The Journal of Experimental Biology 209, 2696-2703
Published by The Company of Biologists 2006
doi:10.1242/jeb.02288

Characterization of 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
*Author for correspondence (e-mail: patwrigh@uoguelph.ca)

Accepted 25 April 2006

Summary

An unusual characteristic of nitrogen excretion in the ammoniotelic mangrove killifish *Rivulus marmoratus* is that urea is excreted (J_{urea}) in a distinct diurnal pattern, whereas ammonia is excreted (J_{amm}) at a steady rate. In this study we tested the hypothesis that the diurnal pattern in *R. marmoratus* is an endogenously generated pattern that is characterized as a circadian rhythm. This hypothesis was tested by measuring J_{urea} and J_{amm} following manipulation of feeding or lighting regimes. The diurnal J_{urea} pattern in food-deprived *R. marmoratus* had a 24 h periodicity under normal conditions of 12 h:12 h light:dark (12:12 L:D) with 72% more urea excreted during 12:00 h and 18:00 h. In contrast, there was no significant pattern in J_{amm}. Fed fish (12:12 L:D) demonstrated a diurnal pattern in both J_{urea} and J_{amm} with up to an eightfold increase in excretion rates compared with rates obtained from food-deprived fish. Patterns of J_{urea} were free running with a 24 h period under conditions of continuous darkness (0:24 L:D). Exposure to an inverse photoperiod (12:12 D:L) resulted in entrainment of the J_{urea} pattern to the new photoperiod, with the highest rates of excretion occurring during midday of the new photoperiod. In contrast to *R. marmoratus*, nitrogen excretion rates in the zebrafish *Danio rerio* remained constant over time. The results of this study show that J_{urea} in *R. marmoratus* demonstrates the characteristics of a circadian rhythm: a 24 h periodicity, a free-running rhythm in continuous conditions, and entrainment to new photoperiods.

Key words: nitrogen excretion, ammonia excretion, photoperiod, *Danio rerio*, *Rivulus marmoratus*, free-running, entrainment.

Introduction

Many behavioral, biochemical and physiological oscillations result in rhythmic circadian events that are often synchronized to various environmental cues. In many cases, these daily oscillations are more than a passive response to variations in the organism's surroundings; they are in fact controlled by an endogenous and complex timekeeping system. The classical view of the circadian timekeeping model describes a hierarchical arrangement of highly specialized pathways connected to a central pacemaker that accepts input from both photic and non-photic influences (Yanelli and Harrington, 2004). Closely coupled biochemical and molecular mechanisms are upregulated in the central pacemaker, triggering neuroendocrine cascades that result in the coordination of various oscillators in the central and peripheral tissues (Jin et al., 1999; Whitmore et al., 2000; Cahill, 2002).

Many catabolic processes are extremely complex. They require an internal temporal organization and integration of various physiological and biochemical pathways. The central pacemaker facilitates the synchronization of these functions. Excretion of nitrogenous wastes requires the coordination of digestive pathways, catabolic processes in the liver and transport proteins in various tissues. The majority of teleosts are ammoniotelic, excreting 70–90% of their nitrogenous waste products as ammonia (Wood, 1993). The remaining 10–30% is largely due to the excretion of urea (Kajimura et al., 2004). The mangrove killifish *Rivulus marmoratus* is no exception to this general rule, although an unusual characteristic of urea excretion (J_{urea}) is the fact that urea is excreted in a diurnal pattern, whereas ammonia is excreted (J_{amm}) at a steady rate (Frick and Wright, 2002a). The majority of urea is excreted during the day, with the peak of J_{urea} occurring between the hours of 12:00 h and 18:00 h. Two other teleosts studied to date, the gobiid fish *Mugilogobius abei* and the marine toadfish *Opsanus beta*, both ureogenic, have a non-uniform pattern of J_{urea}. The ammoniotelic *R. marmoratus* is a small (~100 mg) self-fertilizing fish that inhabits temporal pools and crab burrows in mangrove habitats. They are also known to survive for weeks in moist leaf litter and detritus, as well as tolerating a range of water conditions including variations in water temperature, salinity and oxygen tensions (Abel et al., 1987; King et al., 1989; Davis et al., 1990; Frick and Wright, 2002a; Frick and Wright, 2002b). The purpose of our study was to determine whether

the diurnal pattern of J_{urea} in *R. marmoratus* meets the criteria for a circadian rhythm.

The foremost defining feature of circadian rhythms is a periodicity of approximately 24 h, with only slight deviations from this value. Although biotic and abiotic factors may influence the timing of the phases and length of the period, the rhythm should remain in the absence of these factors. If a diurnal or nocturnal pattern is sustained under constant conditions, the rhythm is defined as free-running. For instance, goldfish *Carassius auratus*, held under conditions of constant darkness, showed free-running rhythms in locomotor and feeding activity with an average of 25.3±1.8 h and 24.4±1.7 h, respectively (Sánchez-Vázquez et al., 1996). Observations on locomotor activity in zebrafish *Danio rerio* under similar constant conditions demonstrated a comparable pattern with a period of 25.5 h (Cahill et al., 1998; Hurd et al., 1998). The third characteristic of a circadian rhythm is that they can be entrained to new environmental stimuli, which can change or reset the phase of a circadian clock. The light:dark cycle clearly appears to be the dominant zeitgeber for all organisms; however, other non-photic stimuli such as feeding can have an equally powerful effect on resetting the clock (Yanelli and Harrington, 2004). Light-sensitive circadian oscillators have been isolated in many peripheral tissues of the zebrafish, including the heart, kidney and liver (Whitmore et al., 1998; Whitmore et al., 2000). The rhythms in zebrafish are entrained fairly quickly, that is, within 2–3 days of exposure to the new photoperiod. Furthermore, cell lines derived from zebrafish embryos have demonstrated that these light-entrainable circadian oscillators are present quite early in development (Pando et al., 2001).

