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Dogmas and controversies in the handling of nitrogenous wastes: Ureotely and ammonia tolerance in early life stages of the gulf toadfish, *Opsanus beta*

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Summary

The marine gulf toadfish (*Opsanus beta*) is an unusual teleost fish as it is able to switch between ammoniotelism and ureotelism in response to a variety of laboratory conditions. The present study integrates field work conducted in Biscayne and Florida Bays, USA with laboratory studies to examine ureotelism during the early life history stages of *O. beta*. Adult toadfish voluntarily nested in artificial shelters placed amongst seagrass beds and were found to be predominantly ureotelic under natural conditions as the internal shelter water had mean urea and ammonia concentrations (*N*=51) of 14.2±1.6 μmol N l^{-1} and 8.9±0.9 μmol N l^{-1}, respectively. Toadfish successfully spawned in shelters, providing eggs, larvae and juvenile toadfish for laboratory study. In the lab, juvenile toadfish were also ureotelic and urea was excreted in pulsatile events that accounted for 62.0±5.9% of total urea-N excreted. Excretion rates of urea-N and ammonia-N were 1.018±0.084 μmol N h^{-1} g^{-1} and 0.235±0.095 μmol N h^{-1} g^{-1}, respectively. Field-collected eggs, larvae and juveniles expressed significant levels of the ornithine–urea cycle enzymes carbamoyl-phosphate synthetase III, ornithine transcarbamylase and arginase and the accessory enzyme glutamine synthetase, all of which increased in activity as toadfish developed through early life stages. In juveniles, the ammonia 96-h LC_{50} value was 875 μmol N l^{-1} and there was a 3-fold increase in ornithine transcarbamylase activity in the 1000 μmol N l^{-1} NH_4Cl treatment. The results are discussed in the context of the causal factor(s) for ureotelism in toadfish. Furthermore, the results of this study suggest it is unlikely that the adaptive significance of ureotelism in toadfish is a means to prevent fouling nests with ammonia and in turn poisoning offspring; however, additional study is warranted.

Key words: ureogenesis, ureotely, *Opsanus beta*, toadfish, ontogeny, ammonia toxicity, ornithine–urea cycle, ornithine transcarbamylase, Florida Bay, Biscayne Bay, Batrachoididae.

Introduction

In most teleost fishes, waste nitrogen is excreted predominantly as ammonia, with trace amounts of urea produced by uricolysis or arginolysis (Wood, 1993; Walsh, 1998). While the genes regulating urea synthesis (ureogenesis) *via* the ornithine–urea cycle (OUC) were thought to be silenced in the more evolutionarily derived teleost fishes (Brown and Cohen, 1960), ureogenesis has been reported in embryonic and larval rainbow trout (Dépêche et al., 1979; Wright et al., 1995; Korte et al., 1997), Atlantic cod (Chadwick and Wright, 1999), Atlantic halibut (Terjesen et al., 1998) and African catfish (Terjesen et al., 2001). This metabolic pathway is apparently suppressed during subsequent developmental stages in most fish. However, ureotely was discovered in several adult teleosts (e.g. Saha and Ratha, 1987; Randall et al., 1989; Mommsen and Walsh, 1989), and the underlying mechanisms have been studied extensively in the facultatively ureotelic gulf toadfish, *Opsanus beta* (see Walsh, 1997; Walsh and Mommsen, 2001).

As adults, *O. beta* express a full complement of OUC enzymes in the liver, which includes carbamoyl-phosphate synthetase III (CPSase III) as opposed to the isoform CPSase I, commonly found in terrestrial vertebrates (Read, 1971; Mommsen and Walsh, 1989). The nitrogen-donating substrate for CPSase I is ammonia directly, while CPSase III is dependent upon glutamine as an intermediary (Mommsen and Walsh, 1989). In the toadfish, glutamine synthetase (GSase) is intimately linked to the OUC and, during facultative shifts to ureotelism, hepatic GSase activities increase 5-fold while white muscle activities double (Walsh et al., 1994; Walsh et al., 2003).

Adult *O. beta* have a high tolerance to elevated external or environmental ammonia, which may be related to ammonia detoxification *via* the OUC, with GSase functioning as an ammonia trap (Wang and Walsh, 2000). In a 96-h lethal concentration (96-h LC_{50}) test, 50% mortality of test subjects occurred at a total ammonia concentration of 9.75 mmol N l^{-1} (166 mg l^{-1}) with a calculated unionized ammonia (NH_3)

fraction of 519 μmol N l^{-1} (8.8 mg l^{-1}) (Wang and Walsh, 2000). The toadfish LC_{50} values are relatively high when compared with NH_3 mean acute toxicity values of 164 and 109 μmol N l^{-1} for 32 freshwater and 17 marine teleost species, respectively (in Randall and Tsui, 2002). Ammonia tolerance in the early life stages of toadfish remains unknown although it is reported that in rainbow trout (Rice and Stokes, 1975), the spotted seatrout (Daniels et al., 1987) and other teleosts (see Steele et al., 2001) that embryonic stages are more tolerant of environmental ammonia than their corresponding adult stages.

The majority of urea produced by adult *O. beta* is excreted in distinct pulsatile events across the gill membrane once or twice daily (Wood et al., 1995; Gilmore et al., 1998). Confined *O. beta* excrete 80% of their nitrogenous waste as urea with pulses ranging from 1192 to 4334 μmol N kg^{-1} in concentration and 0.5–3 h in duration (reviewed in Wood et al., 2003). Furthermore, the oyster toadfish, *Opsanus tau*, was classified as 'moderately' ureotelic when compared with the 'fully' ureotelic *O. beta* (Wang and Walsh, 2000). *O. tau* was found to switch from ammonotely to ureotely upon hatching and through the subsequent larval stage (Stephen and Griffith, 2001), yet little else is known regarding the ontogeny of nitrogen metabolism/excretion in toadfish.

The reproductive ecology of toadfish, however, has been widely studied. *O. beta* are reported to actively spawn when water temperatures range from 15 to 22°C (Breeder, 1941), which tends to occur from March to May in the shallow coastal estuaries of southern Florida. Male toadfish establish nesting sites under stones and in large gastropod shells (Ryder, 1886; Gill, 1907) but are noted to prefer the 'debris of civilisation' such as tin cans and broken jars (Clapp, 1899). Nesting males subsequently attempt to attract mates with courtship vocalizations known as boatwhistles (Gray and Winn, 1961; Breeder, 1968). Eggs are solidly adhered to the nest substrate by females, who leave attending males to brood and guard offspring through the yolk-sac larval stage where larvae remain connected to the substrate by a pedicel until they become free-swimming juveniles (Ryder, 1886; Gudger, 1908).

