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

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SUMMARY

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 h. 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 two- to threefold 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 has an agonistic effect 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.

Key words: toadfish, urea excretion, cortisol, glucocorticoid receptor, mineralocorticoid receptor, metyrapone, RU-486, spironolactone, aldosterone.

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 et al., 1990; Walsh et al., 1994; Walsh and Milligan, 1995). This transition to ureotelism is initiated by a moderate and transient (<24 h) 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 is in contrast to 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 through the gills a few times daily in distinct pulses that last up to 3 h. 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 (Wood et al., 2003; McDonald and Walsh, 2004). 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}) 2–4 h 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 36–96 h (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 glucocorticoid receptor (rtGR1) was isolated from rainbow trout (Ducouret et al., 1995). The discovery of a second trout GR isoform (rtGR2), which 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 (Colombe et al., 2000; Greenwood et al., 2003; Sturm et al., 2005; Alsop and Vijayan, 2008; Stolte et al., 2008b). In tetrapods, the MR is normally activated by the mineralocorticoid aldosterone, and the avoidance of MR activation by cortisol is controlled by colocalization 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., 2007b). 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 more detail the influence of cortisol on urea excretion in the toadfish. In particular, whereas McDonald et al. (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 activity. 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. (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 have different effects 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 h) 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 min) 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., 1997). In the laboratory, toadfish were held in 501 aquaria, at a density of >10 fish per tank, supplied with aerated flowing seawater (18°C–22°C, pH 8.1) under natural photoperiod. Toadfish were fed chopped squid once a week.

Experimental protocol

To induce ureotely, groups of 8–10 fish were relocated to smaller plastic tubs (6 l) as outlined in standard crowding protocols (Walsh, 1987; Hopkins et al., 1995; Wood et al., 1997). Fish were maintained under these conditions for 48 h, until the time of surgery. Caudal artery catheters (Wood et al., 1997; McDonald et al., 2000), and in some cases intraperitoneal (i.p.) catheters (McDonald and Walsh, 2004) (and see below), were implanted in fish anaesthetized with MS-222 (1 g l^{-1}, pH 7.8) and covered in moist towels. 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 h.

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 min. 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 h^{-1} for the remainder of the experiment. Every 24 h, boxes were flushed with fresh seawater for 30 min. Inbetween 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 1

Blocking cortisol synthesis with metyrapone

The influence of metyrapone (methyl-1,2-di-3-pyridyl-1-propanone; Sigma Chemical Co., St Louis, MO, USA) 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. (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* the i.p. catheter once every 24 h. These treatments were followed by a 1.5 μl g^{-1} body mass saline injection to flush the treatments through the catheter into the peritoneal cavity of the fish. Blood samples were withdrawn at 0 and 24 h, and immediately following the 24-h sample fish received their respective treatments; subsequently blood was sampled at 36, 48, 60, 72, 84 and 96 h.

Series 2 and 3: blocking glucocorticoid receptors with RU-486 and mineralocorticoid receptors with spironolactone

The cortisol receptor antagonist RU-486 (mifepristone; Sigma Chemical Co.) was used to determine the involvement of the GR in regulating pulse size in untreated toadfish in Series 2, and in Series 3, spironolactone was used to block the MR. Following a 24-h control period, RU-486 or spironolactone in peanut oil, or peanut oil alone, at a volume of 1.5 μl g^{-1} body mass was administered *via* the i.p. 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 2) or spironolactone (Series 3). These concentrations were chosen based on similar studies in other fish (Sloman et al., 2001; McDonald et al., 2004; Scott et al., 2005). The treatment was followed by an additional 1.5 μl g^{-1} body mass peanut oil to flush the drug or vehicle dose down the length of the 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 (Vijayan and Leatherland, 1989; Sloman et al., 2001). Blood samples were withdrawn at 0, 24, 36, 48, 60 and 72 h.

Series 4: activating mineralocorticoid receptors with aldosterone

The aim of the final series of experiments was to investigate the effects on pulsatile urea excretion of activating the MR by infusion of the tetrapod MR agonist aldosterone. Blood samples were first taken from untreated fish at 0 and 24 h. Following the 24 h 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. (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 h.

