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Review

Pulsatile urea excretion in the gulf toadfish: mechanisms and controls☆

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Abstract

Opsanus beta expresses a full complement of ornithine–urea cycle (OUC) enzymes and is facultatively ureotelic, reducing ammonia-N excretion and maintaining urea-N excretion under conditions of crowding/confinement. The switch to ureotelism is keyed by a modest rise in cortisol associated with a substantial increase in cytosolic glutamine synthetase for trapping of ammonia-N and an upregulation of the capacity of the mitochondrial OUC to use glutamine-N. The entire day's urea-N production is excreted in 1 or 2 short-lasting pulses, which occur exclusively through the gills. The pulse event is not triggered by an internal urea-N threshold, is not due to pulsatile urea-N production, but reflects pulsatile activation of a specific branchial excretion mechanism that rapidly clears urea-N from the body fluids. A bidirectional facilitated diffusion transporter, with pharmacological similarity to the UT-A type transporters of the mammalian kidney, is activated in the gills, associated with an increased trafficking of dense-cored vesicles in the pavement cells. An 1814 kB cDNA ('tUT') coding for a 475–amino acid protein with approximately 62% homology to mammalian UT-A's has been cloned and facilitates phloretin-sensitive urea transport when expressed in *Xenopus* oocytes. tUT occurs only in gill tissue, but tUT mRNA levels do not change over the pulse cycle, suggesting that tUT regulation occurs at a level beyond mRNA. Circulating cortisol levels consistently decline prior to a pulse event and rise thereafter. When cortisol is experimentally clamped at high levels, natural pulse events are suppressed in size but not in frequency, an effect mediated through glucocorticoid receptors. The cortisol decline appears to be permissive, rather than the actual trigger of the pulse event. Fluctuations in circulating AVT levels do not correlate with pulses; and injections of AVT (at supraphysiological levels) elicit only minute urea-N pulses. However, circulating 5-hydroxytryptamine (5-HT) levels fluctuate considerably and physiological doses of 5-HT cause large urea-N pulse events. When the efferent cranial nerves

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to the gills are sectioned, natural urea pulse events persist, suggesting that direct motor output from the CNS to the gill is not the proximate control.

Keywords: Ammonia; Ornithine urea cycle; Glutamine; AVT; UT-A; Facilitated diffusion; Serotonin; 5-hydroxytryptamine; Cortisol; Vesicular trafficking; Pavement cells

1. Introduction

Mommsen and Walsh (1989) reported that two members (*Opsanus beta* and *Opsanus tau*) of the teleost family Batrachoididae (the toadfishes and midshipmen) express a full complement of the ornithine–urea cycle (OUC) in their livers, while Randall et al. (1989) documented the same phenomenon in the Lake Magadi tilapia (*Alcolapia grahami*, formerly *Oreochromis alcalicus grahami*). The data on *O. tau* confirmed the earlier report of Read (1971) on liver biochemistry in this species, which prior to this time had been considered an anomaly, because general belief held that the genes for the OUC were silent or deleted in the teleosts. The presence of the OUC, accompanied by substantial ureotelism (i.e., predominance of urea-N as the major form of N-waste excreted) has subsequently been shown in several other teleost species (reviewed by Walsh, 1997; Anderson, 2001). The phenomenon is now interpreted as retention of an embryonic characteristic, which is probably expressed in the early life stages of most species (e.g., Wright et al., 1995), the majority of which are ammoniotelic later in life (reviewed by Wright and Land, 1998; Wright and Fyhn, 2001). Since ureotelism is metabolically expensive relative to ammoniotelism, costing 2 to 2.5 additional ATP per unit N excreted, expression of this trait in adult fish presumably has adaptive significance. This is most obvious in *A. grahami* where the high water pH (approx. 10) of Lake Magadi prevents ammonia excretion across the gills, and the excretion of urea-N provides a solution to this problem (Wood et al., 1989).

In the gulf toadfish (*O. beta*), the phenomenon has proven particularly interesting, because the species is facultatively ureotelic. When held under 'non-stressful' conditions in the laboratory (Walsh and Milligan, 1995) or when freshly collected from the field (Hopkins et al., 1999) the fish is predominantly ammoniotelic, while 'proxy' biochemical measurements on field-collected animals (Hopkins et al., 1997) indicate that either ammoniotelism, ureotelism or a mixture of the two strategies may occur in wild fish. However, a variety of laboratory treatments will induce a clear switchover to ureotelism. For some treatments, which inhibit ammonia excretion (e.g., air emersion, exposure to high environmental ammonia; Walsh et al., 1990; Wang and Walsh, 2000), the adaptive significance of ureotelism is obvious. However, for others it is not. In particular, when toadfishes are crowded together and/or when individual toadfish are isolated in single small containers, they become ureotelic in 1–3 days even when water flow is maintained so as to keep environmental ammonia at background levels (Walsh et al., 1994a; Walsh and Milligan, 1995). Especially interesting under these circumstances is the fact that the urea excretion is pulsatile, with more than 90% of the fish's waste-N production excreted in one or two short-lasting (0.5–3 h) pulses per day (Wood et al., 1995). In the last few years, we have made significant progress in understanding the mechanisms by which the urea pulsing occurs, though the adaptive significance of this behavior remains elusive.

In the present report, we synthesize our recent published and unpublished findings on these mechanisms in *O. beta*, present new data about the possible involvement of neural and endocrine factors in controlling pulsatile urea-N excretion, suggest promising areas for future research and speculate on the adaptive significance of this behavior. Comparable data on *O. tau* are very limited, but suggest that the same principles apply. For a review of earlier findings, the reader is referred to Walsh (1997).

2. Materials and methods

In general, experiments were carried out on sexually mature specimens (30–400 g) of *O. beta* collected by roller trawl from Biscayne Bay, south Florida, and held initially at low density (<12 g fish per liter seawater; 'ammoniotelic conditions') in sand-covered 80-l aquaria served with flowing seawater (30–33 ppt) at ambient temperature (20–

28 °C). During the first few days in the laboratory, the fish were subjected to one or more prophylactic treatments with malachite green and formalin to kill external parasites. Thereafter, they were fed ad libitum with shrimp or squid three times per week, but starved for 24 h prior to any experimental procedures.

To induce ureotelism, typically 6 toadfish were crowded together for 3 days at a density of >80 g fish per liter seawater, in 6-l tubs served with flowing seawater. In those experiments requiring repetitive blood sampling, the fishes were then anaesthetized in MS-222 (0.67 g l^{-1}, adjusted to pH 8.0 with NaOH) and a chronic indwelling catheter (Barber and Walsh, 1993) filled with Hanks toadfish saline (Walsh, 1987) heparinized at 100 i.u. ml^{-1} was placed in the caudal artery as described by Wood et al. (1997). When required, catheters were also placed into the urogenital papilla for the continuous collection of urine and into the anus for the continuous collection of rectal fluid, as described by McDonald et al. (2000) and Wood et al. (1995), respectively. To maintain ureotelism and to facilitate study of the phenomenon in individual animals, the fish were then transferred (after the initial 3 days of crowding and surgery) to 2–3-l containers served with flowing seawater and aeration, and equipped with shelter (a piece of PVC pipe). Alternately, the fish were transferred from the 80-l holding aquaria directly into these individual containers and left for 3–5 days of confinement before starting experiments. Both treatments ensured that more than 80% of the fish became and stayed ureotelic. All experiments started 24 h after surgery.

