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# Metabolic fate of exogenous $^{15}NH_4Cl$ in the gulf toadfish (*Opsanus beta*)

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**Abstract**

This study was undertaken to determine whether gulf toadfish (*Opsanus beta*) could metabolize ammonia from their environment into other, less toxic products. To this end, gulf toadfish were exposed to 3.8 mM $^{15}NH_4Cl$ in seawater for 24 and 48 h. Liver, kidney, gill, brain and muscle samples were analyzed for distribution of $^{15}N$ within the tissue and among various nitrogen-containing metabolites (ammonia, amino-N, glutamine-N, urea and protein). The data reported here show that the toadfish can indeed take up and metabolize ammonia. Analysis of individual metabolic products of ammonia indicates that the toadfish can convert this toxic chemical into other less toxic metabolites. Ammonia enrichment is significantly different over controls in the kidney, brain and muscle. Urea enrichment is most significant in the brain, with less significant enrichment occurring in the liver and muscle. While accumulation of ammonia into an amino acid pool was not a significant metabolic fate, protein synthesis was significantly enriched in all tissues (with the highest levels occurring in the gill) indicating that amino acid synthesis may be a pathway of ammonia detoxification en route to protein synthesis, and that environmental ammonia can be 'fixed' into protein. Finally, it was found that glutamine-N synthesis occurs at significant levels in the liver, brain and muscle.


*Keywords:* Gulf toadfish; *Opsanus beta*; Ornithine-urea cycle; Hyperammonemia; Hepatic encephalopathy; $^{15}NH_4Cl$

## 1. Introduction

Under normal physiological conditions, the main source of ammonia in mammals and humans is the degradation of proteins and amino acids from either a dietary source during fed states or from tissue 'wasting' during unfed states (Atkinson and Bourke, 1985). Typically, plasma concentrations of ammonia are maintained at $<50$ μM, and ammonia produced in this manner is readily disposed of by conversion to urea in the liver via the ornithine-urea cycle (O-UC) or to a lesser extent, by formation of glutamine (Duda and Handler, 1958; Häussinger et al., 1985; Meijer et al., 1990; Atkinson, 1992). If these mechanisms fail to efficiently remove ammonia, the concentration of ammonia rises to cause 'hyperammonemia'. This condition, under which ammonia concentration is elevated by as little as two- to four-fold, can eventually lead to death (Meijer et al., 1990). There are a number of factors that may lead to hyperammonemia. Among these factors are direct exposures to exogenous ammonia via pollutants

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(e.g. air-borne ammonia gas and ammonium salt particulates) or more commonly due to factors such as genetic disorders and liver disease, which can cause increases in endogenous ammonia (Meijer et al., 1990; Meijer, 1995). Secondary causes may also involve exposure to pollutants, such as carbon tetrachloride ($CCl_4$) and toluene, which may cause liver damage.

Fish and other aquatic organisms face similar sorts of factors which increase endogenous ammonia (e.g. air-breathing/terrestriality), and considerable focus has been given to these effects and the effects of exogenous ammonia as a pollutant in natural and man-made (e.g. aquaculture) environments (Ip et al., 2001; Handy and Poxton, 1993). In this literature, it is often assumed that high environmental ammonia (and/or high environmental pH) will cause ammonia to rapidly cross the respiratory surface (as $NH_3$), elevating plasma ammonia, and that 'metabolism' then acts to detoxify this exogenous ammonia. An alternative hypothesis is that exogenous ammonia also causes proteolysis or amino acid catabolism, which releases tissue ammonia stores (Lim et al., 2001). Due to the lack of convenient and safe radioactive N isotopes, and the expense and tedium of stable $^{15}N$ isotope measurements, very rarely have studies been performed on fish to test this very basic assumption (Iwata and Deguchi, 1995; Iwata et al., 2000).