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. (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. (McDonald et al., 2004) using the following equation:

$$\text{Excretion} = (\Delta C \times V_f)/M ,$$

where ΔC is the increases in concentration (μmol l^{-1}), V_f was the volume of the experimental flux chambers (in liters) and the product of both variables was adjusted to the mass (M) of the individual fish (in kg). These values were used to calculate percentage ureotelism. Nitrogen excretion (μmol N kg^{-1}) values during the treatment periods were averaged from multiple 24-h intervals so they could be compared with the initial 24-h control period. Excretion values were further divided into total urea (μmol N kg^{-1}), pulsatile urea (μmol N kg^{-1}) and non-pulsatile urea (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 concentrations (for Series 4) were measured using commercially available ^{125}I radioimmunoassay kits from MP Biomedicals (Santa Anna, CA, USA) and Beckman Coulter Canada, Inc. (Mississauga, ON, Canada), 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 percentage 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 4 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 1

Saline-infused fish were predominantly ureotelic, excreting urea-N at a rate of 87.1±5.6 μmol N kg^{-1} h^{-1} and ammonia-N at 19.3±10.8 μmol N kg^{-1} h^{-1} (Table 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 h; 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 (Fig. 1A, RM ANOVA, $P=0.99$; Fig. 1B, RM ANOVA, $P=0.98$; Fig. 1C, RM ANOVA, $P=0.99$; Table 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} (Fig. 1D, RM ANOVA, $P=0.96$) and 266.4±16.3 ng ml^{-1} (Fig. 1E, RM ANOVA, $P=0.99$), respectively.

Table 1. Series 1. The percentage of nitrogenous waste excreted in the form of urea and ammonia by toadfish during an initial 24 hour control period followed by a treatment period with either a saline vehicle or metyrapone

	Control period		Treatment period	
Metyrapone concentration (mg ml^{-1})	% 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*

Values are means ± s.e.m.

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

†Saline vehicle only (150 mmol l^{-1} NaCl, 1.5 μl g^{-1} body mass); $N=6$.

‡20 mg ml^{-1} metyrapone (in 150 mmol l^{-1} NaCl, 1.5 μl g^{-1} body mass); $N=7$.