For section of the cranial nerves, large toadfish (>150 g) were selected. The fish were first put through the crowding/confinement protocol and then the denervations were performed. Under MS-222 anaesthesia, the operculum was pushed forward and an incision (approx. 1.0 cm) was cut in the skin, starting from the dorsal end of the first gill arch where it joins the roof of the opercular cavity and progressing backwards past the fourth gill arch. This allowed access to all branchial branches of cranial nerves IX (glossopharyngeal) and X (vagus). The nerves were gently dissected free from the surrounding tissues and sectioned with fine iris scissors. The incision was closed by silk sutures. The procedure was repeated on the other side of the fish. The cardiac and visceral branches of the vagus were left intact. In the sham (control group, $N=6$), the nerves were exposed but not sectioned. All animals were allowed to recover from surgery for 24 h. At the end of the experiment, the fish was killed in an overdose of MS-222 and the denervations were confirmed post mortem by necropsy.

The caudal artery catheters were employed for repetitive blood sampling without disturbance to the fish and for the internal administration of drugs and radioactively labeled compounds. When blood samples were taken, plasma was separated by rapid centrifugation and saved for later analysis, while the red blood cells were resuspended in an equivalent volume of non-heparinized saline and reinfused into the fish. Pharmaceuticals and radiochemicals were injected in typical volumes of 0.2–0.3 $ml.kg^{-1}$ of saline, with a wash-in of an equivalent volume of saline. Circulating concentrations were calculated assuming an extracellular space of 300 $ml.kg^{-1}$ (Perry et al., 1998).

In order to measure the fluxes of urea-N, ammonia-N and radiolabeled compounds from the fish into the environment, water flow to the individual chambers was stopped, the volume set to a known value, typically 1.5–2.0 l, and the aeration set to provide thorough mixing. Thereafter, water samples (5–10 ml) were taken at hourly intervals either by an automated peristaltic pump plus fraction collector system or by manual sampling. In most experiments, colorimetric chemical assays were used to measure the appearance of ammonia-N (Ivancic and Degobbis, 1984) and urea-N (Rahmahtullah and Boyde, 1980; Price and Harrison, 1987) in the water. However, in experiments where there was a need for particularly rapid detection of urea-N pulses, the fish were pre-loaded via the caudal artery catheter with 400 $\mu Ci\ kg^{-1}$ of [^{14}C]urea at least 3 h before the start of the monitoring period. The appearance of [^{14}C]urea cpm in the water, multiplied by the inverse of the measured specific activity of urea-N in plasma samples taken via the caudal artery catheter prior to a pulse event, provided a sensitive and rapid measurement of urea-N flux, one which has been shown to be identical to that measured by colorimetric assay (Wood et al., 1997).

Data are routinely reported as means ± 1 S.E.M. (N). The significance of differences was assessed at the $P \leq 0.05$ level using statistical methods which are either outlined in the original citations

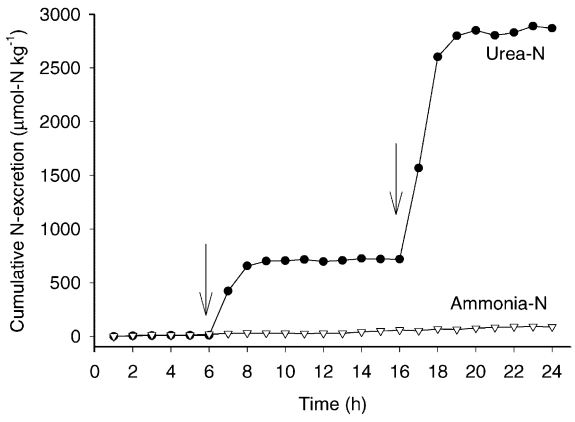

Fig. 1. A representative 24-h record of cumulative urea-N and ammonia-N excretion into the water from a confined ureotelic toadfish (108 g) showing two clear urea-N pulses, marked by arrows. Note the negligible ammonia-N excretion.

for previously published work, or reported in the figure legends for new data of the present report.

For additional details on methods in published studies, please refer to the original citations.

3. Results and discussion

3.1. Rates and routes of pulsatile urea-N excretion

Confined toadfish typically excrete more than 80% of their waste-N in the form of urea at rates averaging approximately 100 μmol-N kg^{-1} h^{-1}; the great majority of this occurs in one or two discrete pulses per day, each of 0.5–3 h duration (e.g., Fig. 1). Absolute values vary somewhat between studies, probably dependent on size, temperature and the nutritional history of the fish. For starved toadfish, mean urea-N excretion rates in different studies ranged from 30 to 220 μmol-N kg^{-1} h^{-1}, mean pulse frequencies from 0.79 to 1.99 pulses day^{-1}, mean pulse size from 1192 to 4334 μmol-N kg^{-1}, percentage ureotelism from 77–94% and percentage of urea-N excreted in pulses from 86–93% (Wood et al., 1995, 1997, 1998, 2001; Walsh and Milligan, 1995; McDonald et al., 2000). Ammonia-N excretion rates are never pulsatile and are kept at minimal levels, indeed undetectable levels, for long periods in some individuals (e.g., Fig. 1). Overall, these rates of N-excretion are quite low for unfed teleost fish (cf. Wood, 1993 for a survey of rates in other species), an observation explained by the fact that the switch to ureotely appears to represent a turning off of ammonia-N excretion rather than a turning on of urea-N excretion (Walsh et al., 1994a; Walsh and Milligan, 1995). Rates of ammonia-N excretion decrease greatly during the period of changeover, while rates of urea-N excretion may actually decrease moderately at this time. Once the changeover is complete, absolute rates of urea-N excretion return close to original values, but ammonia-N excretion is virtually eliminated. As a result, the overall rate of N-excretion during ureotely is only about one third of the original value during ammoniotely.

The pulsatile nature of urea-N excretion in confined toadfish was first noted by Walsh et al. (1990), and subsequently interpreted as pulsatile release of urine from the bladder or of gastrointestinal fluids from the digestive tract (Griffith, 1991; Mommsen and Walsh, 1992; Barber and Walsh, 1993). However, later measurements employing radiolabeling to detect regurgitation of gastric fluids and direct collection of excreted urine and rectal fluid by indwelling urinary and rectal catheters demonstrated that the former does not occur, and each of the latter routes accounts for less than 5% of daily urea-N output (Wood et al., 1995). Most importantly, pulsatile urea-N excretion into the water still occurs despite the presence of the collection catheters. The aglomerular kidney appears to actively secrete urea into the urine (McDonald et al., 2000), while urea movement across the bladder epithelium is by passive diffusion only (McDonald et al., 2002), but these are very small components of overall N-balance.

Wood et al. (1995) also demonstrated that when the fish are placed in divided chambers, the pulsatile urea-N excretion occurs in the compartment containing the head. The axillary pores ('finpits'; unique to the family Batrachoididae) immediately underneath the pectoral fins (Maina et al., 1998) were initially suspected as a possible route, but eliminated when tests showed that pulsatile urea-N excretion is unaffected by their experimental occlusion (Wood et al., 1995). Thereafter, attention focused on the gill, which was essentially proven to be the site of the pulsing by direct sampling of expired water from catheters chronically implanted into the gill pouches (Gilmour et al., 1998). Urea-N pulses always appeared in expired water in correlation with urea-N pulses into the environment. Using indirect calculations of ventilatory flow via the Fick principle, excretion at the gill was large enough to explain the entire urea-N