It is believed that ureotely in toadfish must provide some adaptive significance to counterbalance the bioenergetic cost of 2.5 ATP per unit N excreted (reviewed by Wood et al., 2003). Several viable hypotheses exist pertaining to why toadfish are ureotelic; however, the ultimate causal factor(s) remains unknown. It was suggested that male toadfish are facultatively ureotelic to avoid poisoning progeny with ammonia in confined nests with restricted water flow (Griffith, 1991; Stephen and Griffith, 2001). In the present study, the mechanisms of ureogenesis and patterns of urea excretion across early life history stages of *O. beta* were investigated. In addition, the hypothesis that ureotelism in the toadfish exists as a mechanism to protect offspring from toxic levels of ammonia in confined nests was addressed. Ammonia-N and urea-N were measured in water collected from within *O. beta* shelters at field sites and in containers in the laboratory along with an ammonia 96-h LC_{50} test. Enzymatic activities of eggs, larvae and juveniles were measured for the OUC and accessory enzymes CPSase III, ornithine transcarbamylase (OTCase), arginase and GSase from samples collected in the field and on surviving juveniles from high ammonia exposures.

Materials and methods

Site descriptions and field protocols

Field studies were conducted at Johnson Key Basin in Florida Bay (FB-1) and offshore of Elliot Key in Biscayne Bay (BB-1), located within Everglades National Park and Biscayne National Park, respectively (Fig. 1). Both Florida and Biscayne Bays can be characterized as subtropical estuaries with extensive seagrass coverage. Florida Bay, however, can be further characterized geomorphologically by a web of mangrove islands and mud banks forming basins with restricted water flow. FB-1 served primarily as a site for monitoring spawning activities and is located 78 km WSW of BB-1, which served as a source for early life history stages of *Opsanus beta* Goode and Bean (family: Batrachoididae). Artificial habitats or shelters, patterned after Barimo and Fine (1998), consisted of 25 cm lengths of 5 cm inner diameter polyvinylchloride (PVC) pipe strapped to grey bricks (10×10×20 cm) to provide benthic anchorage. PVC pipe was capped at one end so as to mimic the internal chamber of natural nesting sites such as large gastropod shells. Shelters were deployed at FB-1 and BB-1 at a depth of ~1 m during March 2003 on small patches of sediment among seagrass beds dominated by the halophyte *Thalassia testudinum*. Shelters were grouped in clusters of 6–8 with a density of 1 m^{-2}, thus approximating the natural distribution patterns of *O. beta* (Sogart et al., 1987), and clusters were centred on global positioning system (GPS) decimal-degree coordinates of N25.04033 by W80.91289 and N25.40867 by W80.21895 for FB-1 and BB-1, respectively.

At each cluster of shelters, salinity was determined with a refractometer and water depth was measured with a PVC pole marked in cm increments. Shelters were retrieved by divers (April–May 2003) who capped the shelter's open end, thereby encapsulating nesting toadfish and 'shelter' water. Once onboard the attending research vessel, shelters were opened over a basin, water samples were collected, and pH and temperature were measured with a WTW model pH 331i meter set. To allay concerns that encapsulating toadfish (<2 min) influenced nitrogen excretions, a subset of sampling was compared with shelter water collected rostral to the toadfish by syringe prior to capping. Although the capped samples were slightly higher (3.7±2.3 mmol N l^{-1}) than the syringe samples (2.7±1.7 mmol N l^{-1}), these differences are not significant (P=0.50, N=5) with the paired Wilcoxon signed rank test. The increased values for the encapsulated samples probably represent uniform mixing of the shelter water since ventilated water probably pools caudal to the toadfish at the rear of the shelter. Water-quality samples were also collected from the ambient water column near shelters to access background concentrations of ammonia and urea. Water samples were acidified to pH 2 with 12 mol l^{-1} HCl (2 μl ml^{-1} seawater) to

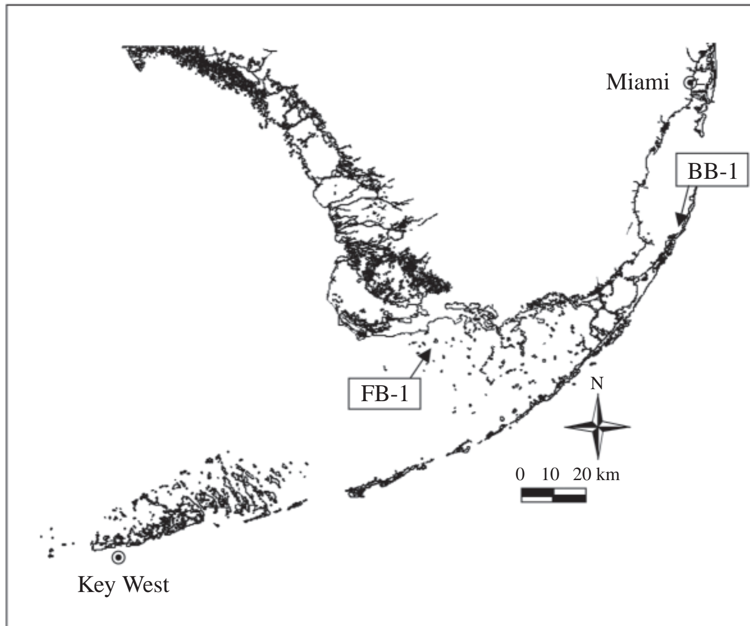

Fig. 1. Map of South Florida and the Florida Keys depicting field locations in Florida Bay (FB-1) and Biscayne Bay (BB-1) where artificial habitats or shelters were deployed. The FB-1 site was used to document toadfish spawning activity and sample water for *in situ* nitrogen excretion within shelters. BB-1 was used to sample early life history stages of toadfish for OUC enzyme analysis and to harvest whole nests (shelter + adult + offspring) for experimental studies.

inhibit bacterial degradation and prevent NH_3 volatilization, stored on ice for 4–6 h and frozen at –20°C upon return to Miami.