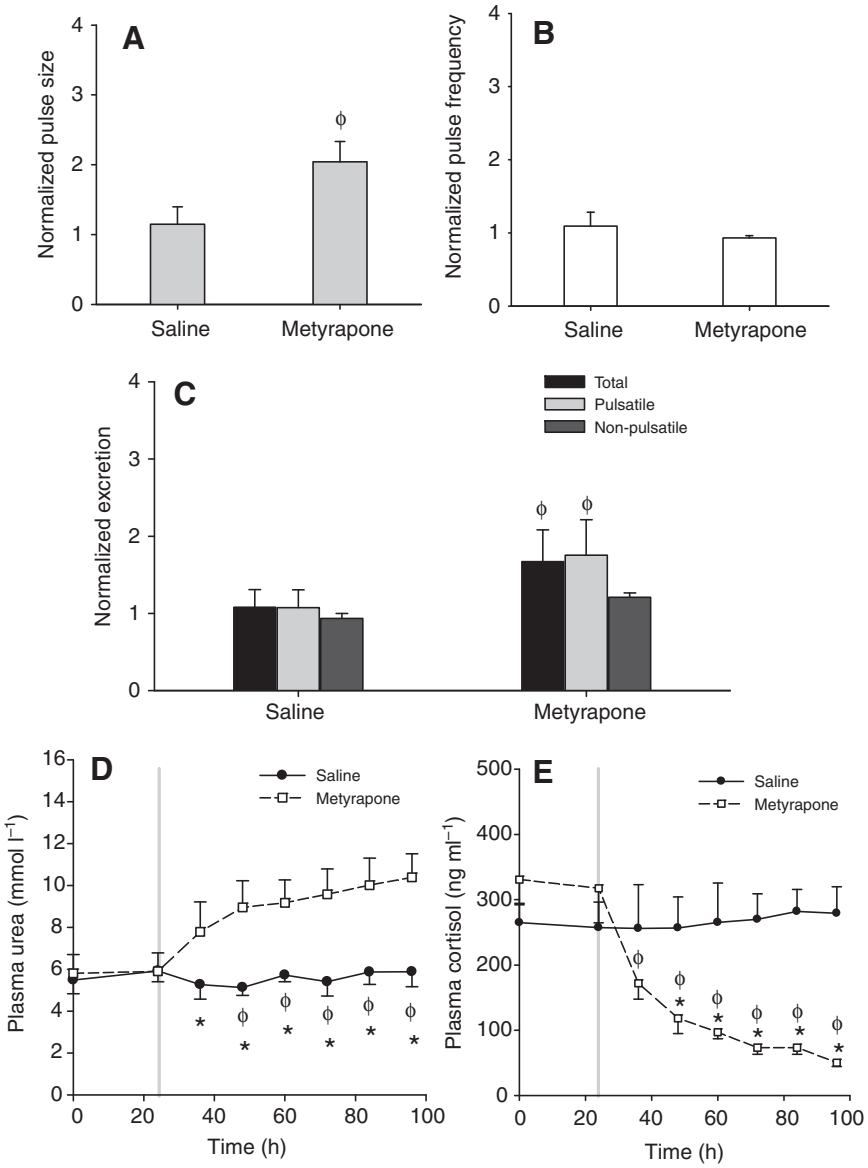

Fig. 1. Series 1. (A,B) The effect of a saline vehicle (N=6) or metyrapone (N=7) on urea pulse size (relative units; A) and frequency (relative units; B), in gulf toadfish. (C) Total, pulsatile, and non-pulsatile components of normalized urea excretion (relative units) of gulf toadfish exposed to either saline vehicle or metyrapone. (D,E) Changes in plasma urea concentrations (mmol l^{-1}; D) and plasma cortisol (ng ml^{-1}; E), 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 in A,B and C are presented as the treatment value normalized to the initial 24 h control period. Values are expressed as means ± s.e.m. Symbols (ϕ,*) indicate, respectively, significant differences within the treatment group from control period (>24 h), and differences between treatment groups at a given time (P<0.05). There were no significant differences in pulse frequency (P>0.05).

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 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 pulses per 24 h. Metyrapone caused a significant 1.7-fold increase in urea pulse size (to 300.8±44.6 µmol kg^{-1}; Fig. 1A, RM ANOVA, P=0.041) but was without effect on pulse frequency (4.3±0.3 per 24 h; Fig. 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 (Fig. 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 h and by 96 h was 1.8-fold higher than the control value (Fig. 1D, RM ANOVA, P=0.03). Plasma cortisol levels, however, had decreased significantly after 12 h of metyrapone treatment and by 96 h were 6.6-fold lower than the initial control values (Fig. 1E, RM ANOVA, P<0.001).

Series 2

Oil-injected fish excreted urea-N and ammonia-N at a rate of 52.9±5.9 µmol kg^{-1} h^{-1} and 13.2±3.5 µmol kg^{-1} h^{-1}, respectively, and the proportion of wastes did not vary over the different phases of the experiment (Table 2; RM ANOVA, P=0.64). Pulsatile urea excretion occurred with a frequency of 3.7±0.4 per 24 h and mean pulse size of 284.2±18.9 µmol kg^{-1}, which did change significantly during the trial (Fig. 2A, t-test, P=0.63; Fig. 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 (Fig. 2C; RM ANOVA, P=0.50). Plasma urea (Fig. 3A; ANOVA, P=0.17) and cortisol (Fig. 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} h^{-1} (mean=84.5±5.8 µmol kg^{-1} h^{-1}) and ammonia excretion ranging from 1.2 to 13.3 µmol kg^{-1} h^{-1} (mean=6.7±

Table 2. Series 2. The percentage of nitrogenous wastes excreted in the form of urea and ammonia by toadfish during an initial 24 hour control period followed by a treatment period with either a peanut oil vehicle or various concentrations of RU-486 administered in the oil vehicle

	Control period		Treatment period	
RU-486 concentration (mg ml^{-1})	% 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*

Values are means ± s.e.m.; *N*=5.
*A significant difference in percentage excretion from the corresponding control period within the treatment group (P<0.05).
†Peanut oil vehicle only (1.5 µl g^{-1} body mass).

