The Journal of Experimental Biology 209, 2704-2712
Published by The Company of Biologists 2006
doi:10.1242/jeb.02289

Metabolic and neuroendocrine effects on diurnal urea excretion in the mangrove killifish *Rivulus marmoratus*

Tammy M. Rodela and Patricia A. Wright*
Department of Integrative Biology, University of Guelph, Guelph, ON, N1G 2W1, Canada
*Address for correspondence e-mail: patwrigh@uoguelph.ca

Accepted 25 April 2006

Summary

In mangrove killifish *Rivulus marmoratus*, urea excretion (J_{urea}) follows a distinct diurnal pattern with the highest rates between 12:00 h and 18:00 h. We investigated the regulating mechanisms that underlie temporal rhythms in J_{urea} in *R. marmoratus*. We hypothesized that the daily pattern of J_{urea} in *R. marmoratus* is (1) due to diurnal changes in urea synthesis rates and ultimately metabolic rate and/or (2) controlled by neuroendocrine messengers. Oxygen consumption and whole body urea content in *R. marmoratus* demonstrated a clear diurnal pattern with maximum rates for both parameters occurring at 12:00 h. A strong synchrony between diurnal patterns of oxygen consumption, whole body urea content and J_{urea} implicated metabolic regulation of the diurnal J_{urea} pattern. Ketanserin, a 5-HT_2 receptor antagonist, and RU-486, a cortisol receptor antagonist, were used to test the second hypothesis. Increasing antagonist concentrations of either ketanserin or RU-486 resulted in dose-dependent decreases in J_{urea}. Application of a single dose of either antagonist significantly decreases J_{urea} for up to 12 and 6 h for ketanserin and RU-48, respectively. Repeated exposure to doses of either ketanserin or RU-486 did not abolish the diurnal pattern in J_{urea}; however, there was a significant decrease in the amplitude of the rates. Taken together, these findings indicate that the diurnal pattern of J_{urea} in *R. marmoratus* are regulated by both metabolic and neuroendocrine factors. We propose that cortisol and 5-HT influence the absolute rate of urea excretion by altering the permeability of the gill membrane to urea and/or the rate of urea synthesis.

Key words: nitrogen excretion, ammonia excretion, oxygen consumption, serotonin, 5-HT, ketanserin, cortisol, RU-486, killifish, *Rivulus marmoratus*.

Introduction

The rate of nitrogenous waste excretion in teleosts is largely dependent on the rate of synthesis in the liver and the rate of transport across body surfaces, which is influenced by concentration gradients and membrane permeability. The majority of fish synthesize and excrete ammonia and urea continuously even when deprived of food (Smith and Thorpe, 1976; Kaushik and de Oliva-Teles, 1985; Alsop and Wood, 1997). In fed individuals, concentrations of nitrogen end products in plasma increase within hours after a meal, followed by a washout period typified by increased rates of excretion (Brett and Zala, 1975; Kaushik and Gomes, 1988; Kaushik and de Oliva-Teles, 1985; Wicks and Randall, 2002). In contrast to most teleosts, nitrogen excretion in the ammoniotelic *Rivulus marmoratus* demonstrates a most unusual pattern. Urea (J_{urea}) but not ammonia excretion (J_{amm}) in *R. marmoratus* undergoes a diurnal rhythmicity, with the highest rates of urea excretion occurring during mid-afternoon (Frick and Wright, 2002a; Rodela and Wright, 2006). Under the influence of circadian clocks, the phase of the J_{urea} cycle was entrained fairly quickly to photic zeitgebers and possibly feeding cues.

One of the aims of the present study was to determine if the diurnal J_{urea} pattern in *R. marmoratus* was related to diurnal changes in metabolism. The ammoniotelic gobiid fish *Mugilogobius abei* also has a distinct daily pattern of J_{urea} under light:dark cycles (Kajimura et al., 2002). The authors postulated that the rate of urea synthesis may vary in a similar diurnal pattern; however, rates of oxygen consumption were not measured. An alternative possibility is that the rhythmic pattern of J_{urea} in *R. marmoratus* is under the control of mechanisms affecting urea permeability across cell membranes.

Urea permeability across lipid membranes is significantly lower than that of ammonia (Collander, 1937; Galluci et al., 1971) and recent studies have shown the presence of urea transporters (UT) on the branchial epithelium of many fish species (Smith and Wright, 1999; Walsh et al., 2000; Walsh et al., 2001; Mistry et al., 2001; McDonald et al., 2002; McDonald et al., 2004). Pulsatile J_{urea} patterns in marine

toadfish *Opsanus beta* are attributed to periodic activation of a facilitated branchial UT, rather than to changes in urea production pathways (Wood et al., 1997; Wood et al., 2003). Recent studies have revealed that the glucocorticoid hormone, cortisol and the monoamine, 5-hydroxytryptamine (5-HT; serotonin) may regulate pulse size and frequency, respectively (Wood et al., 1997; Wood et al., 1998; Wood et al., 2003; McDonald et al., 2004).

Similar control mechanisms may be regulating J_{urea} in *R. marmoratus*. Both 5-HT and cortisol are appealing candidates for neuroendocrine mediators of diurnal urea excretion as these hormones undergo daily rhythms under the influence of a light:dark photoperiod in other species. For instance, circulating cortisol levels reach peak concentrations at or near the onset of light, immediately prior to the initiation of daily locomotor activity in several fishes (Peter et al., 1978; White and Fletcher, 1984; Fivizzani et al., 1984). Daily fluctuations in brain 5-HT levels have been documented in *Anguilla anguilla* (van Veen et al., 1982) and *O. mykiss* (Zuanreiter et al., 1998).

The mangrove killifish *R. marmoratus* are remarkably hardy fish that tolerate a range of environmental extremes (Abel et al., 1987; King et al., 1989; Frick and Wright, 2002a; Frick and Wright, 2002b). In this study we hypothesized that the daily J_{urea} pattern in *R. marmoratus* is due to diurnal changes in metabolic rate, which may in turn alter blood to water urea gradients and ultimately, excretion rates. Tissue concentrations of urea and ammonia, urea excretion rates, as well as whole animal oxygen consumption rates were measured over time. A second hypothesis, that the rate of urea excretion is influenced by neuroendocrine messengers was also tested. We first tested the prediction that RU-486, a cortisol receptor antagonist, will cause a dose-dependent increase in urea, but not ammonia, excretion rates. Second, exposure to ketanserin, a 5-HT_2 receptor antagonist, will cause a dose-dependent decrease in urea excretion rates, but not ammonia excretion rates. Furthermore, we predicted that upon exposure to RU-486 or ketanserin, the amplitude of the diurnal J_{urea} pattern would be increased and decreased, respectively, based on previous studies in the toadfish (McDonald et al., 2004; McDonald and Walsh, 2004).

Materials and methods

Laboratory animals

Mangrove killifish *Rivulus marmoratus* Poey were held at the Hagen Aqualab at the University of Guelph (Guelph, ON, Canada) (Frick and Wright, 2002a). The fish were held in an environmental chamber at 25°C on a photoperiod of 12 h:12 h light (L):dark (D). Mature fish (0.07–0.25 g) were kept in individual translucent containers in 60 ml of 15‰ artificial seawater (pH 8.1), which was changed biweekly. Artificial seawater was made from distilled water and marine salt (Instant Ocean™, Crystal Sea, Baltimore, MA, USA). Fish were fed *Artermia salina* nauplii every other day. To eliminate effects of recent feeding on nitrogen metabolism and excretion, fish were deprived of food 48 h prior to the initiation of an experiment.

