Does Pulsatile Urea Excretion Serve as a Social Signal in the Gulf Toadfish *Opsanus beta*?*

Katherine A. Sloman[1,4,†]
M. Danielle McDonald[2]
John F. Barimo[2]
Olivier Lepage[3]
Svante Winberg[3]
Chris M. Wood[2,4]
Patrick J. Walsh[2]
[1]School of Biological Sciences, University of Plymouth, Plymouth, Devon PL4 8AA, United Kingdom; [2]Division of Marine Biology and Fisheries, National Institute of Environmental Health Sciences Marine and Freshwater Biomedical Sciences Center, Rosenstiel School of Marine and Atmospheric Science, University of Miami, Miami, Florida 33149; [3]Evolutionary Biology Centre, Department of Comparative Physiology, Uppsala University, Norbyvagen 18A, SE-752 36 Uppsala, Sweden; [4]Department of Biology, McMaster University, 1280 Main Street West, Hamilton, Ontario L8S 4K1, Canada

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ABSTRACT

This study evaluated the hypothesis that the pulsatile excretion of urea by toadfish could serve as a social signal. In the first experiment, physiological parameters were measured in pairs of dominant and subordinate toadfish. Subordinate toadfish had elevated concentrations of circulating plasma cortisol, an effect maintained even after cannulation. In the second experiment, one fish of a pair was injected with ^{14}C-urea, and the occurrence of urea pulses during social encounters was documented. Social status did not influence the order of pulsing, that is, whether a dominant or subordinate fish pulsed first during a social encounter. However, in seven out of eight pairs, both toadfish pulsed within 2 h of each other, indicating some form of communication between fish. In the third and final experiment, the response of toadfish to urea (natural or synthetic) was observed. There was a tendency for toadfish to avoid synthetic urea but there was no apparent behavioural response to water containing toadfish urea. Pulsing events do not appear to play an integral role during social encounters as previously hypothesised, but the close timing of pulses in toadfish pairs suggests some transfer of information.

Introduction

The gulf toadfish *Opsanus beta* (Goode and Bean) is a marine benthic teleost fish found on the Atlantic coast of North America from south of Cape Canaveral, Florida, along the Gulf of Mexico to Campeche, Mexico. Toadfish are well known for their acoustic communications, with both males and females producing a variably paced grunt call composed of short-duration notes when disturbed (Thorson and Fine 2002). During the reproductive season, after locating a suitable shelter, males vocalise a characteristic "boat whistle" to attract females (Breder 1968), particularly during the crepuscular period (Thorson and Fine 2002).

Unlike most other teleost fish that are obligately ammoniotelic, *O. beta* possess a functional ornithine urea cycle (O-UC) and can facultatively synthesise urea (Mommsen and Walsh 1989). In the lab, ureotely can be induced in toadfish by confinement or crowding, which results in >80% of waste nitrogen being excreted as urea (Wood et al. 1995). Approximately 90% of this urea excretion occurs as a large pulse (Wood et al. 1995) occurring across the gills (Gilmour et al. 1998; Pärt et al. 1999). Urea pulses are typically 0.5–3 h in duration and occur about once a day (Wood et al. 1997). It is currently believed that in the field, some individual toadfish may excrete a substantial portion of their waste nitrogen as urea (Hopkins et al. 1997; Barimo et al. 2004).

The physiological mechanisms of ureotely in the toadfish have been well studied (reviewed by Walsh 1997 and Wood et al. 2003), and there is strong evidence that pulsing may be under neural and/or hormonal control. The switch from ammoniotely to ureotely is associated with an increase in circulating plasma cortisol concentrations and levels of glutamine synthetase (GSase) activity (Hopkins et al. 1995). The pulsatile nature of urea excretion is, however, dependent on the activation of an excretion mechanism at the gills because metabolic production is continuous rather than pulsatile (Wood et al. 1997). Directly preceding a pulse, a decrease in plasma cortisol

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† Corresponding author; e-mail: katherine.sloman@plymouth.ac.uk.

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is seen (Wood et al. 1997, 2001), but this is believed to be permissive rather than the trigger for a urea pulse (Wood et al. 2003). More recently, it has been discovered that serotonin (5-hydroxytryptamine [5-HT]) can induce urea pulses (McDonald and Walsh 2004), although this probably is a localised effect at the gill rather than direct central nervous system control (Wood et al. 2003).

Does pulsatile urea excretion have adaptive significance? It is an area of great uncertainty, although five hypotheses have been proposed (Walsh 1997; Wood et al. 1997, 2003). The first hypothesis is that if toadfish excrete urea as one daily pulse rather than a continuous excretion of ammonia, then they reduce chemical signals that might attract predation or forewarn potential prey. Second, because toadfish often live in shelters, control of waste product release could avoid fouling of the shelter environment (but see Barimo et al. 2004). Third, ureotely may serve as a strategy to conserve metabolic nitrogen because the transition to ureotelism is associated with a two-thirds reduction in nitrogen excretion (Walsh and Milligan 1995). Fourth, toadfish are commonly found in sea grass beds that are high in ambient ammonia (Serafy et al. 1997), so toadfish may excrete urea as a way of detoxifying exogenous ammonia (Hopkins et al. 1997).

The final hypothesis, posed more recently, is that these pulses of urea may act as an intraspecific social signal (Wood et al. 2003). In an aquatic environment, chemical cues are an important method of communication, and the role of urine in intraspecific communication has previously been documented (Waring et al. 1996; Zulandt Schneider et al. 2001; Breithaupt and Eger 2002). The putative role of cortisol and serotonin in pulse control further suggests a social signaling mechanism because both are involved in the social behaviour of fish (Winberg et al. 1997; Sloman and Armstrong 2002). The series of experiments presented here addresses the hypothesis that the pulsatile nature of urea excretion in the toadfish functions as a method of immediate social signaling during competitive interactions. The first objective was to elucidate any physiological changes in *O. beta* caused by social interaction that may affect urea pulsing, such as those documented in other fish species (see review by Sloman and Armstrong 2002). The second goal was to examine the timing of urea pulses in relation to social behaviour. A final objective was to investigate the ability of toadfish to recognise urea and urea pulses as a chemical signal.

