Direct effects of ambient ammonia on the nitrogen isotope ratios of fish tissues

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Abstract

We tested the assumption that the nitrogen isotope ratios of fish are solely determined by their diet by exposing two species of fish having contrasting nitrogen metabolic profiles to ^{15}N ammonia. Beaugregory damselfish (*Stegastes leucostictus*) representing ammonotelic species and toadfish (*Opsanus beta*) representing ureotelic species were exposed to ^{15}N ammonia for a period of 4 weeks, after which muscle and liver tissues were analyzed for ^{15}N abundance and compared to their respective control group. Both species showed significant ^{15}N enrichment when exposed to ^{15}N ammonia, with the ammonotelic fish showing a greater enrichment compared to the ureotelic fish. We propose that the toadfish showed less enrichment in its muscle tissue because its active ornithine–urea cycle (O–UC) rapidly sequesters ammonia away from the circulatory system and into liver tissue, thereby preventing any substantial exchange between ammonia and muscle tissue. We propose that no such sequestering occurs in the ammonotelic damselfish because they lack a functional O–UC. These results have important implications for studies using nitrogen isotope ratios to delineate trophic structure in aquatic ecosystems.
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1. Introduction

Nitrogen stable isotope analysis is a technique commonly used to evaluate food webs and trophic structure in aquatic and terrestrial ecosystems (Peterson and Fry, 1987). This is

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based on the observation that the $\delta^{15}N$ value of tissue from heterotrophic organisms follows the $\delta^{15}N$ value of their diet by a positive offset of about 3‰ (DeNiro and Epstein, 1976, 1981; Macko et al., 1982; Peterson and Fry, 1987). In aquatic ecosystems, the trophic level of a given fish species have been established by finding the difference between its average $\delta^{15}N$ value and a baseline species and dividing this difference by a constant $\delta^{15}N$ value representing one trophic level (Lake et al., 2001). Although these approaches are useful, they assume that nitrogen isotope ratios of individual species of fish are solely determined by the ^{15}N abundance of their food from the lower trophic level and are not affected by any special characteristics related to their nitrogen metabolism or the nitrogen micro-environment that they frequent. Recent observations indicate that there are several factors, such as age and quality of diet determining the extent of the $\delta^{15}N$ positive offset in terrestrial animals (Hobson et al., 1993; Hobson and Clark, 1994; Fantle et al., 1999). The variable nitrogen metabolism found in marine fish species belonging to the subclass Elasmobranchii, the order Coelacanthiformes, and the infraclass Teleostei (higher bony fish) may be an additional factor modulating the relationship between their nitrogen isotope ratios and their respective diets. Of the teleosts, two species have been studied extensively and their ability to synthesize and excrete urea (ureotely) has been confirmed: the gulf toadfish (*Opsanus beta*) and Lake Magadi tilapia (*Alcolapia grahami*) (for review see Walsh and Mommsen, 2001). The toadfish has a high ammonia tolerance, which may be linked to its ornithine–urea cycle (O–UC) and the associated high glutamine synthetase (EC 6.3.1.2) levels (Wang and Walsh, 2000). It is hypothesized that excess ammonia is sequestered by the toadfish O–UC, giving it a tolerance to high ammonia concentrations. It is not well known why toadfish excrete urea, however, it has been suggested that it may help in minimizing predator encounters due to control over excretion of urea in timed intervals allowing less of a chemosensory trail to be detected (Walsh, 1997). Conservation of nitrogen may be another advantage of ureotely in toadfish (Walsh, 1997).

We report here the labeling patterns of a ureotelic (toadfish) and an ammonotelic (damselfish) fish species exposed to ^{15}N ammonia. We expected that the ureotelic fish would be substantially labeled by the ^{15}N ammonia since it can sequester it into urea and possibly to amino acids destined for protein synthesis. On the other hand, the ammonotelic fish, not having an ammonia sequestering O–UC, would show little if any labeling by ^{15}N ammonia.

