Nitrogen Excretion

in Fish

SYMPOSIUM PROCEEDINGS

Pat Wright

Don M\textsuperscript{ac}Kinlay

International Congress on the Biology of Fish
University of British Columbia, Vancouver, CANADA
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PREFACE

Homer Smith in the 1930s built the foundation of our knowledge in the area of nitrogen excretion in fish. He discovered that teleost fish excrete mostly ammonia across the gills, whereas elasmobranch fish retain urea as an osmolyte and prevent its loss through the kidney. Many studies since have confirmed and extended Smith’s earlier genius, but in the last decade new strides have been made in several areas. The first has been a major advance in our understanding of urea transport. The first three papers in this volume concern membrane proteins that selectively transport urea. In the elasmobranch kidney, urea transporters may be critical for the reabsorption of urea from the filtrate back to the blood. In the rainbow trout gill, urea transporters may facilitate urea elimination.

The second area of interest relates to nitrogenous compounds in protein stabilization and osmoregulation. Most of our knowledge of these interactions comes from studies of adult elasmobranches. There is new information of how organic osmolytes counterbalance the destabilizing effects of urea in early elasmobranch development, as well as counterbalancing the effects of high pressure in deepsea teleosts. Work on the freshwater stingray is also essential in broadening our view of the elasmobranch strategy of ureosmotic regulation.

Urea synthesis is a crucial means of preventing ammonia accumulation in some fish, especially during air exposure or protracted embryogenesis. Attention has also focused, however, on glutamine synthesis, an alternative route for hiding ammonia in a non-toxic form. Molecular studies have revealed that more than one glutamine synthetase gene exists in trout and this has interesting implications for studies of gene expression. Recent studies on mangrove fishes, such as the mudskipper and snake head described here, have alerted our attention to unusual metabolic strategies utilized to cope with air exposure.

Taken together, this collection of papers provides an example of the new view of nitrogen excretion in fish. Although Smith’s early work established many of the basic tenets of the field, new doors have opened inviting physiologists and biochemists to move well beyond the dogma.

Symposium Organizers:

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Don MacKinlay, Fisheries & Oceans Canada, Vancouver BC
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The main organizers of the Congress, on behalf of the Physiology Section of the American Fisheries Society, were Don MacKinlay of DFO (overall chair, local arrangements, program and proceedings) and Rosemary Pura of UBC Conferences and Accommodation (facility arrangements, registration and housing). Thanks to Karin Howard for assistance with Proceedings editing and word-processing; to Anne Martin for assistance with the web pages; and to Cammi MacKinlay for assistance with social events.

I would like to extend a sincere ‘thank you’ to the many organizers and contributors who took the time to prepare a written submission for these proceedings. Your efforts are very much appreciated.

Don MacKinlay
Congress Chair
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REGULATION OF UREA TRANSPORT IN DORSAL AND VENTRAL SECTIONS OF THE LITTLE SKATE KIDNEY (RAJA ERINACEA) – RESPONSE TO CHANGING EXTERNAL SALINITY

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Abstract
The retention of high concentrations of urea in the tissues of marine elasmobranchs is the key to their osmoregulation strategy. During environmental dilution, changes in the mechanisms of retention in the kidney result in the reduction of urea concentrations in the body fluids. In the kidney of the little skate, Raja erinacea, the cDNA of a renal urea transporter (UT) was isolated, with high homology to that of the shark kidney UT (SkUT). This UT was downregulated during environmental dilution, indicating its importance in the ability of the renal tubule to reabsorb urea. Facilitated urea transport was characterized also in brush-border membrane vesicles of renal tubules from the dorsal and ventral sections of the kidney. Although facilitated transport was demonstrated in vesicles from both kidney sections, there were significant differences in inhibitor, analogue and ion responses. These findings provide evidence for the role of facilitated urea transporters in urea retention by the skate kidney.
Introduction

Osmoregulation in marine elasmobranchs is mainly accomplished by the retention of high levels of urea in the body fluids. The kidney is important in this retention because it reabsorbs almost all (>90%) of the urea in the glomerular filtrate (Kempton, 1953). The arrangement of the elasmobranch nephron allows for a counter-current system that may be involved in the passive reabsorption of urea, however it has been speculated that a carrier-mediated process is also present. However, the exact site and mechanisms responsible for urea reabsorption are uncertain. In euryhaline elasmobranchs, dilution of the external salinity results in a marked reduction of urea concentrations in body fluids. During this adaptation, urea excretion is increased, while urea biosynthesis and renal tubule urea reabsorption rates are decreased (Goldstein and Forster 1971). The mechanisms involved in reducing renal urea reabsorption are not known. The purpose of this study was to examine the role of renal urea transporters in urea retention in a marine elasmobranch, the little skate, *Raja erinacea*.

Materials and Methods

Skates were exposed to 100% seawater (control) and 50% seawater (treatment) in a flow-through system for 5 days. The skates were sacrificed, the paired kidneys removed and dissected into dorsal-lateral and ventral sections as described by Hentschel et al. (1986). Using total RNA prepared from the kidney, a partial sequence of a skate urea transporter (SkUT) was isolated using primers from a consensus of fish urea transporter sequences. Relative expression of SkuT mRNA to β-actin mRNA in skates was determined using Northern blot analysis.

In a separate experiment, brush-border membrane vesicles (BBMV) were isolated from dorsal-lateral and ventral sections of the skate kidney using the method of Kipp et al. (1997). Characteristics of $^{14}$C-urea uptake by the BBMV were determined using a rapid filtration method (Fines et al., 2000).

Results

A skate kidney urea transporter (SkUT) was isolated, which has 88% homology with the shark kidney urea transporter (ShUT; Smith and Wright, 1999). High stringency Northern analysis revealed the presence of three SkUT bands at 3.1, 2.8 and 1.6 kb. In skates exposed to 50% seawater, there was a significant
decrease in the relative expression of SkUT in all band in the dorsal segment and in the 3.1 and 1.6 kb bands in the ventral segment (Fig. 1).

There was a linear relationship between urea uptake and urea concentration in both dorsal and ventral BBMV at high urea concentrations (5–370 mM), whereas saturation kinetics was observed at lower urea concentrations (0.2–2 mM). This uptake was inhibitable by phloretin and mercury chloride, and in dorsal BBMV by the urea analog, NPTU. There was no effect on urea uptake with the addition of ATP. In the ventral but not dorsal BBMV, uptake was significantly increased in the presence of a sodium gradient, while there was no effect in the presence of a potassium gradient.

Conclusions

In the skate kidney, the presence of urea transporter mRNA suggests that it may have a role in urea retention. During environmental dilution, SkUT mRNA is down regulated, which may decrease the reabsorption of urea by the renal tubule resulting in a greater rate of urea excretion. The presence of three SkUT bands suggests that there are different isoforms of the urea transporter gene, much like that seen in mammalian UT families. Further work is necessary to complete the molecular analyses of these possible isoforms. Our physiological data using BBMVs indicates that the characteristics of urea uptake differ between the
dorsal and ventral renal sections. There is evidence for the presence of two transporters, one of which is linked to Na⁺ transport. Clearly, the mechanisms of urea reabsorption are complex in the elasmobranch kidney, not surprising in the most structurally complex kidney among the vertebrates.