Material and Methods

Experimental Animals

Gulf toadfish (77.7 ± 3.5 g, 17.4 ± 0.2 cm; $n = 50$) were caught by commercial shrimp fishermen in Biscayne Bay, Florida, from January to April 2003. Toadfish were held in a covered tank at the shrimpers' dock with running aerated seawater for a period of approximately 24 h before collection. Fish were then transported to the laboratory where they were treated immediately with a dip in freshwater followed by Malachite green (0.05 mg L^{-1}) in formalin (15 mg L^{-1}; AquaVet, Hayward, CA) to prevent parasitic infection. Fish were then held in glass aquaria (50 L) with flowing, aerated, and sand-filtered seawater under ambient light regimes. Fish were held at a stocking density of approximately 10 g L^{-1} and were provided with PVC tubes as shelters. All fish were fed ad lib. with thawed squid twice weekly. Before the start of each experiment, toadfish were lightly anaesthetised (0.5 g L^{-1} MS-222; Sigma), and the weight and total length of each fish were recorded. Fish were then marked for individual identification by sewing a combination of small beads (red, yellow, black, or white; maximum of 3 beads) directly below the caudal fin, approximately 2 cm from the end of the tail. Fish were then returned to the holding tank and allowed to recover.

Experiment 1: Cannulation of Dominant and Subordinate Toadfish

Toadfish were allocated to size-matched pairs (mean size difference: 0.3 ± 0.1 cm) and placed together in a 54-L-volume tank supplied with aerated, flowing seawater. Each tank contained one PVC shelter. The light regime consisted of ambient light supplemented with low-level red lighting that remained on throughout the night. The behaviour of the fish was then recorded using an infrared waterproof camera (MVC 2000, WP-LED, Waterproof TubeCam Series; Micro Video Products, Bobcaygeon, Ontario) situated outside of the tank, which was connected to a real-motion time-lapse video recorder (Panasonic). The behaviour of the fish was recorded for the first 24 h of the experiment (starting at 0900 hours) and scored for the whole 24-h period to assign dominance rank (see "Behavioural Observations"). Forty-eight hours after the start of the experiment, fish were removed from the tank and anaesthetised. Caudal vein catheterisation was performed on anaesthetised fish, as in Wood et al. (1997). This procedure was carried out at the same time of day (0900 hours) for the whole experimental series, and an initial blood sample was taken after insertion of the catheter. The sample was transferred to an Eppendorf tube containing 5 μL of a highly heparinised saline solution (200 i.u. mL^{-1}) and centrifuged at 13,000 *g* for 10 min, and the plasma was decanted and frozen in liquid nitrogen for later analysis of plasma cortisol and urea.

Fish were then placed into separate plastic tubs (2.8 L) with lids, where they were supplied with flowing aerated sea water and allowed to recover for 24 h. Tubs were shielded at the front with a wooden board to ensure that the fish were minimally disturbed by the presence of the experimenter. Following the 24-h recovery period, the water flow to the tubs was turned off, and a blood sample (200 μL) was taken from each of the fish via the catheter. Blood samples were treated in the same way as before. A 5-mL water sample was also taken from each

Table 1: Behaviours of toadfish scored every minute during social interaction

Behaviour	Description	Score (min^{-1})
Active swimming	Fish actively swimming in tank	1
Maintaining position	Fish resting on the bottom of the tank but still moving fins	.5
In shelter	In shelter with nose sticking out (i.e., observant)	.5
Sedentary resting	Fish resting on the bottom of the tank without obvious movement	0
Hiding in shelter	Withdrawn into shelter	−.5
Fleeing	Fleeing from the other fish	−1

tub and stored for later analysis of urea and ammonia concentrations. Blood and water samples were then repeated every 2 h for the next 8 h. The first three replicate pairs were then placed in a lethal dose of anaesthetic (5 g L^{-1} MS-222; Sigma). Brains were immediately dissected, separated into telencephalon, hypothalamus, optic tectum, and the remainder of the brain (including the brain stem) and frozen in liquid nitrogen for later analysis of brain monoamines. The sex of the fish was also determined and the weight of the gonads recorded. The liver was removed, weighed, and frozen immediately in liquid nitrogen for later analysis of GSase activity. The remaining replicates ($n = 4$ pairs) were left in the tubs without water flow for a further 16 h, at which time a final water sample was taken and the fish were then sampled as before.

Experiment 2: Measuring Pulsatile Urea Excretion during Social Interaction

Toadfish were allocated to size-matched pairs (mean size difference: 0.2 ± 0.1 cm) and were lightly anaesthetised (0.5 g L^{-1}; MS-222). To determine within each pair the fish from which each pulse emanated, one fish from each pair was injected by caudal venipuncture with 40 μCi ^{14}C-labeled urea (Amersham) in 500 μL of Hanks's saline. The other was given a sham injection of 500 μL of saline. Thus, by determining when pulses of radioactive urea and pulses of "cold" urea occurred throughout the experiment, it was possible to identify the timing of pulses from specific individuals. The fish were allowed to recover from the anaesthetic and were then placed together in their size-matched pairs in 12-L aerated, static tanks containing seawater and a PVC tube for shelter. The behaviour of the fish was recorded for a 48-h period, and the 48 h were scored according to the methods of experiment 1. Here, trials were started at different times of the day (from late morning to early afternoon) depending on availability of tanks, but because behaviour was scored over the full 48-h period, equal numbers of day and night periods were scored. Water samples (5 mL) were taken continuously over every hour by a fraction collector connected to a peristaltic pump; sampling was started at the same time as the video recorder. Water samples were frozen for later analysis of total urea, ^{14}C-labeled urea, and ammonia concentrations. Following 48 h of interaction, fish were removed from the tank and placed into a lethal dose of anaesthetic (5 g L^{-1} MS-222). A blood sample was immediately withdrawn by caudal venipuncture and stored as before for later analysis of total urea and ^{14}C-labeled urea.

Behavioural Observations

In each of the experiments, the behaviour of the toadfish was recorded and played back at a later time. Fish were not fed during behavioural observations. Tapes were observed, and each fish was scored according to its behaviour for the duration of the recording (24 h in experiment 1; 48 h in experiment 2). The activity of the fish each minute was noted (Table 1) and an accurate account of the time spent on each activity compiled. Any aggressive or submissive acts that were carried out by the fish were recorded and scored accordingly (Table 2). The scoring system was based on pilot observations of toadfish social interactions and social behaviours documented in other species of fish (Abbott and Dill 1989; Metcalfe et al. 1989). The weighting of each behavioural parameter is shown in Tables 1 and 2. The behaviour scores for each fish were totaled, and the fish with the highest score within each pair was considered to be dominant.