Adult toadfish collected from shelters were euthanized in the field with an overdose of anaesthetic (clove oil), sexed by the internal examination of gonads, and their gastrointestinal tracts examined for the presence or absence of food items. Eggs, larvae and juveniles were also collected from shelters, blotted dry, wrapped in foil packets, immediately placed in liquid nitrogen and stored at –80°C. Samples were shipped to Guelph, Ontario on dry ice and stored at –80°C for 5 weeks until assays were run for OUC enzyme activities. In addition, four shelters including guardian male and eggs/larvae were transported to the Miami lab in 75-litre insulated coolers supplied with continually refreshed oxygenated seawater. Once a sufficient number of offspring emerged as juveniles, they were used in an ammonia 96-h LC_{50} test and nitrogen excretion experiments.

Based upon observations of a cohort of *O. beta* from a natural spawning event in an 8000-litre mesocosm with a known date of fertilization (J. F. Barimo, personal observations), observed developmental stages of *O. beta* were consistent with previous descriptions of *Opsanus* spp. (Gudger, 1908; Breeder, 1941; Dovel, 1960). Developmental stages were categorized as: (1) egg I stage, 1–7 days post-fertilization, with a distinctive amber colour and no sign of embryonic development; (2) egg II stage, 7–21 days post-fertilization, with a discernible embryo and an orange appearance due to vascularization in the yolk; (3) yolk-sac larvae I stage, 1–7 days post-hatch, noted by the absence of a chorion exposing the yolk-sac, which was adhered to the substrate; (4) yolk-sac larvae II stage, 8–14 days post-hatch, with >50% of the yolk-sac absorption and increased tail flexing and mouth movement but still adhered to the substrate and (5) juvenile stage, where precocious toadfish are free swimming with yolk-sac completely absorbed. Individuals >16 mm total length (*TL*) are still categorically juvenile but were not used since they appear to be less tied to nesting sites. However, juveniles <16 mm *TL* were commonly found inside nesting shelters with adult guardians present. It should be noted that the terms ‘prolarvae’, ‘cling young’ and ‘eleutheroembryo’ have been used by some workers to describe the more common term ‘yolk-sac larvae’.

Experimental design

Standard laboratory prophylactic treatments routinely performed on adult toadfish (Wood et al., 2003) were not used on toadfish nests (shelter + eggs/larvae + guardian male) so as to avoid potential stress to eggs/larvae. Emerging juveniles were fed freshly hatched brine shrimp, *Artemia salina* (10–20 per juvenile), for 1 week, after which they were fasted for 24 h prior to 96-h LC_{50} and nitrogen excretion flux experiments.

Ammonia and urea flux experiments were conducted with individual juvenile toadfish in capped 28×61 mm high-density polyethylene vials with 10 ml of static seawater (35‰) at 23–25°C. Aeration and exhaust (PE-90 tubing) were permitted *via* an access hole in the vial cap, and aeration was adjusted to 1–2 small bubbles s^{-1}. Water samples were manually sampled by pipette (3 ml) at 24 h intervals over the 72 h trial (N=7) to obtain daily waste-N flux rates. Water samples were taken hourly from a second group of toadfish (N=7) for 26 h to determine finer-scale patterns of nitrogen excretion. Given the small water volume in flux chambers, samples were limited to 3 ml and water lost to hourly sampling was replaced with fresh seawater with subsequent dilutions factored into final calculations. Three blank vials were used to evaluate the contribution of nitrogen waste by microbial activity in the sand-filtered seawater utilized.

Ammonia toxicity experiments were designed after Wang and Walsh (2000) with 125, 250, 500, 1000, 2000 and 4000 μmol N l^{-1} treatments based on an initial range-finding test. The corresponding fractions of unionized ammonia (NH_3) and ammonium (NH_4^+) were calculated in accordance with the Henderson–Hasselbalch equation:

$$pH = pK_{Amm} + \log [NH_3]/[NH_4^+] , \qquad (1)$$

where pK_{Amm} is based on temperature and salinity (Cameron and Heisler, 1983).

Toadfish juveniles (N=30 per treatment) were placed in 2-litre polyethylene containers with small PVC shelters and

allowed 24 h to acclimate. The juveniles used were <16 mm *TL* and ranged in wet mass from 27.9 to 48.9 mg. Treatments were spiked with variable volumes of a 3.57 mol N l^{-1} stock solution of NH_4Cl to attain desired concentrations. Water changes (50%, 1 litre) were conducted at 24 h intervals to prevent ammonia degradation and otherwise maintain high water quality. Water temperature, pH and salinity were maintained within a range of 20.0–22.4°C, 8.21–8.25 and 35–36‰, respectively. Water samples were collected at the onset of the LC_{50} test, at 24 h intervals following water changes and at completion of the test to assure that desired ammonia concentrations were maintained. Surviving toadfish were sacrificed and frozen in liquid N_2. Attempts to remove embryos and larvae from the substrate and re-adhere them without subsequent physical damage was unsuccessful and hence these stages were not utilized for ammonia toxicity testing.

Analytical procedures

Water samples were analyzed for total ammonia-N and urea-N within 48 h of collection. Field samples were adjusted to pH 8 with 10 mol l^{-1} NaOH to neutralize the HCl preservative. Urea-N was measured using a standard colorimetric diacetylmonoxime assay (Price and Harrison, 1987). Ammonia-N was determined by a modification to the spectrophotometric indophenol blue method of Ivancic and Deggobis (1984). The indophenol blue method measures total ammonia-N, i.e. ammonia (NH_3) and ammonium (NH_4^+), and total ammonia-N is abbreviated to ammonia-N in subsequent text. Spectrophotometric measures for the ammonia and urea assays were performed using a Molecular Devices Thermo Max microplate reader (Menlo Park, CA, USA).