0.5 µmol kg^{-1} h^{-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), however, RU-486 injections of 75–125 mg ml^{-1} significantly increased the percentage of urea excreted and concomitantly reduced the proportion of ammonia, relative to control values (Table 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 h (mean=4.3±0.2). All RU-486 doses significantly increased urea pulse size by 1.92- to 3.13-fold (Fig. 2A; RM ANOVA, P=0.006), whereas pulse frequency was unaffected (Fig. 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}, resulting in ~2.4- and ~3.5-fold increases in urea excretion, respectively (Fig. 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 (Fig. 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 (Fig. 3B; RM ANOVA, P=0.025), cortisol remained constant among treatment groups and did not vary over time (data not shown).

Series 3

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} h^{-1}) and 13.8±1.4% as ammonia-N (mean=6.5±0.6 µmol kg^{-1} h^{-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 h. Treatment had no effect on pulse size for concentrations up to 100 mg ml^{-1} spironolactone (Fig. 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 (Fig. 4A; RM ANOVA, P=0.027). Spironolactone treatment had no effect on pulse frequency (Fig. 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 (Fig. 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 (Fig. 4C; RM ANOVA, P=0.018). Plasma urea remained constant over time and treatment (Fig. 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 h than the initial sample at 0 h (Fig. 5A; RM ANOVA, P=0.003). Plasma cortisol values remained steady over time and between treatment, with the exception of the 60 and 72 h time points for which the values of 20 and 125 mg ml^{-1}, respectively, were significantly lower relative to the oil only control (Fig. 5B; RM ANOVA, P=0.008).

Series 4

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} h^{-1}) and 14.9±2.5% as ammonia-N (mean=8.5±1.2 µmol kg^{-1} h^{-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 (Fig. 6A; RM ANOVA, P=0.56). Pulses occurred at a frequency of 4.6±0.4 times per 24 h, and did not vary with treatment (Fig. 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. 6C; RM ANOVA, P=0.50). Plasma urea (Fig. 7A; RM ANOVA, P=0.96), cortisol (Fig. 7B; RM ANOVA, P=0.51), and aldosterone (Fig. 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 waste as urea-N (mean=99.0±23.3 µmol kg^{-1} h^{-1}) and 8.2±2.2% as ammonia-N (mean=4.6±1.1 µmol kg^{-1} h^{-1}). Urea excretion occurred in distinct pulses with a mean size of 648.4±152.4 µmol kg^{-1} h^{-1} during the control period and was significantly reduced by 0.9-fold following infusion of aldosterone (Fig. 6A; RM ANOVA, P=0.027). Pulse frequency (mean=3.67±0.27 per 24 h) was unaffected by treatment (Fig. 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 (Fig. 6B; RM ANOVA, P<0.001), with no significant change in non-pulsatile components. Plasma urea (Fig. 7A; RM ANOVA, P=0.96) and cortisol (Fig. 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} (Fig. 7C; RM ANOVA, P<0.001).

