UREA SYNTHESIS/EXCRETION AND AMMONIA TOXICITY IN THE GULF TOADFISH (*OPSANUS BETA*): BASIC AND APPLIED ASPECTS

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The gulf toadfish, *Opsanus beta*, (Fam. Batrachoididae), distributed along the southeastern U.S. through the Gulf of Mexico, becomes ureogenic and ureotelic (>90% of nitrogen excreted as urea) in the laboratory in response to moderate stresses, including crowding/confinement, and ammonia exposure (reviewed by Walsh, 1997). The shift to ureotely also involves the activation of a highly pulsatile facilitated-diffusion urea excretion mechanism at the gills, with >90% of urea being voided in 1 or 2 brief (1-3h) pulses per day (Wood et al., 1998). It is not clear what is the ecological significance of this response, although several hypotheses have been outlined by Walsh (1997) and Hopkins et al. (1997), including nitrogen conservation, chemical crypsis, and habitat ammonia exposure.

The switch to ureogenesis/ureotely involves at least two key biochemical adjustments. First, ammonia excretion is depressed, and thus relative rates of ureogenesis by the ornithine-urea cycle (O-UC) are enhanced, by activation of the liver cytoplasmic ammonia-trapping and nitrogen-feeder enzyme, glutamine synthetase (GSase). Transient increases of the stress hormone, cortisol, are important in triggering GSase activation. Once activated, ureogenesis continues at a relatively constant rate leading to buildup of urea in the body. The second key biochemical event is the activation of a branchial urea transport system involved in pulsatile excretion. Molecular, physiological and morphological evidence is accumulating that a protein similar to the arginine vasopressin- (AVP) activated mammalian renal urea transport protein (UTA-2) is involved in

urea secretion by the toadfish gill. Urea excretion is localized specifically to the gill region (Gilmour et al., 1998; Laurent, Wood, Perry, Gilmour, Part and Walsh, unpublished data) and can be activated by injection of arginine vasotocin (AVT), the piscine analogue of AVP (Perry et al, 1998). Furthermore, a toadfish gill cDNA (1.8 kb) has been cloned and sequenced and shown to have an open reading frame for a protein (525 aa) with high sequence homology (> 60%) to UTA-2 from other vertbrates (Walsh, Heitz, Medina, Wood and Smith, unpublished).

Preliminary studies have shown that gulf toadfish have an exceptionally high tolerance to water ammonia. The present study was undertaken to determine if the ability to make and excrete urea was responsible for this high ammonia tolerance. A comparative toxicological approach was taken, comparing *O. beta* with other members of the family believed to be less ureotelic than *O. beta* based on measurements of O-UC enzyme activities (Anderson and Walsh, 1995).

First, the degree of ureogenesis/ureotely was assessed in two other common members of the family, the oyster toadfish (*Opsanus tau*) occuring along the northeast coast of North America, and the plainfin midshipman (*Porichthys notatus*) occuring along the northwest coast of North America. *P. notatus* was confirmed as a "typical" ammoniotelic teleost species; following confinement/crowding it excreted very little of its nitrogenous waste as urea (<15%), with no urea pulses observed. *O. tau* appears to be intermediate between *O. beta* and *P. notatus* in that it can excrete up to 70% of its waste nitrogen as urea following confinement. However, the switch to ureotely appears to take longer than in *O. beta*. Urea excretion is also pulsatile in this species. Next, the three species were subjected to a broad range of toxic NH_4Cl water concentrations to determine 96h LC_{50} values.

Table 1. 96h LC_{50} values for three species of fishes from the Fam. Batrachoidae.

Species	LC_{50} value (mM) ($\pm$ 95% C.I. where given)	%Ureotely
O. beta	9.75 (8.95/10.61)	>90
O. tau	19.72 (17.60/21.83)	30-70
P. notatus	6.00	<15

All three species exhibited LC_{50} values much higher than those reported for

teleosts examined to date (Table 1). While there was some correlation between degree of ureotely and LC_{50} values, given the exceptionally high tolerance of *P. notatus* relative to other teleosts, there is clearly some component of high ammonia tolerance in the members of this family not associated with ureotely.

Further studies revealed one potential biochemical basis for this adaptation. All three species were subjected to sublethal NH_4Cl exposure (3 mM), and a variety of physiological and biochemical parameters were measured. First, we confirmed that ammonia did enter the plasma and tissues (muscle, brain and liver) to levels which nearly matched water levels. Second, no differences of the activities of glutamate dehydrogenase (Gdh) and glutamine synthetase (GSase) were observed for ammonia-exposed vs control fish. However, 96h LC_{50} values correlated well with brain GSase activities (Table 2).

Table 2. Brain GSase activities (umol/min/g tissue) for three species of fishes from the Fam. Batrachoidae.

Species	Brain GSase Activity
O. beta	109.52 $\pm$ 2.67
O. tau	158.25 $\pm$ 3.79
P. notatus	59.41 $\pm$ 2.30

Our results indicate that brain GSase activity may be an important component of high ammonia tolerance. However, given that other teleosts express brain GSase activities approaching the range of the batrachoidid fishes, other adaptations (e.g., neurochemical) may also be important. These results will also be discussed in the context of aquaculture methodology.

Acknowledgements

Research supported by NSF (IBN9507239) and NSERC Canada. Studies on *P. notatus* were carried out at the Bamfield Marine Station; we gratefully acknowledge the assistance of the BMS staff.

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UREA METABOLISM AND EXCRETION

IN THE LAKE MAGADI TILAPIA ,

OREOCHROMIS ALCALICUS GRAHAMI

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Introduction

The unusual tilapia (*Oreochromis alcalicus grahami*) thrives in the geothermal springs and lagoons of Lake Magadi, Kenya. Arguably, this is the most extreme aquatic environment in which fish life has ever been recorded. Typical water chemistry at Fish Springs Lagoon, our standard collection site, is: pH 10, total CO_2 ($CO_3^{=}$ + HCO_3^{-}) 180 mM, titration alkalinity > 300 mM and osmolality 525 mOsm. Temperatures are up to $42^{O}C$, with an O_2 regime which fluctuates between extreme night-time hypoxia and extreme day-time hyperoxia, due to respiration and photosynthesis of abundant cyanobacteria, the principal food source of the fish.

Previous expeditions have discovered that these fish employ unique strategies of adaptation. For example, it would be difficult or impossible for

the fish to maintain a large enough P_{NH3} gradient across the gills to excrete ammonia into this highly alkaline, well-buffered lakewater. The problem is solved by 100% ureotelism, achieved through full expression of all enzymes of the Krebs ornithine-urea cycle (OUC) in the liver (Randall *et al.*, 1989; Walsh *et al.,* 1993). Prior to this discovery it had been believed that the genes for the OUC were silent or deleted in teleost fish. Other examples have been found, such as the gulf toadfish, *Opsanus* beta (reviewed by Walsh, 1997), but the Magadi tilapia remains the only 100% ureotelic teleost, excreting urea-N at extremely high rates, typically 3000 - 4000 µmol-N. $kg^{-1}.h^{-1}$, but producing no ammonia and no uric acid (Wood *et al.*, 1989).

The high urea-N excretion is attributable to the extremely high metabolic rate (Narahara *et al.*, 1996), mammalian-level body temperature, and the cyanobacterial diet which is rich in N. Very probably, the major route of urea excretion is via the gills, because divided chamber experiments demonstrated that about 80% of urea-N excretion occurred through the head region of the fish (Wood *et al.*, 1994).

Urea Metabolism

The substrates for urea production are

- ammonia, which is supplied to carbamoylphosphate synthetase III either directly or in the form of glutamine via glutamine synthetase, and
- bicarbonate ("base");

thus ureagenesis serves the added benefit of base removal for a fish which is being continually threatened with alkalosis from its carbonate/bicarbonate-rich environment. However ureagenesis appears to be normally saturated with bicarbonate, and therefore unresponsive to further base-loading (Wood *et al.*, 1994). In contrast, it is extremely sensitive to N-loading and therefore poised to detoxify ammonia. Exposure to high environmental ammonia causes an immediate 3-fold stimulation of urea production (Wood *et al.*, 1989). Classic toxicity tests demonstrate that the 48h LC50 (expressed as NH_3 concentration) is at least 5-fold higher than in standard ammoniotelic teleosts.(Walsh *et al.*, 1993). This high ammonia tolerance is likely of real environmental importance, as we have recently found in lagoon areas where NH_3 concentrations are as high as half the LC50 levels because of the presence of extensive deposits of flamingo guano.

Our most recent expedition to Lake Magadi (Jan-Feb., 1997) has cast additional light on urea metabolism in *O. alcalicus grahami*. For example, we have found that urea serves as an osmolyte, responding rapidly to osmotic challenge. When fish were abruptly transferred to 200% lakewater, whole body urea concentration increased 3.5-fold within 5 h. When transferred to 10% lakewater, urea concentration decreased by 35% within 5 h. (Table 1). On a relative basis, these changes were larger and more rapid than those occurring in internal Na^+ and Cl^- levels**.** Fish were also sequentially adapted, over a 2 week period, to progressive dilutions of lakewater ending in 1% (essentially circumneutral freshwater). Metabolic rate, as indicated by O_2 consumption, declined, but urea excretion continued in direct proportion to metabolic rate, and ammonia excretion did not occur. These results indicate that urea production is obligatory rather than facultative in the Magadi tilapia.

Table 1. Whole body urea concentrations (means $\pm$ 1 SEM, N = 6-15) in Magadi tilapia after various periods of exposure to 200% and 10% lakewater. Control fish were maintained in 100% lakewater for the same period. Asterisks indicate significant difference ($P \leq 0.05$) from simultaneous 100% lakewater value.

Time	**100%**	**10%**	**200%**
Pre-exposure	7.75 $\pm$ 0.55	-	-
5 h	8.47 $\pm$ 0.54	4.92 $\pm$ 0.56*	27.26$\pm$0.83*
15 h	5.95 $\pm$ 1.10	5.82 $\pm$ 1.08	25.83 $\pm$ 2.31*
240 h	5.86 $\pm$ 0.43	5.07 $\pm$ 0.79	31.36 $\pm$ 3.05*

Urea Excretion

In as much as plasma urea-N levels are only about 2-4 fold higher than those of standard teleosts, the rate at which urea passes through the gills is exceptional. Fig. 1 compares estimated gill urea permeability values of the Magadi tilapia with that of other fish. Gill urea permeability in *O. alcalicus grahami* is approximately 40-fold greater than in most standard teleosts, which have a permeability of about 10-20 x 10^{-7} cm.sec^{-1}. This is close to the value expected for diffusion through a lipid bilayer (Walsh, 1997). Ureotelic elasmobranchs, which retain very high levels of urea in the body fluids by unknown mechanisms, have values 1-2 orders of magnitude lower than standard teleosts. Recently, physiological, pharmacological, and molecular

evidence has been reported for the presence of a facilitated diffusion urea transporter in the gills of the gulf toadfish similar to that which is present in the mammalian kidney (Walsh, 1997; Wood *et al.*, 1998). In the toadfish, the transporter is periodically activated in a pulsatile fashion to achieve short-lasting bursts of urea excretion at a high rate. Surprisingly, gill urea permeability in the Magadi tilapia is almost 4-fold higher than that for toadfish with full activation of the transport system (Fig. 1). This observation suggests the presence of some mechanism for accelerating urea excretion. We therefore investigated whether a facilitated diffusion transport system is present in the gills of the Magadi tilapia., comparable to that in the gulf toadfish.

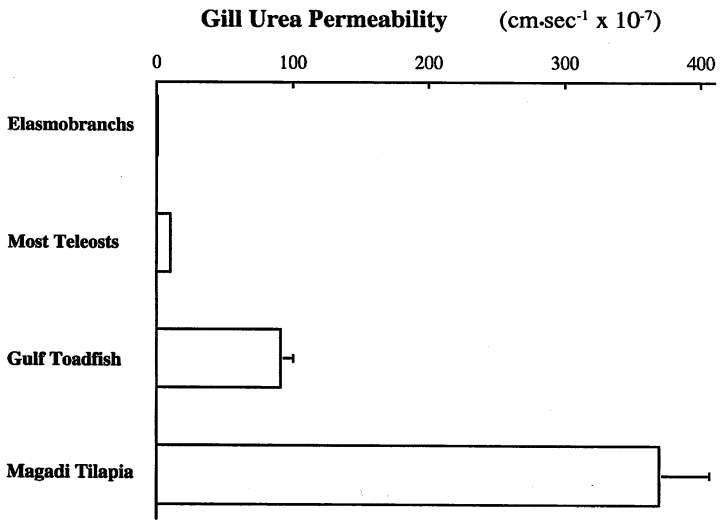

Figure 1. A comparison of gill urea permeability in the Magadi tilapia with that in the gulf toadfish (with urea transporter fully activated; from Wood *et al.*, 1998) and typical ammoniotelic teleost fish and ureotelic elasmobranch fish.