Assays for the enzymes

GSase, CPSase II and III, OTCase and arginase were performed in accordance with Steele et al. (2001) with the following modifications. For enzyme assays, tissue samples from ~4–8 individuals were pooled depending on developmental stage with later stage samples composed of fewer individuals. Samples were homogenized in 17–86 volumes (depending on developmental stage) of ice-cold extraction buffer (0.05 mol l^{-1} Hepes buffer, pH 7.5, 0.05 mol l^{-1} KCl, 0.5 mmol l^{-1} EDTA, 1 mmol l^{-1} DL-dithiothreitol) and sonicated. Supernatants of the centrifuged tissue homogenates (10 min at 14 000 ***g***, 4°C) were passed through Sephadex (G25) columns to removed endogenous substrates and modifiers (Felskie et al., 1998). To account for protein dilution during column purification, a dilution factor was calculated as the pre-column protein concentration divided by the post-column protein concentration. Protein content was assayed by a dye-binding method using a reagent kit from Bio-Rad Laboratories (Hercules, CA, USA). The standards used were 0–0.05 μmole bovine serum albumin. Glutamine was the only substrate provided for the CPSase assay, which measured [^{14}C] carbamoyl phosphate as described by Anderson et al. (1970), since the maximum activity with ammonia as a substrate (data not shown) was minimal and comparable with that found in adults (Anderson and Walsh, 1995). Enzyme reactions in assays were all conducted at 26°C, and reaction times for GSase, CPSase, OTCase and arginase were 10, 30, 10 and 10 min, respectively. All end products of enzyme and protein assays were measured spectrophotometrically using a Perkin Elmer model Lambda 2 UV/VIS spectrophotometer (Norwalk, CT, USA) except for the CPSase assay, which utilized a Beckman Coulter LS 6500 multi-purpose scintillation counter (Fullerton, CA, USA). Toadfish were considered ureotelic if they excreted most nitrogen as urea (>50%) and expressed significant levels of OUC enzymes (as in Anderson, 2001). The term percent ureotelic (as in Wood et al., 2003) is defined as the percent of waste-N occurring as urea-N where:

$$\%\ \text{ureotelic} = \{[\text{urea-N}]/([\text{urea-N}] + [\text{ammonia-N}])\} \times 100\ . \quad (2)$$

Urea-N and ammonia-N are expressed as molar concentrations.

Data analysis

The ammonia 96-h LC_{50} curve was calculated by nonlinear logistic curve regression analysis using Sigma Plot software. SPSS software was used for Student's *t*-test and one-way analysis of variance (ANOVA) with the Student–Newman–Keuls *post-hoc* test to examine differences in OUC enzyme activities among developmental stages and ammonia treatment. All data were tested for normality with the Levene Test for Homogeneity of Variances. Any parameters that were not normally distributed were log transformed and retested for normality before parametric analysis. Statistical analyses performed on proportions were arcsine transformed. All statistical procedures followed recommendations of Zar (1996) and α=0.05. Results are presented as means ± S.E.M.

Results

Field data

O. beta actively recruited to artificial shelters and utilized these structures as spawning sites. The majority of adult toadfish present were guardian males protecting offspring (Table 1). Three gravid female toadfish were found in shelters but in each case were accompanied by a mature male. The numbers of adult and offspring present in nests declined slightly between the sampling dates (Table 1). Guardian males were often found with offspring at different developmental stages, indicating multiple spawning events. Adult males ranged in size from 116 to 231 mm *TL* and were observed to continually fan offspring with caudal and pectoral fin movements, presumably disrupting boundary layers.

Environmental parameters, i.e. salinity, depth, pH and temperature, were relatively consistent between sampling dates except for mean water temperature, which rose 2.8°C (Table 1). Within inhabited shelters, ammonia-N concentration was 8.1±1.0 μmol N l^{-1} on 24 April 2003 and 9.7±1.5 μmol N l^{-1} on 15 May 2003 while urea-N was measured at 17.1±2.6 μmol N l^{-1} and 11.1±1.8 μmol N l^{-1},

Table 1. *Field data from artificial habitats deployed in Florida Bay during the 2003 spawning season of* Opsanus beta

	Sample date	
	24 April 2003	15 May 2003
Number of habitats surveyed	41	42
Toadfish occupancy of habitats (%)	87.8	64.3
Salinity (‰)	33.8±0.3	33.3±0.2
Depth (cm)	108±5.0	120±5.0
pH	8.55±0.02	8.44±0.02
Temp (°C)	29.3±0.2	32.1±0.2
Adult *TL* (mm)	164.5±5.6	167.6±4.6
Adult sex (% male in habitats)	85.7	100.0
Occupied habitats with offspring (%)	96.4	84.0
Nest water urea-N (μmol N l^{-1})	17.1±2.6	11.1±1.8
Nest water NH_4-N (μmol N l^{-1})	8.1±1.0	9.7±1.5
Percent ureotelic* (%)	63.4±3.5	54.0±4.1
Adult toadfish with food in GI tract (%)	3.6	8.0

Samples were only collected from a representative number of occupied shelters (4–5 per cluster) during midday hours while the remainder was left undisturbed. Salinity and depth were measured only once per cluster of toadfish shelters (*N*=6).

*Defined as the percentage of waste-N occurring as urea-N.

Values are means ± S.E.M. unless otherwise stated (*N*=26 on 24 April and *N*=25 on 15 May).

respectively (Table 1). The mean value for waste-N concentration (ammonia-N + urea-N) was 23.0±2.1 mmol N l^{-1}, and the maximum waste-N value was 76.2 mmol N l^{-1}, occurring in a nest containing one male, one female and freshly laid eggs. Ammonia and urea measured within shelters originated from resident toadfish, with background ammonia levels in the adjacent water column being undetectable in all but two cases (both <2 μmol N l^{-1}) and background urea never being detected. Resident toadfish excreted 9.6–100% of waste-N as urea-N; however, the majority of these toadfish were classified as functionally ureotelic (35 of 51). Overall, toadfish with offspring present excreted 58.3±2.8% of waste-N as urea-N (*N*=46) while toadfish without offspring excreted 59.1±12.0% urea-N (*N*=5) and there was no statistical difference between groups (*t*=–0.2080, *P*=0.83, d.f.=51).

The stomachs and intestines of *O. beta* were generally devoid of food, but mud and shell hash was occasionally present, indicating sediment excavation. Only two guardian males had food items present, one with five toadfish eggs (possibly culled) and the other with a 7 cm *TL* toadfish (presumably an unwelcome visitor).