In the present study we hypothesized that the diurnal J_{urea} pattern in *R. marmoratus* follows a circadian rhythm. We predict that J_{urea} in *R. marmoratus* has a periodicity of 24 h, which is sustained under constant conditions, and can be entrained to different photoperiods. J_{urea} and J_{amm} were measured in fasted fish under normal conditions (12 h:12 h light:dark), continuous darkness (0 h:24 h light:dark), and under an inverted photoperiod. Comparisons were made between fasted and fed individuals under normal light:dark conditions to determine if feeding influenced the diurnal pattern. Furthermore, nitrogen excretion rates were measured in zebrafish *Danio rerio*, a tropical fish of similar size and metabolic demand to *R. marmoratus*. In a companion study, we investigated the mechanisms regulating J_{urea} patterns in *R. marmoratus* (Rodela and Wright, 2006).

Materials and methods

Laboratory animals

A colony of mangrove killifish *Rivulus marmoratus* Poey was held at the Hagen Aqualab at the University of Guelph (Guelph, ON, Canada) in brackish water (15‰ seawater) (Frick and Wright, 2002a). Mature fish (0.07–0.25 g) were held in individual translucent containers in 60 ml of 15‰ artificial seawater (pH 8.1) using distilled water and marine salt (Instant Ocean™, Crystal Sea, Baltimore, MA, USA). Water in all containers was changed biweekly. The fish were held in environmental chambers at 25°C on a photoperiod of 12 h:12 h light (L):dark (D). Fish were fed *Artermia salina* nauplii once a day 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 unless otherwise indicated.

Adult zebrafish *Danio rerio* Hamilton (DAP International, Etobicoke, ON, Canada) were kept in static aerated freshwater (pH 8.1, 25°C, 12 h:12 h L:D) in 2 l plastic holding tanks. Zebrafish (0.27–0.37 g) were fed TetraMin fish flakes (Pet Paradise, Guelph, ON, Canada) every other day except 48 h prior and for the duration of the experiments. Treatment of animals followed approved experimental protocols and guidelines of the University of Guelph Animal Care Committee.

Experimental protocol

Diurnal nitrogen excretion rhythms

To investigate the diurnal nitrogen excretion rhythms, five series of experiments were conducted.

Series I: nitrogen excretion measurements under 12 h:12 h L:D in fasted *R. marmoratus.*

Series II: nitrogen excretion measurements under 12 h:12 h L:D in fed *R. marmoratus.*

Series III: nitrogen excretion measurements under 0 h:24 h L:D in fasted *R. marmoratus.*

Series IV: nitrogen excretion measurements under 12 h:12 h D:L in fasted *R. marmoratus.*

Series V: nitrogen excretion measurements under 12 h:12 h L:D in fasted *D. rerio.*

Series I

Initial experiments were conducted on fish under their natural photoperiod (pH 8.1, 25°C, 12 h:12 h L:D) in containers with 30 ml of water. Water samples were collected every 6 h for a 3-day period for measurement of J_{amm} and J_{urea}. Water was changed after each sampling interval to avoid the accumulation of urea or ammonia in the water. To eliminate the stress of handling, the fish were kept in a double container. The inner container was made out of mesh in the exact same configuration as the plastic translucent holding container. When a water change was required, 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 double-walled 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.

Series II–IV

For Series II–V, the fish were acclimated to the experimental conditions 2 days preceding the start of the experiment. For Series II, fish were fed 5 ml of *Artemia* nauplii at 12:30 h daily and the photoperiod remained unchanged (12 h:12 h L:D). In Series III, food-deprived fish were exposed to complete

darkness for 24 h (0 h:24 h L:D). For the final series of experiments (IV) in *R. marmoratus*, the fish were deprived of food and excretion rates were measured under an inverse photoperiod (12 h:12 h D:L).

Series V

As a comparison to other tropical teleost species, both J_{urea} and J_{amm} were measured in food-deprived zebrafish *Danio rerio*, exposed to a 12 h:12 h L:D photoperiod, also kept at 25°C. Water was sampled and renewed as described above for the killifish experiments. Following the termination of each series of experiments, the fish were blotted dry and weighed for calculation of excretion rates.

Analytical techniques

Urea and ammonia analysis

Urea levels in both freshwater and seawater samples were quantified by a colorimetric assay (Rahmatullah and Boyde, 1980) using an Ultrospec 3300 *Pro* spectrophotometer (Biochrom, Cambridge, UK). Ammonia content of seawater samples was measured as described elsewhere (Ivancic and Degobbis, 1984). Freshwater samples were analyzed for ammonia using a colorimetric assay method previously described (Verdouw et al., 1978). The rates of excretion (J) were calculated using the methodology outlined elsewhere (Wright and Wood, 1985).

Control experiments were performed to determine if bacterial contamination from fish sources, water sources or attached to the experimental chamber could have influenced nitrogen excretion rates. Fish were placed in 30 ml of water for a 1 h interval and subsequently removed. Ammonia and urea content were monitored in the water over the following 6 h. Analysis revealed that microbial contamination did not significantly affect nitrogen excretion rates (P>0.05).