Fine time scale measurements revealed that urea excretion was continuous, not pulsatile. We compared the permeability of urea with that of the analogue thiourea, because facilitated diffusion carriers generally transport thiourea at a low rate relative to urea, and are competitively blocked by high

levels of thiourea. Thiourea permeability was linearly correlated with urea permeability in individual fish ($r = 0.89$, $P < 0.001$), but averaged only 0.19 of urea permeability. This ratio was identical to that seen in the gulf toadfish during periods of full activation of its facilitated diffusion transporter, and was substantially different from the ratio of about 1.0 seen in gulf toadfish when the urea transporter is turned off (Wood *et al.*, 1998). A ratio of about 1.0 is expected for simple diffusion of these two molecules of similar size.

In the toadfish, as well as the rat kidney, the urea transporter is bidirectional, and can be made to transport urea into the fish by experimentally raising urea levels in the outside medium higher than than those in the bloodstream. This experiment was performed on Magadi tilapia, with water total urea concentration raised to 20 mM (*versus* 5-10 mM in the blood plasma), and using the movement of [^{14}C] urea to measure unidirectional urea influx into the fish. Unidirectional influx was pronounced, approximately 7000 μmol-N. $kg^{-1}.h^{-1}$, and was significantly inhibited by the presence of three different urea analogues in the external water at a concentration of 60 mM. Acetamide inhibited urea influx by 15%, n-methylurea by 29%, and thiourea by 41%, whereas the addition of 30 mM NaCl (as a control for the elevation of osmotic pressure) had no effect.

These results indicate that a bidirectional facilitated diffusion transporter is present and continuously activated in the gills of the Magadi tilapia, a conclusion supported by histological evidence.

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A REVIEW OF AMMONIA TRANSPORT

IN THE MAMMALIAN NEPHRON

AND ITS REGULATION BY GLUCOCORTICOIDS

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Sites of net ammonia transport in nephrons:

The three most important are proximal tubules (PT), medullary thick ascending limbs (MTAL) and medullary collecting ducts (MCD). In those segments, as well as in the rest of the nephron, ammonia can cross the epithelium in several different ways. Both NH_3 and NH_4^+ can cross epithelia by diffusing through cell-cell junctions, NH_3 can diffuse through the lipid matrix of membranes, and NH_4^+ crosses membranes via several different transporters and channels. After a brief description of junctional permeability and NH_3 diffusion, the main focus of this review will be NH_4^+ transport via cell membrane transporters and channels.

Diffusion across junctions and the membrane lipid matrix:

Junctional permeability to NH_3 and NH_4^+, as well as NH_3 permeation through the lipid matrix have been reviewed recently (Knepper *et al.*, 1989: Good, 1994). Net diffusion of NH_3 and NH_4^+ through cell-cell junctions can be driven by transepithelial gradients of concentration, pH or charge. In nephron segments with relatively "loose" junctions, such as the proximal convoluted tubule, junctional permeability to both NH_3 and NH_4^+ can be significant. Other segments with "tighter" junctions such as the MTAL and the CD establish charge and pH gradients by active ion transport which can drive substantial junctional NH_4^+ or NH_3 diffusion. Careful measurements have shown that NH_3 permeation through the membrane lipid bilayer is less than might be predicted from the low molecular weight and high aqueous

diffusion rate of NH_3. In artificial lipid membranes NH_3 permeability is an order of magnitude lower than CO_2. NH_3 permeability of nephron membranes averages about 0.35×10^{-2} cm/sec, about an order of magnitude lower than that of artificial lipid membranes (Good, 1994).

NH_4^+ transport via carrier proteins and ion channels:

To date no membrane channel or transport protein which exclusively moves NH_4^+ has been identified in vertebrates. NH_4^+ crosses cell membranes by sharing transport proteins with K^+ and Na^+ and by diffusing through K^+ channels. Experiments by a number of investigators focused on different nephron segments provide solid evidence for NH_4^+ substitution for K^+on Na^+/K^+-ATPase, the apical membrane $Na^+/K^+/2Cl^-$ co-transporter (BSC-1), and for K^+ diffusion through apical K^+ channels (ROMK). In renal proximal tubules it has been shown that NH_4^+ can substitute for either Na^+ or H^+ on the apical Na^+/H^+ exchanger (NHE-3).

Proximal tubule NH_4^+ transport: Glucocorticoid effects.

In the PT ammonia is generated by glutamine-dependent ammonia production which is stimulated by glucocorticoids. Adrenalectomy reduces ammonia excretion during chronic metabolic acidosis in rats by about 50% and glucocorticoid replacement returns ammonia excretion to normal levels. NH_4^+ is transferred across the apical membrane into tubular fluid by NHE-3, on which NH_4^+ substitutes for H^+ to enter the tubule lumen. Glucocorticoid stimulation of NHE-3 has been demonstrated *in vivo* (Baum & Quigley, 1993).

Medullary thick ascending limb NH_4^+ transport:

In the MTAL NH_4^+ is absorbed across the apical membrane by secondary active transport via BSC-1 with NH_4^+ substituting for K^+ (Good, 1994). In recent experiments we (Kim *et al.*, 1998) have demonstrated that Na^+ loading, but not acid loading, increased protein expression levels of BSC-1 and Na^+/K^+-ATPase. Acid loading caused significant increases in expression of NHE-3. Transport pathways for exit of NH_4^+ across MTAL basolateral membranes into interstitial fluid are not yet well characterized. I am unaware of any direct studies of effects of glucocorticoids on MTAL

NH_4^+ transport.

Collecting Duct NH_3 and NH_4^+ transport: Glucocorticoid effects.

MTAL NH_4^+ reabsorption establishes a cortico-medullary NH_4^+ gradient which we have shown intensifies during acid loading. NH_3 is transferred from the medullary interstitial fluid across the CD into final urine. This NH_3 transfer is dependent on acidification of urine by apical H^+ ATPase and/or H^+/K^+ ATPase on intercalated and IMCD cells.

Proton pumping in α intercalated cell is regulated by aldosterone, however, glucocorticoids bind to the same receptor with an affinity equal to aldosterone. Circulating glucocorticoid levels are two orders of magnitude higher than aldosterone but the receptors are protected from glucocorticoid binding by the enzyme 11 -HSD2 which oxidizes glucocorticoids, deactivating them. We have demonstrated that acid loading depresses collecting duct 11 -HSD2 activity (Nolan *et al.*, 1996). In recent experiments we treated rats with carbenoxolone (CBX) which inhibits 11 -HSD2. For two days male rats (217-238g) were fed powdered rat chow plus 6.2 mmoles of NaCl. On the third day rats were injected with vehicle (n=6) or CBX (50mg/kg; n=6). They were then fed as on the first two days and urine was collected on ice for the next 6 hours. Blood pH was unaffected by treatment. The following table shows effects of treatment on urinary excretion. All values are means±s.e.m.

	Urine volume		**Urinary ion excretion- mEq/g creatinine**		
Treatment	Urine pH	ml/100 g/hr	Sodium	Potassium	Ammonium
Control	7.33±0.09	0.52±0.05	790±132	441±68	40±6
CBX	6.49±0.08*	0.17±0.04*	131±18*	341±48	76±11*

* denotes significant differences: *t* test- $p < 0.02$.

Creatinine excretion was reduced in CBX treated animals to about ½ that of controls. CBX caused urine acidification, increased NH_4^+ excretion and caused a 6-fold decrease in sodium excretion but did not increase potassium excretion. We hypothesize that those effects were mediated by

glucocorticoids stimulating H^+ secretion but at this point our evidence is indirect.

Generally, it has been hypothesized that low pH in the tubule lumen maintains a diffusion gradient for NH_3 from interstitial fluid into the tubule lumen and that NH_3 simply diffuses across collecting duct cell membranes. In light of recent experiments which show that NH_4^+ may cross basolateral membranes of IMCD cells by substituting for K^+ on Na^+/K^+ ATPase (Wall, 1996), this NH_3 diffusion model might require modification.

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AMMONIA EXCRETION IN THE

MUDSKIPPER, *PERIOPHTHALMODON SCHLOSSERI*

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EXTENDED ABSTRACT ONLY DO NOT CITE

The mudskipper, *Periophthalmodon schlosseri*, can survive air exposure for seven days, but drowns if denied access to air. This fish can tolerate ammonia concentrations of over 100mM NH_4Cl in an external environment of 50% seawater (Peng et al., 1998). Fish exposed to 10 mM NH_4Cl in 50% seawater showed no increase in tissue ammonia concentration after 24 hrs of exposure (Table 1), and only when exposed to 100mM NH_4Cl (446uM NH_3) for 6 days was there an accumulation of ammonia in muscle, liver, brain and plasma. An increase in seawater pH will elevate the NH_3 levels in seawater, even so increases in water pH up to 9.0 had little effect on either ammonia excretion rates or plasma ammonia levels in fish exposed to approximately 2 mM NH_4Cl. Animals exposed to 8 mM NH_4Cl in 50% seawater at pH 9.0, however, all died within 24 hrs. There was no change in ammonia excretion over 24 hrs in the face of high external ammonia (8mM and 30mM NH_4Cl at pH 7.2). When exposed to 10 mM NH_4Cl there was no significant change in ammonia excretion compared to when there is no ammonia in the seawater.(Table 1). The transepithelial potential was 10.3 mV (positive inside) in fish in zero ammonia and decreased to 6.78 mV in fish exposed to 100 mM NH_4Cl in 50% seawater at pH 7.2

Condition (pH)	Ammonia Excretion Rate (μmoles $NH_3 \cdot h^{-1} \cdot g^{-1}$ fish)	Plasma NH_3 Concentration (μmoles $NH_3 \cdot ml^{-1}$ plasma)
Control (pH 7.2)	0.473 ± 0.020	0.221 ± 0.039
pH 7.2	0.497 ± 0.094	0.254 ± 0.009
pH 8.0	0.477 ± 0.038	0.229 ± 0.047
pH 8.5	0.452 ± 0.041	0.172 ± 0.028
pH 9.0	0.479 ± 0.045	0.266 ± 0.018

Table 1. Total ammonia excreted by, and ammonia concentration in the plasma of the mudskipper *Periophthalmidon schlosseri* exposed to 50% seawater with 10mM Tris and ~ 2 mM NH4Cl at pH 7.2, pH 8.0, pH 8.5, and pH 9.0. Results represent means ± SE (n=4). Fish kept for 24 hours in Tris-50% SW with no added ammonia served as controls.

Na^+/K^+-ATPase activity in the gills of *P. schlosseri* was significantly higher than that in *B. boddaerti* (Table 2). NH_4^+, at physiological levels, could substitute for K^+ and Na^+/NH_4^+ ATPase activity was found to be the same as Na^+/K^+ ATPase activity in the gills of both fish. In addition, Na^+/ NH_4^+ ATPase activity was inhibited by ouabain (Table 2). Ouabain inhibited ammonia excretion in *P. schlosseri* held in 50% seawater containing 2 mM NH_4Cl. The inhibition was apparent only after 3 hrs exposure to 0.01 mM ouabain, but within an hr after exposure to 0.1 mM ouabain. Exposure to 2 mM NH_4Cl and 0.1 mM ouabain in 50% seawater for 3 hrs resulted in a significant accumulation of ammonia in the plasma. This concentration of ouabain did not inhibit ammonia excretion when there was no ammonia added to the seawater, presumably because the animal could excrete ammonia by diffusion as NH_3.

ATPase	P. Schlosseri	B. boddaerti
Mg^{2+}	0.42± 0.05	2.051.56± 0.18[a]
Na^+, K^+	0.25± 0.32 (undetectable)	0.88± 0.08[a] (undetectable)
Na^+, NH_4^+	2.05 ± 0.30 (0.10 ± 0.01)	0.69 ± 0.06[a] (0.13 ± 0.07)

Table 2. Specific activities (μmol inorganic phosphate/20 min per mg protein ± SE, n=4) of adenosine triphosphatase (ATPase) activated by Mg^{2+}, Na^+ + K^+ or Na^+ + NH_4^+ from the gills of *Periophthalmodon schlosseri* and *Boleophthalmus boddaerti*. Values in brackets are specific activities following addition of 1 mM ouabain. [a] Significantly different from the corresponding value of *P. schlosseri*

Ammonia excretion was reduced when 0.1 mM amiloride was added to the 50% seawater but 100mM KNO_3 or KCl had no effect on ammonia excretion. NaCl accumulated in the plasma following 6 days exposure to external ammonia concentrations of either 8 mM NH_4Cl or 100 mM NH_4Cl. The increase in plasma NaCl was significantly larger in fish exposed to the higher external ammonia concentration. Inhibition of ammonia excretion and accumulation of ammonia in the plasma with the addition of ouabain to the seawater indicates that Na^+/K^+ ATPase is involved in the branchial excretion of ammonia. Without this pump there is no mechanism in place to remove the ammonia from the blood, resulting in its accumulation. Addition of amiloride to the seawater also resulted in the inhibition of ammonia excretion indicating that a Na^+/H^+ (NH_4^+) exchanger is involved in ammonia excretion.

