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Patterns of nitrogenous waste excretion and gill urea transporter mRNA expression in several species of marine fish

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Abstract Many prior studies of nitrogenous waste excretion in marine fish have examined excretion patterns for short time periods, and with relatively coarse sampling schemes (e.g., an initial and a final sample point). Recent studies of a ureotelic marine fish (the gulf toadfish, *Opsanus beta*) have demonstrated that urea excretion in this species occurs in brief but massive bursts, lasting from 0.5 to 3 h, and often only once per day. The present study sought to determine if prior sampling protocols may have underestimated the amount of urea being excreted by marine fish. A survey of 16 marine species (the teleosts: *Myoxocephalus octodecemspinosus*, *Scophthalamus aquosa*, *Cyclopterus lumpus*, *Lophius americanus*, *Aprodon cortezianus, Cymatogaster aggregatus*, *Parophrys vetulis*, *Microstomus pacificus*, *Hippoglossoides elassodon*, *Bathyagonus nigripinnus*, *Ophiodon elongatus*, *Hemilepidatus spinosus*, *Icelinus terrius*; the elasmobranch: *Raja rhina*; and the hagfish: *Eptatretus stoutii*) was undertaken for ammonia-N and urea-N excretion using a long sampling period (48 h) and hourly sample collection. Apart from the obvious exception of an elasmobranch, ammonia excretion was confirmed to be predominant in marine fish, with urea excretion constituting between 1.4 and 23.8% of the total of ammonia plus urea excreted. Notably, no pulses of urea excretion were detected. Despite the relatively low level of urea excretion, expression of urea transporter-like mRNA (detected using the toadfish urea transporter, tUT, cDNA as a probe) was discovered in gills of many of the species surveyed for nitrogen excretion patterns, although no signal was detected in the hagfish. These results suggest that urea excretion takes place through a specific transport pathway. Finally, more detailed analysis of nitrogen excretion in one of the surveyed species, the plainfin midshipman (*Porichthys notatus*) demonstrates that "total" nitrogen excretion estimated by summing ammonia and urea excretion underestimates true total nitrogen excretion by 37–51%.

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Introduction

It is widely accepted that the vast majority of marine teleost (bony) fishes are ammoniotelic, that is, they excrete the bulk of their nitrogenous waste as ammonia (Wood 1993; Walsh 1998). However, recent studies with the gulf toadfish (*Opsanus beta*) and several other teleost fish suggest that urea may contribute a substantial portion of nitrogenous waste in some fish species. Of particular significance, under certain environmental conditions, the toadfish excretes greater than 90% of its waste nitrogen as urea in a single pulse per day from the gills lasting as little as 30 min (Wood et al. 1995a, 1997, 1998; Gilmour et al. 1998). Given this dynamic pulsatile urea excretion, it is possible that prior studies of marine fish, which often measured excretion for only several hours and/or with only widely spaced water samples, could have missed such pulsatile events and may have substantially underestimated urea-N excretion, potentially impacting nitrogen budget estimates for marine

ecosystems. Furthermore, given that there is uncertainty as to the ecological and evolutionary aspects of why toadfish excrete such a high quantity of urea, and do so in a pulsatile fashion (Walsh 1997; Hopkins et al. 1999), we wished to determine if this pattern is a more general feature of marine fishes. Therefore, the first aim of the present study was to reexamine the proportion of ammonia-N versus urea-N excretion, and the temporal dynamics of this excretion, using long measurement periods (e.g., 48 h) and fine-scale (e.g., hourly) sampling on a variety of common marine fishes.

In this same context, substantial urea excretion has been revealed in the embryonic forms of teleosts that are ammonioteles as adults (rainbow trout, Wright et al. 1995a; cod, Chadwick and Wright 1999), suggesting that urea excretion might be more widespread in teleosts than previously thought. Furthermore, specific gill-facilitated diffusion mechanisms for urea excretion have now been identified in two ureotelic teleost fishes (toadfish, Wood et al. 1998; Lake Magadi tilapia, Walsh et al. 2001). Notably, gill urea transport in the toadfish and the Lake Magadi tilapia appears specifically to involve piscine analogs (Walsh et al. 2000, 2001) of the urea transport proteins (UTs) first discovered in the mammalian kidney (You et al. 1993). Given the availability of piscine UT probes from these studies, and the likelihood of more widespread ureotely in teleost fish, we reasoned that it would be of interest to survey the gills for possible expression of UT mRNA in parallel to the studies of nitrogen excretion patterns. Therefore, the second objective of the study was to determine if UTs are broadly expressed in marine fish gills.