Statistical analysis

The data are presented as mean ± standard error of the mean (s.e.m.). Evaluation of circadian rhythmicity was accomplished using the single cosinor method (Halberg et al., 1972; Nelson et al., 1979). In this model a sinusoidal curve with a predefined period of 24 h is fitted to the data by least squares regression. The cosine function

$$y_i = C_o + C \times \cos\left[(2\pi \times t_i) \div (24)^{-1} + \Phi\right]$$

defines three circadian parameters: C_o represents the mesor, a rhythm-adjusted median value; C is the amplitude (one half the difference between the maximum and minimum values); Φ is the time at which the curve reaches its highest value or acrophase. Time, which is represented by t_i, is measured as a fraction of 24 h. An F-test was used to determine the statistical significance of the circadian rhythm (P<0.05). This test allows subjective analysis of the hypothesis that the rhythm amplitude differs from zero. Furthermore, comparisons of the maximal absolute urea and ammonia excretion values between experimental series were performed using t-tests with either assumptions for equal or unequal variances (P<0.05). Verification of equal variances was accomplished using an F-test (P<0.05).

Results

Series I

Under a normal photoperiod of 12 h:12 h L:D, J_{urea} in food-deprived *R. marmoratus* followed a significant circadian rhythm ($F_{2,9}$=23.8, P=0.0003). The highest rates of J_{urea} occurred during the day between the hours of 12:00 h and 18:00 h with an amplitude of 47% of the meson; the acrophase occurred at 14:30±00:39 h (Fig. 1A). Fasted *R. marmoratus* were predominantly ammoniotelic, excreting 69–89% of their nitrogenous wastes in the form of ammonia. However, in contrast to J_{urea}, ammonia excretion did not demonstrate any significant circadian activity ($F_{2,9}$=0.84, P=0.46; Fig. 1B).

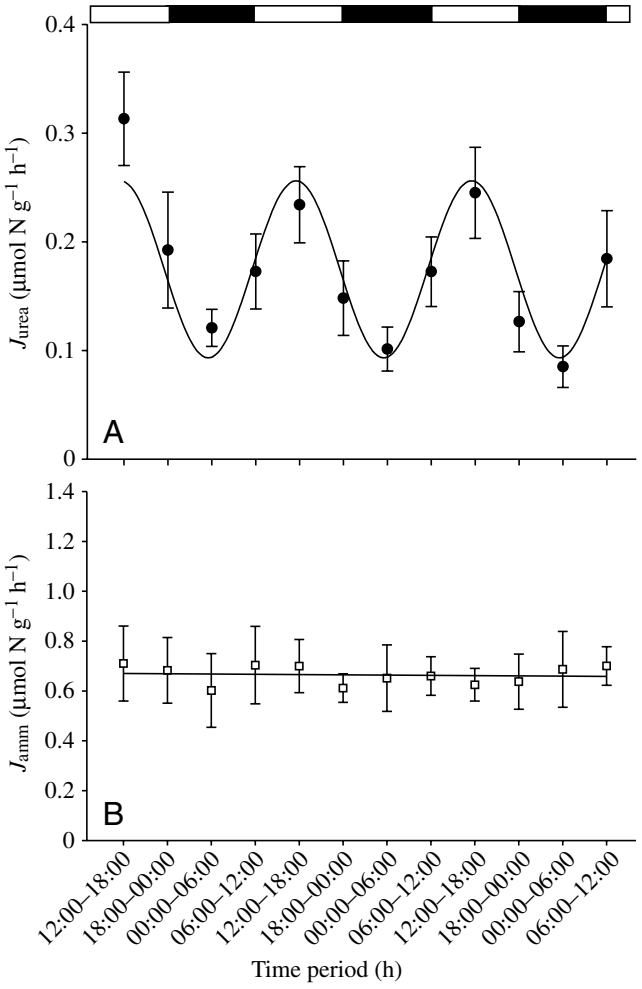

Fig. 1. Diurnal urea excretion (J_{urea}) (A) and ammonia excretion (J_{amm}) (B) in food-deprived *Rivulus marmoratus* exposed to a 12 h:12 h light:dark photoperiod (Series I). White and black bars above the graph indicate periods of light and dark, respectively. Values are expressed as means ± s.e.m. (N=8). J_{urea} values followed a significant rhythm ($F_{2,9}$=23.8, P=0.0003) whereas J_{amm} did not demonstrate a rhythm ($F_{2,9}$=0.84, P=0.46).

Series II

Fed fish demonstrated a significant circadian rhythm in both J_{urea} ($F_{2,9}$=18.3, P=0.0007) and J_{amm} ($F_{2,9}$=11.6, P=0.0032). J_{urea} in fed fish had peaks and troughs at times identical to the pattern observed in fasted fish (Fig. 2A). The amplitude of the oscillation was marked; it was 72% of the meson and the peak of the rhythm occurred at 15:45±00:18 h. In comparison with fasted fish (Fig. 1A), fed fish (Fig. 2A) excreted approximately 2.3–6.3 times more urea, the variations for the minimum and maximum rates respectively. Diurnal J_{amm} comprised 57–85% of total nitrogen wastes excreted in fed fish (Fig. 2B). The amplitude of the diurnal J_{amm} pattern was 88% of the meson with the highest rates of excretion occurring between 12:00 h and 18:00 h and the lowest rates occurring between 06:00 h to 12:00 h, just prior to feeding. The acrophase for the circadian rhythm for J_{amm} occurred at 15:50±0:12 h. J_{amm} rates were 1.4–8.3 times larger compared with values obtained from fasted fish.