In further support of Na^+/NH_4^+ exchange and Na^+/NH_4^+ ATPase being major transporters in branchial ammonia excretion during high environmental ammonia levels, there is an increased accumulation of Na+ in the plasma of *P. schlosseri* with an increase in the ammonia concentration of the seawater. Since KNO_3 had no effect on ammonia excretion it is highly probable that V-ATPases have little involvement in ammonia excretion.

In conclusion, it appears that *P. schlosseri* is able to tolerate high environmental ammonia concentrations by actively excreting ammonium ions across the gills, thereby maintaining low tissue ammonia concentrations.

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SODIUM AND AMMONIA TRANSPORT MECHANISMS

IN ACIDOPHILIC AMAZONIAN FISH

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Introduction

Teleost fish are ammoniotelic and excrete the majority of this ammonia from the gills. How ammonia is transferred across the gill epithelium has been the subject of much debate for over 50 years. In freshwater teleosts two mechanisms of branchial ammonia excretion have received the most attention : 1) passive diffusion of NH_3 down a partial pressure gradient from blood to water, and 2) a linkage with Na^+ uptake via an amiloride-sensitive, electroneutral Na^+/NH_4^+ exchanger located on the apical membrane of gill epithelial cells. However, recent experiments with freshwater rainbow trout suggest that all ammonia excreted may be accounted for by diffusion of NH_3, with no evidence in support of Na^+/NH_4^+ exchange (Wilson *et al*., 1994). Indeed, the currently favoured model for active Na^+ transport involves uptake *via* an apical, amiloride-sensitive Na^+ channel (rather than antiporter) driven by an electro-chemical gradient created by apical proton pumps rather than ammonia excretion (Avella and Bornancin, 1989; Lin and Randall, 1995).

Amazonian fish of the Rio Negro such as the neon tetra inhabit extremely ion-poor and acidic water (Na^+ <10 μM, pH 3.8-4.9; Furch, 1974). Despite the scarcity of external Na^+, and the apparent inability of fish gill proton pumps to function below pH 5.5 (Lin and Randall, 1995), these fish exhibit normal rates of Na^+ uptake for freshwater fish. Under these conditions Na^+/NH_4^+ exchange would be potentially advantageous, *i.e.* using the excretion of endogenously derived NH_4^+ ions as a driving force for Na^+

uptake. In the present study we have therefore investigated whether the current views on mechanisms for ammonia excretion and Na^+ uptake in freshwater fish also apply to the neon tetra, a species adapted to an ion-poor and acidic environment.

Methods

Neon tetras (*Paracheirodon innesi*) were acclimated to artificial soft water (pH 6.5 [Na^+] ~50, [Ca^{2+}] ~50 μmol l^{-1}, 25°C). Fish were not fed for 48 h prior to experiments. The day prior to starting an experiment, fish were transferred to 40 ml flux chambers, 2 fish per chamber, 8 chambers in total, and left overnight to allow metabolic rates and ion fluxes to stabilise. Each chamber was supplied with soft water and aeration during this time.

Each experiment consisted of 3 flux periods of 1 h duration run in succession (control, experimental and recovery). At the beginning of each flux period water flow to chambers was turned off but aeration maintained. In some experiments ^{22}Na was added to each chamber (0.001 μCi/ml) to assess unidirectional Na^+ fluxes. Water samples were taken at the beginning and end of each flux and the chambers were flushed with fresh soft water for 30 min between each flux period. Ammonia excretion, net and unidirectional Na^+ fluxes were calculated by measuring the differences in [total ammonia], [Na] and ^{22}Na in samples taken at the beginning and end of each flux.

To examine the potential mechanisms for ammonia excretion and Na^+ uptake fish were acutely exposed to the following changes in water chemistry during the experimental flux period :

1. *Low and high pH* (nominally 4 and 8). This was to manipulate NH_3 gradients across the gills. Hepes buffer (0.5 mM) was added to maintain the higher pH (8). Without Hepes present atmospheric CO_2 rapidly acidified the artificial softwater due its extremely low buffer capacity.

2. *Low and high external [Na^+]* - (nominally 0 and 150 μM). This was designed to manipulate the Na^+ available for Na^+/NH_4^+ exchange.

3. *Na^+ transport inhibitors* - Amiloride (10^{-4} M) was added to the flux chamber water to inhibit any apical Na^+/NH_4^+ exchange or Na^+ channels. Ouabain (10^{-4} M) was added to the flux chamber water

to look for the possibility of apical Na^+/K^+-ATPase as a strategy for Na^+ uptake in extremely dilute environments.

All values for ammonia excretion and Na^+ flux rates have units of nmol g^{-1} h^{-1}, and are expressed as means ± SEM (n=8). Differences between means were tested using a Student's paired t-test ($P < 0.05$), each fish as its own control, and Bonferroni correction for multiple comparisons.

Results

Effect of water pH

Exposure to low pH (4.14 ± 0.01) significantly and reversibly increased ammonia excretion by 25%, but had no effect on unidirectional Na^+ fluxes (Fig. 1). A change in water pH from 6.8 to 7.9 (buffered with Hepes) caused a 12% reduction in ammonia excretion (Fig. 1). Hepes buffer had no effect when the pH was maintained at the same level as during the control period (6.8).

Manipulation of external [Na^+]

Exposure to low external [Na^+] (11 ± 1 μM) had no effect on the ammonia excretion rate, but reduced Na^+ influx and efflux by 66 and 37%, respectively (Fig. 2). Ammonia excretion was also unaffected by a tripling of the external [Na^+] (150 ± 14 μM; data not shown). The magnitude of the reduction in Na^+ uptake during exposure to low external [Na^+] indicated an extremely high affinity for Na^+ (K_m = 10-20 μM).

Na^+ transport inhibitors

Neither amiloride nor ouabain at 10^{-4} M had any effect on Na^+ fluxes when added to the external medium (data not shown). Ammonia excretion was also unaffected by amiloride (data not shown). However, ammonia excretion could not be measured in the presence of ouabain due to interference with the ammonia assay.

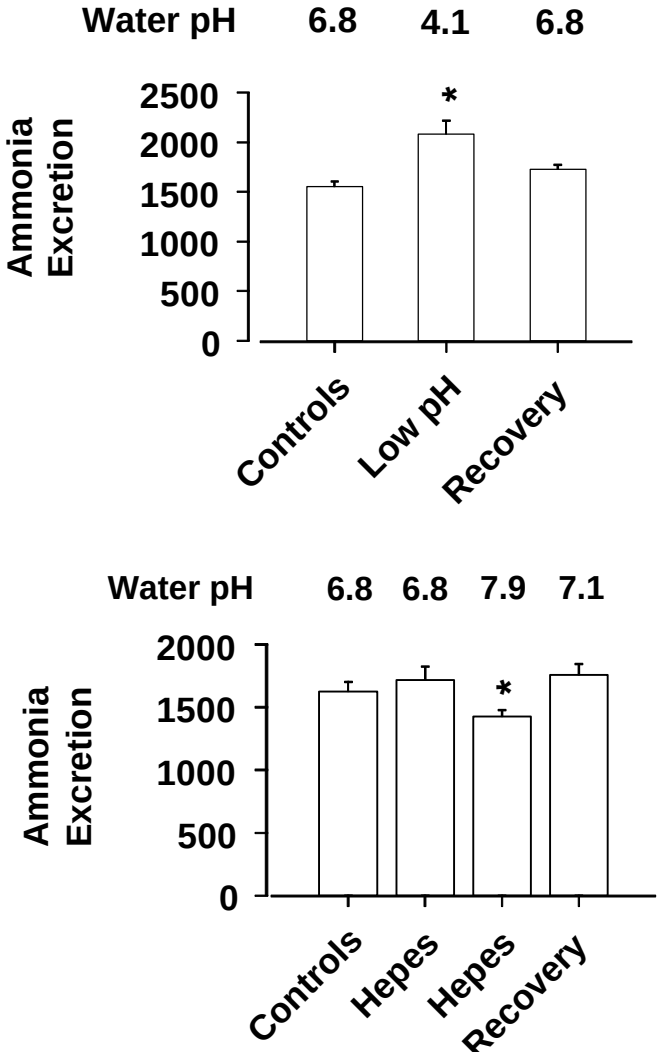

Fig. 1 - The effect of exposure to water of low pH (upper panel) and high pH (lower panel) on ammonia excretion in neon tetras. See text for extra details.

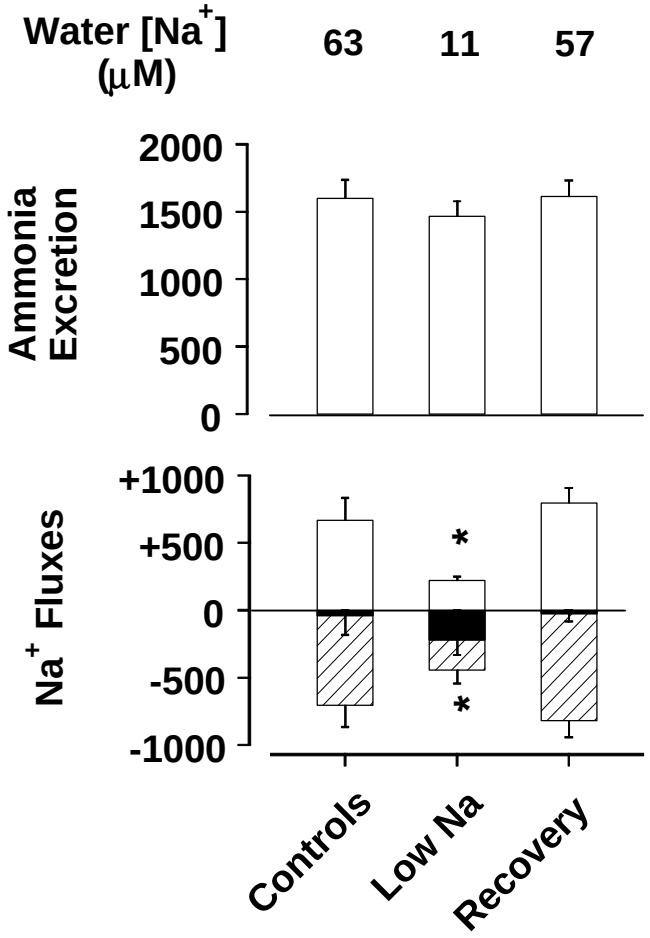

Fig. 2 - The effect of exposure to a very low external Na^+ concentration on ammonia excretion and unidirectional Na+ fluxes in neon tetras.

Discussion

Passive NH_3 diffusion and gill boundary layer acidification

During manipulation of external pH the changes in ammonia excretion (Fig. 1) followed the predicted changes in the NH_3 partial pressure gradient across the gills (low pH reduces PNH_3 in the water, high pH increases it). These results therefore qualitatively support a role for the passive diffusion of NH_3 driving a significant portion of ammonia excretion in neon tetras. A precise quantitative assessment is not possible as blood PNH_3 is currently unknown, so true transbranchial PNH_3 gradients could not be calculated.

It is well established that when inspired water is > pH 6, CO_2 and/or H^+ excretion across the gills create a relatively acidic boundary layer adjacent to the gill surface. Under normal conditions this localised acidification facilitates ammonia excretion by lowering the PNH_3 in the boundary layer thus enhancing the transbranchial PNH_3 gradient (Wilson *et al.*, 1994). The fact that ammonia excretion in neon tetras was unaffected by buffering the boundary layer to pH 6.8 (Fig. 5) suggests that boundary layer acidification either does not occur, or plays little role in facilitating ammonia excretion in these fish.

Do neon tetras use Na^+/NH_4^+ exchange ?