Experimental protocol

Metabolic experiments

Four series of experiments were conducted:

Series I: measurements of tissue urea and ammonia levels over 1.5 days.

Series II: quantification of whole animal oxygen consumption rates over a 3 day period.

Series III: detailed examination of hourly oxygen consumption rates over a 6 h period.

Series IV: hourly nitrogen excretion rates in fasted *R. marmoratus* over a 6 h period.

Series I

In order to determine if changes in the diurnal J_{urea} pattern were due to metabolic changes (i.e. changes in nitrogen production and storage levels), tissue urea and ammonia concentrations were measured. Fish were killed by flash freezing in liquid nitrogen every 3 h during a 1.5-day period. Samples were stored at –80°C for up to 2 months prior to analysis of urea and ammonia content.

Series II and III

Oxygen consumption rates were measured in *R. marmoratus* using closed respirometry techniques. A single fish was placed in approximately 60 ml of brackish water in the respiratory chamber. Fish were acclimated to the respirometry chambers 2 days prior to the start of the experiment (25°C). Additional blank chambers (not containing a fish) were run simultaneously to determine the rate of oxygen consumed by the oxygen probe. The background values were subtracted from the oxygen consumption values of the fish, but were minimal (<10.28% of average fish value). At the onset of the measurements, the chamber was sealed for 1 h, during which changes in oxygen levels were measured in mg l^{-1}. During the measurement, oxygen levels did not fall below 70% saturation in order to ensure that the values obtained were a true representation of normoxic metabolic status in the animal. At the end of the sampling period, the chambers were renewed with fresh aerated water (15‰) for a period of 5 h to allow maximum oxygen saturation levels before the start of the next sampling period. In Series II, experiments involved measuring the change in water oxygen for 1 h intervals every 5 h for 3 days. For Series III, experiments entailed measuring oxygen consumption for every hour between 12:00 h and 18:00 h. At the end of each series of experiments, the fish were removed from their chambers, blotted dry and weighed. Values are presented as μmol O_2 g^{-1} h^{-1}.

Series IV

In order to determine correspondence between J_{urea} and oxygen consumption, J_{urea} was measured for each 1 h interval between 12:00 h and 18:00 h. Fish were held in individual containers with 30 ml of 15‰ artificial seawater (pH 8.1,

25°C, 12 h:12 h L:D) and water samples were collected every hour for a 1.5 day period. The water was changed after each sampling interval to prevent accumulation of nitrogenous wastes. Elimination of handling stress was accomplished through the use of double-walled containers, with an inner mesh container made in the exact same configuration as the plastic translucent holding container. Consequently, during water changes, the inner mesh container with the fish was removed and placed in a new outer chamber containing fresh brackish water. Fish were acclimated to the containers 2 days prior to the start of the experiment. Water samples were frozen at –20°C for up to 1 month and later analyzed for urea and ammonia content.

Pharmacological experiments

Three series of experiment were conducted:

Series V: the dose effects of ketanserin and RU-486 on nitrogen excretion.

Series VI: the effects of a single application of ketanserin and RU-486 on nitrogen excretion.

Series VII: the effects of ketanserin and RU-486 on diurnal J_{urea}.

Series V

Pharmacological agents were used to determine if the diurnal pattern of J_{urea} is controlled, in part, by either cortisol or 5-HT. Specifically, RU-486, a cortisol receptor inhibitor, and ketanserin, a 5-HT_2 receptor antagonist, were used. Initial experiments involved testing the effect of each drug on J_{urea}. Concentrations were chosen based on similar studies on other fish species (Brustein et al., 2003; Dasmahapatra and Lee, 1993; Sathiyaa and Vijayan, 2003). For ketanserin, three different treatment groups of *R. marmoratus* were exposed to concentrations of 10, 30 or 50 μmol l^{-1}. Initially, identical concentrations of RU-486 and ketanserin were used; however, there was a 80% or higher mortality rate associated with these concentrations. Subsequently lower concentrations of RU-486 consisting of 1, 2 or 5 μmol l^{-1} were used. Both receptor antagonists required an ethanol vehicle (0.0003% w/v), consequently control fish were exposed to identical concentrations of the vehicle in the absence of the antagonist. Final water concentrations of ethanol in did not exceed 0.83%. Each drug (and ethanol control) was added to 30 ml of water (15‰). Initial water samples were collected following the addition of the drugs and final water samples were taken 6 h later, and handled as described for Series IV. Due to the small size of the fish (<250 mg), it was not possible to measure the plasma concentration of 5-HT and cortisol.

Series VI

Experiments involved following nitrogen excretion rates over time during and after a 6 h exposure to 30 μmol l^{-1} ketanserin or 1 μmol l^{-1} RU-486. Initial experiments involved measuring both J_{urea} and J_{amm} over 6 h intervals under control conditions for a 24 h period.

As previously described, the control treatment contained ethanol (final concentration less than 0.83%). A single dose of either 30 μmol l^{-1} ketanserin or 1 μmol l^{-1} RU-486 was administered at 12:00 h. Following a 6 h exposure to the receptor antagonists (12:00 h–18:00 h), freshwater (15‰) with ethanol (0.83%) was replaced in each chamber every 6 h period for the remainder of the experiment in order to follow the recovery phase. Water samples were taken every 6 h and were treated as described in Series IV.

Series VII

The final series of experiments involved measuring the diurnal excretion pattern when the fish were either exposed to ketanserin or RU-486. Control excretion rates were measured for the first 30 h and following this period the fish was exposed to 30 μmol l^{-1} ketanserin or 1 μmol l^{-1} RU-486 every 6 h for the next 54 h. Water samples from each experiment were frozen at –20°C for up to 1 month and later analyzed for urea and ammonia content.

Analytical techniques and calculation

Ammonia and urea

Extracts were prepared by grinding the intact fish to a fine powder with a mortar and pestle in liquid nitrogen (Wright et al., 1995). Urea concentrations were analyzed as described previously (Rahmatullah and Boyde, 1980) and values were expressed as mmol N l^{-1}. Samples were analyzed for ammonia content using a modified published method (Kun and Kearney, 1974) for use with a SpectraMax 190 micoplate reader (Molecular Devices Corp., Sunnyvale, CA, USA). The efficiency of the assay was determined by spiking samples with known amounts of ammonia and determining the percent recovery (98.5±1.3% recovery rate). Values for ammonia concentration in the tissues are expressed in mmol N l^{-1}.

Seawater urea concentrations were measured with a colorimetric assay (Rahmatullah and Boyde, 1980) using an Ultrospec 3300 *Pro* spectrophotometer (Biochrom, Cambridge, UK). Ammonia content of water samples were quantified by assay methods described elsewhere (Ivancic and Degobbis, 1984). The rates of excretion (J) were calculated as described previously (Wright and Wood, 1985).

Control experiments were carried out to determine if bacterial contamination from various sources such as the individual fish, the water supply or attached to the experimental chamber could have affected nitrogen excretion rates. Fish were placed in 30 ml of water for a 1 h interval and subsequently removed. Ammonia and urea concentrations were monitored in the water during the next 6 h. Analysis revealed that microbial contamination did not significantly affect nitrogen excretion rates from *R. marmoratus* (P>0.05).