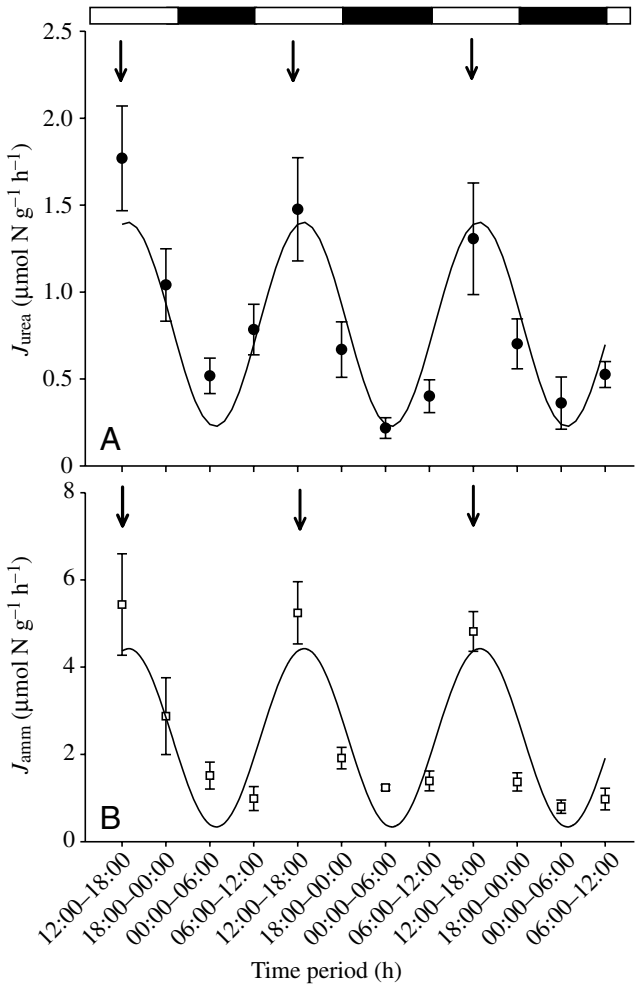

Fig. 2. Diurnal urea excretion (J_{urea}) (A) and ammonia excretion (J_{amm}) (B) in fed *Rivulus marmoratus* exposed to a 12 h:12 h light:dark photoperiod (Series II). White and black bars above the graph indicate periods of light and dark, respectively. Arrows mark the times when the fish were fed. Values are expressed as means ± s.e.m. (N=8). J_{urea} values followed a significant rhythm ($F_{2,9}$=18.3, P=0.0007). J_{amm} also demonstrated a significant rhythm ($F_{2,9}$=11.6, P=0.0032).

Series III

When food-deprived *R. marmoratus* were kept in darkness for 24 h (0 h:24 h L:D) the circadian pattern of J_{urea} persisted ($F_{2,9}$=60.7, P=0.00001; Fig. 3). The highest rates of J_{urea} occurred during 12:00 h and 18:00 h with the amplitude of the oscillation equaling 48% of the meson. The absolute rates of J_{urea} were significantly (1.6–1.8 times) lower relative to values obtained from fasted fish (12 h:12 h L:D). The acrophase occurred at 14:31±00:33 h. J_{amm} was constant over time ($F_{2,9}$=0.003, P=0.99) and excretion rates were not significantly different than values obtained from fasted fish (12 h:12 h L:D) (data not shown).

Series IV

A significant circadian rhythm in J_{urea} ($F_{2,9}$=39.6, P=0.00003) was present in fish acclimated to an inverted photoperiod (12 h:12 h D:L). The period of the single cycle remained at 24 h, however, the occurrence of the J_{urea} peaks and troughs were different from individuals on a normal photoperiod (12:12 h L:D). The highest rates of J_{urea} occurred between the 00:00 h and 06:00 h, with an amplitude 52% of the meson (Fig. 4). The acrophase occurred at 02:16±00:28 h. The absolute values of J_{urea} were significantly lower by 1.6 (peak) to 2.3 (trough) times compared to fish on a normal photoperiod. J_{amm} remained constant over time ($F_{2,9}$=0.48, P=0.63) and there was no

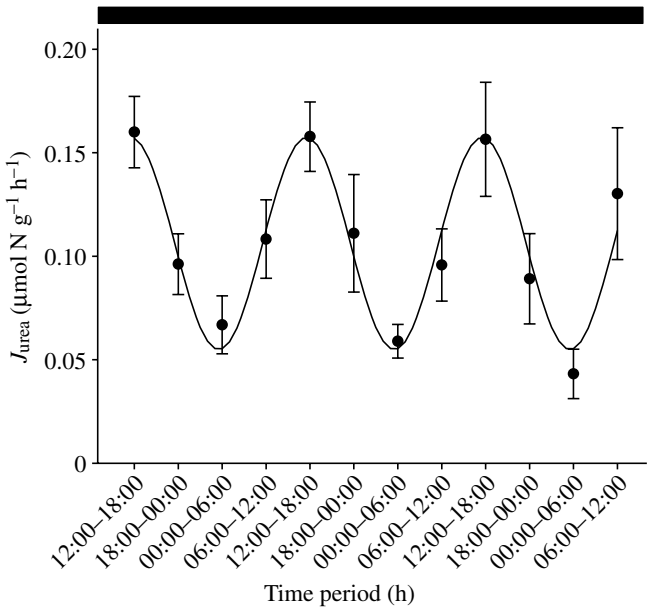

Fig. 3. Diurnal urea excretion (J_{urea}) in food-deprived *Rivulus marmoratus* exposed to a photoperiod of 0 h:24 h light:dark (Series III). The black bar above the graph indicates the dark period. Values are expressed as means ± s.e.m. (N=8). J_{urea} values followed a significant rhythm ($F_{2,9}$=60.7, P=0.00001).