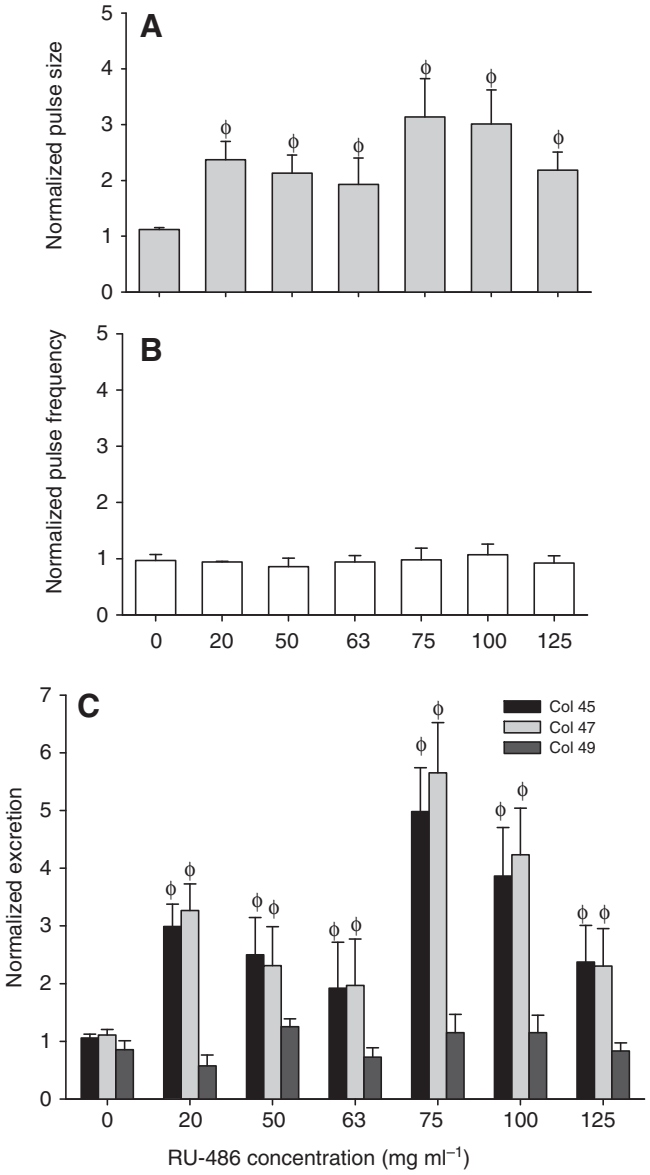

Fig. 2. Series 2. (A,B) The effect of RU-486 (mg ml^{-1}) on urea pulse size (relative units; A) and frequency (relative units; B) in gulf toadfish. (C) Total, pulsatile, and non-pulsatile components of normalized urea excretion (relative units) of gulf toadfish exposed to either oil vehicle (0 mg ml^{-1} RU-486) or RU-486. RU-486 was delivered by intraperitoneal injection in a peanut oil vehicle (1.5 µl g^{-1} body mass). The data are presented as the treatment value normalized to the initial 24 h control period. Values are expressed as means ± s.e.m. The symbol ϕ indicates 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). Col 45, total; Col 47, pulsatile; Col 49, non-pulsatile.

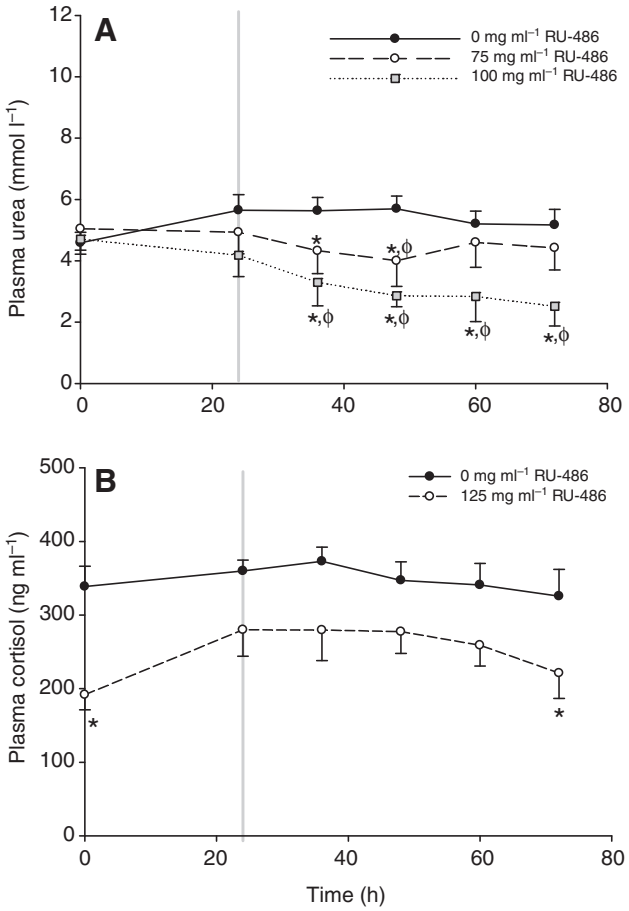

Fig. 3. Series 2. (A,B) Changes in plasma urea concentrations (mmol l^{-1}; A) and plasma cortisol concentrations (ng ml^{-1}; B) in gulf toadfish over time (h) following exposure to vehicle alone or RU-486. RU-486 was delivered by intraperitoneal injection in a peanut oil vehicle (0 mg ml^{-1} RU-486; 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 indicates significant differences (P<0.05) within the group from the initial sample at 0 h, and the asterisk indicates differences between RU-486 and vehicle control (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 and 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 with 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} h^{-1}; ammonia, 1.2–37.8 µmol kg^{-1} h^{-1}) among all individuals in Series 1–4. Although the animals were fasted for *at least* 72 h 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 1 week