Experiment 3: Response of Toadfish to Chemical Cues

To determine whether toadfish could recognise and respond to the urea pulse of another toadfish, a single fish was given the opportunity to choose between two PVC shelters, one provided with a slow flow of clean water and the other with a slow flow of either synthetic or toadfish-derived urea (see below). One fish was taken directly from the stock tank at 1730 hours and placed into a 54-L tank supplied with a flow (50 mL min^{-1}) of aerated seawater. Because preliminary data from experiments

1 and 2 indicated that toadfish were more active at night, it was decided to carry out this experiment overnight. The tank was set up as shown in Figure 1, with two PVC shelters. Each PVC shelter had a hole drilled in the top and was connected to a piece of tubing. Using peristaltic pumps, the shelters were then supplied continuously, one with clean seawater and the other with a test chemical at a flow rate of 0.5 mL min^{-1}. The fish were videotaped as before throughout the experiment. At 0930 hours (after 16 h) the experiment was stopped, the fish was removed from the tank and placed in a lethal dose of anaesthetic, and the weight, length, and sex of the fish were recorded. In eight of the replicates, the experimental shelter (zone 3) was supplied with a stock solution of urea (Sigma) made up in seawater such that the final concentration was 200 mmol N L^{-1}. In the remaining eight replicates, the experimental shelter was supplied with seawater from a container that had housed another toadfish for the 48 h before the start of the experiment.

To create this "toadfish urea," a single toadfish was placed in a plastic tub containing 2.0 L of static, aerated seawater for 48 h. The water was then collected and used as a test chemical for the behavioural experiment, and an aliquot was saved for the measurement of water urea and ammonia. Water from one toadfish tub was used for two behavioural replicates. The fish that had been used to create the conditioned water was then placed in a lethal dose of anaesthetic, and the weight, length, and sex of the fish were recorded. The average concentration of urea stock solution from the confined toadfish was 0.405 ± 0.56 mmol N L^{-1}, and the average ammonia concentration was 0.144 ± 0.108 mmol N L^{-1}.

A trial experiment was carried out to calculate the concentration gradient of urea throughout the tank under conditions identical to those used in the behavioural studies but in the absence of a fish. Urea concentrations were measured in each zone as documented in Figure 1. The two stock concentrations that were used in the experiment were tested (i.e., 200 mmol N L^{-1} and 0.5 mmol N L^{-1}), and water samples were taken periodically from these zones over the time course of the experiment. The synthetic stock solution produced a maximum concentration of 1.2 mmol N L^{-1} in zone 3, and the natural solution produced a maximum concentration of 18 μmol N L^{-1}; the latter was comparable with the concentration of urea measured in toadfish burrows in the field (Barimo et al. 2004).

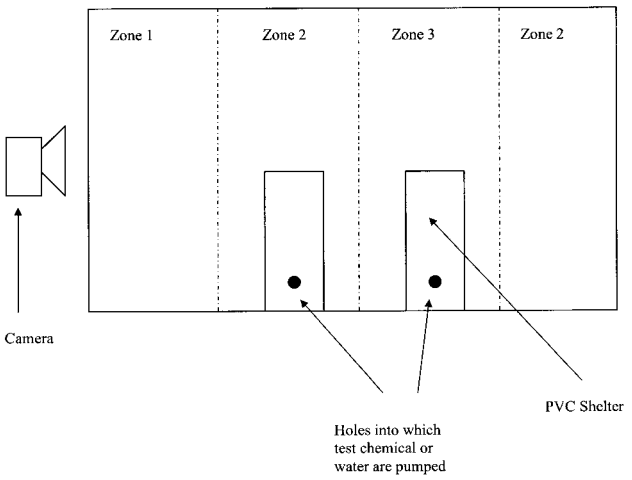

Figure 1. Layout of tank used in experiment 3, designed to observe the response of toadfish to synthetic and natural urea. The two PVC shelters were attached via tubing to peristaltic pumps. The zones represent the areas of the tank used to score the behaviour of the fish. Clean seawater was always pumped into the shelter located in zone 2 and the test chemical into the shelter in zone 3. The location of the shelters was alternated between replicates and the zones of the tank adjusted accordingly; that is, zone 1 was always considered as the area farthest away from the supply of test chemical.

From the videotapes, the time that the fish spent in each zone of the tank was noted. Zone 1 was the farthest from the inflow of urea, and zone 2 consisted of the two areas immediately adjacent to the experimental tube into which urea was pumped, which was located in zone 3 (Fig. 1). The location of the shelter receiving the test chemical (and therefore the zone numbering) was alternated for each replicate to negate any tank effects.

Table 2: Aggressive (A) and submissive (S) behaviours recorded during toadfish interactions

Behaviour (A or S)	Description	Score
Bite (A)	Fish physically bites other fish	20
Mouth fighting (A)	Fish lock mouths and pull back and forth	20
Charge (A)	Fish swims toward other fish with mouth open	20
Mouth display (A)	Fish opens mouth at other fish but no locomotor movement (e.g., from shelter)	20
Fin display (A)	Raises dorsal fin in threat posture	20
Nudge (A)	One fish nudges the other fish	20
Withdrawing (S)	Withdrawing from an aggressive move of another fish	−20