References


IDENTIFICATION OF UREA TRANSPORTER ISOFORMS IN THE ELASMOBRANCH KIDNEY

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In marine elasmobranch fishes, renal tubular urea reabsorption is, in part, responsible for the maintenance of a high plasma urea concentration. Furthermore, the regulation of tubular urea reabsorption is important in the renal response of euryhaline elasmobranchs to changes in environmental salinity. The mechanisms by which tubular urea reabsorption is regulated in the elasmobranch kidney have yet to be elucidated, but both passive and active mechanisms have been proposed. Passive reabsorption of urea occurs via facilitated urea transporter proteins (UT). A facilitated urea transporter (shUT), similar to that of the UT-A2 isoform in mammals, has been cloned from the kidney of the marginal elasmobranch, the spiny dogfish shark, Squalus acanthias (Smith and Wright, 1999). Interestingly, although only a single facilitated urea transporter isoform was identified, Northern analysis suggested that multiple isoforms may be expressed in the elasmobranch kidney. These findings indicate UTs may contribute to renal tubular urea reabsorption in elasmobranchs. We hypothesized that homologous urea transporters, similar to shUT, are also expressed in the kidney of euryhaline elasmobranchs and that these transporters play a role in mediating tubular reabsorption of urea in euryhaline elasmobranchs.
The initial step in testing this hypothesis was to identify UT isoforms expressed by a euryhaline elasmobranch. We utilized molecular cloning (PCR and 5'/3' Rapid Amplification of cDNA ends) and heterologous expression (urea uptake by *Xenopus* oocytes) techniques to identify and characterize cDNAs encoding facilitated UTs from the kidney of the euryhaline Atlantic stingray (*Dasyatis sabina*). The first stingray UT cDNA that we cloned was designated strUT-1. This cDNA is 2.7 kb in length, has an open reading frame (ORF) of 1296 nucleotides (nt) and encodes a 431 amino acid protein (Figure 1). The strUT-1 protein has a distinct carboxy-terminus compared to shUT, but aside from differences in the carboxy-terminus, the first 377 amino acids of strUT-1 were nearly 80% identical to the first 377 amino acids of shUT. By using RT-PCR and flanking primers within the 5' and 3' untranslated region of strUT-1 we also identified a second UT cDNA. This cDNA, which was designated strUT-2, is 3.6 kb in length, contains an open-reading frame of 1137 nt, and is predicted to encode for a UT of 379 amino acids (Figure 1). This shorter isoform is identical to the N-terminal 377 amino acids of strUT-1. Interestingly, even though the strUT-2 transcript was considerably larger than that reported for strUT-1 and shUT, this isoform is almost the same size, has a similar carboxy-terminus, and shares 79% sequence identity that of the shark UT. Both strUT-1 and strUT-2 include a double LP box signature sequence common to all UTs.

![Figure 1. Schematic of strUT-1 and strUT-2 cDNA.](image)

- **5' UTR**
- **ORF – 1296bp**
- **3' UTR**

strUT-1
(2.7 kb)

- **5' UTR**
- **ORF – 1137bp**
- **3' UTR**

strUT-2
(3.6 kb)

*970 bp

* strUT-2 contains a novel nucleotide cassette not present in strUT-1.
Retention of the unspliced cassette in strUT-2 results in an abbreviated ORF.
Characterization of both strUT-1 and strUT-2 in *Xenopus* oocytes demonstrated both isoforms increase $[^{14}C]$-urea uptake. This strUT induced $[^{14}C]$-urea uptake was inhibited by phloretin but was not altered by removal of sodium chloride from the medium. Northern analysis demonstrated transcripts of 2.7 kb and 3.6 kb corresponding to the predicted size of the message for strUT-1 and strUT-2, respectively. Interestingly, strUT expression was specific to the kidney. Further, Northern analysis indicated two other transcripts were expressed in stingray kidney. Following additional PCR optimization of our 5′/3′ RACE reaction, we amplified two 3′ RACE cDNAs 1.4 kb and 3.5 kb in size. The smaller 3′RACE product represents a 3′untranslated region (3′UTR) splice variant of strUT-1, whereas the larger transcript represents a 3′UTR splice variant of strUT-2. These cDNAs were designated strUT-1b and strUT-2b, respectively. The presence of 3′UTR splice variants is similar to that reported for UTs in the mammalian kidney (Bagnasco et al., 2000).

In the second series of comparative studies, we utilized the cloning techniques described above to identify facilitated urea transporters from the kidneys of two batoid species: a subtropical, marginal dasyatid stingray (*Dasyatis sayi*), and a temperate, stenohaline rajid skate (*Leucoraja ocellata*). A single UT cDNA has so far been cloned from the kidneys of both species. The cDNA from the skate (skUT) is 2.1 kb in length, with an open-reading frame of 1134 nt that encodes a 378 amino acid protein. The cDNA from *D. sayi* (UT) is 1.7 kb in length with an open-reading frame of 1140 nt that encodes a 380 amino acid protein. These urea transporters were similar in length and sequence identity to shUT and strUT-2.

We conclude that 1) orthologous facilitated urea transporters are expressed in the kidneys of phylogenetically distinct elasmobranchs, 2) multiple urea transporter isoforms are expressed in the kidneys of at least one species of elasmobranch, and 3) a common urea transporter isoform is expressed in the kidneys of elasmobranchs.

References


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EVIDENCE FOR FACILITATED DIFFUSION OF UREA IN THE GILLS
OF THE FRESHWATER RAINBOW TROUT

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Introduction

Like most other freshwater teiosts, the rainbow trout (Oncorhynchus mykiss) excretes the majority of its nitrogenous wastes across the gills as ammonia, the form in which it is initially produced. However, research in the past decade has uncovered a handful of teleosts that expend energy to detoxify ammonia and excrete urea as their primary waste product. This is made possible by a fully functional ornithine-urea cycle (OUC) in internal tissues (especially the liver) and specialized facilitated diffusion urea transport mechanisms in the gill (UT-type transporters; Walsh et al. 2000, 2001). While adult rainbow trout do not have a full complement of OUC enzymes, they do maintain a surprisingly high level of circulating urea compared to endogenous ammonia concentrations (5–
50x more urea) and urea makes up about 10% of total nitrogen waste excretion. In addition, recent evidence suggests that, as in ureotelic fish, urea excretion is carrier-mediated in ammoniotelic organisms such as the rainbow trout (McDonald and Wood, 1998) and the plainfin midshipman (Walsh et al. 2001).

Therefore, preliminary evidence suggests the involvement of carrier protein in trout branchial urea excretion. The purpose of this study was to investigate the branchial urea transport mechanism hypothesized to be present in the rainbow trout through a combination of \textit{in vivo} and \textit{in vitro} techniques.

\textbf{Materials and Methods}

\textit{In vivo} studies were performed with fish that were surgically implanted with dorsal aortic catheters and allowed to recover for at least 24 hours. In Series \textit{i}, fish were infused with consecutive isoosmotic solutions of urea balanced with NaCl at rates of 0, 15, 60, 240 and 480 \(\mu\text{mol kg}^{-1} \text{ h}^{-1}\) in order to determine the branchial handling of urea in the face of exogenous urea loading. In Series \textit{ii}, fish were injected with enough acetamide or thiourea, two urea analogues, so that analogue concentrations were equal to endogenous urea concentrations in order to determine if the gill preferentially transported urea. \textit{In vitro} studies were performed by using the isolated basolateral membrane vesicle protocol as described by Perry and Flik (1988) where the basolateral membrane of the gill is isolated using differential centrifugation, and transport studies are performed using rapid filtration.

\textbf{Results and Discussion}

\textit{In vivo}, the trout gill effectively cleared the plasma of excess urea during exogenous urea loading, suggesting a role for a facilitated diffusion UT-type transport mechanism for urea. However, saturation of this mechanism was suggested when branchial excretion rate could not keep up with infusion rate (Fig. 1). This apparent saturation resulted in an elevation in plasma urea concentrations, however, observations were complicated by the lethality of the elevated internal urea levels (Fig. 2). In
order to further investigate the saturation kinetics of branchial urea transport in the trout, the isolated basolateral membrane vesicle (BLMV) technique was employed (Perry and Flik, 1988). Basolateral membrane vesicles of the gill (BLMV) demonstrated urea uptake with a saturable component ($K_m = 1.17 \text{ mmol l}^{-1}$; $V_{max} = 0.42 \text{ µmol mg protein}^{-1} \text{ h}^{-1}$) at urea concentrations $< 5 \text{ mmol l}^{-1}$.

Urea analogues, such as acetamide, thiourea and N-methylurea, are useful tools in identifying urea transport mechanisms as the specificity of a transporter leads it to preferentially transport urea over substances that are similar to it. In addition, analogues compete with urea in order to pass through the same transporter. Amongst teleosts where branchial UT mechanisms are present, a consistent pattern of urea and analogue handling is observed where acetamide clearance is 35-60% of urea clearance and thiourea clearance is only 16-19% (McDonald et al. 2000). This highly conserved pattern of differential urea and analogue handling was also observed in the rainbow trout where the ratio of analogue/urea branchial clearance was 48% for acetamide and only 22% for thiourea, strongly suggesting the presence of a UT-type diffusion mechanism. Using BLMV, the basolateral membrane alone was responsible for the differential handling of urea and acetamide, while urea and thiourea were handled similarly. In support of these findings, urea uptake by BLMV was significantly reduced by 73.2% in the presence of thiourea but was not affected by acetamide or N-methylurea.

The uptake of urea by BLMV was reduced by 62.5% when incubated with phloretin, a potent blocker of UT-type transport mechanisms giving strong evidence of carrier mediated diffusion of urea.