2. Materials and methods

Two species of fish with different nitrogen metabolic profiles were each raised for a period of 4 weeks in 37.8 l aquaria having a constantly re-circulating Instant-Ocean© solution passing through an activated charcoal filter having an approximate volume of 200 cm^3. Ammonotely and ureotely was confirmed by monitoring urea and ammonia secretion by the two species in a confined volume. Four replicates of Gulf toadfish (*O. beta*) (obtained from Biscayne Bay, FL by local shrimp trawlers) representing the ureotelic species were raised in two aquaria (two replicates/aquarium) and exposed to ^{15}N ammonium chloride acquired from Isotec, Sigma-Aldrich (treatment). An equivalent number of replicate toadfish in two aquaria were exposed to ammonium chloride having a natural ^{15}N abundance (control). Likewise, four replicates of beaugregory damselfish

(*Stegastes leucostictus*) (obtained from local Florida saltwater aquarium store) representing the ammonotelic species were treated with ^{15}N ammonium chloride and an equivalent number of replicates treated with ammonium chloride having a natural ^{15}N abundance. During 4 weeks, 20 mg of ^{15}N ammonium chloride and ammonium chloride having natural ^{15}N abundances were added to the respective treatment and control tanks on a weekly basis to a concentration of 10 μmol. Toadfish were fed frozen shrimp having a $\delta^{15}N$ value of $+12.2$‰ once a week and the damselfish were fed fish flakes having a $\delta^{15}N$ value of $+13.7$‰ daily. Fish were sacrificed and muscle and liver tissues were dissected and subjected to isotopic analysis at the end of the 4-week period. In addition, five randomly selected muscle tissues were each individually wrapped in nylon cloth and refluxed for a period of 6 h with hot distilled water. All tissues were freeze-dried and pyrolized in an evacuated Vycor ampoule with cupric oxide and copper to generate nitrogen among other gases (DeNiro and Epstein, 1981). Nitrogen from the pyrolysis was cryogenically distilled and frozen on molecular sieve pellets (5A, Aldrich Chemicals), and subjected to mass-spectrometry in a PRISM mass spectrometer (Micromass, England). Nitrogen isotope ratios are reported in δ units relative to atmospheric nitrogen with a precision of ± 0.3‰ (DeNiro and Epstein, 1981).

The $\delta^{15}N$ values of the muscle and liver tissues of the toadfish and damselfish were compared with a three-level nested ANOVA with unequal sample sizes and significant differences between treatments identified using a Tukey–Kramer post hoc comparison test (Sokal and Rohlf, 1995). The nested ANOVA consisted of the following groups: (1) toadfish treated with ammonia having a natural ^{15}N abundance, (2) toadfish treated with ^{15}N ammonia, (3) damselfish treated with ammonia having natural ^{15}N abundance, and (4) damselfish treated with ^{15}N ammonia. Subordinate to these groups were two aquaria per group, and subordinate to the aquaria were the two tissues (liver and muscle) sampled per aquarium. This nested approach is particularly important to determine whether there were any aquarium effects. We used a nested ANOVA with unequal sample sizes since we lost a damselfish in one treatment and one control aquarium. $\delta^{15}N$ values of refluxed muscle tissue were compared with the corresponding non-refluxed muscle tissues using a simple linear regression.

3. Results

There were significant differences between groups, but no aquarium or tissue effects (Table 1). Both species of fish had tissues that were significantly labeled by ^{15}N ammonia

Table 1
Three-level nested ANOVA for toadfish and damselfish treated with natural (controls) and enriched (treatment) abundances of ^{15}N ammonia

