although the genomic regulation of urea transport appears to be different between mammals and teleosts, physiological evidence indicates that toadfish tUT and mammalian UT may be regulated in a similar fashion at the non-genomic level

(Chapters 2 and 3). Thus, questions arise when considering the evolutionary origins of the genomic and non-genomic regulatory mechanisms of urea transporter between fish and mammals. Are the functional GREs in the toadfish UT a derived trait or was there a loss of function for the UT GREs within the mammalian lineage? Are similar regulatory systems present in other teleost species that rely heavily on urea excretion pathways for nitrogen elimination? The most parsimonious hypothesis suggests that the non-genomic pathways involved in UT regulation may have arisen from a common ancestor, whereas the presence of GRE motifs in both mammals and toadfish suggests that the mammalian GRE may have undergone a loss of function. At present, information pertaining to the regulation of non-mammalian UTs is limited to tUT regulation in toadfish. Examination of and comparison with other fish species would clarify the evolutionary relationship of this biological system in vertebrates.

Conclusion

The literature describes the toadfish as a unique teleost species that can modulate its pattern of nitrogen excretion. Previous studies on corticosteroid regulation in toadfish focused on the physiological changes associated with cortisol. This thesis provides new data regarding the underlying molecular mechanisms controlling the unusual nitrogen excretion patterns in toadfish. The interaction of circulating cortisol with the GR is instrumental in regulating the quantities of urea transport protein that are available for pulsatile urea excretion. These changes are brought about through the modulation of both tUT mRNA and protein. The corticosteroid pathway also appears to be important in mediating changes in ammonia

excretion through the combined modulation of ammonia transport mechanisms involving Rh glycoproteins and the ammonia-trapping capacity of GSase. These novel findings illustrated the importance of cortisol for mediating changes in *both* urea and ammonia excretion mechanisms. Taken together, the results of this thesis enhance our understanding of the unique toadfish model for branchial nitrogen transport and provide exciting evolutionary perspectives regarding the integration of biological systems involved in nitrogen excretion in fish.

Figure 7.1: Updated model of nitrogen excretion across the branchial epithelium of the gulf toadfish (*Opsanus beta*). Ammonia excretion occurs through a combination of passive NH_3 and NH_4^+ diffusion through paracellular pathways, transcellular transport through Rhesus glycoproteins (Rhbg, Rhcg1, and Rhcg2), and through K^+ displacement on a basolateral Na^+/K^+-ATPase. A recent study on pufferfish (*Takifugu rubripes*; Nakada et al., 2007a) was used to speculate upon the location Rh glycoproteins in pavement (PV) and mitochondrion rich (MR) cells. The transport of urea across the MR cells is likely facilitated by basolateral UT and possibly an apical mechanism involving an aquaporin (AQP) or another carrier-mediated urea transport mechanism (i.e. tUT). Evidence from morphological studies (Laurent et al., 2001) demonstrated the presence of dense cored vesicles in PV cells that may be involved in mediating urea efflux from the gill. (Modified from Weihrauch et al., 2009).

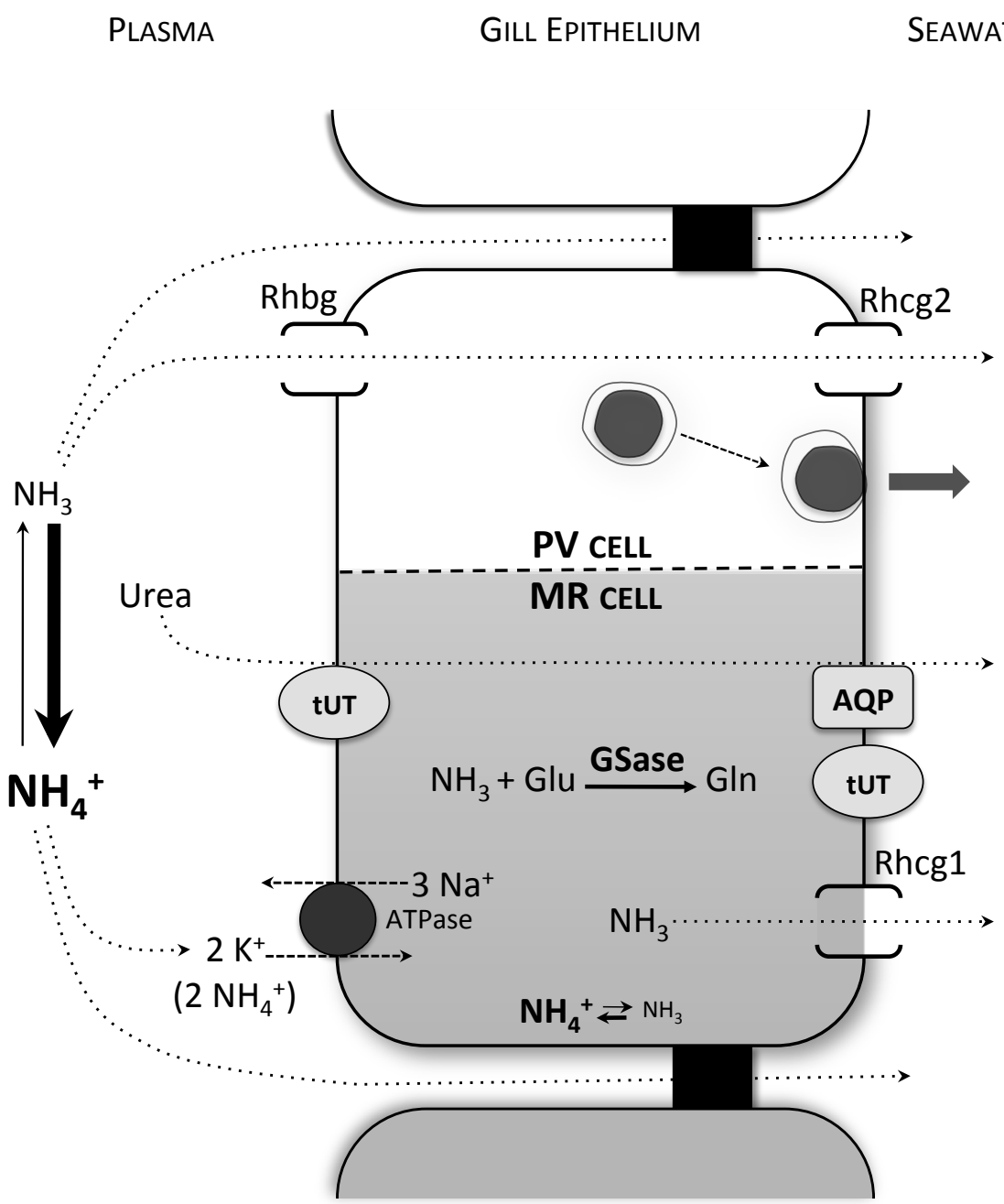
PLASMA
GILL EPITHELIUM
SEAWATER
Rhbg
Rhcg2
NH3
PV CELL
MR CELL
Urea
tUT
AQP
NH4+
NH3 + Glu
GSase
Gln
tUT
Rhcg1
3 Na+
ATPase
NH3
2 K+
(2 NH4+)
NH4+ ⇄ NH3

Figure 7.1

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