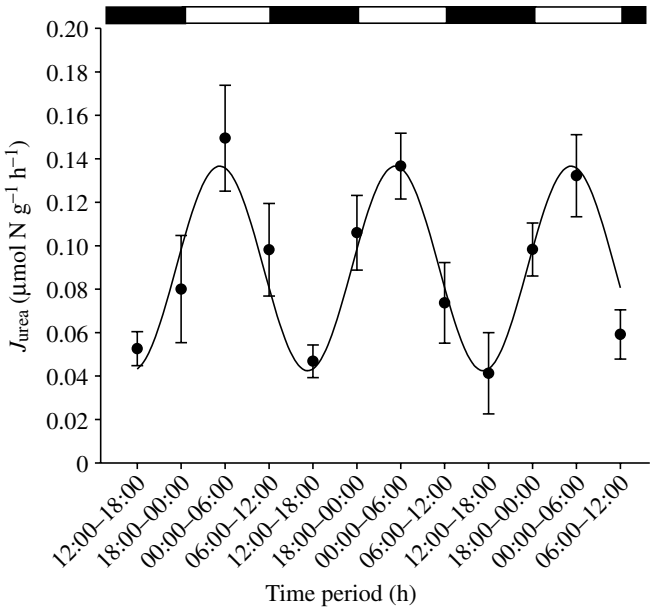

Fig. 4. Diurnal urea excretion (J_{urea}) in food-deprived *Rivulus marmoratus* exposed to an inverse photoperiod of12 h:12 h dark:light (Series IV). White and black bars above the graph indicate periods of light and dark, respectively. Values are expressed as means ± s.e.m. (N=8). J_{urea} values followed a significant rhythm ($F_{2,9}$=39.6, P=0.00003).

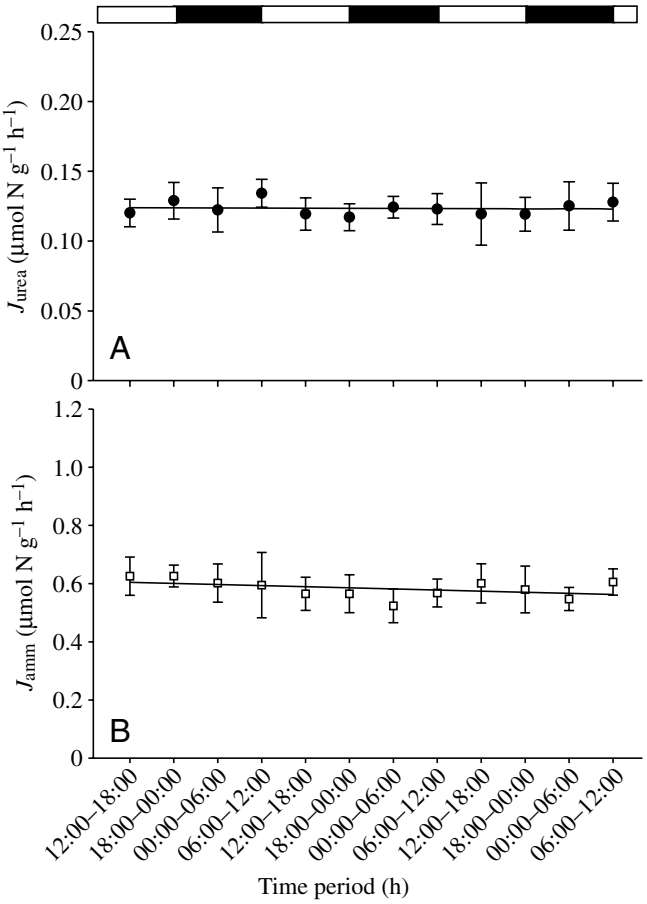

Fig. 5. Urea excretion (J_{urea}) (A) and ammonia excretion (J_{amm}) (B) in food-deprived *Danio rerio* exposed to a 12 h:12 h light:dark period (Series V). White and black bars above the graph indicate periods of light and dark, respectively. Values are expressed as means ± s.e.m. (N=8). Neither J_{urea} nor J_{amm} demonstrated a significant rhythm ($F_{2,9}$=2.33, P=0.15, $F_{2,9}$=1.28, P=0.33).

significant difference between J_{amm} rates in fish on an inverted photoperiod compared to a normal photoperiod (data not shown).

Series V

In fasted *D. rerio*, there was no significant circadian rhythm in urea and ammonia excretion ($F_{2,9}$=2.33, P=0.15, $F_{2,9}$=1.28, P=0.33; Fig. 5A,B). Approximately 83% of total nitrogen in *D. rerio* was excreted in the form of ammonia. J_{urea} was up to 2.6 times higher in *R. marmoratus* compared to *D. rerio* with the largest difference occurring during the daytime (12:00 h to 18:00 h).

Discussion

Free-running rhythms, 24 h periodicity and entrainment are all criteria for classification of a circadian rhythm. Our results support the hypothesis that the diurnal J_{urea} pattern in *R. marmoratus* follows a circadian rhythm. Under various photoperiod manipulations and feeding regimes, J_{urea} in *R. marmoratus* demonstrated a significant rhythmicity with a period of approximately 24 h, the acrophase occurring during mid-afternoon of the subjective day. In contrast, no diurnal pattern in J_{urea} was observed in *D. rerio*. Under continuous conditions of darkness, the J_{urea} pattern in *R. marmoratus* appeared to free-run with an approximate period of 24 h and similar amplitude and acrophase to fish exposed to a normal photoperiod. Furthermore, upon exposure to an inverted photoperiod, the diurnal pattern became entrained to the new conditions (12 h:12 h D:L), with the acrophase of J_{urea} pattern still occurring during subjective afternoon of the photoperiod.