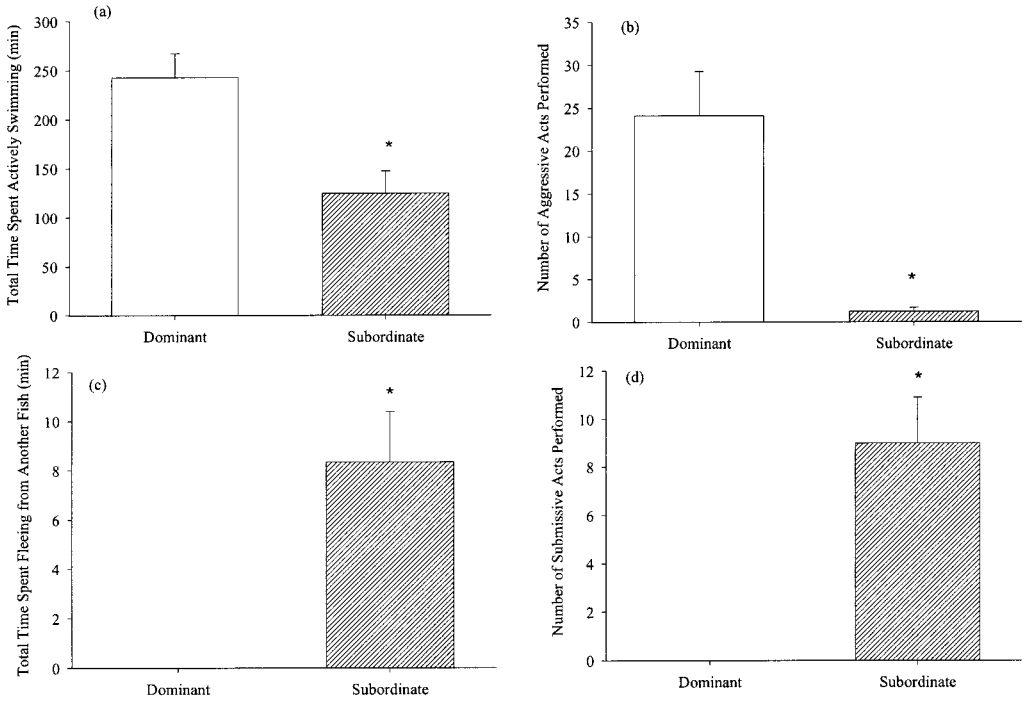

Figure 2. *a*, Amount of time spent swimming in the water column; *b*, number of aggressive acts; *c*, amount of time spent fleeing from another fish; *d*, number of submissive acts performed by dominant (*open bars*) and subordinate (*hatched bars*) in a 48-h period in experiment 2. Asterisks denote statistical differences (unpaired *t*-test; time spent swimming: $t = 3.539$, $P = 0.003$; aggressive acts: $t = 4.416$, $P < 0.001$; time spent fleeing: $t = -4.029$, $P = 0.001$; submissive acts: $t = -4.736$, $P < 0.001$). Data are given as means ± SEM; $n = 8$.

Analytical Techniques and Calculations

For comparative purposes, all urea and ammonia concentrations are expressed in units of nitrogen (Wood et al. 1995, 1997). Water and plasma samples were analysed for urea using the method of Price and Harrison (1987). The method of Ivančič and Degobbis (1984) was used to determine ammonia concentrations in water samples. Both methods have an approximate detection limit of 1–2 μmol N L^{-1}. Concentrations of ^{14}C-labeled urea in both water and plasma samples were determined by scintillation counting using a beta counter (TM Analytic). Plasma samples were diluted with nanopure water, and 5 mL of either plasma dilutions or water sample was added to 10 mL of ACS fluor (ICN Pharmaceuticals) and then counted in a scintillation counter (LKB Rackbeta 1217 Counter). Plasma cortisol concentrations were determined by radioimmunoassay (ICN Pharmaceuticals). For the measurement of GSase activity in liver tissue, samples were sonicated in five volumes of homogenisation buffer (50 mmol L^{-1} Hepes) and centrifuged at 13,000 *g* for 2 min, and the supernatants were analysed by the transferase method described by Walsh (1996).

Brain samples were transferred on dry ice to Uppsala University in Sweden for analysis of brain neurotransmitters by high-performance liquid chromatography. Samples were weighed and homogenised in 4% ice-cold perchloric acid containing 0.2% EDTA, 40 ng mL^{-1} epinine (deoxyepinephrine [internal standard]) using a Potter-Elvenhjem homogeniser (optic tectum and brain stem) or an MSE 100-W ultrasonic disintegrator (telencephalon and hypothalamus). DOPAC (3,4-dihydroxyphenylacetic acid), DA (dopamine), 5-HIAA (5-hydroxyindoleacetic acid), and 5-HT (5-hydroxytryptamine; serotonin) were measured for the calculation of DOPAC/DA and 5-HIAA/5-HT ratios.

In the second experiment, where radiolabeled urea was used to identify urea pulses from different fish, the total amount of urea present in the water in each of the hourly fractions was determined by colorimetric assay, and the amount of radioactivity in the water was determined by scintillation counting. To convert the pulses of radiolabeled urea to actual amounts of urea excreted by the ^{14}C-labeled fish, the initial specific activity of the plasma was required immediately before that pulse. This was calculated by a back-calculation procedure using the following equation for each pulse event:

$$x = \frac{a + (0.8bw)}{c},$$

where *a* is the total number of counts in the water following a pulse, *b* is the plasma activity of the fish following a pulse

(counts mL^{-1}), 0.8 is the constant that reflects extracellular volume (Wood et al. 1997), w is the weight of the fish (g), and c is the concentration of urea in the plasma (mmol N). The back-calculation was performed iteratively starting with the final measurement of plasma urea-N and plasma counts mL^{-1} and assumed that mean plasma urea-N was the same throughout. In practice, changes in plasma urea-N concentrations associated with pulse events and interpulse urea-N accumulation are relatively small (Wood et al. 1997) and therefore would have contributed minimal error to the calculation. The amount of cold urea in each pulse from the radioactive fish could then be calculated by dividing the counts in the water by the immediately preceding plasma specific activity. Once the amount of cold urea excreted by the fish injected with ^{14}C-urea was known, this could be subtracted from the total amount of urea measured in the water and the urea excretion of both fish of a pair determined.

Statistical Analysis

Between pairs of fish, physiological and behavioural values were compared using paired t-tests. Independent t-tests were used to look for overall effects of status on behavioural parameters. Comparisons of physiological values where repeated measurements were taken from the same fish during cannulation studies were made using a repeated-measures ANOVA followed by Tukey tests for multiple comparisons. Where physiological or behavioural parameters were measured once per fish and two factors were considered, for example, social status and time, a two-way ANOVA was used. Linear regression analyses were carried out on both behavioural and physiological data. Comparisons of overall behaviours used Kruskal-Wallis analyses. All data were tested for normality before parametric testing using the Kolmogorov-Smirnov test. Any parameters that were not normally distributed were transformed before parametric analysis. To see whether pulses of paired fish (experiment 2) were dependent on the presence of a conspecific, the percentage of naturally occurring pulses expected in a 2-h period (taken from Wood et al. 2001) was compared, using a χ^2 test, with the percentage of pulses excreted by the second fish of a pair that occurred in the 2 h following a pulse by the first fish. SPSS software was used for statistical analyses, and the limit of significance in all analyses was $P < 0.05$. Data are presented as means ± SEM.