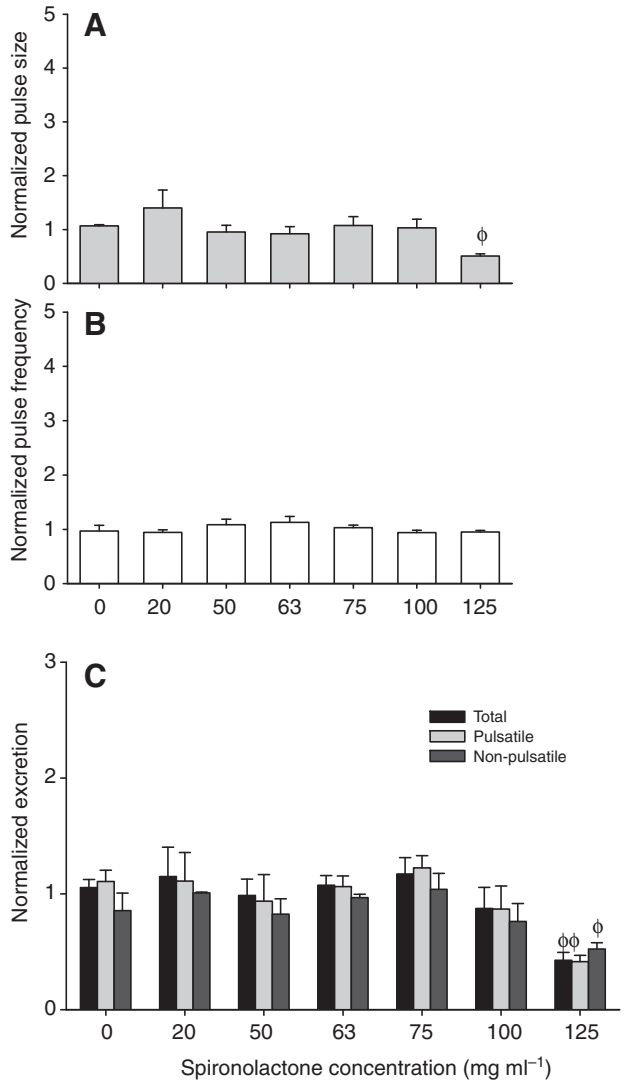

Fig. 4. Series 3. (A,B) The effect of spironolactone (mg ml^{-1}) on urea pulse size (relative units; A) and frequency (relative units; B), in gulf toadfish. (C) Total, pulsatile and non-pulsatile components of normalized urea excretion (relative units) of gulf toadfish exposed to either oil vehicle (0 mg ml^{-1} spironolactone) or spironolactone (mg ml^{-1}). The data are presented as the treatment value normalized to the initial 24 h control period. Values are expressed as means ± s.e.m. The symbol ϕ indicates 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).