Daily nitrogen flux experiment

Juvenile *O. beta* were predominantly ureotelic over the 72 h trial, excreting 81.9±4.0% (*N*=7) of their waste-N as urea-N. In addition, ammonia and urea excretion rates increased

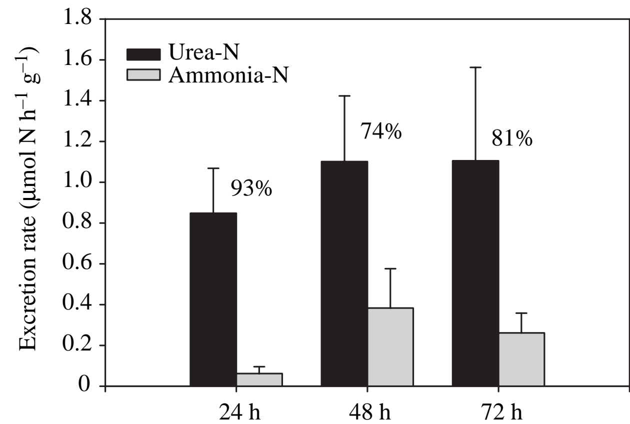

Fig. 2. Urea-N and ammonia-N excretion rates for juvenile toadfish (*N*=7, 14–15 mm *TL*). Juveniles were reared from eggs collected in Biscayne Bay and were used within 1 week of full yolk-sac absorption. The percent of waste nitrogen expressed as urea-N (% ureotelic) appears above bar pairs at each 24 h sampling interval. Values are means + S.E.M.

slightly after the first 24 h interval (Fig. 2). The mean urea-N daily flux rate for juvenile toadfish over the 72 h test was 1.018±0.084 μmol N g^{-1} h^{-1} whereas ammonia-N was 0.235±0.095 μmol N g^{-1} h^{-1}.

Hourly nitrogen flux experiment

Juveniles sampled hourly were also determined to be predominately ureotelic, excreting 65.7±7.4% of waste-N as urea-N over the 26 h experiment. The mean urea-N daily flux rate was 1.275±0.327 μmol N g^{-1} h^{-1} while ammonia-N was 0.726±0.212 μmol N g^{-1} h^{-1}. Background urea or ammonia was not detected in the three blank control vials. All juveniles tested were noted to excrete urea in pulsatile events as do adults; however, urea also appeared to be excreted continually at a lower basal rate similar to that of ammonia excretion (Fig. 3). Urea pulses accounted for 62.0±5.9% (*N*=7 fish) of cumulative urea excreted, and these pulses are defined as short duration increases (>2-fold) in the basal excretion rate.

Ammonia toxicity

The total ammonia 96-h LC_{50} value for juvenile *O. beta* was calculated as 875 μmol N l^{-1} with 13.3% and 60.0% mortality for 500 μmol N l^{-1} and 1000 μmol N l^{-1} treatments, respectively (Fig. 4). At pH 8.23, the corresponding NH_3 concentration was calculated as 38 μmol N l^{-1}. Prior to death, gill opercula tended to flare open, exposing unusually red gill filaments accompanied by a loss of balance and a subsequent loss of buoyancy.

OUC enzyme activities and developmental stages

All developmental stages of *O. beta* expressed significant levels of OUC enzymes. There was an overall trend of increased enzyme activities with development stage and statistically significant differences were noted for each enzyme assayed: GSase (*F*=25.9108, *P*<0.0001, d.f.=23), CPSase

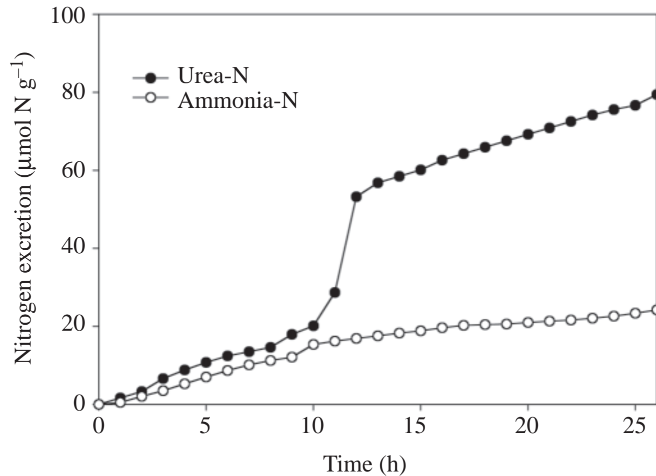

Fig. 3. A representative 26 h record of cumulative urea-N and ammonia-N from an individual juvenile toadfish (14 mm *TL*). Urea pulsing was noted in all juvenile toadfish tested (*N*=7) but the intensity and frequency of the pulse varied among individuals. This individual was collected as a fertilized egg in Biscayne Bay and was used within 1 week of full yolk-sac absorption. Note the urea pulse at 10–12 h and that the ammonia excretion rate was relatively constant.

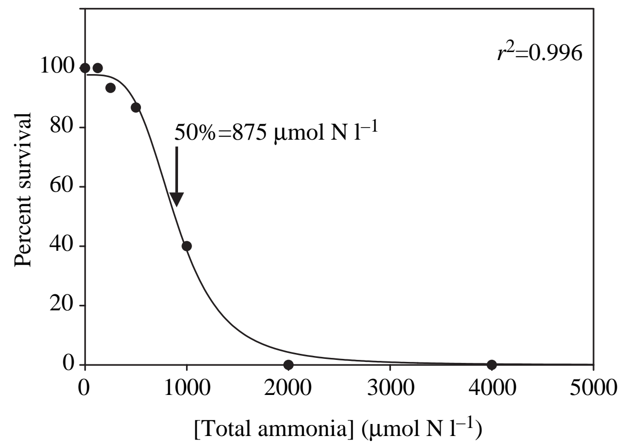

Fig. 4. Results of the ammonia 96-h LC_{50} test plotting survivorship of juvenile fish (<16 mm *TL*) against total ammonia-N concentration (*N*=30 for each treatment group). Juveniles were reared from eggs harvested in Biscayne Bay and were used within 1 week of full yolk-sac absorption. An arrow depicts the calculated LC_{50} value. The test was conducted in individual 2-litre chambers for each treatment group with a daily 50% water change, and ammonia concentrations were verified at t_0 and at 24 h intervals thereafter.

(F=59.2644, P<0.0001, d.f.=23), OTCase (F=12.2383, P=0.0001, d.f.=23) and arginase (F=68.5322, P<0.0001, d.f.=23) (Fig. 5). The majority of total CPSase existed as CPSase III, which is the first enzyme in the teleost OUC. However, CPSase II accounted for 19.1% of total CPSase activity in the egg II stage and gradually declined to 7.7% in the juvenile stage (Fig. 6). These changes in CPSase II and CPSase III activities were statistically significant across developmental stages (F=16.8173, P<0.0001, d.f.=23).