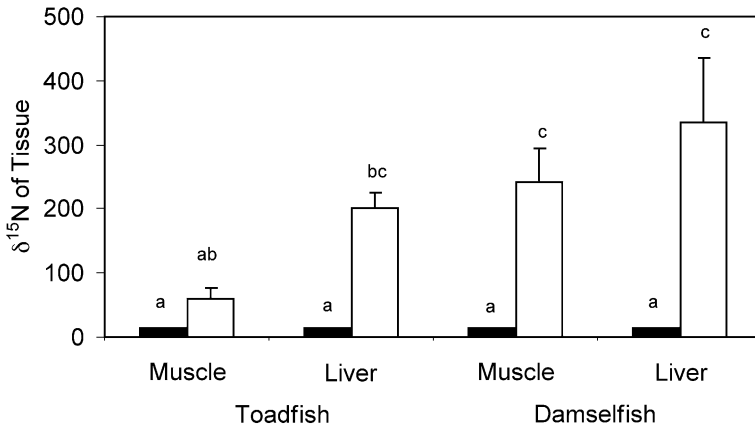| Source of Variation	df	SS	MS	Fs	P
Groups	3	324,122.4	108,040.8	46.95**	$P < 0.002$
Aquaria	4	9204.7	2301.2	0.27	$P > 0.50$
Tissues	8	69,187.6	8648.4	1.89	$P > 0.20$
Replicates	12	54,819.9	4568.3		

Fig. 1. $\delta^{15}N$ values of liver and muscle tissues of toadfish and damselfish exposed to ammonia having a natural abundance of ^{15}N (dark bars) and to ^{15}N ammonia (light bars). Error bars represent $\pm$ SEM. Bars not having a common letter are significantly different at the $P < 0.05$ level.

in the treatment compared to control aquaria. The mean $\delta^{15}N$ value of the treated toadfish liver tissue was $+201.0 \pm 41.6\%o$ ($n=4$, $\pm$ SEM) and significantly higher than liver of control fish $+14.2 \pm 2.3\%o$ ($n=4$, Fig. 1). Toadfish muscle tissue, although having a higher average $\delta^{15}N$ value than that of the control, was not significantly different ($+59.4 \pm 31.6\%o$ vs. $+14.0 \pm 0.1\%o$, $n=4$, Fig. 1). Mean $\delta^{15}N$ values of both liver
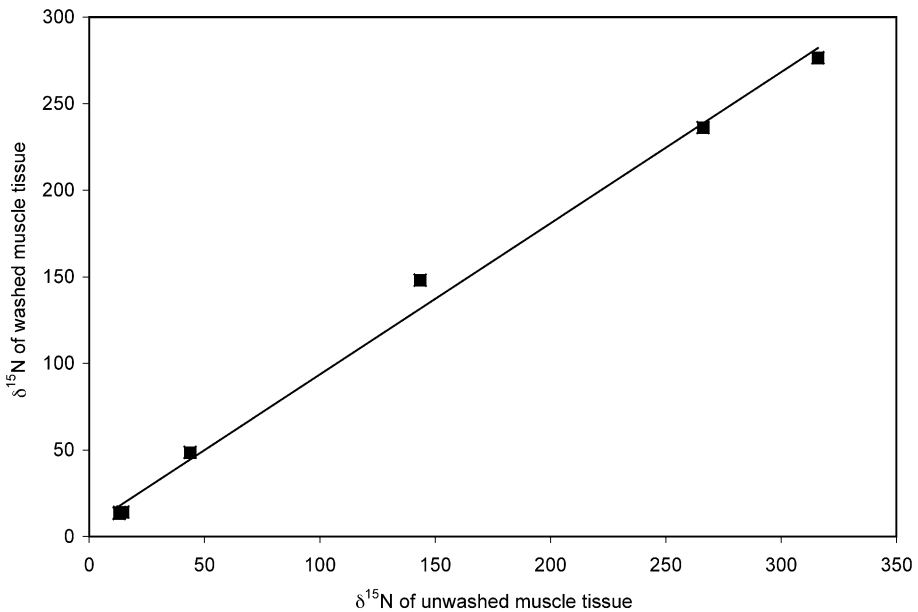

Fig. 2. $\delta^{15}N$ values of muscle tissues washed with hot distilled water for 6 h (vertical axis) vs. the same tissues before refluxing with hot distilled water (horizontal axis).