A long-standing observation in the piscine literature has been that ammonia and urea may not be the only significant components of fish nitrogenous waste (Smith 1929; Wood 1958; Olson and Fromm 1971; McCarthy and Whitledge 1972; Beamish and Thomas 1984; Cockcroft and Du Preez 1989). Yet, ammonia and urea are the sole forms typically measured in studies of nitrogen excretion, likely due to the ease of the chemical assays for these forms. Therefore, a final objective of the study was to determine, for one of the species from the above group, the plainfin midshipman, *Porichthys notatus*, if, and how severely, nitrogen excretion rates are underestimated by this approach.

Materials and methods

Experimental animals and excretion measurements

A variety of marine fish species listed in Table 1 were captured by bottom trawl or hook and line in Bamfield, British Columbia (July 1997), and Salisbury Cove, Maine (August 1998). Following capture, a 48-h acclimation period to laboratory holding conditions in flowing seawater at ambient temperature (~11–13°C) was allowed, during which time fish were not fed. Individual fish were then placed in a measured closed volume of seawater approximately 20 times their volume, and water was sampled continually using a peristaltic pump and fraction collector arrangement that changed sample tubes hourly (Wood et al. 1995a). Water was replaced completely every 12 h, and sampling was continued for up to 48 h. Photoperiod was ambient, and partial submergence of the flux chambers on a wet table ensured temperature control. Hourly samples (10 ml) were examined for urea-N and ammonia-N concentration using previously established spectrophotometric methods (Ivancic and Degobbis 1984; Price and Harrison 1987) in a microtiter plate reader that facilitated the analyses of these large numbers of samples. At the termination of an experiment, all fish were weighed, and one individual of each species was sacrificed with an overdose of MS-222 (tricaine methanesulfonate). Gills were removed and flash-frozen in liquid nitrogen, wrapped in foil, and returned to Miami in a dry shipper for subsequent molecular studies. Ammonia-N and urea-N excretion rates were calculated for each individual fish and normalized to body mass (kg) and time (h), and a twofold correction for urea since it contains two N atoms. Simple means ± SE were calculated for each species and treatment groups and compared by Student's *t*-test when appropriate at the $P<0.05$ level of significance.

In a more extensive series of experiments with *Porichthys notatus*, oxygen consumption rates were measured simultaneously with ammonia-N and urea-N excretion rates. Midshipmen ($n=6$; 30–60 g) were placed individually in shielded 4-l respirometer jars fitted with sampling ports, an aeration line, and a magnetic stir bar for mixing. The respirometers were partially submerged on a wet table to maintain the experimental temperature. After allowing the fish to settle for at least 6 h under flow-through conditions with aeration, the aeration was stopped and the respirometers were sealed. Water samples were drawn at intervals of 0.5–2 h for the measurement of oxygen consumption using a Radiometer pHM 71 gas analyzer and E5046 oxygen electrode in a thermostated cell. The stir bar was activated for 60 s prior to each sampling so as to ensure good mixing. At the end of each 2-h period, the flow remained off but aeration was resumed. Two hours prior to the end of the nitrogen flux period, the respirometer was sealed again and the cycle of oxygen sampling repeated. Measurements of ammonia-N and urea-N excretion were best achieved with a period of > 12 h in this system. Therefore, N excretion was measured over 20 h (and oxygen consumption measurements were made at 0–2 h and 18–20 h).