Elevated ammonia and OUC enzyme activities

The effect of environmental ammonia exposure on OUC enzyme activity was studied in the juvenile survivors of the ammonia 96-h LC_{50} test. Only the control, 500 µmol N l^{-1} and 1000 µmol N l^{-1} treatments had sufficient biomass for analysis. There were no statistical differences between treatments in GSase, CPSase III or arginase activities; however, there was a 3-fold rise in OTCase activity in the 1000 µmol N l^{-1} treatment over the control group, which was significantly different from the control and 500 µmol N l^{-1} treatments (F=5.38, P=0.0138, d.f.=17) (Fig. 7). There was also no significant difference in the activity of either CPSase II or CPSase III (F=2.1420, P=0.1734, d.f.=11) among treatment groups, with the proportion of CPSase III comprising 89.8–92.5% of total CPSase activity.

Discussion

Field data

Most nesting toadfish were found to be functionally ureotelic in the field based on water samples collected inside artificial shelters; however, 30% of those sampled were found to be ammoniotelic. Given the pulsatile nature of urea excretion in toadfish, the urea-N concentrations reported in our study may be an underestimate since the time of actual pulsing was unknown and urea would diffuse out of experimental shelters in <2 h (J. F. Barimo, unpublished data). However, results similar to the present study were calculated from freshly collected toadfish using a glutamine synthetase proxy technique where ~54% of waste-N was excreted as urea-N (Hopkins et al., 1997). In that study, individuals varied in their degree of ureotely and ammonotely, a result that was hypothesized to be a function of heterogeneity in microhabitat ammonia concentrations (Hopkins et al., 1997). The data of the present study are the first to demonstrate as directly as possible that adult toadfish appear to be capable of ureotely in a natural setting.

Environmental parameters measured in Florida Bay were consistent between sampling dates and reflected seasonal weather trends. Water temperatures recorded at spawning sites in the present study were 6°C higher than previously reported for *O. beta* at a site ~200 km NW of our FB-1 (Breeder, 1941). Success of spawning may be less dependent upon actual water temperature than anticipated and thus less susceptible to inter-annual temperature fluctuations unless each population represents a distinct ecotype.

Ureotely in early life history stages

OUC enzyme activities measured in embryonic, larval and juvenile *O. beta* demonstrate that the metabolic pathway for ureogenesis is present during the entire life history of this species. The nitrogen excretion data indicate that juvenile *O. beta* are ureotelic and that urea excretion was primarily pulsatile when juvenile fish were kept in relatively confined

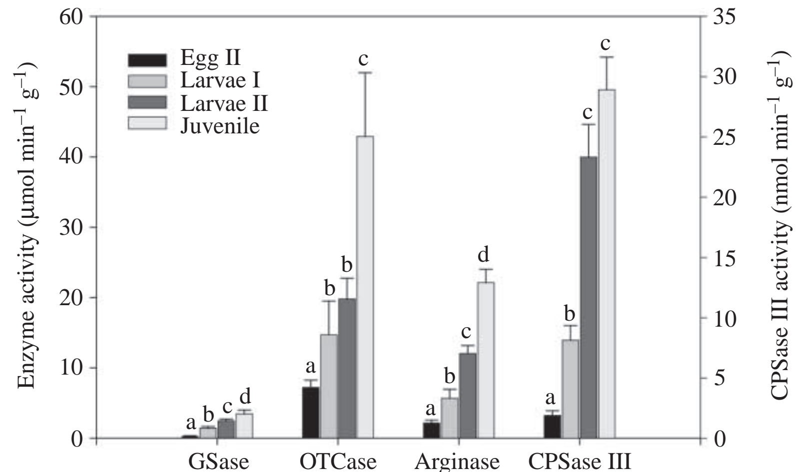

Fig. 5. Enzyme activities for glutamine synthetase (GSase), ornithine transcarbamylase (OTCase), arginase and carbamoyl phosphate synthetase III (CPSase III) measured in homogenized whole toadfish. Developmental stages presented are egg II (10–20 days post-fertilization), yolk-sac larvae I (1–7 days post-hatch), yolk-sac larvae II (8–14 days post-hatch) and juveniles (yolk-sac completely absorbed; <16 mm *TL*). Various developmental stages were collected in Biscayne Bay and flash frozen with liquid N_2. Statistically different groupings (a–d) from one-way ANOVA *post-hoc* analysis are presented above bars for each ornithine–urea cycle enzyme group. Values are means + S.E.M., α=0.05 and N=6 for each developmental stage. Note that CPSase III activity is expressed as nmol min^{-1} g^{-1} (y_2 axis).

conditions. It is likely that juveniles possess the same mechanism for urea excretion as adults, including expression of tUT, a facilitated diffusion urea transporter in the gill (Walsh et al., 2000). Further experiments are necessary to validate this hypothesis.

In the present study, mass-specific OUC enzyme activities were found to increase steadily from the embryonic to the juvenile stage. However, the increased activities probably reflect an initial dilution effect caused by yolk (presumably lacking OUC enzymes), which is gradually consumed during subsequent development. It is likely that OUC enzyme activities are at sufficient levels to synthesize urea *de novo* during each developmental stage. For example, in juveniles, CPSase III activity is 0.028 µmol min^{-1} g^{-1} while urea excretion is 0.008 µmol min^{-1} g^{-1} (Table 2), demonstrating an excess capacity for this apparent rate-limiting enzyme relative to excretion rates. However, the functionality of the OUC remains unknown for embryonic and larval stages of *O. beta*. Additionally, CPSase II accounted for 19.1% of total CPSase activity in the egg II stage and gradually declined to 7.7% by the juvenile stage. This reduction in CPSase II probably represents an ontogenic shift in pyrimidine biosynthesis while toadfish develop through early life stages.

In a study of the congener *O. tau,* yolk-sac larvae and juveniles were found to be ureotelic while embryos were ammoniotelic (Stephen and Griffith, 2001). However, urea may accumulate within the embryo and yolk, which may explain the >5-fold post-hatch increase in urea excretion reported by Stephen and Griffith (2001). In embryonic rainbow trout, Pilley and Wright (2000) suggest that the yolk-sac membrane is relatively impermeable to urea and demonstrate that urea excretion is dependent, in part, on a phloretin-sensitive facilitated urea transporter. It is unknown if such a urea transport mechanism is functional in the yolk-sac membrane of *O. tau*. Additionally, it should be noted from a comparative aspect that juvenile *O. beta* were on average 82% ureotelic during their first week as juveniles while *O. tau* were ~60% ureotelic. These findings are consistent with studies of adults where *O. tau* were classified as 'moderately' ureotelic but where *O. beta* were considered 'highly' ureotelic (Wang and Walsh, 2000).