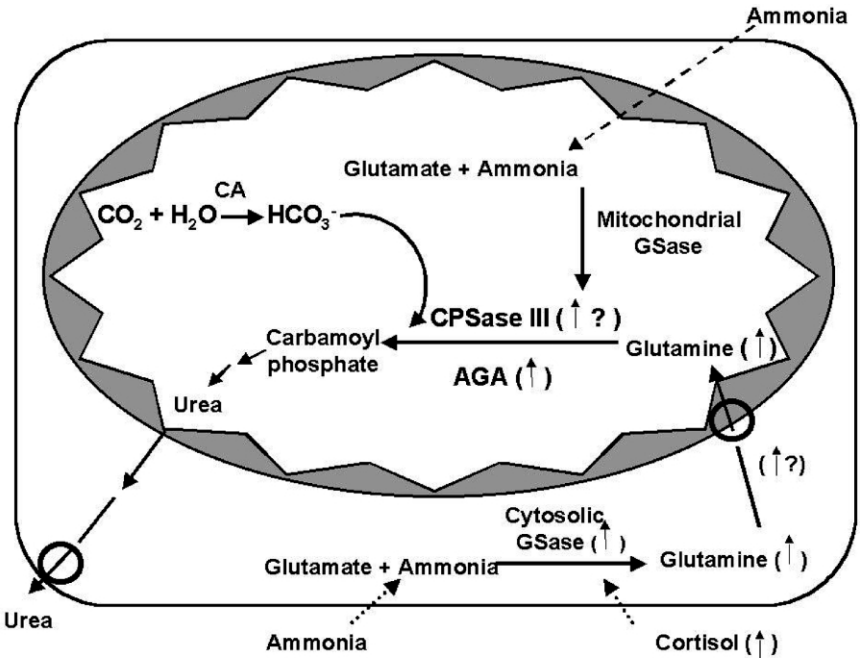

Fig. 2. Schematic representation illustrating the pathways involved in ammonia-N trapping and urea-N synthesis in the toadfish, emphasizing control points which are upregulated (arrows) in the liver, and possibly also in skeletal muscle, during the transition to ureotelism. CA = carbonic anhydrase; GSase = glutamine synthetase; CPSase III = carbamoyl phosphatase III; AGA = *N*-acetylglutamate. See text for additional details.

pulse. Furthermore, spontaneous urea-N pulse events were detected in 4 of 20 perfused toadfish whole-gill preparations (isolated heads detached from the rest of the body), approximating the frequency expected naturally in the perfusion time period for intact toadfish (Pärt et al., 1999). Urea pulse events were never seen (0 out of 23) in perfused heads from non-confined toadfish.

3.2. The mechanism of the transition to ureotelism

Using the crowding/confinement model, a number of biochemical aspects of the transition to ureotelism have now been worked out (cf. Fig. 2), though many questions remain. Physiological analysis of the changeover event has proven difficult and has been essentially limited to flux measurements and terminal samples, because cannulation for repetitive non-invasive blood sampling requires handling and confinement of the animals. In themselves, these treatments raise circulating cortisol levels and initiate the transition (see below). Key to understanding the physiology is that the transition to ureotelism involves a marked reduction in ammonia-N excretion, rather than an increase in urea-N excretion. Key to understanding the biochemistry is the fact that toadfish, like other teleosts and elasmobranchs, which synthesize substantial amounts of urea, employ carbamoyl phosphate synthetase III (CPSase III) as the initial enzyme in the OUC. CPSase III, in contrast to the CPSase I of higher vertebrates, greatly prefers glutamine to ammonia as the N-donor (reviewed by Anderson, 2001). As metabolism produces ammonia, this fact necessitates that glutamine synthetase (GSase) must function in intimate association with the OUC enzymes, serving as the initial ammonia-fixing enzyme (by adding an amino group to glutamate). The GS-CPSase III axis appears to be a critical control point in the turning off of ammonia-N excretion, thereby initiating the switch to ureotelism. The following biochemical events around this axis have been documented in response to the crowding/confinement treatment (Fig. 2).

GSase activities increase up to 5-fold in the liver (Walsh et al., 1994a, 2003; Walsh and Milligan, 1995; Wood et al., 1995; Hopkins et al., 1995), accompanied by comparable increases in GSase protein and mRNA levels (Kong et al.,

2000) and a marked decrease in the hepatic glutamate/glutamine concentration ratio (Walsh and Milligan, 1995). While most attention has focused on the liver, it is now clear that white muscle GSase levels also double at this time (Walsh et al., 2003), while gill GSase activities increase substantially in some studies (Wood et al., 1995), but not others (Walsh et al., 2003). As in the Magadi tilapia (Lindley et al., 1999), the white muscle of the gulf toadfish is probably another ureagenic tissue because it represents a large percentage of the body mass and contains substantial activities of CPSase III and ornithine–citrulline transcarbamoylase (OCTase; Julsrud et al., 1998). However, gill tissue lacks significant levels of the OUC enzymes (Wood et al., 1995).

Curiously, hepatic GSase occurs as two different isoforms in toadfish, one mitochondrial and one cytosolic, both with a very high affinity for ammonia and both apparently the products of the same gene, but with the latter lacking a mitochondrial leader targeting sequence (Anderson and Walsh, 1995; Walsh, 1996; Walsh et al., 1999). However, the increase in GSase activity upon crowding/confinement occurs almost exclusively in the cytosolic fraction (Fig. 2; Walsh and Milligan, 1995; Walsh et al., 2003). Branchial tissue expresses an additional gill-specific GSase, which appears to be the product of a separate gene and is almost exclusively cytosolic because it too lacks a mitochondrial leader targeting sequence (Walsh et al., 2003). Thus, the elevated GSase in both tissues is well positioned to trap excess ammonia-N, keeping it in the cells and preventing its excretion across the gills, but cannot funnel it directly to urea production because CPSase III is exclusively mitochondrial (Fig. 2). Plasma ammonia levels do not change (Wood et al., 1995). Rough calculations (cf. Walsh, 1997) support the idea that there is sufficient GSase activity in the gills to completely prevent ammonia-N excretion despite considerable P_{NH3} gradients from blood to water (Wood et al., 1995; Wang and Walsh, 2000).

If there is greater glutamine production in the cytosol, what happens to it? If this extra glutamine is to donate N to the OUC, the glutamine must enter the mitochondria. Plasma glutamine levels do not change and hepatic glutamine rises twofold at most (Walsh and Milligan, 1995). Henry and Walsh (1997) demonstrated the presence of an inducible glutamine transport system in hepatic mitochondria (Fig. 2). Upon crowding/confinement, V_{max} increases by four-fold, but since Km increases by three-fold (i.e., affinity for glutamine decreases), it is unclear whether glutamine transport into the mitochondria actually increases under in vivo conditions. Km rises from 7.2 to 22.2 mM (Henry and Walsh, 1997), whereas hepatic glutamine levels remain below 6 mM (Walsh and Milligan, 1995). There is similar uncertainty about the capacity of the OUC to use extra glutamine. CPSase III activity is dependent on intra-mitochondrial carbonic anhydrase (CA) activity for HCO_3^- supply (Walsh et al., 1989; Henry and Walsh, 1997). This does not increase during crowding/confinement (Henry and Walsh, 1997), but CA is rarely limiting in biochemical pathways. Activity levels of CPSase III and OTCase measured in vitro (i.e., maximal activities) also do not change (Walsh et al., 1994a; Walsh and Milligan, 1995) but the level of *N*-acetylglutamate (AGA) in the liver, a positive allosteric effector of CPSase III, doubles (Julsrud et al., 1998), providing a probable mechanism for up regulation of activity in vivo (Fig. 2). The stability of maximal CPSase III activity is puzzling, since mRNA levels for CPSase III increase dramatically (5–10-fold) in the livers of crowded fish, but remained unchanged in skeletal muscle (Kong et al., 2000).

A reasonable overall conclusion from these disparate observations is that some upregulation of the capacity for glutamine utilization in the hepatic OUC probably does occur. The most cogent evidence in this regard is a report that the capacity of isolated hepatic mitochondria to synthesize citrulline in the presence of exogenous glutamine increases 10-fold (Henry and Walsh, 1997). This capacity might be exploited in wild, actively feeding fish, but it is not utilized in vivo under the conditions of the crowding/confinement protocol. The principal reason for this conclusion is that the rate of urea-N excretion in vivo does not increase (indeed initially it may decrease), while body urea-N stores rise only slightly (Walsh et al., 1994a; Walsh and Milligan, 1995; Wood et al., 1995). Clearly, there are two parts of the story that have not yet been addressed. The first is the possibility that extra glutamine synthesized may have a fate other than the OUC, for example transamination to other amino acids or deamination by glutaminase. The second is the possibility that the rate of waste-N production is down-regulated during crowding/confinement stress as a result of a shift in fuel usage away from protein and amino acids

to carbohydrate and lipid and/or changes in the relative rates of protein synthesis and degradation. The former could be evaluated by respirometry (cf. Wood, 2001) and the latter by the techniques for measuring protein turnover pioneered by Houlihan and colleagues (cf. Carter and Houlihan, 2001), providing interesting areas for future research.