In separate series directly comparing rates of ammonia-N plus urea-N excretion with rates of total nitrogen excretion, the fish were kept in the closed respirometers (with aeration) for 18–24 h. Water samples were analyzed for ammonia-N and urea-N as above, and aliquots were also frozen and shipped to Hamilton for analysis using an Antek 7000 V N-analyzer, which measures all forms of N in the water sample. In these experiments, fed and unfed midshipmen were compared in which one group ($n=4$) was fed a portion of shrimp equivalent to ~1% of their body weight and immediately placed in chambers for measurements, and the other group ($n=4$) was starved for 72 h prior to measurements.

Northern analysis

Total RNA was isolated from gills of selected species by homogenization of 0.2 g tissue in 1.2 ml phenol-guanidinium thiocyanate (Trizol Reagent, Gibco BRL) followed by standard chloroform extraction and isopropanol precipitation (Sambrook et al. 1989). Equal amounts (10 μg) of total RNA (in ethidium bromide loading buffer) were loaded onto a formaldehyde-agarose gel, electrophoresed, examined under ultraviolet light for loading efficiency and potential RNA degradation, and then transferred to a nylon membrane (Hybond-N, Amersham, Arlington Hts., Ill.) using standard methods (Sambrook et al. 1989). Notably, RNA from two fish species known to excrete urea via the gills, including a gulf toadfish (*Opsanus beta*) positive control, and a sample from the Lake Magadi tilapia (*Alcolapia grahami*), were included on the gel. (Due to sample decay, we were unable to examine *Raja rhina* gill.) Following prehybridization, the membrane was hybridized at 37°C for low stringency analysis with a ^{32}P random-prime-labeled full-length toadfish urea transporter (tUT, GenBank accession number

Table 1 Ammonia and urea nitrogen excretion rates for fish common to the environs of Bamfield, British Columbia, and Salisbury Cove, Maine. Values are means ± SEM (μmol N kg^{-1} h^{-1})

Locale/Species	*n*	Ammonia-N excretion	Urea-N excretion	Percent ureotely	Body weight range (g)
Salisbury Cove					
Myoxocephalus octodecimspinosus (long-horned sculpin)	5	449.8 ± 40.4	75.3 ± 24.9	14.3	180–295
Scophthalamus aquosa (windowpane flounder)	3	298.8 ± 35.6	60.1 ± 16.2	16.8	220–305
Cyclopterus lumpus (lumpfish)	3	518.4 ± 87.2	63.8 ± 13.2	11.0	65–215
Lophius americanus (goosefish)	3	727.8 ± 124.6	94.7 ± 15.9	11.5	220–600
Bamfield					
Aprodon cortezianus (bigfin eel pout)	6	246.3 ± 36.3	76.8 ± 17.6	23.8	64–68
Cymatogaster aggregatus (shiner perch)	6	681.5 ± 94.1	61.4 ± 8.9	8.3	10–19
Parophrys vetulis (English sole)	3	211.1 ± 16.7	11.4 ± 3.1	5.1	108–132
Microstomus pacificus (Dover sole)	3	192.9 ± 50.5	18.4 ± 8.8	8.7	88–123
Hippoglossoides elassodon (flathead sole)	3	127.2 ± 21.1	12.3 ± 7.3	8.8	147–242
Bathyagonus nigripinnus (black-finned poacher)	4	588.9 ± 47.9	104.2 ± 13.7	15.0	4–10
Ophiodon elongatus (ling cod)	3	85.6 ± 16.7	6.7 ± 1.0	7.2	807–950
Raja rhina (longnose skate)	3	145.9 ± 82.1	1620.3 ± 652.1	91.7	63–175
Icelinus terrius (spotfin sculpin)	3	390.0 ± 167.5	5.4 ± 1.7	1.4	28–46
Myoxocephalus octodecimspinosus (long-horned sculpin)	5	164.3 ± 44.2[a]	6.3 ± 1.7[a]	3.7	94–120
Eptatretus stoutii (hagfish)	3	72.7 ± 19.0	5.4 ± 1.7	6.9	110–240
Hemilepidatus spinosus (Irish lord)	3	343.0 ± 96.8	67.5 ± 15.8	16.4	240–480

[a] Significantly different from Salisbury Cove individuals at $P < 0.05$ level

AF 165893) (Walsh et al. 2000). Final washes were in 0.1X sodium chloride, sodium citrate (SSC)-0.1%sodium dodecyl sulfate at 37°C.