The mass-specific urea flux rate for juvenile *O. beta* (0.008 µmol min^{-1} g^{-1}) is an order of magnitude greater than for adults (0.0008 µmol min^{-1} g^{-1}), which was anticipated considering mass-specific metabolic rates are likely to be much higher in the actively growing juveniles if toadfish conform to normal metabolic scaling patterns (Schmidt-Nielsen, 1997). A comparison of mass-specific OUC enzyme activities revealed that GSase, CPSase and OTCase activities in adult livers (Anderson and Walsh, 1995) are double those found in whole juveniles while the reverse is true of arginase (Table 2). For adult toadfish, GSase appeared to be the limiting OUC enzyme

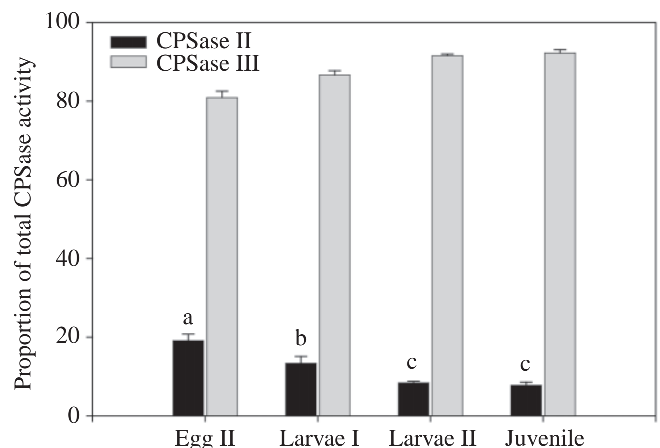

Fig. 6. The proportion of CPSase II and CPSase III occurring in toadfish developmental stages, which include egg II (10–20 days post-fertilization), yolk-sac larvae I (1–7 days post-hatch), yolk-sac larvae II (8–14 days post-hatch) and juveniles (yolk-sac completely absorbed; <16 mm *TL*). Developmental stages were collected in Biscayne Bay and immediately frozen with liquid N_2. Statistically different groupings (a–c) from one-way ANOVA *post-hoc* analysis are presented above bars for each group of developmental stages. Values are means + S.E.M., α=0.05 and N=6 for each developmental stage.

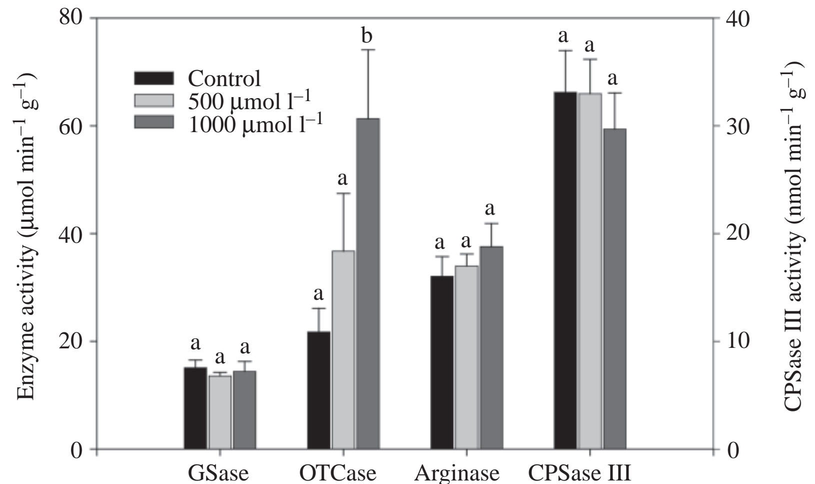

Fig. 7. Enzyme activities for glutamine synthetase (GSase), ornithine transcarbamylase (OTCase), arginase and carbamoyl phosphate synthetase III (CPSase III) were measured in juvenile toadfish exposed to 0, 500 and 1000 μmol N l^{-1} ammonia for 96 h. Data are presented as means + S.E.M. Juveniles were reared from eggs harvested in Biscayne Bay and were used within 1 week of yolk-sac absorption. OTCase was the only enzyme group with a significant difference (P<0.05) among ammonia treatments. The sample size was N=4 for all categories except for the OTCase control and 500 μmol N l^{-1} treatment (N=6). Statistically different groupings (a,b) from the one-way ANOVA *post-hoc* analysis are presented above bars for each OUC enzyme. Note that CPSase III activity is expressed as nmol min^{-1} g^{-1} (y_2 axis).

in terms of activity when GSase transferase activities were converted to biosynthetic rates (Anderson and Walsh, 1995). In juveniles, the biosynthetic rate for GSase is higher than that for CPSase III (Table 2); however, the potential capacity for *de novo* urea synthesis as demonstrated by these activity measurements can account for observed rates of urea excretion.

The adult OUC enzyme activities were measured in liver, the organ traditionally associated with the OUC (Mommsen and Walsh, 1991), even though hepatic tissue comprises only 2.25% of total wet biomass (Hopkins et al., 1997). When comparing adult liver with white muscle (see Table 2), mass-specific CPSase III, OTCase and GSase activities were approximately 100, 78 and 24 times higher, respectively (Anderson and Walsh, 1995; Julsrud et al., 1998; Walsh et al., 2003). However, it is believed that OUC enzyme activities have been underestimated in adults, as recently demonstrated with GSase where liver only accounted for 40% of 'whole body' GSase activity while muscle comprised 28% of activity when using tissue mass to calculate glutamine synthetic potential (Walsh et al., 2003). Approximate (but probably overestimated) enzymatic activities are calculated in a simple model for GSase, CPSase and OTCase in adult toadfish based on the assumption that liver comprises 2% of toadfish biomass with the remaining 98% being muscle tissue (Table 2). Muscle tissue actually represents 70% of wet biomass (Kennedy et al., 1989); however, activities of OUC enzymes in remaining tissue groups are unknown for toadfish but are likely to have low enzymatic activities more similar to muscle than liver, as was largely the case for GSase (see Walsh et al., 2003).