The proximate internal signal triggering the transition to ureotelism appears to be a modest surge in plasma cortisol concentration (Fig. 2). Indeed, treatment of toadfish with dexamethasone, a synthetic glucocorticoid, promotes hepatic ureagenesis (Mommsen et al., 1992) and a putative glucocorticoid-responsive element has been discovered in the 5′ flanking region of the hepatic GSase gene (P.J. Walsh, unpublished data). Hopkins et al. (1995) reported that plasma cortisol (sampled terminally from non-cannulated fish) rose from baseline levels of approximately 10 to 40 ng ml^{-1} by 2 h of a crowding/confinement treatment, returned to resting levels by 24 h and then started to increase again by 72 h. If the fish were pre-treated with the cortisol-synthesis blocker metyrapone, both the initial cortisol surge and the several-fold activation of hepatic GSase at 24 h were eliminated. It is tempting to attribute the long-term maintenance of the ureotelic state to the secondary rise in cortisol at 72 h onwards, but this is uncertain. In another study (Wood et al., 1995), there was no chronic elevation of cortisol in confined ureotelic fish and indeed the fish reverted to ammoniotelism when plasma cortisol rose to approximately 100 ng ml^{-1} in individuals, which became highly stressed as a result of experimental manipulation or disease. However, in toadfish chronically cannulated for repetitive blood sampling, plasma cortisol levels are routinely >100 ng ml^{-1} (Wood et al., 1997, 2001; McDonald et al., 2003b) and the fish are ureotelic. Since in our experience, cannulation in itself tends to chronically raise plasma cortisol levels, intensive terminal sampling from non-cannulated fish is required to resolve this uncertainty.

3.3. The mechanism of pulsatile urea-N excretion

Pulsatile excretion of urea-N from the gill could reflect pulsatile synthesis or pulsatile entry of urea-N into the bloodstream from the site of synthesis or pulsatile release of stored urea-N from gill tissue or pulsatile clearance of urea-N from the bloodstream by the gill or a co-ordination of several of these mechanisms. Present evidence suggests that pulsatile clearance from the blood by the gills is the relevant mechanism. Urea efflux from the liver does appear to be carrier-mediated (Walsh et al., 1994b; Walsh and Wood, 1996), but there is no evidence of urea storage in liver tissue or pulsatile appearance in the bloodstream. Rather, repetitive blood-sampling from cannulated toadfish has revealed that each pulse event of urea-N excretion into the water is accompanied by a significant fall in plasma urea-N concentration (Fig. 3a), the size of which is directly proportional to the size of the excreted pulse (Wood et al., 1997). As urea is more or less equilibrated throughout the body water, the drop in plasma urea-N concentration, multiplied by the body water compartment, quantitatively matches the size of the excreted pulse. In the intervening period between pulses, plasma urea-N levels rise at a steady rate. This rate approximates the overall time-averaged rate of urea-N excretion, indicating that synthesis and entry are more or less constant. Interestingly, pulsatile excretion never lowers plasma urea-N concentration close to zero, and different individual animals appear to ‘regulate’ around different plasma values (as low as 5 and as high as 40 mmol-N l^{-1}) over many days. The reasons behind this individual variation are unknown.

The pulsatile excretion mechanism appears to be very specific for urea. During a pulse event, there is no change in ventilation or cardiac frequency, arterial blood pressure, the excretion of ammonia-N, radiolabelled PEG-4000 (a paracellular permeability marker) or O_2 uptake, and the efflux of ^3H_2O either remains unchanged or increases only very slightly (Wood et al., 1998; Gilmour et al., 1998; Pärt et al., 1999). Furthermore, injections of branchial vasodilating agents such as *l*-isoprenaline and *l*-adrenaline have little or no effect on urea-N efflux (Perry et al., 1998; Wood et al., 1998; Pärt et al., 1999). The only compounds which have been found to ‘pulse’ in concert with urea are radiolabeled thiourea and acetamide, two well-known structural analogs of urea (Wood et al., 1998; McDonald et al., 2000). During non-pulsing periods, the permeabilities of the gills to the three compounds are equal. During pulsing periods, urea permeability increases 25–50-fold, acetamide permeability 18-fold and thiourea permeability only 5-fold (Wood et al., 1998; McDonald et al., 2000). This differential permea-

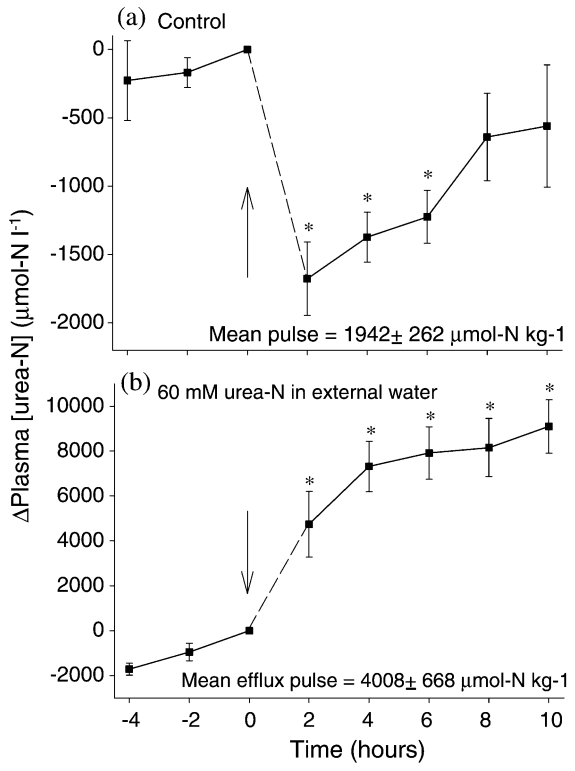

Fig. 3. Changes in plasma urea-N concentrations, as detected by serial sampling via arterial catheter, accompanying natural urea-N pulse events in (a) confined toadfish under control conditions ($N=6$ pulses from five fish; data from Wood et al., 1997) and (b) in confined toadfish exposed to 60 mmol l^{-1} of urea-N in the external water ($N=9$ pulses from eight fish; data from Wood et al., 1998). Values are expressed as differences from the plasma urea-N level immediately preceding the pulse event, taken as 0 at time 0 h. In each case, the mean size of the efflux pulse is shown. Note that under control conditions, plasma urea-N concentration falls by an amount approximately equivalent to the efflux pulse, whereas under 60 mmol l^{-1} of external urea-N, plasma urea-N concentration rises because the net gradient for the facilitated diffusion urea transporter (tUT) is reversed such that urea enters the fish when the transporter is activated. Means ± 1 S.E.M. Asterisks indicate means significantly different ($P \leq 0.05$) from the 0-h value via the Bonferroni test.

bility to various analogs is characteristic of urea transporters in higher vertebrates (e.g., Levine et al., 1973; Chou et al., 1990; Marsh and Knepper, 1992; Gillin and Sands, 1993).