Preliminary experiments were performed to determine if the hormone receptor antagonists used in this study had an effect on the colour development of either the ammonia or urea assays. There was no significant effect of either RU-486 or ketanserin on the ammonia assay, neither was the urea assay affected by RU-486. However, the colour development of the urea assay in the presence of ketanserin was slightly less

intense (10%) relative to samples without ketanserin. Due to the fact that this change was relatively small and underestimated the influence of ketanserin (but did not alter the trends in the data), the values were not corrected for this effect.

Oxygen consumption

Oxygen consumption was measured by closed respirometry with eight double-walled glass chambers connected in series to a water bath to control the temperature inside the chambers. Changes in oxygen levels were measured using an automatic temperature compensated dissolved oxygen sensor with built-in thermistor and amplifier (Vernier Software and Technology, Beaverton, OR, USA) and measurements were recorded in mg l^{-1}. Measurements of water oxygen levels were quantified using the LoggerPro Software (Vernier Software and Technology). Values for oxygen consumption are expressed in (μmol O_2 min^{-1} g^{-1}).

Statistical analysis

The data are presented as means ± standard error of the mean (s.e.m.). For Series I, III and IV, a one-way analysis of variance (ANOVA) was used to determine if tissue levels and oxygen consumption rates varied over time (P<0.05). A Tukey test (SPSS, SPSS Inc., Chicago, IL, USA) was used to test for differences between time intervals (P<0.05).

For Series II, analysis of oxygen consumption values was carried out using the single cosinor approach (Halberg et al., 1972; Nelson et al., 1979; Rodela and Wright, 2006). In this model a cosine function is fitted to the data by least squares regression, defining in the process several parameters of the circadian rhythm: its rhythm-adjusted mean value or mesor; its amplitude, which is one half of the difference between the highest and the lowest values; and the time at which the waveform reaches its peak value or acrophase. Recognition of circadian activity was accomplished by testing a null hypothesis of zero amplitude with an F-test (P<0.05).

For Series V, analysis of the dose-dependence data for both ketanserin and RU-486 was done with a one-way ANOVA (P<0.05) followed by a Dunnett's test to locate statistical differences (P<0.05). For Series VI, a paired t-test was used to test for significance differences between control and treatment values for each individual time period (P<0.05). Due to confounding effects of time and treatment on diurnal nitrogen excretion, a general linear model contrast (SAS, SPSS Inc.) was used to determine if control values during the diurnal antagonist exposure were significantly different from treatment values.

Results

Metabolic experiments

Series I

In fasted *R.marmoratus* kept on their normal photoperiod of 12 h:12 h L:D, whole body tissue urea levels followed a diurnal pattern over time with a definitive a peak and trough over a 1.5 day period (Fig. 1). The period of the cycle was approximately 24 h, with urea accumulating in the tissues overnight to reach maximum concentrations at 12:00 h. Over the next 6 h, tissue urea levels were at their lowest values, 3.5-fold lower that at 12:00 h. In contrast to urea, whole body ammonia concentrations remained constant over time (data not shown), ranging from 6.0 to 6.8 mmol l^{-1}. The highest levels of urea accumulation in the tissues at 12:00 h did not differ significantly from whole body ammonia values at this time. However, following the decline in tissue urea levels (18:00 h), ammonia concentrations were approximately 3.7-fold higher relative to urea.

Series II

Oxygen consumption in *R. marmoratus* followed a significant circadian rhythm ($F_{2,9}$=16.4, P=0.0007). The highest rates of oxygen uptake occurred during the day between 11:30 h and 12:30 h, with an amplitude of 32% of the meson, the acrophase occurred at 13:48±00:34 h (Fig. 2). *R. marmoratus* consume significantly less oxygen between 17:30 h and 18:30 h. Comparisons between the maximum and minimum rates of oxygen consumption yield a 1.9-fold difference between the peaks and troughs.

Series III

A closer examination of oxygen consumption on an hourly basis between 12:00 h and 18:00 h revealed that the definitive peak of oxygen uptake was 2 h in duration and occurred between 12:00 h and 14:00 h (Fig. 3).

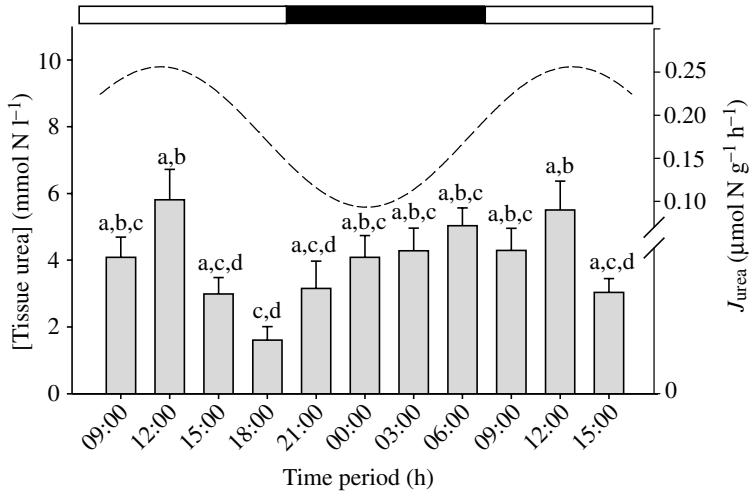

Fig. 1. Urea tissue levels in whole body *R. marmoratus* measured every 3 h over a 1.5-day period (Series I). White and black bars above the graph indicate periods of light and dark, respectively. Values are expressed as means + s.e.m. (N=6). Columns labeled with different letters are significantly different (P<0.05). The broken line represents the J_{urea} pattern under corresponding 12 h:12 h L:D conditions (Rodela and Wright, 2006). The mesor of J_{urea} is 0.175±0.016 μmol N g^{-1} h^{-1} and the acrophase occurs at 14:30±00:39 h.

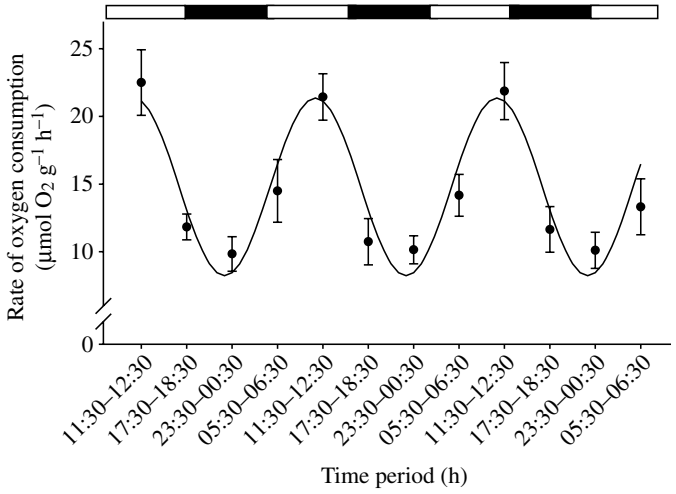

Fig. 2. Diurnal oxygen consumption rates in *R. marmoratus* over a 3-day period measured every 6 h for 1 h. White and black bars above the graph indicate periods of light and dark, respectively (Series II). Values are expressed as means + s.e.m. (N=12). Oxygen consumption values followed a significant rhythm ($F_{2,9}$=15.5, P=0.0012). The mesor of oxygen consumption is 14.79±6.59 μmol O_2 g^{-1} h^{-1} and the acrophase is 13:48±00:34 h.