The adult whole body calculations in Table 2 show that the mass-specific enzymatic activities for GSase and OTCase are approximately an order of magnitude greater in juveniles than adults, which is consistent with the 10-fold difference in mass-specific urea excretion rates. However, juvenile CPSase III activities are only double those of adults. In adults, CPSase III activity is regulated by N-acetyl-L-glutamate as a

Table 2. *Comparison of OUC enzyme activities between juveniles and adults*

	Enzyme activity (μmol min^{-1} g^{-1})			
		Adult		
	Whole juvenile	Liver	Muscle	Whole*
CPSase III	0.028±0.002	0.50±0.02	0.005±0.0005	0.015
OTCase	42.9±9.1	72.3±23.1	0.92±0.13	2.35
Arginase	22.1±1.9	12.3±2.9		
GSase (transferase assay)	3.5±0.5	7.0±3.1	0.29±0.06	0.425
GSase (biosynthetic rate)	0.175	0.35	0.015	0.021
Urea-N flux rate†	0.008			0.0008

Juvenile measurements are based on whole fish while adults were based on livers (Anderson and Walsh, 1995) and muscle (Walsh et al., 2003; Julsrud et al., 1998). Adult whole fish values are calculated based on the approximation that total toadfish biomass is 2% liver and 98% muscle. The GSase biosynthetic rate is calculated from the transferase rate, as in Anderson and Walsh (1995). Additionally, urea-N excretion rates are compared between adults (in Wood et al., 2003) and juveniles.

Enzyme rates are reported as means ± S.E.M. (N=6 for juveniles; N=3 for adult livers; N=8 for adult GSase muscle activity; N=4 for adult CPSase and OTCase muscle activity).

*Whole body enzyme activities assume a 50 g fish with a liver to muscle ratio of 50:1.

†Urea-N excretion rates expressed as μmol min^{-1} g^{-1}.

positive allosteric cofactor, UTP as an inhibitor and phosphoribosylpyrophosphate for some activation (Anderson and Walsh, 1995; Julsrud et al., 1998). In juveniles, the *in vivo* regulatory conditions exerted on CPSase III are unknown and these may account for the differences seen between adults and juveniles for the *in vitro* CPSase III activities.

Nest fouling hypothesis

Juvenile *O. beta* are less tolerant to ammonia than adults when comparing the 96-h LC_{50} value of 0.875 mmol N l^{-1} in this study with 9.75 mmol N l^{-1} reported in adults (Wang and Walsh, 2000). The functional role of the 3-fold increase in OTCase activity when exposed to 1000 μmol N l^{-1} ammonia is puzzling and it appears that OTCase may be more plastic in juveniles than adults. In vertebrates, no other function for OTCase, other than its association with the OUC, has yet been discovered. One possible explanation is that an excess of yolk-derived arginine enters the OUC, is converted to ornithine by arginase and a subsequent build up of ornithine could trigger the upregulation of OTCase during ammonia stress. It is likely that juveniles were subsisting on yolk-derived nutrients in this experiment since active feeding by juveniles on *Artemia* was never observed. Active feeding was unlikely since juveniles failed to survive past 1 month as juveniles except in a fallow 500-litre tank containing a wide assemblage of potential prey items (J. F. Barimo, unpublished data).

GSase activity was unchanged in juveniles with 1000 μmol N l^{-1} ammonia exposure, but adult GSase activity in muscle doubled with high ammonia exposure (Wang and Walsh, 2000), and other factors also upregulate GSase activity in liver (reviewed by Wood et al., 2003). The upregulation of GSase in association with a shift to ureotelism and the relative position of GSase upstream from CPSase III are thought to provide a critical control for the OUC in adult toadfish (Wood et al., 2003). However, these initial results could suggest that either the GSase–CPSase axis does not function as a control point in juveniles, that the OUC is already functioning at maximum potential or that juvenile toadfish are obligately ureotelic.

The level of ammonia measured within nesting shelters appeared to be below the 96-h LC_{50} value for juveniles. In addition, the mean concentration for waste-N (urea-N + ammonia-N) measured in shelters at FB-1 was 23.0±2.1 μmol N l^{-1} (N=51) while the maximum concentration was 76.2 μmol N l^{-1}. Thus, if all excreted waste-N were expressed as ammonia it would still be far below the LC_{50} value for juveniles. However, if the NH_3 fraction is calculated for the previous waste-N values based on field data (pH, salinity and temperature), the mean waste-N value is 3.3±0.3 μmol N l^{-1} with a maximum concentration of 9.6 μmol N l^{-1}. Although these values still fall below the calculated NH_3 96-h LC_{50} of 38 μmol N l^{-1}, the safety margin for the prevention of nest fouling is reduced. Although the ammonia 96-h LC_{50} test was not conducted with embryonic or larval stages of development, their LC_{50} value is not expected to deviate greatly from that of juveniles. In *O. mykiss,* an opposite trend was noted where 85-day-old yolk-sac fry were actually less tolerant to ammonia than both fertilized eggs and alevins by a factor of ~20 (Rice and Stokes, 1975). Therefore, it appears unlikely that male toadfish are facultatively ureotelic in order to avoid poisoning offspring with ammonia excretions in confined nests with restricted water flow, especially since no statistically significant difference was observed in percentage ureotely between males with or without offspring present. This hypothesis also fails to explain why female toadfish are also facultatively ureotelic (Walsh et al., 1990), since they do not brood or guard nests, or that male toadfish are generally fasting while rearing offspring, as determined by examination of gut contents. However, the nest-fouling hypothesis cannot be confidently rejected without ammonia 96-h LC_{50} values for embryos and larvae in addition to data on the chronic effects of ammonia exposure to growth and survivorship. Furthermore, the variability of environmental parameters challenging the toadfish in the field, i.e. pH, temperature and salinity, needs careful consideration.

The data in the present study may, however, lend support to the hypothesis that toadfish produce urea to conserve nitrogen since guardian male toadfish appeared to be fasting. If male toadfish are fasting for long durations, i.e. 4 or more weeks, then such a strategy may help conserve or recycle nitrogen if, for example, mutualistic gut bacteria were harnessing energy by ureolysis (Mommsen and Walsh, 1989; Wood et al., 2003). However, this hypothesis again fails to explain why ureotelism is a characteristic trait of both sexes (Walsh et al., 1990) or even why ureotely occurs across the entire life history of the species, yet it remains a hypothesis worthy of future investigation. Perhaps the most viable remaining hypotheses regarding the adaptive significance of ureotelism in toadfish are: (1) predator avoidance by chemical crypsis since pulsatile urea excretion would enable sessile epibenthic toadfish to avoid emitting a continual chemosensory cue to predators, as would be the case with continual ammonia excretion; (2) chemical communication or pheromones between conspecifics; and (3) a means of coping with high levels of ambient ammonia resulting from the decomposition of vegetation in highly productive seagrass beds, which is the likely habitat of non-nesting (sub-adult) toadfish.

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