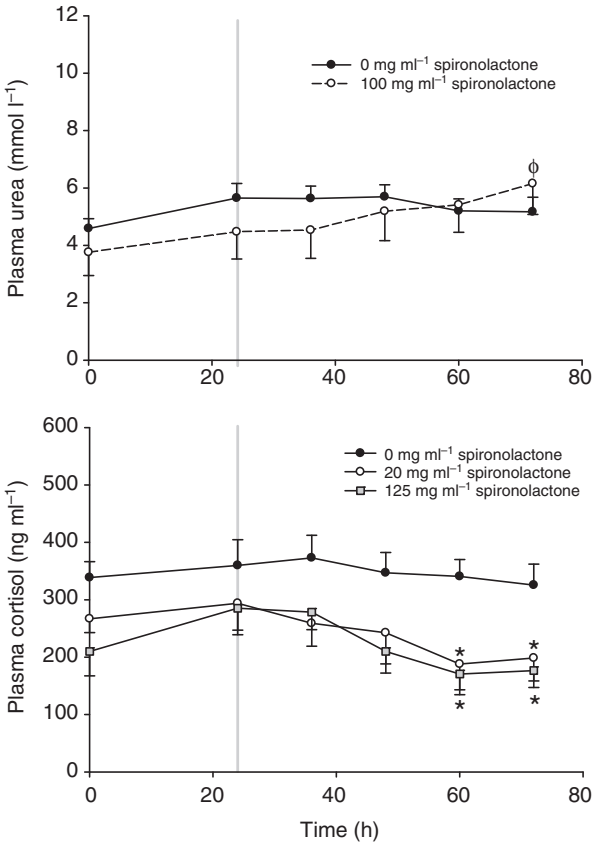

Fig. 5. Series 3. (A) Variation in plasma urea (mmol l^{-1}) in gulf toadfish over time (h) following treatment with spironolactone. (B) Changes in plasma cortisol (ng ml^{-1}) in gulf toadfish over time (h) following spironolactone exposure. The drug was delivered by intraperitoneal injection in a peanut oil vehicle (0 mg ml^{-1} spironolactone; 1.5 µl g^{-1} body mass). The data are presented as the treatment value normalized to the initial 24 h control period. Values are expressed as means ± s.e.m. The symbol ϕ indicates significant differences (P<0.05) within the group from the initial sample at 0 h, and the asterisk indicates differences between spironolactone and vehicle control (P<0.05).

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 (Wood et al., 1995; Wood et al., 1997; Gilmour et al., 1998; McDonald et al., 2004).

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 (Bennett and Rhodes, 1986; Vijayan and Leatherland, 1992; Bernier and Peter, 2001; Milligan, 2003; McDonald et al., 2004; Aluru and Vijayan, 2007; Killerich et al., 2007a). Although 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 upregulation of urea excretion rates. A recent study documented that toadfish increase tUT mRNA in response to infusion with cortisol with associated decreases in urea

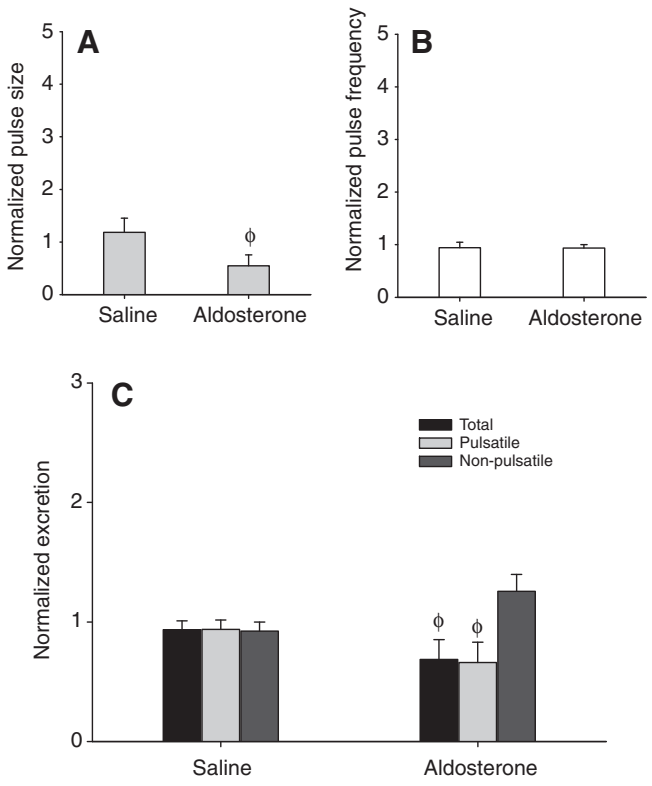

Fig. 6. Series 4. (A,B) The effect of a saline vehicle (N=8) or aldosterone (N=14) on urea pulse size (relative units; A) and frequency (relative units; B) in gulf toadfish. (C) Total, pulsatile, and non-pulsatile components of normalized urea excretion (relative units) of gulf toadfish exposed to either 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 are presented as the treatment value normalized to the initial 24 h control period. Values are expressed as means ± s.e.m. The symbol ϕ indicates significant differences from the initial control period within the treatment group (A,C) (P<0.05). There were no significant differences in pulse frequency (P>0.05).

excretion (McDonald et al., 2009). However, cortisol-mediated effects on urea excretion rates in toadfish occur fairly rapidly (<60 min) *in vivo*, and in less than 4 h 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 Wehling, 1997; Borski, 2000). 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., 1997; 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 by 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., 2008a). 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 et al., 1994; Walsh and Milligan, 1995). 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 h 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-synthesising enzymes may lag behind removal of the corticosteroid stimulus. One other study has reported a similar trend of toadfish lacking a chronic elevation of cortisol, and those individuals reverting to ammoniotelism (Wood et al., 1995). Therefore, it is possible that cortisol, mediated by a specific GR isoform, 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 but may influence other aspects of metabolism (reviewed by Mommsen et al., 1999).

