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

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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 permissive declines in circulating cortisol concentrations. The current study examined toadfish urea transporter (tUT) and glucocorticoid receptor (GR) transcript levels in toadfish gill following chronic (days) and acute (hours) changes in corticosteroid activity. Experimentally lowering circulating cortisol did not significantly alter tUT mRNA abundance but increased GR mRNA. On an acute timescale, a 6.2-fold upregulation of tUT mRNA occurred 12 to 18 h following a urea pulse event with no change in GR mRNA. *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. *In vivo* reporter assays of the tUT promoter fragment demonstrated relative luciferase activity was enhanced 3.4- and 9.8-fold following exposure to moderate (*via* a 48 h crowding stress) and high (*via* infusion for 48 h) cortisol. We conclude that a GRE-mediated upregulation of mRNA 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.



## 1. Introduction

The Gulf toadfish (*Opsanus beta*) has become one of the best-characterized piscine models with respect to the excretion of urea, and 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., 2004, 2007, 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 (GS) mRNA and activity (Walsh et al., 1994; Hopkins et al., 1995; Walsh and Milligan, 1995; Kong et al., 2000; Esbaugh and Walsh, 2009). GS shuttles ammonia as glutamine into the piscine ornithine–urea cycle (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 h (Wood et al., 1995, 1997; Gilmour et al., 1998). 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 (Wood et al., 1997; Walsh et al., 2000; McDonald et al., 2009). In addition to the strong correlation between urea production capacity via GS 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; Wood et al., 1997; McDonald et al., 2004; Rodela et al., 2009). When circulating cortisol is maintained at a high concentration (>500 ng $mL^{-1}$) by chronic infusion, there appears to be an inhibition of urea transport, 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 h 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 a minimum coinciding with the peak of the pulse event, and rise rapidly thereafter (Wood et al., 1997, 2001). On occasion, a drop in plasma cortisol occurs without an associated pulse, indicating that cortisol is a permissive rather than

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causative agent of urea pulses (Wood et al., 2001, 2003). It is postulated that the acute pre-pulse cortisol decline enables the monoamine neurotransmitter, serotonin (5-HT; 5-hydroxytryptamine), to initiate a pulse event by activating tUT through 5-$HT_2$ receptor-mediated phosphorylation (Walsh et al., 2000; McDonald and Walsh, 2004). 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 regulating the activity of tUT at the gills through rapid 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; Nelson et al., 1999; Bury and Sturm, 2007). 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 (Schulte et al., 2000; Esbaugh and Walsh, 2009). 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., 2009). However, the molecular mechanisms of tUT regulation remain unclear.

The main goal of this study was to examine tUT expression following chronic (days) and acute (hours) changes in circulating cortisol. 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. The first objective focused on characterizing tUT transcript levels in gill samples collected from an earlier *in vivo* pharmacological examination (Rodela et al., 2009) that experimentally *lowered* corticosteroid activity in crowded toadfish. For the second objective, the 5' flanking region of the tUT gene was examined for the presence of hormone response elements that may be important in the transcriptional regulation of tUT. Specifically, we hypothesized that cortisol-induced changes in tUT mRNA expression is attributed to the activity of GRE motif(s) within the promoter region of the tUT gene. This hypothesis was tested using an *in vivo* luciferase reporter assay first described in fish by Schulte and colleagues (Schulte et al., 2000). The final objective sought to characterize changes in tUT mRNA transcript levels over the pulse cycle, as it is uncertain if acute changes in cortisol influences tUT mRNA expression over a short period of time.

## 2. Materials and methods

### 2.1. Animals

For *Series I* and *III*, samples were collected during a previous physiological study (Rodela et al., 2009). In brief, Gulf toadfish (*Opsanus beta*, Goode and Bean) were acquired from commercial shrimp fishermen in Biscayne Bay, FL, 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 (Rodela et al., 2009). For *Series II*, toadfish were obtained from Gulf Specimen Marine Lab in Panacea, FL, 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 recirculating 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.

### 2.2. Experimental protocol

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

To investigate the effects of cortisol on gene expression, three series of experiments were conducted:

Series I: *Chronic changes in tUT and GR expression following* in vivo *manipulation*
Series II: *The regulatory role of the tUT promoter*
Series III: *Acute temporal changes in tUT and GR expression*

#### 2.2.1. Series I: chronic changes in tUT and GR expression following in vivo manipulation

The purpose of *Series I* was to ascertain whether samples collected from an earlier *in vivo* experiment (Rodela et al., 2009) 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., 2009). In brief, caudal artery catheters (Wood et al., 1997; McDonald et al., 2004), 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 h.

For the first 24 h 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-Aldrich 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 h, up to 96 h; 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., 2004; Rodela et al., 2009) 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., 2009). 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 h for metyrapone, and 72 h for RU-