Recently, our laboratory has documented that *Opsanus beta* and other members of the family Batrachoididae are in fact rather tolerant of exogenous ammonia exposure. Ninety-six hour $LC_{50}$ values range from 6 mM for the plainfin midshipmen (*Porichthys notatus*), to 10 mM for the gulf toadfish (*O. beta*), and to 20 mM for the oyster toadfish (*Opsanus tau*) (Wang and Walsh, 2000), whereas these values are often below 1 mM for many teleost fish (Ip et al., 2001). These studies also demonstrated that exogenous ammonia caused an increase in toadfish tissue ammonia (again presumably through ammonia entry) such that sensitive organs (e.g. brain, liver) had ammonia concentrations at these millimolar levels (Wang and Walsh, 2000). In addition, it was found that there is a direct correlation across these species between 96 h $LC_{50}$ and brain glutamine synthetase activity, indicating that the synthesis of glutamine and perhaps other nitrogen-containing species, such as urea, play an important role in the extreme ammonia tolerance of these fish (Wang and Walsh, 2000). Given the high tolerance to ammonia in these fish, one of the goals of our laboratory is to develop the toadfish as a model for hyperammonemia in mammals.

The possible metabolic fates of exogenous nitrogen in the ureotelic toadfish, can be predicted using the ornithine-urea cycle (O-UC). Based on this cycle, excess nitrogen may be found as either free ammonia or converted to less toxic compounds such as urea and glutamine, or possibly into other amino acids (e.g. arginine), which can then be incorporated into proteins. In addition, to the types of nitrogen waste products generated, the location of these waste products prior to excretion is also of interest. The O-UC typically occurs in the liver, which suggests that a large portion of the metabolites may be found there. However, in ureotelic fish including toadfish, several recent studies suggest that muscle is also a potentially important site of urea synthesis (reviewed by Anderson, 2001). It has also been shown that the primary site of urea excretion in the toadfish is the gills (Gilmour et al., 1998; Wood et al., 2003). In mammals, the conversion of ammonia to glutamine is a main line of defense against neurotoxicity in the brain (Meijer et al., 1990). Therefore, elevated levels of glutamine-N may be found in brain tissue, as shown in the brain of mudskipper fish exposed to high ammonia concentrations (Iwata and Deguchi, 1995). Finally, any waste ammonia converted to amino acids or protein would most likely be found in muscle tissue where these molecules are primarily found, and which may be a potentially large sink for excess nitrogen in fish (Chew et al., 2003; Saha et al., 2000, 2002).

With this background in mind, we studied the fate of exogenous $^{15}NH_4Cl$ in gulf toadfish first to validate the assumption that exogenous ammonia enters the fish and is incorporated into metabolites within the fish. Additionally, we wished to more fully characterize the metabolites to which ammonia is converted.

## 2. Materials and methods

### *2.1. Toadfish*

Gulf toadfish, *O. beta*, were obtained from local fishermen who capture the fish in roller trawls during shrimping. After transfer to the University of Miami, fish were held in 45-l aquaria supplied with flowing seawater at ambient temperature from

Biscayne Bay. Each aquarium contained a bed of beach sand and polyvinylchloride tubes for shelter. Fish were fed squid twice a week and were starved for 48 h prior to experimentation.

### 2.2. Exposure to $^{15}NH_4Cl$

Fish were divided into two groups—an experimental group and a control group. In both cases, fish were 'crowded' in individual 2-l tubs with flowing seawater to induce ureotely (Wood et al., 1997). In the case of the control group, the fish were left in 2 l seawater and flow stopped for either 24 h or 48 h. In the case of the experimental fish, fish were exposed to standing seawater containing 3.8 mM $^{15}NH_4Cl$ (99% $^{15}N$; Isotech) and fish were exposed for 24 h or 48 h. Temperature was $23 \pm 1$ °C. Ammonia assays of the seawater indicated that the ammonia levels in the water did not fluctuate significantly over the 48 h time period. As previously reported (Wang and Walsh, 2000), these conditions generate a final seawater pH of 7.7–7.9.

### 2.3. Tissue collection and isolation of nitrogen metabolites

After exposure, fish were anesthetized with MS-222. The entire liver, kidneys, gills, brain and a sample of muscle tissue were then dissected from each fish. Tissues were immediately frozen in liquid nitrogen. For whole tissue analysis, a portion of the frozen tissue (5–10 mg) was lyophilized and processed for isotope ratio analysis as described below. For determination of specific metabolic products, the remaining tissue was treated with 7% perchloric acid and centrifuged to separate the protein and non-protein fractions. Protein samples were washed twice with deionized water in order to dissolve any absorbed ammonia and to remove excess acid. Protein fractions were then lyophilized and processed for isotope ratio analysis as described below.