Natural 24 h light:dark cycles play an important part in circadian rhythms as daily biological patterns often parallel environmental changes. In many cases, this synchrony between the animal and its external milieu gives the individual the ability to anticipate temporal changes in the surroundings. However, a true endogenously generated rhythm persists even in the absence of abiotic cues and continues unperturbed until the animal is presented with a new external cue that acts to reset the central clock (Whitmore et al., 2000; Pando and Sassone-Corsi, 2002). Locomotor activity in several fish species continues in a diurnal fashion with a periodicity of 24.4–25.5 h under constant dim or darkened conditions (Sánchez-Vázquez et al., 1996; Cahill et al., 1998; Hurd et al., 1998; Gerkema et al., 2000). Free-running rhythms in darkness have been recorded in electric discharge of the gymnotid electric fish, *Eigenmannia virescens* (Deng and Tseng, 2000). Furthermore, a daily pattern in J_{urea} in *M. abei* was maintained in darkness (Kajimura et al., 2002). In *R. marmoratus*, the peak

of J_{urea} in constant darkness occurred during the time of subjective day of the previous photoperiod (12 h:12 h L:D). Aschoff demonstrated that the free-running rhythm in diurnal animals tends to be slightly longer than 24 h, whereas the period length in nocturnal animals under free-running conditions is somewhat shorter than 24 h (Aschoff, 1981). As measurements for J_{urea} and J_{amm} in *R. marmoratus* were made over 6 h intervals, a precise estimation of period length was not possible.

An intriguing feature of the free-running rhythm (0 h:24 h L:D) in fasted *R. marmoratus* is that the absolute rates of J_{urea} were significantly lower (1.6–1.8 times) than values obtained from fasted fish held under a normal photoperiod (12 h:12 h L:D). Usually, under constant conditions, when some peripheral oscillators are removed from the light-sensitive influence of the master pacemaker the amplitude of the circadian rhythm becomes diminished over time and eventually ceases. For example, circulating thyroxine levels in red drum *Sciaenops ocellatus* (Leiner and MacKenzie, 2001) and melatonin rhythms in the pineal organ of the ayu *Plecoglossus altivelis* are reduced over time in constant darkness (Iigo et al., 2004). As well, circadian rhythms of *Clock* mRNA in the kidney and heart of zebrafish oscillated with a lower amplitude until they disappeared after a period of 2–3 days in darkness (Whitmore et al., 2000). Urea excretion rates in *R. marmoratus* were measured for three complete cycles after a 2-day acclimation period. It is possible that J_{urea} is under control of a peripheral oscillator and perhaps over time, a period of several weeks, the rhythm may become dampened and eventually cease under conditions of continuous darkness.

The transition between dark and light appears to be the dominant zeitgeber for all organisms. Lighting cues appear to play an important role in resetting the clock that controls J_{urea} in *R. marmoratus*. Like most animals, *R. marmoratus* can become entrained to new photoregimens within a day or two (Pohl, 1978). Measurements of zebrafish *Clock* gene expression in the heart and kidney revealed inherent and independent light-sensitive circadian oscillators that entrained to altered photoperiods within 2–3 days (Whitmore et al., 1998; Whitmore et al., 2000).

Under the influence of an inverted photoperiod, the amplitude of J_{urea} in food-deprived *R. marmoratus* was significantly lower (by twofold) relative to fish exposed to a normal photoperiod (Table 1). There are several possible explanations for this difference in amplitude. Non-photic stimuli such as feeding may act as an important cue regulating and resetting the clock (Yanelli and Harrington, 2004). It is possible that while light may have a role in entraining the J_{urea} pattern in *R. marmoratus* there may also be some confounding influences from a prior non-photic zeitgeber that may be resisting the change to the new photoperiod. Stokkan et al. reported that rhythmicity of certain genes in the liver of rats retain a phase relation to previous restricted feeding schedule even during fasting (Stokkan et al., 2001). Furthermore, there is a partial coupling between food-entrained and light-entrained activity in sea bass, *Dicentrarchus labrax* (Sánchez-Vázquez et al., 1995). When goldfish were held under conflicting zeitgebers (light and feeding cycles), activity rhythms were initially synchronized to the light cycle but gradually feeding time assumed greater importance (Aranada et al., 2001). In *R. marmoratus*, J_{urea} may depend on temporal regulation of the rate of food digestion, urea synthesis pathways, and changes in transport mechanisms that may ultimately be under control of light and food entrainable circadian clocks.

Table 1. *Values obtained from cosinor analysis of J_{urea} and J_{amm} rates from R. marmoratus in Series I–IV and D. rerio in Series V*

Series	C_o	C	Acrophase time (h)
J_{urea}			
I	0.175±0.016	0.0815±0.0097	14:30±00:39
II	0.815±0.114	0.587±0.087	15:45±00:18
III	0.106±0.0072	0.0513±0.0045	14:31±00:33
IV	0.090±0.0054	0.0537±0.0047	02:16±00:28
V	0.123±0.0067	NR	NR
J_{amm}			
I	0.664±0.044	NR	NR
II	2.43±0.18	2.14±0.19	15:50±00:12
III	0.645±0.0330	NR	NR
IV	0.713±0.045	NR	NR
V	0.584±0.0277	NR	NR

C_o, mean level of oscillation (μmol N g^{-1} h^{-1}); C, amplitude of the rhythm (μmol N g^{-1} h^{-1}).

Acrophase is the timing of the crest of the waveform function (24 h timescale).

Series I: food-deprived, 12 h:12 h L:D photoperiod; II: fed, 12 h:12 h L:D; III: food-deprived, 0 h:24 h L:D; IV: food-deprived, 12 h:12 h D:L; V: food-deprived, 12 h:12 h L:D.

NR denotes absence of a significant circadian rhythm (P>0.05).