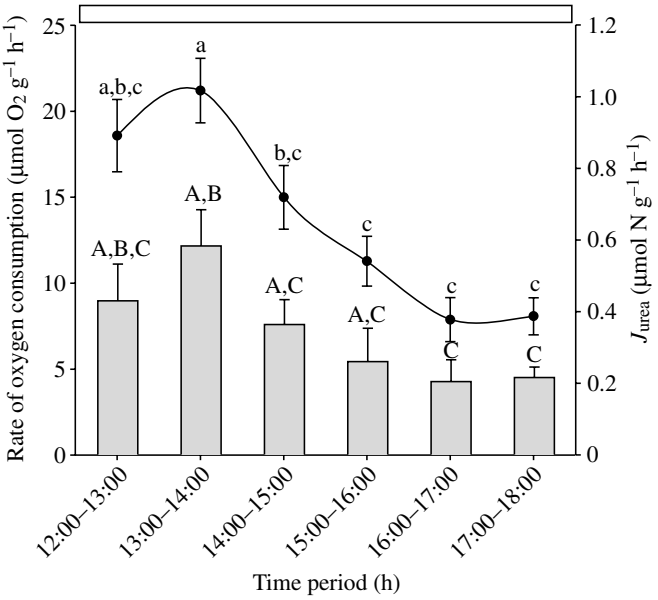

Fig. 3. Oxygen consumption rates (solid circles) for *R. marmoratus* measured for 1 h intervals over a 6 h period (Series III). The white bar above the graph indicates constant light. Values are expressed as means + s.e.m. (N=12). Columns labeled with different lowercase letters are significantly different (P<0.05). J_{urea} (grey bars) for *R. marmoratus* measured for 1 h intervals over a 6 h period (Series IV). Values are expressed as means + s.e.m. (N=8). Columns labeled with different uppercase letters are significantly different (P<0.05).

Oxygen consumption decreased over the afternoon until rates reach their lowest values at 18:00 h.

Series IV

When J_{urea} rates were examined over the same 1 h intervals between 12:00 h and 18:00 h, the pattern was similar to the hourly oxygen consumption measurements (Fig. 3). J_{urea} was significantly higher between 13:00 h and 14:00 h relative to the subsequent hourly intervals. By 17:00–18:00 h, approximately 72% less urea was excreted compared to values obtained between the hours of 13:00 h and 14:00 h. Over the same 6 h intervals, there was no significant difference in ammonia excretion rates (data not shown).

Pharmacological experiments

Series V

Both receptor antagonists required the use of an ethanol vehicle to facilitate solubility; however, ethanol concentrations in the water did not significantly affect either urea or ammonia excretion rates (*t*-test, P<0.05). Application of the ketanserin, a 5-HT_2 antagonist, caused a dose-dependent inhibition of J_{urea} (data not shown). A concentration of 50 μmol l^{-1} ketanserin caused a 61% inhibition of J_{urea}. Excretion of ammonia remained unaffected by the application of ketanserin (data not shown). Exposure to RU-486, a cortisol receptor antagonist, also produced a dose-dependent decrease in J_{urea} (data not shown). Exposure to 5 μmol l^{-1} RU-486 resulted in a 65% inhibition of J_{urea} (data not shown). Ammonia excretion remained unaffected by RU-486 (data not shown).

Series VI

Under control conditions a diurnal cycle in J_{urea} was present (data not shown). The highest rates of urea excretion occurred between 12:00 h and 18:00 h, and were 78% higher than rates between 00:00 h and 06:00 h. Exposure to 30 μmol l^{-1} of ketanserin between 12:00 h–18:00 h resulted in a 40% inhibition of J_{urea} relative to control values (Fig. 4A). During the first 6 h of recovery (18:00 h–0:00 h), urea excretion continued to be depressed (50%) in post-treatment fish relative to control values. In the subsequent 6 h interval (0:00 h–6:00 h), however, J_{urea} returned to control levels. Ammonia excretion rates remained unaffected by the application of ketanserin (data not shown). RU-486 (1 μmol l^{-1}) exposure resulted in a 49% inhibition of J_{urea} rates (Fig. 4B). Following recovery in fresh brackish water, J_{urea} returned to control values during the next 6 h interval. Ammonia excretion did not change in response to RU-486 (data not shown).

Series VII

A strong diurnal cycle in urea excretion was present in fish exposed to the ethanol control prior to repeated applications of 30 μmol l^{-1} ketanserin (Fig. 5). Application of ketanserin did not affect the properties (i.e. period, timing of peaks and troughs, degree of change between peaks and troughs) of the diurnal cycle, however, the absolute values on the rates of J_{urea} were affected (Fig. 5). Exposure to ketanserin resulted in a 39–72% inhibition in J_{urea} rates. Ammonia excretion remained constant over time (data not shown). For control fish, J_{amm}

constituted 72–85% of $J_{nitrogen}$ whereas in the ketanserin-treated fish, J_{amm} constituted 81–95% of $J_{nitrogen}$.

Exposure to repeated doses of 1 μmol l^{-1} RU-486 resulted in a 30–42% inhibition in the absolute rate of J_{urea} (Fig. 6). Similar to ketanserin-treated fish, the period and the timing of the peaks and troughs did not change in RU-486-treated fish; only the absolute values of the rates were affected. Ammonia excretion remained steady for the duration of the experiment (data not shown). The majority of wastes excreted during the experiment were in the form of ammonia.

Discussion

Urea, but not ammonia, excretion in *R. marmoratus* undergoes a diurnal rhythmicity with the highest rates of urea excretion occurring during mid-afternoon between 12:00 h and 18:00 h (Rodela and Wright, 2006). We first hypothesized that the diurnal J_{urea} pattern in *R. marmoratus* is due to metabolic changes. In particular, fluctuating tissue urea concentrations alter the plasma-to-water urea gradient and these variations are reflected in the pattern of J_{urea}. Measurements of oxygen consumption in fasted *R. marmoratus* demonstrated a diurnal pattern with the highest rates occurring during midday. Diurnal fluctuations in urea, but not ammonia tissue concentrations paralleled rhythms observed in oxygen uptake and urea excretion. The peak of each variable occurred between 12:00 h and 14:00 h. Taken together, these results suggest that urea excretion rates in *R. marmoratus* may be under metabolic influence.