Na^+-coupled active urea transport, which has now been reported in mammals (e.g., Kato and Sands, 1998a,b), was first described in elasmobranchs for the reabsorption of urea-N in the kidney (Schmidt-Nielsen et al., 1972) and very recently for the retention of urea-N at the gills by 'back-transport' across the basolateral membranes (Fines et al., 2001). If such a mechanism exists in the toadfish gill and is periodically turned off, pulsatile excretion would occur. However, modification of the external water by removal of Na^+ or addition of high levels of competitive analogs provided no evidence for the presence of an active 'back-transport' mechanism (Wood et al., 1998). Rather, there is now overwhelming evidence for the presence of a facilitated diffusion transporter for urea homologous to the UT-A family in mammals (Sands et al., 1997; Smith and Rousselet, 2001; Walsh and Smith, 2001; Sands, 2003; Bagnasco, 2003).

The UT-A transporters are electroneutral, bidirectional transporters which behave in a similar manner to channels; they cannot net transport urea against a gradient, but they greatly speed the diffusion of urea in proportion to the relative concentrations on the two sides (Sands et al., 1997; Sands, 1999; Bagnasco, 2003). In the toadfish, this property was revealed by an experiment that reversed the normal urea-N concentration gradient at the gills by placing 60 mmol l^{-1} urea-N in the external water (Fig. 3b). Pre-equilibration of the internal urea-N pool with [^{14}C]urea allowed the detection and quantification of 'efflux' pulse events, while repetitive blood sampling by catheter allowed measurement of internal urea-N levels (Wood et al., 1998). Now, instead of plasma urea-N levels falling during each natural pulse event as would happen under control conditions (cf. Fig. 3a), they rose sharply as the facilitated diffusion mechanism was forced to run backwards on a net basis, transporting urea-N from water to blood (Fig. 3b). Indeed, the sizes of the unidirectional 'influx' and 'efflux' pulses could be calculated and when factored by the relevant urea-N concentration (water for 'influx', blood plasma for 'efflux'), were found to be linearly related on a 1:1 basis. Furthermore, the presence of a two-fold greater concentration of thiourea in the external water inhibited the influx component by 73% (Wood et al., 1998). These characteristics are all hallmarks of UT-A transporters (Chou and Knepper, 1989; Marsh and Knepper, 1992; Sands et al., 1997; Smith and Rousselet, 2001).

Proof of the presence of a UT-A type transporter was provided by Walsh et al. (2000) who cloned an 1814 base pair cDNA from toadfish gill which putatively codes for a 475 amino acid residue protein ('tUT'). Northern blot analysis of gill,

liver, kidney, intestine, skin and red blood cells using a full-length tUT cDNA probe detected the presence of tUT mRNA only in gill tissue, even under low stringency conditions. This suggests that the urea transporter in the liver (Walsh et al., 1994b; Walsh and Wood, 1996) must be rather different. Interestingly, a Northern blot survey with the tUT cDNA probe of gill tissue mRNA from a variety teleosts revealed that related UT-A type transporters occur widely, even in ammoniotelic teleosts (Walsh et al., 2001b). The tUT protein exhibits approximately 62% homology at the amino acid level to UT-A transporters in higher vertebrates and in the elasmobranch dogfish kidney ('shUT'; Smith and Wright, 1999) and approximately 75% homology to two other recently identified teleostean urea transporters, mtUT and eUT from the gills of the *Magadi tilapia* (Walsh et al., 2001a) and the Japanese eel (Mistry et al., 2001), respectively. When the cRNA for tUT was expressed in *Xenopus* oocytes, it caused a many-fold increase in urea permeability that could be inhibited by the aglycon phloretin (Walsh et al., 2000), another hallmark of facilitated diffusion urea transporters (Chou and Knepper, 1989). When intact toadfish were treated with phloretin, pulsatile urea-N excretion was abolished, but interpretation was clouded by toxicity caused by the drug (Wood et al., 1998).

Semi-quantitative Northern analysis of tUT mRNA in the gills (relative to β-actin) demonstrated that the signal was more or less the same in ammoniotelic non-crowded fish and in confined ureotelic fish killed during actual pulse events (detected with [^{14}C]urea) or at various times up to 18 h thereafter (Walsh et al., 2000). This suggests that if tUT protein activity is regulated in relation to pulsing, this regulation occurs at a point beyond the mRNA level. An unusual feature of tUT from other UT-A proteins is a much longer C-terminal sequence containing potential amidation and phosphorylation sites. Potentially, these could be activated to cause rapid upregulation of urea-N transport during a pulse event.

Pronounced ultrastructural changes accompanying the pulse event have been detected in the gill epithelia of the both *O. beta* and *O. tau* (Laurent et al., 2001). The pavement cells of toadfish, which make up the majority of the branchial surface, are unusually rich in Golgi membranes and contain a substantial number of dense-cored vesicles (50–200 nm diameter) in the cytoplasm. These vesicles are lacking from the chloride cells, and are rare in the pavement cells of other species, even in the related plainfin midshipman *Porichthys notatus*, which is obligately ammoniotelic (Wang and Walsh, 2000). However, they are abundant in the pavement cells of the *Magadi tilapia*, another ureotelic teleost with a facilitated diffusion urea transporter (mtUT) in its gills (Walsh et al., 2001a). The numerical abundance of these vesicles in the pavement cells is doubled in toadfish sacrificed during a pulse event (detected with [^{14}C]urea), and a much greater percentage are found close to the apical membrane (Laurent et al., 2001). The vesicles appear to bud off the Golgi as clear structures, then grow in size and fill with electron-dense material as they move outwards, eventually contacting the apical membrane. The vesicles do not appear to fuse with the membrane, but may discharge their contents through a narrow pore. This trafficking is reminiscent of the vasopressin-regulated trafficking and apical membrane insertion of aquaporins (Nielsen et al., 1995) and UT-A1 (Nielsen et al., 1996) reported in inner medullary collecting duct cells (IMCD) of the rat kidney, though the UT-A1 trafficking was later refuted (Inoue et al., 1999). In this regard, treatment of intact ureotelic toadfish with colchicine, a blocker of microtubule assembly and, therefore, of vesicular trafficking, abolished pulsing and caused internal urea-N accumulation (Gilmour et al., 1998). At present, it is unclear whether the vesicles are carrying tUT, urea-N, other substances or some combination thereof, though preliminary immunogold cytochemistry with tUT-specific antibodies supports the presence of tUT in the vesicles. We speculate that this trafficking is a significant component of the post-transcriptional events mediating pulsatile excretion of urea-N.

3.4. The trigger for pulsatile urea-N excretion

The relative constancy of plasma urea-N levels in individual toadfish might suggest that pulses are triggered when threshold concentrations are surpassed, but this does not appear to be the case. When a bolus of urea was re-injected into the bloodstream via an arterial catheter immediately after a natural pulse event, thereby raising plasma urea-N concentration above the pre-pulse level, another pulse was not elicited and indeed, there were no further pulses for at least 8 h (Wood et al., 1997). Furthermore, continuous infusion of

urea, which raised plasma urea-N more than four-fold (to 75 mmol-N l^{-1}!) had a negligible effect on branchial urea-N excretion (no change in pulse frequency, marginal rise in pulse size; McDonald et al., 2003a). In light of these results and our general observations on toadfish over the years, we favor a behavioral explanation (conscious choice) rather than an internal urea-N threshold as the trigger for a pulse event. Just as humans maintain relatively constant internal fluid, ionic and urea-N levels but consciously choose when and how much to urinate, we speculate that toadfish may do the same with urea-N pulsing. In confined fish in the laboratory, we have never seen any correlation with time of day, photoperiod, the tidal cycle in the nearby bay from which they were collected, activity patterns, or use of shelters (cf. Walsh, 1997; Gilmour et al., 1998). However, we have noted that pulsing tends to occur more frequently when people are not present in the lab–, i.e., when the general level of disturbance is lower. This line of thought suggests that pulsing may be under neural and/or hormonal control(s).