In order to decrease sample loss from sample manipulation, two sets of samples were obtained—one set was used to isolate ammonia and urea-N, and the other set was used to isolate Gln-N and amino-N. The non-protein fraction was treated with 10 M NaOH and vacuum distilled into an acid trap to isolate ammonia-N (Ta and Joy, 1986). After ammonia-N removal, the sample was titrated to pH 7 using hydrochloric acid and then diluted with 2 ml 10 mM HEPES, pH 6.5, 3 mM EDTA. Samples were treated with six units urease (Sigma) to release urea-N as ammonia and allowed to react overnight at room temperature. Samples were then treated with 10 M NaOH and ammonia was collected as before.

For Gln-N isolation, ammonia-N was removed from the sample as described above. The sample was adjusted to pH 5 with hydrochloric acid. Samples were treated with two units of glutaminase (Sigma) at 37 °C overnight to release Gln-N as ammonia. Samples were treated with 10 M NaOH and ammonia was collected as before.

After Gln-N removal, the pH of the samples was adjusted to five. The samples were diluted with 1 ml sodium acetate buffer (3 M, pH 5.0). Amino-N was isolated by formation of a ninhydrin complex followed by release of amino-N and ammonia from the complex (Kennedy, 1965). A 2 ml aliquot of a 3% ninhydrin solution in water was added to the sample. The sample was heated at 100 °C for 10 min. After 10 min, 4 ml 2 N HCl was added to the sample and heated at 100 °C for 10 min more. A 0.25 ml of hydrogen peroxide was then added to the sample and heated at 100 °C for a further 10 min.

The acidic distillates for each sample were lyophilized and processed as described below for isotope ratio analysis. Percent recovery of ammonia by distillation was approximately 60% based on ammonia assays conducted during method development with ammonia standards.

### 2.4. Sample preparation for isotope ratio mass spectrometry

Lyophilized samples (5–10 mg) were combusted at 800 °C in sealed Vycor ampoules with 1 g of copper and 1 g of cupric oxide. Prior to combustion, 5–8 mg glycine was added to each sample to increase detectability of the non-protein distillates. Combusted samples were cryogenically separated to separate C-containing and N-containing products (Minagawa et al., 1984). The carbon products were discarded and the nitrogen samples were analyzed by isotope ratio mass spectrometry using a Micromass Prism II isotope ratio mass spectrometer.

### 2.5. Data analysis

Data are reported in terms of $\delta N \pm$ S.D. Calculation of $\delta N$ following addition of glycine to each

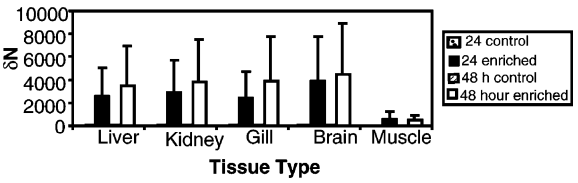


Fig. 1. Mean whole tissue data for toadfish exposed to 3.8 mM $^{15}NH_4Cl$ for 24 h and 48 h. Error bars represent S.D.

sample was calculated in two steps as outlined below:

$$R_{sample} = \text{atom \% } N_{sample} = \frac{(\text{atom \% } N_{sample+glycine}) \times (mg_{sample+glycine}) - (\text{atom \% } N_{glycine}) \times (mg_{glycine})}{mg_{sample}} \quad (1)$$

$$\delta^{15}N = \frac{R_{sample} - R_{air}}{R_{air}} \times 1000 \quad (2)$$

where $R_{air} = 0.003663$

Significance is measured using either Student's *t*-test or ANOVA. Differences are considered significant at $P < 0.05$ unless otherwise noted.

## 3. Results

### 3.1. $^{15}N$-enrichment of whole tissue samples

Three distinct analyses of the data shown in Fig. 1 and Table 1 for each time period were carried out. Firstly, for each tissue, a *t*-test was used to compare the $\delta N$ values for the control and experimental samples at 24 h. It was found that for all tissues, there was a significant difference between the enriched and control samples after 24 h of exposure. The same analysis was carried out for the 48 h samples, and it was found again, that all samples were enriched over control after 48 h of exposure.