Despite the potential for using outwardly directed NH_4^+ gradients to drive Na^+ uptake, there was no dependence of ammonia excretion on Na^+ uptake (Fig. 2). Thus there is no evidence for coupled Na^+/NH_4^+ exchange as a route for ammonia excretion and Na^+ uptake in the gills of neon tetras. It is also worth noting that exposure to low external [Na^+] induced simultaneous reductions in both Na^+ uptake and efflux (Fig. 2). The reduction in Na^+ efflux indicates rapid compensatory control of the paracellular permeability in response to inhibtion of Na^+ uptake. The fact that ammonia excretion remained unaffected under these conditions indicates that ammonia excretion is therefore a predominantly transcellular process.

Mechanism of Na^+ uptake

Na^+ uptake was insensitive to external acidification to pH 4 (Fig. 1). Under these conditions the H^+ gradient across the apical membrane of gill cells should be sufficient to prevent functioning of either proton pumps or Na^+/H^+ exchangers (Lin and Randall, 1995). In addition, all current models for Na^+

uptake in freshwater animals incorporate an amiloride-sensitive apical component (either Na^+ channels or a Na^+/H^+ antiporter). It was therefore surprising to observe that Na^+ uptake in neon tetras was totally insensitive to 10^{-4} M amiloride, a concentration which reduces Na^+ uptake by 80-100% in other freshwater teleosts (Wilson *et al.*, 1994). The possibility that Na^+ uptake is achieved by sodium pumps (Na^+/K^+-ATPase) located on both apical and basolateral membranes can be discounted due to the lack of inhibition in the presence of 10^{-4} M external ouabain.

In summary, there was no evidence for a linkage (direct or indirect) between ammonia excretion and Na^+ uptake in neon tetras. A qualitative assessment suggests a major role for passive diffusion of NH_3, as in trout, and that this is predominantly transcellular. However, ammonia excretion was surprisingly unaffected by boundary layer acidification, suggesting that somewhat different processes for NH_3 transfer may operate in the gills of these acidophilic fish. In addition, the impressive insensitivity of Na^+ uptake to low pH is similar to, although more extreme than, that of another Amazonian blackwater fish the blackskirt tetra (Gonzalez et al., 1997). This together with the lack of effect of traditional Na^+ uptake inhibitors such as amiloride, suggest that further study of these acidophilic fishes could potentially reveal novel mechanisms of ion transport.

Acknowledgements

This work was funded by a grant from the University of Exeter Research Fund.

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TISSUE AMMONIA LEVELS AND SWIMMING PERFORMANCE OF BROWN TROUT EXPOSED TO COPPER IN SOFT ACIDIC WATER

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Background

Exposure to sub-lethal concentrations of copper in soft acidic water significantly reduced sustained swimming performance (Ucrit) of brown trout, *Salmo trutta* (Beaumont *et al*. 1995a). Such a reduction in Ucrit had previously been attributed to disruption of oxygen transfer at the gill or a decrease in the oxygen carrying capacity of the blood. However, despite considerable gill damage resulting in a 2-4 times expansion of the blood/water diffusion barrier (Taylor *et al*. 1996), there was little indication from measured blood parameters of a limit to oxygen uptake. Indeed, the oxygen content of arterial blood was unchanged or even elevated following exposure to copper in soft acidic water (Beaumont *et al*. 1995a).

A consistent effect of exposure to copper in acidic water was the elevation of plasma total ammonia, Tamm (Beaumont *et al*., 1995b). Ammonia and ammonium ions have a number of possible physiological effects that may directly influence swimming performance (see Beaumont *et al*. 1995b). It may interfere with central or peripheral nervous integration, transmission at the neuromuscular junction, excitation/contraction coupling or muscle electrophysiology. Alternatively ammonia may affect the metabolic status of the muscle as a regulator of certain enzymatic pathways. In addition, while neither oxygen uptake nor carrying capacity were limiting, oxygen transport to the tissues may have still been disrupted. Osmotic loss of plasma water and an increase in number and size of red cells could elevate blood viscosity

subsequrntly reducing oxygen transport to the tissues and local circulation through the peripheral capillaries.

The current study.

Both local hypoxia and elevated ammonia may result in alterations to the metabolic organisation of the swimming musculature and the data presented in this paper are preliminary findings from our continuing investigation of the effects of copper exposure in soft acidic water upon adult brown trout. Fish were exposed for 96 hours to 0.08 µmol l^{-1} Cu^{2+} at pH 5 and 10°C before being either swum to Ucrit or left at rest. A control group was treated in a similar manner except the pH of the water was left at 7 and no copper was added. Arterial blood samples were taken from the dorsal aorta *via* chronically indwelling cannulae and tissue samples rapidly freeze clamped for analysis of metabolite concentrations.

Just as at 5 and at 15°C, swimming performance was significantly reduced in the copper/acid exposed fish (Ucrit 1.11 ± 0.2 bl s^{-1}) compared to the untreated controls (Ucrit 2.02 ± 0.1 bl s^{-1}). Similarly, as in those previous studies, there was seemingly no effect of this sub-lethal level of copper/acid upon the ability of fish to extract oxygen from the water, both arterial oxygen partial pressure and content were unaffected.

Tissue metabolic status

There were no significant changes in glycogen, ATP or CrP concentration in the red muscle of resting copper exposed trout and only glycogen was depleted in white muscle. Lactate concentration was unaltered in either tissue at rest and while white muscle lactate increased significantly with exercise in control animals, it did not do so in those that had been expsed to copper at low pH. Significantly, whilst in our earlier study (Beaumont *et al*., 1995a) the haematological data was somewhat equivocal, the results from the current study are not. No changes in Hct, [Hb] or plasma protein concentration occurred to indicate increased blood viscosity. Moreover, Gallaugher *et al*. (1995) have now shown that, despite a positive relationship between Hct and viscosity, polycythemia up to 55% Hct does not reduce Ucrit but rather increases it. Oxygen supply to the exercising muscles would appear to not be a problem.

Ammonia distribution and muscle electrical properties

Plasma Tamm rose with copper and acid exposure and this was mirrored by rises in both red and white muscle. We currently do not have an explanation for this increase in plasma ammonia. It may simply be due to a large increase in its production, perhaps as part of a cortisol mediated stress response, but why this potentially toxic load is not excreted is unclear, particularly when conditions (low 'bulk water' pH and [Tamm]) are apparently ideal for outward diffusion of NH_3 across the gill.

In control trout at rest, the ratio of ammonia concentration between tissue and extracellular fluid ([Tamm]i/[Tamm]e) was 28.4 ± 6.1 and 33.6 ± 7.8 for white and red muscles respectively. These high values agree with many other observations for white muscle and suggest that the ratio of relative membrane permeability for NH_3 and NH_4^+ in both tissues is relatively low and hence that the distribution of ammonia is a function of muscle membrane potential (E_m). Indeed, predictions of the [Tamm]i/[Tamm]e ratio using the hypothesis of non-ionic diffusion fall far short of the measured ratio, whilst those calculated from the Nernst equation (with E_m -85 mV) are close to it.

Reversing this analysis and assuming that membrane permeability to NH_3 and NH_4^+, or at least the ratio of permeabilities, remain unchanged by copper/low pH exposure, the calculated E_m of red and white muscle from trout at rest after exposure to copper in low pH were -66.3 ± 3.7 and -57.5 ± 3.2 mV respectively. If this analysis is correct, both muscles are significantly depolarised with respect to values calculated for control fish at rest (-83.2 ± 5.9, -79.4 ± 5.5 mV).

There are a number of assumptions underlying this analysis however and the use of arterial blood in estimating the Tamm ratio may introduce significant error (Wang *et al*., 1996). However, we have been able to subsequently measure K^+, Na^+ and Cl^- concentrations in white muscle samples from some of the fish and estimates of E_m using the Goldman-Hodgkin-Katz equation also indicate that this muscle is depolarised after copper/acid exposure. In addition, preliminary data from experiments to measure E_m directly using microelectrodes are revealing depolarisation of similar magnitude to these estimates. Heald (1975) demonstrated the functional significance of such a depolarisation in rat skeletal muscle with a reduction in tension as fibres became electically inexcitable.

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DEVELOPMENTAL ASPECTS

OF NITROGENOUS WASTE METABOLISM

IN THE SEA LAMPREY (*PETROMYZON MARINUS)*

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Introduction

Sea lampreys typically spend the first 3-7 years of their life cycle as sedentary filter-feeding larvae (ammocoetes) that live burrowed in stream substrate. The larval phase is followed by a complex metamorphosis into more active, freely swimming parasitic lampreys which migrate downstream to the sea or to freshwater lakes where they primarily feed on the blood of teleosts (see Youson, 1994 for recent review) . Based on these shifts in activity level, habitat, and food preferences, we hypothesized that patterns of nitrogenous waste (N-waste) production and excretion reflect the sea lamprey's stage of development.

In the last several years our knowledge of nitrogenous waste production and excretion in teleosts has increased substantially (see Wright, 1995; Wilkie, 1997 for recent reviews). In most fishes, ammonia is the predominate waste product with urea comprising about 10-20 percent of the total nitrogenous

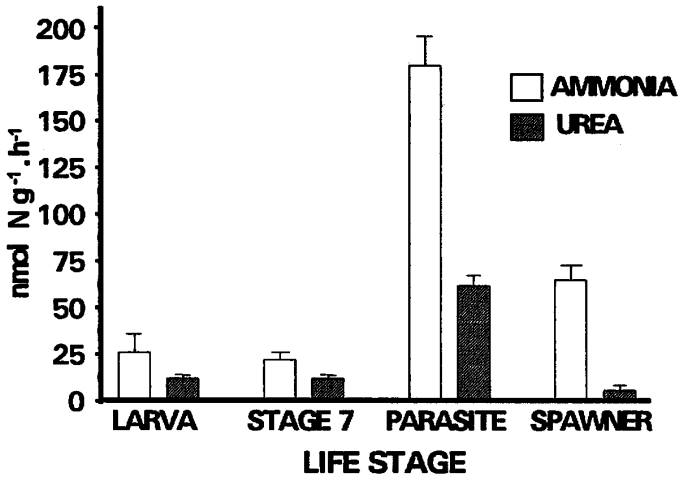
wastes ($J^{N\text{-waste}}$) excreted. Only a few studies have examined N-waste production and excretion in the jawless fishes (Superclass: Agnatha), and these suggest that this group lacks the ability to produce physiologically relevant amounts of urea. The work by Read (1968) demonstrated that urea excretion only comprised about 1 percent of $J^{N\text{-waste}}$ in adult Pacific lamprey (*Entosphenus tridentatus*). In the present study, the patterns of N-waste excretion and production were examined in larval, transforming (stage 7), parasitic and spawning lampreys collected from the Great Lakes watershed. In the case of larval and parasitic sea lampreys, food was withheld in the 10 d preceding experimentation to ensure that basal excretion rates were measured; transforming and spawning lamprey do not feed.

Results & Discussion

Basal $J^{N\text{-waste}}$, the sum of ammonia plus urea excretion, in larval and transforming lamprey was about 50 nmol $N \cdot g^{-1} \cdot h^{-1}$, but $J^{N\text{-waste}}$ was 6-fold greater in the parasites (Fig. 1). The greater $J^{N\text{-waste}}$ in the parasites is likely related to their higher metabolic rate which accompanies a more active life style. The greater $J^{N\text{-waste}}$ in the parasites was accompanied by comparably greater glutamate dehydrogenase (GDH) activity, which was approximately 25 $\mu mol \cdot min^{-1} \cdot g^{-1}$ liver tissue compared to about 4.0 $\mu mol \cdot min^{-1} \cdot g^{-1}$ liver tissue in larval and spawning lampreys. We suggest that the greater GDH activity in the parasites enhances their ability to deaminate excess amino acids, at a time when the animals are likely to be ingesting high amounts of protein rich blood and soft tissue from fishes. The 70 percent lower $J^{N\text{-waste}}$ (Fig. 1) and GDH activity in spawning lampreys supports this hypothesis.

When some fish are subject to conditions that impede ammonia excretion (e.g alkaline lakes, air exposure), urea may be the predominate N-waste product (e.g. Randall *et al.*, 1989; Saha and Ratha, 1989). In such fishes, urea may be produced via the ornithine urea cycle (OUC) but in most ammoniotelic fishes, the degradation of excess purines via uricolysis accounts for the majority of urea production (Wright, 1995). In spawning Pacific lampreys, which excrete very little urea, the key enzymes of the OUC are absent, and to date the full complement of uricolytic enzymes have not been detected in lampreys (Florkin and Duchâteau, 1945).

Figure 1: Ammonia & Urea Excretion at Different Life Stages of the Sea Lamprey.