**Conclusions**

Thus, branchial excretion of urea in adult rainbow trout appears to be mediated by a UT-type facilitated diffusion transport mechanism.
Acknowledgements

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References


NITROGENOUS SOLUTES AS PROTEIN-STABILIZING OSMOLYTES: COUNTERACTING THE DESTABILIZING EFFECTS OF HYDROSTATIC PRESSURE IN DEEP-SEA FISH

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Abstract

Hydrostatic pressure can inhibit protein functions. Many deep-sea proteins have evolved resistance to this, but not all. Recently we have found unusual osmolyte compositions in deep-sea fishes and other animals. The protein-stabilizer trimethylamine N-oxide (TMAO), under 80 mmol/kg wet wt in most shallow teleosts, increases with depth in white muscles of several families of teleosts (up to 288 mmol/kg at 2900 m). Similarly, deep-sea skates have more TMAO (and less urea) than do shallow relatives. Here we report that red muscle of deep-sea macrourids contains 93-118 mmol/kg wet wt TMAO (less than white muscle, but more than shallow species) plus large amounts of a possible (but unidentified) methylamine. We hypothesized that methylamines counteract effects of pressure on proteins, and have tested TMAO and pressure with lactate dehydrogenase, pyruvate kinase, and actin, all pressure-sensitive proteins. 200-250 mM TMAO (but not glycine) protected ligand binding, protein stability, and polymerization against pressure inhibition. Here, to test TMAO in living cells, we grew yeast under pressure. After 1 hr at 71 MPa, 3.5 hr at 71 MPa, and 17 hr at 30 MPa, 150 mM TMAO (but not glycine) generally doubled the number of cells that formed colonies. These results support the hypothesis.
Introduction

Most marine animals osmoconform with seawater at about 1000 mOsm, but their cells do not have high salt levels. Rather, they prevent osmotic shrinking with intracellular organic osmolytes, which add up to about 600 mOsm to cells (the other 400 mOsm coming from basic inorganic and organic cell solutes). In invertebrates, organic osmolytes are neutral free amino acids (e.g., glycine, taurine), sometimes with lesser amounts of methylamines, especially glycine betaine and trimethylamine N-oxide (TMAO). In fishes, hagfish osmoconform using free amino acids, glycine betaine and TMAO, while cartilaginous fishes and coelacanths use urea, TMAO, and glycine betaine (Yancey 2001).

Except for urea, these solutes are probably used because, in contrast to common inorganic ions, they do not perturb proteins, and thus serve as "compatible" osmotic effectors (Brown and Simpson, 1972). But beyond this, many osmolytes—especially methylamines—enhance functional and structural properties of proteins such that they can offset negative effects of perturbants such as urea and NaCl. Such “counteracting” osmolytes have universal effects, working on proteins regardless of source, and are typically present at high levels only in cells subjected to protein perturbants. Well-studied marine examples are the elasmobranchs, with the protein destabilizer urea typically at 300-400 mM and TMAO at 150-200 mM in muscle cells (Yancey et al., 1982). At this ratio (about 2:1), urea’s inhibitory effects are often fully offset by TMAO’s stabilizing effects. Coelacanths have similar contents of these osmolytes. Another (non-marine) example is the mammalian kidney, where urea is the main nitrogenous waste, and methylamines (glycerophosphorylethanolamine, glycine betaine) are major cellular osmolytes. Like TMAO, renal methylamines can counteract urea’s effects on protein functions (Yancey, 1994, 2001).

In contrast to the common pattern of osmoconforming, teleosts are typically hypo-osmotic regulators. In most shallow marine teleosts, TMAO can be a major cellular solute, but at less than 80 mmol/kg (Hebard et al., 1982), leaving the animals hypo-osmotic—typically at about 350-400mOsm. An exception to this pattern are polar teleosts, some of which have glycerol up to 400 mM or TMAO up to about 150 mM (Raymond, 1994; Raymond and DeVries, 1998).

The above information is based on shallow-water organisms. Our recent studies have revealed striking differences in deep-sea animals, providing new insight into adaptation to this unique habitat. In particular, we discovered a linear increase in TMAO contents of white muscle with depth in several families of
teleosts (up to 288 mmol/kg wet wt in a morid cod from 2900m), as well as in some crustaceans and other invertebrates. In deep-sea skates, TMAO was also higher than in shallow elasmobranchs. In the osmoconformers, TMAO in effect replaced the major osmolytes of shallow species (glycine in shrimp, urea in skates). Interestingly, the urea:TMAO ratio in the deepest skates was about 1:2, a reversal of the typical shallow-water pattern (Gillett et al., 1997; Kelly and Yancey, 1999).

Our studies have focused on white muscle, the best studied tissue in terms of osmolytes in marine animals. Here we report on osmolytes in red muscles of macrourid teleosts, showing that they have high TMAO contents, but not as high as white muscle; additional osmotic pressure is due to other unusual osmolytes.

Why would methylamines be accumulated in the deep? The only environmental factor that is also linearly correlated with depth is hydrostatic pressure, which can inhibit protein folding and reaction kinetics. We hypothesized that TMAO (as a general protein stabilizer) might serve to counteract these effects (Gillett et al., 1997). Some deep-sea proteins have been selected to be less affected by high pressure than are homologues from shallow species (Siebenaller and Somero, 1989). But some deep-sea proteins exhibit significant pressure sensitivities and thus seem incompletely adapted. For cofactor (NADH) K_m, a lactate dehydrogenase (LDH) homologue from a deep-sea fish showed less pressure sensitivity than did a homologue from a shallower congener, but the deep homologue nevertheless had some sensitive (Siebenaller and Somero, 1978). We found that TMAO at appropriate levels can fully offset this inhibition, restoring K_m to the control (unpressurized) value (Gillett et al., 1997). Similarly, deep-sea LDH homologues had greater stability under prolonged pressure than did shallow-derived homologues, but the former still lost activity. They also had pressure-enhanced sensitivity to tryptic digestion, suggesting that subtle conformational changes occur under pressure. For all LDH homologues tested (from shallow and deep fish, and cow), TMAO but not glycine was able to counteract pressure destabilization (Yancey and Siebenaller, 1999).

We have also examined other deep-sea proteins that have sensitivity to pressure: actin in assembly of G- to F-form, and pyruvate kinase (PK) and its ADP K_m. Actin homologues in deep species are less sensitive, but not fully resistant to pressure (Swezey and Somero, 1982); deep homologues of PK show no pressure adaptation (Mustafa et al., 1971). We found that TMAO (but not glycine) partly or fully offset pressure effects on both systems (Yancey et al., 2001).
To test more directly the postulated protective effects of TMAO and the universality thereof, we chose a living model system—yeast cells grown under pressure. Other researchers have shown that yeast are protected from pressure by DMSO, D$_2$O (Komatsu et al., 1991), and trehalose (Iwahashi et al., 1998).

**Materials and Methods**

*Fish Muscle Osmolytes*

Deep-sea macrourid teleosts (*Coryphaenoides* species) were collected from 2900 m off Oregon and stored as previously described (Gillett et al., 1997). White and red myotomal muscles were analyzed for TMAO and other osmolytes by spectrophotometry and HPLC as previously described (Wolff et al., 1989; Kelly and Yancey, 1999). An unidentified solute detected by HPLC was further analyzed by $^1$H-NMR as previously described (Yin et al., 2000) and by standard ninhydrin procedure.

*Yeast*

Yeast (*Saccharomyces cerevisiae*) were tested according to Komatsu et al. (1991), with minor modifications. Cells were grown in medium containing 0.3% yeast extract, 0.3% malt extract, 0.5% peptone, and 1.0% glucose (YM medium); 2% agar was added to the YM medium for plates. Cultures were grown in test tubes (with culture caps) in a shaking water bath at 25°C; after suitable growth, cell densities were determined with a hemocytometer. To determine if TMAO had any effects on yeast growth under standard conditions at 1 atmosphere, cultures were grown at 0, 50, 100, 150, and 200 mM TMAO, then serially diluted and plated. TMAO up to 150 mM had no effect on number of successful colonies, but was inhibitory at 200 mM. For pressure tests, cells were grown for 24 hr in YM medium with or without 100 or 150 mM TMAO, collected by gentle centrifugation, and resuspended at $10^7$ cells $l^{-1}$ in medium with or without TMAO. 70 µL samples of each culture (in quadruplicate for each test condition) were sealed in airless polyethylene tubes, then incubated at atmospheric and high pressures at 25°C in water-filled high-pressure steel cylinders. After varying times, they were rapidly depressurized, released from the tubes, and serially diluted by factors of 10, 100 and 1000. 50 µL aliquots were plated in quadruplicate, and resulting colonies were counted after 2-3 days at 26°C in an incubator. 150 mM glycine was tested similarly.
Results

Red Muscle Osmolytes

In macrourids from 2900 m, red muscle differed somewhat from white. Unlike the latter, in which TMAO occurred at 170-183 mmol/kg wet wt, red muscle had 93-118 mmol kg/wet wt (Table 1) and 5-10 mmol/kg of scylo-inositol. Over half of the rest of the osmotic pressure came from a solute that eluted near glycine betaine on the HPLC, but which did not match any known carbohydrate, methylamine or amino acid standard. It reacted weakly with ninhydrin, consistent with a mono-methylamine (this and amino acids react with ninhydrin, while di- and trimethylamines do not). In the NMR, this solute had a large singlet peak at 2.73 ppm, in the range of N-methyl hydrogens on mono-methylamines (e.g., N-methyl hydrogens of sarcosine and methyltaurine are at 2.71 and 2.79 ppm, respectively) (Agar et al., 1991; Yin et al., 2000). Another singlet peak was at 3.37 ppm and was unidentifiable. This solute was not seen in muscles of shallow-water cods (Gadus macrocephalus; macrourid relatives) in previous research.