3.5. Possible endocrine controls on pulsatile urea-N excretion

Since injections of catecholamines via arterial catheter do not cause pulsing (Wood et al., 1998; Perry et al., 1998; Pärt et al., 1999), the stress hormone which obviously comes to mind is cortisol, the hormone involved in the original transition to ureotelism (Hopkins et al., 1995). When repetitive small blood samples are taken by catheter under conditions intended to minimize disturbance, plasma cortisol levels fall markedly (from approximately 120 ng ml^{-1} down to 40 ng ml^{-1}) in the 2–4-h period prior to a pulse event and then rise rapidly thereafter. This pattern has now been seen in two separate studies (Wood et al., 1997, 2001). However, cortisol levels will sometimes decline without urea-N pulses occurring. Interestingly, when the cortisol-synthesis blocker metyrapone is used to pharmacologically lower plasma cortisol, tiny pulse events (<100 nmol-N kg^{-1}, much smaller than natural pulses) usually occur in the ensuing 30–90 min (Wood et al., 2001). Therefore, we interpret the cortisol decline as a correlate, likely a permissive correlate (see below), rather than a causative event. An anthropomorphic interpretation is that the fall in circulating cortisol reflects the fact that the fish 'feels safe' to excrete a urea-N pulse. At a mechanistic level, there may well be a parallel with the regulation of UT-A transporters in the mammalian kidney. Glucocorticoids are known to down-regulate urea-N transport by reducing the amount of UT-A1 type protein but without changing the level of UT-A1 mRNA in the rat kidney (Marsh and Knepper, 1992; Naruse et al., 1997; Klein et al., 1997; Sands, 1999–but see Peng et al., 2002), so potentially a fall in cortisol could upregulate tUT in the toadfish gill.

Teleost fish have only one major corticosteroid hormone, cortisol, which is classically considered a glucocorticoid, and lack the aldosterone-type mineralocorticoid hormones of higher vertebrates (Mommsen et al., 1999). Nevertheless, it has recently come to light that there are two different cortisol receptors in teleosts, a glucocorticoid receptor (cloned by Ducouret et al., 1995) and a mineralocorticoid receptor (cloned by Colombe et al., 2000). At least one function has already been attributed to the latter, cortisol-stimulated proliferation of gill chloride cells in freshwater trout exposed to extremely dilute freshwater (Sloman et al., 2001). Traditionally, mineralocorticoid receptors can be blocked by spironolactone and glucocorticoid receptors can be blocked by RU-486 (Bertagna et al., 1984; Gaillard et al., 1985), lipid-soluble agents that are best administered by intraperitoneal injections in vegetable oil.

With this background in mind, McDonald et al. (2003b) recently investigated whether experimental maintenance of high plasma cortisol levels will suppress urea-N pulsing and the nature of the receptors potentially involved. When toadfish are chronically infused with sufficient cortisol to 'clamp' their circulating concentrations at a very high value (>500 ng ml^{-1}), urea excretion is greatly reduced and plasma urea-N concentrations rise over the following 48–96 h. The effect is not due to a change in pulse frequency (Fig. 4b), but rather to about a 70% decrease in pulse size (Fig. 4a). The effect persists in toadfish injected with peanut oil alone (shams) but is reversed in toadfish treated with RU-486–indeed, pulse size greatly increases. Spironolactone has little effect. Thus, the inhibitory action of cortisol is clearly mediated through glucocorticoid receptors and not through mineralocorticoid receptors. Since the occurrence of urea-N pulsing is not reduced, but rather just the size of the pulses, these data suggest that the activity of tUT is down-regulated by high cortisol,

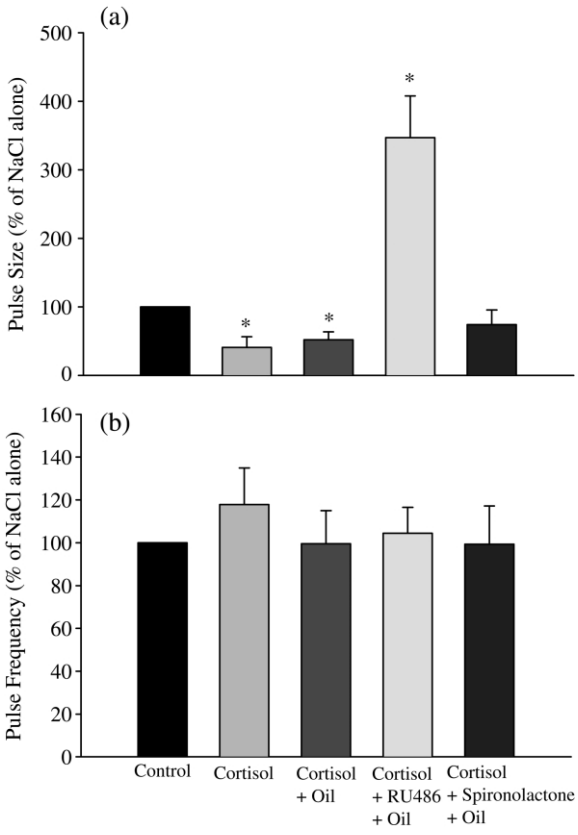

Fig. 4. Summary of the effects on urea-N pulsing of continuous cortisol (hydrocortisone semisuccinate) infusion via arterial catheter at a rate (270 μg cortisol kg^{-1} h^{-1}) sufficient to hold plasma cortisol levels at a plateau of >500 ng ml^{-1} in confined toadfish. (A) Mean pulse size as percent of pretreatment control value; and (B) mean pulse frequency as percent of pretreatment control value. The fish were first infused with vehicle alone (140 mM NaCl) for 48 h to establish the control pattern and then experimental treatments were instituted for a further 48–96 h. The glucocorticoid blocker RU-486 and the mineralocorticoid blocker spironolactone were administered as intraperitoneal injections (5 mg in 0.3 ml peanut oil) at 12-h intervals during the experimental period. Note that cortisol alone or cortisol plus oil, significantly inhibited the size of urea-N pulses, while treatment with RU-486 reversed this response such that pulse size increased greatly, whereas spironolactone had little effect. Pulse frequency was unaffected by all treatments. Means ± 1 S.E.M. (N=6–10 per treatment). Asterisks indicate means significantly different from the control value ($P \leq 0.05$; ANOVA on transformed data followed by Tukey's test). Data from McDonald et al. (2003b).

but that the proximate signal eliciting pulses is unaffected. Thus, low cortisol prior to a pulse may up-regulate tUT (i.e., a permissive action) but something else triggers the pulses. The result is in accord with the situation in mammals where the amount of transporter is down-regulated via chronic stimulation of glucocorticoid receptors (Marsh and Knepper, 1992; Naruse et al., 1997; Klein et al., 1997; Peng et al., 2002), but other agents cause the rapid activation of transport.

The best-known in this regard in mammals is the neurohypophysial nonapeptide arginine vasopressin (AVP, 'antidiuretic hormone') which within 40 s can activate urea-N transport via a V_2-receptor in IMCD cells (Knepper and Star, 1990; Wall et al., 1992; Sands, 1999). Adenylyl cyclase is turned on and there is a cAMP-mediated post-translational action involving protein kinase A mediated phosphorylation of the UT-A1 protein, an effect which increases the activity, but not the total protein level, of UT-A1 in the apical membrane of the renal IMCD (Nielsen et al., 1996; Sands et al., 1997; Shayakul et al., 1997; Inoue et al., 1999; Zhang et al., 2002). In addition, AVP has long been known to activate urea transport in the toad bladder (Levine et al., 1973; Eggena, 1973). As the teleost homolog of AVP is arginine vasotocin (AVT), these findings in higher vertebrates make it an obvious candidate in toadfish.