Fish are incapable of synthesizing significant amounts of aldosterone (Sangalang and Uthe, 1994; Jiang et al., 1998), 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 (Sturm et al., 2005; Prunet et al., 2006). Expression studies on the common carp (*Carassius auratus*) and rainbow trout MR indicate that this receptor is more sensitive to aldosterone and 11-deoxycorticosterone (DOC), whereas the GR is more sensitive to cortisol (Sturm et al., 2005; Prunet et al., 2006; Stolte et al., 2008a). 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. (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

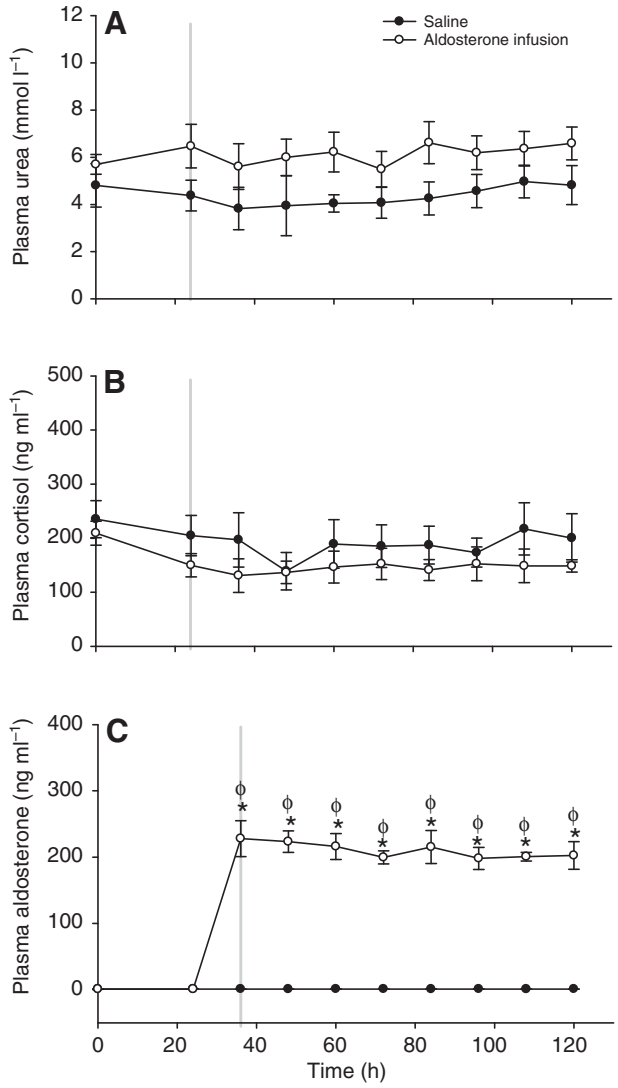

Fig. 7. Series 4. (A) Variation in plasma urea (mmol l^{-1}) from gulf toadfish over time (h) following aldosterone infusion. (B) Changes in plasma cortisol (ng ml^{-1}) of gulf toadfish over time (h) following treatment with aldosterone. (C) Plasma aldosterone levels (ng ml^{-1}) over time (h) in gulf 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 indicates significant differences (P<0.05) within the group from the initial sample at 0 h, and the asterisk indicates 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).

that aldosterone infusion in toadfish may have activated pathways that are normally insensitive to cortisol activation (which is also an agonist of the MR) as a result of 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.

Owing 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). Although 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 (Sturm et al., 2005; Prunet et al., 2006; Stolte et al., 2008a). With this information in mind, McDonald et al. (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 dose of spironolactone (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} mol l^{-1}) 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 behavior in piscine and mammalian systems and the data should be interpreted with caution.

Although 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 (T.M.R., K.M.G., P.J.W. and M.D.M., in preparation). 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 in 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.

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