The present study demonstrates for the first time that Agnathans do excrete significant quantities of urea. In larval, transforming and parasitic lampreys, urea excretion comprised about 10-30 percent of $J^{N\text{-waste}}$ (Fig. 1). Moreover, urea production appears to be via uricolysis since the key enzymes, uricase and allantoicase, were active in the liver of larval lampreys. The absence of significant carbamoyl phosphate synthetase III (CPS III) activity and low arginase activity indicate that a working ornithine urea cycle is not present in larval, parasitic or spawning sea lampreys. Further, attempts to induce urea production by subjecting larval lamprey to high environmental ammonia (2 mM total ammonia) or emersion (> 90 percent humidity), only had minor effects on the patterns of urea excretion and production.

Conclusions

We conclude that life cycle dependent changes in N-waste metabolism and excretion in the sea lamprey are related to changes in their feeding preferences, habitat and life style. The ability of phylogenetically ancient lampreys, which are the modern day representatives of the original jawless

vertebrates, to produce and excrete significant amounts of urea suggests that the uricolytic pathway was present in the earliest vertebrates. Since the activities of certain key ornithine urea cycle enzymes were below detection or very low, the possibility that the earliest vertebrates also had an active ornithine urea cycle remains unresolved.

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ACUTE AND SUBLETHAL GROWTH EFFECTS

OF UN-IONIZED AMMONIA

TO NILE TILAPIA *OREOCHROMIS NILOTICUS*

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Abstract

This study investigated the acute effects of un-ionized ammonia (NH_3-N) on 11 g fingerlings of Nile tilapia *Oreochromis niloticus* at two temperatures (23 and 33^{o}C); and at 28^{o}C with two sizes of fish, 3 and 45 g. In addition, sublethal effects of un-ionized ammonia on growth of Nile tilapia at 28^{o}C were evaluated. All tests were conducted in flow-through bioassay system.

In acute toxicity tests at 23 and 33^{o}C, the 96-h LC50's (±SD) were 2.25 ± 0.07 and 2.51 ± 0.16 mg/L NH_3-N, respectively. There was not a significant effect between the two temperatures on acute toxicity of NH_3-N in these tests (P 0.05). Estimates of the 96-h LC50's (±SD) at 28^{o}C were 1.36 ± 0.45 mg/L NH_3-N for the small fish and 2.65 ± 0.09 mg/L NH_3-N for the large fish. In these tests, there was a significant difference between the two sizes of fish tested (P 0.05). In 35-day study of the effects of sublethal concentrations of NH_3-N at 28^{o}C, there was a linear decrease in fish weight gain with increasing un-ionized ammonia concentrations (r^2 = 0.90, P 0.001). The concentrations of un-ionized ammonia that cause no reduction in growth, 50% reduction in growth, and 100% reduction in growth were 0.06, 0.73, and 1.46 mg/L NH_3-N, respectively. These data suggest that Nile tilapia has a tolerance to un-ionized ammonia similar to that of tilapia species, somewhat greater than that of channel catfish, and greater than that of many other warmwater fish and salmonids.

Introduction

Nile tilapia *Oreochromis niloticus* is a widely cultured warm-water fish that efficiently converts food web products growing in response to manure fertilizers and feed into high quality protein while resisting relatively poor water quality and diseases (Balarin and Haller, 1982). However, accumulation of ammonia has been recognized as a major constraint to the survival and production of tilapia species in aquaculture systems (McGeachin and Stickney, 1982; Abdalla et al., 1996). Total ammonia nitrogen (TAN) in water is present primarily as the relatively nontoxic ionized form (NH_4^+-N) and the highly toxic un-ionized form (NH_3-N) (Meade, 1985). Principal sources of ammonia in aquaculture ponds are fertilizers, feeds, and fish excretion. In addition, source water used to fill ponds and raceways may also contain ammonia from nitrogenous materials in sewage treatment plant effluent, industrial effluent, and runoff from fertilized agricultural land and animal feedlots (Khaleel et al., 1980).

Fish exposed to acute concentrations of un-ionized ammonia suffer disturbances of the nervous system, and they exhibit hyperexcitability, an increase in gill ventilation, a loss of equilibrium, convulsions, coma, and finally death (Russo, 1985; WHO, 1986). In general, reported 96-h median lethal concentrations of un-ionized ammonia range from 0.32 to 0.84 mg/L for salmonids, and from 0.40 to 3.1 mg/L for non-salmonids (Ruffier et al., 1984). However, fish exposed to lower sublethal concentrations of un-ionized ammonia suffer histological and physiological changes in different organs (Smith and Piper, 1975; Thurston et al., 1986; Oppenborn and Goudie, 1993), with an overall result of decreased weight relative to unexposed fish (Colt and Tchobanglous, 1978).

Temperature and fish size are among many factors that can modify the toxicity of ammonia on aquatic organisms (Sprague, 1985; WHO, 1986). No data were available in the literature for the effect of temperature or fish size on the acute or sublethal concentrations of un-ionized ammonia on Nile tilapia. With this background of information, the present study was undertaken to determine the median lethal concentrations (LC50) of un-ionized ammonia on Nile tilapia at two different temperatures (23 and 33^o C), and with two different sizes of fish at 28^{o}C. We also determined the sublethal effects of increased un-ionized ammonia on growth of Nile tilapia at 28^{o}C.

Materials and Methods

1. The toxin delivery system

A municipal well-water tap entered a head tank that held approximately 650-L water, aerated to maintain dissolved oxygen near saturation, and treated daily with sodium thiosulfate (Boyd, 1979) to get red of chlorine. Ammonia concentrations were established by continuous addition of appropriate volume of ammonium chloride stock solution delivered by peristaltic ten-channel variable-flow Autoclude pump to duplicate 1-L vacuum flasks (mixing chamber) that also received dilution water from the head tank. Mixing the water and ammonia solution was facilitated by mounting flasks on a shaking table. Each vacuum flask drained to a 38-L glass aquarium (bioassay chamber). An air pump provided constant aeration to each aquarium. A flow of about 150 mL/min was delivered continuously to each aquarium during assays. Fluid replacement time in aquaria was approximately 4.2 hr. Each test consisted of a control that received only water flow-through and five experimental concentrations of un-ionized ammonia in duplicate aquaria. However, three aquaria were used as a control at the sublethal test. The concentrations used in each acute test were chosen to give from 0% to 100% mortality of Nile tilapia within 96 h based on results of preliminary trials. Sublethal ammonia concentrations were chosen based on results from acute tests. Fluorescent lights in the laboratory were placed on a 14 h light and 10 h dark light cycle during assays.

2. Water quality

Temperature in the aquaria was controlled by regulating temperature in the head tank. This was obtained by regulating the rate that cool (14-17^{o}C) well-water overflowed the tank, and by heating the water with immersed heating coils operated by mercury-switching thermoregulator. However, additional small heaters were required in each aquarium to maintain temperature during the acute test at 33^{o}C.

Un-ionized ammonia in aquaria was determined from measurements of TAN, pH, and temperature (Emerson et al., 1975). Water quality analyses were conducted with procedures described in Standard Methods (APHA, 1985). Total ammonia nitrogen (ammonia electrode), pH (Orion model 221), temperature (mercury thermometer), and dissolved oxygen (YSI model 54 A) were measured once daily in each aquarium in the acute tests and once every other day in the sublethal toxicity assay. Total alkalinity (acid

titration), total hardness (EDTA titration), and nitrite-nitrogen (sulfanilamide method) were determined in each aquarium at the start and end of acute experiments, and once each week in the sublethal experiment. Total chlorine concentrations were measured and monitored daily in the source water and head tank using HACH test kit.

3. Fish and experimental conditions

Adult fish were obtained from Auburn University and spawned continuously in the laboratory (Balarin and Haller, 1982). Fish were acclimated for two weeks at each test temperature before beginning assays. Fish were not fed two days prior to or during the acute tests, and fed purina trout chow (38% protein) twice daily to satiation in the sublethal test. Ten fish were used per aquarium, except in the assay using large fish for which six fish were used per aquarium. One day prior to start any test, fish were netted from the acclimation tanks, individually weighed, and randomly distributed among aquaria. In acute tests, dead fish were removed and counted at 24, 48, 72, and 96 h intervals. However, at the end of 35-days sublethal exposure to un-ionized ammonia concentrations, fish were weighed individually from each aquarium and average net weight gain per fish per aquarium was calculated by the difference between average initial and final fish weight.

4. Statistical analyses

Median lethal concentrations (LC50) and associated 95% confidence intervals for each acute toxicity test were estimated at 24, 48, 72, and 96 h by the method of binomial probability (Wardlaw, 1985), and a mean LC50 (±SD) for each time period was calculated from the duplicate aquaria. LC50 values were tested for differences with regard to temperature or fish size effects at different time intervals by two-way analysis of variance (temperature and time & fish size and time). Least significant difference (LSD) tests were conducted to identify statistical differences between means (Sokal and Rohlf, 1981). In sublethal experiment at 28^{o}C, average net weight gain of fish at each un-ionized ammonia concentration was evaluated by linear regression. Analyses were conducted using the statistical software package Minitab. Statistical significance is assumed at P 0.05. Means are given with ± 1 SD.

Results

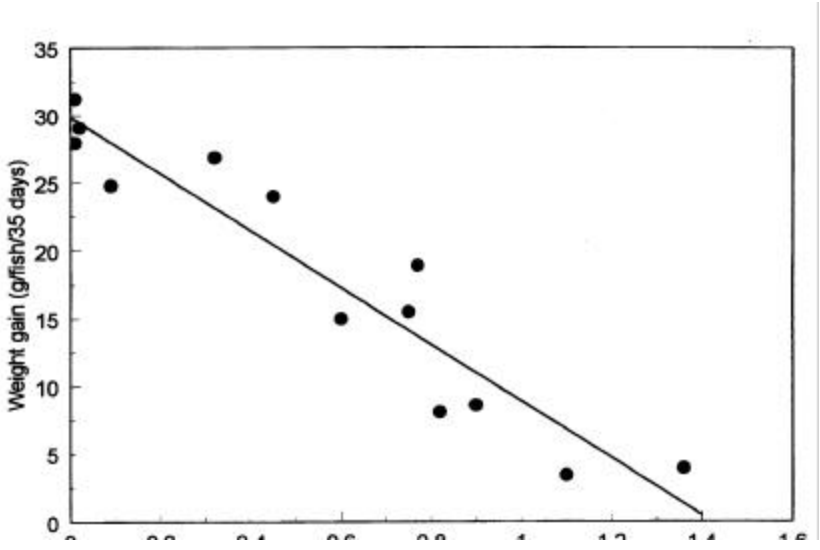
Average total chlorine concentrations in the source water was 0.01 ± 0.01 mg/L (ranged from 0.00 to 0.05 mg/L), and it was zero in the head tank after aeration and sodium thiosulfate treatment. Acute and sublethal tests were conducted under conditions shown in Table 1. The pH of water in aquaria ranged from 7.6 to 8.5.

Dissolved oxygen concentrations ranged from 92% to 115% of saturation. Nitrite concentrations were well below the 96-h LC50 of 16.2 mg/L reported by Polachek and Tomasso (1984) for blue tilapia *O. aurea*. Alkalinity or hardness were always higher than 300 mg $CaCO_3$/L.

Actual concentrations of un-ionized ammonia used in the acute and chronic tests are shown in Table 2. They ranged from 0.02 to 4.78 mg/L NH_3-N in acute tests, and from 0.02 to 1.31 mg/L NH_3-N in the sublethal test. No manipulation of pH was attempted, and un-ionized ammonia concentrations were between 2.1 and 16.0 % of total ammonia present in all tests. Mean coefficients of variation (C.V.) for un-ionized ammonia concentrations in aquaria during assays (C.V % = variance/mean) varied from 5 to 37% in all tests (Table 2).

No mortalities or sign of diseases were observed in controls of any of the acute experiments. Prior to death, all fish showed general symptoms of loss of equilibrium, convulsions, and coma. Estimates of LC50 at 24, 48, 72, and 96 h time intervals are presented in Table 3. No significant differences (P 0.05) were found among LC50 values at any time intervals when comparing results at 23 and 33°C for 11 g fish. The 96-h LC50 values were 2.25 ± 0.07 and 2.51 ± 0.16 mg/L NH_3-N at 23 and 33°C, respectively.