Initial results with AVT were encouraging (Perry et al., 1998): injected doses (via arterial catheter) calculated to produce circulating AVT levels of 10^{-10} and 10^{-9} M elicited urea-N pulses of several 100 μmol-N kg^{-1} in confined toadfish. However, Pärt et al. (1999) were unable to detect any effect of 10^{-10} M AVT on urea-N excretion in isolated–perfused gills of confined toadfish. In a later in vivo study (Wood et al., 2001), the threshold for effect was higher than reported by Perry et al. (1998): only 10^{-9} M AVT consistently induced pulse events and the pulses were smaller (approx. 100 μmol-N kg^{-1}). AVP (10^{-9} M) had the same effect in approximately 60% of the trials, while isotocin (10^{-9} M; the other fish homolog) was never effective. Overall, these results are equivocal, considering that natural urea-N pulses in these same animals were much larger (>2000 μmol-N kg^{-1}) and that the threshold for effect in both studies was above the normal circulating AVT levels reported in fish (10^{-12}–10^{-11} M; Warne et al., 2002).

Using a sensitive radio-immunoassay (Warne et al., 1994; Wood et al., 2001) then confirmed that circulating AVT concentrations in confined toadfish really were very low (10^{-12}–10^{-11} M) and more importantly that there was no consistent fluctuation of plasma AVT levels accompanying

spontaneous pulse events, in contrast to the clear fall in plasma cortisol. A possible explanation in toadfish for the small urea-N pulses in response to high doses of AVT is that aquaporins are activated. Aquaporins have recently been identified in fish gills (Cutler and Cramb, 2002) and are known to transport urea weakly in other systems (Borgnia et al., 1999). Indeed, Perry et al. (1998) noted that tritiated water efflux tended to increase during AVT-induced pulses. Alternatively, pulses could be an artifact of the intense pressor response caused by 10^{-10}–10^{-9} M AVT in toadfish (Perry et al., 1998). Interestingly, in rat kidney medulla, AVP-sensitivity is seen only in the larger transcript (UT-A1) and not in the smaller UT-A2 (Sands, 1999), whereas the predominant tUT transcript in the toadfish gill is small (1.8 kB). At present, we conclude that circulating AVT is probably not the trigger eliciting normal urea-N pulses in toadfish gills. However, we cannot eliminate the possibility that very high AVT levels released locally from neurons could be involved. Furthermore, it must be remembered that in teleosts, AVT is one of the two hypothalamic peptides (the other is CRH) that control the release of ACTH from the pituitary, which in turn mobilizes cortisol from interrenal cells (Baker et al., 1996).

Another neuroendocrine agent of interest is 5-hydroxytryptamine (5-HT; serotonin) and we here report new data on responses to 5-HT in confined toadfish. Our interest was drawn to this indoleamine because it is known to be stored in high concentrations in both neurons and neuroepithelial cells (NECs) of the gill (Bailly et al., 1989, 1992; Jonz and Nurse, 2003) and because the gills are known to avidly extract 5-HT from the entering venous blood (Olson, 2002). Using a commercial ELISA (American Laboratory Products), we have found 5-HT to be present at surprisingly high concentrations (10^{-7}–10^{-6} M) in the circulating blood plasma sampled by arterial catheter from confined toadfish. While plasma levels fluctuate considerably over time, it is presently unclear whether these fluctuations are correlated with pulse events. Nevertheless, when 5-HT was injected via arterial catheter at a dose calculated to yield circulating concentrations of 3×10^{-6} M (i.e., close to physiological levels), substantial pulses resulted in 57% of the fish ($N=70$), averaging 872 ± 158 μmol-N kg^{-1}, with some pulses as high as 3000 μmol-N kg^{-1}. Furthermore, when the data were re-analyzed to eliminate any fish where a natural urea-N pulse had occurred in the 6-h period preceding the 5-HT injection, the success rate for pulse elicitation increased significantly to 73%. Thus, 5-HT-induced pulses are comparable in size to spontaneous pulse events and there may be a 'refractory period' after the latter. Fig. 5 illustrates a large 5-HT-induced pulse, and the inset compares the mean size of natural pulses with those induced by AVT (10^{-9} M) or 5-HT (3×10^{-6} M). Because 5-HT is known to have potent effects on branchial blood flow, vasoconstricting the arterio-arterial pathway and dilating the arterio-venous pathway in the gills (Sundin and Nilsson, 2002), we were concerned that pulses might result from non-specific effects. However, there was no change in ammonia excretion (e.g., Fig. 5) and no increase in [^{3}H]PEG-4000 permeability ($N=18$) accompanying 5-HT-induced pulses.

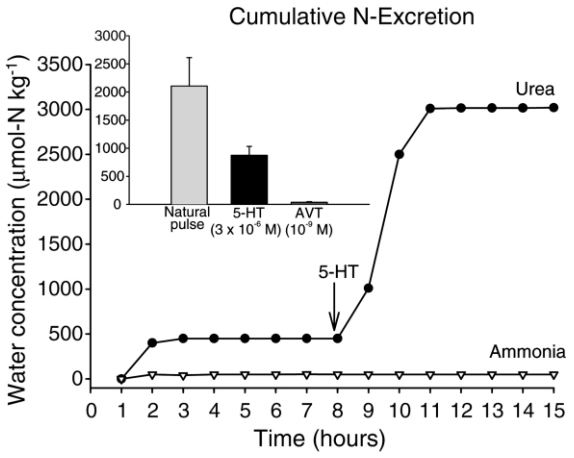

Fig. 5. A representative 15-h record of cumulative urea-N and ammonia-N excretion into the water from a confined ureotelic toadfish (60 g) showing a large urea-N pulse induced by a bolus injection of 5-HT calculated to produce circulating levels of 3×10^{-6} M. Compare with the natural urea-N pulse event of Fig. 1. Note the complete lack of effect on ammonia-N excretion, which remained negligible. Inset compares the mean size of spontaneous natural pulses ($N=27$) with those induced by bolus injections of 5-HT (3×10^{-6} M; $N=40$) and AVT (10^{-9} M; $N=18$). Means $\pm$ 1 S.E.M.

While these findings are preliminary, they are exciting because there are now known to be powerful interactions between 5-HT, the hypothalamo-pituitary-interrenal (HPI) axis responsible for cortisol mobilization, and behavior in fish. For example, in rainbow trout, elevated brain 5-HT levels activate the HPI axis, stimulating cortisol mobilization and causing behavioral inhibition in

subordinate fish (e.g., Winberg et al., 1997; Winberg and LePage, 1998). If urea-N pulsing in toadfish has social signaling significance (see below), we might expect both 5-HT and cortisol to be involved. These considerations suggest a range of future experimental approaches: careful time course monitoring of 5-HT levels in plasma and brain tissue relative to the timing of pulses; pharmacological analysis of the 5-HT response; immunocytochemical studies to determine if degranulation of 5-HT stores accompanies pulsing (e.g., Bailly et al., 1992); investigation of the effects of 5-HT on plasma cortisol and the HPI axis; and investigation of pulsing behavior in relation to dominance/subordination relationships. Clearly, they also suggest that checking for direct