Results and discussion

Of the 16 species of fish examined in the current study, representing a number of taxonomic groups, only one, the elasmobranch (*Raja rhina*), excreted substantial quantities of urea, and this was to be expected from prior studies of elasmobranchs where urea excretion predominates (e.g., *Squalus acanthias*, Wood et al. 1995b), and where a urea transporter has been identified in the kidney, and with apparent slight expression in the gill (Smith and Wright 1999). The percent of the ‘‘total’’ nitrogen (ammonia plus urea) excretion that was as urea in the other non-elasmobranch species ranged from 1.4% to 23.8%. Even the upper end of this range is well short of the arbitrary definition of ureotely, namely, when an organism excretes 50% or more of its nitrogenous waste as urea (Huggins et al. 1969). Furthermore, in none of the time course traces of hourly samples of urea and ammonia excretion did we detect any pulses of nitrogen excretion; rather, nitrogen excretion was as a continuous stream (results not shown). Given the long sampling period of the current studies (48 h), and the fine-scale sampling time points, we conclude that these fish can be clearly classified as ammonotelic, and that none of them exhibit pulsatile urea excretion. One note of caution, however, is that our approach would not pick up the extremely fine scale events, such as the apparent ‘‘co-pulsing’’ of ammonia-N and urea-N at intervals of far less than an hour as reported by Sayer (1988) in the blenny (*Blennius pholis*).

When the RNA from the gills of selected members of the above group were examined for potential expression of a specific urea transporter, it was apparent that many species expressed UT-like RNA (Fig. 1). As in mammals, the toadfish expresses multiple mRNA isoforms of UT, with a predominant band at 1.8 kb, and a minor band at 3.5 kb. In mammals, these isoforms are believed to be splice variants of a single gene (Sands et al. 1997). Therefore, it is not surprising that a range of molecular weight signals was obtained for the species surveyed. The relative signal strength of the UT-like mRNA is strong for plainfin midshipman (PN), long-horned sculpin (MO), windowpane flounder (SA), ling cod (OE), and unidentified cod (GC); moderate for goosefish (LA), lumpfish (CL), Lake Magadi tilapia (AG) (a 100% ureotelic teleost with exceptionally high urea-N excretion rates; Walsh et al. 2001), long spine sculpin (LS), and Irish lord (HS); and weak to negative for hagfish (ES), eel pout (AC), black-finned poacher (BN), Dover sole (MP), and shiner perch (CA). There does not appear to be any consistent correlation between the extent of ureotely (Table 1) in these species and signal strength in the tUT northern blot (Fig. 1), although this is to be expected given the potential for variable cross-reactivity of the tUT probe with less closely related species. In this regard, it is not surprising that the signal strength is greatest for the closest relative to the toadfish, the plainfin midshipman (*Porichthys notatus*), which belongs to