In tests conducted at 28°C using both small (3 g) and large fish (45 g), estimates of LC50 at any time interval were always significantly lower (P 0.05) with the smaller fish tested. The 96-h LC50's were 1.36 ± 0.24 and 2.65 ± 0.11 mg/L NH_3-N for the small and the large fish, respectively. Fish of approximately 6 g size were used in tests of effects of sublethal concentrations of un-ionized ammonia on growth at 28° C (Fig. 1). No fish



mortalities were observed in the control or at any un-ionized ammonia concentration tested. Regression analysis of fish weight gains (g/fish/35 days) and un-ionized ammonia concentrations (mg/L NH_3-N) revealed a significant negative correlation in all aquaria ($r^2 = 0.90$; $P \leq 0.001$). The equation describing this relationship is (fish weight gain) = 29.9 - 21.• (un-ionized ammonia concentration). Extrapolating to zero un-ionized ammonia concentration, the average fish weight gain over 35 days is 30 g/fish; and extrapolating to zero fish weight gain, the concentration of un-ionized ammonia at which growth stops is 1.42 mg/L NH_3-N.

Figure 1. The effect of increased un-ionized ammonia concentrations (NH_3-N) on average weight gain of Nile tilapia at 28°C for 35-day exposures. Ten fish were used per each aquarium.

Table 1. Fish sizes and water quality variables for tests of the acute and sublethal toxicity of un-ionized ammonia to Nile tilapia. Acute tests lasted for 96 h and sublethal test lasted for 35 days. Tests 1 and 2 had fish at 23 and 33°C, while test 3 had the small fish and test 4 had the larger fish. Values represent the mean ± SD.

	ACUTE TOXICITY				
Variable	Test 1	Test 2	Test 3	Test 4	Sublethal Test
Fish Weight (g)	11 ± 2	11 ± 1	3 ± 0	45 ± 3	6 ± 1
Temperature (°C)	23 ± 1	33 ± 1	28 ± 1	28 ± 1	28 ± 1
pH (units)	8.2 ± 0.1	8.2 ± 0.1	8.1 ± 0.1	8.0 ± 0.1	7.8 ± 0.1
Dissolved oxygen (mg/L)	7.9 ± 0.3	8.3 ± 0.4	8.0 ± 0.2	8.0 ± 0.3	8.2 ± 0.3
Nitrite-N (mg/L)	0.07 ± 0.01	0.14 ± 0.06	0.11 ± 0.07	0.11 ± 0.05	0.03 ± 0.02
Alkalinity (mg/L $CaCO_3$)	341 ± 12	326 ± 6	334 ± 13	332 ± 10	334 ± 17
Hardness (mg/L $CaCO_3$)	354 ± 16	335 ± 12	342 ± 17	346 ± 13	353 ± 11

Table 2. Mean ± SD actual concentrations of un-ionized ammonia in the acute and sublethal toxicity tests with Nile tilapia. Values for coefficient of variation (C.V. %) for un-ionized ammonia concentrations for each test are reported as mean (ranges).

	Test 1	Test 2	Test 3	Test 4	Sublethal Test
Control	0.02 ± 0.00	0.13 ± 0.01	0.11 ± 0.01	0.07 ± 0.01	0.02 ± 0.00
1	1.22 ± 0.37	1.23 ± 0.24	1.17 ± 0.25	1.71 ± 0.57	0.21 ± 0.16
2	2.25 ± 0.26	2.29 ± 0.33	2.38 ± 0.28	2.28 ± 0.19	0.53 ± 0.11
3	2.81 ± 0.43	2.92 ± 0.20	3.13 ± 0.33	3.15 ± 0.41	0.77 ± 0.02
4	3.36 ± 0.19	3.68 ± 0.24	3.97 ± 0.29	4.14 ± 0.65	0.86 ± 0.06
5	4.01 ± 0.66	4.34 ± 0.73	4.78 ± 0.52	4.62 ± 0.68	1.23 ± 0.19
C.V. (%)	15 (11-23)	17 (10-30)	18 (11-29)	17 (12-37)	18 (5-27)

Percent decrease in fish weight gain relative to controls (calculated from Figure 1) was significantly linearly correlated to un-ionized ammonia concentrations ($r^2 = 0.92$; $P \leq 0.001$). The equation for the regression analysis is (% decrease in fish weight gain) = - 4.2 + 71.8 • (un-ionized ammonia concentration). The no reduction in growth effective concentration (EC0), the median effective concentration (EC50), and the no weight gain effective concentration (EC100) of un-ionized ammonia were predicted from the equation for conditions when the percent decreases in fish weight gain relative to controls were 0, 50, and 100 %, respectively. They were 0.06, 0.73, and 1.46 mg/L NH_3-N, respectively.

Discussion

Most information in the literature for warmwater fish suggests a decrease in toxicity of ammonia with increased temperature. Thurston et al. (1983) found the toxicity of ammonia decreased with increased temperature from 12 to 22°C for fathead minnow *Pimephales promelas*. The same trend was reported for bluegill *Lepomis macrochirus*, channel catfish *Ictalurus punctatus*, and largemouth bass *Micropterus salmoides* over temperature range from 22 to 30°C (Roseboom and Richey, 1977). The 96-h LC50 values for channel catfish in a static toxicity testing system were 2.4, 2.9, and 3.8 mg/L NH_3-N at 22, 26, and 30°C, respectively (Colt and Tchobanoglous, 1976).

Table 3. Median lethal concentrations (LC50) of un-ionized ammonia nitrogen (mg/L NH_3-N) at 24, 48, 72, and 96 h for Nile tilapia. Tests 1 and 2 had fish at 23 and 33°C, while test 3 had the small fish and test 4 had the larger fish. Values represent mean ± SD for two replicates.

Time	Test 1	Test 2	Test 3	Test 4
24 h	3.07 ± 0.11	3.45 ± 0.36	1.83 ± 0.32	3.85 ± 0.21
48 h	2.76 ± 0.15	3.11 ± 0.13	1.70 ± 0.42	3.15 ± 0.46
72 h	2.50 ± 0.14	2.62 ± 0.15	1.45 ± 0.49	2.65 ± 0.09
96 h	2.25 ± 0.07	2.51 ± 0.16	1.36 ± 0.24	2.65 ± 0.09

However, it was demonstrated in this study that though the LC50 values were always higher at 33°C than at 23°C (Table 3), the effect of temperature on toxicity of un-ionized ammonia to Nile tilapia was not significant as evidenced by the similarity of

LC50 values at any time intervals for the 23 and 33°C acute toxicity tests (P 0.05). This might be due to deviation of both temperatures tested from the optimum range of 26-30°C recommended for tilapias (Hepher and Pruginin, 1981). In the only report of acute ammonia toxicity for species of tilapia found in the literature, Redner and Stickney (1979) obtained a 48-h median lethal concentration (LC50) for *O. aurea* of 2.4 mg/L NH_3-N at 27°C. The 48-h LC50 for Nile tilapia in this study was somewhat higher for all fish larger than 3 g (Table 3).

Results in this study show that, in the optimum range of temperature for growth, small Nile tilapia are more susceptible to acute ammonia toxicity than larger fish. There was a significant differences (P 0.05) among LC50 values at all of four 24-h intervals. Daniels et al. (1987) found the 24-h LC50 values for un-ionized ammonia were 0.34, 1.68, 2.40 mg/L NH_3-N for larvae, 1-month-old juveniles, and 4-month-old juveniles of spotted seatrout *Cynoscion nebulosus*. Also, the work of Straus et al. (1991) demonstrated that the toxicity of un-ionized ammonia to freshwater prawn *M. rosenbergii* decreases as animals increase in size and age. As fish grow, they develop physiological detoxification pathways and reduce the ratio of gill surface area to body weight (Rand and Petrocelli, 1985). In addition, small fish receive a larger dosage per unit body weight than larger fish, and thus are more susceptible to the toxicant.

In this study, it was observed that increased sublethal concentrations of un-ionized ammonia caused a linear reduction in growth of Nile tilapia, mainly because fish appetite were suppressed with increased un-ionized ammonia concentrations. Robinett (1976) found that 0.12 mg/L NH_3-N caused a reduction in growth and gill damage for channel catfish. Channel catfish suffered gill lesions when average un-ionized ammonia concentrations in ponds ranged from 0.02 to 0.08 mg/L and average daily maxima ranged from 0.08 to 0.22 mg/L (Soderberg et al., 1984 a, b). In a comparable study with channel catfish, Colt and Tchobanoglous (1978) found a linear decrease in fish growth with increased un-ionized ammonia concentrations. They reported an EC50 of 0.52 mg/L NH_3-N for channel catfish compared with 0.73 mg/L NH_3-N for Nile tilapia; and an EC100 of 0.97 mg/L NH_3-N compared with 1.46 mg/L for Nile tilapia. These data with results from similar work in the literature suggest that Nile tilapia has a tolerance to un-ionized ammonia similar to that of blue tilapia (Redner and Stickney, 1979), somewhat greater than that of channel catfish (Robinett, 1976; Colt and Tchobanoglous, 1978), and greater than that of many other warmwater fish and salmonids (Thurston et al., 1984; WHO, 1986; Oppenborn and Goudie, 1993).

Acknowledgements

The research is a component of the Pond Dynamics/Aquaculture Collaborative Research Support Program (PD/A CRSP) supported by U.S. Agency for International Development grant DAN-4023-G-00-0031 and by contributions from the Agricultural Experiment Station at Michigan State University. We thank Dr. Donald L. Garling for contributions to the test apparatus design, Ralph Bebee with laboratory setup, And Dr. Ted R. Batterson for aid during many situations of this work.

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THE EFFECT OF TEMPERATURE ON AMMONIA EXCRETION

OF UNDERYEARLING LAKE INARI ARCTIC CHARR

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Introduction

Ammonia excretion by fish is an indicator of the intensity of protein catabolism (Brett & Zala, 1975; Wood, 1993), and ammonia excretion may also be an indirect indicator of feed intake because protein catabolism is related strongly to protein intake (Paulson, 1980; Medale et al., 1995). Studies of ammonia excretion are less frequent than those of oxygen consumption, and the influence of temperature on ammonia excretion has been studied for relatively few fish species.

The study of rates of ammonia excretion has practical significance because ammonia excretion is central to the calculation of water requirements within intensive culture. The current study was conducted to examine the influence of temperature upon rates of ammonia excretion in groups of actively feeding and growing Arctic charr (*Salvelinus alpinus* (L.)).

Material and Methods

Eyed eggs of Lake Inari Arctic charr were brought to Laukaa Aquaculture and Fisheries Research Station. Eggs were incubated, and following hatch the fish were reared under standard culture conditions at ambient water temperature. At the beginning of October the fish (N = 2360), of mean weight c. 6 g, were divided among

twelve 67 l plastic tanks. Water temperature in all tanks was held at 11.0 °C for a further 22 days.

After 22 days, constant temperature treatments were established at 11.0 °C, 14.4 °C and 17.7 °C within a couple of hours. A fourth treatment group was subjected to a fluctuating temperature: a daily mean of 14.3 °C with daily fluctuation of $\pm$ 1 °C. A 12L:12D photoperiod was established and was synchronised with the temperature fluctuation so that the high temperature phase was during daylight (600-1800 hours) and the cold temperature phase was during the dark part of the photoperiodic cycle (1800-600 hours). The fish were fed with commercial salmon feed (Tess Nutra G, 2.0 mm: 44.5 % protein and energy content 22.7 kJ g^{-1}) in excess using belt feeders. Feeding started at 600 hours, simultaneously with `lights on`, and lasted for four hours.

All fish were weighed before transfer to the experimental tanks (day = 0) and 17-18, 37-38 and 52-53 days thereafter in order to estimate tank biomasses and their changes.

Ammonia excretion was measured using the tanks as flow-through metabolism chambers. Measurements were made on 5 days over a period of 31 days following the change. The sampling system is described previously in Lyytikäinen & Jobling 1998. Ammonia concentrations in water samples were determined using a spectrophotometric method (Suomen standardoimisliitto, 1976).

Results

Daily average ammonia excretion rates ranged from 7.31 to 10.54 mg kg^{-1} h^{-1}. Temperature had a significant influence on ammonia excretion rates (Kruskall-Wallis, $P<0.05$) and excretion was lowest in fish held at 11.0 °C. The temperature effect on daily mean ammonia excretion (NH_4-N, mg kg^{-1} h^{-1}) could be described by an exponential model ($\pm$SE):

$$N = 4.655(\pm 1.305)e^{(0.048(\pm 0.018)T)},$$

where N is mean daily ammonia excretion rate, $R^2 = 0.51$, $n = 9$.

Ammonia excretion showed a diel pattern at all temperatures and excretion was

highest during the middle of the day and lowest at 100-400 hours (Figure 1).