Table 1. Contents of TMAO and an unidentified possible methylamine in white and red myotomal muscles of Coryphaenoides species from 2900 m off Oregon. Means and s.d. values are in mmol/kg wet wt, from 3 to 5 specimens each. Contents of the unidentified solute were estimated by refractive index detection (HPLC) using a refractive index typical of other methylamines (Wolff et al., 1989).

<table>
<thead>
<tr>
<th>Species</th>
<th>TMAO</th>
<th>Unidentified (estimate)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>White</td>
<td>Red</td>
</tr>
<tr>
<td>C. armatus</td>
<td>179 ± 12</td>
<td>105 ± 21</td>
</tr>
<tr>
<td>C. filifer</td>
<td>170 ± 9</td>
<td>93 ± 16</td>
</tr>
<tr>
<td>C. leptolepis</td>
<td>183 ± 18</td>
<td>118 ± 9</td>
</tr>
</tbody>
</table>

Yeast

Several combinations of time and pressure were used on yeast cultures. First, we tested for concentration effects of TMAO. As shown in Fig. 1A, baroprotection did occur and was dependent on concentration: survival was considerably higher in 150 mM compared to 100 mM TMAO. 50 mM had no significant effect (not shown).

Second, we tested 150 mM TMAO for 1 hr at 71 MPa (700 atm), 3.5 hr at 71
MPa, and 17 hr at 30 MPa (300 atm). TMAO improved survival of cells after pressurization in all cases, although not significantly in the 1 hr test (Fig. 1B). 150 mM glycine gave no significant protection (not shown). These results are similar to previous studies on DMSO and D$_2$O, which also gave baroprotection (Komastu et al., 1991).

![Figure 1](image)

Figure 1. Effects of TMAO on yeast growth following pressurization for times and at MPa values indicated. A: Effects of 100 and 150 mM TMAO on growth after 3.5 hr at 71 MPa (700 atm). B: Effects of 150 mM TMAO on yeast grown under different pressure conditions and times. *significantly less than unpressurized controls; †significantly higher than pressurized samples without TMAO (p < 0.05).

**Discussion**

Our results with red muscle show that TMAO contents are higher in red muscle of deep-sea macrourids than in tissues of shallow-living teleosts, but not as high as in white muscle. However, scyllo-inositol and another methylated amine not present in white muscle—and not one of the common biological amines—makes up the rest of the osmotic pressure expected. Thus, again an unusual osmolyte composition occurs in another tissue in the deep sea.

Our results with living cells add to our previous results with proteins in support of our hypothesis: that high levels of TMAO in some deep-sea animals have been selected to offset inhibitory effects of high hydrostatic pressure.
Accumulation of TMAO could facilitate adaptation to the deep sea by protecting cellular functions and perhaps decreasing protein turnover, reducing the need to evolve pressure-resistant proteins. However, since the latter clearly have evolved in some cases, protein evolution is probably necessary since TMAO is unlikely to counteract all effects of pressure completely. Similarly, TMAO does not counteract all effects of urea on proteins (Yancey, 1994). We have speculated elsewhere on the possible mechanism of TMAO in counteracting pressure (Gillett et al., 1997; Yancey et al., 2001). Briefly, high hydrostatic pressure inhibits reactions with a positive volume change, which can result from the release of water molecules densely bound to some ligands and protein side groups (Siebenaller and Somero, 1989). Stabilizing osmolytes tend to disfavor the formation of bound water (hydration water) around ligands and proteins, thus favoring protein folding and ligand binding (which reduce hydration water compared to unfolded and unbound states) (Timasheff, 1992; Wang and Bolen, 1997). The effects of pressure and TMAO may simply oppose each other in an additive manner.

It should be noted that, while TMAO was better than glycine at pressure counteraction in our tests, other solutes can offset pressure effects. Trehalose, DMSO and D$_2$O have been shown to protect non-marine microorganism growth from pressure (Komatsu et al., 1991; Iwahashi et al., 1998), and sugars and polyols can protect non-marine proteins from pressure degradation (Athès et al., 1998; Ashie et al., 1999). Thus there may be other osmolytes in deep-sea organisms that counteract pressure. Indeed, not all deep-sea animals contain high levels of TMAO. We have found high levels of the following in other deep-sea taxa: (1) scylo-inositol in echinoderms (Yin et al., 1999); (2) this polyol plus glycine betaine and sarcosine (N-methylglycine) in gastropods, octopods, polychaetes and pycnogonids (Yin and Yancey, 2000); (3) N-methyltaurine and an unidentified methylated solute in vestimentiferans (Yin et al., 2000); and (4) a novel osmolyte made of serine, phosphate and ethanolamine in vesciomyid clams (from seeps in Pacific trenches) (Fiess et al., 2001). These solutes are markedly different from the common osmolytes of shallow invertebrates. They could relate to dietary and metabolic differences or to other environmental stresses such as sulfides and temperature, but may be adaptations to high pressure. The unidentified solute (if a methylamine) and/or scylo-inositol in red muscle of macrourids may be as well.

There are other hypotheses for the occurrence of high TMAO in marine animals in addition to counteraction, including diet and counteraction of NaCl (Kelly and Yancey, 1999). Another hypothesis concerns buoyancy: TMAO solutions are
less dense than seawater and solutions of other common osmolytes (Withers et al., 1994). However, this property would not explain why TMAO increases linearly with depth in shrimp, since shallow shrimp should benefit as much as deep shrimp from TMAO’s buoyancy properties (Kelly and Yancey, 1999). Another explanation concerns lipid metabolism. Seibel and Walsh (2002) have shown a correlation between acylglycerol metabolism and TMAO production, proposing that the latter arises as a byproduct in proportion to the former. However, even if this is correct, such metabolism and/or retention of TMAO could still be selected for in species that use its counteracting properties, so this proximate hypothesis is not mutually exclusive with an evolutionary one.

Acknowledgements

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References


dehydrogenases of congeneric fishes living at different depths. Science 201:255-257


OSMOREGULATION AND NITROGEN EXCRETION IN THE

EMBRYONIC LITTLE SKATE (Raja erinacea)

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EXTENDED ABSTRACT ONLY - DO NOT CITE

Abstract

Some adult elasmobranchs are able to adapt to dilute seawater environments by adjusting tissue levels and excretion of nitrogenous osmolytes. Embryonic elasmobranchs undergoing rapid development and feeding endogenously may not yet have developed the same osmoregulatory abilities as adults. We tested the hypothesis that embryonic skates (Raja erinacea) would differ in their osmoregulatory characteristics relative to adult skates. Under control conditions, the ratio of urea: TMAO and other organic osmolytes was 2.1:1 in 3 month embryos, 2.3:1 in 3 month yolk, and 2.5:1 in 8 month muscle tissue. Detectable levels of the ornithine urea cycle enzymes ornithine transcarbamoylase (OTCase), carbamoyl phosphate synthetase III (CPSase III), and arginase, as well as the accessory enzyme glutamine synthetase (GSase), were found in 3 month embryos and 8 month muscle tissue. When exposed to dilute 75% (25 ppt) seawater versus 100% seawater (33 ppt), skate embryos at 4 and 8 months significantly increased excretion of urea to the environment for up to 24 h after exposure. There was a significant decrease in tissue urea (-18%) and TMAO (-20%) levels and yolk urea (-17%) and TMAO (-17%) levels in 4 month embryos exposed to 75% seawater for 120h. These findings contradict our hypothesis and suggest that osmoregulatory mechanisms similar to adult skates are present at very early stages of development.
Introduction and Objectives

Marine elasmobranchs maintain high levels of organic osmolytes in their tissues to remain isoosmotic to their seawater environment. High tissue levels of urea, the predominant osmolyte in elasmobranch tissues, are maintained by production in the liver and retention mechanisms in the gill and kidney. Trimethylamine oxide (TMAO), generally the second most predominant osmolyte in elasmobranch tissues, is sometimes produced in the liver (often obtained in the diet) and is also reabsorbed in the kidney. Some elasmobranch species, including the little skate, can adapt to dilute seawater environments by down-regulating the production of urea and excreting more urea and other osmolytes to the environment (for review see Yancey, 2001). Embryonic elasmobranchs undergoing rapid development and feeding endogenously may not possess the same mechanisms as adults to produce and maintain osmolytes at the appropriate concentrations under dilute seawater conditions. The objectives of this study were to determine 1) if embryonic little skates have similar osmolyte concentrations and urea cycle enzyme activities as adults and 2) if they are able to down-regulate urea and TMAO tissue concentrations in dilute seawater environments.