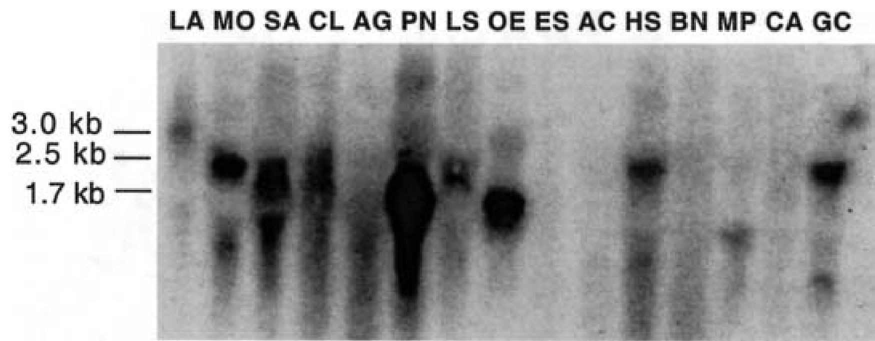

Fig. 1 Urea transport protein (UT) mRNA expression in marine fish. *Left to right*, lanes contained 10 ug total RNA from goosefish (*LA*, note that abbreviations are the first letters of the Latin names), long-horned sculpin (*MO*), windowpane flounder (*SA*), lumpfish (*CL*), Magadi tilapia (*AG*), midshipman (*PN*), long spine sculpin (*LS*), ling cod (*OE*), hagfish (*ES*), eel pout (*AC*), Irish lord (*HS*), black-finned poacher (*BN*), Dover sole (*MP*), shiner perch (*CA*), and unidentified cod (*GC*). Blots were probed with ^{32}P-tUT and exposed for 48 h under low stringency conditions (37°C hybridization and washes). The gulf toadfish positive control was omitted from the figure as the autoradiograph signal was much more intense than for these other species. Sample loading amount was within 10% for all samples as judged by the intensity of 18S and 28S rRNA bands on ethidium bromide stained gels. Similarly, these controls and the positive toadfish control revealed no substantial RNA degradation

the same family, Batrachoididae. However, we are relatively confident that the signals on the northern blot represent UT analogs, because even the moderate signal intensity of the Lake Magadi tilapia (*Alcolapia grahami*) is now known to in fact represent a true UT with approximately 80% amino acid sequence homology with the toadfish tUT protein (Walsh et al. 2001; GenBank accession number AF 278537). Notably, the mobility of the Lake Magadi tilapia band is the same, but the signal intensity is stronger, when it is probed with the tilapia UT probe (Walsh et al. 2001). Thus, it is possible that species showing weak or no expression with the tUT probe may be expressing a UT that could be visualized using an authentic probe, or a probe from a more closely related species. One caveat should be added to these observations, namely, that mRNA expression does not necessarily reflect protein expression, and the development of antibody probes to the tUT protein would be useful in establishing the degree of UT protein expression.

An important area for future experimentation will be to reconcile the low level of urea excretion, and negative pharmacological data (e.g., see below), with the apparent presence of specific urea transporters. It has been suspected for many years that the small amount of urea excreted by most teleosts occurs as a result of a non-specific "leak" at the gill, perhaps across the lipid bilayer, or via other, non-specific pathways (reviewed by Walsh and Smith 2001). For example, Wright et al. (1995b), using a variety of pharmacological and unidirectional flux approaches, were unable to show any evidence for the presence of a facilitated diffusion carrier for urea in the gills of Pacific tidepool sculpin (*Oligocottus maculosus*), a species with low ureotely (8–17%) comparable to that of many of the species surveyed here (Table 1). They concluded that only simple diffusion occurred. In contrast, our molecular results suggest that this excretion may occur via specific facilitated diffusion pathways and, by extension, is a process that can be dynamically regulated, even in fish that are not classically considered to be ureoteles. Perhaps the extremely fine scale pulsing of urea-N (and ammonia-N) excretion reported in the blenny (Sayer 1988) is a manifestation of this process? Clearly, in light of these new molecular data, there is a need to revisit experimentally the physiological mechanisms of urea-N excretion in ammoniotelic teleosts.

A further interesting point is brought out by the data of Table 1 for the long-horned sculpin, *Myoxocephalus octodecimspinosus*. Notably, the Salisbury Cove population excreted far more nitrogen than the Bamfield population, despite similar measurement temperatures and post-absorptive status. It is yet to be determined if there is some genetic basis to this observation, or if acclimation state (e.g., particularly with respect to "stress" status) differed in the two groups.

In one group of plainfin midshipmen (*P. notatus*, $n=6$), we conducted simultaneous measurements of the rates of oxygen consumption, ammonia-N excretion, and urea-N excretion on two subsequent time periods covering approximately 20 h (Table 2). The ratio of summed nitrogen excretion rates (ammonia-N + urea-N) to oxygen consumption rates yielded a nitrogen quotient (NQ) of 0.068–0.107 (Table 2). This is in the normal range of NQ values reported in non-fed fish (reviewed by Wood 2001). Given that an NQ of 0.27 represents the condition in which aerobic respiration relies entirely on protein as a metabolic fuel (van den Thillart and Kesbeke 1978), the measured NQ for *P. notatus* indicates that it falls in the range of most fish that exhibit 14–36% protein oxidation (i.e., NQs between 0.04 and 0.10; see Wood 2001), though values of 44–85% may be calculated from the data of Kutty (1972) and Sukumaran and Kutty (1977) on Mozambique tilapia and Madurai catfish, respectively.