Results

Toadfish Behaviour (Experiments 1 and 2 Only)

In both experiments 1 and 2, dominance was determined according to the fish within each pair with the highest behaviour score at the end of the observation period (24 h in experiment 1; 48 h in experiment 2). Therefore, as expected, dominant fish generally had higher behavioural scores than subordinate fish (experiment 1: $P = 0.01$; experiment 2: $P < 0.001$). No effect of gender on behaviour score was seen (experiment 1: $P = 0.481$; experiment 2: $P = 0.431$). However, this total behaviour score could then be broken down into the various behavioural components to examine more closely differences in behaviour between dominant and subordinate fish. In experiment 1, the duration of behavioural observation was too short to reveal any significant differences in behaviour between dominant and subordinate toadfish, except that subordinate animals carried out more submissive acts ($P = 0.044$; see Tables 1 and 2 for a more detailed description of behaviours). However, during the 48 h observation of experiment 2, it was possible to determine more about the behavioural differences of dominant and subordinate fish. Dominant fish spent more time actively swimming around the tank ($P = 0.003$; Fig. 2*a*) and performed a larger number of aggressive acts ($P < 0.001$; Fig. 2*b*) than submissive animals. Subordinate toadfish spent more time fleeing from a more dominant fish ($P = 0.001$; Fig. 2*c*) and performed more submissive acts ($P < 0.001$; Fig. 2*d*) than their dominant counterparts. Overall, toadfish were more active during the night and the crepuscular period (dawn and dusk) than during the day (Fig. 3; $P < 0.001$).

Experiment 1: Cannulation of Dominant and Subordinate Toadfish

Subordinate fish had significantly higher plasma cortisol concentrations both before and after cannulation (Fig. 4; $P = 0.048$). There was also a significant effect of cannulation on plasma cortisol concentration, levels being higher following

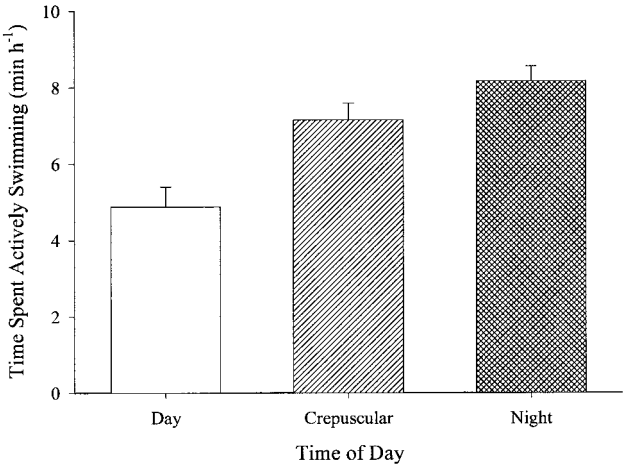

Figure 3. Time spent actively swimming by toadfish in a 48-h period during day, night, and crepuscular (dawn and dusk) periods in experiment 2. Time is presented as minutes in an hour to account for the varying lengths of these periods. There is a significant effect of time of day on swimming activity (Kruskal-Wallis; $\chi^2 = 24.041$, df = 2, $P < 0.001$). Data are given as means ± SEM; $n = 8$.

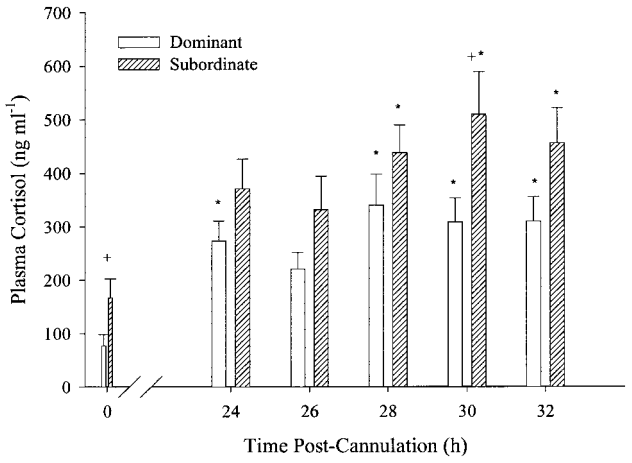

Figure 4. Plasma cortisol concentrations of dominant (*open bars*) and subordinate (*hatched bars*) toadfish before and after cannulation in experiment 1. Fish were allowed 24 h to recover from cannulation and then sampled for blood every 2 h for the next 8-h period. Asterisks denote significant differences between pre- and postcannulation values, and crosses denote significant effects of social status (repeated-measures ANOVA; social status: $F = 5.450$, df = 1,8, $P = 0.048$; time: $F = 13.280$, df = 1,7, $P < 0.001$; time × status: $F = 2.50$, $P = 0.937$). Data are given as means ± SEM; $n = 7$.

cannulation in both dominant and submissive fish ($P < 0.001$). However, the difference in plasma cortisol between dominants and subordinates was maintained following cannulation. There was no significant interaction of gender with plasma cortisol concentration ($P = 0.904$). No significant relationship was found between total behaviour score and initial plasma cortisol concentrations ($P = 0.101$) or between the number of aggressive acts performed and initial plasma cortisol concentrations ($P = 0.907$). However, the number of submissive acts was positively correlated with higher plasma cortisol concentrations before cannulation ($r^2 = 0.36$; $P = 0.03$). There were no differences in plasma urea between dominant and subordinate animals ($P = 0.589$; Table 3), and there were no differences in plasma urea before and after cannulation ($P = 0.966$).

There was a significant increase with time in both cumulative urea-N and ammonia-N excretion when toadfish were left in their flux boxes overnight, following the 8-h sampling period (Fig. 5; urea excretion: $P = 0.002$; ammonia excretion: $P = 0.001$). It is likely that the increase in urea-N was the result of urea pulses, whereas the increase in ammonia-N was caused by a more constant excretion. However, no significant effect of social status on the cumulative excretion of either urea-N or ammonia-N was seen in the flux box water (urea-N: $P = 0.944$; ammonia-N: $P = 0.271$). When urea-N and ammonia-N were combined to consider total cumulative nitrogen excretion, there was still an effect of time (Fig. 5*c*, $P = 0.001$) but no overall effect of social status ($P = 0.061$). Both dominant and subordinate fish were ureotelic, with dominant fish excreting 75% of nitrogen as urea and subordinates excreting 57%. The percentage of nitrogen excreted as urea was not significantly affected by social status ($P = 0.142$). There was no significant difference in liver GSase activity, hepatosomatic index (HSI), or brain monoamine ratios between dominant and subordinate toadfish (GSase: $P = 0.695$; HSI: $P = 0.104$; DOPAC/DA: telencephalon, $P = 0.219$; hypothalamus, $P = 0.665$; optic tectum, $P = 0.851$; brain stem, $P = 0.429$; 5-HIAA/5-HT: telencephalon, $P = 0.186$; hypothalamus, $P = 0.583$; optic tectum, $P = 0.881$; brain stem, $P = 0.788$; Table 4). Gender did not significantly affect liver GSase activity, HSI, or the majority of brain monoamine ratios (liver GSase activity: $P = 0.314$; HSI: $P = 0.626$; DOPAC/DA: telencephalon, $P = 0.698$; hypothalamus, $P = 0.818$; optic tectum, $P = 0.890$; brain stem, $P = 0.230$; 5-HIAA/5-HT: telencephalon, $P = 0.758$; hypothalamus, $P = 0.807$; brain stem, $P = 0.193$). However, there was a significant effect of sex on 5-HIAA/5-HT in the optic tectum ($P = 0.025$), with males having significantly lower 5-HIAA/5-HT than females (0.52 ± 0.07 and 0.73 ± 0.04, respectively).