Secondly, for each tissue, a *t*-test was used to compare the $\delta N$ values of the 24 h and 48 h experimental samples. It was found that there was no significant difference in enrichment between 24 and 48 h for tissues, except for the gill.

Finally, an ANOVA analysis was carried out on the 24 h enriched samples to determine if there was a significant difference in incorporation over all five tissues. If the muscle data is included, there is a significant difference in incorporation among the tissues. However, when the muscle data are excluded, there is no significant difference in enrichment among the liver, gill, brain and kidney. The same analysis was carried out for the 48 h samples, with the same results as the 24 h analysis.

### 3.2. $^{15}N$-metabolic product distribution

Ammonia-N was significantly enriched over the controls for muscle, brain and kidney samples (Table 2 and Fig. 2). Liver and gill samples were not significantly enriched over controls.

Urea-N was significantly enriched in brain samples, and slightly enriched in liver and muscle. Liver and muscle samples were not significantly enriched using a $P = 0.05$ criteria for ANOVA

Table 1
Nitrogen enrichment in whole tissue samples after 24 h and 48 h exposures to 3.8 mM $^{15}NH_4Cl$

| Tissue | 24 h | | 48 h | |
|---|---|---|---|---|
| | Control | Enriched | Control | Enriched |
| Liver | 23.261 ± 9.022 ($n$=9) | 2531.952 ± 815.050* ($n$=9) | 33.525 ± 17.895 ($n$=7) | 3464.271 ± 1810.918* ($n$=6) |
| Kidney | 24.277 ± 5.631 ($n$=8) | 2852.663 ± 984.207* ($n$=7) | 28.736 ± 13.907 ($n$=7) | 3763.221 ± 1151.191* ($n$=6) |
| Gill | 25.825 ± 6.782 ($n$=7) | 2359.235 ± 852.615* ($n$=7) | 22.463 ± 12.047 ($n$=7) | 3901.810 ± 917.541* ($n$=6) |
| Brain | 54.879 ± 11.931 ($n$=3) | 3870.018 ± 440.283* ($n$=3) | 24.691 ± 15.104 ($n$=9) | 4477.034 ± 688.948* ($n$=6) |
| Muscle | 18.041 ± 4.498 ($n$=8) | 609.565 ± 212.140* ($n$=8) | 13.822 ± 3.736 ($n$=8) | 470.222 ± 352.568* ($n$=7) |

Significant differences between experimental and control samples ($P < 0.05$) are marked with an asterisk.
Data is represented as Mean ± S.D.
$n$ = number of samples.

Table 2
Nitrogen metabolic product enrichment in control and experimental samples

| Tissue | Ammonia-N | | Urea-N | | Amino-N | | Protein-N | | Gln-N | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Control | Enriched | Control | Enriched | Control | Enriched | Control | Enriched | Control | Enriched |
| Liver | 23.899 ± 120.741 ($n=5$) | 888.116 ± 1652.249 ($n=5$) | 2.048 ± 28.098 ($n=4$) | 3659.213 ± 3907.754 † ($n=4$) | −26.407 ± 30.276 ($n=3$) | 298.227 ± 1216.722 ($n=8$) | 14.766 ± 1.764 ($n=5$) | 608.796 ± 329.066* ($n=6$) | 94.009 ± 84.155 ($n=4$) | 3519.6 ± 2137.307* ($n=3$) |
| Kidney | 122.901 ± 107.458 ($n=4$) | 4801.902 ± 1285.835* ($n=3$) | N/A | 430.043 ± 688.671 ($n=2$) | −56.926 ± 29.844 ($n=4$) | 175.285 ± 410.057 ($n=5$) | 11.064 ± 0.587 ($n=3$) | 776.303 ± 309.623* ($n=4$) | 26.168 ± 33.369 ($n=4$) | 42.101 ± 30.768 ($n=5$) |
| Gill | 13.636 ± 31.039 ($n=4$) | 2410.076 ± 4222.062 ($n=5$) | −324.034 ± 249.228 ($n=4$) | 2318.446 ± 4108.149 ($n=5$) | −19.941 ± 17.344 ($n=5$) | 82.892 ± 616.868 ($n=6$) | 13.109 ± 0.405 ($n=4$) | 1259.457 ± 192.664* ($n=5$) | 42.194 ± 26.338 ($n=4$) | 538.487 ± 688.556 ($n=4$) |
| Brain | −138.531 ± 85.831 ($n=5$) | 4072.691 ± 85.83146* ($n=5$) | −101.112 ± 94.910 ($n=5$) | 753.608 ± 596.224* ($n=5$) | −43.890 ± 24.691 ($n=3$) | −116.759 ± 185.955 ($n=5$) | 13.122 ± 3.169 ($n=3$) | 402.202 ± 150.898* ($n=5$) | 77.819 ± 62.864 ($n=5$) | 255.145 ± 169.334* ($n=5$) |
| Muscle | −58.545 ± 51.197 ($n=5$) | 6192.042 ± 3700.815* ($n=5$) | 734.689 ± 893.092 ($n=5$) | 3376.495 ± 3708.709 ‡ ($n=5$) | −58.989 ± 141.230 ($n=5$) | 613.595 ± 1268.163 ($n=6$) | 11.476 ± 0.809 ($n=4$) | 223.063 ± 71.029* ($n=4$) | 21.098 ± 222.409 ($n=5$) | 354.934 ± 240.723* ($n=5$) |