tissue ($+335.6 \pm 174.4$‰, $n = 3$) and muscle tissue ($+241.9 \pm 89.3$‰, $n = 3$) of damselfish were significantly greater than those of control ($+14.4 \pm 0.5$‰ and $+13.6 \pm 0.9$‰, $n = 3$, Fig. 1). $\delta^{15}N$ values of muscle tissue refluxed with hot distilled water had a nearly 1:1 relationship with those of tissues that were not refluxed ($\delta^{15}N_{not\text{-}refluxed} = 0.87 * \delta^{15}N_{refluxed} + 6.2$, $r^2 = 0.99$, $p < 0.01$, Fig. 2).

4. Discussion

Both toadfish and damselfish were labeled by ambient ^{15}N ammonia, therefore our expectation that the ureotelic species would be the only species labeled by ambient ammonia was not shown to be true. In fact, the muscle tissues of the damselfish were labeled significantly more than those of their respective control, whereas those of the toadfish were not (Fig. 1). Muscle from both fish species were labeled at the protein level since high $\delta^{15}N$ values were observed even in tissues washed for several hours with hot distilled water (Fig. 2), which removes free amino acids and residual ^{15}N ammonia chloride from the tissues. Both species, however, had liver tissue with significantly higher $\delta^{15}N$ values than the controls. The significant labeling of liver tissue in the toadfish is expected based on the observations that the O–UC sequestering ammonia from the circulatory system occurs in the liver (Walsh, 1997). Presently, we are investigating whether this label is predominantly in the form of urea or other compounds (Rodicio et al., to be published). One possible explanation for the higher labeling of muscle tissue in the ammonotelic fish compared to the ureotelic fish involves the ammonia concentration in their respective circulatory system. We propose that the liver of the ureotelic fish efficiently scavenges ammonia from its circulatory system, therefore minimizing exchange between ammonia and amino acids during protein synthesis in muscle cells via the glutamine synthetase reaction. Since no such mechanism is present in the liver of the ammonotelic fish, the labeled ammonia concentration in its circulatory system could be sufficiently high to exchange with the amino acid pools in muscle tissue. Another possible explanation is that ureotelic fish effectively "traps" most of the ambient ammonia in the form of the "dead-end" metabolite urea, therefore preventing exchange between ammonia and muscle tissue via glutamine synthetase.

The findings reported here have important implications in ecosystem studies that use nitrogen isotope analysis of tissue to trace food webs and delineate trophic levels. The underlying assumption in this methodology is that $\delta^{15}N$ values of tissue from heterotrophic organisms follows that of their diet with a positive offset of about 3‰ (DeNiro and Epstein, 1976, 1981), hence the commonly used statement: "you are what you eat plus a few per mil" (DeNiro and Epstein, 1976). We have unequivocally shown that ambient ammonia labels the tissues of two fish species having contrasting nitrogen metabolisms. Further, we have shown that proteins are being labeled (Fig. 2). Particularly noteworthy is that these patterns are not peculiar to a ureotele, but are also seen in an ammonotele, which appears to be the typical nitrogen metabolic pattern of the vast majority of marine and freshwater teleosts (Walsh and Mommsen, 2001). We note that ammonia concentrations higher than those of our experiments have been observed in sediment pore waters of seagrass beds (Fourqurean et al., 1992) where toadfish and other species are commonly

found. Therefore, fish that frequent burrows and other micro-habitats close to seagrass beds may be exposed and labeled by ambient ammonia. The relative amount of nitrogen labeled by ambient ammonia cannot be determined from our measurements because the amount of new protein synthesized during the 1-month treatment period and the net fractionation factor for the exchange reaction between ammonia and amino acids destined toward protein synthesis is unknown. Nevertheless, the results presented here show that the nitrogen metabolism of each species and its respective micro-environment may be an important consideration in delineating food webs and trophic levels by the ^{15}N analysis method.

Acknowledgements

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