Experiment 2: Measuring Pulsatile Urea Excretion during Social Interaction

In each of the replicate pairs of fish ($n = 8$), at least one pulse of urea excretion was seen during the 48-h observation period. By subtracting the amount of radioactive urea from the total amount of urea in the water, the accumulated urea excretion of dominant and subordinate fish could be calculated (e.g., Fig. 6). The volume of the tank was relatively large to ensure that the fish had sufficient room for social interaction and that the subordinate could escape if aggression from the dominant fish became too severe. The small fluctuations in urea seen in Figure 6 were caused by mixing of a large volume of water in a closed system. The first pulse of each fish was used to calculate the average pulse size of dominant and subordinate fish during

Table 3: Plasma urea-N concentrations before and after cannulation of dominant and subordinate fish in experiment 1

Time Post-cannulation (h)	Plasma Urea (mmol N)	
	Dominant	Subordinate
0	7.92 ± 1.2	7.06 ± 1.7
24	10.36 ± 2.62	9.54 ± 2.56
26	11.38 ± 1.98	9.28 ± 1.98
28	10.78 ± 1.56	9.16 ± 2.3
30	9.52 ± 1.84	8.92 ± 2.02
32	10.38 ± 2.02	9.64 ± 1.98

Note. Data are given as means ± SEM, $n = 7$. No statistical effects of social status or cannulation were found ($P > 0.966$).

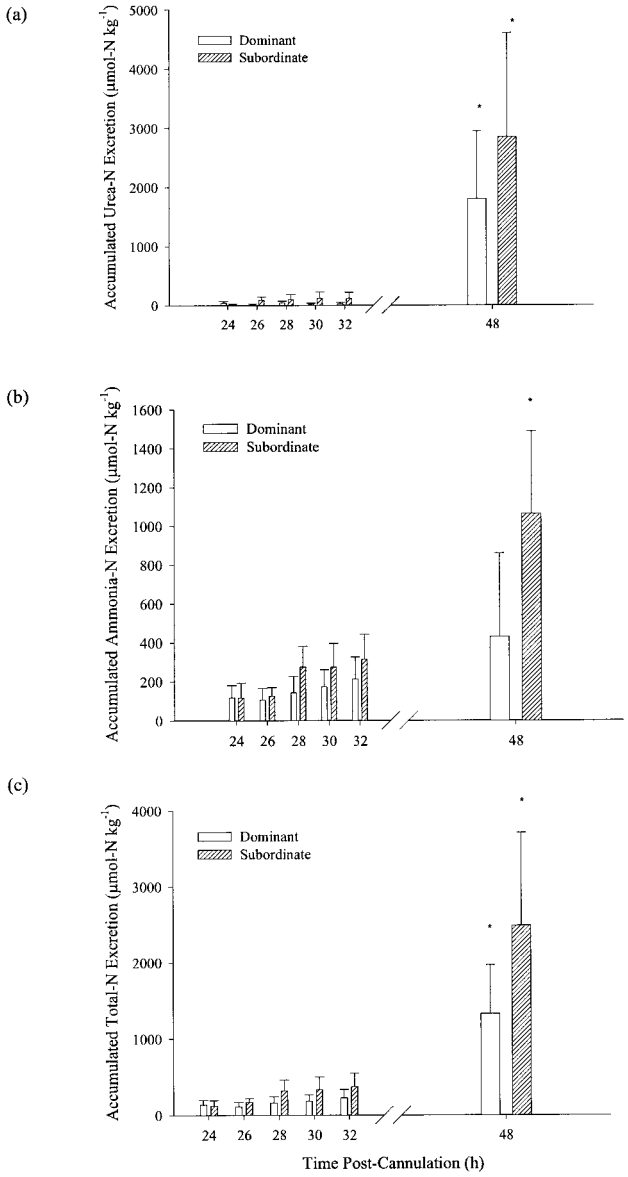

Figure 5. Accumulated urea-N (*a*), ammonia-N (*b*), and total-N (*c*) excretion of dominant (*open bars*) and subordinate (*hatched bars*) toadfish in experiment 1 following cannulation and confinement in flux boxes with no through-flow of water ($n = 7$ for times 24–32, and $n = 4$ for time 48). Data are given as means ± SEM. Asterisks denote post hoc (Tukey) significant effects of time (repeated-measures ANOVA; urea: social status, $F = 0.353$, df = 1,6, $P = 0.574$; time, $F = 4.937$, df = 1,6, $P = 0.002$; status × time, $F = 0.234$, $P = 0.944$; ammonia: social status, $F = 1.469$, df = 1,6, $P = 0.271$; time, $F = 5.287$, $P = 0.001$; status × time, $F = 0.885$, $P = 0.503$; total-N: social status, $F = 5.306$, df = 1,6, $P = 0.061$; time: $F = 7.973$, $P < 0.001$; status × time: $F = 0.659$, $P = 0.658$).

social encounters. Toadfish were ureotelic, excreting on average 65% of their nitrogen waste as urea-N. There was no significant effect of social status or time of day on pulse size (two-way ANOVA; social status, $P = 0.244$; time of day, $P = 0.618$; status × time, $P = 0.354$). Fifty percent of pulses occurred at night, 38% were during the day, and 12% were during the crepuscular period (dawn or dusk). There was no significant correlation between activity of the fish and amount of urea-N excretion ($P = 0.741$).

Subordinate fish pulsed first in 25% ($n = 2$) of the pairs, as did dominant fish ($n = 2$). In the remaining four pairs (50%), it was not possible to distinguish which fish pulsed first because they pulsed within <1 h of each other, which was beyond the resolution of water sampling. Regardless of social position, the timing of the pulse of the second fish in a pair was dependent on the pulsing of the first fish. Wood et al. (2001) demonstrated that the percentage of natural pulses in an isolated toadfish occurring in any 2-h period was 7%. Taking this to be representative of a random distribution of pulses (i.e., not influenced by the presence of other toadfish), this value was compared with the percentage of pulses from the second fish of a pair that occurred in a 2-h period following a pulse from the first fish. The timing of the pulse from the second fish in each pair was significantly different from random ($P < 0.001$), demonstrating that the timing of the second fish's pulse was dependent on the timing of the first fish's pulse.