Significant differences between experimental and control samples ($P<0.05$) are shaded and marked with an asterisk.
Data is represented as Mean ± S.D.
$n$ = number of samples.
† $P=0.06$.
‡ $P=0.08$.

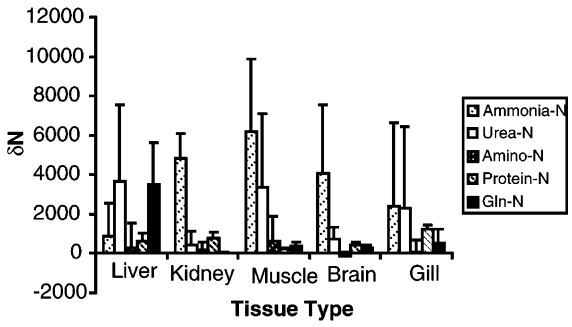


Fig. 2. Mean metabolic product distribution among tissues for toadfish exposed to 3.8 mM $^{15}NH_4Cl$ for 24 h. Error bars represent S.D.

analysis. However, at $P=0.06$ and $P=0.08$, liver and muscle enrichment were significant, respectively. In some cases, the enrichment in muscle and liver was higher than that observed in brain tissue, but the variability was such that the enrichment was only marginally different than in the respective control. There was no significant enrichment in the gill samples. In the case of kidney samples, urea was either entirely absent or barely detectable in both control and enriched samples. Therefore, there were not enough sample points to determine whether the differences were significant.

In the case of amino-N, there was no significant enrichment in any of the tissues from the experimental group. In contrast, all experimental tissues were significantly enriched in protein-N over controls.

Gln-N was found to be significantly enriched in liver, brain and muscle tissues. Gill and kidney tissues were not significantly enriched in Gln-N.

In terms of metabolic product distribution within the tissues, there is a significant variance among product formation in all tissues except the gill (Fig. 2). In the liver, it appears that the majority of the metabolic products are in the form of urea and glutamine. In the kidney, muscle and brain, ammonia enrichment is highest. Urea formation is also prevalent in the muscle.

## 4. Discussion

The first goal of this study was to confirm using stable isotope data, whether or not toadfish take up excess ammonia from their environment into various tissues. The data reported here show that the toadfish can indeed take up excess ammonia from their environment. The whole tissue data (Table 1 and Fig. 1) indicate that at both 24 and 48 h, all tissues are permeated by $^{15}N$. The difference in $^{15}N$ enrichment between the muscle and other tissues is probably due to the lower perfusion of the toadfish muscle tissue relative to the other organs isolated. A previous study in our lab concluded that ammonotelic damselfish exposed to $^{15}N$ showed higher enrichment in muscle tissue compared to toadfish exposed to the same levels (Moeri et al., 2003). This finding indicates that ammonia is quickly sequestered and metabolized by ureotelic fish. Our findings here in terms of muscle enrichment support this previous observation. The presence of $^{15}N$ in all tissues in this portion of the study indicates that the toadfish does not simply 'keep out' the excess ammonia from their environment.