In *R. marmoratus*, urea is derived from the routine turnover of uric acid or arginine hydrolysis, rather than the ornithine–urea cycle (Frick and Wright, 2002b). One possibility to explain the diurnal urea excretion pattern in fasted *R. marmoratus*, is that urea is produced periodically. The highest concentrations of urea occurred at 12:00 h (~6 mmol l^{-1}) and lowest levels were observed at 18:00 h (~1.6 mmol l^{-1}). This is a profound change (~ 3.8-fold) in whole body urea levels in a relatively short period of time. Although plasma urea concentrations change by approximately sevenfold over 2 h in gulf toadfish (*O. beta*) due to their peculiar pattern of pulsatile urea excretion (Wood et al., 1997), this is not surprising given that the extracellular fluid volume is approximately 5% of the mass of the fish. Whether or not similar dramatic changes occur in the intracellular compartment in *O. beta* is unknown, to our knowledge. Smaller diurnal changes in body urea (~ twofold) content were observed in the ureogenic gobiid *M. abei*, which also has a diurnal urea excretion pattern (Kajimura et al., 2002). The activity of several hepatic enzymes related to urea synthesis in *M. abei* did not show any diurnal changes. Although it is unlikely that enzymes would be rapidly up- or downregulated within a few hours of each day, temporal changes in substrate concentrations may influence the rates of urea synthesis and ultimately affect urea excretion rates. Hence, the results generally support the 'metabolic' hypothesis stated above, but upon closer examination of the data, it is clear that other factors

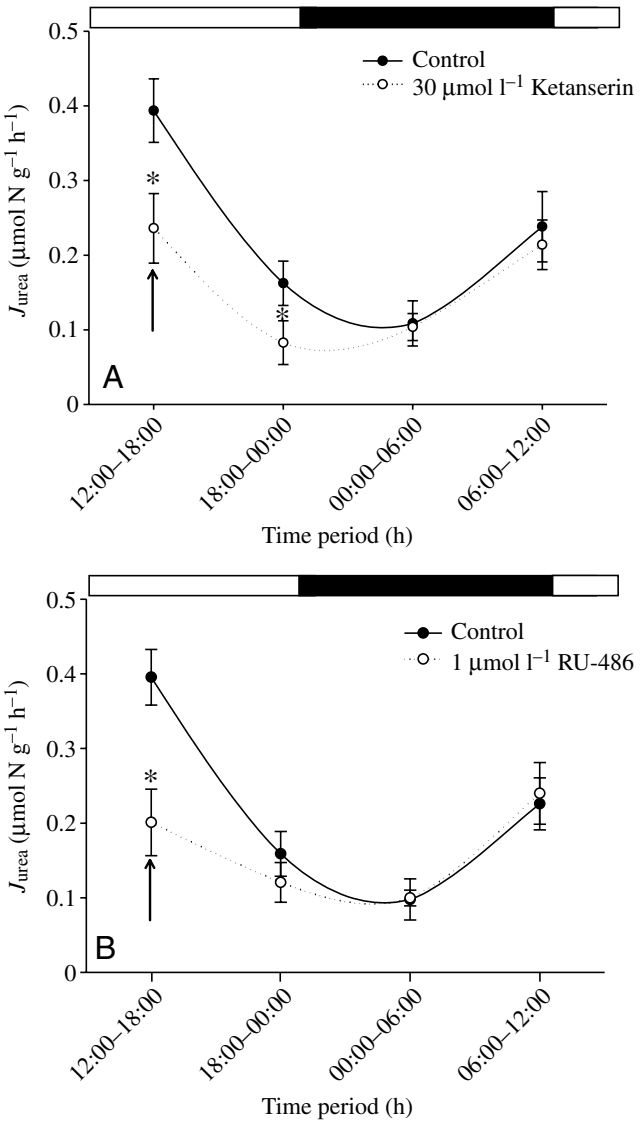

Fig. 4. The effect of a single 6 h exposure to 30 μmol l^{-1} ketanserin (A) and 1 μmol l^{-1} RU-486 (B) on J_{urea} in *R. marmoratus* (Series VI). Values are expressed as means ± s.e.m. (N=8). The arrows indicates the time at which the single dose of the drug was administered. Treatment values (dotted lines) labeled with an asterisk are significantly different from control values (solid lines) at corresponding time intervals (P<0.05). White and black bars above the graph indicate periods of light and dark, respectively.

are involved in regulating the diurnal pattern of urea excretion in *R. marmoratus*.

Measurements of oxygen consumption from fasted *R. marmoratus* revealed a 24 h diel pattern with approximately a twofold increase in oxygen consumption rates during midday (12:00 h) compared to values obtained at 18:00 h. Clear diurnal patterns in oxygen consumption have been observed in other fish species (Brett and Zala, 1975; Ghosh et al., 1986; Ghosh et al., 1990) but the peaks in oxygen consumption are usually indicative of active periods. The oxygen uptake rates obtained in the present study potentially reflect daily changes in routine

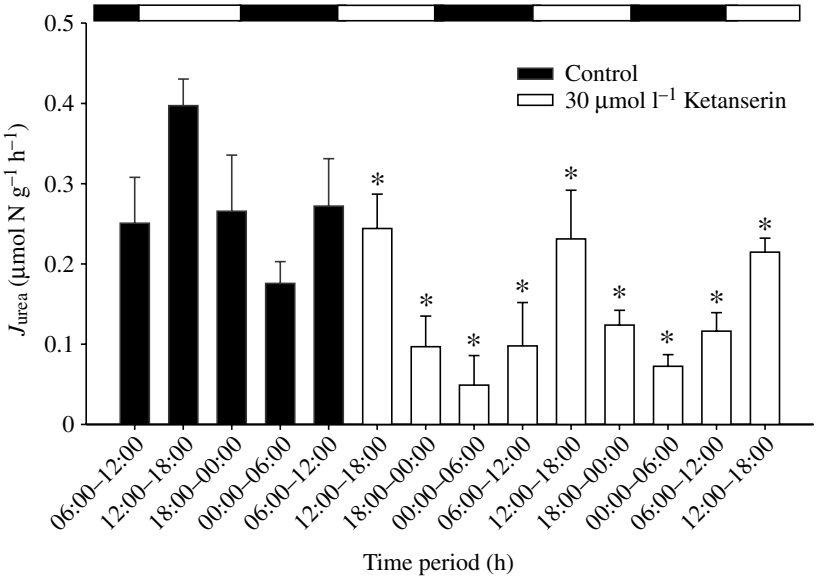

Fig. 5. The effect of 30 μmol l^{-1} ketanserin on the diurnal cycle of J_{urea} in *R. marmoratus* (*Series VII*). For each 6 h time interval following the first exposure to ketanserin (12:00–18:00 h), fresh 30 μmol l^{-1} solutions were added to the chamber. White and black bars above the graph indicate periods of light and dark, respectively. Values are expressed as means + s.e.m. (*N*=8). Treatment values (white bars) labeled with an asterisk are significantly different from control values (black bars) at corresponding time intervals (P<0.05).

and active metabolic rate (Rao, 1968; Sims et al., 1993). Hence, the daily variation in urea excretion may not be due to diurnal changes in the rates of all metabolic processes. This idea is supported by the lack of diurnal variation in ammonia content and excretion rates.

There is indirect evidence in *M. abei* that diurnal fluctuations in urea excretion and urea content are correlated with changes in the permeability of membranes to urea (Kajimura et al., 2002). Hence, the diurnal changes in J_{urea} in *R. marmoratus* may be partly explained by diurnal changes in gill urea permeability. Our second hypothesis stated that regulation of J_{urea} in *R. marmoratus* is influenced by 5-HT and cortisol, that is, changes in the levels of these hormones results in a change affecting rates of urea excretion. Indeed, ketanserin inhibited J_{urea}, but not J_{amm}. The 5-HT antagonist, therefore, probably did not alter protein catabolism but was specific to J_{urea} mechanisms.