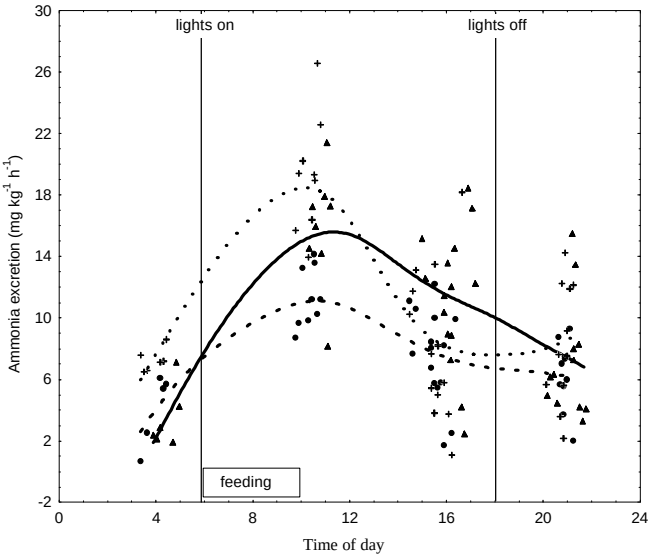

Figure 1. The diel variation in ammonia excretion rates (mg $kg^{-1}h^{-1}$) of underyearling Lake Inari Arctic charr (*Salvelinus alpinus* (L.)) at constant temperatures. Symbols: cross = 17.7 °C, triangle = 14.4 °C and circle = 11.0 °C. Lines indicate distance weighted least square fits for the data: dotted line = 17.7 °C, solid line = 14.4 °C and broken line 11.0 °C. Feeding period and the times when lights were switched on or off are indicated.

Thermal fluctuation had no significant influence on ammonia excretion rates, the ammonia excretion rate and the diel pattern of ammonia excretion being similar under constant (14.4 $°C_{const}$) and fluctuating (14.3 $°C_{fluc}$) thermal conditions (Figure 2).

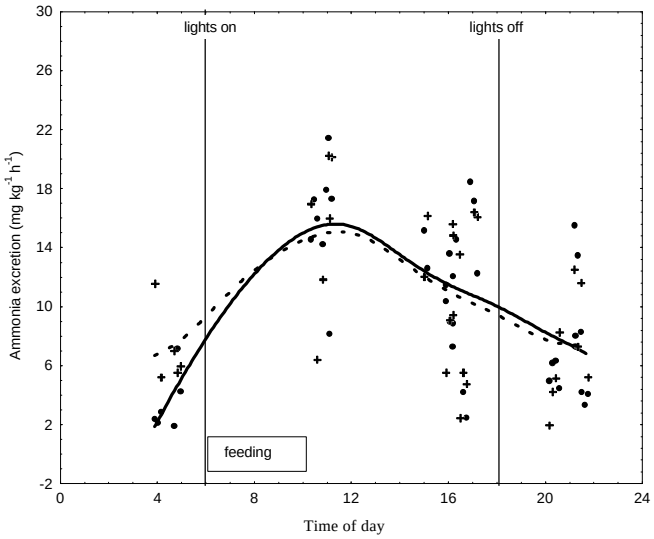

Figure 2. The diel variation in ammonia excretion rates (mg $kg^{-1}h^{-1}$) of underyearling Lake Inari Arctic charr (*Salvelinus alpinus* (L.)) at constant (14.4 °C) and fluctuating (14.3 ±1 °C) temperatures. Symbols: circle = constant temperature and cross = fluctuating. Lines indicate distance weighted least square fits for the data:, solid line = constant temperature and broken line = fluctuating temperature. Feeding period and the times when lights were switched on or off are indicated.

Discussion

The Arctic charr showed a diel pattern of ammonia excretion and peak excretion was recorded after the termination of feeding. The temperature coefficient in the exponential model for ammonia excretion was close to those reported for rainbow trout and brook trout (Paulson, 1980). The results confirm that ammonia excretion of Arctic charr is temperature dependent in a similar manner as to other salmonids (Brett & Zala, 1975, Medale et al., 1995).

There were no differences in ammonia excretion between fish under fluctuating and constant temperature conditions, so thermal fluctuation seemed to have a negligible influence on exogenous and endogenous nitrogen excretion of Arctic charr in the present study.

Acknowledgements

This work was financed by Finnish Game and Fisheries Research Institute and The Graduate School of Fisheries and Fish Biology of Finland.

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EXPRESSION OF GLUTAMINE SYNTHETASE AND ORNITHINE CARBAMOYLTRANSFERASE IN THE COURSE OF *PIARACTUS MESOPOTAMICUS* HOLMBERG 1887 (TELEOSTEI, CHARACIDAE) DEVELOPMENT

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Introduction

Knowledge of ornithine urea cycle (OUC) enzymes during initial stages of development may be the clue for understanding several points observed in fish. Among that, the nitrogen excretion, the acid-base balance, the aminoacid metabolism and the production of amino derivative compounds are the prime.

Usually ammonia excretion is not a problem for aquatic animals. While terrestrial have to synthesize urea through OUC, those one are able to excrete ammonia to the environment. However, the role of OUC enzymes could be not strictly related to urea excretion as it happened with anaplerotic cycles. Therefore, urea synthesis can be relegated to some second alternative facing environmental disturbances or during the larval phase of teleostean (Wright et al, 1995).

Two of the OUC enzymes, glutamine synthetase (GS) and ornithine carbamoyl transferase (OCT), play important role in the nitrogen metabolism. Such enzymes are responsible by glutamine synthesis and addition of nitrogen into ornithine. Glutamine,

through CPS_{III}, is the nitrogenous molecule involved in carbamoyl phosphate synthesis (Anderson, 1980).

The role of OUC in the synthesis of nitrogenous intermediaries supposed to participate in developmental process as well as its role in adaptive strategies facing environmental changes have called our attentions particularly regarding ontogenetic process. The present paper is concerned the activity of OCT and GS in *P. mesopotamicus* along ontogenesis aiming to infer their role during developmental process in this species.

Material and Methods

The experiments were carried out employing eggs, embryos in several degrees of development and alevins of *P. mesopotamicus*. Induction of fish ovulation was done and the eggs as well as the fertilized one were immediately collected. Such procedure was done along four weeks at suitable time intervals. The samples were promptly transferred to liquid nitrogen and lyophilized for posterior analysis. All such experiments were done at CEPTA IBAMA-Pirassununga-SP.

Enzyme analysis:

Samples were mechanically disrupted in a Potter homogenator under a cold bath. Biological material was used in the proportion of 100 mg/ml of glicine buffer at pH 7.0, with glycerol 50%. The extracts were centrifuged at 1000 rpm for 1 minute and the supernatants were used as enzyme extract.

Glutamine synthetase

The activities of GS were determined in 50 mM HEPES pH 7.0 in a reaction mixture containing 20mM K_2HAsO_4; 60mM hydroxilamine; 0.4mM ADP and 3mM $MnCl_2$. The incubation temperature was 25^o C running for 30 minutes. After incubation period the reaction was stopped and developed by chloride reagent. Such reaction consisted of the development of γ-glutamyl hydroxamate, the product of reaction, by addiction of 0.2N $FeCl_3$ in 50% HCl and 24% TCA.
After that, the mixture was centrifuged at 12,000 rpm for 2 minutes and the absorbance of the supernatant read at 540 nm. This method was proposed by Woolfolk (1966).

Ornithine carbamoyl transferase.

The activity of OCT was determined in a reaction system containing 50mM of HEPES pH 8.5; 10mM of ornithine and 10mM of carbamoyl phosphate. The reactions were incubated at 25^{o} C for 30 minutes and stopped by 70% TCA addition. The reaction mixtures were centrifuged at 12,000 rpm for 2 minutes. Citrulline resulted as reaction product was colorimetricaly determined at 464 nm as proposed by Rahmatullah (1980).

Results and Discussion

Ammoniotelism is observed in adult samples of *P. mesopotamicus* as in the most teleosts. However, we have observed the highest specific activity of GS and OCT along the larval phase. Activity of GS started to grow from egg phase and its peak was reached around the tenth day. Maximum value observed for such enzyme was near to 400 µmols per gram of protein (fig.1). Several short peaks of GS were observed along development.

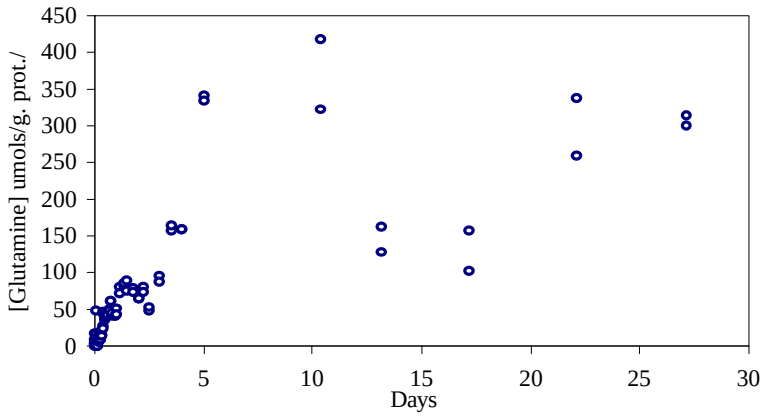

Figure 1. Activity of GS of *P. mesopotamicus* during the developmental phases. The activities were carried out employing eggs, embryos and alevins.

OCT increased along all the phases to a maximum value around 120 µmols per gram of protein (fig. 2). Differently of GS, this activity started from a basal value nearly 40 µmols of citrulline per gram of protein per hour.

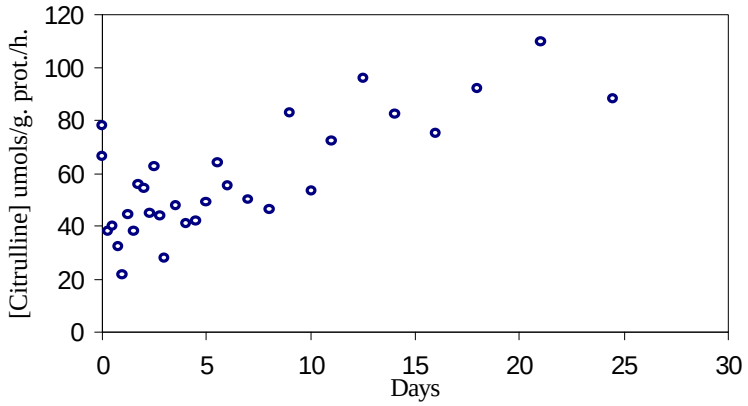

Figure 2. Activity of OCT of *P. mesopotamicus* during the developmental phases. The activities were carried out employing eggs, embryos and alevins.

The absence of GS when compared to OCT let us to suppose that OUC is not complete along all developmental phases of *P. mesopotamicus.* Moreover, ARG was detected from the fourth day following the hatching (not showed data). It is difficult to assume ureotelism in larva of *P. mesopotamicus* under such results. However, the excretion of urea and ammonia must to be measured before any conclusion.

Other roles have been proposed to justify the presence of OUC in fish independently the life phase. Besides urea synthesis from ammonia, detoxification and the acid-base balance should be claimed (Atkinson 1995). For such purpose, catabolic path for ornithine has to be active and this aminoacid has to be shifted. This function would lead to constant production of citrulline. However, the increase of the specific activity of OCT along the fish development should be consequence of its graduate activation. Polyamines, particularly putrescin, should be responsible for such increase of OCT activity. High production of polyamines is reported for tissue growth and development (Hyvönen et al., 1088; Pegg, 1988). We have observed that putrescin, if employed at the same concentration of carbamoyl phosphate (10mM) in OCT reaction, increases the enzyme affinity (fig.3). Values of Km change from 2.32 mM in the absence to 1.00

mM in the presence of putrescin. Several authors reported involvement of polyamines into cell differentiation process. Following the developmental processes the increase of polyamine should result into OCT increase.

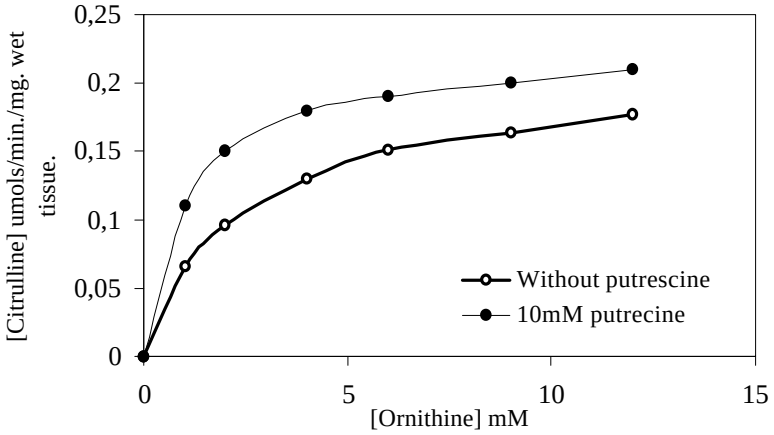

Figure 3. Effect of ornithine concentration in OCT of the liver of *P. mesopotamicus* in the presence and absence of putrescin. Km_s were respectively 1.00mM and 2.31mM. The concentration of carbamoyl phosphate was 10mM for both determinations.