The second portion of this study was aimed at determining the metabolic fate of ammonia in the toadfish. To this end, various nitrogen-containing compounds that were thought to be likely stores of excess nitrogen were analyzed. Analysis of individual metabolic products of ammonia indicates that the toadfish can indeed convert this toxic chemical into other less toxic metabolites (Table 2 and Fig. 2).

The first compound analyzed was free ammonia. Our previous studies indicated that high levels of ammonia are present in toadfish even after the onset of ureotely. Here, it was found that ammonia enrichment is significantly different over controls only in the kidney, brain and muscle. The lower levels of ammonia enrichment in the liver are probably due to the rapid uptake of ammonia and incorporation into glutamine and subsequently into the O-UC cycle for conversion into other products. In the gill, ammonia is most likely released back into the surrounding water. The presence of such high levels of ammonia indicate that although some excess ammonia is metabolized to other products as described below, the toadfish is still able to tolerate high levels of this toxic substance in many of its tissues.

The second compound analyzed for the presence of labeled nitrogen was urea. The fact that toadfish are ureotelic under high ammonia concentrations makes urea a likely suspect to contain excess nitrogen. Our findings here were quite surprising. Urea enrichment is most significant in the brain, and it will be interesting to determine in future studies if this is reflected by an increase in the

total content of urea and other osmotically important solutes in the brain, with one possible explanation being to offset osmotic water gains caused by ammonia detoxification to glutamine (Cooper, 2001; Brusilow, 2002). Liver and muscle samples were not significantly enriched using a $P=0.05$ criteria for ANOVA analysis. However, at $P=0.06$ and $P=0.08$, liver and muscle enrichment were significant, respectively. The liver is the site of the O-UC cycle and it was expected that the urea enrichment levels would be higher. It is possible that at the time-scale of these experiments, some of the urea synthesized in the liver was excreted prior to tissue collection. The muscle has been found to contain the enzymes required for the O-UC cycle (Julsrud et al., 1998). The presence of urea in the muscle supports previous findings that toadfish may synthesize urea in the muscle.

The third compound analyzed for $^{15}N$ enrichment was amino-nitrogen, followed by protein-nitrogen. As stated in Section 1, other researchers have found that amino acid synthesis actually increases during exposure to exogenous ammonia (Chew et al., 2003; Saha et al., 2000, 2002). While the amino acid fraction was not a significant pool for accumulation of excess ammonia, protein synthesis was significantly enriched in all tissues, with the highest levels occurring in the gill. This finding indicates, that at the time scale of these experiments any excess nitrogen converted to amino or amide nitrogen may be subsequently incorporated into protein. This is an interesting observation in light of a study showing that even a modest level of environmental ammonia (i.e. 70 μM) can stimulate growth in rainbow trout (Linton et al., 1998). Our results demonstrate that 'fixation' of nitrogen into protein, possibly through glutamate dehydrogenase and glutamine synthetase, is possible in fish, and may provide a pathway underlying the observations of Linton et al. (1998). However, further enzymatic analyses are required to support this assumption, and to examine if amine groups can also be transferred from glutamate and glutamine to other amino acids prior to incorporation into protein.

The last compound to be analyzed for incorporation of exogenous ammonia was glutamine. As stated in Section 1, GSase levels drastically increase in *O. beta* during exposure to ammonia and other stressors (Wood et al., 2003). It was found that Gln-N synthesis occurs at significant levels in the liver, brain and muscle. These results are not surprising in light of our previous studies demonstrating high GSase levels in many toadfish tissues after ammonia exposure, particularly in the brain. In addition, the presence of the O-UC in liver and muscle would necessitate the production of glutamine.

A final note is that some of the $\delta N$ values for some control samples seem to be depleted in $^{15}N$ (Table 2). The cause of this depletion is most probably due to dilution of $^{15}N$ by addition of the glycine standard.

In summary, we have shown that the Gulf toadfish can indeed take up excess ammonia from its environment and convert it to a number of metabolic products in addition to tolerating elevated levels of ammonia in its tissues.

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