Rates of nitrogen excretion in fed fish are typically correlated with dietary protein content and the proportion of nitrogen wastes excreted as urea *versus* ammonia remains constant (e.g. Wright, 1993; Walsh and Milligan, 1995; Verbeeten et al., 1999). Indeed, the ratio of nitrogen waste products did not vary under different feeding regimes in *R. marmoratus*. Both J_{urea} and J_{amm} demonstrated up to a six- and eightfold increase, respectively, in absolute rates and this is consistent with trends described by other studies (Brett and Zala, 1975; Kaushik, 1980; Kaushik et al., 1983; Wright, 1993; Alsop and Wood, 1997).

J_{urea} and J_{amm} in zebrafish were steady over time, whereas J_{urea} in captive (this study) and wild *R. marmoratus* (Frick and Wright, 2002a) demonstrate a circadian rhythm. This disparity between nitrogen excretion strategies may relate to the ecology and life history of each species. Zebrafish are primarily a freshwater species that prefers to inhabit lotic bodies of water, such as rivers and streams, and they will often remain near the surface of the water column in large groups (Talwar and

Jhingran, 1991). *R. marmoratus* tend to exhibit a more solitary and fossorial lifestyle, often seeking shelter in detritus and leaf litter; however, they also frequently inhabit the burrows of land crabs in estuarine environments (Taylor, 1988; Davis et al., 1990). Limited water flow and elevated ammonia concentrations appear to be major shortcomings for the cryptic burrowing strategy used by *R. marmoratus* (Frick and Wright, 2002a). A few exceptional species shift towards ureotelism under these conditions (Forster and Goldstein, 1966; Gordon et al., 1978; Ramaswamy and Gopalakrishna Reddy, 1983; Saha and Ratha, 1990; Wood et al., 1998), but this is not the case for *R. marmoratus*. Exposure to ammonia concentrations up to 10 mmol l^{-1} does not change the proportion of ammonia *versus* urea excreted in *R. marmoratus* (Frick and Wright, 2002a). An alternative explanation may relate to predation. *O. beta*, a facultative ureotelic teleost, is a preferred prey item of the grey snapper (*Lutjanus griseus*). Preliminary data from behavioral assays indicates that the grey snapper has a stronger attraction to ammonia than either urea alone or an ammonia/urea mixture (Barimo and Walsh, 2005). Hence, it is possible that the circadian J_{urea} pattern in *R. marmoratus* is correlated with potential predator activity but further ecological data is required to verify this hypothesis.

In conclusion, this study provides strong evidence that the diurnal J_{urea} pattern in *R. marmoratus* meets the criteria for a circadian rhythm, namely a 24 h periodicity, a free-running rhythm in constant darkness, and entrainment to a reversed photoperiod. The findings suggest that light cues may have an important role in coordinating the various physiological processes involved in nitrogen metabolism, primarily catabolism of proteins, urea synthesis and urea transport in *R. marmoratus*. The absence of a diurnal pattern of J_{urea} in *D. rerio* is consistent with the majority of teleost fishes.

The authors would like to thank Dr David Noakes for the donation of the fish and Dr Don Stevens for his assistance with the statistical analyses. 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. H. and Wood, C. M. (1997). The interactive effects of feeding and exercise on oxygen consumption, swimming performance and protein usage in juvenile rainbow trout. *J. Exp. Biol.* **200**, 2337-2346.

Aranda, A., Madrid, J. A. and Sanchez-Vazquez, F. J. (2001). Influence of light on feeding anticipatory activity in goldfish. *J. Biol. Rhythms* **16**, 50-57.

Aschoff, J. (1981). Freerunning and entrained circadian rhythms. In *Handbook of Behavioral Neurobiology: Vol. 4, Biological Rhythms* (ed. J. Aschoff), pp. 81-93. New York: Plenum Press.

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.

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

Cahill, G. M. (2002). Clock mechanisms in zebrafish. *Cell Tissue Res*. **309**, 27-34.

Cahill, G. M., Hurd, M. W. and Batchelor, M. M. (1998). Circadian rhythmicity in the locomotor activity of larval zebrafish. *Neuroreport* **9**, 3445-3449.

Davis, W. P., Taylor, D. S. and Turner, T. J. (1990). Field observations of the ecology and habits of mangrove Rivulus (*Rivulus marmoratus*) in Belize and Florida (Teleostei: Cyprinodontiformes: Rivulidae). *Environ. Res. Lab.* **2**, 1-14.

Deng, T. S. and Tseng, T. C. (2000). Evidence of circadian rhythm of electric discharge in *Eigenmannia virescens* system. *Chronobiol. Int.* **17**, 43-48.

Forster, R. P. and Goldstein, L. (1966). Urea synthesis in the lungfish: relative importance of purine and ornithine cycle pathways. *Science* **153**, 1650-1652.

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.

Gerkema, M. P., Videler, J. J., de Wiljes, J., van Lavieren, H., Gerritsen, H. and Karel, M. (2000). Photic entrainment of circadian activity patterns in the tropical labrid fish *Halichoeres chrysus*. *Chronobiol. Int.* **17**, 613-622.

Gordon, M. S., Ng, W. W. and Yip, A. Y. (1978). Aspects of the physiology of terrestrial life in amphibious fishes. III. The Chinese mudskipper *Periophthalmus cantonensis*. *J. Exp. Biol*. **72**, 57-75.

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.

Hurd, M. W., Debruyne, J., Straume, M. and Cahill, G. M. (1998). Circadian rhythms of locomotor activity in zebrafish. *Physiol. Behav*. **65**, 465-472.

Iigo, M., Fujimoto, Y., Gunji-Suzuki, M., Yokosuka, M., Hara, M., Ohtani-Kaneko, R., Tabata, M., Aida, K. and Hirata, K. (2004). Circadian rhythm of melatonin release from the photoreceptive pineal organ of a teleost, ayu (*Plecoglossus altivelis*) in flow-thorough culture. *J. Neuroendocrinol.* **16**, 45-51.