Experiment 3: Response of Toadfish to Chemical Cues

There was no significant difference in time spent in each zone of the tank in the presence of synthetic urea ($P = 0.08$), although there was a trend toward less time spent in the zone where urea was added. When toadfish-derived urea was added to the tank, there was no significant difference in the time spent in the different zones ($P = 0.707$). No effect of sex of either the experimental fish ($P = 0.8$) or the fish used to collect the natural urea ($P = 0.7$) was found.

Discussion

Toadfish were more active during the night and crepuscular periods, when they would actively swim about the tank and interact with each other. Dominance hierarchies formed between all pairs of toadfish observed, with dominant fish being more active than submissive animals and performing more aggressive attacks. The higher activity and aggression levels associated with dominance are in agreement with other studies (Peters et al. 1980; Winberg et al. 1996; O'Connor et al. 1999; Øverli et al. 1999; Sloman et al. 2001).

Subordinate fish had elevated concentrations of plasma cortisol, as documented previously in subordinates of other fish species (e.g., Peters et al. 1980; Laidley and Leatherland 1988; Fox et al. 1997). This elevation indicates that submissive individuals are subjected to chronic stress levels as a result of social encounters (Sloman and Armstrong 2002). Interestingly, even after the stress of cannulation resulted in a doubling of plasma cortisol, there was still a marked difference in cortisol

Table 4: Liver GSase activities, hepatosomatic indices (HSI), and brain monoamine ratios for dominant and subordinate toadfish in experiment 1

Physiological Parameter	Dominant	Subordinate
Liver GSase activity (μmol min^{-1} g^{-1})	3.01 ± .72	2.68 ± .44
Hepatosomatic index	1.81 ± .19	1.42 ± .12
3,4-dihydroxyphenylacetic acid/dopamine (DOPAC/DA):		
Telencephalon	.35 ± .05	.24 ± .07
Hypothalamus	.10 ± .03	.12 ± .04
Optic tectum	.22 ± .09	.20 ± .04
Brain stem	.31 ± .05	.28 ± .08
5-hydroxyindoleacetic acid)/5-hydroxytryptamine (5-HIAA/5-HT):		
Telencephalon	.22 ± .05	.15 ± .08
Hypothalamus	.20 ± .03	.18 ± .02
Optic tectum	.64 ± .09	.62 ± .06
Brain stem	.39 ± .06	.47 ± .08

Data are given as means ± SEM, $n = 7$. There were no significant effects of social status (GSase, $P = 0.695$; HSI, $P = 0.104$; DOPAC/DA: telencephalon, $P = 0.219$; hypothalamus, $P = 0.665$; optic tectum, $P = 0.851$; brain stem, $P = 0.429$; 5-HIAA/5-HT, telencephalon, $P = 0.186$; hypothalamus, $P = 0.583$; optic tectum, $P = 0.881$; brain stem, $P = 0.788$).

levels between dominants and subordinates. Plasma cortisol values of cannulated dominant toadfish were similar to those seen in previous cannulation studies (Wood et al. 2001) and were higher in subordinates.

Other studies that have measured plasma cortisol in fish of different social status have used either terminal or single blood samples (Øverli et al. 1999; Sloman et al. 2000) to avoid complications of raised cortisol in response to sampling. In toadfish, a drop in circulating plasma cortisol concentrations is seen directly before a pulse (Wood et al. 1997, 2001; McDonald et al. 2004), and therefore, a single blood sample from a ureotelic animal may not be a good indicator of blood cortisol levels because it would be influenced by previous or impending pulsing events. In this study, plasma cortisol was measured over a longer period of time by cannulation to avoid this possibility. It is clear that the cannulation procedure was stressful but the differences between dominant and subordinate cortisol concentrations remained. The persistence of this socially mediated difference in plasma cortisol following invasive surgery has not been documented before, to our knowledge.

When cannulated toadfish were left undisturbed in tubs for 24 h following the 8-h sampling period, there was a significant accumulation of both urea-N and ammonia-N in the water. The amount of total nitrogen waste excreted as urea-N indicates that both dominant and subordinate fish were ureotelic (Walsh and Milligan 1995), with no difference in ammonia-N, urea-N, or total N excretion between dominant and subordinate fish, although the total N excretion difference was nearly significant. Chronic exogenous loading of cortisol over a 48-h period resulted in circulating cortisol concentrations of 500 ng mL^{-1} and caused a decrease in urea-N pulse size but had no effect on the frequency of pulsatile excretion (McDonald et al. 2004). However, in this study the chronic elevation of plasma cortisol in subordinate fish did not appear to have an effect on pulse size, despite endogenous cortisol concentrations exceeding 400 ng mL^{-1}. Ammoniotelic toadfish have levels of liver GSase activity around 2–3 units g^{-1} and ureotelic toadfish that have been acutely stressed, around 10–11 units g^{-1} (Hopkins et al. 1995; Walsh and Milligan 1995). In this study, levels of GSase activity in the liver were more reflective of ammoniotelic fish at 1–3 units g^{-1}. However, Hopkins et al. (1995) note that levels of chronic stress, for example, those associated with social stress, eliminate relationships between nitrogen excretion, cortisol, and GSase activity. GSase activity did not differ with social rank.

No differences in the ratios of DOPAC/DA or 5-HIAA/5-HT were seen between dominant and subordinate fish. Brain monoamine activities are often estimated by calculating the ratio of catabolite to the parent neurotransmitter concentration as a more direct index of neuronal activity than measuring total levels of monoamines (Shannon et al. 1986). In both mammals and other fish species, brain monoamine activities can be influenced by social status. Øverli et al. (1999) demonstrated elevations of both DOPAC/DA and 5-HIAA/5-HT in the brains of subordinate rainbow trout following staged fights for dominance, and elevated brain 5-HT activity is seen in subordinate Arctic charr of stable dominance hierarchies (Winberg and Nilsson 1993). It is possible that the lack of difference in brain monoamine activities between dominant and subordinate toadfish could reflect terminal sampling of ureotelic fish. The hy-