Of the seven different families of receptors, the 5-HT_2 receptor subtype family is the lone category of 5-HT receptors that display sensitivity to ketanserin (Barnes and Sharp, 1999). Evidence from numerous studies on teleosts have revealed that ketanserin blocks 5-HT-mediated locomotor activity in zebrafish (Brustein et al., 2003) and 5-HT-mediated gonadotropin release in goldfish (Somoza et al., 1988). Furthermore, 5-HT-induced increases in plasma urea concentrations and excretion rates in *O. beta* were abolished, in a dose-dependent fashion, by ketanserin (McDonald and Walsh, 2004). These findings indicate that the effects of ketanserin specifically target 5-HT receptors in teleost fish, but whether these physiological responses are specifically mediated by the 5-HT_2 subtype in teleosts remains to be ascertained.

Repeated exposure to ketanserin for 2 consecutive days revealed that the diurnal J_{urea} pattern still remained under these conditions; however the absolute rates of excretion were significantly diminished. In *O. beta*, injections of a 5-HT_2 receptor agonist elicited substantial urea pulses of comparable size and duration to natural pulses, whereas a 5-HT_2 receptor antagonist inhibited pulsatile urea excretion implicating 5-HT_2 receptors in the rapid activation of the toadfish UT (McDonald and Walsh, 2004). Based on the ketanserin experiments in the present study, it is possible that 5-HT may have a role in regulating putative urea transport proteins in *R. marmoratus*; however, further experiments are required to explore this idea.

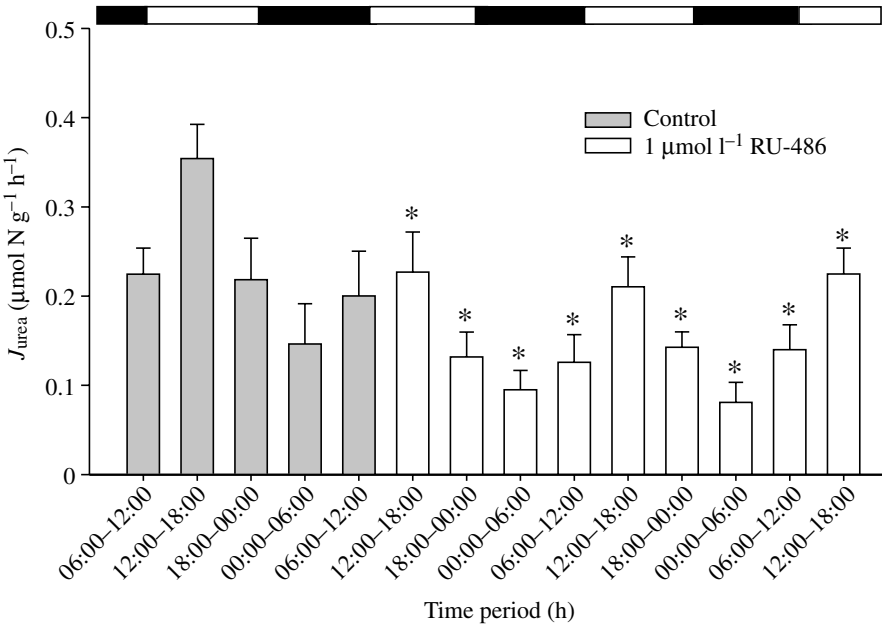

Fig. 6. The effect of 1 μmol l^{-1} RU-486 on the diurnal cycle of J_{urea} in *R. marmoratus* (Series VII). For each 6 h time interval following the initial exposure to RU-486 (12:00–18:00 h), fresh 1 μmol l^{-1} solutions were added to the chamber. White and black bars above the graph indicate periods of light and dark, respectively. Values are expressed as means + s.e.m. (*N*=8). Treatment values (white bars) labeled with an asterisk are significantly different from control values (black bars) at corresponding time intervals (P<0.05).

Ketanserin may be affecting other neuroendocrine cascades that ultimately influence urea excretion pathways. In rats, brain serotonergic activity increases corticosterone release (Fuller, 1996). In rainbow trout, 5-HT agonists elevate plasma cortisol levels in a dose-dependent manner, suggesting that this hormone has an important role in cortisol mobilization (Winberg et al., 1997). It is possible that fluctuations in 5-HT activity in the brain in *R. marmoratus* affect circulating cortisol levels specifically through the HPI axis, which in turn influences various aspects of urea metabolism and excretion (see below).

Exposure to RU-486 resulted in an inhibition of urea, but not ammonia excretion in *R. marmoratus*. As with ketanserin, the inhibition of urea excretion following exposure to a solitary dose of RU-486 was reversible, therefore confirming that the drug was entering the fish and influencing J_{urea} pathways. J_{urea} rates returned to control levels during the next 6 h period post-exposure. Similar to ketanserin, repeated application of RU-486 did not alter the timing of the diurnal cycle; however, the absolute rates of urea excretion were significantly depressed. Therefore, blocking cortisol appears to significantly decrease urea excretion in *R. marmoratus*, in contrast to other studies pertaining to urea transport regulation in fish and mammals by glucocorticoids (Marsh and Knepper, 1992; Wood et al., 1997; Wood et al., 2001; Peng et al., 2002; McDonald et al., 2004).

Inhibiting cortisol receptor activity by RU-486 may alter the rate of urea synthesis in *R. marmoratus*, targeting enzymes in the arginolytic and/or uricolytic pathways. Exposure to exogenous cortisol triggers a twofold increase in arginase activity accompanied by an increase in allantoicase activity in the ammoniotelic sea raven, *Hemipterus americanus* (Vijayan et al., 1996). An increase in arginase activity would enhance arginine catabolism to form urea, whereas an increase in the uricolytic enzyme, allantoicase, would enhance the synthesis of urea from uric acid. *In vivo* exposure to cortisol in the rainbow trout resulted in an increase in circulating urea concentrations accompanied by an increase in branchial and renal excretion rates due to an altered blood-to-water urea gradient (McDonald and Wood, 2004). Thus, cortisol may specifically target urea metabolism in *R. marmoratus*. Furthermore, coupled with the evidence that 5-HT may also increase cortisol mobilization in fish (see above), it is possible that these two hormones act in tandem to increase arginolysis and uricolysis and therefore affect plasma urea concentrations and excretion rates.

Regulation of urea synthesis and excretion by hormones such as 5-HT and cortisol may have an adaptive significance in relation to predation risk in *R. marmoratus*. Many studies have implicated the involvement of cortisol in modifying behavioural processes and social rank in fish (e.g. Pottinger and Pickering, 1992; Gregory and Wood, 1999; DiBattista et al., 2005). Recent studies have shown that predation or threat of predation significantly raised cortisol levels or serotonergic activity in a number of fish species (Winberg et al., 1993; Woodley and Peterson, 2003). It is possible that predation risk or perceived predation risk by *R. marmoratus* is greatest between 12:00 h and 14:00 h, and elevated hormone levels, in turn, alter nitrogen excretion rates. Such a strategy may help to evade predators. Preliminary findings from Barimo and Walsh (Barimo and Walsh, 2005) have shown that *O. beta* may be excreting pulses of urea as a form of chemical crypsis to avoid predator detection by the grey snapper (*Lutjanus griseus*). There is very little ecological information, however, on *R. marmoratus* in the wild.