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.

Jin, X., Shearman, L. P., Weaver, D. R., Zylka, M. J., de Vries, G. J. and Reppert, S. M. (1999). A molecular mechanism regulating rhythmic output from the suprachiasmatic nucleus. *Cell* **96**, 57-68.

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.

Kajimura, M., Croke, S. J., Glover, C. N. and Wood, C. M. (2004). Dogmas and controversies in the handling of nitrogenous wastes: the effect of feeding and fasting on the excretion of ammonia, urea and other nitrogenous waste products in rainbow trout. *J. Exp. Biol.* **207**, 1993-2002.

Kaushik, S. J. (1980). Influence of nutritional status on the daily patterns of nitrogen excretion in the carp (*Cyprinus carpio* L.) and the rainbow trout (*Salmo gairdneri*). *Reprod. Nutr. Dev.* **20**, 1751-1756.

Kaushik, S. J., Dabrowski, K. R., Dabrowska, H., Olah, E. and Luquet, P. (1983). Utilization of dietary urea in rainbow trout. *Ann. Nutr. Metab.* **27**, 94-106.

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.

Leiner, K. A. and MacKenzie, D. S. (2001). The effects of photoperiod on growth rate and circulating thyroid hormone levels in the red drum, *Sciaenops ocellatus*: evidence for a free-running circadian rhythm of T(4) secretion. *Comp. Biochem. Physiol.* **130A**, 141-149.

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

Pando, M. P. and Sassone-Corsi, P. (2002). Unraveling the mechanisms of the vertebrate circadian clock: zebfrafish may light the way. *BioEssays* **24**, 419-426.

Pando, M. P., Pinchak, A. B., Cermakian, N. and Sassone-Corsi, P. (2001). A cell-based system that recapitulates the dynamic light-dependent regulation of the vertebrate clock. *Proc. Natl. Acad. Sci. USA* **98**, 10178-10183.

Pohl, H. (1978). Comparative aspects of circadian rhythms in homeotherms, re-entrainment after phase shifts of the zeitgeber. *Int. J. Chronobiol*. **5**, 493-517.

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.

Ramaswamy, M. and Gopalakrishna Reddy, T. (1983). Ammonia and urea excretion in three species of air-breathing fish subjected to aerial exposure. *Proc. Indian Acad. Sci*. **92**, 293-297.

Rodela, T. M. and Wright, P. A. (2006). Metabolic and neuroendocrine effects on diurnal urea excretion in the mangrove killifish *Rivulus marmoratus*. *J. Exp. Biol.* **209**, 2704-2712.

Saha, N. and Ratha, B. K. (1990). Alterations in excretion pattern of ammonia and urea in a freshwater air-breathing teleost, *Heteropneustes fossilis* (Bloch) during hyper-ammonia stress. *Indian J. Exp. Biol.* **28**, 597-599.

Sanchez-Vázquez, F. J., Zamora, S. and Madrid, J. A. (1995). Light-dark and food restriction cycles in sea bass: effect of conflicting zeitgebers on demand-feeding rhythms. *Physiol. Behav.* **58**, 705-714.

Sánchez-Vázquez, F. J., Madrid, J. A., Zamora, S., Iigo, M. and Tabata, M. (1996). Demand feeding and locomotor circadian rhythms in the goldfish, *Carassius aurutus*: dual and independent phasing. *Physiol. Behav*. **60**, 665-674.

Stokkan, K. A., Yamazaki, S., Tei, H., Sakaki, Y. and Menaker, M. (2001). Entrainment of the circadian clock in the liver by feeding. *Science* **291**, 490-493.

Talwar, P. K. and Jhingran, A. G. (1991). *Inland Fishes of India and Adjacent Countries, Vol. I.* New Delhi: Oxford and IBH Publishing Co.

Taylor, D. S. (1988). Observation on the ecology of the killifish *Rivulus marmoratus* (Cyprinodontidae) in an infrequently flooded mangrove swamp. *Northeast Gulf Sci.* **10**, 63-68.

Verbeeten, B. E., Carter, C. G. and Purser, G. J. (1999). The combined effect of feeding time and ration on growth performance and nitrogen metabolism of greenback flounder. *J. Fish Biol*. **55**, 1328-1343.

Verdouw, H., van Echteld, C. J. A. and Dekkers, E. M. J. (1978). Ammonia determinations based on indophenol formation with sodium salicylate. *Water Res.* **12**, 399-402.

Walsh, P. J. and Milligan, C. L. (1995). Effects of feeding and confinement on nitrogen metabolism and excretion in the gulf toadfish *Opsanus beta*. *J. Exp. Biol*. **198**, 1559-1566.

Whitmore, D., Foulkes, N. S., Strahle, U. and Sassone-Corsi, P. (1998). Zebrafish *Clock* rhythmic expression reveals independent peripheral circadian oscillators. *Nat. Neurosci*. **1**, 701-706.

Whitmore, D., Foulkes, N. S. and Sassone-Corsi, P. (2000). Light acts directly on organs and cells in culture to set the vertebrate circadian clock. *Nature* **404**, 87-91.

Wood, C. M. (1993). Ammonia and urea metabolism and excretion. In *The Physiology of Fishes* (ed. D. Evans), pp. 379-425. Boca Raton: CRC Press.

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.

Wright, P. A. (1993). Nitrogen excretion and enzyme pathways for ureagenesis in freshwater tilapia (*Oreochromis niloticus*). *Physiol. Zool*. **66**, 881-901.

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

Yanelli, P. and Harrington, M. E. (2004). Let there be 'more' light: enhancement of light actions on the circadian system through non-photic pathways. *Prog. Neurobiol*. **74**, 59-76.