Materials and Methods

A resident breeding colony of little skates were housed in the Hagen Aqualab, University of Guelph. Little skate embryos were removed from their egg cases and placed in individual chambers of either 12L of 100% (33 ppt) or 75% (25 ppt) artificial seawater. Water was aerated and changed every 48 h to prevent the accumulation of excreted wastes. Animals were kept on a 15h:9h light:dark photoperiod. Excretion rates of urea and ammonia were measured every 24 h for 5 days (120 hours), at which point the animals were weighed and tissues were isolated for later analyses of urea, ammonia, TMAO, and OUC enzymes. Urea in water and tissue samples was measured using the method of Rahamatullah and Boyle (1980). Tissue ammonia was measured using a Sigma diagnostic kit (170-C), and water ammonia was measured using the method of Ivancic and Deboogis (1984). Tissue TMAO levels were measured using the method of Weckell and Barnett (1991). OUC enzymes were measured using the methods described by Steele et al. (2001).

Results

Under control conditions, 4 month post-conception embryos had tissue levels of urea and TMAO of 330 and 106 mM respectively, while in the yolk levels are 231 and 71 mM. Levels of other osmolytes taken together
(e.g. sarcosine, betaine, and β-alanine) were 51mM in the tissue and 30 mM in the yolk. Muscle tissue of 8 month old embryos was found to contain 345 mM urea, 62 mM TMAO, and 76 mM of other organic osmolytes. Detectable levels of three OUC enzymes and one accessory enzyme were found in the embryonic tissue of 3 month and 8 month embryos (Table 1).

**Table 1**: Activities of ornithine urea cycle enzymes in the tissues of 3 and 8 month post-conception little skate (*Raja erinacea*) embryos. Units are umol/g wet weight/min for OTCase, GSase, and arginase, nmol/g wet weight/min for CPSase III. Values are mean ± SEM (n = 6)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>3 month embryo</th>
<th>8 month muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPSase III</td>
<td>17.02 ± 1.54</td>
<td>7.51 ± 1.26</td>
</tr>
<tr>
<td>OTCase</td>
<td>9.07 ± 1.10</td>
<td>10.20 ± 1.97</td>
</tr>
<tr>
<td>Arginase</td>
<td>3.93 ± 0.42</td>
<td>1.22 ± 0.17</td>
</tr>
<tr>
<td>GSase</td>
<td>1.68 ± 0.29</td>
<td>1.26 ± 0.42</td>
</tr>
</tbody>
</table>

Urea excretion was significantly increased at the onset of exposure to 75% seawater in both 4 (10.1 ± 1.3 umol N/g/h versus control, 1.0 ± 0.3 umol N/g/h) and 8 (0.5 ± 0.1 umol N/g/h versus control, 0.1 ± 0.01 umol N/g/h) month old embryos. Urea excretion remained significantly higher in 8 month old embryos after 24 h in 75% seawater, while values in 4 month old embryos returned to control levels at 24h. Ammonia excretion could not be detected in 4 month old embryos. In 8 month old embryos, ammonia excretion rates were approximately 0.15 umol N/g/h and did not differ between 75% and 100% seawater. Tissue levels of urea and TMAO were both decreased in 4 month old embryos exposed to dilute seawater (Fig 1).
Figure 1. Stacked tissue levels (mmol/kg + SEM) of urea and TMAO in the embryo and yolk of 4 month post conception embryos of the little skate.

Preliminary Conclusions

The data indicate that at as early as 3 months of development, little skate embryos express detectable levels of OUC enzyme activities that are approximately 5% of levels expressed in the adult liver. The ratio of urea to TMAO and other osmolytes in tissues and yolk were very similar to those reported in adults. Embryonic skates can also down regulate tissue levels of urea and TMAO in response to dilute seawater. This down regulation can be attributed, at least in part, to an increase in the rate of urea excretion to the environment.

References


THE FRESHWATER STINGRAY *HIMANTURA SIGNIFIER*

IS ABLE TO INCREASE UREA PRODUCTION
IN RESPONSE TO BRACKISH WATER (UP TO 20 PPT),
BUT APPARENTLY HAS A LIMITED CAPACITY
TO RETAIN UREA

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EXTENDED ABSTRACT ONLY-DO NOT CITE

The river Batang Hari originates from the Barisan Range, flows eastwards through the whole of Jambi, Indonesia, and drains into the South China Sea. *Himantura signifier* (Dasyatidae) is a stingray found in the Batang Hari basin in Jambi, Sumatra. It is believed to occur only in freshwater. In the laboratory, *H. signifier* could survive in freshwater (0.7 ppt) indefinitely or in brackish water (20 ppt) for at least two weeks. In freshwater, the blood plasma osmolality (416 mosmolal) was maintained hyperosmotic to that of the external medium (38 mosmolal). There was approximately 44 mM of urea in the plasma, with the rest of the osmolality made up mainly by Na+ (167 mM) and Cl− (164 mM). In freshwater, it was not completely ureotelic, excreting at most 45% of its nitrogenous waste as urea. It had a functional ornithine-urea cycle in the liver. The hepatic carbamoylphosphate synthetase III and glutamine synthetase activities were similar to those of other marine elasmobranchs.
When it was exposed to a progressive increase in salinity (0.7 ppt→ 5 ppt→ 10 ppt→ 15 ppt→ 20 ppt→ 20 ppt→ 20 ppt→ 20 ppt) through a 8-d period, there was a continuous decrease in the rate of ammonia excretion. After exposure to 20 ppt for 4 d, the ammonia excretion rate was only 1/5 that of the freshwater control. In 20 ppt water, there was no change in the ammonia content in the muscle and plasma, but a decrease was observed in the liver. Presumably, ammonia was used as a substrate for urea synthesis and storage for osmoregulation at higher salinities. Indeed, in 20 ppt water, urea levels in the muscle, brain and plasma increased significantly. In addition, certain free amino acids were used as intracellular osmolytes in the muscle (β-alanine, glycine and sarcosine) and the brain (β-alanine, glycine, glutamate and glutamine).

In the blood plasma, osmolality increased to 571 mosmolal, in which, urea, Na⁺ and Cl⁻ contributed 83, 231 and 220 mM, respectively. This was almost isoosmotic to the external medium (540 mosmolal). The total amount of urea accumulated in the tissues of the specimen exposed to 20 ppt water was equivalent to the deficit in ammonia excretion through the 8-d period, indirectly indicating an increase in the rate of urea synthesis at higher salinities would have occurred. However, no induction in the activity of carbamoylphosphate synthetase was observed. It is possible that the carbamoylphosphate synthetase activity was enhanced in vivo by an increase in the concentration of N-acetylglutamate which was not determined in this study. There was also a significance decrease in the rate of urea excretion during passage through 5, 10 and 15 ppt water. However, the rate of urea excretion increased back to the control value (3.5 μmol day⁻¹ g⁻¹) when the stingray reached 20 ppt water on the 5th day, presumably resulted from the steeper urea gradient built up between the plasma (83 mM) and the external medium (0 mM). In comparison, the local marine stingray, *Taeniura lyamma*, maintained a urea excretion rate of 4.7 μmol day⁻¹ g⁻¹ in full strength sea water (30 ppt), with a plasma urea concentration of 380 mM. Therefore, *H. signifer* appeared to have reduced its capacity to retain urea in order to survive in the freshwater environment. Consequently, it could not survive well in full strength sea water, although it was more euryhaline than the South American freshwater stingray, *Potamotrygon motoro*. Different from *P. motoro*, *H. signifer* retained the capacity to produce urea, as demonstrated by the capability of *H. signifer*, but not *P. motoro*, to detoxify ammonia to urea during ammonia loading.