Our data concerning OCT and GS are in agreement with that observed for rainbow trout (Wright et al, 1995). The enzymes of OUC working on the larval phase suggest a role of them other than nitrogen excretion particularly considering their hormone activation. The hormone 3.3'-5-triiodine-l-thyronine (T3) acts on the metamorphosis at molecular level resulting cellular, morphological and biochemical changes (Cohen, 1970; Tata, 1993). Synthesis of OUC enzymes, as arginase (ARG) for instance, is induced by T3 in amphibian (Callery, 1996). Increase of all OUC enzymes is observed in *Rana catesbeiana* related to ammoniotelism-ureotelism transition (Brown, 1960). Increase of ARG and argininosuccinate synthetase-argininosuccinate lyase was observed during the larval phase of *P. mesopotamicus* (data not shown) and of *Oncorhynchus mykiss* (Wright et al, 1995). Considering that both teleosts have no transition from aquatic to terrestrial life such results should come from hormonal action. Activation of OUC by T3 during metamorphosis along the ontogenesis of *P. mesopotamicus* may work coupled to polyamine production through ornithine

decarboxilase (ODC). Genes for ODC are constitutive and regulated post-transcription by putrescin or other polyamine concentration through the frame-shifting mechanism (Matsufuji at al.,1996). Wherefore, ornithine should be working as the pivotal point to shunt ornithine toward citrulline or putrescin pursuant to the cell need.

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NITROGENOUS EXCRETION PATTERN

OF THE FRESHWATER FISH

HOPLERYTRINUS UNITAENIATUS:

A FACULTATIVE, AIR-BREATHING

NEOTROPICAL TELEOST

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Abstract

Ammonia is usually the nitrogenous excretion form of aquatic organisms. Fish are commonly reported as ammonia produces. However, marine elasmobranchs and some teleostean fish are able to synthesize urea in significant amounts, through ornithine urea cycle (OUC) enzymes, for different purposes. The air-breathing fish *Hoplerytrinus* has been studied concerning urea production as a strategy employed under stressing conditions to face the air exposition. All OUC enzymes were studied and ammonia, urea and uric acid were also estimated. Our results suggest this species ammoniotelic and ureogenic as well as environmental changes lead to different responses.

Introduction

Aquatic animals are usually more tolerant to high levels of plasma ammonia if compared to terrestrial animals. Total ammonia concentration on plasma may change from 0.05 to 1.0 mmols.l^{-1}(Wright et al., 1993) but if such level reach 2 mmols.l^{-1} a flaccid paralysis is observed in arctic char (Lumsden et al., 1993). In contrast, plasma levels higher than 0.05 mmol.l^{-1} may be highly toxic to central nervous system of several mammals (Meijer et al., 1990).

Most fish waste ammonia as main product through gill diffusion or exchanges it by Na^+. However, the whole ornithine urea cycle (OUC) enzymes have been detected in the liver of many species followed by significant ureogeneses (Cvancara, 1969; Read, 1971; Casey & Anderson, 1982; Campbell & Anderson, 1991; Mommsen & Walsh, 1992; Wright, 1995).

The role of OUC as well as its enzymology for freshwater teleosts is scarcely known. Such cycle seems to be functional for many species but is apparently inoperative for most of the teleosts (Anderson & Walsh, 1995). According to several authors is very questionable and improbable the preservation of the OUC-enzyme-genes if they have no role or codify functional proteins (Wright et al., 1995).

The species *H. unitaeniatus* is a freshwater teleostean fish and belongs to Erythrinidae family. It is well known its behavior running relatively long routes on land changing from ponds to ponds. For such, it uses the vascularized swimbladder to endure the trip (Graham, 1995) and may be classified as an air-breathing species.

Material and Methods

Experimental design

Seven specimens of *H. unitaeniatus* captured at Mogi Guaçu River SP, near to Cachoeira de Emas Pirassununga, were carried to large tanks inside lab. The animals were kept in such conditions for seven days for complete recovery under pO_2 130 mmHg at 25°C and starvation. After recover the animals were weighted, rinsed with distilled water and transferred to excretion chambers containing suitable volume of natural and autoclaved water. The water pH was kept constant at 7.0 during all the experiment. After introducing the animal in the chambers, samples of water were collected in regular time intervals and the concentration of ammonia, urea and uric

acid were immediately determined. After the last sampling of water, the animals were removed from the chambers. A sample of blood was withdraw and immediately centrifuged at 7,000g for 3 minutes at 4°C. Plasma were separated from red cells, added to perchloric acid 0.6N at the ratio 1:3, centrifuged at 12,000g for 3 minutes and kept for subsequent analysis of ammonia, urea and uric acid. After blood withdrawal, the animals were anesthetized with MS222 and killed by punching the spinal cord. The liver was immediately excised and kept under liquid nitrogen for posterior enzyme analysis.
Cell Extract.

Hepatic tissue slices were put into 10mM phosphate buffer pH 7.0 containing 50% glycerol and submitted to mechanic disruption for 30 sec. in a Potter homogenator. The extract was used for enzyme analysis. All studied enzymes were colorimetricaly assayed by end-point procedures.

Glutamine synthetase (GS).

The activities of GS were determined in 50 mM HEPES pH 7.0 in a reaction mixture containing 20mM K_2HAsO_4; 60mM glutamine, 15Mm hydroxylamine; 0.4mM ATP and 3mM $MnCl_2$. The incubation temperature was 25° C running for 30 minutes. After incubation period the reaction was stopped and developed by acid ferric chloride reagent. Such reaction consisted of the development of γ-glutamyl monohydroxamate, the product of reaction, by addiction of 10% $FeCl_3$ in 0.2N HCl and 50% TCA (Verhaben et al., 1973). After that, the mixture was centrifuged at 12,000g for 2 minutes and the absorbance of the supernatant read at 540 nm.

Carbamoyl phosphate synthetase (CPS)

CPS was determined in cell extracts containing: 50mM HEPES pH 8; 20mM ATP; 10mM glutamine; 10mM ornithine; 5mM sodium bicarbonate; 24mM sodium sulfate; 5 IU of OCT from Streptococcus faecalis. Citrulline was estimated in the supernatant as described by Boyde & Ramatullah (1980).

Ornithine carbamoyl transferase

The activity of OCT was determined in a reaction system containing 50mM of HEPES pH 8.5; 10mM of ornithine and 10mM of carbamoyl phosphate. The reactions were incubated at 25° C for 30 minutes and stopped by 70% TCA addition. The reaction mixtures were centrifuged at 12,000g for 2 minutes. Citrulline resulted as reaction

product was colorimetricaly determined at 464 nm as proposed by Boyde & Ramatullah (1980).

Argininosuccinate synthetase-argininosuccinate lyase (ASS-ASL)

ASS-ASL system was measured by arginine, the end product, through the Sakaguchi reaction. The reaction mixture contained: 50mM HEPES pH 7.5; 16mM ATP; 30mM citrulline; 90mM aspartic acid; 5mM $MgCl_3$. After incubation at 25 ^oC for 45 minutes the reaction was stopped by 70 % PCA and centrifuged at 10,000g. Hence, the reaction product was estimated.

Arginase (ARG)

The activity of ARG was determined in 50 mM HEPES pH 11 in a reaction mixture containing 10mM $MnCl_2$ and 278mM arginine. The reaction mixture was run for 15 minutes at 25° C. The reaction was killed by adding perchloric acid 70% in a ratio of 1:10. After centrifuging at 12,000g for 2 minutes, urea was determined in the supernatant (Boyde & Ramatullah 1980).

Results and Discussion

Ammonia is usually the nitrogenous excretion form of aquatic organisms. Fish are commonly reported as ammonia producers. However, marine elasmobranchs and some teleostean fish are able to synthesise urea in significant amounts, through ornithine urea cycle (OUC) enzymes, for different purposes (Atkinson, 1992). The air-breathing fish Hoplerythrinus unitaeniatus has been studied concerning urea production as a strategy employed under stressing conditions to face the air exposition (unpublished results). In spite of the strategy for facing such hard situation, is interesting to know about nitrogenous form of excretion under normal conditions.

We have observed that total nitrogen excreted by H. unitaeniatus, under normoxic conditions, was fundamentally ammonia. Uric acid was not detected as a nitrogenous form of excretion for the present experimental conditions. After two hours of excretion, the total amount of nitrogen was near 4.8µmols/g of fish (fig. 1). Ammonia reached 4.7 while urea concentration was less than 0.1 µmols/g of fish. Considering a linear excretion on the coarse of the experimental determinations, we can say that only 1.5% of total N excretion was in the urea form. However, such ratio is not the same for plasma (tab. I). These results suggest a preferential ammonia excretion but

significant amount of urea is synthesised.

Production of urea from arginine metabolism is plausible particularly for carnivorous fish, however the OUC enzymes were studied considering the chances of any adaptive response from this set of enzymes against severe environmental situation. Enzyme determinations showed the presence of all OUC enzymes and correlates (Table. II).

As may be observed high values of arginase were detected and the amount of hepatic CPS is very low. However, detection of all enzymes is a clear indicative of urea synthesis ability in H. unitaeniatus. Our results suggest this species is ammoniotelic and ureogenic. This attempts to admit the use of such enzyme set to face any adverse condition.

Table I. Plasma nitrogenous compounds of *H. unitaeniatus* under normoxic conditions. Uric acid was not colorimetrically detected.

Nitrogenous product	Plasma contents μmol/ml	Statistic
Ammonia	1.402	± 0.08 (N=8)
Urea	0.555	± 0.04 (N=8)
Ammonia/urea	2.551	± 0.34 (N=7)

Table II

OUC enzyme	**Specific activity** (μmoles/g/min)	Statistic
Glutamine synthetase (GS)	1.691	± 0.05 (N = 7)
Carbamoyl phosphate synthetase (CPS)	0.0424	± 0.03 (N = 5)
Ornithine carbamoyl transferase (OCT)	0.688	± 0.017 (N = 7)
Argininosuccinate synthetase–Argininosuccinate lyase (ASS-ASL)	0.1045	± 0.004 (N = 5)
Arginase (ARG)	16.337	± 4.85 (N = 8)

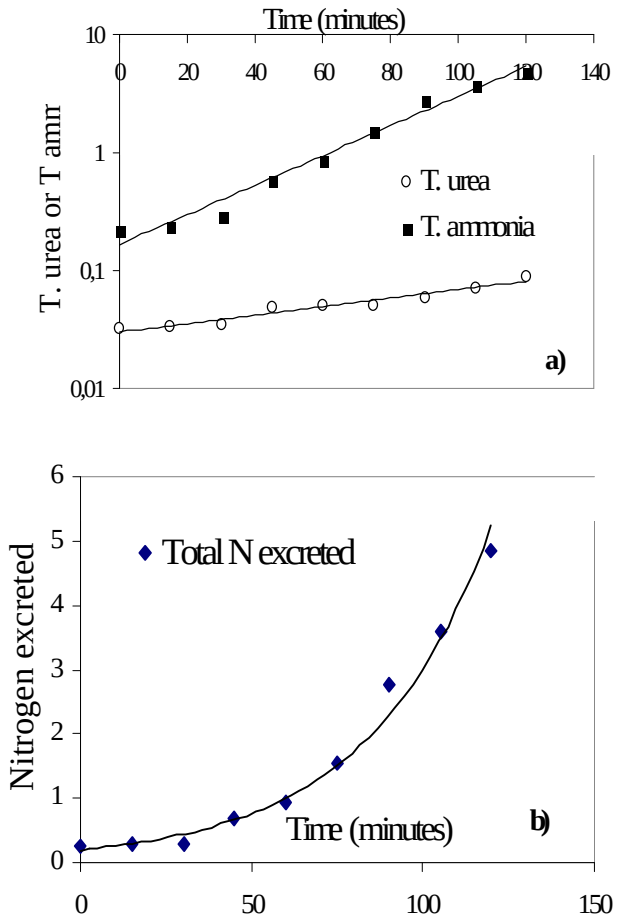

Figure 1. Nitrogenous excretion pattern of *H. unitaeniatus* under normoxia. Urea and ammonia are expressed as a total amount (T) in µmols per gram of fish (a). Total nitrogen excretion is expressed in µgrams per gram of fish (b).

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