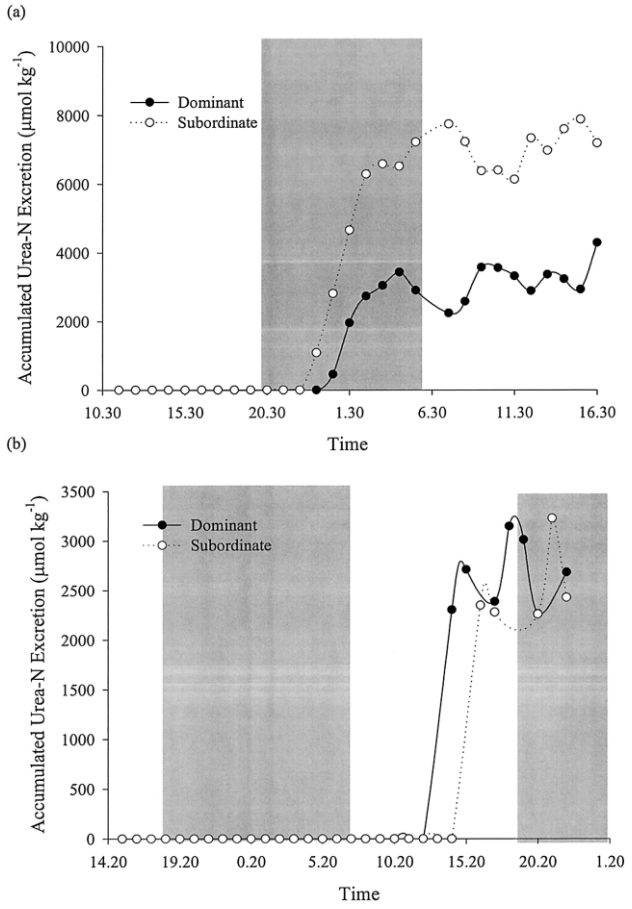

Figure 6. Examples of pulsatile urea-N excretion of dominant (*closed circles*, *solid line*) and subordinate (*open circles*, *dotted line*) toadfish (replicate pair numbers 1 [*a*] and 5 [*b*]), given as accumulated urea excretion in experiment 2. Shaded areas represent night in the diurnal cycle.

pothalamo-pituitary-interrenal axis (resulting in the release of cortisol) and brain serotonin levels are closely linked (Winberg and Lepage 1998; Øverli et al. 1999). It is therefore plausible that fluctuations in cortisol that occur during the pulsing cycle are mirrored in brain serotonergic activities. If fluctuations are seen in association with urea pulsing, then the terminal sampling of fish at different times in their pulsing cycles could generate variation great enough to mask any differences associated with social status. Although serotonin is known to induce pulses, this is presently believed to be a localised effect at the gill rather than under direct central nervous system control (Wood et al. 2003), and the potential for brain monoamine levels to fluctuate around pulsing events remains to be tested.

Traditionally, ureotely can be induced in the lab by crowding or confinement of fish (Wood et al. 2003), and yet in experiment 2, urea pulses were detected in relatively large tanks (12 L) with only two fish. The stress associated with social interaction may have induced ureotely, as cortisol is known, to stimulate hepatic GSase in toadfish and is believed to be a contributing factor in the switch from ammoniotelism to ureotelism (Hopkins et al. 1995). Measurements of both GSase activity (Hopkins et al. 1997) and urea (Barimo et al. 2004) have demonstrated that *Opsanus beta* are ureotelic in the field. It is also possible that because the toadfish were held in stock tanks before these experiments, they were already ureotelic as a result of previous confinement.

Pulse sizes were independent of social status, and pulsing did not appear to correlate well with time of day or activity of the fish. No relationship between the immediate behaviour of toadfish and the timing of pulsatile excretion of urea was found, and within each pair, social status did not predict which fish pulsed first. However, social status aside, the first pulse released by one fish of a pair triggered the second fish of the pair to pulse, and the percentage of pulses where fish of the same pair pulsed within 2 h of each other was significantly higher than would be expected if pulsing was random.

For a urea pulse to act as a social signal, it would be necessary for the receiver to be able to detect it, and it was hypothesised in experiment 3 that detection of urea would result in a behavioural response. Toadfish did show a tendency to avoid the zone supplied with synthetic urea, but water containing natural toadfish urea did not elicit a detectable behavioural response. For a urea pulse to act as a social signal, it would need to be detected at concentrations occurring after release by another fish into an environment affected by water currents. Therefore, this study used concentrations of natural urea that would be relevant to the natural environment of the fish that were significantly lower than the concentration of synthetic urea but similar to those measured in toadfish burrows in the field (Barimo et al. 2004). It is most probable that the tendency of fish to avoid synthetic urea and not natural urea is caused by differences in concentration.

In summary, subordinate toadfish display characteristic elevations of plasma cortisol also seen as a result of social stress in other vertebrates. However, parameters such as brain monoamine concentrations were not affected by social status, perhaps caused by natural fluctuations in these parameters masking individual differences. Pulsing events did not appear to play an integral role during social encounters. Toadfish are able to communicate acoustically without a large metabolic cost (Amorim et al. 2002), and an alternative, immediate, signaling system may therefore not be necessary. The presence of urea (either natural or synthetic) did not seem to alter the behaviour of isolated toadfish.

However, this study clearly demonstrates that urea pulses have the potential to communicate information even if the "stimulus" and "response" may not be so closely linked as to act as an immediate signal during social encounters. So what information is being exchanged during pulsing events? It is not known whether urea pulses contain other compounds as well

as urea. Laurent et al. (2001) demonstrated the presence of vesicles in the branchial pavement cells of the gulf toadfish that are believed to be involved in urea excretion from the gills, and so it is possible that other substances are released with the urea from vesicles. Pulses may therefore carry social information of a more general nature, for example, about the condition or sexual status of a pulsing fish. While we now discount the hypothesis that pulsatile urea excretion serves as a direct social signal during aggressive encounters in the toadfish, pulses could still convey social information.

Alternatively, the close timing of pulses in toadfish pairs draws attention to the first hypothesis mentioned in the introduction, that toadfish excrete urea as one daily pulse rather than a continuous excretion of ammonia to reduce chemical signals that might attract predators or forewarn potential prey. In the field, it may be advantageous for toadfish to pulse at the same time as their neighbours if pulses act as homing signals for potential predators. Toadfish may choose a "safe" time to pulse, and detection of a pulse by another toadfish indicates that this is a "safe" time to pulse as well. A group of toadfish all pulsing at once may also function to confuse a predator to the location of a single toadfish. Alternatively, once one toadfish in the near vicinity has released a pulse, then any potential prey may be forewarned, and so the release of further pulses among neighbouring fish would have no detrimental effect. This study has only just begun to unravel the mystery of why toadfish pulse urea and the complexity of aquatic communication systems in general, coupled with the complexity of the physiological mechanisms behind toadfish pulsatile urea excretion, create a system about which many questions remain unanswered.

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