In freshwater containing 10 mM NH₄Cl at pH 7, ammonia accumulated in the muscle, brain and plasma of *H. signifer*. The primary strategy adopted was to allow ammonia to build up internally, especially in the plasma, to slow down the
influx of exogenous ammonia. This was reflected by the unaltered urea excretion rate (3 \( \mu \text{mol day}^{-1} \text{ g}^{-1} \)) in specimens exposed to ammonia for the first day, during which ammonia excretion (7.3 \( \mu \text{mol day}^{-1} \text{ g}^{-1} \)) was presumably impeded totally. However, the urea excretion rate increased continuously to 7.4 \( \mu \text{mol day}^{-1} \text{ g}^{-1} \) by the 4th day of ammonia exposure, with no change to the muscle urea content, indicating that it was able to release the excess urea without creating a problem for osmoregulation.
EVOLUTION OF GLUTAMINE SYNTHETASE IN VERTEBRATES:
MULTIPLE GLUTAMINE SYNTHETASE GENES EXPRESSED IN
RAINBOW TROUT (ONCORHYNCHUS MYKISS)

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EXTENDED ABSTRACT ONLY – DO NOT CITE

Glutamine synthetase (GSase; L-glutamate:ammonia ligase (ADP forming);
E.C. 6.3.1.2) catalyses the ATP-dependent formation of glutamine from
ammonia and glutamate. In fish, GSase is a multifunctional enzyme. GSase is a
critical enzyme in detoxification of the highly mobile and toxic ammonia (for
review, see Ip et al. 2001). The enzyme is also key to the ‘fish type’ ornithine
urea cycle, with glutamine as the N-donor substrate for the initial step catalysed
by carbamoyl phosphate synthetase III (CPSase III) (for review, see Anderson
2001). The regulation of urea synthesis, at least in the facultatively ureogenic
marine toadfish (Opsanus beta), is upstream of the urea cycle and presently
attention is focused on GSase (e.g. Walsh et al. 1999). The objective of this
study was to isolate and characterize the GSase gene(s) in the rainbow trout,
Oncorhynchus mykiss.

Our data show direct evidence for four GSase genes in rainbow trout. Using
GSase specific primers, the polymerase chain reaction (PCR) was found to
amplify four expressed GSase sequences from a rainbow trout cDNA library
constructed from mRNA isolated from the combined gill and kidney tissues of
12 trout. In a separate experiment, three of the four sequences isolated above
were amplified via RT-PCR from a total RNA sample pooled from the isolates
of six rainbow trout alevin. For two of these sequences, we characterized the
full length coding regions (Onmy-GS01, GS02), and for two others, we described partial sequences, lacking only the 5' end of the coding sequence (Onmy-GS03, GS04).

A maximum likelihood radial phenogram, based on the alignment of CDS nucleotides, was constructed (Fig. 1). This phylogeny shows strong bootstrap support (>70%) for four monophyletic clades, one containing all mammalian and avian genes, a second containing all teleostean fish genes, a third containing *Xenopus* and a single *Fugu* (possibly pseudogene) sequence and a fourth containing the shark sequences. The relationship among these four clades is not as well supported (62%). Among the bony fish genes, the present phylogeny suggests that there are two rainbow trout lineages, an *Onmy-GS01/3* and an *Onmy-GS02/04* clade. These clades are highly divergent and do not form a monophyletic grouping suggesting that these two lineages arose prior to
speciation. We speculate that two functional GSase lineages were present in the common ancestor of all salmonids prior to the tetraploidization event that preceded the rise to the modern salmonids. We also report the existence of two distinct GSase genes in zebrafish. The relationship of the zebrafish genes is not well resolved in the phylogeny, but their duplication appears to be independent of the duplication of the two trout lineages.

The sequence similarity of Onmy-GS01 to Onmy-GS03 and Onmy-GS02 to Onmy-GS04 could either represent allelic diversity or alternatively reflect a relatively recent duplication event. To investigate these hypotheses, we conducted a sequence comparison of intron 4 variation amplified from a single fish. Four unique intron sequences, 146, 256, 120 and 113 bp corresponding to Onmy-GS01 – GS04, respectively, were found. Reflective of the maximum likelihood phylogeny, a dot plot analysis shows a slightly higher level of similarity between the Onmy-GS01/GS03 genes sequences and also between the Onmy-GS02/GS04 sequences. As expected, this similarity is most noticeable around the splice boundaries. For most of the remaining intron sequence no significant similarity exists, arguing against an allelic relationship between the sequence pairs. The analysis of the 3' UTR sequences agrees with the intron analysis. We conclude that the similarity within these gene sequence pairs reflects a recent gene duplication event. This predicts at least four GSase loci in trout. Consistent with this interpretation, a Southern blot of a single individual hybridised with a probe made up of both the Onmy-GS01 and Onmy-GS02 genes revealed five to seven bands in Hind III and Alu I digests, respectively.

The genetic distance estimates between the GSase gene pairs, Onmy-GS01/03 and Onmy-GS02/04 (0.0948 and 0.0825 substitutions/site, respectively), are greater than those observed between Mus and Rattus (0.0798, Fig. 1). Assuming a molecular clock for bony fish GSase genes, the Onmy-GS01/03 and Onmy-GS02/04 values are, therefore, consistent with the duplication of these loci during the ancestral tetraploidization event estimated to have occurred 25 - 100 mya (Allendorf and Thorgaard 1984).

From total RNA extracted from ten adult trout tissues the relative expression of Onmy-GS01 and Onmy-GS02 was studied using semi-quantitative PCR (Fig 2). This analysis shows 1) Onmy-GS02 is expressed at higher levels relative to Onmy-GS01 in most adult tissues, 2) the highest level of expression is in the brain, and 3) the largest difference between expression of the two genes is found in the intestine. Clearly, rainbow trout possess multiple GSase loci with differing levels of tissue expression, implying manifold potential routes of
regulation. Our data also indicate that caution should be taken when interpreting mRNA expression data of a single gene, unless multiple genes have been ruled out.

![Image of semi-quantitative PCR results]

**Fig. 2.** Semi-quantitative PCR of glutamine synthetase genes *Onmy-GS01* and *Onmy-GS02* in adult tissue. An equal amount of each amplification was run on a 1.5% agarose gel. The reactions are organized according to tissue sample, in groups of four. The order of samples is *Onmy-GS01* (01), 30 cycles and 25 cycles total, and *Onmy-GS02* (02), 30 cycles and 25 cycles total.

**References**


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EARLY DEVELOPMENTAL EXPRESSION OF TWO GLUTAMINE SYNTHETASE GENES IN RAINBOW TROUT  

(ONCORHYNCHUS MYKISS).

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EXTENDED ABSTRACT ONLY - DO NOT CITE

Introduction and Objectives

Most adult teleosts excrete nitrogenous wastes primarily as ammonia, mainly across the gills. Ammonia diffusion from the teleost embryo, however, may be hindered by the lack of gill ventilation and the presence of an unstirred boundary layer (Rahaman-Noronha et. al., 1996), resulting in a potentially toxic accumulation of this molecule (Wright et. al., 1995). Alternatively, ammonia may be converted to less toxic compounds, such as glutamate, glutamine or urea. Glutamine synthetase (GSase) catalyzes the conversion of glutamate and ammonia to glutamine, and in fish, functions in ammonia detoxification, and urea and pyrimidine synthesis (Ip et. al., 2001). Besides its roles in urea and pyrimidine synthesis, we propose that GSase plays a critical role in the detoxification of ammonia via storage in the glutamine molecule. Our study attempts to answer the following questions: 1. When are GSase genes first expressed and what is the pattern of expression during development and in different adult tissues? 2. How does the increase in expression of GSase genes correlate with activities and with increases in tissue ammonia and glutamine?
Recent work has revealed the presence of at least four GSase genes in rainbow trout (Murray et al., manuscript submitted). We measured the expression of two of these GSase genes, Onmy-GS01 and Onmy-GS02, in early life stages of rainbow trout, ranging from 3 - 80 days post fertilization (dPF), compared to expression in six rainbow trout adult tissues. As well, GSase enzyme activities, ammonia excretion rates, and ammonia concentrations were measured.

Materials & Methods

Rainbow trout embryos were purchased on the day of fertilization. Hatching occurred 26 - 30 dPF and total yolk absorption occurred at 50 dPF. To characterize the pattern of mRNA expression of Onmy-GS01 and Onmy-GS02 during early development and in adult tissues, we designed and constructed radiolabelled RNA probes for these transcripts for use in ribonuclease protection assay (RPA) (Ambion, Austin, Texas). Total RNA from six early developmental stages (3, 10, 21, 31, 60 and 80 dPF) and from six adult tissues (brain, spleen, white muscle, large intestine, skin and liver) was also extracted for use in the RPA. GSase enzyme assays for early developmental stages were conducted on whole animals, as described by Chadwick and Wright (1999). Ammonia excretion rates were measured over 3 h, at each of the 6 early developmental stages (Wright et al., 1995). Ammonia levels were measured using a Sigma diagnostic kit on frozen tissue. Ammonia concentrations were measured on either whole animals or separate yolk and tissue fractions.