We wondered whether there might be additional products of nitrogen metabolism (other than ammonia-N and urea-N) excreted by the fish. In two separate se-

Table 2 Comparison of ammonia and urea excretion for a 20-h period with simultaneous oxygen consumption measurements in hours 0–2 and hours 18–20 in the plainfin midshipman, *Porichthys notatus*. Values are means ± SEM (μmol N or O_2 kg^{-1} h^{-1}); $n = 4$

	Ammonia-N excretion	Urea-N excretion	Percent ureotely	Ammonia-N + urea-N	Oxygen consumption	Nitrogen quotient (N excretion/O_2 consumption)
0–20 h	208.6 ± 51.8	10.1 ± 2.9	4.6	218.7		
0–2 h				3123.3 ± 461.2	0.068 ± 0.012	
18–20 h				2011.8 ± 321.3*	0.107 ± 0.017	

*Significantly different from 0–2 h value at $P < 0.05$ level

Table 3 Comparison of total nitrogen excretion with ammonia and urea excretion in the plainfin midshipmasn, *Porichthys notatus*.

Values are means ± SEM (μmol N kg^{-1} h^{-1}); $n = 4$

Condition	Ammonia-N excretion	Urea-N excretion	Percent ureotely	Ammonia-N + urea-N	Total nitrogen excretion	Total-(ammonia-N + urea-N)
Unfed for 72 h						
24-h flux period	68.9 ± 23.7	3.7 ± 0.8	5.0	72.6	99.5 ± 35.9	+ 26.9 (137%)[a]
Immediately post-feeding						
18-h flux period	177.2 ± 26.9	6.4 ± 5.5	3.5	183.6	278.8 ± 31.8*	+ 95.2 (152%)[a]

*Significantly different from corresponding unfed values at $P < 0.05$ level
[a]Percent values are the percent nitrogen excretion measured in the total N measurements compared to the sum of ammonia plus urea

ries ($n = 4$ each), one using fish starved for 72 h, the other using fish that had been fed an approximately 1% body weight meal of shrimp and then measured for a subsequent period of 18 h, the rates of total N excretion were directly compared to the rates of ammonia-N plus urea-N excretion. As expected, total N excretion rates, as well as the sum of ammonia-N and urea-N excretion rates, were significantly higher in the fed fish. More importantly, these analyses (Table 3) clearly indicated that the total nitrogen excreted by this species is significantly higher (by 37–52% depending on conditions) than that measured as the sum of ammonia-N and urea-N by chemical assay.

This discrepancy is comparable to that of earlier reports on carp (30–45%; Smith 1929), Pacific staghorn sculpin (20%; Wood 1958), rainbow trout (32%; Olson and Fromm 1971), two species of anchovies (15%–23%; McCarthy and Whitledge 1972), and surf steenbras (14%; Cockcroft and Du Preez 1989). According to the detailed chemical assays of Wood (1958), only a very small fraction of the discrepancy in the Pacific staghorn sculpin could be accounted for by creatine-N, creatinine-N, trimethylamine-N, and trimethylamineoxide-N, and more than three-quarters of it remained unaccounted for. Interestingly, Olson and Fromm (1971) were able to account for virtually all of the discrepancy in the rainbow trout as the excretion of protein-N. Clearly, more work is needed in this area, because if protein (i.e., a non-oxidized product) is excreted (perhaps as mucoprotein?), this does not enter into aerobic fuel use calculations but does enter into energy budgets. However, if the discrepancy represents oxidized end-products of nitrogen metabolism, they must be included in both aerobic fuel use calculations and energy budgets.

In summary, our results show that although the species studied do not appear to be highly ureotelic (with the obvious exception of elasmobranchs), urea transporter-like mRNA is expressed in the gills of many species. These results suggest that although not quantitatively important, urea likely exits by specific membrane protein pathways in many species. Our results further confirm that conventional measurement of urea and ammonia may substantially underestimate total nitrogen excretion rates in fish.

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