To conclude, there is a strong synchrony between the diurnal patterns of oxygen consumption, whole body urea content and urea excretion rates in *R. marmoratus*. These results support the hypothesis that the diurnal urea excretion pattern is under metabolic control, but several factors suggested a more complex regulatory process. Cortisol and 5-HT receptor antagonists inhibited the absolute rates of J_{urea} but did not influence the timing of the J_{urea} pattern. We propose that 5-HT may regulate the rate of J_{urea} by affecting the permeability of the gill to urea *via* a putative gill urea transport protein and/or indirectly altering urea synthesis mediated by other neuroendocrine agents. We further propose that changes in cortisol levels may alter the rate of urea synthesis, which in turn alters the blood-to-water urea gradient, ultimately influencing the rate of urea excretion in *R. marmoratus*.

The authors would like to thank Dr David Noakes for the donation of the fish. Furthermore, the authors would like to extend their gratitude to Dr Don Stevens for his help with the statistical analyses and Dr Nick Bernier for his guidance in the experimental design. This work was supported by a NSERC Discovery Grant to P.A.W.

References

Abel, D. C., Koenig, C. C. and Davis, W. P. (1987). Emersion in the mangrove forest fish *Rivulus marmoratus*: a unique response to hydrogen sulfide. *Environ. Biol. Fishes* **18**, 67-72.

Alsop, D. and Wood, C. M. (1997). The interactive effects of feeding and exercise on oxygen consumption, swimming performance and protein usage in juvenile rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.* **200**, 2337-2346.

Barimo, J. F. and Walsh, P. J. (2005). Ureotely in the gulf toadfish, *Opsanus beta*: a means of predator avoidance by chemical crypsis? *Integr. Comp. Biol.* **44**, 520.

Barnes, N. M. and Sharp, T. (1999). A review of central 5-HT receptors and their function. *Neuropharmacology* **38**, 1083-1152.

Brett, J. R. and Zala, C. A. (1975). Daily pattern of nitrogen excretion and oxygen consumption of sockeye salmon (*Oncorhynchus nerka*) under controlled conditions. *Can. J. Fish. Aquat. Sci.* **32**, 2479-2486.

Brustein, E., Chong, M., Holmqvist, B. and Drapeau, P. (2003). Serotonin patterns locomotor network activity in the developing zebrafish by modulating quiescent periods. *J. Neurobiol.* **57**, 303-322.

Collander, R. (1937). The permeability of plant protoplasts to nonelectrolytes. *Trans. Faraday Soc*. **33**, 985-990.

Dasmahapatra, A. K. and Lee, P. C. (1993). Down regulation of CYP 1A1 by glucocorticoids in trout hepatocytes in vitro. *In Vitro Cell. Dev. Biol. Anim.* **29**, 643-648.

DiBattista, J. D., Anisman, H., Whitehead, M. and Gilmour, K. M. (2005). The effects of cortisol administration on social status and brain monoaminergic activity in rainbow trout *Oncorhynchus mykiss*. *J. Exp. Biol.* **208**, 2707-2718.

Fivizzani, A. J., Speiler, R. E. and Noeske, T. A. (1984). The influence of ambient temperature on the daily variation of serum cortisol in the banded killifish, *Fundulus diaphanus*. *J. Interdiscipl. Cycle Res.* **15**, 3-8.

Frick, N. T. and Wright, P. A. (2002a). Nitrogen metabolism and excretion

in the mangrove killifish *Rivulus marmoratus*. I. The influence of environmental salinity and external ammonia. *J. Exp. Biol.* **205**, 79-89.

Frick, N. T. and Wright, P. A. (2002b). Nitrogen metabolism and excretion in the mangrove killifish *Rivulus marmoratus*. II. Significant ammonia volatilization in a teleost during air-exposure. *J. Exp. Biol*. **205**, 91-100.

Fuller, R. W. (1996). Serotonin receptors involved in regulation of pituitary-adrenocortical function in rats. *Behav. Brain Res*. **73**, 215-219.

Galluci, E., Micelli, S. and Lippe, C. (1971). Non-electrolyte permeability across thin lipid membranes. *Arch. Int. Physiol. Biochem*. **79**, 881-887.

Ghosh, T. K., Moitra, A., Kunwar, G. K. and Munshi, J. S. (1986). Bimodal oxygen uptake in a freshwater air-breathing fish, *Notopterus chitala*. *Japan J. Ichthyol*. **33**, 280-285.

Ghosh, T. K., Kunwar, G. K. and Munshi, J. S. D. (1990). Diurnal variation in the bimodal oxygen uptake in an air-breathing catfish, *Clarias batrachus*. *Japan J. Ichthyol*. **37**, 56-59.

Gregory, T. R. and Wood, C. M. (1999). Interactions between individual feeding behaviour, growth, and swimming performance in juvenile rainbow trout (*Oncorhynchus mykiss*) fed different rations. *Can. J. Fish. Aquat. Sci*. **56**, 479-486.

Halberg, F., Johnson, E. A., Nelson, W., Runge, W. and Sothern, R. B. (1972). Autorhythmometry: procedures for physiologic self-measurement and their analysis. *Physiol. Teach.* **1**, 1-11.

Ivancic, I. and Degobbis, D. (1984). An optimal manual procedure for ammonia analysis in natural waters by the indophenol blue method. *Water Res.* **18,** 1143-1147.

Kajimura, M., Iwata, K. and Numata, H. (2002). Diurnal nitrogen excretion rhythm of the functionally ureogenic gobiid fish *Mugilogobius abei*. *Comp. Biochem. Physiol.* **131B**, 227-239.

Kaushik, S. J. and de Oliva-Teles, A. (1985). Effect of digestible energy on nitrogen and energy balance in rainbow trout. *Aquaculture* **50**, 89-101.

Kaushik, S. J. and Gomes, E. F. (1988). Effect of frequency of feeding on nitrogen and energy balance in rainbow trout under maintenance conditions. *Aquaculture* **73**, 207-216.

King, J. C., Abel, D. C. and DiBona, D. R. (1989). Effects of salinity on chloride cells in the euryhaline cyprinodont fish *Rivulus marmoratus*. *Cell Tissue Res*. **257**, 367-377.

Kun, E. and Kearney, E. B. (1974). Ammonia. In *Methods of Enzymatic Analysis.* Vol. 4 (ed. H. U. Bergmeyer), pp. 1802-1806. New York: Academic Press.

Marsh, D. J. and Knepper, M. A. (1992). Renal handling of urea. In *Handbook of Physiology: Section 8 Renal Physiology* (ed. E. E. Windhager), pp. 1317-1348. New York: Oxford University Press.

McDonald, M. D. and Walsh, P. J. (2004). Dogmas and controversies in the handling of nitrogenous wastes: 5-HT_2-like receptors are involved in triggering pulsatile urea excretion in the gulf toadfish, *Opsanus beta*. *J. Exp. Biol.* **207**, 2003-2010.

McDonald, M. D. and Wood, C. M. (2004). Evidence for facilitated diffusion of urea across the gill basolateral membrane of the rainbow trout (*Oncorhynchus mykiss*). *Biochim. Biophys. Acta* **1663**, 89-96.

McDonald, M. D., Walsh, P. J. and Wood, C. M. (2002). Branchial and renal excretion of urea and urea analogues in the plainfin midshipman, *Porichthys notatus*. *J. Comp. Physiol.* **172**, 699-712.