Results

Early Developmental Stages

Expression of Onmy-GS01 mRNA during early developmental stages resembled one oscillation, with a peak at 21 dPF. The pattern of expression of Onmy-GS02 during early developmental stages also oscillated, with two peaks, one at 21 dPF and another at 60 dPF. Overall, Onmy-GS02 was expressed at a higher level than Onmy-GS01. GSase activities were observed at the first early developmental stage examined, rising steadily to a maximum value of 0.49 μmol/g/min at 80 dPF, with a significant increase in activity at 31 dPF. Ammonia concentrations in whole embryos increased gradually from 3 dPF (0.97 nmol N/g) to 21 dPF (1.29 nmol N/g). When embryos were dissected from yolk at 31 dPF, ammonia tissue levels were 1.11 nmol N/g, whereas yolk ammonia levels were significantly higher, at 2.47 nmol N/g. Ammonia excretion steadily rose during development to 0.67 μmol N/g/h at 80 dPF, with a significant increase observed at 21 dPF.
Adult Tissues

Overall, *Onmy-GS02* was more highly expressed in adult tissues than *Onmy-GS01*. Expression of both *Onmy-GS01* and *Onmy-GS-02* was higher in the brain of adult rainbow trout when compared with all other tissues examined. Adult tissue expression of *Onmy-GS01* was, in descending order of intensity, brain >> white muscle > skin > large intestine > spleen/liver, and of *Onmy-GS02*, brain > white muscle > large intestine > liver/skin > spleen. The largest difference in expression of these two GSase genes was in the brain. GSase activity was higher in the brain than any other adult tissue assayed (~200 times greater than the next highest tissue, the liver). After the brain, activities were next highest in the liver, followed by spleen > large intestine > skin > white muscle.

Summary & Conclusions

We have characterized the early pattern of expression of two GSase genes in rainbow trout, and the data indicate that *Onmy-GS01* and *Onmy-GS02* are differentially expressed during early development, as well as in adult tissues. Significant increases in both *Onmy-GS01* and *Onmy-GS02* mRNA expression at 21 dPF, coupled with increasing levels of GSase activities at 31 dPF (just after hatching) may reflect the importance of this enzyme, particularly in early developmental stages, in ammonia detoxification. Low levels of ammonia in embryonic tissue, but not in yolk, at 31 dPF support this proposal. GSase may be important in both glutamine storage and urea synthesis (via the O-UC) in the embryo (Wright et al., 1995). Further work on tissue glutamine and urea levels during development will be valuable.

References


Murray, B.W., Busby, E.R., Mommsen, T.P and P.A. Wright. Evolution of glutamine synthetase in vertebrates: Multiple glutamine synthetase genes expressed in rainbow trout (*Oncorhynchus mykiss*). (Manuscript submitted)

ORNITHINE-UREA CYCLE IN THE
BRAZILIAN CATFISH, PSEUDOPLATYSTOMA CORUSCANS
DURING EARLY-LIFE STAGES

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Introduction

To maintain the best physiological performance to face the toughness of environmental conditions the vertebrates selected the best strategies in the course of the evolutionary history. The outcome is the biological diversity as vast as the ensemble of molecular adaptations. The freshwater fishes in particular are usually exposed to many external changes and chemicals, as ammonia can be very stressing in some conditions. Internal ammonia is as harmful as the external one. Aquatic organisms can directly clear off this waste product from the special metabolism of nitrogenous substances. However, some environmental circumstances may impair that clearance. The main is the water availability but as much important as it is the concentration of $H^+$. Conversion of ammonia ($\text{NH}_3$) to the ammonium ($\text{NH}_4^+$) ion is a form to detoxify it. Moreover, the ammonium is usually rather excreted than ammonia. Any physiological condition accountable for the imbalance of the nitrogenous excretion forms should result in inappropriate cell medium and in the start of alternate steps to reestablish the equilibrium.

Growth and development bring a very special set of cell conditions, which impose a particular metabolic profile. Changes of nitrogenous metabolism during the fish embryonic development were early reported in rainbow trout Oncorhynchus mykiss, (Smith, 1947). While the fish dry weigh and the nitrogen excretion increased, the yolk content decreased. Recently, ammonia and urea
excretion were correlated to ornithine-urea cycle (Wright et al., 1995) during the rainbow trout development. The higher activities of OUC enzymes take place in the larval period, between the 53\textsuperscript{th} and 71\textsuperscript{st} day. The expression of the genes for such enzymes would reduce the toxic effects of ammonia from the plentiful protein metabolism of the embryo. Those genes are expressed during the development and some are silent in adult fish. The OUC enzymes were quite active during the embryonic and larval stages of the Atlantic cod Gadus morhua, but only arginase and glutamine synthase were kept very active (Chadwick & Wright, 1999) in adult fish. These should be expected for the most teleost species. However, those from warm waters present distinct physiological characteristics. The developmental period is very short compared to cold water fish. Such difference is positively related to the metabolic activity. Due to the faster processes concerning the ontogenesis we suppose the time of exposition to ammonia it be less injurious in tropical fish eggs and embryos than in cold water fishes, and this would imply that OUC enzymes are less active. We have studied the OUC enzyme expression in the Brazilian catfish “pintado” Pseudoplatystoma coruscans, in the whole developmental life stages.

Material and Methods

Eggs of P. coruscans were obtained from induced spawning. These experimental steps were performed at the Centro Nacional de Pesquisas Tropicais (CEPTA-IBAMA), Pirassununga, SP (21°59’46” S; 47°25’33” W). Two adult females and six adult males (15 ± 8 kg) were used. Eggs were fertilized at 24°C and incubated in well-aerated water in 100L fiberglass conical tanks, provided constant water renewing. Samples were collected by suction, transferred to special sieves, frozen at −10°C and lyophilized. The time interval of sampling are depicted in the table I. The OUC enzyme activity of lyophilized liver and kidney of adult fish were compared with eggs, embryos and larvae.

Biological samples (whole animal for eggs, embryos and larvae) were mechanically homogenized with a motor driven pestle into cold-water bath. Homogenates were done into 20mM glycine 10mM phosphate pH 7.0 in glycerol 1:1. Tissues, eggs and embryos were disrupted in the ratio 16.67mg per mL of homogenization buffer.
Table I.
Time interval of sampling *Pseudophytomma coruscans* during the early stages of life.

<table>
<thead>
<tr>
<th>Δt</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0h</td>
<td>---</td>
</tr>
<tr>
<td>2h</td>
<td>Morula</td>
</tr>
<tr>
<td>4h</td>
<td>Blastula</td>
</tr>
<tr>
<td>6h</td>
<td>Gastrula</td>
</tr>
<tr>
<td>8h</td>
<td>Blastopore</td>
</tr>
<tr>
<td>10h</td>
<td>---</td>
</tr>
<tr>
<td>12h</td>
<td>Neurula</td>
</tr>
<tr>
<td>14h</td>
<td>Embryo (no movements)</td>
</tr>
<tr>
<td>16h</td>
<td>Embryo (movements)</td>
</tr>
<tr>
<td>17h</td>
<td>Fast movements</td>
</tr>
<tr>
<td>17h:30</td>
<td>Hatching</td>
</tr>
<tr>
<td>23h:30</td>
<td>---</td>
</tr>
<tr>
<td>29h:30</td>
<td>---</td>
</tr>
<tr>
<td>35h:30</td>
<td>---</td>
</tr>
<tr>
<td>41h:30</td>
<td>Barbels and eyes</td>
</tr>
<tr>
<td>47h:30</td>
<td>Mouth</td>
</tr>
<tr>
<td>53h:30</td>
<td>Irregular opercular beats</td>
</tr>
<tr>
<td>59h:30</td>
<td>Fast swimming</td>
</tr>
<tr>
<td>65h:30</td>
<td>---</td>
</tr>
<tr>
<td>71h:30</td>
<td>---</td>
</tr>
<tr>
<td>77h:30</td>
<td>---</td>
</tr>
<tr>
<td>83h:30</td>
<td>Fins</td>
</tr>
<tr>
<td>89h:30</td>
<td>Feeding (Arthemia sp)</td>
</tr>
<tr>
<td>113h:30</td>
<td>Arthemias in the gut</td>
</tr>
<tr>
<td>Five-days from hatching</td>
<td>Larvae length 1.0 cm</td>
</tr>
<tr>
<td>12 day</td>
<td>Eating artificial feeding</td>
</tr>
<tr>
<td>19 day</td>
<td>Larvae length 2.5 cm</td>
</tr>
<tr>
<td>28 day</td>
<td>Larvae length 3.0 cm</td>
</tr>
</tbody>
</table>

The next OUC enzymes were assayed: ornithine carbamoyl transferase (OCT) as Nakamura & Jones (1970), arginase (ARG) as Rahmatullah & Boyde (1980) and carbamoyl-phosphate synthetase III (CPSIII) as Saha *et al.*,1997. Protein contents of the samples were determined by UV-photometry (Warburg & Christian, 1941).
Results

Ornithine carbamoyl transferase was present in embryos since the egg phase. The enzyme activity in the ovule was 0.426 nmol of citrulline/min/mg of protein, and it oscillates (Fig. 1-a) until the larval stage (17:30 min). From this moment to the 53h:30 min the activity increased eight times. At this phase the first opercular movements were observed. Again, the activity decreases until 113:30 min as the first arthemias were observed in the gut. From then on, the activity enhances to the maximum values observed (Fig 2-a). Arginase activity was present since the unfertilized egg with a sharp peak around 18 h (beginning of the larval phase) and decreased to the lowest level. From this phase, the arginase activity trend was of constant raise. The activity of carbamoyl phosphate synthetase was observed from the fourth hour of development. This enzyme activity increased, oscillating with large peaks since the eighth to the hundredth hour. From that moment, it was observed a constant decrease to near by zero at the third week of development.