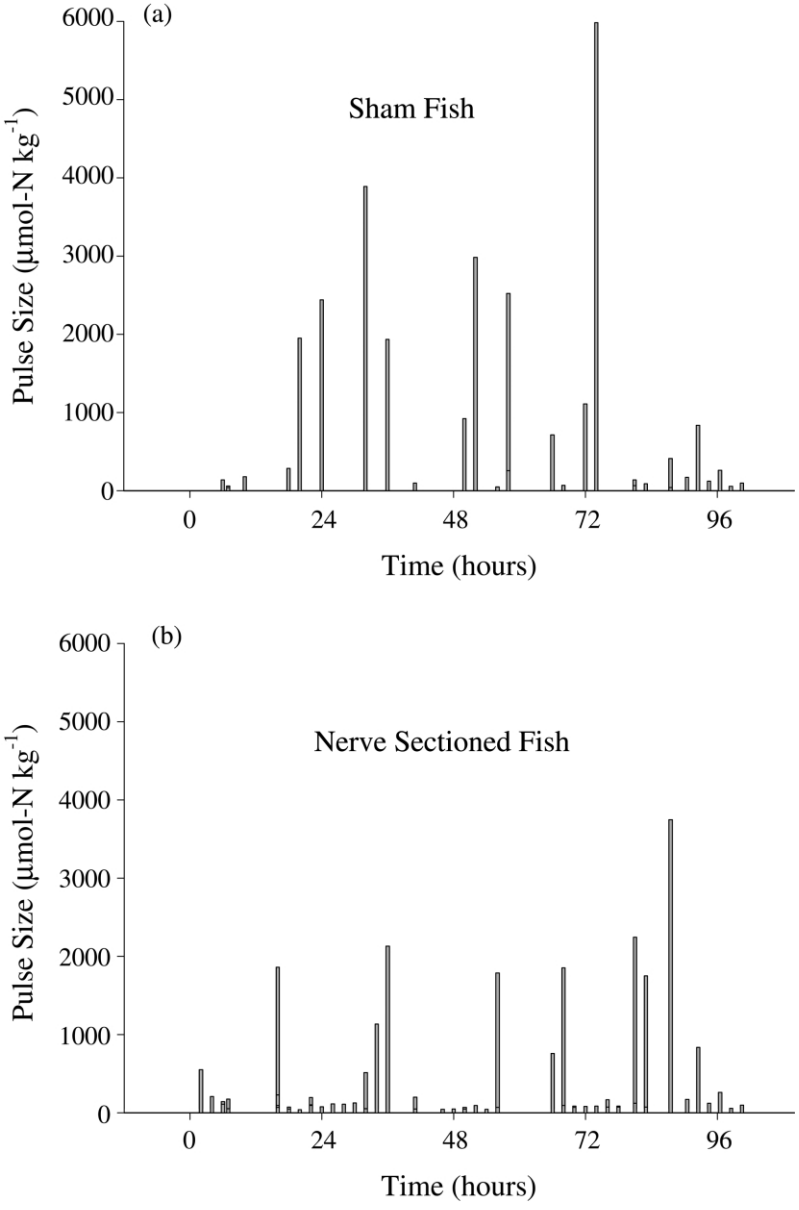

Fig. 6. A record of the individual sizes and occurrence of all urea-N pulse events over a 100-h monitoring period in (a) six confined toadfish that had been subjected to sham surgery and (b) six confined toadfish that had been subjected to bilateral surgical section of all branchial branches of cranial nerves IX (glossopharyngeal) and X (vagus). Note the persistence of urea-N pulses in the nerve-sectioned fish.

neural activation of pulsing should be a high priority. We can already shed some light on this issue.

3.6. Possible neural controls on pulsatile urea-N excretion

Cranial nerves IX (glossopharyngeal) and X (vagus) provide virtually all motor and sensory innervation to the gills in teleosts (Sundin and Nilsson, 2002). Here, we report new data on the responses to bilateral surgical sectioning (see Section 2) of all branchial branches of these nerves in ureotelic toadfish that had been crowded and then confined. Sham animals were put through identical surgery but without nerve sectioning. The key finding is that pulsatile urea-N excretion persisted throughout a 100-h monitoring period after the branchial nerves were cut ($N=6$; Fig. 6) and total urea-N excretion remained unchanged relative to shams ($N=6$). There were subtle changes in the pattern of pulsatility, with a significant two-fold increase in frequency compensated by a non-significant 50% reduction in mean pulse size (Fig. 7). This is perhaps not surprising, as the animals were clearly affected by the procedure. Ventilation became very labored, likely reflecting the loss of proprioceptive and chemosensory feedback, as well as motor control to the branchial muscles (Sundin and Nilsson, 2002). Apart from the caveat that cranial nerves VII (facial) could not be sectioned since they innervate the muscles of breathing, these results present strong evidence against direct neural control of the pulse event coming from the central nervous system. However, they do not eliminate the possibility of endocrine or paracrine agents acting on nerve networks, individual neurons, or NECs in the gills to activate pulsing. For example, in addition to the earlier reported extrinsic serotonergic neurons and NECS (Bailly et al., 1989, 1992) Jonz and Nurse (2003) have recently identified a network of intrinsic serotonergic neurons in the gills of the zebrafish. These survive in explant culture when removed from central nervous influence.

3.7. The adaptive significance of pulsatile urea-N excretion

This remains the area of greatest uncertainty. We believe it most unlikely that the crowding/confinement protocol is non-specifically eliciting

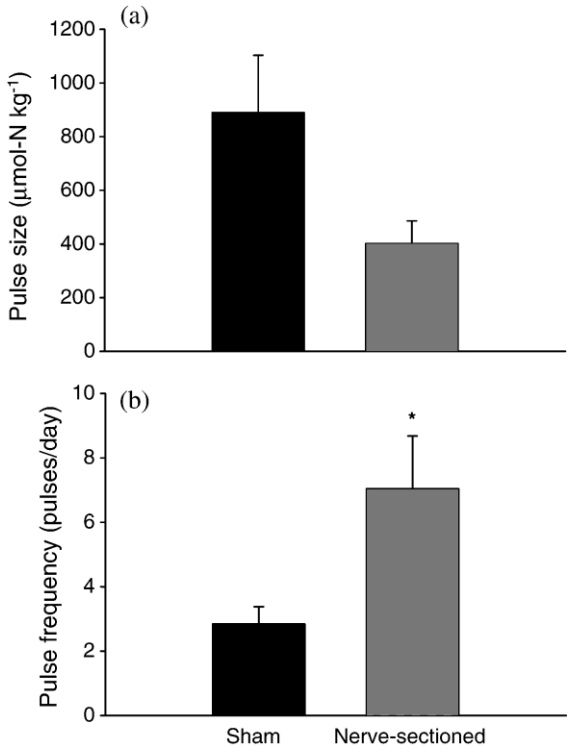

Fig. 7. A summary of (a) mean urea-N pulse size and (b) mean urea-N pulse frequency in confined toadfish that had been previously subjected to either bilateral section of all branchial branches of cranial nerves IX (glossopharyngeal) and X (vagus) or sham surgery. Means ± 1 S.E.M. ($N=6$ per treatment). Asterisks indicate means significantly different ($P \leq 0.05$) via Student's *t*-test.

a behavior intended solely to cope with high environmental ammonia or air emersion. Rather, we suspect that it mimics some natural situation that the toadfish encounters from time to time in its natural environment for which pulsatile urea-N excretion is adaptive. Earlier, we suggested three possibilities (Wood et al., 1995; Walsh, 1997), to which we can now add a fourth. The first is chemical crypsis. By shutting off ammonia-N excretion and restricting urea-N excretion to short periods when the animal perceives the environment to be 'safe', the toadfish avoids giving off a signal to olfactory predators such as sharks, as well as to prey. This would be most important when the toadfish are crowded at high density in one area. The second is housekeeping. Toadfish spend much of their time in burrows and under rocks; the absence of ammonia-N efflux combined with short forays to voluntarily 'pulse' out stored up urea-N would avoid fouling of home base during this

natural confinement. The third is N-conservation. At times of food scarcity (e.g., intense competition), the transition to ureotelism which reduces N-excretion by two-thirds may be part of an overall strategy to minimize protein breakdown and conserve metabolic N. Our new suggestion, which stems from our findings on 5-HT and cortisol, is that pulsatile urea-N excretion may provide a message for intra-specific signaling of social status under crowded conditions. For example, high 5-HT levels may both foster behavioral inhibition in a subordinate fish and stimulate a urea-N pulse to transmit that fact to conspecifics. These hypotheses are neither exhaustive nor mutually exclusive, but provide a rich area for future behavioral and ecological investigations.

Acknowledgments

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