McDonald, M. D., Wood, C. M., Grosell, M. and Walsh, P. J. (2004). Glucocorticoid receptors are involved in the regulation of pulsatile urea excretion in toadfish. *J. Comp. Physiol. B* **174**, 649-658.

Mistry, A. C., Honda, S., Hirata, T., Kato, A. and Hirose, S. (2001). Eel urea transporter is localized to chloride cells and is salinity dependent. *Am. J. Physiol.* **281**, R1594-R1604.

Nelson, W., Tong, Y. L., Lee, J. K. and Halberg, F. (1979). Methods for cosinor-rhythmometry. *Chronobiologia* **6**, 304-323.

Peng, T., Sands, J. M. and Bagnasco, S. M. (2002). Glucocorticoids inhibit transcription and expression of the UT-A urea transporter gene. *Am. J. Physiol.* **282**, F853-F858.

Peter, R. E., Hontela, A., Cook, A. F. and Paulencu, C. R. (1978). Daily cycles in serum cortisol levels in the goldfish: effects of photoperiod, temperature, and sexual condition. *Can. J. Zool*. **56**, 2443-2448.

Pottinger, T. G. and Pickering, A. D. (1992). The influence of social interaction on the acclimation of rainbow trout, *Oncorhynchus mykiss* (Walbaum) to chronic stress. *J. Fish Biol.* **41**, 435-447.

Rahmatullah, M. and Boyde, T. R. C. (1980). Improvements in the determination of urea using diacetyl monoxime; methods with and without deproteinisation. *Clin. Chem. Acta* **107**, 3-9.

Rao, G. M. (1968). Oxygen consumption of rainbow trout (*Salmo gairdneri*) in relation to activity and salinity. *Can. J. Zool*. **46**, 781-786.

Rodela, T. M. and Wright, P. A. (2006). Characterization of diurnal urea excretion in the mangrove killifish, *Rivulus marmoratus. J. Exp. Biol.* **209**, 2696-2703.

Sathiyaa, R. and Vijayan, M. M. (2003). Autoregulation of glucocorticoid receptors by cortisol in rainbow trout hepatocytes. *Am. J. Physiol. Cell Physiol.* **284**, C1508-C1515.

Sims, D. W., Davies, S. J. and Bone, Q. (1993). On the diel rhythms in metabolism and activity of post-hatching lesser spotted dogfish, *Scyliorhinus canicula*. *J. Fish Biol.* **43**, 749-754.

Smith, C. P. and Wright, P. A. (1999). Molecular characterization of an elasmobranch urea transporter. *Am. J. Physiol*. **276**, R622-R666.

Smith, M. A. and Thorpe, A. (1976). Nitrogen metabolism and trophic input in relation to growth in freshwater and saltwater *Salmo gairdneri*. *Biol. Bull.* **150**, 139-151.

Somoza, G. M., Yu, K. L. and Peter, R. E. (1988). Serotonin stimulates gonadotropin release in female and male goldfish, *Carassius auratus* L. *Gen. Comp. Endocrinol.* **72**, 374-382.

van Veen, T., Laxmyr, L. and Borg, B. (1982). Diurnal variation of 5-hydroxytryptamine content in the pineal organ of the yellow eel (*Anguilla anguilla* (L.)). *Gen. Comp. Endocrinol.* **46**, 322-326.

Vijayan, M. M., Mommsen, T. P., Glémet, H. C. and Moon, T. W. (1996). Metabolic effects of cortisol in a marine teleost, the sea raven. *J. Exp. Biol.* **199**, 1509-1514.

Walsh, P. J., Heitz, M. J., Campbell, C. E., Cooper, G. J., Medina, M., Wang, Y. S., Goss, G. G., Vincek, V., Wood, C. M. and Smith, C. P. (2000). Molecular characterization of a urea transporter in the gill of the gulf toadfish (*Opsanus beta*). *J. Exp. Biol.* **203**, 2357-2364.

Walsh, P. J., Grosell, M., Goss, G. G., Bergman, H. L., Bergman, A. N., Wilson, P., Laurent, P., Alper, S. L., Smith, C. P., Kamunde, C. et al. (2001). Physiological and molecular characterization of urea transport by the gills of the Lake Magadi tilapia (*Alcolapia grahami*). *J. Exp. Biol.* **204**, 509-520.

White, A. and Fletcher, T. C. (1984). Radioimmunoassay of serum cortisol in the plaice (*Pleuronectes platessa* L.). *Gen. Comp. Endocrinol.* **53**, 410-417.

Wicks, B. J. and Randall, D. J. (2002). The effect of feeding and fasting on ammonia toxicity in juvenile rainbow trout, *Oncorhynchus mykiss*. *Aquat. Toxicol.* **59**, 71-82.

Winberg, S., Myberg, A. A. and Nilsson, G. E. (1993). Predator exposure alters brain serotonin metabolism in bicolour damselfish. *NeuroReport* **4**, 399-402.

Winberg, S., Nilsson, A., Hylland, P., Soderstom, V. and Nilsson, G. E. (1997). Serotonin as a regulator of hypothalamic-pituitary-interrenal activity in teleost fish. *Neurosci. Lett*. **230**, 113-116.

Wood, C. M., Hopkins, T. E. and Walsh, P. J. (1997). Pulsatile urea excretion in the toadfish (*Opsanus beta*) is due to a pulsatile excretion mechanism, not a pulsatile production mechanism. *J. Exp. Biol.* **200**, 1039-1046.

Wood, C. M., Gilmour, K. M., Perry, S. F., Part, P., Laurent, P. and Walsh, P. J. (1998). Pulsatile urea excretion in gulf toadfish (*Opsanus beta*): evidence for activation of a specific facilitated diffusion transport system. *J. Exp. Biol*. **201**, 805-817.

Wood, C. M., Warne, J. M., Wang, Y., McDonald, M. D., Balment, R. J., Laurent, P. and Walsh, P. J. (2001). Do circulating plasma AVT and/or cortisol levels control pulsatile urea excretion in the gulf toadfish (*Opsanus beta*)? *Comp. Biochem. Physiol*. **129**, A859-A872.

Wood, C. M., McDonald, M. D., Sundin, L., Laurent, P. and Walsh, P. J. (2003). Pulsatile urea excretion in the gulf toadfish: mechanisms and controls. *Comp. Biochem. Physiol*. **136**, B667-B684.

Woodley, C. M. and Peterson, M. S. (2003). Measuring responses to simulated predation threat using behavioral and physiological metrics: the role of aquatic vegetation. *Oecologia* **136**, 155-160.

Wright, P. A. and Wood, C. M. (1985). An analysis of branchial ammonia excretion in the freshwater rainbow trout: effects of environmental pH and sodium uptake blockade. *J. Exp. Biol.* **114**, 329-353.

Wright, P. A., Part, P. and Wood, C. M. (1995). Ammonia and urea excretion in the tidepool sculpin (*Oligocottus maculosus*): sites of excretion, effects of reduced salinity and mechanisms of urea transport. *Fish Physiol. Biochem.* **14**, 111-123.

Zuanreiter, M., Brandstatter, R. and Goldschmid, A. (1998). Evidence for an endogenous clock in the retina of rainbow trout: circadian rhymicity of serotonin metabolism. *Neuroreport* **9**, 1475-1479.