Discussion

At the present time, the ureogenesis during the initial phases of fish development has being generally accepted. This concept comes from the set of data for cold water fish (Wright et al, 1995; Chadwick & Wright, 1999). Just a few are available for tropical freshwater fish (Monzani, 1999; Terjesen et al, 2001). The Brazilian catfish pintado express the OUC enzymes at the first steps of development. However, compared with trout and Atlantic cod some differences are observed. Arginase and OCT of pintado are active since the unfertilized egg. Protein metabolism from adult fish during mating phase should be active enough to transfer these metabolic features to the generation.

To understand the OCT activity in the embryos of pintado three relevant events must be considered: the hatch, the beginning of opercular beats and the exogenous feeding. From the hatch to the opercular beats the OCT of pintado increased ten times. The hatch efforts should increase the metabolic demands and the absence of the gill apparatus during this period (Rombough, 1988) impairs the waste of ammonia propitiating the urea synthesis. The metabolic requirements at the opercular-beating phase also demand muscle work, and the consequent breakage of adenilates resulting in large amounts of ammonia. Similar biochemical response is also observed in adult fish submitted to exercise (Wright et al, 1988). Therefore, is reasonable to think that the increase of urea synthesis in this life phase of pintado occurs to detoxify ammonia. Decrease of
OCT is remarkable from the 53 h, precisely the moment of the beginning of the opercular beats. That means the gill started to work, probably in addition to ammonia excretion. Moreover, the yolk sack was practically reabsorbed. From the 113 h the enzyme activity of OCT raises constantly. That moment matches to a new event in the life cycle of pintado, it starts to feed on artemias. The large income of protein is likely the cause of such enzyme response. Correlation between OCT and CPS was observed particularly before the hatching.

Carbamoyl phosphate synthetase III must work in consonance with OCT as a substrate supplier. This enzyme is strictly related to urea synthesis and the presence in white muscle of many teleosts (Korte et al 97; Felskie et al. 1998; Chadwick & Wright 1999) reinforce the assumption of many fishes are ureogenic. However, under circumstances where ammonia toxicity does not threat the organism, the spare of ureogenesis is expected. This should not be the case of many developmental phases (Felskie et al, 1998). Like OCT ability to express the arginase activity seems to be bequeathed from the parental cell.

Arginase is a key enzyme of the intermediary metabolism. It is directly involved in the protein metabolism, in the synthesis of creatinine, nitric oxide, polyamines and proline/glutamate (Jenkinson et al, 1996). The enzyme product of arginase is urea and ornithine. This amino acid is the precursor of polyamines (putrecine, spermine and spermidine), which are related to cell division and differentiation (Tabor & Tabor, 1984). In spite of the arginase activity and the developmental phases of pintado seem to be not straightly related its activity affords ornithine in the course of the growth and differentiation processes. Furthermore, the increase of arginase follows the food intake and larger growth of the larvae.

Comparison of warm and cold-water fish concerning the expression of OUC enzymes in the course of ontogenesis depicted the same pattern, and the specific enzyme activities are also similar. The time of residence of ammonia within the embryos is likely less important in the expression of OUC enzymes than are many other cell development mechanisms. The fishes’ ancestors probably bequeathed the OUC enzymes and the species did not lose them in the course of history. They are silenced until new circumstances demand the use.

References


BOTH *PERIOPTHALMODON SCHLOSSERI* (MUDSKIPPER) AND *CHANNA ASIATIA* (SNAKEHEAD) ACCUMULATE ALANINE DURING AERIAL EXPOSURE, BUT ONLY *P. SCHLOSSERI* CAN SUSTAIN LOCOMOTORY ACTIVITY ON LAND THROUGH PARTIAL AMINO ACID CATABOLISM

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**EXTENDED ABSTRACT ONLY-DO NOT CITE**

*Periophthalmodon schlosseri* is a mudskipper, which can be suffocated when submerged in water and is very active on land. When it was exposed to terrestrial conditions under a 12 h:12 h light:dark regime, the fish could be very active, and levels of total free amino acids increased significantly in the muscle and plasma. Alanine level increased three-fold in the muscle, four-fold in the liver, and two-fold in the plasma. From these results, we concluded that *P. schlosseri* was capable of partial catabolism of certain amino acids to support activity on land. The amino groups of these amino acids were transferred directly or indirectly to pyruvate to form alanine. The resulting carbon chain was fed into the Krebs cycle and partially oxidized to malate, which could replenish pyruvate through the function of malic enzyme. This favourable ATP yield from partial amino acid catabolism was not accompanied by a net release
of ammonia. Such an adaptation would be advantageous to *P. schlosseri* confronted with the problem of ammonia excretion during aerial exposure.

Indeed, when *P. schlosseri* were forced to exercise on land after 24 h of aerial exposure, the alanine level in the muscle increased significantly, with no apparent change in glycogen content. In addition, there was no significant change in the ATP level and energy charge of the muscle. In contrast, when *Boleophtalmus. boddaerti*, another species of mudskipper, were exercised on land glycogen levels in the muscles decreased significantly, and lactate levels increased. In addition, muscle energy charge was not maintained and the ATP level decreased significantly. Hence, it can be concluded, that when *P. schlosseri* were active on land, they were capable of using certain amino acids as a metabolic fuel and avoided ammonia toxicity through partial amino acid catabolism. Such a strategy is the most cost-effective way of slowing down internal ammonia build-up without involving energy expensive ammonia detoxification pathways. Furthermore, an examination on the balance between nitrogenous excretion and accumulation in a 70 g *P. schlosseri* revealed that degradation of amino acids in general was likely to be suppressed to slow down the build-up of ammonia internally.

The fresh water snakehead, *Channa asiatica*, is an obligatory air-breather that resides in slow-flowing streams and in crevices near riverbanks. In its natural habitat, it may encounter bouts of aerial exposure during the dry seasons, but it cannot maintain activity on land like *P. schlosseri*. In the laboratory, the ammonia excretion rate of *C. asiatica* exposed to terrestrial conditions in a 12 h:12 h dark:light regime was ¼ that of the submerged control. Consequently, the ammonia contents in the muscle, liver and plasma increased significantly. Alanine increased 4-fold to 12.6 µmol.g⁻¹ in the muscle after 48 h of aerial exposure. The accumulated alanine could account for 70% of the deficit in ammonia excretion during this period, indicating that partial amino acid catabolism would have occurred. This would allow the utilization of certain amino acids as energy sources and, at the same time, minimize ammonia accumulation. There was a reduction in the aminating activity of glutamate dehydrogenase from the muscle and liver of specimens exposed to terrestrial conditions, presumably facilitating the entry of α-ketoglutarate into the Krebs cycle.

However, *C. asiatica* was unable to reduce the rates of proteolysis and amino acid catabolism as in mudskippers. The reduction in nitrogenous excretion during 48 h of aerial exposure was completely balanced by nitrogenous
accumulation in the tissues. Exercise on land led to a decrease in glycogen content with no significant effect on the ammonia and alanine contents in the muscle of *C. asiatica*. Hence, unlike the mudskipper *P. schlosseri*, *C. asiatica* was incapable of increasing the rate of partial amino acid catabolism to sustain locomotory activities on land. Taken altogether, it can be concluded that alanine accumulation through partial amino acid catabolism may be widely adopted by obligatory air-breathing fishes to avoid ammonia intoxication during aerial exposure, but not all of them can use it to fuel muscular activities.